PREFACE

The excitement of pursuing a new frontier is unequalled. The microbiology of deep terrestrial subsurface environments is an area that has been woefully neglected by the microbiologist in general and microbial ecologists in particular. While the oil and gas industry have, over the past five decades, been concerned about selected microorganisms fouling and corroding subsurface structures, they have concentrated on selected microorganisms such as the sulfate-reducing bacteria and the sulfur-oxidizing organisms rather than defining the scope, density, distribution, and diversity of microbial communities that exist in the subsurface. It is the desire of the organizers of the First International Symposium on Microbiology of the Deep Subsurface to bring together international investigators that are actively pursuing microbiological research of deep terrestrial sediments. It is our hope to foster a mechanism for communication, interaction and technical exchange in the scientific community that spans disciplines of microbiology, geology, hydrology, commercial applications and government administrators. These proceedings are a tribute to and in appreciation for the efforts of the investigators associated with the Department of Energy's Microbiology of Subsurface Environments Program who contributed so magnificently for the success of the symposium.

These proceedings contain the papers presented at the First International Symposium on the Microbiology of the Deep Subsurface, January 15-19, 1990, at Delta Court of Flags in Orlando, Florida. The symposium was sponsored by Westinghouse Savannah River Company, the United States Department of Energy, and Graves Environmental and Technical Services of Jackson, SC, and to them we express our appreciation.

The papers in this collection have been edited in such a way as to bring the papers into somewhat of a conformity as to citations and biographical information, but a relatively light hand was used for stylistic and grammatical concerns. We have chosen to let the authors' work stand substantially as written. Of course, we have corrected errors of spelling and sometimes altered the order of words when clarity was at stake. Additional thanks are expressed to Leonard G. O'Neal, technical editor, Westinghouse Savannah River Company, for his assistance in editing and formatting these proceedings.

A note of explanation is needed for the Plenary Sessions. Each Plenary Session was audiotaped at the time of presentation. Some of the speakers did not provide manuscripts of their Plenary talks and thus their papers were transcribed from the audiotapes. It was the editors' task to develop manuscripts for inclusion in this volume by carefully comparing the typescript with the audiotapes. It is desired that the developed manuscripts are in accord with both the views and the high standards of each of the authors, though of course, only the editors are responsible for any miscommunication as a result of this process. During the Plenary presentation, data and information slides were presented and are so noted positionally in the text.
although they are not included in the manuscript. Each manuscript that was prepared in such a fashion is noted by a symbol (†) before the title.

Editors:

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HISTORICAL PERSPECTIVE: NATIONAL AND INTERNATIONAL RESEARCH PROGRAMS

Convener: W. C. Ghiorse

Plenary Session

ONE
HISTORICAL PERSPECTIVE: NATIONAL AND INTERNATIONAL RESEARCH PROGRAMS

Convener: William C. Ghiorse

Introduction

The First International Symposium Microbiology of the Deep Subsurface signaled the beginning of a new era of increased interest around the world in the microbial ecology of deep subsurface environments, especially those vast unexplored environments at depths that are not easily sampled without using drilling fluids. Details of the most recent research are provided in the papers that were presented in the following scientific sessions. The purpose of the opening session was to provide an overview of past and present subsurface microbiological research. The session was organized to learn more about who is doing what in subsurface microbiological research, and why.

Speakers were invited from the three main funding agencies of the United States whose research programs include major efforts in subsurface microbiological research – the United States Department of Energy (DOE), the United States Geological Survey (USGS), and the United States Environmental Protection Agency (EPA). Speakers were also invited from other countries with established or planned subsurface microbiological research programs. The foreign speakers, including representatives from the Soviet Union, Great Britain, United Germany and Australia, were encouraged to give an historical overview with insight into the reasons for the development of the research activities.

More details on the individual research programs and lists of publications resulting from the research can be obtained by writing to the individual speakers at the addresses given under each title.
Subsurface Microbiological Research at the United States Department of Energy

Dr. Frank J. Wobber, Office of Health and Environmental Research, Program Manager, Subsurface Science Program, U.S. Department of Energy, ER-75, Germantown, MD 20545.

Abstract

Deep subsurface microbiology is one of eight principal areas of research interest (subprograms) of the Subsurface Science Program within the Department of Energy's (DOE's) Office of Energy Research. DOE has been conducting deep subsurface microbiological research for about five years, primarily concentrating on basic exploratory research to characterize the microbial populations and activities in deep sediments and groundwater systems. Initial emphasis is on investigations in uncontaminated areas so that fundamental hypotheses concerning microbial-environmental interactions can be explored; such information is essential in the long term for in situ bioremediation. So far, attention has been given to determining microbial presence and to natural environmental processes that control microbial survival, abundance, and diversity, which in turn can affect the degradation of contaminant mixtures including the mobilization or immobilization of radionuclides.

Explorative, deep microbiology drilling began with three boreholes at the Savannah River Site (SRS); a fourth hole was drilled to depths of 1734 feet downslope and generally downgradient from SRS at Allendale, South Carolina. Innovative drilling and sampling technologies were developed and research was conducted by interdisciplinary teams of microbiologists, geohydrologists, and geochemists from about 30 universities and national laboratories. Initial scientific results were published in a special issue of the Geomicrobiology Journal Vol. 7, 1989. A DOE subsurface microbiology culture collection was established at Florida State University and plans are being made to make isolates generally available. A majority of the papers presented at the symposium describe results obtained by investigations from samples obtained at SRS.

Future research plans are to develop a comparative environmental database by investigating uncontaminated hydrogeologic systems now, and contaminated zones later. Sampling is planned at the Idaho National Engineering Laboratory and the Hanford Site in the state of Washington in 1990-1991 for comparison to the SRS data. The goal is to broaden fundamental understanding of natural environmental (hydrogeologic, geochemical) factors at depths that affect microbial abundance, distribution, and activities. Research plans and other documents are available that describe the research program and future research directions. These documents are available free on written request.

No paper submitted.
Subsurface Microbiological Research at the United States Geological Survey

I. Francis H. Chapelle, Research Hydrologist, Water Resources Division,
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Abstract

The United States Geological Survey (USGS) has a Congressional mandate to conduct studies of hydrologic systems and water chemistry. This includes studies of the geochemistry of groundwater systems influenced by natural phenomena and stressed by human activities. Groundwater microbiology, as determined from well water samples, has been studied by USGS scientists for many years, going back to the beginning of this century.

Of interest to the participants of the symposium were the results of several recent projects that focused on how microbial processes impact groundwater geochemistry. This includes work on CO₂ generation by heterotrophic bacteria in the Atlantic Coastal plain system of South Carolina, as well as studies on toxic substance hydrology at several locations.

In these studies, the traditional geochemical mass balance approach was applied to specific microbiological questions. For example, water-chemistry data clearly indicated a source of CO₂ to groundwater in deep coastal-plains aquifers. This observation poses the question of whether the CO₂ source is the result of microbial processes or the result of abiotic decarboxylation reactions. Recent studies have shown that bacteria are the principal CO₂-producing agents, coupling organic matter oxidation with the reduction of electron acceptors, O₂, Fe oxides, and SO₄ in different parts of the Atlantic Coastal Plain.

In groundwater systems stressed by human activities, the influence of microorganisms on the transport and the fate of organic contaminants under aerobic and anaerobic conditions in surficial aquifers is being investigated in several locations. In addition, a sandy aquifer in Cape Cod, Massachusetts is being studied intensively to explore fundamentals of subsurface microbial ecology, including movement of bacteria through aquifer sediments, their attachment to particles, stratification of subsurface activity zone, and grazing pressure of subsurface protozoa on bacterial population density and activity.

No paper submitted.
Subsurface Microbiological Research at the United States Environmental Protection Agency

Dr. John T. Wilson, Research Microbiologist,
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Abstract

The historical roots of current subsurface microbiology research programs at the United States Environmental Protection Agency (EPA) go back to the late 1960s when a chemist (Bill Dunlap) at the Kerr Laboratory noted that DDT was dechlorinated in aquifers. Biological activity was suspected, but little information was available in the literature on subsurface biological activity in shallow aquifers. This led to the development, primarily by a microbiologist (James McNabb) during the 1970s, of a protocol for retrieving core samples uncontaminated by exogenous microorganisms.

The major impetus for developing the early aseptic sampling devices and protocols and, subsequently, for conducting a series of fundamental groundwater microbiology research studies in the early 1980s, was to convince regulatory personnel that indigenous microorganisms and their activities are important in groundwater aquifers. A direct and positive outcome of this work is the current interest at the U.S. EPA, and at other agencies represented at the symposium, in applying bioremediation technologies to clean up contaminated aquifers.

In other U.S. EPA laboratories, besides the Kerr Laboratory, subsurface microbiological research is being conducted by programs on the effects of hazardous waste injection and the behavior of contaminants in landfills. When appropriate, microbiological approaches that include in situ bioremediation technologies are also being considered within the Super-Fund program. The U.S. EPA is mandated to deal with enormous contamination problems; bioremediation technology alone cannot be expected to solve them. More collaborative, interdisciplinary research, involving field work and laboratory studies, is needed to determine how far and how well bioremediation technologies can be applied to major environmental contamination problems.

No paper submitted.
Abstract

Subsurface microbiological research in the Soviet Union goes back to the early work of petroleum microbiologists in the 1920s, who studied the distribution of microorganisms in deep sedimentary rocks and stratal waters of oil bearing formations. In the 1950s and 1960s, Soviet geological microbiologists showed that high numbers of hydrocarbon-oxidizing, hydrogen-oxidizing, and sulfate-reducing bacteria existed in deep underground aquifer systems. Early evidence of bacterial participation in sulfur underground transformations, especially in association with mining operations, was obtained using radioisotope studies. This work was later confirmed by isotope fractionation studies in natural samples. Much of the early work of Soviet geomicrobiologists was summarized in a book by S. I. Kuznetsov, M.V. Ivanov, and N. N. Lyalkova Introduction to Geological Microbiology, English translation, McGraw-Hill, NY, 1963). During the 1970s, the microbial populations and the activities of methane-oxidizing bacteria in saline oil field formations, and the changes in microbial populations after injection of freshwater for oil recovery, were also investigated. Recent work on hydrocarbon-oxidizing bacteria in oil field waters is described later in the paper by Balyaev (Plenary Session 4).

The past work of Soviet microbiologists shows clearly that different physiological groups of microorganisms are widespread in underground environments and that subsurface microorganisms are capable of significant geochemical activity. In recent years, we have concentrated on harnessing the activities of microorganisms in their underground environments for useful purposes. For example, microorganisms are now being exploited to enhance oil recovery and to reduce the concentrations of methane in coal mine air. In the latter example, methane-oxidizing bacteria are grown in fermenters and are then injected into lateral core holes within a coal mine. This process has been shown to reduce the methane concentrations in the air by 50-60% in one month, thus reducing the risk of explosion and fire. This type of work has led to the definition of a new research area in the Soviet Union called Biogeotechnology.

†Oral presentation.
Introduction

Studies on distribution of microorganisms in deep underground water began in the Soviet Union in the mid-1920s. Dr. Ginsburg-Karagicheva told me about cells of sulfur-reducing bacteria in the formation waters of all deposits in the Bachu districts at depths of 800-1000 m. The report by Ginsburg-Karagicheva, as well as the report by Bastin, an American microbiologist, both published in 1926, did not attract any special attention at that time. These works were not recognized until 1945 when a mining engineer, Rubenchik, noticed the unusual pink color of formation waters of the Bachu oil deposits. Microbiological investigations by this field worker showed that this color was due to the vast development of sulfur bacteria. The field water was coming out of the oil drills for six months and the total daily water output from only one well was over 10,000 pounds. This was very impressive for a microbiologist. According to the best known Soviet microbiologist, Dr. Isachenko, these data have shown that sulfur bacteria are not some unusual contaminant, but rather a normal inhabitant of some oil strata. That is why during the war years Isachenko demanded systematic investigation of the distribution of microorganisms in rocks and underground water. As a result, before the second world war, it had been shown that the entire profile of our soil was not sterile at least to the depth of 2000 m. Bacteria were cultured in both the underground water and in their sediments. This was made possible due to specific nutrient media and addition of anaerobic conditions.

The second period of Soviet studies of subsurface microbiology began fairly recently. When papers by the American scientist, Claude Zobell, came to my attention, I became interested in the investigations of microflora of all living deposits of sediment. Studies of Professor Kuznetsov, who was my teacher and doctoral advisor, should be mentioned also in this respect. According to these data, distribution of microflora enumerated by direct counting demonstrated the viability of the sediment. The bacteria numbers in these sediments varied from 37 to 170 million cells/g, even when growth had not been observed after 60 days of incubation.

Studies on the subsurface microbiology were conducted during the 1950s and 1960s in both the Academy of Science and in the Institute of the Minister of Geology. A series of government reports during these investigations of underground waters and their central ideas are of significant interest. According to these data, sediment microflora could only be detected by direct counting. Thus, water could not have been at this location.

The composition of stratal water of all deposits acquired from the data of several investigations were considerably more diverse. In particular, according to the data of Korshunova, hydrocarbon-oxidizing bacteria were found in all waters. During that same year, information began to appear that at all depths the oil extraction process had led to changes in microflora in all deposits. For example, Dr. Shvurt showed that the total number of microorganisms in the stratal water from all deposits increased by one order of magnitude. Unfortunately during these years, microbiologist had enumerating techniques but did not have techniques to quantify the geochemical activity of microorganisms.
In most cases, this led to confusion about geochemistry of the underground water, since assumptions had been made on the basis of indirect data. In some cases, however, it still demonstrated the great importance of microbiological forces in changes of the geochemistry of the stratal water, especially in the deposits undergoing secondary flooding. A good example of these are the data of Dr. Gasanov, who analyzed hydrogen sulfide content in underground waters of the Kosha-Naur oil deposits. Only 70 days after the oil stratum was flooded with high sulfate water to increase oil output (slide), the calculation of the intensity of the sulfate reduction process in several wells showed them to be producing up to 1-2 mg of hydrogen sulfide per liter. Activation of the sulfate-reducing process during secondary flooding of the oil deposits with sea water could sometimes be observed when freshwater was also used for flooding.

Such situations have been described by Dr. Kuznetsova for sediment deposits in the petroleum fields of Central Volga region. Formation water of this deposit contained significant sulfate oxidation. However, the sulfate-reducing process in this water was restricted by the lack of organic compounds available for sulfate-reducing bacteria. During flooding with fresh water, the reduction in total salinity of the water was favorable for the development of many different microorganisms. Furthermore, different amounts of dissolved oxygen necessary for the oxidation activity of hydrocarbon-oxidizing bacteria was also connected with the injection of fresh water.

Due to the activity of these hydrocarbon-oxidizing microorganisms, low level organic acids and short-chained hydrocarbons have been formed from the oil. These products have then been used by the sulfate-reducing microorganisms. The hydrogen sulfide investigations of such sites has shown that these microorganisms are widely distributed in underground waters in different regions of the Soviet Union. This represented the first significant evaluation of the total number of microorganisms in underground water and in the rocks, as well as the type of data about distribution of such specific bacterial groups as sulfate-reducing, hydrocarbon-oxidizing, and others. Finally, the first quantitative evaluation of the geochemical activity of microorganisms in regards to sulfate-reducing bacteria, based on the indirect method, has begun to appear in literature.

There is a method of determination of the geochemical activity of microorganisms in the subsurface based on the usage of radioactive isotopes that was introduced at the end of the last year. The first experiments of this addition of radiolabelled sulfates to samples of stratal waters of the sulfur and oil deposits of Shor-Su have been completed (slide). The evidence from these experiments have shown that microbiological sulfate-reducing processes have achieved mineralization rates of up to 100 g/l. This data has also provided information about the high geochemical activity of sulfate-reducing bacteria in brine. There have been brine depositions in sulfur deposits of Kosha-Naur, Central Volga, and some oil deposits in Central Asia and other regions. From similar stratal water, Dr. Kuznetsova isolated two-thousand cultures of sulfate-reducing bacteria. Detailed examination of the water found in the
sulfate-reducing process has also been done on the sulfate deposit of the Cis-Carpathian region. It has been shown that the number of sulfate-reducing bacteria increases significantly in the pores of the underground waters more than in the infiltration zone of the sulfur deposits. The intensity of the sulfate-reducing process, as determined by the techniques of Roger Little, has been increasing (slide).

The organic substances used by sulfate-reducing bacteria in the underground sulfate-containing water has been proven to be indigenous. Our experiments have shown that these microorganisms will grow on two mineral media in the presence of additives that have been extracted from the sulfur deposits. Cultures of sulfate-reducing bacteria have also been grown on the extracts of these rocks. Therefore, it has been shown that the sulfate-reducing processes have proceeded on the account of the indigenous organic substrates disseminated in the rock.

Work on the sulfur deposits in the Carpathian region in the summer of 1971 were substantial. Another series of proofs of hydrogen sulfide were obtained in the underground water of sulfur deposits. The results of isotopic studies of hydrogen sulfide and sulfate in water of different chemical compositions showed that hydrogen sulfide in all samples was enriched in light sulfur isotopes as compared to sulfate. At the same time, the rate of formation of delta S of hydrogen sulfide was rather dependent on the sulfate amount accumulated by bacteria. If bacteria accumulate a lot of sulfate (as in cap rocks), then the isotopic content of hydrogen sulfide is rather heavy, as opposed to the bacteria use only part of the sulfate reduction system and the isotopic content of hydrogen sulfide is rather low, physically.

Investigations of sulfate reduction in density, associated with the flow of changes of hydrogen sulfate concentrations in underground water, have allowed me to carry out the quantitative evaluation of sulfate reduction processes. Almost 2000 tons on biogenic hydrogen sulfide have been formed during four years in the underground water of the Shor-Su sulfur deposits. Only sedimentary deposits of natural compounds have been studied for the distribution of microorganisms in different rocks. In many cases, samples for analysis have been taken directly from sulfur mines; therefore, the possibility of surface contamination of this microflora can not be excluded. Unfortunately, this sort of doubtful information has always been neglected by microbiologists in favor of drilled cores. Sulfate-reducing bacteria have been formed only in water-bearing sulfur ores, as seen by isolations from these deposits (slide). When the deposit lacked water, it did not contain any microflora at all. Similar data was found for the Cis-Carpathian sulfur deposits. Summing up the results of the distribution of sulfate-reducing bacteria in different rock showed that the bacteria have been found in 90% of all sulfate-reducing formations, but they have been completely absent from the water recharge areas of these sulfur formations (slide).

During consideration of the role of microorganisms, information has been discovered concerning the sedimentary deposits of nitrous sulfur. Thus, great attention has been given to analyze the distribution of the geochemical activity of
primordial bacteria of the Yazov deposits in the Cis-Carpathian region. The Rozdol deposits also show that the bacteria have been practically absent from water in oil fields, but at the same time they have been found in relatively poorly mineralized water in the formations of native sulfur. On the basis of several sulfur deposits located in broken-up, oil-bearing structures, it was discovered that primordial bacterial have been active in the second step of the evolution of epigenic sulfur deposits in oxidation of biogenic hydrogen sulfide to native sulfur. In hydrogen sulfide sometimes the water of the stratal epigenic deposits, limited amounts of bacteria have been found as rule only in water that has no concentration of hydrogen sulfide dispersed through the deposit. These bacteria are not active in the water's geochemical process until the draining of the sulfur-bearing rock begins. When the sulfate-bearing rock has been taken out of hydrogen sulfide-containing water's protection, either due to nutrients from natural processes or as a result of the exploitation of these rocks, the intensive multiplication of bacteria begins in these rocks, leaving the complete oxidation of the sulfate to native sulfur. The number of polluting bacteria in the sulfur ore of one particular natural deposit have reached up to ten million cells/g of rock (slide). It is not uninteresting to know that during 20 years of exploitation of this sulfur deposit, about 20,000 tons have been lost due to oxidation process.

This part of my report has been devoted to the investigation of the role played by microorganisms in the formation of sulfur deposits. It is necessary to state that all of my conclusions about the key role of microorganisms in the formation of these deposits might be the beginning of a system for the basis of biogeochemical studies. My latest studies, which dealt with the examination of isotopic composition of sulfur deposits of different origin helped complete this work. Data from these studies show that absolutely all sulfur deposits contain sulfur that is isotopically light compared to sulfate (slide). The residual sulfate of the sulfur ores has been more or less significantly high in the region of the heavy isotope, sulfur 34. Moreover, all of the data show that the total amount of sulfate was used during intensive sulfur reduction in the last industrial deposit (slide). Before this, the isotopic composition of the native sulfur in the industrial ore was close to that of the original sedimentary sulfate. On the other hand, in other known industrial deposits, the sulfate reduction process was not so active and attacked only a part of sedimentary sulfate. Before the native sulfur in the mined industrial deposits was intensively enriched in the light sulfur isotopes by sulfate reduction processes, it was initially high in the heavy isotopes.

Matters of direct investigation of the intensity of microbiological forces in subsurface conditions that were originally developed for studies of the sulfur-reducing bacteria have also begun to be used in the investigation of geochemical activity of heterogeneous methane-oxidizing bacteria. Since methane has been formed by the microorganism's reduction of one or more different organic compounds, two kinds of experiments were carried out during the course of our methanogenic activity investigation. A radiolabelled compound and a specifically labelled methyl group were used as an example (provided by Dr. Belyaev). The
forces of microbial methane formation in underground water of different ages was investigated. The central process of methane formation in sediment was biogenic and the bulk of microbial action was by methanol reduction. Data on the rate of methane formation and the result of analysis of isotopic composition of methane and mineral carbon deposits in underground water was gathered (slide). In some samples of the underground water investigated by Dr. Belyaev methanogenic bacteria formed in association with labelled methane-oxidizing bacteria. In order to find out where the process of methane oxidation was occurring, they were incubated for different periods of time—after the addition of radiolabelled methane—with samples from underground water. The second product of methane oxidation has not yet been formed in this experiment (slide). The data also showed that the oxygen was a limitation of the development of methane-oxidizing bacteria. Almost all of the water samples that were incubated together and labelled methane and oxygen had measurable methane oxidation occurring. A part of the sample was separated into viable microorganisms.

By the middle 1970s, Soviet microbiologists had found rather significant new materials that could provide nutrients for this geological group of microorganisms and that these were widespread in the underground waters. The use of radiolabelled sulfur compounds and the relative data on the isotopic composition of these nutrients helped provide proof that these microflora were capable of carrying out many of the geochemical processes, which meant they could change the chemical composition of underground water. For example, in investigations of the flooded oil deposits and sedimentary sulfur deposits, it has been shown that the rate of the geochemical activity of microorganism in sulfur formation is significant and it is necessary to keep them in mind during exploitations of these deposits. All of these studies have also helped us focus our investigations on the use of the geochemical activity of microorganisms for a practical purpose. In the beginning of this paper, some data was presented about the successful use of geochemical activity of microorganisms for detection of oil and in underground water of oil deposits for enhancement of oil recovery.

I would now like to speak about the use of methane-oxidizing bacteria for altering the methane content of air in coal mines. High concentrations of methane in coal mines is very dangerous for miners because methane forms an explosive mixture in air. As soon as the methane content in the coal layer has reached dangerous concentrations, its removal from the atmosphere of the mine by ventilation is not always enough protection.

Two field experiments, using cultures of methane-oxidizing bacteria, were done to see if the methane content in the coal layer and in the walk out area could be reduced. In the first experiment, a suspension of methane-oxidizing bacteria was injected under pressure directly into the coal layer through a special borehole. This part of the coal layer had been intensively aerated for two weeks. The extent of the air concentration sample after each application was removed through each hole.
Some holes in the coal layer were not treated and thus served as a control (slide). Methane content during the exploration of this station was monitored as they tried to obtain coal. One coal section was treated with water to dissolve microorganism that might have developed during aeration of this station (slide). The methane content in this section doubled as compared to the control section that did not received methane-oxidizing bacteria, water, or air. Another section was treated with the suspension of methane-oxidizing bacteria and continuous aeration. In this section, a significant decrease of methane content was found. However, there was an increase in CO₂ concentration, which was the process of microbial oxidation of methane in this section.

According to the application of this technology, suspensions of methane-oxidizing bacteria were used to treat the crushed rock walkout area behind the working miners. The use of this technology did not require additional aeration because the walkout area was aerated by normal mining ventilation. In order to follow the changes in the methane content in the atmosphere of mines created by the activity of microflora in the walkout area, several different methods were tried. The most convincing data were obtained when the systematic analysis had success in determining the isotopic composition of methane and carbon calcite. Before the beginning of the experiment with the microorganisms, the methane content in the mine varied rather significantly, but its isotopic composition was quite stable, about 48% C13. After application of the suspension of methane-oxidizing bacteria into the rock, the total methane content decreased and its isotopic composition had significantly changed to 40% C13. At the same time, the isotopic composition of CO₂ had no pronounced changes. This undoubtedly pointed to the method of methane oxidation.

**Conclusion**

It is necessary to mention that the methane oxidation of the isotopically light metals by microorganisms has been advanced for five years in detail by our laboratory experiments with 2000 culture of the methane-oxidizing bacteria. Because of limited time, only two mine experiments on the use of microorganisms for lowering the methane content in mines have been discussed in this paper. I have two volumes of information about our laboratory investigations. In the Soviet Union, several thousand mine experiments have already been carried out. This has shown that by using microbiological technology it is possible to lower the methane content in both the coal air and in the walkout area by 60% of its original content after only one month of treatment. The special difficulties connected with obtaining sufficient biomass of methane-oxidizing bacteria have been overcome by using rumen fluid.
Q and A

**M. Ivanov:** Most Soviet scientists feel that mine organic carbon substance for sulfate-reducing processes is not hydrocarbon, but instead a process of hydrocarbon oxidation, such as fatty acids, some alcohols and others. This is the reason for the intrusion of sulfate-reducing processes in oil fields when they use surface water for injection in these deposits. In the Soviet Union, this injection water contains dissolved oxygen, which activates the hydrocarbon-oxidizing bacteria. As a result of this activation, these bacteria produce small organic molecules that can be used by sulfate-reducing bacteria if the water contains sulfate ions, or methane-generating bacteria. I am aware of some new data from a German microbiologist who had a report in Moscow last September about the possibility of sulfate-reducing bacteria using hydrocarbons. I am aware of many publications on this topic, but I am not sure I should speak of them now.

**T. Hazen:** I would like to thank you for an excellent presentation. I am wondering about your application of methane-oxidizing bacteria. Were those pure cultures, and if they were, did you identify what they were or were they consortia?

**M. Ivanov:** In our lab experiments, different pure cultures of methane-oxidizing bacteria were used. In our mine experiments, a few cultures of methane-oxidizing bacteria that were grown in our laboratory were used in some cases. In other cases, biomass, not culture, of methane-oxidizing bacteria that was produced in our plant and belongs to our Minister of Biotechnology were used. In the USSR, microbial cells are widely used as a source of protein for animals. Furthermore, people in microbial industries produce biomass methane-oxidizing bacteria in two places of the country.

**Z. Filip:** Again, with regards to the practical use of the methane autotrophic bacteria. Is there any report in your institute concerning the possible use of methanotrophic bacteria as a means to degrade some halogenated hydrocarbons in the underground?

**M. Ivanov:** People from the Institute of Biochemistry and the Institute of Microorganisms use mixotrophic bacteria for degradation of some methylated components.

**G. Matthes:** I have found in a Russian textbook a concept in which calculations were made for the breakdown of a natural gas reservoir or a petroleum reservoir due to bacterial degradation. Do you think this was possible since you mentioned that they cannot live on the hydrocarbons?
M. Ivanov: It is not so easy to answer that question because it depends on the geological structure of the deposits. If the structure is open and there is infiltration of water, and if a small quantity of oxygen and aerobic bacteria are present in the oil field or gas field, it is possible. However, I have another interesting story about the role of microorganisms in gas storage. I have some extremely interesting data about the changes of gas composition in a gas storage unit that was used for about six months. A part of this gas content was from sea water matter. During the storage of gas in the reservoir, anaerobic microorganisms changed the content of this gas material, increasing the hydrogen content, decreasing the sea water hydrogen content, increasing the methane content and forming a lot of acetate. Six grams per liter of acetate was formed under the groundwater in this gas storage during the six months. It was a real anthropomorphic biogeochemical problem.

W. Ghiorse: I have one final question that might interest the entire audience. It is a very general question about a report that I have heard concerning the Super Deep Well that is more than 3000 m deep. I heard that microbiology might be occurring in the Soviet Union's Super Deep Well. Could you comment on that for us?

M. Ivanov: There is not much data in this field right now. I believe that the upper limit of distribution of microorganisms depends on the movement of this ecosystem. It is a rather slow project.
†Subsurface Microbiological Research in the United Kingdom
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Abstract

Much of the study of deep subsurface microbiology in the United Kingdom has been closely linked with environmental concerns, such as the geological disposal of radioactive waste and the quality of potable groundwater. Emphasis has been placed on understanding the development of microbial systems in the subsurface by studying the controls on growth (nutrient and energy availability, and environmental constraints), thereby determining their effects. For the past 10 years, research efforts have been focused on processes affecting transformations of nitrogen, sulfur, and iron compounds and the likelihood of biodegradation of synthetic organic pollutants in fissured, microporous, water-supplying aquifers down to a depth of approximately 100 m. The work on microbially-mediated denitrification in the Chalk aquifers of Norfolk has led to additional work on groundwater quality in the London basin. However, this work has been limited in scope as a result of severe financial constraints.

Microbiological research is also included in deep radioactive waste disposal programs in the United Kingdom and other countries in Europe. Low budgets, limited personnel, and proper equipment have restricted this work, but some progress has been made in studies of the microbiology of deep geological formations penetrated by mine shafts. Indigenous and colonizing microorganisms are included in these studies. Questions on the influence of microbial activities on the deep radioactive waste environment are being addressed. Specifically, these research programs focus on the occurrence, survival, and activities of microorganisms in the deep repository environment and the long-term effects of microbially-mediated processes such as biodeterioration of casing materials, backfill degradation, and change in radionuclide speciation. Microbiological activity data are employed in current safety assessment models that are being developed in Switzerland to predict nearfield and farfield waste-barrier interactions. Groups in Canada, France, the United States, and Sweden are also contributing to these investigations.

†Oral presentation.
Introduction

This paper describes the characteristics of the work in the UK that is occurring at the moment and also, the characteristics of some related collaborative programs in Europe. Now on the whole, we have extremely low budgets, particularly when compared to the deep subsurface programs in the United States. We only have about $100,000 a year going toward these programs and that is for the microbiological aspects of the work. We usually have to work on limited time, therefore, a lot of the projects are only a year's duration. Sometimes they go up to two or three years and thus, we have tended to concentrate on total ecosystems and controls. In fact, a black box system has been utilized and the effects have been observed.

Discussion

So how did we get to this situation? I am going to explain this by discussing the worksite that was involved. This will allow us to take note of the effects of microbiology on the geological contaminants of radioactive waste, which has had a very long and checkered history. In general terms, I will talk about the work from the UK perspective. Such work has been an uphill battle in Britain and also in many parts of Europe. Minimum funding has been obtained, even lower than the $100,000 per annum that was mentioned earlier. This funding comes from the commissions, the European communities, the UK Department of the Environment, and naturally, from a group in Britain called NICRA, which is responsible for radioactive waste containment in that country.

If one looks at a repository, as shown in the diagram (slide), buildings can be seen rising near the repository. Vertical shafts, which may be as much as a kilometer in length, descend into the subsurface. Out from the shafts that are going down, there are attics where the waste is to be placed. Again, this varies from one country to the next, but this is the general concept of the geological disposal. The waste is placed in a canister of some kind (slide), and it can be very high-level and very radioactive (very hot) to very low activity. Furthermore, the waste can be very homogeneous or it can be very heterogeneous; it can be made up of vitrified material or it can be a mixture from components of organic and inorganic waste or ion exchange resins.

As for the canister, it can be steel, iron, or so on, depending on the country. Generally, the canister is placed in a backfill (slide), and when it is put into the attics, it is surrounded by a backfill that could be bentonite, concrete, or a number of other things. The whole thing is surrounded by a host rock (called the Bar Field), which could be granite, clay, salt, etc. The area known as the Near Field is where the so-called engineered barriers are. It is necessary to get these bits of jargon out of the way now, because they will be referred to later.

One can imagine my problem as a biologist coming into a radioactive waste program. Most of the scientists involved in this work were geologists, physicists, and chemists. Most of them had very little biological training. Therefore, they confronted me with a series of questions which I had to answer. The first question of concern was whether or not microorganisms occur in these kinds of environments. Therefore, a survey of the various geological formations that were of interest, not only in Britain, but elsewhere in Europe, had to be initiated. Granite, clay, salt, and
other environments were sampled through the use of old mines. Cave mines, which are being built for the radioactive waste program, became underground laborato-
ries. It was decided that the kind of microorganisms that were present did not need
to be taken into account because a repository under construction would be
contaminated anyway.

A colleague and myself went down into a mine in Southwest England in a town
called Cornwall (slide). It was a tin mine in granite and it was approximately 600
m in depth. All of the red slimy material shows the kind of environment it was
(slide). Everything that was sampled from this mind was full of iron and sulfur
oxidizers. That was one mine that was sampled in the UK.

An abandoned mine in Britain was also explored, which was rather scary
(slide). Some of the mines were incredibly dangerous. One particular mine was
first excavated in 1792. It was in a limestone formation and they were trying to
retrieve iron-metal compounds until it was closed in 1916. It was approximately
100 m in depth, and we were one of the first to go down and to take samples in order
to see if microorganisms could exist after human contamination had stopped. It was
a rather unpleasant environment. Once again, there were two attics going out and
a lot of the red material, which was evidence of sulfur and iron-oxidizing bacteria
(slide).

Samples were also taken from boreholes. One borehole was drilled in granite
in the northern part of Scotland in Case Nest (slide). Some groundwater samples
were taken using standard procedures just to see if microorganisms were present.
Unsurprisingly, there were, but the process still had to be completed in order to
demonstrate to our funding body that microorganisms were present. Apart from
these sites, studies were also done in Sweden, Germany, Belgium, and Italy.
Sampling was done from a variety of sources such as granite, chert, limestone, etc.

In summary, three types of groups of microorganisms were found. Due to a lack
of funding, however, the bacteria were identified only to the group level. The
groups obtained were aerobic and anaerobic heterotrophs, sulfate reducers, and
sulfur and iron-oxidizers. Another group, which was called the metal-precipitating,
nonoxidizing bacteria, was also obtained. The relevancy of these groups was
placed next to each one: the aerobes and anaerobes for their breakdown of organic
materials, thus indicating in some ways how much contamination is occurring;
sulfate-reducing bacteria for their anaerobic corrosion; sulfur oxidizers for
corrosion; and iron oxidizers for corrosion and their possible influence on
absorption processes in the repository.

There is an interest in how many radionuclides are coming out and in any
factors that may influence the speed of radionuclide movement from the repository.
The iron-oxidizers and the other metal bacteria may in fact inhibit the absorption
process. Then again, they may promote it. Therefore, the next question that was
asked was, "Okay, you found these bugs, but can they survive the environment"? A
good question, can they survive? According to the literature, a variety of ranges
and tolerances can be obtained. (Temperature, pH, pressure and radiation were
listed.) Some of the results had question marks because there was a debate about whether they were naturally surviving at high temperatures or not. These ranges included conditions produced in a repository. Therefore, the literature did not say the microorganisms would not survive. Consequently, work was done to look at the isolates that were found from the various sites. Their ability to tolerate temperatures, pressures, radiation and so on was tested. Some of them did not survive, some did. Most of the ones that did survive were the sulfate reducers.

Some work has also been done in other countries. An analog site has been sought for in Aman in the Middle East because a repository would produce heat if it was surrounded by concrete in very alkaline conditions. The Middle East, with its natural alkaline springs coming up to the surface provides such a setting. Therefore, studies were done at the sites to see whether bacteria would be present and once again, sulfate reducers were found. A body of evidence was beginning to build to confirm that bacteria probably could survive a repository environment.

The next question that was asked was, "What can they do down there?" One can see that all of these questions seem very consistent, but actually, the geologists, the physicists, and the chemists had to be taken along every step of the way. "What can microorganisms do?" Well, they can do many things (slide). They can produce gas, which is actually quite important in a repository. There can be methanogens down there, which can make it very explosive. They can produce acid. They can actually use the waste as a source of nutrients and energy. They can cause physical disruption of the waste and sequester the radionuclides. They can enhance external absorption of the isotopes and take it up intracellularly. If they do this and migrate away from the repository, say in the groundwater, then very severe implications can occur because they may carry the radionuclides away from the repository faster than what is normally anticipated.

In slightly more serious terms, if one were to look at what happened at the repository over time (slide), starting with containment, one could see that the breakdown greatly changed due to groundwater infiltrating into the repository and the migration of radionuclides through the backfill and the Bar Field, (the host rock), and into the biosphere known to man. Each one of the following can be influenced by microbial activity: repository deterioration; alteration of groundwater chemistry; factor of biodegradation; radionuclide migration; radionuclide absorption of the microorganisms; movement into the rock by microorganisms; changes in nuclide speciation; and radionuclide input into the food chain. Therefore, it is evident that they can do quite a lot.

On to the next question, which asked, "How do we evaluate it?" One of the priorities of radioactive waste is the ability to make a model in the laboratory that shows what microorganisms will do. This is called modelling for a safety specimen. A safety specimen must be produced before one can dispose of the waste. This is the final aim of every experimental plan, the aim of evaluation. There presently exists computer models based on nutrient and energy availability for radioactive waste
problems. Although they are very simplistic, and very conservative, they do exist. They give some feel as to whether the microbial activity will be a problem. This approach is being adopted by us in the UK, by the Swiss, and by the Canadians.

The model, if one can call it a model, is being taken further and the concept of what is happening in a repository is depicted in a diagram (slide). The system has components that interact among the waste and engineered barriers, nutrients, microbial activity, microbial biomass, biodegradation, and the geochemistry. As the groundwater infiltrates the system and the Bar Field, it may cause all sorts of effects. These effects, which have already been discussed, include nuclide leaching, gas formations, microbial enhancement, and in fact, changing of the overall geochemical environment by deterioration of the waste-engineered barrier.

If one is looking at this from an experimental point of view, (i.e., obtaining information for the model that is being developed), it is important to keep in mind that this model is still in its early stages. There is still a lot of work to be done, but an experimental program must be initiated with a model. This can be looked at in the following two ways: one can look at the individual processes and try to work out how each is being controlled, which is being adopted by some of my colleagues; or one can look at the whole thing as a black box, which is what I have done in my particular laboratory. One can look at the interaction as a whole and not really worry about what is occurring physically, yet still monitor the effects. This so-called black box system can be explained in a diagram (slide).

When there is a black box simulating a repository environment, one knows what nutrients, what radionuclides, and what microorganisms are inputted into the system. One can sample the time, the gases, and leachate, using radionuclide leaching adsorption experiments. One can monitor the system, the Eh, the pH, and at the end, the data sets can be observed to see what happened to all of the contaminants that were put into the system. This experiment is running at the moment. There are six circle cells and black boxes in water baths and various mechanisms for sampling each one of the cells. There are other cells that also contain radionuclides, but this system is up and running at the moment. If the information obtained from this experiment can be plugged into the models that are being developed in Switzerland, then there can be a lot of interaction between various groups within Europe.

Individual processes that are occurring were also mentioned. Other colleagues of mine in Britain and elsewhere have decided to concentrate on the factors that control each one of the processes. Gas production and physical integrity is being studied by a group at Harwell in Britain. Corrosion by deterioration is being studied in southern France at a place called Catarash, and also by the University of Zurich in Switzerland. They have been funded by the same group that funded our studies, the NICRA. Absorption is being looked at in West Germany, by myself in the UK, and also at Los Alamos in the United States. Concerning site characterization, the Swiss investigators have actually decided to study the sites without looking at the
microbial content, and thus, they have gone back to studying what microorganisms are present in case something was overlooked. The modeling is being done by my colleagues in the UK, Switzerland, and in Canada as well.

Since there is such a dissipated lot working with the microbiology of radioactive waste in the subsurface, a group called Microbes for Nuclear Waste Disposal has been set up. An attempt is made to have regular meetings in order to talk about what will be done, but we are terribly aware that we are very isolated. Therefore, any suggestions or comments one has concerning this group and their work would be very appreciated.

Thus far, I have only talked about radioactive waste disposal and microbiology because it is my main area of work. Now I am going to talk about a couple of other subjects that are going on within the UK, ones in which I am partly involved. Once again, there are the same funding problems as with radioactive waste. There is very little money and the contracts are very short-term. This may seem very consistent again from the American point of view, but I know we are very far behind their work. I will, however, discuss these subjects for your interests.

First of all, there is a very large concern for denitrification of the groundwater in UK. There is also concern about the total groundwater quality in the UK. The British Geological Survey certainly has a large investigative input into such areas as organic pollutants and pesticides. The funding came from our water company, the UK Department of the Environment, and from the Overseas Development Agency, whose governing body is located in the UK. The groundwater quality portion of this work (i.e., microbiology), is only beginning to be recognized as an important issue.

Finally, I would like to talk a little about denitrification. There are two main aquifers within the UK: the Norfolk Chalk (Norfolk is in eastern England), and the Lincolnshire limestone, which is in eastern England, but slightly further north. These two main aquifers are not very deep (approximately 50 m). In the aquifers, a lot of the nitrate is present in the groundwater in excess of national limits (1.3 mg nitrogen per liter) as a result of increased leaching from cultivated soils. The British government is about to be taken to the European Court for exceeding these limits. Therefore, remediation needs to be provided quite rapidly, but again, there is very little money.

Using techniques that were developed here in the United States, it has been demonstrated that there is denitrifying potential in these aquifers. However, observations of the rates are just now getting underway, which makes apparent the fact that our work is very much behind what is being done in the United States. Therefore, any help from the United States would be very gratefully accepted by the British government. Otherwise, there are going to be some difficult times ahead.

Another area of work that resulted from a hydrogeological survey is the groundwater quality. Work is being done in part of the London basin, particularly in the northeast, to depths of approximately 50 m (slide). Once again, techniques developed through work that was done in the United States are being used. The company that is funding this work is Thames Water. They are very concerned with
the effects of recharging this part of the London basin because past experience suggests that the water is unpalatable after recharge, probably because of the nitrogen and sulfur iron levels. We, the microbiologists, are looking at the biogeochemistry of these cycles. Since it is a multidisciplinary study, cooperation is needed between ourselves, the geologists, the hydrogeologists, and so on. Again, it has been demonstrated that the microorganisms are present, but only nitrogen and sulfur cycling appears to be occurring. Furthermore, the reaction rates must be observed before one starts looking into the models, in order to tell Thames Water exactly what the problem is going to be, thus allowing for modification of the system. This project is in the beginning stages so I cannot really tell you very much more than that.

**Conclusion**

To summarize, our work is characteristic to that in the UK, and to some extent, within Europe. The environmental problems must be corrected if any work is to be done on subsurface microbiology. We have to be very, very applied. Generally, these studies are very interdisciplinary and involve several countries. Furthermore, the contracts are usually short-term and the budgets are very low. Therefore, everyone must work together to get it straight. Thank you.
Q and A

W. Ghiorse: What is the oxygen status of the deep nitrifying aquifers?

J. West: The oxygen levels vary with depth. I do not have the specific details with me, but I could let you know.

P. J. Boston: With respect to the black box set-up that you showed us the picture of, what are you controlling? Are you controlling pH and temperature?

J. West: No controls are being done at all. It is just sitting there doing its thing, and we want to know what happens.

P. J. Boston: Are you simulating some features of the waste repository environment, for example, pressures or ...

J. West: No, nothing like that, because it would have made the experiment too difficult. It has been quite difficult already, so we are just letting them go.

B. Russell: You mentioned that you had been studying the effects of temperature, pressure, pH, and so forth. Have you done any work to look at what happens when you have the effect of rapid change in temperature or pressure on the microbial population?

J. West: The experiments done on pressure were from samples. They were in special little autoclaves used for testing reactions to the fusing of rocks and waters, but they were used for microorganisms as well. When they were subjected to rapid depressurization, they were still alright, which was evident from the viability checks that were done afterwards.
Subsurface Microbiological Research in United Germany

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Abstract

Early, deep drill hole studies on microorganisms in oil field waters were conducted in West Germany during the 1960s. This topic was recently taken up again in a broader interdisciplinary program organized by the German Scientific Association for Petroleum, Natural Gas and Coal (Deutsche Wissenschaftliche Gesellschaft für Erdöl, Erdgas und Kohle - DGMK), and funded by the German Federal Ministry of Science and Technology. Research has focused on detrimental and beneficial microbiological activities associated with extraction, treatment, piping and storage of fluid raw materials (groundwater, petroleum, and natural gas). Detrimental activities include uncontrolled gas formation (H₂S, CH₄), organic and inorganic clogging of formation pores and pipelines, corrosion, and degradation of useful products. Beneficial activities include waste water treatment, controlled synthesis of biogases, metal leaching, and microbi ally-enhanced oil recovery. The DGMK research program aims to achieve a better understanding of microbial populations and their living conditions to enable effective control and utilization of microbial activities. There is also a plan to explore for microorganisms in the Deep Continent Oil Drilling ultra deep (10 km) borehole.

Microbiological research has also been included in interdisciplinary studies of shallow aquifer systems, having been supported by the state governments of Hesse and Wiesbaden and by the federal government since the mid 1970s. These studies include research on microbial effects on groundwater at solid waste disposal sites. They focus on the distribution of waterborne and attached microorganisms in polluted groundwater plumes and in nearby polluted zones to show their contribution to chemical pollutant degradation. In addition, the Federal Environmental Protection Office and the Federal Ministry of the Interior have supported research on migration and persistence of bacteria and viruses in groundwater with regard to the definition of well head protection zones.

The German Research Foundation (Deutsche Forschungs Gemeinschaft - DFG) has supported interdisciplinary research programs, including the microbiological research in the framework of their Priority Research Program on "Hydrogeochemical Processes in the Hydrogeological Cycle within the Unsaturated and Saturated Zones". In this program, microbiologists, geochemists, and hydrogeologists worked in close cooperation to conduct the first ever systematic research on biotopes in shallow, unsaturated and saturated zones. The microbiologists investigated the resident microflora and their activities in the subsurface biotopes. Conditions were defined for microbial life in different hydrogeological and geochemical environments and the influence of microorganisms on the kinetics of important geochemical processes were also investigated, especially transformations of sulfur, iron, manganese, phosphorous, and nitrogen.
This Priority Research Program led to a subsequent DFG program on "Detrimental Substances in Groundwater", which began in 1985 to address the specific problems in contaminated groundwater systems. Ongoing microbiological research in this program includes experimental and field studies in contaminated groundwater aquifers and riverbank filtered systems, as well as experimental work on man-made groundwater plumes.

No paper submitted.
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Abstract

Subsurface microbiological studies are being planned as part of the Bureau of Mineral Resources (BMR) regional hydrogeological research program that is investigating the Murray Basin, which lies within the states of New South Wales, Victoria, and South Australia. The "hydrological" Murray Basin, which can be divided into eastern and western zones by a north-south-lying permeability barrier that extends through the center of the Basin, consists of four major aquifer systems. Recharge occurs at the margins (by stream leakage to all aquifers) and across the Basin (by rainfall infiltration to the unconfined aquifers). Discharge is either via seepage to the Murray River, the only outlet from an essentially closed groundwater basin, or by direct evaporation at the ground surface. Hydraulic gradients are characteristically very low. Groundwater salinity increases along flow lines toward the central west of the Basin and it is characterized by an inverted salinity profile, which may be particularly steep in saline discharge zones found in the center and west of the Basin.

A major problem being addressed by BMR and other federal and state agencies is how to deal with high salinity groundwater. The salinity problems, resulting from long-term, natural hydrological variations, are exacerbated by agricultural activities, particularly land clearing, and the application and recycling of irrigation water. Microbiological research will include studies of microbial activities that may influence the geochemical cycling of nutrients and other elements, the degradation of pesticides and other agricultural chemicals, and the effect of salinity on such processes in the subsurface waters and sediments.

Limited human and financial resources have constrained studies of groundwater microbiology in Australian sedimentary basins, causing a substantial lag behind sustained programs in geology, and more recently, hydrology. Various applied research programs have incorporated studies of microbial activities, though few of these have microbiological research as their primary focus. Microbiological investigations previously undertaken, or currently in progress, by non-BMR researchers include the following: accession and survival of bacteria and viruses to shallow water tables; organic and nutrient pollution by septic tanks; land application of treated waste water, and dairy wastes; naturally occurring, nitrate-rich groundwaters; corrosion and iron deposition in borehole casings; microbiologically-enhanced oil recovery; and the microbiology of thermal bore waters. Further details of these non-BMR activities are provided in the final section of the paper by Bauld et al. in Plenary Session 2 (Murray Basin, Southeastern Australia: Hydrogeological Framework and Prospectus for Subsurface Microbiology).

*Oral presentation.
Introduction

This paper will provide information on a somewhat different environment in which I will be working in the near future, along with some ideas of what might take place. In conclusion, one or two names of people that I am personally aware of at this stage who are carrying out such work will also be provided.

I have become associated with what is essentially a hydrogeological program. That has some advantages and disadvantages. One of the major advantages is that I arrived at the time when the hydrogeological and hydrochemical framework for this particular basin, which is the Murray Basin in southeastern Australia, was in place or at least moving toward some early stages of completion. The information that is provided here was carried out by a number of people within the Bureau of Mineral Resources (BMR), a small number I might add, who have worked very hard over the last decade to acquire this information.

The Murray Basin, located in southeastern Australia, is a sedimentary basin which has an area of approximately 300,000 square kilometers (slide). For those of you in North America, this means an area that is somewhat larger than the state of Nevada and somewhat smaller than the state of California; this gives you an idea of the scale involved. The Basin covers about 10% of Australia's land surface area. The total catchment, which includes the surrounding fractured rock zone, is about 450,000 square kilometers. Most of the surface water flow and discharge of groundwater from the basin is via the Murray River into the Gulf. This is an area of major economic importance to Australia. It contains about 75% of Australia's irrigated agriculture, nearly half of its crop land production, half of its sheep and wool, and about a quarter of its cattle production. In all, it accounts for some 30-40% of natural productivity from natural resources.

Productive farmland occurs in the marginal recharge areas of the Murray Basin (slide). This is in contrast to those areas which are characteristic of the Central West Basin. First of all, it is characterized by very flat, low-lying topography, and it is also characterized by groundwater discharge areas. Until a few weeks ago, these discharge areas were always thought of as ephemeral salt lakes. In this particular case, they are very saline waters. This points to a major problem in this particular basin in terms of economics. Information gained by my hydrogeological and chemical friends back at BMR, along an east/west transect, showed the floodial profile of the aquifers, and also some of the chemical properties. Some interesting characteristics are apparent (slide). First of all, there are a number of geological formations which enclose these aquifers. Since these proceedings are devoted primarily to deep subsurface, I will concentrate on the Redmark Group aquifer as an example, which extends across the basin from east to west. The formation basement is sealed by Cretaceous weathering, which is very impermeable. The center of the basin is characterized by arcs of basement highs, which effectively cut the sedimentary basin into two hydrological regimes. This basement high system affects the regional flow of groundwater from the marginal discharge zones in the east, through the system to where it emerges in various parts of the basin.
Groundwater flow is also affected by a formation known as the Gera Plain. It is highly impermeable, but not totally. It does the following two things: it deflects groundwater flow moving east to west through the Redmark Group up into the overlying aquifers and eventually to the surface; and it allows partial transmission of water from the deeper Ring Mark aquifers into the Murray limestone aquifers and also, eventually to the surface. The hydraulic pressure developed into this system is such that water cannot force its way through. A plan view of this flow, displays recharge, regional groundwater flow, and recharge from the margins of the basin, with some eruptions through the center of the Redmark Group at the center of the basin (slide). Most of it, however, appears in the central west of the basin which is really in the deposition center.

One characteristic of this basin is the very low hydraulic gradient, which is approximately 1 m for every 50 km. This also results in very slow groundwater transmission rates that are approximately 0.5 mm/day. These bring some special problems to the system. Again, from viewing the lower section in this particular diagram transect (slide), C-D is a floodal profile of salinity in an approximately east/west section across the basin. What we have is freshwater moving in from the marginal recharge areas with increasing salinity as the flow moves east to west across the basin, and in certain areas of the basin, even on a regional scale. Thus, there are inverted salinity profiles that occur in the sediments and aquifers at the basin. In the discharge zones, salinity profiles are in the surface aquifers and they are very steep gradients indeed.

One question about this system pertains to how we arrived at this situation of having a very high saline content in groundwaters. There are two parts to the story. One is the long-term geological and hydrological cycles, while the other one is the interference by man. Prior to European colonization of Australia, the local vegetation, the rain flow, and recharge and discharge were approximately in balance in the long-term and the water table was relatively low (slide). Human intervention brought the clearing of substantial areas of natural vegetation for dry land farming, which immediately lowered the removal of water from the groundwater table by the vegetation. To add insult to injury, we provided significant irrigation areas, which not only brought in additional water to a system that had lost the capacity to remove it, but in many cases water that was marginally saline was brought in. This is a major problem with rising groundwater tables, and in an area like the Malee (the western area of the Murray Basin), for example, where less than 10-15% of the original vegetation still occurs across the basin, the main groundwater level is rising at the rate of about 20 cm annually. This brings large areas of the basin very close to having the groundwater tables moving into the capillary zone. As a result, there exist areas such as the major saline groundwater discharge zone known as the Rockonka, which is present within the Malee regions of western Victoria.

That brings us to one of the major problems, one of the major political and economics drivers of studies of the Murray Basin, which is to ameliorate or to deal
with this salinity problem. A plan view of a salinity map of the Murray Basin shows that time is running out on us to solve this problem (slide).

In terms of dealing with the problem, we could look at it from a conceptual point of view, a technical point of view, or an overriding political point of view. First of all conceptually, a very straightforward operation is being dealt with. This is our use of a friendly bathtub model. Thus, it boils down to two things: one can either decrease the inflow of groundwater into the system recharge, or one can increase the outflow from this system to lower the water table. At a technical level, decreasing inflow can be achieved by decreasing irrigation, which has immediate economic consequences. The outflow can be decreased by revegetating or by intercepting and pumping saline waters out of the system. All of these have an economic cost and their efficiency in economic terms within a necessary time scale is probably not good. We are dealing with what is really a minor perturbation on a long-term geological cycle. The situation is further complicated because of the large number of federal and state agencies that are involved in the water resources of Australia. This is a significant issue in first coming to an agreement and then achieving some action. Furthermore, since water rights are invested within the states and not the federal government, there are some immediate problems in terms of coordinating and convincing. However, we continue to travel hopefully, and if hydrogeologists can walk on salt water (slide) then perhaps we will be able to achieve some mutual agreement down the track.

Conclusion

These are a number of active researchers that I am aware of who are involved in groundwater studies in Australia: Regg Waldson and his group at the Engineering and Water Supply Laboratory in South Australia in Adelaide, are interested in iron deposition in boreholes in South Australia. Boreholes within the Murray group aquifer provide a major source of water for both rural and urban areas in south Australia. Alan Shea at the Canberra College for Advanced Education, has initiated work which is funded through CSIRO and by a private company on Microbial Enhanced Oil Recovery (MEOR). This has been carried out in another basin in Australia, the Iramanga Basin. I was involved in this program peripherally as the consultant and advisor, but not in any hands-on way. There is someone at Griffith University who I know is interested in thermophiles in the greater Artesian Basin borewaters, where very hot geothermal waters coming from depths as great as 3000 m.
Q and A

G. Matthess: Let me mention that recently there was a program in West Germany organized and granted by the German Ministry of Research and Technology whose purpose was to study the possibility of remediation of heavily polluted tides. We are presently involved jointly with a private company and with the Technical University of Berlin that deals with the remediation of oil refinery tides. It is a joint project supported by the Prime Minister, and it deals with the behavior of genetically engineered microorganisms in the underground. Once again, our laboratory is involved in it along with the Institute of Genetics of the University of Birought and the University of Ounbourh. A great deal of the program dealt with the resistance of autochthonous microorganisms in the underground.

W. Ghiorse: Thank you very much.

B. Bennison: I know that there are some activities occurring at the University of Lund in Sweden at the Department of Environmental Sciences. I do not even know the amount of facilities that they have, but they had some problems.

W. Ghiorse: One further comment here.

R. Lindqvist: My name is Roy Lindqvist, and I am from the University of Lund. As far as I know, the activity Dr. Benson is referring to is not a very thorough program of research. It is funded by seed research funds.

W. Ghiorse: Thank you, thank you very much.

W. Ghiorse: Are there any further comments?

J. Wilson: There is a laboratory in Leon, France that works on the microbiology of the subsurface that I think deserves some recognition. There has been some work done in Austria on crustaceans and higher organisms that are actually an integral part of subsurface ecosystems. In the United States, we are in the second round of the rediscovery of this. When I first came to the agency, we were in the first round and it had all been forgotten. Now it has been rediscovered again. Certainly there is more to the biology subsurface environments than bacteria.

W. Ghiorse: We should not forget the Swiss Federal Water and Resources or the Research Laboratory either. I think the crustacean story is really interesting in river bank aquifers. Perhaps that interest echoes along with the Edwards Aquifer in Texas where the blind catfish live in underground caves.
Plenary Session

Included within:
Contributed Papers 1, Posters
Contributed Papers 1, Oral
Round Table 1
An Aseptic Procedure for Soil Sampling in Heaving Sands  
Using Special Hollow-Stem Auger Coring  
Lowell E. Leach¹ and Robert S. Kerr²  
¹U. S. Environmental Protection Agency  
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Abstract

Hollow-stem auger coring has generally been accepted as the conventional method of collecting unconsolidated in situ soil samples for contaminant and microbial characterization of RCRA and CERCLA sites. However, depth discrete soil sampling in cohesionless heaving sands below the water table continues to plague drillers and is often virtually impossible. A new method of hollow-stem auger drilling using a clam shell capped bit, which prevents artesian flow of sands into the auger annulus, allows sample accessibility in heaving sands. A uniquely designed core barrel fitted with a wireline and actuated with an internal suction piston has proven to be extremely reliable for collection of in situ cohesionless saturated cores when inserted through the clam-shell capped auger. These extremely fluid samples are retrieved under the piston's vacuum, aseptically extruded from the core barrel inside an anaerobic environmental glovebox, and preserved in the field. Laboratory physical, chemical and biological analysis of duplicate core sections from the same vertical horizons have verified the reliability and integrity of this sampling procedure for accurate horizontal and vertical contaminant distribution. The variability has also been determined between adjacent boreholes sampled at the same time.
Introduction

Conventional hollow-stem auger coring has generally been accepted as the most efficient and reliable method of collecting unconsolidated in situ soil samples for contaminant and microbial characterization of hazardous waste sites.

There have been a number of recent articles written on hollow-stem auger drilling procedures.\textsuperscript{3,7,9,11} Soil sampling equipment developed by essentially all the major hollow-stem drill manufactures perform extremely well even below the water table where the unconsolidated materials contain sufficient clay to maintain cohesive properties.\textsuperscript{2,10}

However, an accurate definition of contaminant plume geometry has remained a challenge for the engineer, especially in unconsolidated saturated zones where various solvents or hydrocarbon contaminants and their associated microflora exhibit concentration change both laterally and vertically through time. Physical collection of samples for snapshot characterization with conventional hollow-stem auger equipment in heaving sediments has been virtually impossible. When the inner string of tools are raised, artisan pressure forces cohesionless sand into the annulus of the hollow auger. Once this occurs, conventional sampling can no longer be managed since the materials are too fluid to be held in the core barrel during retrieval, subsequently, the sample integrity is destroyed.

New hollow-stem auger sampling techniques have been developed to resolve these difficulties.\textsuperscript{7,8} These tools utilize the basic principal of vacuum piston sampling with special modifications to the piston and sample tube.\textsuperscript{13} The alterations were made to insure noncontamination of the sample from the tools and to enhance sample retrieval. Retrieved samples were transferred from the piston sampler into sealed sterile containers by placing one end of the sampler inside a specially constructed anaerobic nitrogen flow-through field glove box.

Materials and Methods

Conventional Hollow-Stem Auger Sampling. Conventional hollow-stem auger soil sampling is performed by drilling to a desired sample depth with two strings of tools, a pilot inner string with a lead bit inside the hollow auger and the auger itself which has an outer bit and helical flighting to carry the cuttings to the surface (Figure 1). The inner pilot assembly can be removed when the desired depth is reached, leaving the hollow auger in the borehole to serve as a temporary casing. Sampling can then be easily done by inserting a split spoon or barrel sampler down the auger annulus and pushing or driving the sampler to the desired sample depth or until it has filled (Figure 2).

An alternate method of hollow-stem auger coring can be performed by using an in line bearing assembly on the drill spindle. This bearing allows the core barrel on the inner pilot assembly to remain stationary as the outer auger drills to the desired depth. The core barrel is carried downward with the augers as the drill advances and samples are pared as they are pushed into the core barrel. The advantage of this method of sampling is that a continuous profile of core material can be collected by retrieving the core barrel each time it fills and reinserting an empty barrel.

The above procedures work extremely well in unconsolidated soils in the
unsaturated zone and in the saturated zone as well when enough clay is present to maintain sufficient cohesive properties that soil stability is retained. However, numerous unsuccessful drilling techniques to capture totally cohesionless aquifer material below the water table have been tried. These soil conditions are routinely encountered in the saturated zone where artisan conditions exist. During hollow-stem auger drilling, when the inner string of tools are raised, artisan pressure can force cohesionless sand up into the annulus of the hollow auger. Once this occurs, conventional sampling methods can no longer be managed since the soil materials are too fluid to be retained in the sampler during retrieval and the sample integrity is destroyed by the soil movement. This problem prompted innovative modifications of hollow-stem auger drilling as well as the development of a special wireline sampler.

Technical Modifications for Hollow-Stem Auger Sampling. The special wireline piston sampler originally designed and tested by the Institute of Water Research, University of Waterloo, Ontario, Canada, has been reasonably successful in collecting heaving aquifer soil materials. Several modifications were made in Waterloo's piston design since the authors desired to collect and seal aseptic samples in the field in sterile containers and in a sterile environment. The aluminum sleeve canister used by Waterloo was discarded in sampler design modifications since samples were intended to be aseptically collected and preserved in the field. A standard 10 cm diameter by 150 cm long barrel sample tube with a drive cap containing an internal ball valve was selected as the tool for modification (Figure 3). During advancement of the sampler, the cutting shoe pares the sample as it is pushed into the tube. Fluids and air trapped inside the tube above the sample are vented out through the ball valve thus relieving internal pressure which could retard entry of the sample into the core barrel.

A wireline piston similar to Waterloo's was built to fit tightly inside a conventional thin wall barrel sampler. Several modifications were made in Waterloo's piston design since the author desired to collect and seal aseptic samples in the field. The piston was attached to a wireline with a special swivel-locknut which prevents kinking of the wireline during disassembly. The wireline passes through the drive cap to the surface where it was rigidly attached to the drill rig. The piston was constructed with eight compression screws instead of six to improve compression adjustment of the neoprene seals inside the core barrel. Additionally, teflon and stainless steel plates were added to the bottom of the piston to prevent organic contamination of samples from the neoprene seals (Figure 4).

Initially, a hardened steel drive shoe without a core catcher basket, as designed by Waterloo, was tested with a piston positioned flush with the cutting shoe. However, when tested in very fluid heaving sands, the piston would not consistently create sufficient suction to hold the cored sample in the core barrel when raised above the heaving material. Assembly with the original manufactures core catcher basket and cutting shoe and initially positioning the piston on top of the core catcher basket
resolved this problem. An excess of 95% core recovery in totally saturated unconsolidated heaving sands has been possible as a result of the modifications described.

Waterloo sampling has been performed by flooding the borehole with drilling mud or fresh water during drilling to establish hydrostatic equilibrium of the unconsolidated aquifer material.15 The introduction of fluids into the borehole, especially drilling mud, is not desirable. Drilling fluids and many other chemicals, when introduced into the borehole, can significantly affect pore water and soil geochemistry as well as soil microflora. Therefore, extensive modifications of the hollow-stem auger bit and drilling procedures were made to minimize the disturbance of unconsolidated subsurface materials and prevent heaving in the annulus before sampling.

A special clam-shell cap was designed to cover the annulus of the lead hollow auger (Figure 5). This device allows bore hole construction to desired depths in heaving soils, preventing hydraulic movement of soils until sampling can be completed. The hinged clam-shell doors are closed at the surface and carefully held in place as the auger is forced into the soil during initial drilling of the bore hole. Constant vertical pressure insures the doors will remain in place until the desired sampling depth is reached. During drilling, none of the inner tools such as the center head or sample tube attached to the center rods are used. When the borehole is completed, the augers are decoupled from the spindle assembly at the surface and left open until the special wireline piston sampler can be inserted. Since the clam-shell cap remains closed and in place, the integrity of the saturated unconsolidated soil below the bit is retained until the piston sampler is in proper position for sample collection.

Positioning is accomplished by lowering the piston sampler inside the auger with the center rods while maintaining slack in the wireline attached to the piston inside the sampler. The sampler is lowered until it contacts the inner face of the clam-shell doors. The center rods are then decoupled and attached to the drilling spindle of the rig and a slight vertical pressure is applied to the center rods to prevent upward movement of the sampler as the clam-shell doors are opened. The decoupled auger string can then be raised about 30 to 45 cm while the piston wireline remains slack, thus opening the clam-shell doors with the cutting shoe of the sampler. The auger is then pinned with an auger fork at the ground surface preventing rotation or movement back down the bore hole which could damage the clam-shell doors. This procedure allows the piston sampler to instantly contact the soil interface at the bottom of the borehole before aquifer heaving can occur. The aquifer can then be sampled by hydraulic reciprocal hammering or pushing the wireline piston core barrel ahead of the pinned auger.

Once the clam shell doors have been opened and the piston sampler has made contact with the fluid soil sample, slack in the piston wireline is taken up. The wireline is held taut by maintaining tension with a wireline reel or fixing it firmly to
the rig structure. The wireline is then marked at some reference point, usually at the top of the open auger, so that during sampling its position can be observed. If the piston moves during driving of the sampler, less sample will be collected than indicated by depth of penetration. Once the piston wireline is marked, and the center rod is marked to indicate the depth of sampling that will be performed, the sample tube is filled by percussion driving or pushing as described above.

The piston sampler is then retrieved using a technique very similar to that described for conventional sampling. The sampler is slowly removed from the soil material by a cable attached to the center rod. No tension should be placed on the piston wireline during retrieval. Upward movement of the piston inside the sample tube before removal of the sampler from the soil will result in additional soil being sucked into the sampler. Likewise, if the sampler has been retrieved above the soil but is in water or air and the piston is moved, water or air can be pulled into the sample. The slack in the piston wireline is retrieved with minimum tension as the sampler is raise. The cutting shoe on the sampler is immediately covered with plastic wrap or PVC to minimize aeration as it is lifted from inside the auger. The sampler drive cap is then removed and the piston is pulled from the sampler while keeping it vertical in the retrieved position. This position keeps the fluidized sample intact until a 10 cm long, tightly fitted stainless steel plug can be quickly inserted and pressed down onto the sample, trapping it tightly inside.

The temporarily preserved sample can then be extruded from the sample tube and collected for analysis. A special hydraulic powered core extruder is used as part of the sampling equipment in this experiment. This tool is located beneath the derrick on the back of the truck. The core barrel can be screwed into the sample extruder and the stainless steel plug can be used as a foot to extrude the fluid sample. No stainless steel plug is required for extruding unsaturated cores. During routine geotechnical sampling there is little concern for protecting cores from natural atmospheric exposure. To remove contamination from the outside of the core, which may have been caused by contact with the core barrel, the cutting shoe is removed from the sample tube and is replaced with a specially designed stainless steel paring device which peals away the outer 2.5 cm of core as it is extruded (Figure 6). Geotechnical samples can be collected and preserved in the field as they are pared.

Sample Collection Using an Aseptic Glove Box. Characterization of the oily phase hydrocarbon distribution in the subsurface soils or volatile organics and associated microflora in the soils at other sites, requires a special aseptic and oxygen free environment for capturing the samples as they are extruded out of the core barrel. If samples are extruded from a core barrel in the natural atmospheric environment, unstable organics instantly volatilize and the sample adsorbs oxygen, destroying in situ integrity. This problem can be surmounted by inserting the sealed cutting shoe end of the core barrel inside a special constructed portable 1.0 cm thick plexiglass glovebox with dimensions of 60 x 90 x 120 cm (Figure 7). The box is equipped with a special self-closing iris diaphragm for insertion of the core barrel.
The glove box can be prepared for sample collection in approximately 30 minutes by filling it with the desired number of presterilized sample containers and sterile stainless steel core paring devices and purging it with nitrogen gas to reduce the oxygen level below detectable limits.

In preparation for field sampling, a sufficient number of quart and pint glass sample containers are sterilized in the laboratory. Sterilization is done by washing the containers and sealable lids and autoclaving at a temperature of 120°C at 1 atmosphere pressure for 60 minutes. As the containers and lids are removed from the autoclave, they are placed in a laboratory environmental chamber or glove box. When filled to capacity, the chamber is sealed and the interior air is flushed from the box by purging with pressurized nitrogen gas for 30 minutes using a flow rate of 2500 L/hr at a pressure slightly in excess of atmospheric. This procedure displaces gases inside the sample containers and fills them with nitrogen as the chamber fills. After 30 minutes purging, the sample containers and lids are wrapped in aluminum foil in the chamber while under a nitrogen atmosphere and the lids are screwed hand tight. The chamber is then opened and the containers are removed and packed for transport to the field. Stainless steel paring devices are rinsed in distilled water then wrapped in foil for transport to the field.

Quality assurance tests of the field glove box were conducted by measuring a series of 1000 microliter samples of vented gas with a Varian Model 90-P gas chromatograph equipped with a thermal conductivity detector. These tests verified that the air-oxygen level inside the box after 30 minutes purging with nitrogen is less than 0.02% on a volume per volume basis.

In the field, the glovebox is loaded with a sufficient number of presterilized sample containers and sterile stainless steel core paring devices to collect a minimum of 300 cm of cored sample (three separate 100 cm samples). About 10 minutes prior to placement inside the glove box, at least three of the paring devices are rinsed in a 95% ethanol bath, placed in a stainless steel pan and ignited to fire-burn dry the excess ethanol. They are then carefully wrapped in sterile aluminum foil and placed in the glove box. The glove box is then closed and purged with pressurized nitrogen gas as previously described for laboratory work, reducing oxygen levels below detectable limits in about 30 minutes. A positive pressure of nitrogen flowing through the box is maintained during all sampling activities.

After insertion of the extruder mounted core barrel into the glove box through the iris diaphragm, the cutting shoe and core catcher basket are removed. A sterilized, foil-wrapped paring tool and holding bracket are unwrapped and screwed onto the core barrel. About 10 cm of soil is then extruded through the 5 cm diameter paring tool. This part of the core is carefully broken away exposing an aseptic face. Cores are then routinely collected, sealed and numbered inside the glove box. Paring of the core is necessary to peel away the slick exterior skin of the 10 cm diameter core since it is in contact with the interior of the core barrel and becomes disturbed and contaminated during sample collection.
Once three 100 cm sampling events have been collected and preserved in the
glove box, the box must be opened, samples removed, the box thoroughly cleaned,
and prepared for repurging. Normally if the samples are to be analyzed for oily phase
organics or volatile compounds or used in microbiological studies, they are exited
from the box through the iris diaphragm, labeled, placed in an ice chest, and covered
with ice for transport to the laboratory.

It is not possible to close the clam-shell doors on the lead auger and continue
drilling as presently designed, nor is it desirable since contaminated soils generally
are inside the doors and annulus of the lead auger. Therefore, if deeper samples are
desired, the entire flight of augers must be carefully removed from the borehole
without rotation. The annulus of the augers, exterior flighting, the clam-shell doors,
and all components of the piston sampler must be thoroughly high pressure steam
cleaned to insure the integrity of sequential samples. The borehole can then be
backfilled with clean sand or uncontaminated cuttings and then redrilled to the next
desired sample depth. In many situations, researchers prefer to move the rig a few
feet and drill a new hole to the next sample depth with the clam-shell auger bit.
Admittedly, the process is slow, but the tools must be clean, and the clam-shell doors
properly reclosed if high integrity sampling is to be consistently obtainable.

Piston Sampler Core Replication. There has been considerable speculation
concerning the capability of the piston sampler to collect duplicate soil samples from
the same stratigraphic horizons in boreholes located in the same proximity. Three
boreholes were drilled at a jet fuel spill site in Traverse City, Michigan. The
boreholes were drilled in a triangular pattern, about one meter apart, and each was
sampled at three identical depth intervals to determine the repeatability of in situ
coring with the specially designed piston sampler.

Cohesionless soils were collected, both above and below the water table. Grain
size distribution analysis of three cored intervals in the separate boreholes are
compared in Figure 8. The shallowest depth interval was approximately 60-80 cm
above the water table while the intermediate and deepest intervals were 60-120 and
300-365 cm, respectively below the water table. The data show that the sampler has
excellent capability of collecting cores of replicate physical properties in heaving
noncohesive soils, supporting the theme that integrity in situ soil sampling can be
performed with the special piston sampler and clam-shell auger equipment.
References


Figures

Figure 1. Auger Column Containing Pilot Assembly.

Figure 2. Capped Tube Sampler Plugging Auger Annulus.
Figure 3. CME Standard Thin Wall Sample Tube.

Figure 4. Modified Wireline Piston Design.
Figure 5. Clam-Shell Fitted Auger Head.

Figure 6. Core Paring Tool.
Figure 7. Field Sampling Glove Box.
Figure 8. Grain Size Distribution (Three Boreholes/Three Depths).
Q and A

G. Matthey: Could you drill holes very fast since the heat of friction increases as you go? What was the temperature of your sampling tools that you push down?

L. Leach: Sampling was done below the auger. The doors were open and then the sample tool was driven below the auger such that the sample was collected at the in situ temperature.

G. Matthey: Did you push down the tool?

L. Leach: Deeper, below the auger?

G. Matthey: Right, so the pushing heats the head. How do you measure what you observe?

L. Leach: It is possible for a minute temperature gradient down the rod by hammering, but I can not imagine that it would be a significant factor because both of the samples would be, in this situation, below the water table. Therefore, one obtains the insulating effect of the water as well as the distance down to the water table.

J. Wilson: I can not resist one comment. Mr. Leach referred to collecting heaving material. Actually, what is collected is the undisturbed pristine material out in front of the augers. It is collected in a way that does not mix or stir inside the core barrel before it is distributed. This technique preserves microlamella, those layers that have been sitting down on the deposits, very nicely.

Audience: We use the same technique; however, care is taken for the temperature, and by moving very slow, one takes time to let the drill cool down. I am not sure, but the temperature may effect the microorganisms.

C. Litchfield: Have you used or contemplated to use any tracers to test exactly how pristine the samples are?

L. Leach: Some analysis has been done on quality control of various types of hydrocarbons and a very good correlation of those samples were obtained as well. One must, however, take into consideration the factorability of the samples and their location. Nothing has been done yet, at least as a study project to evaluate the replicability of biological samples.

C. Fliermans: How deep do you go with the drilling rig that you described? You showed data from 6.5 m, how deep do you think you can go?
L. Leach: That particular rig was designed to drill to 120 feet. There are other auger rigs now available that can go to 300 feet. Sampling has been done using this equipment down to 95 feet.

C. Fliermans: How long does it take to pull the drill string or the drill rod once you are down at 100 feet, and then bring it back?

L. Leach: It takes a while. That is one of our biggest time constraints. However, it is the only way to get integrity samples because once the door is opened, one can be sure that the annulus has been contaminated.

T. Phelps: Thank you very much, that was a very interesting modification, very unique.
Development of Sampling Techniques to Measure In Situ Rates of Microbial Processes in a Contaminated Sand and Gravel Aquifer
Richard L. Smith and Ronald W. Harvey
United States Geological Survey
Denver, CO and Menlo Park, CA.

Abstract
Denitrification was assayed by the acetylene blockage technique in samples obtained from a nitrate-contaminated sand and gravel aquifer to test the utility of various sampling and incubation procedures for assessing microbial processes that occur in groundwater. Steep vertical gradients of specific conductance, nitrate, and oxygen were evident from groundwater samples taken from multilevel sampling devices, and they demonstrated that close-interval vertical sampling was first necessary to locate potential zones where activity might be occurring. Aquifer solids were obtained with both a split-spoon and a piston core barrel; denitrification was assayed in sediment slurry incubations in the first case and directly within intact cores in the latter case. Slurry incubations resulted in rate estimates that were nine-to fifteen-fold higher than within core incubations. The intact core technique was more representative of in situ conditions, but required a great deal of core material, while slurries were more versatile for screening samples for potential activity and testing the response of denitrification to different amendments and perturbations. No single combination of coring and incubation technique was ideal, yet collectively, they can provide a great deal of information about microbial processes in the subsurface.
Introduction

The distribution and relative rates of microbial processes in groundwater systems have not been well characterized, despite growing evidence that microorganisms control much of the geochemistry of groundwater.\textsuperscript{7} Much of the reason for this lack of information is the difficulty in assessing \textit{in situ} rates of a given microbial process and often in determining whether that process is even occurring. These difficulties are usually the direct result of the nature of the subsurface. It is now widely accepted that microorganisms are predominantly found attached to solid surfaces in groundwater environments, which necessitates the collection of core material for activity measurements. However, obtaining undisturbed and uncontaminated subsurface core samples can be problematic, even in the most accessible situations, and as a result, samples are often compromised to some unknown degree. There are also the difficulties of maintaining \textit{in situ} conditions during activity measurements. The nutrient regime in the subsurface represents extreme oligotrophy, even in many contaminated environments, which usually necessitates lengthy incubation periods and usually tests the lower limit of detection for most assay procedures.

Together, all of these factors contribute to a large degree of uncertainty when interpreting the results of activity assays for microbial processes in subsurface samples. Consequently, there have been very few studies which have attempted either to directly measure the \textit{in situ} rate of any microbial process in a groundwater environment or to test the various sampling and assay techniques that might be used for conducting such measurements. Therefore, a systematic study was initiated at the United States Geological Survey’s Cape Cod Groundwater Contamination site to compare and contrast the ability of various sampling and assaying techniques available at the site to assess \textit{in situ} rates of a selected microbial process. Because the site is a contaminant plume of sewage origin, inorganic nitrogen compounds and the processes that affect these compounds are important aspects of the plume composition.\textsuperscript{12} Thus, denitrification was selected as the microbial process to assay during this comparative study. The results indicate that no single technique was ideal for assaying denitrification. Collectively the techniques provided more information than any single approach, while the range of \textit{in situ} rate estimates can be used to judge the validity of the individual sampling methods that were tested.

Materials and Methods

\textit{Study site.} The study site was situated on a glacial outwash plain located in the southwestern portion of Cape Cod, MA near the town of Falmouth. The unconfined sand and gravel aquifer at this location has been contaminated by the continuous disposal of treated sewage onto rapid infiltration sandbeds for more than 50 years. The sewage effluent percolates into the ground to the water table and has consequently generated a contaminant plume in the groundwater that is more than 3.5 km long and 1 km wide. The nature of the contaminant plume and the hydrogeology of the aquifer have been previously described.\textsuperscript{4,10,11}
**Sampling.** Groundwater and core samples were obtained at well location F347, which is located within the contaminant plume 0.25 km downgradient from the sand infiltration beds. Groundwater was obtained from 5-cm diameter monitoring wells with a submersible pump and from multilevel sampling devices with a peristaltic pump as described by Smith et al. For activity measurements, one-liter glass bottles were flushed with 3-5 liters of sample, completely filled, and capped with stoppers to avoid exposure to air. For nitrate determinations, samples were filtered through 0.45-μm membrane filters and frozen until analyzed.

Aquifer sediments were collected using two different types of core barrels fitted onto an auger drilling rig. In both cases the core material was obtained through a hollow-stem auger fitted with a knockout plug in the cutting bit. The core was taken by displacing the knockout plug and driving the core barrel into the undisturbed aquifer below the leading edge of the cutting bit. Split-spoon core barrels were fitted with precleaned aluminum liners prior to coring. The core material was immediately transferred into glass jars as each core was brought to the surface, after first discarding approximately 10 cm from the top and bottom. Because the split-spoon core barrels did not retain the interstitial pore water within the core, the sediment material in the glass jars was resaturated with groundwater taken from the same location and the same depth as the core.

The second type of core barrel was a piston-type core barrel. This core barrel, which was also fitted with a precleaned aluminum liner, was capable of obtaining a 1.5 m core with the interstitial pore water intact. Upon retrieval of this core barrel, the core liners were first capped with tight-fitting plastic endcaps, then approximately 10 cm were cut off from each end with a pipe cutter and discarded. The remainder of the core segment was cut into desired lengths; for intact, whole core incubations, the length of each section was 30 cm. After each cut, the newly exposed ends were immediately capped with endcaps and sealed with polyvinyl chloride tape.

**Incubations.** All microbial incubations were initiated on the same day in which the samples were collected. Denitrification was assayed using the acetylene blockage technique with several different incubation methods. The first method was a sediment slurry incubation as described by Smith and Duff. Briefly, triplicate 125-ml Erlenmeyer flasks were flushed with oxygen-free N₂, then approximately 30 ml of sediment and 50 ml of groundwater taken from the same location and depth were added to each flask. The flasks were flushed an additional 15 minutes with the N₂ and then sealed with recessed butyl rubber stoppers. The sediment slurries were preincubated six hours at room temperature on a reciprocating shaker and then 9 ml of acetylene was added by syringe to each flask. The flasks were incubated on the shaker and 10-500 ml of headspace gas was removed at various time intervals and assayed for nitrous oxide.

For one set of samples, a modification of the sediment slurry technique was used. In this case the flasks (six per sample) were completely filled with groundwater after the sediment had been added so that no gaseous headspace was in the flasks. The flasks were incubated as described above, 14 ml of acetylene were added by
displacing 6 ml of water from the flask through a syringe needle. Three flasks for each sample were immediately sacrificed at the zero time point; the remainder were incubated at room temperature with shaking for 36 hours. The N₂O concentration in each flask was determined by adding N₂ to create a headspace.

Another incubation technique that was used to assay denitrification was to introduce acetylene directly into intact core segments. A hole was cut into the plastic endcap on each end of the core segments with a cork borer. Screened fittings that were connected to tygon tubing were then inserted into the holes (Figure 1). Groundwater that had been collected from the same depth as the core material and amended with acetylene (100 ml/l) was pumped through the core segment with a peristaltic pump. The volume of groundwater removed from the storage vessel was replaced with oxygen-free N₂. Direction of flow was from the bottom of the core segment upwards, and effluent water was collected in a syringe. Three pore volumes of groundwater were pumped through each core segment. The third volume was collected separately, transferred to a 100 ml stoppered serum bottle, and assayed for N₂O at the zero time point. The tube fittings were then removed from the endcaps and replaced with butyl rubber stoppers, after which the cores were incubated at room temperature for approximately four days. At the end of the incubation period, the cores were reconnected to the pumping apparatus and slightly less than one pore volume of distilled water was slowly pumped into the core to displace the pore water of the core into a syringe. The pore water collected in the syringe was not diluted by the distilled water, as determined by specific conductance, and was subsequently assayed for N₂O in the same manner as the samples.

**Analytical techniques.** Samples for oxygen analysis were collected in 300-ml BOD bottles and analyzed by the iodometric technique with the azide modification. Specific conductance was assayed by electrode, while nitrate was assayed with a flow injection analyzer. Nitrous oxide was analyzed with a gas chromatograph that was equipped with a 63Ni electron capture detector.

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**Results and Discussion**

The first step towards assaying for a microbial process is to identify potential zones where the activity might be occurring. This involves sampling the groundwater and measuring the physical and geochemical constituents appropriate for the microbial process of interest. The need for this information is obvious, but the importance of characterizing the habitat as thoroughly as possible to provide a framework within which to interpret the results of the activity measurements cannot be over-emphasized. For example, at the Cape Cod site, we are finding with increasing frequency the need for close-interval vertical sampling. Much of the contaminant plume is characterized by relatively steep vertical gradients, which dictate the microbial processes occurring at a given location and depth. Consequently, we have been using multilevel sampling devices to delineate these gradients.

For this study, samples were collected from a typical site where these gradients were evident (Figure 2). At this site, the contaminant plume extended from
approximately 2-22 m below the water table. The upper margin of the plume contained a steep gradient of dissolved O₂, which decreased from greater than 300 μM to essentially anoxic conditions within less than a 2 m vertical interval. Oxygen values less than 25 μM in Figure 2 are overestimates due to O₂ diffusion into the polyethylene tubing of the well and the peristaltic pump tubing (R. L. Smith, unpublished data). Immediately beneath the O₂ gradient was a nitrate-containing zone, which itself was only about 6 m thick (Figure 2). The highest nitrate concentration was slightly greater than 1 μM in groundwater obtained from 3.4 m below the water table. The lower portion of the nitrate-containing interval also contained relatively high nitrous oxide concentrations. The combination of low O₂ and high NO₃ and N₂O suggested that this depth interval was a potential zone of denitrification. This was indeed the case, but only within the nitrate-containing zone (Figure 3). No activity was evident above or below this zone. Interestingly, before a multilevel sampling device had been installed at this site, which was responsible for detecting the nitrate-containing zone, we had previously failed to find denitrifying activity because a sample had not been taken from this depth interval. This scenario emphasizes the need for vertical, close-interval sampling of groundwater before core samples are taken for activity assays.

The aquifer sampled for this study is composed of cohesionless sands and gravel, with a clay content of <0.1%. This particular composition has proven to be a difficult obstacle for obtaining intact cores from aquifers of this type. Only two of the coring techniques that have been tested at the Cape Cod Site were able to obtain aquifer core material that was suitable for assaying microbial processes. The first successful coring technique was the use of a split-spoon core barrel through the center of a hollow stem auger. This technique does not require drilling muds and can obtain several continuous cores from a single hole. Thus, a large amount of material can be obtained very quickly and the technique is compatible with close-interval sampling. Since the cores do not retain the interstitial pore water, it is necessary to immediately slurry the core material with groundwater from the same depth to restore the nutrient regime. This latter step more or less dictates that activity measurements for microbial processes in core material collected with a split-spoon core barrel will use a sediment slurry incubation.

Although they represent a significant perturbation relative to in situ groundwater conditions, sediment slurry incubations have some distinct advantages. This type of incubation can be used to demonstrate whether a particular microbial process is occurring within an aquifer at a given location. For example, denitrifying activity was evident in core material taken from the nitrate-containing interval at site F347 (Figure 3). There was a linear increase in N₂O concentrations in flasks containing samples taken from this depth interval with no apparent lag time. This would indicate that denitrification was an active process in this depth interval within the aquifer. In contrast, there was very little activity evident in flasks containing material taken from above or below the nitrate-containing interval (Figure 3). The ability to reach these conclusions was enhanced because the sediment slurry incubations produced several
time courses. It is also relatively easy to simultaneously set up several incubation flasks with this technique, which allows the flexibility to test the response of the activity of interest to a variety of incubation conditions. Smith and Duff use this approach to differentiate where denitrification was carbon-limited as opposed to nitrate-limited within the Cape Cod contaminant plume.

The rates of a microbial activity can be estimated from the slope of a time course plot. With sediment slurries there is a large degree of uncertainty as to whether these rates are indicative of in situ conditions. Therefore, they are often called potential rates so as to avoid misrepresenting them as in situ rates. Potential rate estimates can be useful in a comparative manner, however. A depth profile of the potential denitrification rates calculated from the time course in Figure 3 is compared in Figure 4 with a similar profile for sediment slurries incubated in flasks with no headspace. Both types of incubations indicate that there was a zone of active denitrification at this site that was approximately 6 m thick and a peak of activity at 4.5 m below the water table. The depth of this peak corresponds to the depth of the nitrate peak at this site (Figure 2). Although the shape of the two profiles were similar, there was over a seven-fold difference in the peak rate estimate between these two incubation conditions.

Slurry assays with the anoxic headspace almost certainly overestimate the in situ rate of denitrification, possibly because the sparging of the flasks to create the anoxic headspace during setup removes low levels of O₂ that are actually limiting the in situ rate of the process. This would not be the case for the flasks with no headspace since they were stoppered immediately after being filled with groundwater. Thus, these two different sediment slurry assays suggest that threshold concentrations of O₂ may be at least one factor controlling in situ rates of denitrification.

The second, and much more recent coring technique that has successfully obtained core material from the Cape Cod aquifer was the use of a piston-type core barrel. This core barrel overcomes the primary limitation of the split-spoon core barrel because the piston holds the interstitial groundwater intact within the core. The result is a relatively undisturbed core that can easily be cut into sections without removing the core material from the core liner. The availability of this kind of core made it feasible to conduct incubations directly within intact cores in a manner similar to the techniques developed for assaying processes such as sulfate reduction and denitrification in surface water sediments. The technique was successfully modified to assay denitrification in aquifer cores by pumping acetylene-amended groundwater into each core. A depth profile of denitrification rates at site F347 (Table 1) illustrates the results using these direct, intact core incubations. Again, there was a narrow zone of denitrifying activity (3-5 m) with a peak located at the 4.4-4.7 m interval. These depth intervals corresponded very well with the intervals delineated by the split-spoon, sample-sediment slurry assays. In general, the intact core technique is much more cumbersome and time consuming to set up than the sediment slurry incubations and it uses a much greater amount of core material. It is also a single time point assay.
The rates of denitrifying activity determined by the various combinations of coring and assaying procedures are compared in Table 2 for core material taken from the depth of peak activity at F347. In general, there is a fifteen-fold range of estimates between the highest and lowest rates. Significantly higher rates were evident for samples assayed by the standard sediment slurry incubation technique (with an anoxic headspace), irrespective of the coring method used. In contrast, the lowest rate estimate was obtained from the intact core incubations. Interestingly, there was very close agreement in the rate estimates for split-spoon samples incubated in flasks without a gaseous headspace and the intact core incubations, despite the rather large overall difference between these two incubations. Intuitively, the intact core incubations would appear to be much closer to an in situ setting than the sediment slurries. In fact, the results of these types of incubations are frequently inferred to be in situ rates in surface water sediments. However, it must be noted for these groundwater samples that: (1) drilling and coring probably represent a greater disturbance than manually obtaining a surface water core; (2) incubation times are much longer and (3) the physical aspect of groundwater flowing through the aquifer is not present during the incubation techniques.

The best method for ultimately obtaining an accurate in situ rate measurement in a groundwater environment will involve assaying the activity directly within the aquifer. This approach would involve injecting a tracer into an aquifer, letting groundwater flow transport the tracer through an undisturbed section of the aquifer, and sampling for the tracer at downgradient rows of multilevel wells. Tracer tests such as this have been conducted at the Cape Cod site to study the transport of bacteria and to measure methane oxidation. It is our goal to assay denitrification in this manner as well, the results of which can be used as a standard against which the ability of the various techniques in Table 2 to estimate in situ rates can then be judged.

Conclusion

In summary, two different coring methods and a variety of incubation techniques have been utilized to study denitrification in a groundwater environment that has been contaminated by a dilute sewage effluent. These results emphasize the need for close-interval vertical sampling of both groundwater and core material when assaying for microbial processes in such situations. For the present, it is not known which coring and incubation technique is best suited for obtaining in situ rate estimates of a given microbial process. However, it evident that a combination of sampling and assaying techniques can collectively maximize the information obtained about the factors within the subsurface that affect the activity of interest.

Acknowledgements

I would like to thank M. Brooks, M. Ceazan, and J. Duff for their technical assistance, D. LeBlanc and S. Garabedian for the managing field site logistics, B. Howes for the use of a gas chromatograph, and R. Harvey and D. Lovley for their manuscript review.
References


### Table 1. Rates of denitrification during intact whole core incubations.\(^a\)

<table>
<thead>
<tr>
<th>Depth Interval Below Water Table (m)</th>
<th>Pore Water N(_2)O ((\mu)mol/l)</th>
<th>Rate of N(_2)O Production ((\mu)mol per gram dry sediment per day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 day</td>
<td>4.25 days</td>
</tr>
<tr>
<td>2.6-2.9</td>
<td>6.8</td>
<td>6.2</td>
</tr>
<tr>
<td>2.9-3.2</td>
<td>4.5</td>
<td>9.0</td>
</tr>
<tr>
<td>3.2-3.5</td>
<td>13.1</td>
<td>25.9</td>
</tr>
<tr>
<td>3.8-4.1</td>
<td>14.5</td>
<td>40.8</td>
</tr>
<tr>
<td>4.1-4.4</td>
<td>14.6</td>
<td>46.2</td>
</tr>
<tr>
<td>4.4-4.7</td>
<td>17.1</td>
<td>53.9</td>
</tr>
<tr>
<td>4.7-5.0</td>
<td>19.3</td>
<td>49.4</td>
</tr>
<tr>
<td>5.3-5.6</td>
<td>19.7</td>
<td>26.7</td>
</tr>
<tr>
<td>5.6-5.9</td>
<td>19.3</td>
<td>17.3</td>
</tr>
<tr>
<td>5.9-6.2</td>
<td>17.4</td>
<td>25.0</td>
</tr>
<tr>
<td>6.2-6.5</td>
<td>7.1</td>
<td>16.7</td>
</tr>
</tbody>
</table>

\(^a\)Cores were obtained from site F347 and incubated at ambient nitrate concentrations.

### Table 2. Comparison of denitrification rates for the different sampling and incubation techniques.\(^a\)

<table>
<thead>
<tr>
<th>Coring Method</th>
<th>Incubation Method</th>
<th>Denitrification Rate ((\mu)mol N(_2)O produced per gram dry sediment per day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Split-spoon</td>
<td>sediment slurry,</td>
<td>18.7</td>
</tr>
<tr>
<td></td>
<td>anoxic headspace</td>
<td></td>
</tr>
<tr>
<td>Split-spoon</td>
<td>sediment slurry,</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>no headspace</td>
<td></td>
</tr>
<tr>
<td>Piston</td>
<td>sediment slurry,</td>
<td>30.4</td>
</tr>
<tr>
<td></td>
<td>anoxic headspace</td>
<td></td>
</tr>
<tr>
<td>Piston</td>
<td>whole core</td>
<td>2.0</td>
</tr>
</tbody>
</table>

\(^a\)Core material and groundwater were collected from site F347, 4.5 m below the water table and incubated at ambient nitrate concentration.
Figure 1. Diagram of intact core incubation setup.
Figure 2. Vertical profile of dissolved constituents at site F347.
Figure 3. Time course of nitrous oxide production by slurried core collected from site F347 at indicated depths below the water table. Samples were incubated at ambient nitrate concentrations.
Figure 4. Vertical profile of rates of denitrification potential in slurried core material obtained from site F347 and incubated at ambient nitrate concentrations.
Q and A

_T. Phelps:_ How many screen zones do you estimate you have at your site?

_R. Smith:_ That is a good question Tom. I think there are 450 of those multilevel samplers, each of which has 15 ports. Of course, there will probably be another 150 of the more standard monitoring wells.

_J. Sufita:_ Dick, I wonder if you could clear up a point for me concerning the denitrification potential assays. Were those whole core assays or were they slurry tests?

_R. Smith:_ When I say a potential rate, I usually mean the indigenous rate. Therefore, the depth profiles that you saw were indigenous incubation.

_D. Boone:_ What do you think was going on. What was the reason for the big differences with and without a headspace?

_R. Smith:_ Well, there was a couple of things. I think one sort of gets into the problem that we have of measuring dissolved oxygen at the site. We know that the dissolved oxygen is very low and it was hard estimating whether there was microaerophilic conditions or true anoxic conditions. If there were microaerophilic conditions, then there was a difference with the flushing flask in one situation, significantly more than another. An oxygen control may have been removed. Another explanation is that one could have a static situation, where one does not have mixing occurring in the sediment itself but does in the other. Therefore, it is one of those two or a combination of the two.

_D. Boone:_ Did you check to make sure that there was not a pH change that occurred when you flushed those samples with nitrogen, CO₂,...

_R. Smith:_ Yes, that is something that one has to be aware of and careful of. That and there is not a significant effect.

_M. Jacobsen:_ I was just curious if you could clarify a point for me also. Did you make any attempt to seal the annulus in the well so that you did not have mixing on the outside? I understand you had a lot of intervals that were 0.5 m apart.

_R. Smith:_ That was not done. We found that there was no need to do that. However, a lot of testing was done at this site to make sure that sampling was being done at specific intervals with our multilevel samplers without getting channeling up or down the annulus.
Drilling and Coring Deep Subsurface Sediments for Microbiological Investigations
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Abstract
Subsurface sediments have been recovered from depths greater than 500 m using rotary drilling techniques, bentonite drilling fluids, and modified CP-wireline systems. Sediments were extruded, pared, and processed aseptically under anaerobic conditions in a glove bag. Numerous quality assurance protocols measured contamination from drilling tools, drilling fluids, or paring equipment. Lithological examination by on-site geologists assured sediments were of the proper types. Rhodamine dye, potassium bromide, and perfluorocarbons were added to drilling fluids and provided chemical indicators of drilling fluid intrusion into the sediments. Micron-sized magnetic beads, total plate counts, and coliform analyses served as biological indicators of drilling fluid intrusion into cores. The quality assurance protocols provided multiple measures for detecting milligram levels of drilling fluid contamination per kilogram sediment for different types of subsurface materials. These sampling protocols enabled the evaluation of microbial biomass, activities and potential capabilities of microorganisms residing in deep subsurface sediments.
Introduction

Recent studies have established the presence of microorganisms in deep subsurface environments and determined they could influence groundwater chemistry.\textsuperscript{1-13} Anaerobic, as well as aerobic microorganisms, have been isolated and many microorganisms are different from known species or those isolated from surface soils.\textsuperscript{1,6,7,12}

The majority of subsurface studies have been performed in shallow, unconsolidated sands employing samplers that are pushed into sediments by hydraulic pounding.\textsuperscript{5,11-13} Samples have also been obtained from coring devices which allowed drilling fluids to surround the sample,\textsuperscript{2,8} making contamination from drilling fluids a major problem to control and quantify. Biological agents have been used as tracers during subsurface sampling.\textsuperscript{4,13} but it is unlikely that future studies will allow large concentrations of foreign microorganisms mixed into subsurface aquifers. The use of ionic species as conservative tracers of groundwater flow is well established\textsuperscript{9,8} and applicable to drilling fluids. Recent studies have incorporated fluorescent beads approximating the size of bacterial cells.\textsuperscript{5} Unfortunately, their use in 15,000 liters of drilling fluids to monitor mg/kg levels of contamination is cost prohibitive.

This report describes sampling procedures and quality assurance protocols developed for the Microbiology of Deep Subsurface Program sponsored by the United States Department of Energy. These protocols enabled sampling of subsurface sediments greater than 500 m in depth in a variety of unconsolidated sedimentary formations. Multiple quality assurance measures were incorporated with some measures being field applicable while others were performed independently. The quality assurance program enabled detecting drilling fluid contamination of 0.01-10 mg/kg sediment.

Materials and Methods

Site Description. Core holes were located within the Upper Atlantic Coastal Plain on the Aiken Plateau and beside the Savannah River as described previously.\textsuperscript{8,10} Unconsolidated sediments extended to depths of 400-600 m and were underlain by crystalline metamorphic rock or consolidated mudstone.

Coring tools. Conventional wireline coring tools (Christensen Diamin Tools Inc., Salt Lake City, UT) were modified. Specially designed shoes were manufactured to increase the distance from the drill bit on the outer barrel to the punch corer which decreased contamination by drilling fluids.

Lexan core liners were used for the 3-inch and longer shoes, but PVC sewer pipe was the core liner for 1.5 inch and shorter shoes. A welded anti-jam sampler was the wireline version of the phosphate barrel.\textsuperscript{8} Both the inner barrel and outer barrel rotated and the shoe was also welded. Core catchers were not easily adaptable. All of the systems were compatible with the pinned plungers that resided at the leading edge of the shoe.
**Sampling.** The drill rig and all of the rods were steam cleaned three times before the start of the project. The drilling fluid system was an enclosed, continuously recirculated tank, which contained the bentonite viscosifier. All water was obtained from a thoroughly flushed municipal hydrant to which chlorine was added to provide a 3-5 mg/l free chlorine residual after twelve hours. Coring was conducted after steam cleaning the desired tools. The bottom of the shoe was covered with a whirlpak bag and a teflon cover and then taped. The sample tool was placed into the top of the drill rod and clamped. The inner barrel was filled with chlorinated water. The latch was assembled and the coring barrel dropped into the hole. Gentle pump pressure was applied to force the sample barrel downhole. The sample interval was cored and the inner barrel was retrieved. Subsamples were taken for rhodamine analyses to ascertain the merit of further processing. All equipment and the decontamination table was steam cleaned before and after each use.

**Field studies.** Retrieved materials were removed from the sampler and the core liner was immediately carried into the Mobile Microbial Ecology Laboratory (MMEL). Sediments were extracted from the core liners using an extruding device (Model P-107, Soiltest, Evanston, IL). Materials exiting the core liners automatically entered a N₂ flushed glove bag. The N₂ flushed bag was a commercial anaerobe bag that was 2 x 4 x 2 ft and contained two pairs of sleeved gloves, an extruder port, and a 12-inch diameter air lock (Coy, Ann Arbor, MI). All manipulations were performed in the bag using double layers of gloves. Paring of the samples was accomplished with flame sterilized paring tools and the work was performed in the anaerobic glove bag using the first pair of sleeves. Approximately one half of the outermost portions of the samples were pared in addition to all of the portions that appeared to be contaminated with drilling fluids. After the paring was completed, the aseptic sample was placed into another flame sterilized pan and quartered using the second set of glove bag sleeves, new gloves and flame sterilized tools. Samples were mixed and transferred into sterile whirlpak bags. Bags were weighed and placed into quart canning jars. To minimize oxygen contamination, the storage jars were flushed with N₂ gas via a cannula. Jars were sealed and exited the bag through the airlock. Between each sample the bag was dismantled for thorough cleaning and disinfection.

Within 30 minutes of sample recovery aseptic, materials were generally disbursed into sample jars, subsamples were handed to analytical chemists, frozen for lipid analyses, used for initiation of on-site activity measures, prepared for express shipment to program participants, or archived. Samples for shipment were packed on ice, bagged, and then boxed. Cores exiting the ground by 1:00 p.m. were prepared for transit by 4:00 p.m. and in greater than 90% of the instances, samples arrived in the appropriate laboratory by 10:00 a.m. the following morning.

**Quality assurance.** The on-site geologist was responsible for maintenance of the lithological logs and comparing logs from the preliminary hole with the observed
lithologies. Tracers were monitored as an indicator of contamination. Potassium bromide was added at 500 mg/l as a conservative tracer of drilling fluid contamination and assayed independently. Rhodamine WT (Crompton and Knowles, Green Hills, PA), which is a fluorescent dye, was added at 20 mg/l as a tracer and assayed on-site to insure that highly contaminated materials were not disbursed to participating labs. Rhodamine was assayed by mixing equal volumes of water and pared sediments and then examining the supernatant by fluorometry, using an excitation wavelength of 546 nm. The fluorescence was measured at 590 nm using a Turner fluorometer.8

Fluorescent beads, approximately 2 μm in diameter (Polyscience Inc., Warrington, PA), were examined at selected intervals. Four perfluorocarbons were also added to the drilling fluids at 1 mg/l and assayed by gas chromatography using pyrolysis-electron capture detection at Brookhaven National Laboratory (Senum, this volume). In addition to the added tracers, the drilling fluids naturally contained high levels of ammonium which was detected at the 10⁴ dilution. Total bacterial plate counts were performed independently to monitor the level of microbial colony forming units (CFU) in both drilling fluids and each sample. Coliforms common to surface soils proliferated in the drilling fluids and provided a biological control (Sulfita, this volume). Additionally, CFUs were picked and twenty-one biochemical tests were performed to establish whether the microorganisms were similar to those of other formations or drilling fluids. Phospholipid fatty acids were compared between drilling fluids and subsurface samples providing another measure of sample contamination by microorganisms from the drilling fluids (White et al., this volume).

Results

Obtaining subsurface sediments suitable for microbiological studies at 12 separate laboratories from depths of 100-500 m proved to be a formidable task. The date in Table 1 show the quality assurance protocols used to measure contamination of the samples. Upon extrusion of the sediment material from the core liners, the on-site geologist verified that the retained materials were, in fact, from the selected location. This was accomplished by comparing sediments with known properties of the formation previously determined with a preliminary hole cored continuously in a manner suitable for geologic studies. The preliminary hole was logged lithologically with written descriptions and photographs.

The on-site microbiologist examined the pared materials for visible contamination by the rhodamine dye. If no dye was visible, subsamples were diluted in equal volumes of water and the supernatant was assayed for the presence of dye by fluorometry. If the sample was from the correct formation, and free of rhodamine dye, the materials were distributed. Rhodamine dye analyses were performed in the field and allowed for a quality assurance protection factor of 10⁴, or approximately 10-100 mg of drilling fluids per kilogram of sample.

All other quality assurance protocols were performed off-site by independent
laboratories. Potassium bromide was added to drilling fluids and was detectable at 10 ug/kg, thereby providing a log protection factor of five.

Four perfluorocarbons were added at 1 mg/l to drilling fluids (Table 1) and the assays were determined by Brookhaven National Laboratory. The perfluorocarbon tracers allowed a protection factor of eight logs, and consequently, most samples contained traces of perfluorocarbons. The chemical tracers provided multiple means of detecting drilling fluid intrusion into sediments, but they did not necessarily relate to microbial contamination. Evidence suggested (Garland et al., this volume) that ionic tracers moved rapidly through cored sediments. The goal was to provide a six-log protection factor which may have been exceeded with respect to microbial contamination in many samples. Few samples obtained a six-log protection factor for ionic species in expressed pore waters.

Biological tracers and surrogates of microbial cells were also used as quality assurance measures (Table 1). Bacterial sized beads were used in four sampling attempts with two successes. In both instances, the beads moved less than 1 cm into the cored materials giving a log protection factor of four. This suggested that the movement of bacteria-sized particles was considerably less than for ionic species. Total CFU's on bacterial plate-count media was performed on each sample and drilling fluid. Twenty isolates from each sample were then screened for twenty-one biochemical and physiological tests. As a general rule, an isolate from one location was rarely seen at any other location in that borehole or in another borehole (Balkwill, this volume). In addition, the sediments exhibited CFU enumerations of 10^3-10^5/g, often providing a 10^6 protection factor for contamination of clays that exhibited less than 10^2 CFU/g. Coliforms from surface soils proliferated in drilling fluids and provided a three-log protection factor. Samples, drilling fluids and shavings were examined by Sufita et al. (this volume), which suggested that there was negligible microbial contamination of samples by microorganisms in the drilling fluids. Additionally, membrane phospholipids (White et al., this volume) and biochemical tests that were performed on isolates provided a two-log protection factor, and suggested that the cultured microorganisms did not resemble the microorganisms in the surface soils or the drilling fluids.

The data in Table 2 show the results of on-site rhodamine analyses and the sampling tools employed. Pared shavings typically contained more rhodamine than the disburned samples, and whenever rhodamine levels were greater than 2 ug/l of supernatant, warnings were issued to the investigators that the sample may be compromised because the four-log protection factor was not being met. Whenever the rhodamine level was between 20 and 50 ug/l, warnings were issued that the sample was compromised. Samples that contained greater than 50 ug/l rhodamine were not dispersed. Sample G-17, a control, was centrifuged to decrease the rhodamine and then compared to G-16, which came from the same depth. In one instance, background fluorescence masked the rhodamine analyses and a warning was issued.
As noted in Table 2, several types of coring tools were utilized. All tools used represented modifications to commercially available equipment. All sampling shoes longer than 1.5 inches were especially prepared by the manufacturer. PVC sewer pipes were used as liners for some shoes that were two inches long because they more closely matched core diameter, and thus eliminated the volume of drilling fluids surrounding cored sediments. The welded anti-jam represented a wireline version of the phosphate barrel that rotated in the sediments and cored in front of the drilling bit. Welded anti-jam tools were particularly useful in clayey sediments that did not require retention by core catchers. Pinned plungers kept drilling fluids from entering the core liner until coring commenced. Pressure from the cored sediments broke the teflon liner and the pin, allowing the plunger to slide through the core liner. Sediments then entered the core liner, dramatically reducing the intrusion of drilling fluids.

The data in Table 3 show the spatial distribution of microorganisms in retrieved subsurface sediments. If microbial contamination from the drilling fluids influenced the samples, then the innermost subsamples (7 and 8), would have exhibited fewer CFU than subsamples 1, 2, 3, 4 and 6, which were located within 2 cm of the core section. Eight samples were analyzed in detail, three of which had CFUs that were too low to evaluate. As shown in Table 3, subsamples 7 and 8 of the Congaree sands showed fewer CFUs than subsamples 1-6. Congaree sediments contained aquarium gravel-sized sands which contained greater than 1 mg/l rhodamine. Due to rhodamine contamination, samples of Congaree sands were not distributed as a general sample, and those investigators which specifically requested them were notified that the sediments were “grossly contaminated”. In other sediments, rhodamine contamination was not severe and subsamples showed no differences in CFU.

Discussions

Crucial to subsurface microbiology studies is the quality assurance of the sediment samples used for microbiological analyses. By the very nature of coring, extruding, and disbursing large volumes of subsurface sediments, contamination occurs. A program goal was to minimize contamination and to incorporate a series of methodologies to ascertain the extent of contamination.

The combination of quality assurance protocols incorporating rapid field analyses and substantiating the quality with independent verification resulted in a comprehensive program to limit and quantify contamination. Biological quality control agents such as recognizable bacterial morphologies or yeasts would prove ideal, but permission to add such agents to groundwaters is difficult and sufficient fluorescent beads in 15,000 liters of drilling fluids would be economically prohibitive.

Conclusion

The information in Table 4 shares points that should be considered for future subsurface investigations. Most importantly, interdisciplinary scientists and subcontracting specialists should work together on-site and problems should be resolved through organized meetings at the site. Chemical contamination is problematic and multiple screen zones with packers may be required for determining groundwater
chemistries. Multiple tracers, quality assurance programs, blanks and sanitation are all essential to quantify contamination and rectify any crises. Redundancy in quality assurance and experimentation are required to maximize the success of expensive coring and on-site activities. Rapid initiation of experiments assures the best possible results and information transfer.

The protocols listed in Table 4 are capable of providing multiple measures of 10 mg/l contamination but the lack the sensitivity to ascertain pg/kg levels of contaminants. With the above combination of protocols for contamination control and the finding that microorganisms did not appear in multiple formations, our assertion is strengthened that it is feasible to obtain subsurface sediments suitable for microbiological investigations, and furthermore, to assure that microorganisms observed from subsurface habitats are in fact from such habitats rather than from drilling contamination.

Acknowledgements

The authors would like to acknowledge the program managers; Frank Wobber of DOE, Carl Fliermans and Jack Corey of the Savannah River Plant. Thanks are due Horace Bledsoe, the Site Liaison, and Ken Sargent the Site Geologist. Special gratitude is extended to Carl Prigin and Rick Frye of Graves Well Drilling Co. of Jackson, SC. This work was funded by United States Department of Energy, contract DE-FG05-88ER60702, SRP, and the Idaho National Engineering Laboratory.
References


# Tables

Table 1. Quality assurance protocols used to evaluate drilling fluid intrusion into cored sediments.

<table>
<thead>
<tr>
<th>Chemical Tracer Tests</th>
<th>Concentration in Drilling Fluids (mg/l)</th>
<th>Detection Limits in Sediments (ug/kg)</th>
<th>Protection Factor (log)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhodamine</td>
<td>20</td>
<td>0.5-2.0</td>
<td>4</td>
</tr>
<tr>
<td>Bromide</td>
<td>500</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Potassium</td>
<td>300</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Perfluorocarbons</td>
<td>1</td>
<td>0.00001</td>
<td>8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Biological Tracer Tests</th>
<th>(log/ml)</th>
<th>(log/ml)</th>
<th>(log)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial Sized Beads</td>
<td>10</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Plate Counts</td>
<td>3-6</td>
<td>0-1</td>
<td>2</td>
</tr>
<tr>
<td>Coliforms</td>
<td>3-5</td>
<td>0-1</td>
<td>3</td>
</tr>
<tr>
<td>Membrane Phospholipids</td>
<td>(ng/g)</td>
<td>(pg/g)</td>
<td>2</td>
</tr>
<tr>
<td>Biochemical Tests (on colony morphologies)</td>
<td>-</td>
<td>-</td>
<td>2</td>
</tr>
</tbody>
</table>
Table 2. Rhodamine Tracer Results and Tools used in Coring Samples.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Rhodamine (ug/l) in Supernatants of Samples(1)</th>
<th>Sampling Tool Employed</th>
<th>Br Sample Origin (2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-1</td>
<td>&lt; 1.0</td>
<td>C-L-6</td>
<td>A</td>
</tr>
<tr>
<td>G-2</td>
<td>&lt; 0.5 (S &gt; 1.0)</td>
<td>AJ-L-6</td>
<td>A</td>
</tr>
<tr>
<td>G-3</td>
<td>&lt; 0.3</td>
<td>AJ-L-4</td>
<td>A</td>
</tr>
<tr>
<td>G-4</td>
<td>&lt; 1.0</td>
<td>AJ-L-4</td>
<td>A &amp; S</td>
</tr>
<tr>
<td>G-5</td>
<td>2 - 3 (S &gt; 5.0)</td>
<td>C-PVC-2</td>
<td>A &amp; S</td>
</tr>
<tr>
<td>G-6</td>
<td>&lt; 2.0 (S &gt; 5.0)</td>
<td>C-L-2 1/2</td>
<td>A &amp; S</td>
</tr>
<tr>
<td>G-7</td>
<td>Autoclaved sands</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G-8</td>
<td>1.0 (S &lt; 5.0)</td>
<td>WAJ-L-5</td>
<td>A &amp; S</td>
</tr>
<tr>
<td>G-9</td>
<td>&lt; 2.0 (S = 25)</td>
<td>WAJ-L-5</td>
<td>A &amp; S</td>
</tr>
<tr>
<td>G-10</td>
<td>&lt; 1.0</td>
<td>C-PVC-2</td>
<td>A</td>
</tr>
<tr>
<td>G-11</td>
<td>5</td>
<td>P</td>
<td>A</td>
</tr>
<tr>
<td>G-12</td>
<td>&lt; 5.0 (S &lt; 15)</td>
<td>C-PVC-2</td>
<td>S</td>
</tr>
<tr>
<td>G-13</td>
<td>&lt; 5.0</td>
<td>C-PVC-2</td>
<td>A</td>
</tr>
<tr>
<td>G-14</td>
<td>&lt; 2.0</td>
<td>C-N-1/2</td>
<td>A</td>
</tr>
<tr>
<td>G-15</td>
<td>&lt; 2.0</td>
<td>WAJ-L-2 1/2</td>
<td>A</td>
</tr>
<tr>
<td>G-16</td>
<td>50 (measured 10)</td>
<td>C-PVC-2*</td>
<td>S</td>
</tr>
<tr>
<td>G-17</td>
<td>cen 100 (measured 20)</td>
<td>C-PVC-2*</td>
<td>A</td>
</tr>
<tr>
<td>G-18</td>
<td>50 (measured 10)</td>
<td>C-PVC-2*</td>
<td>S</td>
</tr>
<tr>
<td>G-19</td>
<td>Visible</td>
<td>Unpoured Wood Chips</td>
<td>A</td>
</tr>
<tr>
<td>G-20</td>
<td>&lt; 2.0</td>
<td>C-N-1/2</td>
<td>A</td>
</tr>
<tr>
<td>G-21</td>
<td>40</td>
<td>C-PVC-2*</td>
<td>S</td>
</tr>
<tr>
<td>G-22</td>
<td>masked by color</td>
<td>C-N-1/2</td>
<td>S</td>
</tr>
<tr>
<td>G-23</td>
<td>Visible on rock</td>
<td>C-N-1/2</td>
<td>A</td>
</tr>
</tbody>
</table>

1Similarly treated drilling fluids containing 20 mg/l rhodamine typically exhibited 10-15 mg/l rhodamine.
2This information provided to assist in interpretation of bromide tracer results.

Notations: G = Generally Disbursed Sample; C = Conventional wire line barrel; AJ = Anti-jam option; WAJ = Welded anti-jam; P = Phosphate barrel; L = Lexan liner; PVC = PVC liner; N = No liner; A = sample sent to investigators; S = Shavings; * = pinned plunger; Numerical notation is the length of shoe in inches.
Table 3. Spatial Distribution of Microorganisms from Subsurface Cores.

<table>
<thead>
<tr>
<th>Subsample Number</th>
<th>Congaree Sands (^a)</th>
<th>Pee Dee Sands</th>
<th>Pee Dee Sands</th>
<th>Pee Dee Clay (^b)</th>
<th>Black Creek Sands</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.6</td>
<td>6.7</td>
<td>6.5</td>
<td>&lt;2</td>
<td>6.1</td>
</tr>
<tr>
<td>2</td>
<td>4.6</td>
<td>6.8</td>
<td>6.4</td>
<td>&lt;2</td>
<td>5.8</td>
</tr>
<tr>
<td>3</td>
<td>4.8</td>
<td>6.5</td>
<td>6.3</td>
<td>&lt;2</td>
<td>6.5</td>
</tr>
<tr>
<td>4</td>
<td>5.5</td>
<td>6.1</td>
<td>6.5</td>
<td>&lt;2</td>
<td>6.5</td>
</tr>
<tr>
<td>5</td>
<td>4.7</td>
<td>6.3</td>
<td>6.5</td>
<td>2.3</td>
<td>6.3</td>
</tr>
<tr>
<td>6</td>
<td>4.7</td>
<td>6.4</td>
<td>6.5</td>
<td>&lt;2</td>
<td>6.5</td>
</tr>
<tr>
<td>7</td>
<td>3.6</td>
<td>6.3</td>
<td>6.5</td>
<td>2</td>
<td>6.1</td>
</tr>
<tr>
<td>8</td>
<td>3.8</td>
<td>6.6</td>
<td>6.4</td>
<td>2</td>
<td>6.4</td>
</tr>
</tbody>
</table>

\(^a\) Mud soaked. Sample was labeled "grossly contaminated".
\(^b\) All plates had less than 30 CFU.

Six-inch core sections were obtained with the conventional barrel and 2-inch shoes were split in three directions. CFU’s were performed on eight subsamples. Subsamples 1, 2, and 3 were from a plane one inch beneath the surface of the core, approximating the outermost area typically used in dispersed samples. Samples 4, 5, and 6 were from a cross section one inch from the end containing subsample 1. Subsample 6 was closer to the edge than the sample used for distributed core. Subsample 4 was 1/2 inch from the surface and rarely in an area used for disbursement. Sample 5 was from the center of the core segment. Samples 7 and 8 were the centermost portions of the core section.
Table 4. Considerations for Subsurface Microbiological Investigations.

- Documented statement of work and program plan.
- Expert review of workplan and quality assurance procedures.
- On-site geologists, hydrologists and microbiologists.
- Ability to differentiate chemical from biological contamination.
- Access to background groundwater data from sample units.
- Minimize impact to pore water chemistry or develop alternative sampling strategies.
- Use of controls and blanks for tools and sediments.
- Employment of multiple drilling fluid and downhole tracers.
- Sterilization, steam cleaning or disinfection of all equipment.
- Use of HEPA filters when air drilling.
- All sample materials to be pared or sub-cored.
- Pare on-site until tracers reduced by >3 orders of magnitude.\(^6\)
- All experimentation to begin within 72 hours of sample collection (samples refrigerated <10°C during storage).
- Key experiments repeated at a second laboratory.
Novel Drilling Technologies Applicable to Deep Subsurface Tunnels
Roger Jacobsen
Nevada Test Site, NV.

Abstract
No abstract provided.

Introduction
The Nevada Test Site is referred to as “the tunnels” (slide). They are really not tunnels; they are attics. There is approximately 20 miles of these attics that are mined with a cross section that has a diameter anywhere from 6-12 feet. It was originally proposed that this might be an interesting area for subsurface microbiological investigations because one of the things available at the Nevada Test Site is small diameter drill holes.

Discussion
Most of wells that are drilled there range from 2000 to 4000 feet. This would probably classify in most people’s definition of deep. There are 10-20 inch diameter wells that are referred to as the “slim” holes. These holes are drilled in various locations from which core and groundwater samples can be collected. There are also larger diameter drill holes that are 8-12 feet in diameter, and it is possible to obtain cores from these holes as well. To get cores from the bottom of the hole to the top very rapidly, a pressure vacuum system is used that brings the rock up at just under subsonic velocities and then captures it in a little glove box on the surface. It is being referred to as the supersonic rock sampler; if one wants to get a sample from the bottom to the top very quickly, then that is the way to do it. One does not have to worry about the 16 or 20 hour turnaround times. In a couple of seconds, the sample will be in your hand.

The last thing to discuss at this point is the mine tunnels. They go back in underneath the area where considerable testing is done. A cross section of these mines shows that they are basically the standard sort of western flat-top areas (slide). These mines range from 1000 to 1500 feet from land surface down to the tunnel levels, which provide an opportunity to study the areas in two-fold (slide). The regional water table is deeper but because of the way the tunnels are constructed, because of the tuck units in the mine, there is locally perched water. Therefore, saturation levels can be obtained that are basically 100% saturated, down to something that is perhaps around 50-75% saturated. In that way, one can vary the conditions considerably as far as the moisture, yet the mine is still below the surface enough that one will probably consider this a fairly deep environment.

When looking at a tunnel complex, one can see that there are varying depths of material over the tunnels (slide). Thus, one can have different vegetation zones. This is sort of the basic setting and, depending on where one is in the mine, there may also be a somewhat different geology and different amounts of water that leak down into the system.

†Oral presentation.
A fairly basic way to collect a sample is to chisel into the system a short distance. The contamination in the tunnels from people's activity, and so on, appeared to be gone after going in only a few inches. In other words, the surface microbial populations were much different than they were at just a few inches. Therefore, in this particular environment, one could easily go in a foot or three and most likely obtain any environment needed for testing. However, one could most likely be into what is considered to be a more negative type population.

There are also techniques that use drill rods to drill into the tunnel's ceiling. These are basically used for rock bolts but in this kind of a technique, one can also put in a hollow-stem drill rod to take cores (slide). Once again, questions arise concerning the build-up of temperature and things of that type, which one needs to be concerned about.

For short cores, however, one can use a system that has a little air-driven motor, similar to what people use to put air in the tires of their car (slide). This kind of system is basically a drilling machine that can drill anywhere from 10 feet to approximately 400-500 feet. With this technique, horizontal drilling can be done where the core is cut. The United States Geological Survey, as part of their system for Yuka Mountain, has instrumented some of these bits to measure temperature and to look at the temperature build-up under different drilling rates, in different materials, and with different air flow so that one can try to keep temperatures under control. In this particular system, one can filter the air that is being used and sterilize the equipment. Again, however, this is a fairly mobile unit, and in most mining operations one will have something like this available.

Various depth holes are made to check the geology, and they often bring out continuous or fairly continuous cores (slide). These cores can be quite different in sizes, from the small 2-4 inch diameter cores with virtually any conceivable length.

The last drilling technique which seemed to be everyone's favorite was called the Alpine Miner. The business end of this drill turns at several hundred rpm. It is composed of a large number of carbide tips that look like little prongs. Basically, it just chews right into the rock and it runs back over to the conveyor belt. With the Alpine Miner, the object is to mine into the system, leaving a little lip, and then just breaking the samples off into a sample container that can then be subcored.

The Alpine Miner may be utilized in other applications as well. The last thing to do, which is the one thing that is hard to do in utilizing a well, is to examine the spatial variability in a 3-D block of rock that is several meters on the size. In our study, in that same tunnel complex, three different locations that were several hundred feet apart were sampled and they looked quite different. Once again, in the cross section the proposal was basically an over coring technique. If one wants to get underground, there are a lot of ways to do it.

The other things that would be interesting for this community to look at would be other opportunities such as caves and caverns, which are clearly interesting
environments. The big concern with caves and caverns is contamination, because they have been open for a long time. From the tunnels that have opened up for many years, there is still uncertainty as to whether contamination gets in there. If one has water moving out of the walls, perhaps it is not that big of a problem.

Deep mining areas all around the world, where people are down hundreds, perhaps thousands of feet, should have deep mining applications. Our friends from Germany are familiar with deep salt mines and other kinds of mines of that type. There is an opportunity in many of those mines to perhaps get in and obtain good samples. Depending again on how the mining is done, one may be able to obtain some very good samples in those particular environments. Information concerning such environments was heard from Great Britain as well.

**Conclusion**

When one considers sampling right now, the drilling must be looked at very carefully. Drilling is clearly an area where a lot of progress can be made. Additionally, when one looks at potential sampling opportunities, the interest in developing new drilling techniques and technologies is sometimes overlooked. The real mission, however, is to understand the microbiological population. Therefore, whenever the opportunity arises to get underground, we should take that opportunity. Thank you.
**Q and A**

*J. Frederickson*: What exactly was the gradation in the water content of the wall material? Is that just the nature of that photograph or was that actually the case in that sample? If that was the case, did you look at the microbiological distribution in the different water content?

*R. Jacobson*: I did not take the photo. However, in the other areas that were observed, one could see different water contents, clearly one of the parameters. When more sampling is to be done, then one would want to check the wetter areas rather than the drier areas. They certainly do exist.

*J. Frederickson*: I do not recall seeing it. We have not looked at that.

*R. Jacobson*: Of the three sites taken, sampling could be done on the first one. They were all fairly wet. Someone said that in dry areas you will never find anything. That was a direct quote from some famous microbiologist. Therefore, observations were made in the areas that were basically seeping water or had a high water content.
A Conceptual Low-Cost Approach to Obtaining Contamination-Free Solid- and Fluid-Phase Samples from the Deep Subsurface Environment

James L. Ruhle
California State University, Fullerton, CA.

Abstract

An innovative and conceptual drilling and sampling technology, which features a downhole pump, nonrotational linear-motion kinetics, and intercalated reverse-circulation Newtonian hydraulics, is described. Since the deep subsurface formation is not subjected to any drilling-fluid pump pressure, any excess drilling-fluid hydrostatic pressure, or any drilling-fluid pressure surges, this technology would make it possible to recover solid- and fluid-phase samples from the deep subsurface without any contamination by the drilling fluid and without any invasion of the formation surrounding the borehole by drilling fluid solids, or reworked formation solids.

The downhole pump/reverse-circulation hydraulics and linear-motion kinetics make it possible to continuously pump to the surfacer segmented punch-core samples from Hanford's Ringold formation or any other sections of incompetent rock. It would also make it possible to continuously pump to the surface large impact-excavated chunk samples from INEL's Snake River basalt, or any other sections of competent rock. Furthermore, it may also be possible to perform large-volume pump tests of formation fluid at any time during the drilling operation and to accurately measure in situ hydraulic conductivity or formation permeability. Special wireline, conveyed samplers have also been designed for uncontaminated solid- and fluid-phase sampling from any desired sidewall location within deep subsurface boreholes that are excavated by means of this hydraulically underbalanced technology and methodology.

No paper submitted.
DRILLING AND SAMPLING TECHNIQUES, TOOLS, AND METHODS

Contributed Papers 1
Posters
Characterization and Bioavailability of Dissolved Organic Carbon in Deep Subs surfaced Surface Waters

A. V. Palumbo,1 P. M. Jardine,1 J. F. McCarthy,1 and B. R. Zaidi2
1Environmental Sciences Division, Oak Ridge National Laboratory
Oak Ridge, Tennessee 37831-6036
2Department of Marine Sciences, University of Puerto Rico, Mayaguez, PR.

Abstract

The bioavailability of chemical fractions of dissolved organic carbon (DOC) from deep well at the United States Department of Energy’s Savannah River Site (SRS) was characterized and compared with those from South Carolina surface waters. DOC levels of 0.30-0.40 mg of carbon per liter were found in groundwater from wells at P24, P28, and P29 at SRS. Water from these deep wells was fractionated by XAD-8 chromatography into hydrophilic (H1), hydrophobic acid (HbA), and neutral (HbN) components, and was found to be 100% HbN. In contrast, surface waters had a much higher total organic carbon content (30 mg of carbon per liter, and the percentage composition was 29% H1, 50% HbA, and 21% HbN. The composition of the organic solutes in the deep groundwater differed, probably, as a result of differences in their sorption to aquifer constituents. Binding affinity increased in a series from HbN < H1 < HbA.

Experiments with three bacterial cultures, Corynebacterium sp., Pseudomonas sp., and a bacteria isolated from the surface water, indicated that the bioavailability of the carbon in the near surface water may be limited by inorganic nutrients. Also, at comparable levels of carbon (20 mg of carbon per liter), the Corynebacterium grew to greater densities when grown in the unfractionated water than when grown on the H1 fraction. Thus, there may have been an organic fraction present in the whole water that was more available than the H1 fraction.
Introduction

The abundance, nature, origin, and biological significance of natural organic matter in deep subsurface environments has not been well characterized. Yet it is clear that, in most environments, organic matter plays a central role in microbial productivity, serving as a source of nutrients such as carbon, nitrogen, phosphorus, and sulfur. It can also complex with and alter the mobility of other biologically important materials, including environmental contaminants.

Dissolved organic compounds may be grouped into humic and nonhumic substances. The latter group includes those substances whose physical and chemical characteristics are still recognizable, such as carbohydrates, proteins, peptides, amino acids, fats, waxes, and low-molecular-weight organic acids. Most of these compounds are assimilated relatively readily by microorganisms and therefore, they usually have fast turnover times. Because they are rapidly utilized in soils or in surface waters, they are unlikely to be transported significant distances through the subsurface. In contrast, the more environmentally refractory substances (humic material) no longer exhibit the specific physical and chemical characteristics (e.g., exact elemental composition or defined infrared spectrum) that are normally associated with well-defined organic compounds.

The purpose then of this preliminary investigation was to improve our understanding of the organic matter in groundwater by characterizing the natural organic matter in water recovered from different formations in the Microbiology of the Deep Subsurface Program and by determining if the natural organic carbon can support growth of bacterial populations. The characterization was directed at elucidating the properties of dissolved or colloidal organic matter that are relevant to the transport and mobility of the organic matter (and contaminants sorbed to the organic matter). Additionally, the potential role of organic matter in groundwater as a nutrient source for supporting microbial productivity in the deep subsurface was evaluated. A secondary objective of this study was to determine the factors limiting microbial growth in surface waters and near surface groundwaters, and to determine the response of the microbial community to a mixing of these waters.

The information developed in this project supports the objectives of the United States Department of Energy’s Office of Health and Environmental Research Subsurface Microbiology Program, specifically in characterizing the physical-geological setting of subsurface microbiota (organic matter as a nutrient for microorganisms and changes that occur in this potential nutrient source with depth and lithology). The deep microbiology corings and wells provide a unique opportunity to obtain carefully sampled water and cores from defined depositional zones with extensive characterization of the samples by a variety of other investigators with a range of expertise. Analyses of this natural material will enhance the scientific information base on the character of subsurface colloidal material and improve DOE’s capabilities to predict contaminant transport in deep subsurface environments.
Materials and Methods

Water was obtained from wells P24, P28, and P29 from the Savannah River Site at the level of the Middendorf formation. Samples of aquifer material were obtained from several different levels during drilling of the C10 hole in Allendale, SC. Total particulate organic carbon and total (organic and inorganic) carbon were measured in these sediment samples.

Total organic carbon (TOC, expressed as percent by weight) was measured by using the ampule method in an OI Corp. Model 700 Total Carbon Analyzer. The weighed sample of sediment was acidified and purged to remove inorganic carbon; organic carbon was then oxidized using sodium persulfate. Carbon, as CO₂, was analyzed with an infrared detector. Total carbon (organic and inorganic) was measured with a Leco inductive furnace carbon analyzer, based on incineration of the solid sample and detection by infrared. Concentrations of DOC in adsorption and fractionation studies were measured with the OI Corporation Model 700 Carbon Analyzer.

Batch equilibrium adsorption of DOC was measured for material from levels G-6 (McBean), G-13 (Black Creek clay), and G-21 (Cape Fear sand) of the C10 Hole. Sediments were selected to encompass a range of mineralogies. The DOC for the sorption experiments was from a well-characterized source of organic matter that was from a stream in Hyde County, NC, and had been used in other sorption studies. Sediment was weighed into 50-mL glass centrifuge tubes with a solution of DOC. The sediment-water solutions were agitated on a reciprocating shaker for two days, which is sufficient time to reach equilibrium. Mixtures were centrifuged and filtered through 0.45-μm polycarbonate nucleopore filters. Supernatant was analyzed for pH and DOC. DOC from these experiments and from the wells was chemically characterized by using XAD-8 chromatography as described by Leenheer and Huffman.

Sorption of specific fractions of the dissolved or colloidal organic matter to the deep sediments was also measured. Solutions of the natural DOC from the stream in North Carolina were equilibrated with various sediment samples for two days. The equilibrium solution was fractionated into its hydrophilic (HI) and hydrophobic solutes (HB; Total HB equals the sum of hydrophobic acid [HbA] and hydrophobic-neutral [HbN] DOC) by using XAD-8 resin chromatography. The percent of different fractions of carbon sorbed to the solid was calculated based on differences in composition of the stock DOC solution and the final equilibrium solution.

Potential for microbial utilization of the HbA, HI, and HbN fractions was determined by following the growth of three species of bacteria in separate batch experiments. The three species of bacteria used were a Corynebacterium sp., a Pseudomonas sp. (both isolated from surface waters), and a bacterium (GT-1) isolated from the Georgetown site surface waters. The bacteria were enumerated by plating on agar plates containing 0.3% Tripticase soy and 1.5% agar using the drop plate method. Microbial growth in deep well water, containing only HbN, was compared to growth in well water from a near-surface site and to growth in UV-oxidized mineral salts media with added HbA and HI.
Results and Discussion

Analysis of Sediments. The results of analysis of the sediments collected from different depths during drilling of the C10 hole are shown in Table 1. These data indicate that the deep subsurface sediments have low particulate matter (POM) concentrations. The range of TOC was only from 0.0077% to 1.5%, and most (13 of 15) samples had less than 1% TOC. Total carbon content was higher, ranging up to 12.2%, indicating substantial quantities of inorganic carbon in some of the samples.

Adsorption Isotherms for DOC-Sediment Interactions. For the batch equilibrium adsorption of DOC studies (Figure 1), results were again similar to those observed for sorption of the aqueous organic matter with clay and sand subsoils. The carbonates of the McBean and the sands of the Cape Fear formation had a low affinity for binding organic matter. Isotherms were comparable to those for sands from a shallow unconsolidated aquifer at the Baruch Forest Science Institute in South Carolina (P. M. Jardine, unpubl. data). The Black Creek clay material had a much higher affinity for binding the organic matter and was comparable to the sorption observed when the organic matter was equilibrated with kaolinitic subsoil clays from Tennessee.

The results of the adsorption studies of the different organic fractions indicated that there were differences among the sediments in the ability to sorb the different fractions (Table 2). The G-6 (McBean) sample sorbed little of the HI fraction or the HbN fraction, and this sediment had the highest inorganic carbon content (12%). Both the G-13 (Black Creek clay) and G-21 (Cape Fear sand) fractions sorbed more of the HI fraction, but only the G-13 sample sorbed any of the HbN. The sorption to the aquifer material will probably affect the availability of the organic matter to the bacteria.

Analysis of Water from Wells P24, P28, and P29. Water was collected from wells in the Middendorf formation at well clusters (P24, P28, and P29) from the Savannah River Site, and the organic carbon was analytically fractionated into hydrophobic and hydrophilic solutes. Total amounts of DOC in the water from the three wells were 0.30 mg of carbon per liter in P24, 0.39 mg of carbon per liter in P28, and 0.40 mg of carbon per liter in P29. The organic matter present in the water from all three wells was 100% HbN. There was no HI or HbA present in any of the wells. In contrast, the percentage composition was 29% HI, 50% HbA, and 21% HbN in surface waters with organic carbon concentrations of 30 mg of carbon per liter. The HbA fraction would be expected to be highly retarded in the aquifer because of its high affinity for sorbing to the aquifer material, especially the shallower McBean formation. In contrast, the HI and HbN fractions would be expected to be more mobile and might be expected to be present in well water from a deep formation. Thus, the absence of the HI fraction may be due to initially low concentration in the source water, to utilization by the bacteria during transport, or, despite its greater mobility than the HbA, to sorption to the aquifer material over long transport distances.
In summary, preliminary analyses of organic matter from the Middendorf formation in wells P24, P28, and P29 indicated that organic solutes differed in their sorption to aquifer constituents, with binding affinity increasing in a series from HbN < Hl < HbA, and all the organic matter in the well water was HbN.

**Microbial Growth in Organic Fractions and Well Water.** The densities of a Corynebacteria sp., isolated from near-surface groundwater, increased from an initial inoculation of $2.0 \times 10^3$ to $1 \times 10^6$/ml in well water from all three deep wells containing only HbN (Figure 2a). This growth represents >6 doublings of the original population. By comparison, the Corynebacteria sp. (Figure 2b), the Pseudomonas sp. (Table 3), and the GT-1 bacteria (Table 3) did not grow in unamended surface waters (GHF) containing approximately twice the amount of organic carbon. Some growth, to densities of $1 \times 10^5$/ml with GT-1, was observed in these near-surface waters upon the addition of inorganic nutrients, but none was observed when all the nutrients except the phosphorus were added. In fact, densities declined in treatments without added phosphorus. Densities of the Pseudomonas sp. and the Corynebacteria sp. did not reach over $5 \times 10^4$/ml in any of the treatments.

Similar experiments with the Pseudomonas sp. (Table 3) and Corynebacteria sp. (Figure 2c) resulted in substantial microbial growth, reaching densities of $10^7$/ml in surface water samples (GSSW) and in mixtures of surface water and GHF groundwater. Again, there was a decline in bacterial numbers in the treatment containing only the near-surface GHF groundwater. Thus, the deep well water supported microbial growth better than the near surface well water. The greatest growth, however, was seen in the surface water (GSSW), which also had the highest organic carbon content.

Growth of the Corynebacteria sp. in mineral salts medium that was amended with Hl and HbA fractions was also substantial, reaching densities of $>10^7$/ml in both the Hl fraction and the HbA fraction (Figure 2d). Also, these preliminary experiments indicated that there were no substantial differences in the ability of the organic fractions to support microbial growth.

**Conclusion**

These data are consistent with the hypothesis that sorption to the aquifer matrix attenuates the HbA, thereby accounting for its absence and contributing to the predominance of unretarded HbN organic matter in the deep well water. However, these experiments do not yet establish the reason for the absence of the Hl organic matter in the deep well water. The preliminary data indicate that the HbN fraction can support some microbial growth. Thus, it does not appear to be the only fraction present due to its high resistance to microbial degradation. Although the Hl fraction is more mobile than the HbA fraction, the Hl fraction has a higher affinity for the aquifer material than does the HbN fraction. Therefore, it too may be removed by sorption as it is transported over long distances. Thus, the sorption of the other fractions to the aquifer material may be the key to the dominance of the HbN matter in the deep well water.
Acknowledgements

We thank Tom Phelps for providing the core samples, Terry Hazen for providing the Savannah River Site water samples and Harry Boston, Lee Shugart and other members of the staff of ORNL, for providing critical reviews of the manuscript. This research was sponsored by the Subsurface Science Program, Ecological Research Division, United States Department of Energy under contract DE-AC05-84OR21400 with Martin Marietta Energy Systems, Inc. publication number 3468 of the Environmental Sciences Division, Oak Ridge National Laboratory.
References


Table 1. Total and Organic Carbon Concentrations on Deep Aquifer Solids.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Depth Feet</th>
<th>TOC %</th>
<th>Total C %</th>
<th>Color</th>
</tr>
</thead>
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<tr>
<td>G-0</td>
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<td>0.36</td>
<td>0.69</td>
<td>tan, grey</td>
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<tr>
<td>G-4</td>
<td>184</td>
<td>0.014</td>
<td>0.098</td>
<td>yellow</td>
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<tr>
<td>G-5</td>
<td>231</td>
<td>0.052</td>
<td>12.242</td>
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</tr>
<tr>
<td>G-8</td>
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<td>0.20</td>
<td>1.077</td>
<td>dark grey</td>
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<td>G-11</td>
<td>838</td>
<td>0.14</td>
<td>0.216</td>
<td>white</td>
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<tr>
<td>G-12</td>
<td>952</td>
<td>0.16</td>
<td>0.396</td>
<td>light grey/silver</td>
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<tr>
<td>G-13</td>
<td>994</td>
<td>0.35</td>
<td>1.192</td>
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<tr>
<td>G-15</td>
<td>1240</td>
<td>&gt;0.026</td>
<td>0.070</td>
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<tr>
<td>G-16</td>
<td>1325</td>
<td>0.057</td>
<td>0.139</td>
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<tr>
<td>G-19</td>
<td>1300</td>
<td>1.5</td>
<td>6.43</td>
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<tr>
<td>G-20</td>
<td>1432</td>
<td>1.1</td>
<td>3.179</td>
<td>grey</td>
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<tr>
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<td>0.037</td>
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<tr>
<td>G-22-23</td>
<td>muds</td>
<td>0.27</td>
<td>0.65</td>
<td>light brown w/red</td>
</tr>
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Table 2. Original composition of the stock dissolved organic carbon (DOC) in the adsorption experiments, total DOC adsorbed, and percent of the total DOC adsorbed that was hydrophilic (H1), total hydrophobic (total HB), hydrophobic-acid (HbA), and hydrophobic-neutral (HbN).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total mg/kg</th>
<th>%H1</th>
<th>%Total HB</th>
<th>HbA (% of Total HB)</th>
<th>HbN</th>
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<tr>
<td>Stock/DOC</td>
<td></td>
<td>28</td>
<td>72</td>
<td>62</td>
<td>10</td>
</tr>
<tr>
<td>G-6</td>
<td>468.2</td>
<td>1.6</td>
<td>98.4</td>
<td>100</td>
<td>0</td>
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<tr>
<td>G-13</td>
<td>1048.7</td>
<td>23.4</td>
<td>76.6</td>
<td>93</td>
<td>7</td>
</tr>
<tr>
<td>G-21</td>
<td>260.2</td>
<td>35.1</td>
<td>64.9</td>
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Table 3. Results of experiments with different water sources and nutrient additions.

<table>
<thead>
<tr>
<th>Treatments and Bacteria</th>
<th>GT-1 Results</th>
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</thead>
<tbody>
<tr>
<td>GHF, INO, NOP, NON</td>
<td>No Growth in GHF or in NO PO₄</td>
</tr>
<tr>
<td></td>
<td>• MAX 1.7 x 10⁴</td>
</tr>
<tr>
<td>(Pseudomonas sp.</td>
<td>No Growth in GHF or in NO PO₄</td>
</tr>
<tr>
<td>GHF, INO, NOP, NON</td>
<td>• MAX 1.0 x 10⁵</td>
</tr>
<tr>
<td>GT-1</td>
<td>Little Growth in OMM</td>
</tr>
<tr>
<td>OMM, H₁, HbA</td>
<td>• MAX 3 x 10⁶</td>
</tr>
<tr>
<td>Pseudomonas sp</td>
<td>No Growth in GHF MAX on GSS + GHF</td>
</tr>
<tr>
<td>GHF, GSS</td>
<td>• MAX 5 x 10⁶</td>
</tr>
<tr>
<td>Pseudomonas sp</td>
<td>No Growth on GHF MAX on GSS</td>
</tr>
<tr>
<td>GHF, GSS</td>
<td>• MAX 2 x 10⁷</td>
</tr>
</tbody>
</table>

*Georgetown Hobcaw Field (GHF), oxidized mineral media (OMM), hydrophilic acid (HL), hydrophobic-acid (HbA), hydrophobic-neutral (HbN), Georgetown stream water (GSS).

*Inorganic nutrients (INO), inorganic nutrients without phosphorus (NOP), inorganic nutrients without nitrogen (NON).
Figure 1. Isotherm for sorption of dissolved organic carbon (DOC) on sediment from the C10 hole at constant ionic strength (NaCl, I=0.1), pH (initial pH=6.0), and temperature (298°K). See Table 1 for description of sediments.
Figure 2. Growth of the *Corynebacterium* sp. in water containing different sources of DOC.
A. Growth in water samples from all three deep wells at the Savannah River Site.
B. Growth in groundwater samples from the near surface (G.H.F.) with and without added nutrients.
C. Growth in surface water (G.S.S.W.) and shallow subsurface water (G.H.F.).
D. Growth in H1 and HbA fractions of the surface water.
Phylogenetic Diversity Among Subsurface Microorganisms
David A. Stahl, Rebekah Key, and David L. Balkwill
University of Illinois and Florida State University, FL.

Abstract
Comparative 16S rRNA sequencing was used to infer the phylogenetic diversity among a study group of nine subsurface isolates. All organisms were isolated from deep subsurface sediments obtained at five different depths from drilling site P29 at the Department of Energy's Savannah River Plant near Aiken, SC (Middendorf formation). Depth of isolation and culture numbers were: 181 m, C0484; 187 m, C0528, C0553, C0564; 193 m, C0570, C0580; 200 m, C0651, C0652; and 213 m, C0682. All but one (C0484) were derived from transmissive, sandy, aquifer-bearing sediments. Seven were isolated on a dilute Peptone-Tryptone-Yeast extract-Glucose (1% PTYG) medium and the remaining two (C0528 and C0678) on a more concentrated medium. Five of the isolates (C0484, C0528, C0553, C0564 and C0651) comprised a closely related assemblage of organisms, including Arthrobacter globiformis, that were affiliated with the high G+C subdivision of the Gram-positive bacteria. All share greater than 90% sequence similarity. This association was also reflected by similar physiological profiles (API Rapid NFT system). The remaining four isolates were members of the beta subdivision of the purple bacteria. Isolates C0580 and C0652 (approximately 80% sequence similarity) were grouped with the alpha subdivision. No organisms were specifically related to these later four isolates (at greater than 90% sequence similarity). This sequence information should serve for the initial design of hybridization pres for studies of environmental abundance and characterization of additional subsurface isolates.
Particle-Size Relationship to Heterotrophic Microbial Community Characteristics of Two Deep Subsurface Samples

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Colorado State University, Fort Collins, CO.

Abstract

Two deep subsurface samples (GS9 at 213 m and GS20 at 437 m) from Allendale, SC were analyzed for heterotrophic microbial community responses in relation to particle-size fractions. Four particle fractions were obtained from each sample by using gravity and centrifugation, with microbial responses differing not only between the subsurface samples but also between fractions within each of the samples. Aerobically incubated subsurface sediments showed significantly greater microbial numbers than anaerobically incubated sediments (p=0.001), with GS9 exhibiting the largest difference in viable populations between the two treatments. It was also found that aerobic and anaerobic organisms from the sandier material (GS9) displayed a greater number of high-nutrient responsive bacteria while the sediment with a higher percentage of clay (GS20) contained a greater relative population of low-nutrient responding microorganisms. The larger particle fractions from both GS9 and GS20 had larger populations of high nutrient bacteria, while smaller particle fractions showed a larger response on low-nutrient media. Bacterial isolates from these fractions were examined for differences in substrate utilization patterns of sugar and tween compounds, miscellaneous organic compounds and organic acids, and amino acids using Biolog plates. Substantial differences in carbon-source utilization occurred not only between samples from different depths, but also between fractions of a given sample. These studies emphasize the necessity of understanding physical properties of subsurface material, such as particle characteristics and its relationship with the distribution and biochemical characteristics of microbial communities within deep subsurface environments.
Introduction

The deep subsurface has been found to have a diverse and active microbial community. These organisms play an important role in nutrient cycling and can potentially contribute to the transformation of toxic compounds and bioremediation of many anthropogenic pollutants. Many geological processes, previously considered to be abiotic, are being reevaluated to better understand possible involvement of subsurface organisms in these transformation processes.

Hattori and Hattori have described the relationship between soil microorganisms and the terrestrial physical environment as being quite complex, with neighboring microsites varying in (O2), pH, Eh, percent moisture, and many other variables. In addition, investigation of soils showed that marked differences in nutrient distribution can occur between different particle fractions. To our knowledge, no studies involving particle characteristics and microbial communities of deep subsurface materials are available. In this study, the metabolic characteristics of microorganisms isolated from four particle-size fractions of two deep subsurface sediments were examined, along with the examination of these particle fractions by scanning electron microscopy.

Materials and Methods

Sample Collection and Storage. Subsurface sediment samples were obtained from the C10 hole of the Department of Energy’s Microbiology of the Deep Subsurface project located at Allendale, SC. Aseptic sampling procedures were used during handling and processing of the core. After arrival at Colorado State, the samples were placed in an oxygen-scavenged anaerobic hood (Bactron I Anaerobic Hood, Anaerobe Systems, San Jose, CA) with an atmosphere of N2:H2:CO2 at 85:10:5 and kept at room temperature. The use of a reducing atmosphere was based on the observation of methane and hydrogen in this type of coastal sediment formation, and the sensitivity of these samples to oxidation even with trace levels of oxygen present. Two samples were investigated in this study: GS9 from 231 m below ground level, and GS20 from 437 m.

Sample Fractionation. Sample fractionation for the aerobic studies was performed by placing 0.5 g of sediment into 4.5 ml of sterile deionized water, following the method of Genrich and Bremner with alterations. The sample was dispersed by shaking at 300 rpm for one hour. For analysis of anaerobic populations, the sample was similarly dispersed by using a Virtis S34 tissue homogenizer with a standard microblade (Virtis cat. # 147140). To achieve similar mixing, the sediment was blended for three minutes at the display setting of 50. Aerobic and anaerobic samples were then fractionated in a vertically-held microcolumn (25 x 1 cm hollow glass tubing). Four fractions were collected: F1, 1 x G for 10 minutes; F2, 1 x G for 24 hours; F3, pelleted sediment at 1970 x G for 10 minutes; and F4, supernatant from F3 centrifugation.

Bacterial Enumeration and Characterization. Fractions were diluted and inoculated by spread plating onto PTYG medium (10 g of glucose, 5 g of trypticase soy broth, 10 g of yeast extract, 5 g of bacto peptone, and 15 g of bacto agar per liter) and 1% PTYG medium. These two media types were used to culture higher and lower
level nutrient responding organisms, respectively. Plates were incubated aerobically and anaerobically at room temperature for three weeks before enumeration. Representative isolates of different colony types were characterized (colony morphology, size, pigmentation and Gram stain), purified and preserved with 5% dimethyl sulfoxide (DMSO) in PTYG or 1% PTYG. Attempts were also made to examine these materials by direct microscopic procedures using the technique of Kogure et al. 13

Isolate substrate utilization patterns were evaluated using Biolog Microplates (Biolog Inc., Hayward, CA). Isolated colonies were uniformly suspended by rolling a sterile cotton swab over a colony and twirling the swab in 20 ml of sterile 0.85% saline (to obtain a suspension within a specific density range). Then 150 µl of the cell suspension was inoculated into each well of the microplate and incubated at room temperature. Substrate utilization patterns were read with a Dynatech MR600 microplate reader and analyzed for differences between fraction utilization patterns of sugars and tween compounds, miscellaneous organic compounds including organic acids, and amino acids.

Results

Bacterial Enumeration. The viable populations of aerobic and anaerobic bacteria in GS9 and GS20 includes microorganisms capable of growth on PYTG and dilute PTYG (DPTYG). Average counts per fraction ranged from 1.89 to 7.00 log CFU for GS9, while GS20 contained 4.19 - 8.51 log CFU.

Both aerobic and anaerobic bacteria from GS9 showed higher viable populations on DPTYG medium in the smaller particle fractions, while the larger particle fractions gave greater numbers on the PTYG medium. The GS20 sample displayed a more equal distribution between the organisms growing on both media types with no obvious correlation being shown with particle size. When all fractions were considered, GS9 showed larger numbers of high nutrient organisms while GS20 contained more organisms on the lower nutrient medium. Both samples demonstrated greater numbers of microorganisms under aerobic than anaerobic incubation, but GS9 showed a significantly greater difference between aerobic and anaerobic bacteria than GS20 (p<0.001).

Attempts to utilize microscopic procedures were not successful due to the low levels of bacterial cells in relation to background particle levels.

Bacterial Characterization. Results from the Biolog microplate analysis demonstrated different carbon utilization patterns between fractions within a sample and also between samples from different depths. Fractions from GS9 showed major differences in the utilization of sugar and tween compounds, and of amino acids, while GS20 fractions demonstrated substantial differences of all three substrate groups. These varied substrate utilization patterns indicate that bacterial communities with differing metabolic characteristics occur in association with different particle fractions of deep subsurface sediments.
Discussion

Most investigators who examine particle-associated characteristics utilize chemicals or harsh physical dispersion and fractionation procedures. These approaches can influence microbial responses within a fraction by either direct contact with chemicals used during fractionation, or by dissociation of the microorganisms from particles by violent physical fractionation. The procedure used in this study, based on prior work by Genrich and Bremner, utilized no chemicals or harsh physical means to fractionate the sediment.

It has been assumed in the past that microbial populations decrease in numbers with an increase in depth. Many studies, as well as this one, have shown that depth, of itself, is not the major factor influencing the presence and characteristics of microbial communities in terrestrial environments. However, the type and amount of nutrients present as well as the properties of the physical environment do influence the presence and characteristics. GS9 aerobes and anaerobes, when cultured on high- and low-nutrient media, demonstrated that low-nutrient responding organisms were present at higher levels in the smaller particle fractions and that high-nutrient colonies were more dominant in the larger particle fractions. In GS20, these two types of microorganisms were more evenly distributed between the larger and smaller fractions. GS20 also contained higher numbers of bacteria in the smallest fraction and fewer in the largest fraction than GS9.

This can be explained in terms of particle size distribution. The two samples were markedly different, with GS9 being >80% sand while GS20 was >47% clay and <16% sand (personal communication J. M. Thomas, Battelle NW Laboratories). The sandier the sample, the greater the potential permeability of the sample. This allows an increased flux rate for soluble nutrients and ions, resulting in a higher percentage of high nutrient organisms in GS9. GS20, in contrast, contained a greater amount of clay. Clay particles not only pack tighter, but they also have a greater adsorption constant than sand so that nutrients and ions are not freely available to microorganisms. This will lead to a shift in organisms capable of growth under lower nutrient conditions. These assumptions are based on considerations presented by Hattori and Hattori and are supported by this investigation.

Because the core sample’s chemical profile showed that a majority of the material was deposited in an oxidizing environment, it is not surprising to find more aerobes than anaerobes in these samples. The fact that GS20 demonstrated a lesser degree of difference between aerobes and anaerobes than GS9 is believed to be due to the fact that clays restrict the movement of not only liquids but also gases such as O₂, H₂ and CH₄, thus giving a greater predominance of anaerobic microenvironments in GS20.

Conclusion

The substrate utilization patterns, based on the Biolog analysis, demonstrated that the microbial community of a given particle-size fraction was significantly different from those of other fractions. The differences in the capability of the bacterial isolates from each fraction to utilize sugar and tween compounds, miscellaneous organic compounds and amino acids suggests that differences in available
substrates and substrate flux rates may be occurring in the environment of the
different particles. Although the Biolog analyses have not been processed to yield
isolate identifications, it would appear that different microbial communities are
present in the different samples and sample fractions. Because these organisms seem
to be distinctive for each sample, vertical movement of the microorganisms might
be restricted and thus contamination at each depth from bordering areas may be
limited. This study supports the suggestion of Fliermans and Balkwill\(^6\) that indig-
enous organisms were probably concurrently deposited with the sediments at the
time of deposition.

These investigations suggest that to best understand the microbial communities
and their metabolic characteristics in these materials, it will be necessary to consider
particle size ratios and particle characteristics. This level of resolution will provide
information useful in basic studies of subsurface microbial populations, and will also
assist in bioremediation efforts.

**Acknowledgements**

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helped support these studies.
References


Table 1. Microbial populations of fractionated samples of GS9 and GS20.

<table>
<thead>
<tr>
<th>Fraction Number</th>
<th>Fraction Weight</th>
<th>Log CFU 1%PTYG</th>
<th>Log CFU PTYG</th>
<th>Fraction Weight</th>
<th>Log CFU 1%PTYG</th>
<th>Log CFU PTYG</th>
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</thead>
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<tr>
<td><strong>Aerobic</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F1*</td>
<td>0.35 g</td>
<td>5.56</td>
<td>5.42</td>
<td>0.56 g</td>
<td>4.57</td>
<td>4.09</td>
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<td>4.20</td>
<td>6.25</td>
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<td>5.94</td>
<td>6.51</td>
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<td>6.82</td>
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<td><strong>Anaerobic</strong></td>
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<td></td>
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</tr>
<tr>
<td>F1</td>
<td>0.35 g</td>
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<td>2.96</td>
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</tr>
<tr>
<td>F2</td>
<td>0.20 g</td>
<td>4.33</td>
<td>1.54</td>
<td>0.18 g</td>
<td>4.40</td>
<td>3.50</td>
</tr>
<tr>
<td>F3</td>
<td>0.20 g</td>
<td>1.78</td>
<td>2.97</td>
<td>0.24 g</td>
<td>3.44</td>
<td>3.27</td>
</tr>
<tr>
<td>F4</td>
<td>0.25 g</td>
<td>4.59</td>
<td>4.88</td>
<td>0.02 g</td>
<td>4.44</td>
<td>4.44</td>
</tr>
</tbody>
</table>

F2 = 1 x G for 24 hours.
F3 = pellet from 1970 x G for 10 min.
F4 = supernatant from F3 centrifugation.
Table 2. Physiological characterization of bacterial isolates from fractionated samples of GS9 and GS20 by use of Biolog. Results expressed as percent utilization of various substrate groups.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Substrate group</th>
<th>Percent utilized by fraction</th>
<th>Percent utilized by all isolates</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Aerobic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GS9</td>
<td>sugars and tween compounds</td>
<td>26.4</td>
<td>9.1</td>
</tr>
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<td></td>
<td>misc. organic compounds, incl. organic acids</td>
<td>24.2</td>
<td>7.0</td>
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<td></td>
<td>amino acids</td>
<td>17.3</td>
<td>38.0</td>
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<td>51.7</td>
<td>45.0</td>
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<td>20.2</td>
<td>21.5</td>
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<td>amino acids</td>
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</tr>
<tr>
<td>GS9</td>
<td>Not Determined</td>
<td></td>
<td></td>
</tr>
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<td>GS20</td>
<td>sugars and tween compounds</td>
<td>12.1</td>
<td>10.6</td>
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<tr>
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<td>misc. organic compounds, incl. organic acids</td>
<td>7.0</td>
<td>8.1</td>
</tr>
<tr>
<td></td>
<td>amino acids</td>
<td>0.0</td>
<td>0.0</td>
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</tbody>
</table>
Can Groundwater Bacteria Facilitate the Transport of Hydrophobic Aromatic Compounds?

Roland Linqvist

University of Lund, Sweden.

Abstract

Bacteria, if mobile, could potentially facilitate the transport of aromatic compounds through saturated soil by acting as a dissolved organic and living phase. To study the partitioning of hydrophobic compounds to bacteria, batch sorption isotherms with labeled hexachlorobenzene or DDT, and groundwater bacteria, were run. The sorption and desorption data were fitted atop a linear sorption isotherm. The hydrophobicity of the bacteria was measured using hydrophobic interaction chromatography. Finally, a soil/water partition coefficient for a bacterial strain was quantified, and the transport of this strain was quantified. The transport of this strain through a saturated soil column was studied as well. The partition coefficient for hexachlorobenzene to bacterial biomass of one of the strains was much lower than the $K_{ow}$ would suggest. Autoclaved bacteria of this strain sorbed more hexachlorobenzene and DDT than living cells, and were also more hydrophobic than living cells. Bacteria moved through the soil column with the same velocity as the water, even though the soil/water partition coefficient was as high as 12.

No paper submitted.
Groundwater Systems of the Murray Basin, Southeastern Australia
John Baud, W. R. Evans, and J. R. Kellett
Groundwater Branch, Bureau of Mineral Resources, Canberra, Australia.

Abstract

The Murray Basin, which extends over 300,000 km$^2$ of southeastern Australia, is a shallow, saucer-shaped, intracratic depression. It is infilled with approximately 200-600 m of Tertiary unconsolidated sediments and sedimentary rocks which contain several major regional layered aquifer systems separated by low permeability layers. Groundwater flow within the Murray Basin is towards the area of deepest basement subsidence in the central west. However, lateral flow through the Renmark Group and Pliocene Sands aquifer systems is disrupted by a mid-Tertiary, low permeability barrier which extends through the center of the Basin in an arcuate belt, and by aquifer thinning over concealed basement ridges. The thin cover of sediments is largely saturated with ground water (4.6 x 10$^{12}$ liters) and has little additional storage capacity.

Groundwater discharge is either via seepage to the lower reaches of the Murray River, which provides the only outlet from an essentially closed groundwater basin, or by direct evaporation in places where the water table reaches the ground surface. The latter has led, during the last 0.5 Ma of arid to semi-arid climate, to the development of saline groundwater discharge lake complexes. Basin aquifer systems generally exhibit an “inverted salinity profile”, the most saline waters occurring toward the top of the sequence, and fresher waters occurring at the bottom. Laterally, salinities increase downflow toward the Basin depocenter, ranging from $<$100 mg/l of total dissolved solids (TDS) in the marginal recharge zones up to approximately 300,000 mg/l in some groundwater discharge zones. Land clearing and irrigation have accentuated the salinity problem and have contributed to the rising water tables found over most of the Basin. Microbiological investigations of the groundwaters and subsurface sediments of the Murray Basin and other Australian sedimentary basins are few, and have been concerned primarily with nitrate contamination, borehole corrosion and encrustation, and microbiologically enhanced oil recovery.
Introduction

The Murray Basin is a roughly circular, shallow sedimentary basin which occupies 300,000 km² of inland southeastern Australia (Figure 1), an area comparable to the state of New Mexico or to the nation of Italy. The catchment area, which includes the surrounding fractured rock aquifer system, is approximately 450,000 km² in area. Surface water flow and discharge from the Basin is dominated by the Murray River and its larger tributaries (Figure 1). The Murray Basin is of considerable economic significance to Australia, because it contains much of Australia’s irrigated agricultural land, cropland production, and sheep, lamb, wool and cattle production. In total, it contains a major proportion of the nation’s natural resource productivity. The sustainability of this regional economic activity and the quality of the natural environment is under increasing threat from degradation wrought by salinization of formerly productive land due to rising groundwater tables, particularly in major irrigation areas.⁶

Significant areas of the Murray Basin rely on groundwater for irrigation and for livestock and human consumption. The salinity problem is ground water-related,¹⁵ which has inexorably led to the realization that an essential prerequisite to its solution is an understanding of the hydrogeological framework for the regional groundwater aquifer systems.¹⁶ Considerable progress has been made in documenting and analyzing the regional geology, sedimentology, hydrology and hydrochemistry of the Murray Basin²,⁵,⁸,¹⁵,¹⁶,²² and a synopsis of these achievements forms the basis of this paper. By contrast, microbiological investigations of the groundwaters and subsurface sediments of the Murray Basin by the Bureau of Mineral Resources (BMR) are just commencing and discussion of this aspect relies on consideration of the possibilities for such research in the light of known or probable environmental perturbations. These are placed within the context of other such activities taking place within other Australian groundwater systems.

Geological Framework

Understanding the relationships between the development of surface salinity problems and the distribution of surface discharge features, groundwater flow, recharge and aquifer geometry requires a knowledge of the subsurface structural and stratigraphic framework of the basin.⁵ The geometry and stratigraphic development of the Murray Basin, including the location of major sedimentary depocenters, is determined by the tectonic and topographic framework of the underlying pre-Tertiary basement rock.⁹ Though the basement is now tectonically stable, subtle differential subsidence of arcuate pre-Tertiary terranes within the craton, together with intersecting fold and fault trends and fracture sets, have produced variations in the extent of sediment accumulation across the basin.²,²²

Depositional history, the evolving stratigraphic framework, and paleoenvironmental interpretation of the Murray Basin sediments have been documented in considerable detail²,³³ but the essentials are as follows: despite the considerable areal extent of the Murray Basin, Tertiary sediment accumulation has provided only an extremely thin veneer over the pre-Tertiary basement because rather than a “conventional” basinal succession, the Murray Basin developed through several partial marine incursions from the southwest, which produced
shallow epicontinental seas over a topographically flat, low-lying platform. During
the past 60 Ma there have been three major depositional sequences which occurred
during the Paleocene-Lower Oligocene (mainly Eocene), Oligocene-Middle Mio-
cene, and Upper Miocene-Pliocene. Each sequence includes laterally extensive,
shallow-marine to paralic sediments in southwestern to central areas (correlating
with major periods of high global sea level), which intercalate with the fluvio-deltaic
silt, sand, clay, and carbonaceous sediments predominating in the east and north.

These stratigraphic units form the regional aquifer systems, confining layers,
and permeability barriers to groundwater flow (Figure 2). Fluvio-lacustrine sedi-
mentation continued intermittently throughout the Quaternary in the east and north
of the Murray Basin, giving rise to the modern Riverine Plain. To the west, Tertiary
sediments are partly overlain by a thin veneer of Pleistocene lacustrine clay and
minor dolomitic limestone which was deposited in Lake Bungunnia, a Pleistocene
megalambo ponded within the Murray Basin some 2.5-0.7 Ma ago. During the period
0.7-0.4 Ma, Lake Bungunnia emptied and dried, coincident with the onset of seasonal
aridity in southern Australia and the consequent development of salinization and
present-day aeolian features in the Murray Basin.

On the basis of surface geomorphology and structurally controlled depocenters,
the Murray Basin can be divided into the following two sub-regions: an eastern zone
underlying the Riverine Plain and a western zone underlying the Mallee region
(Figure 1). Stream leakage recharges the deeper, confined and the shallow,
unconfined aquifer systems for a distance of up to approximately 100 km into the
Basin. Recharge to the unconfined aquifers also occurs by rainfall infiltration over
the Basin surface and by upward leakage, through confining layers, from the deeper
aquifer systems. Groundwater flow is internal and directed towards the central-west
depocenter of the Basin (Figures 2, 3). Sediments are mostly water-saturated and
excess groundwater discharges either to the Murray River, which provides the sole
conduit for the transport of ground water and salts out of the Basin, or directly to the
land surface, where evaporation increases the concentration of salts that reflux into
the underlying aquifers.

The major aquifers of the Murray Basin are the Renmark Group, the Murray
Group, Pliocene Sands, and the Shepparton Formation (Figure 2). The major
impediment to regional flow is the mid-Tertiary low-permeability barrier (Geera
Clay), which represents the maximum extent of the Late Oligocene-Miocene marine
transgression into the Murray Basin. The Geera Clay extends in an arc approximately
100 km wide through the centre of the Basin, where it provides a barrier to lateral
troughflow within the Renmark Group aquifers and a low-permeability base to the
Loxton-Parilla Sands of the Pliocene Sands aquifer.

The Murray Group aquifer comprises mid-Tertiary marine limestone and
calcarenite. It is the most intensively exploited regional aquifer in the Murray Basin.
The Shepparton Formation aquifer, which lies at the top of the Tertiary Murray Basin
sediments under much of the Riverine Plain in the east, is the unit most susceptible
to damage by waterlogging because its soils are intensively irrigated by large
volumes of surface water. Ground waters in the uppermost aquifer systems have become increasingly saline as salts were trapped within the Basin during the several periods of high groundwater levels over the past 0.5 Ma of arid to semi-arid climatic conditions. For example, the Pliocene Sands aquifer (consisting mostly of Loxton-Parilla Sands), which forms the unconfined aquifer in western areas (Figures 2, 3c), generally contains highly saline groundwaters (approximately 40,000 mg/l; Figure 5) and is dotted with numerous widespread saline groundwater discharge zones.

Distinctive lateral and vertical salinity trends are evident (Figures 4, 5). Laterally, salinities generally increase along flow lines toward the Basin depocenter, ranging from <100 mg/l TDS in marginal recharge zones up to approximately 300,000 mg/l TDS in some groundwater discharge zones. However, irregularities do occur in this regional trend (e.g., the acceleration in the rate of groundwater salinity increase near the western edge of the Riverine Plain). Vertically, the Basin aquifer systems exhibit an inverted salinity profile in which fresher waters occur at the bottom of sedimentary sequences while the most saline waters are present at the top. Preliminary chlorine-36 measurements in the Murray Basin have shown significant differences in $^{36}$Cl/Cl ratios between saline discharge zones and the deeper Murray Group limestone ground waters. The data are considered to indicate that recent atmospheric accession is a major source of salts to the latter, through significant rainfall recharge to the aquifer, but not to the discharge zones where salts concentrated by evaporation reflux into the underlying aquifers.

Before European settlement, the Murray Basin existed under a hydrological equilibrium in which the mean water table was considerably lower than now, and river discharge, though highly variable, was on average less than now. The margins of the Basin were forested and admitted less recharge from rainfall than cleared areas do today. Agricultural development, in particular the clearing of trees over large areas, has significantly perturbed this hydrological equilibrium. As a consequence of this, and also of intensive irrigation, regional water tables have risen dramatically. Accompanying this rise has been a remobilization of salts and evaporative concentration of groundwater in areas where the capillary fringe of the water table, or the water table itself, intersects the ground surface.

Salinization of the Murray Basin is occurring both in irrigation districts and at sites of former groundwater discharge. Indeed, the paleoenvironmental record shows that salinization in the Murray Basin has been widespread during the past 0.5 Ma and considerably more severe than that being presently experienced.

A record of past fluctuations in groundwater levels is evident in the surficial Quaternary geology of the Basin. Many currently active and fossil groundwater discharge sites have developed as a consequence of subtle tectonic subsidence together with the flow disruption at subsurface permeability barriers. The latter has occurred where underlying aquifers were substantially thinned over concealed basement features or by lateral changes in lithology. For example, the Hatfield discharge complex, an arcuate belt of presently inactive saline lake and lunette complexes on the western Riverine Plain, overlies an area where the Renmark Group
aquifer system thins significantly (from >300 m to <50 m), due to the structural influence of upfaulted but concealed basement ridges. Groundwater is forced to flow to the southwest where it is further impeded by the thick, mid-Tertiary, low-permeability barrier of the Geera Clay.5,7

By contrast, many of the salinas and stranded lake deposits of the western Murray Basin occur in low-lying areas of tectonic subsidence. The deeper Renmark Group aquifer system is fully saturated and groundwater flow toward the central west is over the thick (120-160 m), concealed permeability barrier of the Bookpurnong beds and the Geera Clay, and into the overlying near-surface Pliocene Sands aquifer system (Figure 2). Groundwater flow is sufficient to maintain the water table above the land surface within low-lying areas, where it forms the presently active discharge lake complexes,5,16 that are effective14 “windows” into the Pliocene Sands aquifer.

Sustainable, regional economic activity in the Murray Basin will depend upon adequate salinity management strategies since the presence of large volumes of near-surface saline water is inevitable. Evans14 points out that an effective saline water disposal strategy for the entire Murray Basin is of the highest priority in dealing with the salinization problem. Detailed economic and environmental analysis suggest that reuse and controlled outfall (often with holding basins) via the Murray River are the most attractive options for low salinity water. Evaporation basins, though somewhat controversial, become more economic as salinity increases. Environmental effects are becoming a key factor in discriminating between available options.14

Subsurface Microbiology in Australian Sedimentary Basins

A major focus of Murray Basin research at the moment is the groundwater-related salinization of land and water resources with its consequent deleterious effects on the regional and national economies. Though microbial activities can be expected to continue in aqueous habitats ranging in salinity from that of fresh water to that approximating halite saturation23 there is evidence suggesting that increasing salinities beyond, say approximately 120,000-150,000 mg/l, may significantly slow microbial nutrient recycling.36 The capacity of aquifer microbial populations to degrade xenobiotic compounds such as pesticides and herbicides, leaching through the vadose zone to the water table, may also be inhibited as salinity increases, with resultant toxin accumulation. Increasing salinities will also increase sulfate levels and may stimulate significant bacterial hydrogen sulfide generation and metal precipitation, while methanogenic bacteria may be inhibited under these conditions. So, in addition to the direct salinity problem, secondary effects arising from microbial stimulation or constraint may also impinge on groundwater quality.

Paradoxically, while it is the quality of the water recovered from aquifer systems that is of overriding importance to the end user, it seems probable that in most cases this quality is in fact determined by the activity of microbial populations resident on the aquifer surfaces past which the ground water flows.17,18 However, the abundance of unattached bacteria appears to increase in the presence of organic contamination.18 In a recent summary of groundwater contamination incidents in
Australia, Jacobson and Lau\textsuperscript{19} found that shallow, unconfined aquifers underlying regions of intensive urban, industrial, or agricultural development were most at risk. They reported that several important regional aquifers were affected by a range of contaminant sources including industrial effluent, sewage and landfill leachate. The range of problems is comparable to those affecting groundwaters in the United States and Europe,\textsuperscript{29} though the scale and severity of Australian groundwater contamination would appear to be considerably less. Perhaps as a consequence of this, the microbiological aspects of groundwater contamination, both in terms of bioremediation and of toxicity to biogeochemical cycling, have received very little attention in Australia.

As in other natural environments, microorganisms may be expected to play an important role in the biogeochemical cycling of the nonconservative elements (C, S, N, P, H, and O), and metals such as Fe and Mn, within groundwaters and subsurface sediments.\textsuperscript{1,34} Despite the significant advances in our understanding of the geology and hydrology of Australian sedimentary basins,\textsuperscript{24} the populations and in situ activities of microorganisms resident in various Australian groundwater aquifers are essentially unknown, as is their impact on aquifer geochemistry under either pristine or perturbed conditions. A limited number of prior or current studies have been brought to our attention.

Whelan and Parker\textsuperscript{35} investigated bacterial (fecal coliforms) and nutrient pollution of a shallow groundwater table by septic tank effluent and concluded that this might pose a serious problem in coarse, sandy sediments with a high water table. Artificial recharge to the shallow, unconfined aquifers of the Swan Coastal Plain (Perth Basin), resulting from land application of treated waste waters, has been investigated with respect to the removal of nitrogen by bacterial nitrification-denitrification during infiltration through the vadose zone\textsuperscript{27} and to the movement and survival of viruses.\textsuperscript{20,21} The survival and growth of contaminant bacteria and phage in groundwaters is being investigated by S. Ragusa and colleagues (CSIRO Division of Water Resources), who have also been studying nitrification and denitrification within nitrate-contaminated groundwaters of the Gambier Limestone aquifers of the Otway Basin in SE South Australia (S. Ragusa, S. B. Richardson and P. J. Dillon, in prep).

The occurrence of nitrate-rich groundwaters is widespread in Australia, invariably at shallow depths (<100 m) and commonly where the unsaturated (vadose) zone exhibits high hydraulic conductivity and heterogeneity, and prominent fracture porosity.\textsuperscript{25} Anthropogenic recharge includes both point (animal and industrial waste, and sewage effluent) and diffuse (agricultural fertilizers and leguminous pastures) sources, most commonly as organic-N or NH\textsubscript{4}-N, which are then metabolized to nitrate by nitrifying bacteria.\textsuperscript{1} In the arid zone of central Australia, there is evidence that sources of high groundwater nitrate concentrations include free-living, nitrogen-fixing cyanobacteria\textsuperscript{32} and possibly symbiotic nitrogen-fixing bacteria associated with the roots of native flora.\textsuperscript{25} It seems probable that, once produced, nitrate removal from groundwaters is constrained by very low concentrations of the dissolved organic carbon required by denitrifying bacteria.
Corrosion and encrustation of groundwater bores looms as a major water supply problem in several Australian sedimentary basins and Dr. Reg Walters (State Water Lab, South Australia) is currently leading a project to determine the environmental controls on borehole fouling by iron bacteria in the Gambier Limestone aquifers of the Otway Basin. Thermophilic sulfate-reducing bacteria, and other heterotrophs including *Thermus* sp., have been isolated from Great Artesian Basin bore waters by Dr. B.K.C. Patel (Griffith University, Brisbane) and are currently under study in his laboratory. A variety of thermophilic anaerobic heterotrophs have been recovered from 2000 m deep, oil-bearing strata of the Surat Basin by Dr. K. McLean and G. Grassia (University of Canberra) in the course of investigations related to the microbially enhanced recovery of oil (MEOR). These MEOR studies were initiated by Bubela and are being continued, in collaboration with private industry, under the supervision of A. J. Sheehy at the University of Canberra.

**Acknowledgements**

We thank G. Grassia, K. McLean, B.K.C. Patel, S. Ragusa, and R. P. Walters for permission to discuss their current research. This paper is published with the permission of the Director, Bureau of Mineral Resources, Canberra.
References


Figures

Figure 1. Locality map of the Murray Basin.

Figure 2. Aquifer systems of the Murray Basin.
Figure 3. Flow regimes in major Murray Basin aquifer systems.
Figure 4. Cross-section showing groundwater salinity distribution along and approximate east-west flow line.

Figure 5. Map of groundwater table salinity for the Murray Basin.
Abstract

Bacterial isolates from deep sediment samples from three sites, P24, 28, and 29 at the Savannah River Site (SRS), near Aiken, SC were studied to determine their microbial community composition and genetic structure by total DNA hybridization and mol %G+C. Standard phenotypic identification underestimated the bacterial diversity at the three sites, since bacteria with the same phenotype have different genetic composition. The mol %G+C of deep subsurface bacteria ranged from 20 to 75%, with more than 60% and 12% of the isolates tested showing values similar to the Pseudomonas sp. and Acinetobacter sp., respectively. No significant difference was found between the average mol %G+C content of isolates from different sites, which suggests that the same bacterial genus was the most abundant in all sites. Total DNA hybridization and mol %G+C analysis of deep sediment bacterial isolates showed that each formation was comprised of different microbial species. No isolates from deeper formations showed the same genetic structure of isolates from upper formations, although geological age of the sediment appeared to limit the diversity of bacterial genera. The Pseudomonaceae and Acinetobacteraceae were the only bacterial families found in deeper formations, suggesting a long period of adaptation to the environmental conditions of the deep subsurface does exist.
Introduction

Deep subsurface microbial communities have been found at the Savannah River Site, near Aiken, SC, that have shown higher bacterial densities and diversity than previous studies done at shallow depths. A diverse microbial community was found across the geological profile, including very different physiological groups, (i.e., methanogens, sulfate and nitrate reducers, and heterotrophic bacterial populations). Moreover, the diversity of the heterotrophic bacterial populations did not decrease with depth. A large number of different plasmids were found that were encoded for antibiotic and metal resistance. Plasmids larger than 200 kb were most frequently found at deeper aquifers. Plasmid frequency increased with greater depth suggesting a different bacterial composition across the depth profile. A great majority of these communities were aerobic or facultative chemoheterotrophic bacteria, and most of them were oxidative (82%) rather than fermentative (4%). Considerable bacterial diversity was observed even within defined geological formations based upon the Rapid API-NFT test.2

The identification of deep subsurface bacteria using standard biochemical assays had brought out a number of unidentified strains from the three sites already sampled. However, several studies have shown that phenotypic tests alone are not an accurate way to identify and separate bacterial populations in natural environments (i.e., those bacteria that are identified by these assays may not be identified correctly). Accurate identification, detection, and discrimination of bacterial communities in nature have been done by molecular techniques such as nucleic hybridization and the mol %G+C. Microbial ecologists and bacterial taxonomists have used the mol %G+C and DNA hybridization for the classification of bacterial isolates from clinical and natural environments.

Since significant microbial populations occur in deep subsurface sites, it is very important to know the composition and genetic structure of these microorganisms. Moreover, they will play a significant role in the transformation and mobilization of different pollutants in the deep subsurface and in the formation of the geological layers.

A number of studies have been reported regarding the phenotypic diversity of subsurface bacteria, but none have presented information about the genetic structure and diversity of these communities. No effort has been given to assessing genetic changes across a depth profile as well. Using molecular analysis (e.g., mol %G+C and total DNA homology), the genetic structure and diversity of deep subsurface bacteria were studied across a geological profile, and the phenotypic identification and genetic content of these communities were compared in order to understand the community structure of deep subsurface bacterial communities.

Materials and Methods

Study site. Subsurface sediments were obtained at three different sites, (P24, P28, and P29) at the Savannah River Site, near Aiken, SC. For a more complete description of the site, sampling procedures, and geological profiles, see Balkwill et al. and Fliermans and Balkwill.
Bacteriology. Deep subsurface bacterial isolates used in the genetic studies were provided by Dr. David Balkwill from Florida State University, Tallahassee, Florida, from the Subsurface Microbiology Culture Collection (SMCC). For further details on the isolation of deep subsurface bacteria and media used, see Balkwill et al. After receiving all of the isolates, they were analyzed again using the API-NFT Rapid tests to corroborate previous identification.

DNA melting point. More than 65 bacterial isolates were randomly chosen from all three sites across the geological profile and their DNA was isolated using a variation of the Marmur technique as described by Bermudez and Hazen. A preparation was considered pure when it had an optical density at 260 nm/optical density at a 280 nm ratio of 1.8. The thermal denaturation method was used for measuring DNA base composition. Samples were prepared as described before. Standard DNA’s (e.g., Escherichia coli (ATCC 11773) and Pseudomonas aeruginosa (ATCC 9721)) were used in each run as a reference standard. The melting profiles were determined with a 300 DMS Varian UV spectrophotometer (Varian Instrument Co., Texas) and connected to a thermal program from an Apple IIe computer. The mol %G+C of each bacterial isolate was determined by using the Marmur and Doty equation.

DNA homology. Whole chromosomal bacterial DNA was labeled in vitro using a nick translation kit (New England Nuclear, Boston, MA) with H-thymidine (specific activity: 75 Ci/m mol). The DNA probe was precipitated as described by Crouse and Amorese. Specific activity of the nucleic acid probe was measured by the method of Maniatis et al. The probe was fragmented by sonication, denatured at 100°C for five minutes, and rapidly chilled on ice.

Denatured DNA from bacterial isolates was immobilized as previously described. DNA hybridization and washings were done as described before by Bermudez and Hazen. After being washed, individual membranes were removed from the wells in the filtration plate, air dried, and counted in a liquid scintillation analyzer model 2000 CA (United Technologies Packard, Virginia). Percentage of homology was calculated as described elsewhere. Similar mol %G+C values and ≥70% DNA homology were used to discriminate among different bacterial species.

Vertical distribution of bacterial genotypes across the geological profile. To assess the distribution of bacterial genotypes across the geological and depth profile, more than 65 bacterial isolates from the three sites were selected and their mol %G+C values were determined as described above. This allowed for preliminary genetic comparisons of the different bacterial species present in each formation at the three sites. In addition, whole chromosomal probes of four bacterial isolates were prepared as described before to measure the DNA relatedness of bacteria from different sites and geological formations and to assess the genetic relatedness across the depth profile. Pseudomonas aeruginosa (ATCC 9721) DNA was extracted, labeled by nick translation, and hybridized against deep subsurface isolates identified as P. aeruginosa to determine the reliability of the API system and to determine known bacterial species.
Data analysis. Programs developed for a Macintosh computer were used for all statistical analyses. Analysis of variance was used to determine differences between sites and formations in the DNA homology and base composition analysis. Data were subjected to the appropriate transformation before statistical analysis as described by Zar (1987). Any statistical probability less than or equal to 0.05 was considered significant.

Results

Total DNA hybridizations and DNA base content of isolates from deep sediments. The identification of deep sediment isolates randomly chosen for the DNA tests from the three sites by the Rapid API-NFT identification system showed that 28% were unknown species. Based on these physiological tests, 40% of the isolates were identified as *Pseudomonas* sp. and 25% as *Acinetobacter* sp. (Table 1). The species *Pseudomonas luteola* was found across the geological profile at all of the sites, while *Acinetobacter* sp. was found in all formations at sites P24 and P28, with the exception of the Tobacco Road. Unknown species were found throughout the geological profile at all sites.

The guanine and cytosine content of deep subsurface bacterial isolates that were randomly chosen from the three sites ranged from 20 to 76% (Table 2). More than 60% of the isolates have their values between 58 and 75%. Of the isolates not identified by the phenotypic tests, 55% showed mol G+C values between 57 and 70% while 27% of the isolates had values between 38 and 49% (Tables 1 and 2). The mol G+C of bacterial isolates from site P28 ranged from 32 to 72% while sites P24 and P29 showed ranges of 36-60% and 43-74%, respectively. The average mol G+C of bacteria from site P28 was 58.67 ± 3.1%, whereas a 53.4 ± 16.2% was found in site P24. On the other hand, site P29 showed an average of 59.3 ± 3.1%. There was no significant difference between sites in the total mol G+C values found.

Three whole chromosomal probes were used to study the molecular relatedness between and within site isolates A0481 (site P28), B0703 (site P24), and C0397 (site P29). Isolates A0481 and B0703 had the same phenotype and were identified as *Pseudomonas luteola* using the API-NFT code (Table 1); however, their mol G+C was different (66.0 and 64.0) and the DNA homology between the two was less than 50% (Table 3). Thus, they could not be the same species and were misidentified as *P. luteola*. Probe C0397 had a mol G+C of 61.0 and was identified as *P. acidovorans* by the API-NFT code (Tables 1 and 2). Since all three probes were different species, they were used as diversity probes against isolates from different sites and formations.

The average DNA homology of probe A0481 (P28) against isolates from site P24, P28 and P29 were 32.5 ± 10.3, 19.5 ± 7.3, and 19.7 ± 14.2, respectively. Probe B0703 showed DNA homologies against isolates from the same three sites of 20.1 ± 6.2, 26.6 ± 10.9, and 20.2 ± 14.4. Probe C0397 showed DNA homologies of 29.0 ± 6.7, 37.6 ± 15.9, and 29.2 ± 23.5. Overall there was no significant difference in DNA relatedness between isolates from different sites and formations. DNA
relatedness between the same formations and sites were not significant at the three sites. Nevertheless, no bacterial isolate from upper formations or different sites had the same genetic structure of isolates from deeper stratas, since none of them showed $\geq 70.0$ DNA homology under stringent conditions or similar mol %G+C values (Tables 2 and 3).

Bacteria with the same phenotype from different geological formations did not show the same genetic composition. Therefore, since phenotypic tests did not suggest the genetic differences between deep subsurface bacteria, several isolates from the same geological formation and phenotype were analyzed using total DNA hybridizations and base composition to see whether this was a common pattern in these communities. All isolates were hybridized against a phenotypically identical probe (A0481) from site P28 with a mol %G+C of 66.0%, and none showed more than 52.0% DNA homology. Their DNA base composition was significantly different ($F=217.9, df=1$ and 6, $P<0.05$). Isolates from site P28 showed an average of 71.4% of G+C, while isolates from site P29 showed an average of 31.6% (Table 4). The same reaction was found with isolates from site P24. Although they come from the same site and formation and have the same phenotype, their DNA homology against isolate B0703 was not equal to or higher than 60% (Table 4).

The accuracy of the phenotypic tests to describe known bacterial species was determined by using a whole chromosomal DNA probe from Pseudomonas aeruginosa type strain ATCC 9721. The mol %G+C of the type strain was 67.9%. However, when the mol %G+C of deep subsurface bacteria identified as P. aeruginosa (by the API-NFT) was done, they showed values from 46.8 to 64.7%. None of the isolates showed similar values with the type strain. Total DNA hybridization of the type strain against these isolates showed DNA homologies lower than 50% (Table 5).

To study the effect of depth on the distribution of bacterial genotypes in deep subsurface environments, at least three randomly chosen bacteria from each formation were tested across the depth profile at site P24 (Table 6). These bacteria were hybridized against one isolate each from the Middendorf and Tobacco Road formations. In addition, the mol %G+C of each bacterial DNA was determined. In that way, the genetic distance between isolates from upper and deeper aquifers was measured. Isolates from the Tobacco Road formation showed a broader range of mol %G+C (29-73%), whereas the Middendorf isolates had a narrower range (64-71.6%). No isolate showed a significant hybridization (e.g., $\geq 70\%$) against the two probes tested.

**Discussions**

Three of the four probes used were in the genus *Pseudomonas*, since they all were Gram-negative, glucose nonfermentative rods with mol %G+C of 61-66%. Although two of these probes were phenotypically identical, they did not share the same genotype (i.e., less than 50% DNA homology and different mol %G+C). The mol %G+C of deep subsurface bacteria ranged from 20 to 75%, with more than 60% of the isolates between 57-75%. According to Palleroni, this is the range of the family Pseudomonaceae. Moreover, of the 28% unknown bacteria not identified by
the API-NFT system, 55% showed mol %G+C values within this range; therefore, since they were all Gram-negatives rods, oxidase positive, glucose nonfermentors, they probably should be classified as Pseudomonas sp. Based on the physiological tests and the mol %G+C analysis, it can be concluded that the Pseudomonas spp. were the most abundant heterotrophic bacteria in the isolates tested.

Nevertheless, a great majority of the isolates that were identified by the API-NFT system as Pseudomonas luteola did not show the reported mol %G+C values for this species. Holmes et al. divided the Pseudomonas luteola species into two groups based on their differences in DNA base composition, Flavimonas spp. with a mol %G+C of 63 ± 1.6% and Chryseomonas spp. at 56.8%. Bacterial isolates B0428, B0703, and B0725 showed similar mol %G+C values with the Flavimonas spp. These two new species were created to separate this genetically heterogeneous group in different genera. Since deep subsurface bacterial communities showed a number of unknown phenotypic types, it is possible that a number of new species are present in the deep subsurface. An extensive number of phenotypic tests and electron microscopy studies have to be done to gather enough information about it. Also, different rRNA probes are currently available that can be used to identify some of these isolates to the species level.

Deep subsurface isolates from the three sites identified as P. aeruginosa did not have the same mol %G+C value of the type strain, and their homology was less than 50% against a type strain. Thus, they were misidentified as P. aeruginosa. Bacterial isolates A0474, A0735, B0344, B0103, B0121, B0444, and B0457, were identified as Acinetobacter spp. by the API-NFT test. Since they all had mol %G+C values that ranged from 38 to 49%, they could be Acinetobacter spp. Overall, DNA homology studies did not show any positive correlation with phenotypic identification for the identification of bacterial species, because bacteria with the same phenotype from the same or different formations not only showed less than 50% of DNA homology but different mol %G+C as well. Thus, phenotypic tests alone underestimated the bacterial diversity at the three sites and did not provide an accurate assessment of the structure and composition of heterotrophic bacteria from the deep subsurface. Busse et al. found that the API-NFT system may not yield valuable results even with isolates characterized as true Pseudomonas spp. due to the limited number of reference strains and because of the ill-defined status of this genus. Indeed, some of the Pseudomonas species listed in the API code book have been placed in separate genus and did not reflect the actual status of the family Pseudomonaceae.

When the Middendorf (deepest) and Tobacco Road (upper) geological formations were compared, they did not show any mol %G+C content in common. Moreover, no isolate from different geological formations and sites belonged to the same species (i.e., showed more than 70% DNA homology and same mol %G+C value). Therefore, each geological formation seems to have a distinct microbial community. Nevertheless, bacterial isolates tested from the Middendorf formation showed narrower mol %G+C values than Tobacco Road isolates, suggesting that a single bacterial genus, probably Pseudomonas sp., was dominant in the deepest
stratas. It seems that the deeper the formation, the narrower the range of mol %G+C values found. This suggests that greater distances from the recharge zones and older depositional sediments diminish the diversity of bacterial genera, since only mol %G+C values similar to the Pseudomonas and Acinetobacter sp. were found in deeper aquifers. Therefore, it seems that based on these genetic tests and those reported by Balwkwll et al., each geological formation has its own microbial community that was laid down during the deposition of the sediments, and that specific bacterial species were selected by particular conditions such as stratigraphy, geochemistry, and hydrology in each formation.

Deep subsurface bacterial species probably reflect phenotypic and genetic adaptations to the prevailing climatic and abiotic conditions of the deep subsurface. Similar results were reported by Yayanos et al. when deep sea bacteria were closely related to, but distinct from, members of the genus Vibrio. Olsen et al. reported that Thiobacillus spp. 5S rRNA sequences are the most predominant species living in symbiosis inside worms in deep hydrothermal vents. They stated that these new species are related to, but different from other Thiobacillus species based on the divergence in the RNA sequences.

The family Pseudomonaceae has been found as part of the predominant heterotrophic flora in the Schirmacher Oasis in the Antarctica based on phenotypic characteristics and mol %G+C of bacterial isolates. However, the differentiation of the Pseudomonaceae and Acinetobacteraceae at the species level in ecological studies as shown in this paper is difficult due to their phenotypic and genetic heterogeneity. Woese has shown that both families are comprised of phylogenetically distant strains and that they are spread over more than five different rRNA homology groups. The metabolic versatility of the genus Pseudomonaceae has been well documented and their widespread distribution in the environment is a result of this characteristic. Since more than 70% of deep subsurface bacteria tested are in those two families the potential uses of these communities for subsurface bioremediation strategies are excellent. This is due to the reported metabolic capacity of these two genera to degrade a wide range of xenobiotics.

**Conclusion**

In a single Pseudomonas species, there may be several different pathways by means of which dissimilar organic compounds are broken down. Deep subsurface bacteria are an ideal choice to be used for environmental detoxification studies due to their metabolic and genetic capability, and they may offer new strategies for in situ bioremediation of deep aquifers and unsaturated vadose zone sediments. This capability can be increased by gene amplification and increased expression of the degradative gene. Further genetic studies on deep subsurface bacteria will provide additional evidence to understand the variability, evolution, establishment, and survival of these communities in deep subsurface environments.
Acknowledgements

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References


### Table 1. API Identification of deep subsurface bacterial isolates used in the genetic tests.

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Table 2. DNA base composition as determined by DNA thermal melting point of deep subsurface bacteria.

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Table 3. DNA homologies between deep subsurface bacterial isolates.

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NA = Not applicable.
ND = Not determined.
Table 4. DNA homologies and base composition of bacteria with identical phenotypic profile classified as *Pseudomonas luteola*. API-NFT Code 0-476-642.

**PROBE A0481**

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**PROBE B0703**

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Table 5. Total DNA hybridizations of *Pseudomonas aeruginosa* and isolates phenotypically identified by API-NFT as *P. aeruginosa*.

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Table 6. DNA base composition and homology of bacterial isolates across the geological profile in site P24.

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<th>Mol %G+C</th>
<th>DNA Homology BO703</th>
<th>BO169</th>
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Restriction Endonuclease Analysis of Deep Subsurface Bacterial Isolates

J. Y. Reeves, R. H. Reeves, and D. L. Balkwill

Florida State University, Tallahassee, FL.

Abstract

More than 50 bacterial isolates were analyzed with a combination of restriction endonuclease analysis and restriction fragment length polymorphisms. The isolates, all aerobic chemoheterotrophs, were obtained from aquifer sediments at the United States Department of Energy's Savannah River site near Aiken, SC. Isolates from the Congaree aquifer (sediment sample from 91 m) were compared to those from the Middendorf aquifer (samples from 244, 259, and 265 m). Four common six-base cutting endonucleases, BamHI, EcoRI, HindIII, and PstI, were used to digest DNA that was prepared from each isolate. Four DNA fingerprints for each isolate were generated by agarose gel electrophoresis, and a ribosomal-DNA probe (plasmid pST32) was employed (by Southern blotting and hybridization) to identify a set of the ribosomal-DNA bands that were characteristic of each isolate and restriction endonuclease.

The above procedure allowed for the following: (1) the identification of identical or closely related isolates, (2) the selection of isolates to be used for 16S rRNA sequencing, and (3) the comparison of isolate patterns to a database of DNA fingerprints that were generated in like manner from a large set of type strains. In general, the isolates from one depth were not closely related to those from the other three depths. Those from one depth, however, often clustered into small, related groups.

No paper submitted.
16S Ribosomal RNA Sequencing Analysis of Phylogenetic Relatedness Among Aerobic Chemoheterotrophic Bacteria in Deep Aquifer Sediments from a Site in South Carolina
K. P. Stim, G. R. Drake, S. E. Padgett, and D. L. Balkwill
Florida State University, Tallahassee, FL.

Abstract

Aerobic, chemoheterotrophic bacteria were isolated (by plating on concentrated and dilute media) from the topsoil and from two deep aquifers (92 and 260m) at the United States Department of Energy's Savannah River Site near Aiken, SC. Isolates from each sample were tested for their reactions to 21 physiological tests (nine specific enzymatic activities and the use of 12 compounds as sole carbon sources), and the results of these tests were examined with a multivariate analysis algorithm designed for numerical taxonomy. This analysis identified two clusters of isolates (one containing seven strains and the other containing eight) that appeared to be closely related on the basis of their physiological characteristics. A variation of the dideoxy DNA-sequencing technique was then used to partially sequence 16S ribosomal RNA from each isolate. The resulting sequence data were aligned with those for Escherichia coli and Bacillus subtilis (as outgroups) and analyzed with parsimony and distance matrix algorithms in order to derive phylogenetic trees for selected groups of isolates. The results were subjected to additional statistical analysis (bootstrap analysis) to obtain confidence intervals for the branching patterns seen in the phylogenetic trees. The isolates in the two clusters of physiologically similar strains (above) were not closely related phylogenetically. Although some of these isolates were very closely related, others appeared to be very distantly related. These results indicate that the isolates were more diverse than suggested by the 21 physiological tests. In addition, the physiological tests were of limited value for the study of phylogenetic relationships among the types of bacteria that were examined in this study.

No paper submitted.
DRILLING AND SAMPLING TECHNIQUES, TOOLS, AND METHODS

Contributed Papers 1
Oral
Studies on the Utilization of Turbidity and Particulate Loadings in Groundwater as Correlatable Factors with the Bacterial Population
Abimbola Abiola,¹ D. Roy Cullimore,¹ and Marina Mnushkin,¹
and Neil Mansuy²
¹Regina Water Research Institute, University of Regina, Regina, Saskatchewan, Canada
²Layne-Western Company, Inc., Shawnee Mission, KS.

Abstract
A significant component in the groundwater microflora consists of particulate associated sessile bacteria. These bacteria, unlike the truly planktonic organisms, were not dispersed completely in the aquatic matrix. The colonized particulates may have arisen from sheering, which caused the detachment of parts of the biofilm. Monitoring the incumbent organisms within the E.C.P.S.-based particulates was difficult using traditional spread plate techniques. Various techniques were applied to directly measure these suspended particles by laser-driven particle counting, turbidometry, and reflectance measurement (for pigmented particulates). High levels of correlations were obtained between turbidity N.T.U. and color (pH indicator) changes that occurred in a range of inoculated enteric media, although no visible colonial growth was observed. This leads to the postulation that a group of particulate-related bacteria existed within the water that could not be directly enumerated as colonial growths on a range of 74 media tested to date, but could, however, initiate biochemical reactions on enteric media leading to pH shifts. Such bacteria may form a major and, as of yet, unrecognized component in groundwater systems.

No paper submitted.
Hydrogen Concentrations in Groundwater as an Indicator of Bacterial Processes in Deep Aquifer Systems
Francis H. Chapelle and Derek R. Lovley
United States Geological Survey, Columbia, SC.

Abstract

Concentrations of hydrogen gas dissolved in groundwater were measured at two well-cluster sites, with PVC-cased wells screened in four distinct aquifers at each site, in the coastal plain of South Carolina. The lower aquifer in the Pee Dee formation, which exhibited evidence of ferric iron reduction, had hydrogen concentrations in the 0.1-0.2 nmolar range. The upper aquifer in this unit was characterized by active sulfate reduction and had hydrogen concentrations in the 1-2 nmolar range. Finally, a well that was screened just below an organic-rich claystone and showed active methanogenesis had hydrogen concentrations in the 10 nmolar range. The observed hydrogen concentration ranges for iron reduction, sulfate reduction, and methanogenesis were virtually identical to those observed in studies of aquatic sediments. These results suggest that hydrogen concentrations are a potentially useful tool in documenting predominant, bacterial electron-accepting processes in groundwater systems, as well as in aquatic sediments. Hydrogen concentrations measured in the Black Creek and Middendorf aquifers along flow paths from the outcrop recharge areas to the Atlantic coast suggest that sulfate reduction (hydrogen concentrations approximately 1-4 nmolar) is the primary terminal electron-accepting process in the aquifers. However, a few sites showed hydrogen concentrations in the 10-15 nM range, methane concentrations in the 20-40 µM range, and sulfate below 30 µM. This suggests that methanogenesis also occurs in these aquifers when sulfate is depleted.

No paper submitted.
Possible Microbiological Origin of Reduction Spots in Red Beds

Beda A. Hofmann*

U.S. Geological Survey, Denver Federal Center, Denver, CO.

Abstract

Reduction spots are bleached spheroids that are found worldwide in continental and marine red beds and in altered, hematite-stained crystalline rocks. Reduction spots are formed by diffusive outward migration of a reducing agent from a point source. Centers of reduction spots are enriched in vanadium, uranium, sulfide-S, and many other elements. Occasionally, they contain kerogen-like organic matter. Typically, reduction spots form about 100 Ma after sedimentation of their host rocks at depths of 100 - 1000 m. The genesis of reduction spots has been attributed to the presence of synsedimentary organic detritus or to droplets of migrated oil. Occurrences of reduction spots in crystalline rocks and of fracture-bound spots in red beds are arguments against an origin due to detrital organic matter. Because current nonbiological models for the origin of reduction spots are inadequate, the following microbiological model is considered: reduction spots may be the result of former bacterial activity in deeply buried red beds. This model is supported by the following arguments: (1) reduction spots are sites of in situ catalyzed production of an active reductant or chelator from a kinetically inert reductant; (2) sulfides formed at low temperature in oxidized rocks are likely due to in situ biogenic sulfate reduction; (3) the accumulation of bioelements; (4) the possible biogenic structures in reduction spots; and (5) the immobility of causing agents in rocks with pore sizes smaller than typical bacteria (1 μm). Oxygen or sulfate are the most easily available electron acceptor. Possible carbon and energy sources include low molecular-weight hydrocarbons and organic acid anions.

*Present address: Museum of Natural History, CH-3005 Bern, Switzerland.
Introduction

Often, evidence of former activity of microorganisms in the deep subsurface can only be deduced indirectly from geochemical signatures that are characteristic for microbial activity. The involvement of microorganisms in the formation of certain geochemical anomalies in deep subsurface environments has often been suspected, but clear proof is usually lacking. One type of mineral deposit where the involvement of sulfate-reducing bacteria at depth is likely is certain sandstone-hosted uranium-(vanadium) deposits. \(^{10,17,18}\)

The geological features investigated in this paper are reduction spots in hematite-rich rocks (Figure 1). Reduction spots are sites where local dissolution of hematite occurs in combination with the deposition and reduction of trace elements. Reduction spots have a spherical to spheroidal shape. This shape is likely the result of diffusive outward migration of a hematite-dissolving agent (reductant or chelator) from the center of the structures. The boundaries between the bleached haloes and hematitic host rocks are usually very sharp, indicative of a rapid rate of reaction between dissolving agents and hematite. Reduction spots consist of a hematite-free halo (1-10 cm diameter) surrounding a core (0.1-1 cm diameter) that contains high concentrations of vanadium, uranium and many other elements including precious metals and rare earths.\(^9\)

Reduction spots occur in continental and marine red beds and in altered, hematitic crystalline rocks of Precambrian to Tertiary age. The frequency of occurrence of reduction spots in rocks is extremely variable. Spots may occupy up to 2 vol.% of a rock, but typically much less (0.01-0.1 vol.%).

Occurrences in crystalline rocks,\(^5,9\) inside pebbles in sediments,\(^13\) and in continental red beds of Precambrian age,\(^19\) preclude an origin due to detrital organic particles as is often proposed.\(^{11,16,21,22}\) Mass-balance calculations showed that no organic particle of sufficient size could initially be present (B. Hofmann, Chem. Geol., in press). An origin due to migrated liquid hydrocarbons was unlikely, because most occurrences were not related to oil source rocks. The reductant that caused the formation of the spots was not detrital organic matter or oil, and therefore, it must have been generated during diagenesis at the site of the present spots from a kinetically inert, primary reductant present in the formation water. This primary reductant did not react with hematite. Only at the sites now represented by the centers of reduction spots did redox reactions take place between the primary, inert reductant and oxidized species, producing secondary reactants that caused reduction of uranium, vanadium and hematite dissolution. Inert primary reductants that may have been present in the pore water are methane and other hydrocarbons, organic acid anions, and hydrogen.

Because there is no published nonbiological model that explains the genesis of reduction spots, a microbiological model is considered. This paper is an attempt to present arguments and evidence for a microbiological origin of reduction spots.

The red bed environment poses some special problems for microbial life. Metabolizable solid organic matter is absent in red beds. Due to the absence of reductants, electron acceptors are abundant in red beds in the form of iron oxides and
Materials and Methods

The results in this paper are based on mineralogical and geochemical analyses of reduction spots from Switzerland (Permian and Triassic host rocks), Germany (Permian), Great Britain (Devonian, Permian, Triassic), Oman (Upper Jurassic - Lower Cretaceous) and several U.S. localities in Colorado, Utah, Oklahoma (Carboniferous to Permian) and Pennsylvania (Ordovician).

The mineralogy of reduction spot cores was determined by reflected light microscopy and Scanning Electron Microscopy (SEM), combined with energy dispersive x-ray analysis. Organic carbon was determined with a Leco carbon analyzer on decarbonated (6N HCl) samples. Sulfur was determined as sulfate on aqua regia leachates by ion chromatography, phosphorous by inductively coupled plasma spectroscopy (ICP), and ammonium-nitrogen with an ion-selective electrode following dissolution of the sample in HF/HCl and basic distillation.

Arguments for a microbiological origin of reduction spots. Reactions between a kinetically inert reductant and oxidized elements in the pore water and in the rock took place only at isolated sites that were structurally indistinguishable from the bulk rock. Thus, at the sites of the reduction spots, a catalyst must have supported this conversion. No host rock heterogeneity was evident at the site of the spots. Bacteria are well known for their ability to catalyze kinetically inhibited reactions such as the reduction of sulfate. Thus, bacteria are likely candidates for the catalyst involved.

Presence of reduced sulfur compounds. Sulfides of Cu, Fe, Ag and Pb are common among the mineral phases formed in the center of reduction spots. Hydrogen sulfide reacts rapidly with iron oxides, precluding any transport of hydrogen sulfide in red beds. The presence of sulfides in reduction spots must therefore be due to the in situ reduction of sulfate. Estimated formation temperatures of reduction spots range from 30° to 70°C (B. Hofmann, Chem. Geol., in press). In this temperature range, sulfate reduction is exclusively biologically mediated. Experimental evidence indicates that thermochemical sulfate reduction is important only at temperatures above 200°C, especially in the absence of preexisting sulfide that is acting as a catalyst (W. L. Orr, Geol. Soc. Am. Abstr. with Progr., pp 580, 1982). There is evidence, however, that thermochemical sulfate reduction in nature occurs at temperatures between 100° and 200°C, but in these cases, catalyzation by preexisting hydrogen sulfide is likely.

Because reduction spots were formed at relatively shallow depths and at temperatures well below 100°C, no preexisting hydrogen sulfide could have been present in the red beds. Thus, a biological in situ origin of sulfide in the cores is the most likely explanation. Apart from the presence of sulfide minerals, indirect evidence of the former presence of hydrogen sulfide was provided by minerals that
contained U\textsuperscript{IV} (uraninite) and V\textsuperscript{III} (roscoelite). At diagenetic temperatures well below 100°C, hydrogen sulfide is the only agent known to reduce hexavalent uranium and tetravalent vanadium to the tetravalent and trivalent state, respectively. Because sulfides were present in minor amounts only, both assimilatory or dissimilatory sulfate reduction may have been responsible for the presence of reduced sulfur.

Enrichment of bioelements in reduction spots. Evidence of the former presence of microorganisms can be obtained from concentrations of the bioelements carbon, sulfur, nitrogen, and phosphorous, assuming that these elements were fixed in or near the spots after the death of microorganisms.

Organic matter is present only in a relatively minor number of the investigated cores. Based on pyrolysis-GC/MS results (B. Hofmann, in preparation), this organic matter was an insoluble aromatic polymer formed from precursor compounds by irradiation from associated uranium minerals. Due to the strong alteration, molecular information about the organic precursor could not be obtained from this material. Stable carbon isotopes showed a large range of values from -23 to -50 permil (PDB), which indicated that a wide variety of precursor materials were involved.

Enrichments of sulfur were common in reduction spots from Switzerland, South Devon (U.K.), and from several localities in Colorado. Phosphorous is not generally enriched, but a few typical reduction spots from northern Switzerland had cores that were rich in apatite, indicating a strong enrichment of phosphorous. Analysis for ammonium-bound nitrogen showed an enrichment in the core in three of the eight analyzed pairs of cores and host rocks. Although none of the bioelements (C, S, P and N) were consistently enriched, all of them seemed to be involved in the genesis of reduction spheroids.

Possible biological structures. Usually, the minerals in the centers of reduction spots are very fine-grained and do not exhibit organized structures. In some cases, however, spherical structures outlined by ore mineral like uraninite and niccolite (NiAs) have been found (B. Hofmann, Chem. Geol., in press). These spherical structures have a diameter of 5-15 µm and resemble artificially mineralized bacterial cells.\textsuperscript{1,2}

Mobility of causing agent depends on porosity. The development of spherical cores and haloes around the center of reduction spots demonstrated that whatever caused their formation was immobile in the pores of the rock. Bacteria are typically larger (about 1 µm) than the pore space of fine-grained sediments (<0.11 µm) in which most reduction spots occur. Therefore, it can be assumed that bacteria were immobile in these rocks. Growth of colonies might have been possible due to mineral dissolution at the interface between bacteria and the minerals. Reduction phenomena in rocks with pores larger than the size of bacteria, such as clay-poor sandstones, have mineralized cores and haloes of irregular shape. Therefore, the irregular core shape was an indication that the causing agent was mobile in these cases, an observation that is in agreement with the proposed bacterial origin.
Discussion

A microbiological model for the origin of reduction spots. The proposed microbiological model for the formation of reduction spots is schematically presented in Figure 2. Bacteria in the center of the spots use a kinetically inert, dissolved source of carbon and energy (primary inert reductant) and dissolved electron acceptors (oxygen, sulfate). A bacterially produced, secondary reactive reductant (e.g. hydrogen sulfide or sulfur species of intermediate valence) or a chelator (siderophore) diffuses outward until it reacts with hematite to form dissolved ferrous iron or a soluble ferric iron complex. Rare elements (dominantly vanadium and uranium) are concentrated in the cores by adsorption followed later by reduction.

Although Fe$^{3+}$-oxides are abundant in red beds, their use as electron acceptors by iron-reducing bacteria is limited due to the nonavailability of the iron oxide pigments to bacteria living in the center of reduction spots. If the conditions of the formation are anaerobic, then the most likely electron acceptor is sulfate since this ion is most abundant in red bed formation waters. Nitrate has been detected in minor quantities in water-soluble extracts of several red beds (0.3-22 ppm nitrate compared to 37-500 ppm sulfate) and, therefore, it was also a possible electron acceptor.

Possible carbon and energy sources were organic acid anions (acetate, propionate) and low-molecular-weight hydrocarbons such as methane. Because of the low organic carbon content of red beds, it is most likely that these compounds were introduced from outside the red beds by groundwater flowing in more permeable (sandstone) horizons, followed by diffusion into low permeability clays.

The enrichment of rare elements in the cores of reduction spots may not have been due to reduction only, but may be better explained by adsorption, probably to bacterial cell walls$^{1,6,15}$ followed by reduction. Reduction of dissolved uranyl ions, present in sub-ppm concentrations by hydrogen sulfide, is possible only after prior adsorption of the uranyl to a surface.$^{7,14}$ The fact that the highly mineralized cores of reduction spots were separated from the hematite-dissolution front by several centimeters of a nonmineralized zone indicates that vanadium, uranium etc., were not precipitated from solution by the agent that caused dissolution of the hematite. This was probably due to low concentrations of these elements. Reduction may have only been possible after adsorption to bacterial cells or other material in the center of the spots.

Origin of bacteria in red beds. The presence of bacteria in deep red beds at depths of several hundred meters can be explained in two different ways: (1) introduction into the sediment during sedimentation, or (2) introduction with flowing groundwater during advanced burial of the sediment. Because the formation of reduction spots postdated the sedimentation of the host rock by as much as 100 Ma (B. Hofmann, Chem. Geol., in press), a later introduction seems more likely. Secondary introduction is the only possible way to explain the microbiological origin of fracture-bound reduction spots in crystalline rocks.
Conclusion

From the available evidence, it is proposed that reduction spots were formed by microbiological activity at depths of burial of the host rocks of 100-1000 m. Among the possibly involved microorganisms, sulfate-reducing bacteria are the most likely candidates. Bacteria were probably introduced into the rocks by flowing groundwater along fractures and through highly permeable layers. Possible carbon and energy sources include organic acids and low-molecular hydrocarbons.

If the bacterial origin of reduction spots can be further substantiated, the very widespread occurrence in red beds implies that bacterial activity is very common during advanced stages of red bed burial. Mapping and dating of reduction spots on a local to regional scale might then be used to obtain more information about this type of bacterial activity in the subsurface. In combination with fluid inclusion and stable isotopic studies, temperature constraints for the activity of reduction-spot-forming bacteria in the subsurface can be established. A bacterial origin of reduction spots would imply that bacteria can be very effective under subsurface conditions in the concentration and the immobilization of many trace elements that have to be considered in radioactive waste disposal such as uranium and, as actinide-analogues, the rare earth elements.

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References


Figures

Figure 1. Typical reduction spots in clay-rich sandstone. The bleached haloes are depleted in ferric iron due to hematite dissolution. Dark centers (cores) are enriched in vanadium and uranium. Permian continental red beds, Riniken well (depth 837 m), northern Switzerland. Scale bar = 1 cm.

Figure 2. Schematic presentation of microbiological model for the origin of reduction spots.
Effect of Dissimilatory Iron-Reducing Bacteria on the Geochemistry of Iron and Organic Contaminants in the Deep Subsurface

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Abstract

The oxidation of organic matter to CO₂ coupled with the reduction of Fe³⁺ to Fe²⁺ is one of the most important redox reactions affecting the geochemistry of deep aquifers. Although studies on the geochemistry of iron in groundwater have typically treated Fe³⁺ reduction as an abiological reaction that is a function of redox potential and pH, recent studies in surface water environments have demonstrated the existence of respiratory Fe³⁺-reducing microorganisms that are capable of enzymatically catalyzing the complete oxidation of organic compounds with the reduction of Fe³⁺. Therefore, the potential for the activity of respiratory Fe³⁺-reducing bacteria in pristine and contaminated deep subsurface environments was investigated. Respiratory Fe³⁺-reducing bacteria were recovered from the sediments of pristine deep aquifers in which Fe³⁺ was undergoing reduction, but such organisms were not recovered from sediments in which Fe³⁺ was not being reduced. The Fe³⁺-reducing bacteria were capable of reducing the Fe³⁺ present in the subsurface sediments that were deposited approximately 80 million years ago. Studies with aquifer sediments and with chemically defined systems demonstrated that Fe³⁺-reducing bacteria could oxidize most naturally occurring organic compounds with the reduction of Fe³⁺, but few organic compounds abiotically reduced Fe³⁺. Microbially catalyzed Fe³⁺ reduction was found to be an important process for the oxidation of aromatic contaminants in a polluted aquifer. Enrichment cultures capable of oxidizing a wide range of aromatic contaminants with the reduction of Fe³⁺ were obtained, and an Fe³⁺-reducing organism capable of oxidizing several important aromatic contaminants such as toluene, phenol, and p-cresol was isolated. These results demonstrate that respiratory Fe³⁺-reducing bacteria can have a significant impact on the inorganic and organic geochemistry of deep subsurface environments.
**Q and A**

*J. Tiedge:* Have you done enough samples to work with iron cultures to know how commonly nitrate reduction is correlated with the iron reduction in regard to the aeromatic oxidation?

*D. Lovley:* I do not know what enough samples would be. We have not done very many, so I would say no.

*J. Tiedge:* Your gut feeling then.

*D. Lovley:* It seems to be associated, but I should point out that it not nitrate reduction to ammonia. It is a form of nitrate reduction, not denitrification.
A Model of Sulfate Diffusion and Bacterial Production of CO₂ in the Black Creek Aquifer, South Carolina
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Abstract

Isotope and mass balance calculations on dissolved inorganic carbon in the Cretaceous Black Creek aquifer of South Carolina show that bacterial CO₂ is added to the groundwater at a rate of 10⁵ to 10⁴ millimoles CO₂/l/year. This addition accounts for 20% of the total input of organic carbon to the aquifer. Measured changes in the concentrations of dissolved oxygen, ferrous iron, sulfate, and methane in the aquifer suggest, however, that bacterial CO₂ accounts for less than 1% of the carbon input. This apparent lack of a terminal electron acceptor in the aquifer to account for bacterial CO₂ production is widely observed in Cretaceous aquifers of the Atlantic Coastal Plain.

A model is presented in which dissolved sulfate diffuses from overlying marine clays into the Black Creek aquifer, where it is utilized by sulfate-reducing bacteria to produce CO₂. This model is substantiated by (1) measured concentrations of dissolved sulfate in the marine clays exceeding 2400 ppm, (2) calculated rates of diffusion of dissolved sulfate out of the clay which are in equilibrium with rates of CO₂ production in the aquifer, (3) the identification of sulfate-reducing bacteria in the aquifer, and (4) concentrations of dissolved hydrogen in the aquifer which suggest sulfate reduction is an active terminal electron-accepting process. Reports of high concentrations of dissolved sulfate in marine clays in the Coastal Plains of New Jersey and Maryland suggest that this process may be widespread in aquifers of the Atlantic Coastal Plain.

*Oral presentation.*
Introduction

This paper uses an example of the problem from the Black Creek aquifer to help propose a model that can provide the additional electron acceptor needed to produce the CO$_2$. The Black Creek aquifer consists of sands and clays of upper Cretaceous age. The aquifer outcrops in a narrow band in the upper coastal plains of South Carolina and generally dips to the Southeast. The aquifer is overlain by the Tertiary aquifer system and it is underlain by the Middendorf aquifer, which is also upper Cretaceous in age. The objectives of this paper are as follows: (1) to show that the amount of dissolved oxygen, ferrous iron, sulfate, and methane in the Black Creek aquifer is insufficient to account for CO$_2$ production, and (2) to present a model of sulfate diffusion that can account for the additional electron acceptor needed.

Discussion

There are two ways to estimate bacterial CO$_2$ production in groundwater based on geochemical mass balance techniques. One is to do a mass balance on the major dissolved constituents in the aquifer. The other is to do a mass balance on the products and reactives of bacterial metabolisms. Both mass balance approaches were used to estimate bacterial CO$_2$ production along a flow path in the Black Creek aquifer. First the major ion mass balance.

Four constituents, dissolved sodium, inorganic carbon, calcium and magnesium, make up over 95% of the total dissolved solids along the flow path. If one does a mass balance on these constituents between any pair of wells along the flow path, the end result will be reactions that describe the changes in concentrations of these constituents downgradient. This mass balance also provides an estimate of the amount of CO$_2$ entering the system. Therefore, if a mass balance is done between each pair of wells, one can estimate how much CO$_2$ is entering along the flow path. A graph of CO$_2$ in mM/l between each pair of wells showed that CO$_2$ production increased from about 0.1 mM/l to a total of about 5.0 mM/l downgradient (slide).

In the electron acceptor mass balance, one can also estimate CO$_2$ production by coupling the change in concentrations of each of the products or reactives of bacterial metabolism with its corresponding terminal electron-accepting reactions. For example, for each mM of sulfate that is consumed, 2 mM of CO$_2$ will be produced. If one does this for each of the constituents between each pair of wells, then the CO$_2$ production along the flow path can be estimated. By doing this, one obtains a CO$_2$ production that increases from less than 0.1 mM/l to a total of approximately 0.5 mM/l downgradient.

Comparing CO$_2$ production from the two methods then shows that there is a deficiency of electron acceptors in the aquifer. There is more CO$_2$ production based on major ion mass balance than there is in the electron acceptor mass balance. This is true even if one assumes in the high ion zone that all of the CO$_2$ is from iron reduction. The electron acceptor mass balance still underestimates by approximately one order of magnitude.
If the electron acceptor is not present in the aquifer, then it may be present in the confining units. Therefore, pore water from confining units collected at the Florence and the ASR boreholes were analyzed for dissolved sulfate. The Florence borehole is located in the high iron zone and consists predominantly of terrestrial sediments. The ASR borehole consists predominantly of marine sediments. At the Florence borehole, concentrations of dissolved sulfate in the clays and sands were about the same, indicating that there does not appear to be any additional source of electron acceptors in the clays of the terrestrial zone. However, at the Myrtle Beach borehole, concentrations of dissolved sulfate in the clays were much higher than in the sands. In fact, some of the clays had concentrations of dissolved sulfate near 20 mM.

Based on these results, a model was proposed in which sulfate diffuses from zones of high concentration in the confining units into zones of low concentration in the aquifers, where it is then utilized by sulfate-reducing bacteria to produce CO₂. A buildup of hydrogen sulfide in the aquifer is not evident, so the proposal can be furthered by suggesting that the sulfide that is produced is tied up in the sediments as pyrite. This model can be tested by comparing the rate of CO₂ production from the mass balance approach with a rate of CO₂ production based on the diffusion of sulfate in the sand. If one uses a range of values for the diffusion variables—diffusivity, concentration gradient, porosity in clay and sand, and the thickness of the sand—then rates of CO₂ production from the diffusion model will range from approximately 10⁻⁴ to 10⁻³ mM of CO₂ per liter per year. The rates based on the geochemical and the mass balance models are in the order of 10⁻⁴ mM of CO₂ per liter per year. Therefore, based on the values for the variables that have been used, the diffusion model could at least provide sulfate at a fast enough rate to account for CO₂ production in the aquifer.

Some evidence does exist concerning the potential for sulfate reduction in other sediments. Geochemical evidence that sulfate reduction is occurring is provided by concentrations of dissolved hydrogen gas in the aquifers, (Chapelle, this volume). Downgradient in the marine section of the Black Creek aquifer, where there are high concentrations of sulfate in the confining unit, concentrations of hydrogen gas are in the 1-4 nM range. This is characteristic of sediments in which sulfate reduction is the predominant, terminal electron-accepting process. Evidence of the potential for sulfate reduction can also be seen by the distribution of sulfate-reducing bacteria as a function of depth in the Florence borehole (slide). Sulfate-reducing bacteria were found in just about all of the sandy intervals, yet they were not found in any of the clay-rich intervals.

Results of some mercury injection tests provide some reasoning for this particular distribution of sulfate-reducing bacteria. These tests provide the distribution of pore throat sizes in sediments. In one particular clay, over 80% of the pore throat had radii less than 0.05 micron. The smallest bacteria that has been reported was approximately 0.1 micron, so these data would suggest that the pores in these clays were just too small to harbor bacteria. In fact, time series incubations of this particular sediment were done. Radiolabelled acetate was added and the sediment
was then observed for the accumulation of radiolabelled CO$_2$ over time. No radio-
labelled CO$_2$ production was found after 40 days. In a sandy sediment, however,
there were a large number of pore throats that had radii much greater than 0.1 micron.
In fact, at one studied depth, the metabolism of radiolabelled acetate was observed
and the accumulation of radiolabelled began within 24 hours of incubation. Therefore,
it appears that sedimentary structure is an important component in looking at the
distribution of bacteria in sediments. One final piece of evidence which suggests that
sulfate reduction is occurring in the downgradient sediments is the occurrence of
secondary pyrite in these sediments.

**Conclusion**

In conclusion, geochemical data show that there is insufficient dissolved
oxygen, ferrous iron, sulfate, and methane in the aquifer to account for the all of the
CO$_2$ production that is seen from mass balance calculations. There is a large pool
of dissolved sulfate that exists in the confining beds and based on a number of
arguments, it appears that this sulfate in confining beds can provide the additional
electron acceptor needed for CO$_2$ production in the Black Creek aquifer. Thank you.
Comparison of Bacteria from Deep Subsurface Sediment and Adjacent Groundwater

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Abstract

Samples of groundwater and the enclosing sediments were compared for densities of bacteria using direct (acridine orange direct staining) and viable (growth on 1% PTYG medium) count methods. Sediments to a depth of 550 m were collected from boreholes at three sites on the Savannah River Site near Aiken, SC using techniques to insure a minimum of surface infiltration. Clusters of wells screened at discrete intervals were established at each site. Bacteria densities in sediment were higher by both direct and viable count than they were in groundwater samples. Differences between direct and viable counts were much greater for groundwater samples than sediment samples. Densities of bacteria in sediment ranged from less than 1.00 x 10⁶ bacteria/g dry weight (gdw) up to 5.01 x 10⁸ bacteria/gdw for direct counts, while viable counts were less than 1.00 x 10³ CFU/gdw to 4.07 x 10⁷ CFU/gdw. Bacteria densities in groundwater were 1.00 x 10³ - 6.31 x 10⁴ bacteria/ml and 5.75 - 4.57 x 10² CFU/ml for direct and viable counts, respectively. Isolates from sediment were also found to assimilate a wider variety of carbon compounds than groundwater bacteria. The data suggests that oligotrophic aquifers have unique and dense bacterial communities that are attached and not reflected in groundwater that is found in the strata. Effective in situ bioremediation of contamination in these aquifers may require sediment sampling.
Introduction

Organic xenobiotic chemical contamination of groundwater has become the most important pollution problem of industrialized nations of the world. More than 15% of community drinking water supplies in the United States have been found to be contaminated with carcinogenic, chlorinated hydrocarbons.\textsuperscript{7} Identification of previously unknown waste disposal sites that are impacting groundwater occurs almost daily, and thus, the extent of the problem is undoubtedly greater than what any of the current data suggest. Indeed, our reliance on groundwater in the United States has steadily increased over the past 30 years, not only for drinking water but also for industrial processes, agricultural irrigation, etc.\textsuperscript{7} As sources of clean surface water steadily decline, our reliance on groundwater will undoubtedly continue to increase far into the next century. Thus, with increasing urgency we have been seeking ways to clean up (i.e., remediate, contaminated groundwater). Since many aquifers are quite deep and contain tremendous volumes of water with slow turnover times, \textit{in situ} bioremediation is very attractive and in some cases may be the only recourse.

\textit{In situ} bioremediation has been practiced for more than 30 years by petroleum industries, (e.g. petroleum land farming).\textsuperscript{4} The initial process of inorganic nutrient infiltration of groundwater to stimulate biodegradation by indigenous bacteria in groundwater contaminated with petroleum was patented by R. L. Raymond in 1974 (U. S. Patent 3,846,290). However, widespread application of this technology has not occurred due to limited successes. This was due in part to the paucity of knowledge concerning the microbial ecology of subsurface sediments.\textsuperscript{12} During the 1980s, several laboratories, including ours, began studying the biogeochemistry of subsurface sediments in order to understand and control biodegradation processes in groundwater.

The United States Department of Energy’s Office of Health and Energy Research began a comprehensive program to study subsurface microbiology in 1985.\textsuperscript{9} During this program, four sites were chosen at the DOE’s Savannah River Site near Aiken, SC. Sediments were sampled using special recovery techniques from the surface to bedrock, 550 m in the deepest borehole.\textsuperscript{23} (For a comprehensive description of these studies see Fliermans and Balkwill,\textsuperscript{9} and volume seven of the \textit{Geomicrobiology Journal}.) At three of the sites, a series of well clusters (6-12) were established so that groundwater would be recovered from discrete strata. The purpose of the present study was to compare the microbiology of the sediments with the adjacent groundwater. These studies were undertaken in order to determine the efficacy of using groundwater to monitor the microbiology of aquifers, especially as it may apply to \textit{in situ} bioremediation.

Materials and Methods

\textbf{Study sites.} Sediment samples were taken by aseptic coring from three sites (P24, P28, P29) at the Savannah River Site, near Aiken, SC. (For a thorough description of the geology and hydrology of these sites and this area of South Carolina, see Sargent and Fliermans.\textsuperscript{26} For a general description of the U. S. Department of Energy’s Deep Probe Project findings, see Fliermans and Balkwill\textsuperscript{9} and volume 7, numbers 1 and 2 of the \textit{Geomicrobiology Journal}.) Water samples
were taken from a cluster of wells at the same site. All of these wells were established within 20 m of the borehole used for sediment sampling. The wells were built using carbon steel casing, stainless steel screens, gravel packs, and dedicated, 9 gpm pumps. Wells in each cluster were single screened at specific geological formations over a 3-m interval to provide water from specific aquifers or segments of aquifers. Table 1 provides a listing of wells, their physical description, and representative physical-chemical data.

*Sediment analysis.* Bacteriological data from sediment analysis were taken from Balkwill and Sinclair and Ghiorse. For a detailed description of sediment sampling techniques, see Phelps et al.

*Water analysis.* Wells were flushed by pumping until at least three well volumes of water had been evacuated and the pH and conductivity were stable, (i.e., less than 1% change in one hour; sterile, one liter bottles were filled with water for bacteria counts), and all samples were placed on ice and transported to the laboratory for analysis within 1-3 hours. Viable counts were determined by filtering 1, 10, and 100 ml of water through 0.45 μm pore size, 47 mm, HA type, membrane filters (Millipore Corp., Bedford, MA). Filters were placed on 1% PTYG medium and incubated at 23°C for two weeks. Bacterial colonies were counted with the aid of a stereo microscope. Total cell counts were determined by direct count (AODC) methods using acridine orange. Activity was estimated by dividing the log density of AODC's by the log density of viable counts on 1% PTYG.

*Bacteria Isolation and Assimilation.* Deep subsurface bacterial isolates from sediments used in the physiological studies were provided from the Subsurface Microbiology Culture Collection (SMCC) by Dr. David Balkwill of Florida State University, Tallahassee, FL. (For further details on the isolation of deep subsurface bacteria from sediment and media used, see Balkwill et al. and Balkwill and Ghiorse. Water isolates were obtained from random colony selections from 1% PTYG medium. Both water and sediment isolates were analyzed for physiological capabilities using the API-NFT Rapid tests as described by Balkwill et al. and Bone and Balkwill.

*Data analysis.* The data were analyzed by using prepared programs for Macintosh computers. Factorial analyses of variance were used to test for differences between sites, samples and methods. Data were subjected to the appropriate transformation before statistical analysis by the method of Zar. Any probability less than or equal to 0.05 was considered significant.

**Results**

Direct enumeration of bacteria in sediments using AODC revealed from less than 1.00 x 10^6 bacteria/gdw up to 5.01 x 10^8 bacteria/gdw (Table 2). Viable counts using 1% PTYG medium varied from no detectable growth (i.e., less than 1.00 x 10^3 CFU/gdw) up to 4.07 x 10^7 CFU/gdw. Due to variations between samples within sites and within geological formations, a factorial analysis of variance demonstrated no significant differences between sites or between geological formations. However, differences between viable counts and direct counts were significant (F = 66;
DF = 1.85; P < 0.0001). Direct counts were usually the same, but for some samples they were two orders of magnitude higher than viable counts (Table 2). (For further analysis of sediment enumeration techniques and geochemistry see Balkwill and Sinclair and Ghiors.27

Groundwater AODC measurements ranged from $1.00 \times 10^3$ to $6.31 \times 10^4$ bacteria/ml (Table 3), while bacteria densities measured by viable counts on 1% PTYG ranged from 5.75 to $4.57 \times 10^2$ CFU/ml. As with the sediment enumeration, a factorial analysis of variance revealed that variability within sites and within geological formations prevented density differences between sites and between geological formations from being significant. Differences between direct counts and viable counts were very significant ($F = 155; \ DF = 1, 36; P < 0.00001$). The average difference between viable and direct counts for sediment samples ranged from two to three orders of magnitude.

Comparison of viable counts from the sediment and water demonstrated very significant differences (Table 4; $F = 85; \ DF = 1, 36; P < 0.00001$). Differences ranged from two to five orders of magnitude, with an average of three. Comparison of direct counts from the sediment and water also revealed even greater differences (Table 5; $F = 232; \ DF = 1, 36; P < 0.00001$). Sediment direct counts were from three to five orders of magnitude (average = 4) higher than adjacent groundwater densities.

As an index of activity, the ratio of direct to viable counts (D/V) was calculated for both sediment and water and compared (Table 6). The D/V ratio was significantly higher for water ($F = 14.5; \ DF = 1, 36; P < 0.001$). Indeed, all but three of the samples had a higher water D/V than the sediments and only one of these was significant. The average D/V was 75% larger for water samples. D/V was not significantly different by site or geological formation as analyzed by factorial analysis of variance. Isolates from both water and sediment collected at the same sampling interval were compared in terms of their physiological abilities using the API-NFT test (Table 7). The first nine metabolic tests were not significantly different between water and sediment isolates. However, significantly higher proportions of sediment isolates were able to utilize the carbon compounds that were assayed ($F = 3.32; \ DF = 1, 22; P < 0.01$). Eleven of 13 compounds were assimilated at a higher frequency by the sediment isolates.

Discussion

The detailed studies done by several investigators on the sediment samples described in this study have defined many microbiological parameters.8,9 Great care was taken to collect samples with a minimum of surface and drilling mud contamination.23 Sixteen separate lines of evidence using a variety of tracers and microbiological assays suggests that fewer than 10% of the sediment samples were compromised.8 The sediment samples demonstrated that overall bacteria were present in high densities ($10^7$ AODC or CFU/gdw) and were able to rapidly utilize a wide array of carbon compounds. This was indicated by the close agreement of viable and direct counts (1-2 orders of magnitude) and the greater frequency of isolates capable of assimilating carbon compounds found in the API-NFT assay.
Related studies of these same isolates have also demonstrated their ability to degrade a variety of toxic compounds, such as trichloroethylene, quinoline, phenol, and 4-methoxybenzoic acid.\textsuperscript{6,10,16} Poindexter\textsuperscript{24} suggested that bacteria adapted to oligotrophic conditions might be expected to have broader uptake systems, having adapted to utilization of a broader range of substrates. Similar findings have been reported for oligotrophic bacteria in Antarctica.\textsuperscript{29} The sediments and water in the present study were oligotrophic based on the low concentrations of phosphorous and nitrogen and recalcitrant organic matter since dissolved organic carbon was between 3-9 ppm.\textsuperscript{11} Thus, even though the sediment bacteria in our study may be living in an oligotrophic, recalcitrant environment, they were able to maintain a high standing biomass in a quiescent state and could almost immediately respond to small increases in nutrients like those found in the low nutrient, 1% PTYG medium. This is quite different from oligotrophic aquatic systems where differences between direct and viable counts for the same samples are typically 4-5 orders of magnitude.\textsuperscript{18,25} This was further supported by the data of Tunlid et al.,\textsuperscript{28} who showed that the phospholipid fatty acid profiles of bacteria in these sediments were under nutrient stress. Balkwill\textsuperscript{11} and others\textsuperscript{2,5} showed that bacteria from these deeper sediments had greater metabolic flexibility than surface sediment bacteria, growing rapidly on both low nutrient and high nutrient media. Other studies on the same isolates\textsuperscript{12} showed an increasing frequency of plasmids among isolates with increasing depth at these sites. This suggested that as the environment becomes more recalcitrant it becomes more metabolically economical to increase the plasmid burden in order to increase the number of degradable compounds.

In contrast to sediments, groundwater adjacent to the sediments had direct counts that were 2-3 orders of magnitude lower and viable counts that were 3-5 orders of magnitude lower than sediment densities. The ratios of direct to viable counts were much greater for groundwater samples. Thus groundwater bacteria were not only much lower in density, but they were under greater stress because they were much less capable of responding to nutrient input (i.e., growth on low nutrient medium: 1% PTYG). This was further supported by the lower frequency of isolates that could assimilate API-NFT compounds.

Studies in Germany\textsuperscript{20,21} comparing sediment and groundwater bacterial communities also found that densities of bacteria in the sediment by viable counts were 2-3 orders of magnitude higher than nearby groundwater. In their studies, viable counts for water were quite similar to the results in this study; however, sediment densities were significantly lower. Combined with the present study, these findings suggest that these aquifers are comprised of epilithic communities. In other words, the microbial communities of these aquifers are nearly all attached. It appears that the microorganisms that are free in the groundwater are either dead or stressed attached bacteria or organisms that are being transported through the aquifer having their origins in the recharge zone of the aquifer. It is unlikely that these planktonic bacteria exist as a free living community due to their poor culturability and the extremely low nutrient levels of the water.\textsuperscript{25}
Support for a recharge zone or sediment independent origin of the groundwater bacteria is further evidenced by our laboratories finding that three DNA probes from sediment bacteria, one from each site,\textsuperscript{19} did not show significant homology, < 26\%, with any of 23 groundwater isolates. API-NFT assays could not be used for identification of sediment bacteria because isolates with identical phenotypes by these assays did not have similar DNA homologies or even similar mol\% G+C composition of the DNA.\textsuperscript{19} Identification comparison of sediment and water isolates will therefore not be possible until sediment phenotypes can be differentiated and further characterized.

Conclusion

The present work has demonstrated that deep oligotrophic aquifers have large attached communities of potentially very active indigenous bacteria that are not reflected in the groundwater from that aquifer. This has serious implications for the \textit{in situ} bioremediation of contaminated aquifers, since monitoring of groundwater is the principal method used to characterize and control biodegradation by indigenous bacteria stimulated by nutrient infiltration. Groundwater monitoring will not indicate community or population numbers, or physiological activity of the sediment attached microorganisms, which are the principal biologically active component of these aquifers. Harvey et al.\textsuperscript{15} and Harvey and George\textsuperscript{14} have shown that shallow, eutrophic, rapidly moving aquifers, behave quite differently, in that there are no significant differences between groundwater and attached sediment communities. This is reasonable because attachment in such an environment would have no significant advantage, unlike the oligotrophic deep aquifers.

Thorough characterization and control of deep aquifer communities, necessary for any bioremediation effort that utilizes stimulation of indigenous bacteria, may require deep sediment sampling. Deep sediment sampling and recovery of uncontaminated samples for microbial analysis may make this type of \textit{in situ} bioremediation cost prohibitive. A better understanding of biogeochemistry, microbial composition and synecology of these deep aquifers would seem warranted before any severe manipulation by man is tried.

Acknowledgements

We are grateful for the technical assistance of W. Fulmer, J. J. Foreman, and C. Betivas. The information contained in this article was developed during the course of work under contract DE-AC09-76SR00001 between the U.S. Department of Energy and Westinghouse Savannah River Company and in part by contract DE-AC05-760R000033 between the U.S. Department of Energy and Oak Ridge Associated Universities. The U.S. Department of Energy, Office of Health and Environmental Research supported several investigators that provided ancillary information through their Subsurface Science Program, under the direction of F. Wobber.
References


Table 1. Physical and Chemical Descriptions of Cluster Wells.

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<th>Cond (μmols)</th>
<th>Redox (mv)</th>
<th>DO (mg/l)</th>
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* Screen = Screened interval of well below surface, Temp = Temperature, Cond = Conductivity, DO= Dissolved Oxygen, ND = Not Done.
Table 2. Sediment Direct vs. Viable Counts for Selected Sediment Samples.

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All values are log$_{10}$ densities, per gram dry weight soil.
$^1$From Sinclair and Ghirose (1989).
$^2$From Balkwill (1989).
NG = No growth on lowest dilution ($10^{-3}$).
Table 3. Water Direct vs. Viable Counts for Selected Water Samples.

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<tr>
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All values are log_{10} densities per ml water.
Table 4. Water vs. Sediment Samples Using Plate Counts on 1% PTYG.

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All values are log₁₀ densities, per gram dry weight soil or per ml water.

*From Balkwill (1989).

NG = No Growth on lowest dilution (10⁻³).
Table 5. Water vs. Sediment Sample Using Acridine Orange Direct Counts.

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<td>-</td>
<td>-</td>
<td>3.8</td>
<td>7.6</td>
<td>4.2</td>
<td>6.6</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>3.5</td>
<td>7.3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Middendorf</td>
<td>4.3</td>
<td>7.4</td>
<td>3.4</td>
<td>6.7</td>
<td>3.5</td>
<td>7.4</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>3.3</td>
<td>7.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>3.4</td>
<td>7.3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>3.2</td>
<td>&lt;6.0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3.4</td>
<td>7.8</td>
</tr>
<tr>
<td>Mean</td>
<td>3.51</td>
<td>6.88</td>
<td>3.43</td>
<td>7.15</td>
<td>4.01</td>
<td>7.09</td>
</tr>
</tbody>
</table>

All values are log_{10} densities, per gram dry weight soil or per ml water.

*From Sinclair and Ghiorse (1989).
Table 6. Ratio of Direct to Viable Counts for Selected Water and Sediment Samples.

<table>
<thead>
<tr>
<th>Formation</th>
<th>P24 Water</th>
<th>Sediment*</th>
<th>P28 Water</th>
<th>Sediment</th>
<th>P29 Water</th>
<th>Sediment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface Soil</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tobacco Road</td>
<td>1.46</td>
<td>1.69</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Dry Branch</td>
<td>1.39</td>
<td>1.00</td>
<td>-</td>
<td>-</td>
<td>2.38</td>
<td>1.08</td>
</tr>
<tr>
<td>McBean</td>
<td>2.08</td>
<td>1.37</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Congaree</td>
<td>3.86</td>
<td>1.11</td>
<td>-</td>
<td>-</td>
<td>1.37</td>
<td>1.13</td>
</tr>
<tr>
<td>Williamsburg</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ellenton</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2.15</td>
<td>2.17</td>
</tr>
<tr>
<td>Pee Dee</td>
<td>1.71</td>
<td>1.85</td>
<td>2.33</td>
<td>1.07</td>
<td>2.22</td>
<td>1.48</td>
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<td></td>
<td>1.37</td>
<td>1.08</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Black Creek</td>
<td>-</td>
<td>-</td>
<td>2.71</td>
<td>1.47</td>
<td>1.80</td>
<td>2.20</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>2.45</td>
<td>2.23</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Middendorf</td>
<td>4.94</td>
<td>1.68</td>
<td>2.38</td>
<td>1.17</td>
<td>4.61</td>
<td>1.25</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>2.23</td>
<td>1.05</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>3.47</td>
<td>2.94</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td></td>
<td>-</td>
<td>-</td>
<td>2.30</td>
<td>1.54</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4.20</td>
<td>1.13</td>
</tr>
<tr>
<td>Mean</td>
<td>2.537</td>
<td>1.592</td>
<td>2.400</td>
<td>1.422</td>
<td>2.674</td>
<td>1.492</td>
</tr>
</tbody>
</table>

All values are ratios (AODC/PTYG) of log_{10} densities per gram dry weight soil or per ml water.

*From Sinclair and Ghiorse (1989) and Balkwill (1989).
Table 7. Percent of Bacterial Isolates from Sediments and Groundwater that Tested Positive for API-NFT Compounds.

<table>
<thead>
<tr>
<th>Metabolic Tests</th>
<th>Sediment</th>
<th>Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrate reductase</td>
<td>58</td>
<td>51</td>
</tr>
<tr>
<td>Tryptophanase</td>
<td>&lt;1</td>
<td>0</td>
</tr>
<tr>
<td>Arginine Dihydrolase</td>
<td>5</td>
<td>15</td>
</tr>
<tr>
<td>Urease</td>
<td>42</td>
<td>16</td>
</tr>
<tr>
<td>Esculin Hydrolysis</td>
<td>54</td>
<td>ND</td>
</tr>
<tr>
<td>Gelatinase</td>
<td>32</td>
<td>37</td>
</tr>
<tr>
<td>β-Galactosidase</td>
<td>56</td>
<td>44</td>
</tr>
<tr>
<td>OxidaseE</td>
<td>39</td>
<td>37</td>
</tr>
<tr>
<td>Glucose Fermentation</td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Aerobic assimilation tests</th>
<th>Sediment</th>
<th>Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Glucose</td>
<td>86</td>
<td>64</td>
</tr>
<tr>
<td>L-Arabinose</td>
<td>51</td>
<td>36</td>
</tr>
<tr>
<td>D-Mannose</td>
<td>56</td>
<td>36</td>
</tr>
<tr>
<td>D-Mannitol</td>
<td>61</td>
<td>47</td>
</tr>
<tr>
<td>N-Acetyl-D-Glucosamine</td>
<td>35</td>
<td>45</td>
</tr>
<tr>
<td>Malosee</td>
<td>69</td>
<td>60</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>70</td>
<td>48</td>
</tr>
<tr>
<td>Caprate</td>
<td>15</td>
<td>20</td>
</tr>
<tr>
<td>AdipateE</td>
<td>53</td>
<td>31</td>
</tr>
<tr>
<td>L-Malate</td>
<td>76</td>
<td>53</td>
</tr>
<tr>
<td>Citrate</td>
<td>44</td>
<td>42</td>
</tr>
<tr>
<td>Phenylacetate</td>
<td>36</td>
<td>11</td>
</tr>
<tr>
<td>Total Number of Isolates</td>
<td>430</td>
<td>120</td>
</tr>
</tbody>
</table>

Values are a percentage of total isolates that tested positive, all three sites (P24, P28, P29) combined.
Figures

Figure 1. Map of Savannah River Site and Well Clusters P24, P28, and P29 (continued on following page).
Round Table 1
Drilling and Sampling Technology: How Good is the Current State-of-the-Art?
Conveners: Brent F. Russell and Tommy J. Phelps

NO ABSTRACTS
Density and Distribution of Aerobic, Chemoheterotrophic Bacteria in Deep Southeast Coastal Plain Sediments at the Savannah River Site
David L. Balkwill, Florida State University, Tallahassee, FL.

Abstract

Samples of topsoil and subsurface sediments (to depths of 500 m) were aseptically obtained at four drilling sites at or near the United States Department of Energy's Savannah River Site in Aiken, South Carolina. Aerobic, chemoheterotrophic bacteria in these samples were characterized by enumeration of viable cells (plate counts on several media), analysis of colony morphologies appearing on enumeration plates, and determination of the physiological traits of isolated strains. Substantial numbers of viable bacteria (10^3-10^8 cells/gdw) were detected in nontransmissive, aquifer-bearing sediments, and their numbers did not decrease with depth. Considerably fewer viable bacteria (less than 10^3 cells/g) were detected in nontransmissive, confining layers (between the aquifers). The highest viable counts were obtained on dilute media, but 10-50% of the bacteria in the aquifer sediments could also grow rapidly on concentrated, nutrient-rich media. Analyses of morphological and physiological traits (responses to 21 selected biochemical tests: 9 specific enzymatic capabilities, and the ability to use 12 different compounds as sole carbon sources) indicated that the subsurface bacterial flora was quite diverse (10-60 morphologically and/or physiologically distinct types isolated from most aquifer sediments). Diversity did not decrease with depth, but the specific composition of the microflora (based on physiological traits of the isolates) varied extensively from one geological formation to another. The composition of the microflora in any given geological formation differed from one drilling site to another (i.e., laterally) as well. Physiological testing of isolated strains also indicated that (1) the subsurface microorganisms differed markedly from those in the overlying topsoil, and (2) distinct strains of subsurface microorganisms were isolated on concentrated and dilute plating media.

Oral presentation.
Introduction

The work described in this paper was carried out in conjunction with the United States Department of Energy's research program, called the Microbiology of the Deep Subsurface. The program has been concerned with the microbiology and the microbial ecology of relatively deep subsurface environments, including deep aquifers. Deep is more or less defined as more than 30-50 m or so below the earth's surface.

The work I will talk about was done as part of the recently completed first phase of the Deep Microbiology Program. This phase of the program addressed some relatively basic questions about the microbiology of subsurface environments. When the program was started in the mid-1980s, of course, it was well known by then that substantial numbers of microorganisms were present in comparatively shallow subsurface environments, as shown primarily by the Environmental Protection Agency from the studies that were headed by John Wilson. There were also several groups in West Germany working on it, as well as in other countries. Therefore, it was quite well known that organisms were in relatively shallow subsurface environments.

In the mid-1980s, however, relatively little had been done on deeper environments, and certainly very few studies in the way of in-depth, detailed microbiological examinations of the deep subsurface. As this program was getting started, people began to notice it. In the 1980s, there were papers out of West Germany that began to look in detail at some of the deeper sediments. Furthermore, just as the sampling activities at Savannah River were getting started, some of the first papers were written by Dr. Frank Chapelle and his co-workers at United States Geological Survey. Therefore, people were now beginning to look at the deep sediments. However, not too much was known at that point and so the initial phase of the DOE program was designed to address some very basic questions such as the following: Were microorganisms commonly present in these deeper environments as they were known to be in the shallow environments? If so, how many were there? How diverse were the populations? What different types of organisms were present? How were they distributed throughout the subsurface? How might the composition of the microflora vary from one location to another? The physiological traits and the metabolic capabilities of these organisms needed to be studied. The principle interest to DOE, because of the contamination problems at the National Lab Sites, was whether these organisms may be of some use in the bioremediation of contaminated groundwater.

Discussion

The primary activity of the program during the first phase has just been completed. Several sets of deep sediments were obtained from DOE's Savannah River Site, which is situated on top of eastern, coastal plain sediments, with several major aquifers located one on top of another. The aquifers are composed primarily of transmissive sandy materials. They are separated and sometimes intersected by relatively dense, clay-confining zones. During the study, four deep boreholes were drilled for sampling purposes through these aquifers. The first three, sites P24, 28,
and 29, were drilled in 1986, and then C10 was drilled in the summer of 1988. At each of those four drilling sites, approximately 15 depths were sampled (slide). The idea was to obtain materials from the same geological formations at each drilling site in order to obtain a vertical and a lateral survey of these sediments and any microorganisms that might be present in them. Once the samples were obtained, they were examined by a multidisciplinary group of investigators, including microbiologists, chemists, geologists, hydrologist, statisticians, and so on. I think the fact that we have been able to have that kind of multidisciplinary interaction has been one of the major strengths of the program.

In our laboratory at Florida State, the particular task at hand was to enumerate, isolate, and to some extent, characterize aerobic, chemoheterotrophic bacteria that might be present in the samples. As it turned out, and perhaps surprisingly so, those organisms were the most abundant forms in the sediments. They represented approximately 95% or more of the different types of bacteria that were found. Many other types of microorganisms were detected in comparatively lower numbers. Some of those other types of organisms will be described in other papers in this session as well as throughout the rest of the proceedings. The aerobic, chemoheterotrophic bacteria were, however, the predominant forms, and therefore, I will continue on with the work that was done with those particular organisms.

Initially, a little information on some of the physical and chemical characteristics in the sediments must be given. The aquifers were composed primarily of sand and relatively small amounts of clay. The confining layers contained considerably more clay. Moisture content ranged from 12-40%; groundwater temperature at the site was 23-26°C year round, depending on the depth; and the pH was approximately 5.0-7.5 in the aquifer, which was a little lower than that found in the confining material. Perhaps one of the more interesting features about the samples was that none of them were highly reduced. There were certainly anaerobic zones, but they were small zones within some of the samples. By-in-large, they were not truly anaerobic environments.

The job at hand was to look at the aerobic, chemoheterotrophic bacteria present in the samples. Enumerating those organisms with viable counts or plate counts was the first task chosen. General protocol for such a task is to blend the samples in a sodium pyrophosphate solution. It has been found that this provides somewhat higher and more reproducible counts than handshaking and some of the other techniques that might be used. After dilution with phosphate buffered saline, the samples were spread-plated and incubated aerobically. The plates were counted after two weeks or so for some of the concentrated media, and after 4-6 weeks for the dilute media. Initially, a fair variety of different media and incubation temperatures were used so a number of combinations could be tried in the process of looking at the samples. Over the first few months, the highest counts and perhaps the most interesting results were obtained on the Peptone-Tryptone-Yeast Extract-Glucose (PTYG) medium. Among the dilute media, a 1:100 dilution of PTYG medium
provided the most consistent results. Although the data for many different combinations was obtained, I will confine the following material to what was obtained on the two media at 23°C. Just for a reference, the PTYG is a very rich medium, and the one percent is merely a 1:100 dilution of the organic components of that medium.

Results that were obtained from one of the four drilling sites, (P29) indicated that the viable counts or plate counts, expressed as the log of the number of colony forming units per gram of sediment, were roughly equal to the log of the number of viable cells per gram of sediment (slide). Comparisons can be made with total microscopic counts, the Acridine Orange Direct Counts (AODC), obtained by Bill Ghirose at Cornell (slide). Fairly substantial numbers of viable, aerobic chemoheterotrophs were obtained in many of the samples, from 10^5, 10^6 and, sometimes close to 10^7 plateable cells per gram of sediment (slide). The results in most of the deep sediments were perhaps an order of magnitude or so lower than one would expect to find in a nice, rich top soil. However, the results were obviously high enough for the organisms to exert a considerable influence on the chemistry of the environment, if and when they were metabolic active. But of course, from the standpoint of bioremediation and DOE's interest, it was important to know whether the organisms were naturally occurring populations that were there to begin with and whether they were large enough to influence the chemistry, which appeared to be the case.

The results did vary from one sample to another. They tended to be a little lower in some zones than they were in either the top soil or many of the aquifers, and the plateable numbers were quite low in the clay confining zones. Sometimes it was impossible to grow any cells out of the samples. Within the transmissive materials of aquifers, the number of microorganisms was consistently high. They did not appear to decrease with depth and given the right conditions, one might have expected to find considerable numbers at greater depths than what was sampled. It should be noted that even though the plateable numbers in the confining zones were quite low, there did appear to be substantial cell numbers present. The total counts were still relatively high, and isolating these organisms or growing them in the lab has not been very successful.

In contrast, and also very interesting, was the fact that the plateable numbers in the aquifers transmissive material were sometimes almost as high as the total direct counts. Sometimes 90% or more of the organisms seen in the total count were recovered on the plates, which was somewhat unusual for these nutrient poor environments. It is an old rule of thumb that only 1-10% of the organisms seen by direct counts are ever cultured in the laboratory. However, these deep subsurface organisms were unusually easy to grow and isolate in the lab. Because the plate counts were sometimes in the same order of magnitude as the total counts, it was felt that some of the information obtained from the organism isolated on the plate counts was fairly representative of the total microflora that was present in the system. Basically, the results were the same at all four sites (slide). Substantial numbers of microorganisms were obtained in the transmissive materials, and the numbers were always low in the confining zones. There was really not that much variation from
one site to another. Therefore, at this point, it was evident that there were large numbers of aerobic chemoheterotrophs in these samples.

Continuing on with the first phase of the program, questions concerning how much diversity existed between organisms and how many different types of organisms were being seen took precedence. These questions were addressed by looking at all of the different types of colonies that grew up on the sets of plates for each sample. Describing all of the colonies with the usual morphological characteristics and with the aid of a computer database management program allowed for the comparison of the colony descriptions of the organisms on a set of plates. The data indicated how many visible, distinct types of colonies were seen on each set of plates, even though it was a very rough indication of microbial diversity in the samples. The number of distinct colony types, as based on colony morphology when grown on 1% PTYG, indicated a fair amount of diversity in many of these samples, such that 15-25 different colony types were frequently visible on PTYG plates. There were somewhat fewer types on the 1% PTYG plates, but still a reasonable amount of diversity did exist. The diversity was low only in the clay layers where, of course, the plate counts were very low to begin with. Within the aquifers, however, there was a fair amount of diversity and it did not seem to decrease with depth. The results were fairly consistent from one sight to another. There were a couple of geological formations where the diversity might have been a little lower, although in general, the results seemed to be about the same. One thing that needed to be done was to preserve the organisms for future use by isolating as many of the different colony types as possible. To date, approximately 4500 strains have been isolated from the 60 or so sediment samples that were obtained from the Savannah River Site. Those, along with isolates from the other investigators that were involved in that part of the program, have been preserved mostly in frozen forms. These microorganisms are in the DOE's Deep Subsurface Microbiology Culture Collection (SMCC) that is currently housed in my lab at Florida State University. Mechanisms have been put into place to make the cultures available to researchers who would like to work with them.

To learn a little more about the organisms in the process of preserving them, isolating them, and putting them away, a look was taken at some of the gram morphologies. A small percentage of the isolates, <2%, were fungi. Some fungi were seen scattered throughout the samples. Most of them were bacteria, approximately 3.5% of which were clearly streptomycyes bacteria; however, the rest of them were not. The nonstrepotmecyes were mostly rods in a wide variety of lengths and widths, some coccoid rods and some pleomorphic organisms. There were a few filaments that did not seem to be streptomycyes. This could have been others in the actinomycyes group. Typically most of the organisms were Gram-negative, and if one wanted to summarize what the usual isolate was, it was a Gram-negative rod. However, there were many other things along with them.

More needed to be known about the physiological characteristics of these isolates, and better information was needed concerning diversity. Therefore, one had to discover whether those little variations seen in the colony morphology were really
different strains of organisms? Furthermore, better ways of comparing the groups of isolates from different sources, different depths, and different drilling sites were needed. Even trying to compare them with the help of a computer program (i.e., trying to compare relatively subjective descriptions of colonies, like differences in color and so on) was obviously a difficult thing to do. It was felt that some information on physiology would be more useful for comparative purposes, and it would provide more concrete information to work with. Therefore, specific physiological traits of some of the isolates were determined with the API-NFT rapid test strips. These products were produced by Analytab Products, and were really designed for rapid identification of nonfermentative, Gram-negative rods of clinical significance. The products test for nine different properties and then for the ability to use selected compounds as a sole source of carbon. As one might expect, they do not do a terribly good job of identifying isolates from the deep subsurface. It was not expected that they would, and therefore they were not used for that purpose.

What was needed was a rapid way to get some kind of physiological data on the organisms as a rapid way of allowing comparison of the isolates from different groups. At the time, a number of physiological kits were available, all of which were designed for clinical purposes. This particular kit seemed to work about as well as anything else (slide). After the work was complete, more alternative kits have come out on the market, for example, the Biolog kit that tests for 96 biochemical properties. We are in the process of evaluating different kits and making decisions as to what can be used the next time around. There are investigators who have used API’s or some of the other systems, who may be experimenting with the Biolog plates as well.

Subsurface isolates (more than 1000) were taken from three of the four drilling sites and compared to 200-250 isolates from the top soil at the Savannah River Site. Subsurface isolates were looked at first, and only 4% of them fermented glucose, whereas 82% of them used it aerobically as a carbon source. Most of these isolates appeared to have an oxidative metabolism rather than a fermentative metabolism. They were reasonably versatile with respect to their ability to use different carbon compounds. These results and the results from some of the other members of the team, as well as looking at the morphology of the organism, indicated that many of these organisms are in the general pseudomonads type of group. This could be of interest with respect to bioremediation as well. The group is well known for its abilities to degrade organic compounds.

The surface isolates sometimes responded quite differently to the tests, and given the sample sizes, the differences were often statistically significant. Thus, there was a hint that the surface soil contained a fairly different group of organisms from the subsurface. Responses of groups of isolates to the tests were studied, but one can also study and compare the response of individual isolates (slide). A small sampling of such data showed that each isolate produced a series of plus or minus reactions to the tests. With the aid of the computer database system (slide), one can ask. "How many unique series of responses to the test occurred, and how many distinct physiological types can there be in the group of organisms?"
tests, they could not have been recorded in form of API-types, which were not very
good. However, they could have been thought of as distinct physiological types, just
like distinct colony types.

The computer system could have also been used to compare two different groups
of organisms, and furthermore, to determine if there were identical reactions in the
two groups or if the strains in the two groups were completely different in their
response to the tests. Some general results from that sort of analysis are reported.
First of all, it was confirmed that there was a lot of diversity among the isolates.
Looking at a group of over 1100 isolates, there were at least 626 distinct responses
to the 21 biochemical tests. That does not mean the isolates were actually different.
Some of them of course, were very similar, differing only by one test or by 10 or 12
of the 21 tests. However, there were certainly a lot of distinct strains within that group
just based on the physiology.

For the PTYG versus the 1% PTYG medium, interestingly enough, there were
many different isolates from those two media. Only an 11% overlap occurred, and
it seemed to be worthwhile, at least in the case of these sediments, to use both a
concentrated medium and a dilute medium to get a better feel for the diversity in the
sample. Of course, the results meant that we may see more diversity as we use more
media. Furthermore, there was a very sharp difference between the subsurface
isolates and the isolates from the top soil: there was only about a 3% overlap. That
is not to say that the top soil organisms were not in the subsurface. Remember, these
were isolates from plating, and therefore, they were numerically predominant forms
that were picked up at the highest dilutions. Things that might have been present in
relatively low numbers were not being detected.

Finally, more on the topic of distribution of the organisms throughout the
subsurface. A lot diversity existed in the samples; 15, 25, 30, or more physiologically
distinct isolates were obtained from each aquifer sample. How the isolates from one
sample compared to the isolates from the Congaree or the Middendorf aquifer
became the next area of concern. If one has looked at different depths within the
Middendorf, then one must question how they were compared. Furthermore, did the
composition of the microflora vary from one location to another?

In this study variations with depth were looked at first. For each of the four
sample sites, the total number of distinct response patterns was added to those
physiological tests (with the aid of the computer program), which provided a total of
what was called number physiologically distinct types. Then the question was, "At
how many depths could one isolate each of those physiological types"? At all four
sites, more than 80% of the distinct physiological types were detected in only one of
the 15 sampled depths that were taken at that site. Approximately 10% were in two
samples, less than 5% in three, and nothing showed up in more than eight of the 15
depths that were sampled. Therefore, the implication was that as one went from one
depth to another (one often goes from one geological formation to another), distinct
microbiological strains were obtained. How different they may have been was not
clear, but distinct physiological groups of organisms were being obtained at each
depth. When the chemical and significant environmental factors changed from one
geological formation to another, it was not surprising then to see slightly different organisms that were adapted to each part of the environment.

The only analysis that has been completed so far was to look at the total number of distinct physiological types among all of the isolates from all four sample sites and to then determine at how many sample sites was that particular physiological type detected (slide). A great majority of them (approximately 80%) were found at only one of the core sample sites, and less than 2% were present at all four. So there did appear to be a shift in the composition of the microflora when going from one drilling site to another. Specific geological formations still need to be looked at in more detail.

**Conclusion**

To summarize then, substantial populations of aerobic, chemoheterotrophic bacteria were found in all of the deep aquifer sediments from the Savannah River Site. Viable numbers were much lower in the clay confining zones, although the organisms appeared to be there according to direct counts. There seemed to be a considerable amount of diversity among the aerobic, chemoheterotrophs that could be detected both by morphological and physiological types of analyses. It is still unknown how much diversity there was in the phylogenetic sense, whether a large number of closely related strains were looked at or a great variety of genera, families, or classes were there. Finally, detailed composition of the microflora did seem to vary quite a bit from one location to another. Again, there was the question of how great the variations were, and whether different strains or completely different groups of organisms were being obtained. Some of this will become more apparent as the molecular and genetic analyses continue.
Q and A

Z. Filip: You indicated that you found a similar number of the bacteria samples from the surface layer (0-8 m thick) and down to 300-500 m. Now did you also perhaps check the concentration of the other nutrients at depth? Perhaps these nutrients did not change in their concentration or the stability of the bacteria was very high within these different environments. Another question before you answer deals with the high proportion of isolates that you were able to grow at both high and low nutrient conditions. Would you consider the deeper isolates being oligotrophic bacteria?

D. Balkwill: To answer the first question on the nutrient levels, a variety of chemical measurements were made on the pore water from the samples, and as far as I could tell, there was sufficient organic carbon in the system to support the number of organisms we found, even all the way down to the lower depths. There was clearly sufficient nutrients.

Audience: Did you check for the carbon, nitrogen, and phosphorous concentration?

D. Balkwill: Most of those things were checked. Bill, do you wish to comment?

W. Ghiorse: Actually, the problem is very complex because we are looking at pore water chemistry and we do not necessarily have all the information we need on total amounts of carbon, nitrogen and phosphorus, because we did not always get both analyses done at the same time as the pore water analysis. The question of nutrient availability and nutrient cycling will be addressed in a talk by my post doc. It is actually a very complex issue, and it is a situation that is not easy to unravel in terms of analysis.

D. Balkwill: We are still trying to understand some of the results that we saw concerning the growth on the rich medium and the dilute medium. It was very surprising to me to see large numbers of organisms growing in the concentrated medium from deep aquifers and it is something that I am still thinking about in terms of what it really means. They appear to be fairly distinct sets of isolates. Some of the organisms that were obtained on the dilute media would not grow well on the contaminated media; they could not adapt to it. Where the organisms have come up on the concentrated medium, from what little we have done with this, they seemed to also grow at very dilute nutrient concentrations, although very slowly. They seemed to be unusually versatile in terms of handling different levels of nutrients and adjusting their growth accordingly, whereas the other group of organisms that only grew under low nutrient conditions did not seem to have that ability to deal with the higher nutrient environment.

J. Wiegel: Could you comment a little more on your results of growth at various incubation temperatures?
D. Balkwill: At 55°C, the numbers were generally very low. There was either no growth on the plates or it was in the $10^2$ CFU/gdw range, where one would obtain just a couple of colonies on the plates. The top soil produced much higher counts at 55°C, nearly $10^4$-$10^5$/gdw. In the subsurface samples, the counts were very low and some did not produce enough results to make the comparison between concentrated and dilute media. If one incubates the plates at 4°C for 4-6 months, the counts eventually approach the ones seen at 23°C, but one does not see things coming up quickly at 4°C (within say, a couple of weeks). So there did not seem to be very many true psychrophilic organisms in there, just organisms that grew very slowly at low temperatures.

S. Kellogg: I am curious; since nearly all of your isolates are based on colony morphology, did you ever check on the same plate of colonies with similar morphologies to see if they had the same physiological tests?

D. Balkwill: No, that was not done in any systematic way. Our initial problem was handling up to 15 sets of plates for each of the 45 samples in a very short period of time. That was, however, something that should be done. It has been done on occasion, mainly by accident. It turns out that the colonies were really not that different. As one looks back at the colony description, one can see that they were basically identical. Sometimes the isolates had different physiological traits, but that is something that would be better done the next time around.

T. Hazen: David, the question we get quite often is how were the isolates picked, and what bias might there be?

D. Balkwill: We simply went for as much diversity as we could see in terms of the colony morphology. From viewing the plates, it is now known which of the isolates were the numerically predominant forms. In some cases, there would be five or 10 different types that were present at the high dilution, and all of them would be at about the same level. In those cases, an isolate that was really predominant could not be picked. In other cases, there may have been one or two organisms that were present at 10 or 50 times the level of everything else that was seen. In the database, those organisms that were in the predominant forms were marked. The selection, however, was strictly to obtain as much diversity as possible. It was not a random selection off of the plates. I know one can make an argument either way.

C. Fliermans: David, in soil microbiology we often see a lot of spore formers. Can you comment on that with regard to the 80°C treatment?
D. Balkwill: Yes, that is right. Heat shock samples were done prior to plating to look for spores, and the numbers were very low in most of the subsurface sediments. The top soils were again 10^2-10^4/gdw with an occasional colony, which was not statistically valid. With many of the samples, especially the deeper aquifer samples, there was no growth at all after heat shock. Spores did not seem to be a major factor in the system.

Z. Filip: Did you look for slow-growing organisms?

D. Balkwill: We did not really look for those in our lab. The closest that we came was with the medium that was nothing more than Bacto-agar and distilled water. Those were incubated for relatively long periods of time, but we did not isolate from them. Those colonies were very small, but they had no differentiation, no color, or anything else. Those plates do produce slightly higher counts than the 1% PTYG.

N. Monir: I have two little questions. Did you try to cluster the organisms you isolated and, which method did you adopt to have all of the composites of the thousands of investigations and biochemical data put together?

D. Balkwill: In response to the first question, no type of formal cluster analysis has been done at this point, although there is some work that will be done on that so a statistical analysis of the data can be obtained. The API product is the NFT System, which is strictly for nonfermentative Gram-negative rods, simply because it is about all that was available at the time.
Composition of Aerobic, Heterotrophic Bacterial Communities in a Sandy, Gravelly Aquifer in the Lower Rhine Region in the Federal Republic of Germany

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Abstract

Fifty-two sediment and 49 water samples were obtained from different depths of seven bore sites to investigate the composition of aerobic, heterotrophic bacterial communities in the saturated groundwater environment. Total and viable cell numbers were determined. About 2,700 bacterial strains were isolated randomly from 60 of these samples. Each strain was characterized with regard to 155 morphological and physiological properties and compared to other strains by similarity analysis. Communities isolated from different depths of one bore site, from different bore sites, and from water and sediment were examined by cluster analysis for similarities in community composition.

Viable cell numbers in water samples from deeper than 5 m below surface were less than 10^2 CFU/ml. In sediment samples, CFU numbers ranged mostly between \(10^2\) and \(10^4\)/gdw, but varied widely with depth. Total cell counts usually yielded 100- to 1000-fold higher cell numbers than viable cell counts. Each of the communities obtained from the seven bore sites was composed of many different bacterial morphotypes and physiotypes. Community composition varied markedly from one bore site to another, as well as from one depth to another at the same bore site. Cluster analyses demonstrated very clearly that no higher similarities existed among sediment communities or among water communities, than were detected between sediment and water communities.

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Introduction

In the present study on aerobic, heterotrophic bacterial communities in the saturated groundwater environment, four questions are of particular interest: What are the cell numbers in this environment? How diverse are the compositions of such communities? Are there similarities or differences between communities isolated from sediment or water, from different bore site in a test field, and from different depths of the same bore site? Which physiological capabilities can be observed with these bacteria? These questions were investigated by the way of community analysis resting on numerical taxonomy. This method has become more and more important during the last decade and was successfully employed in studies on various aquatic biotops, such as fresh water, brackish water, estuaries, and drinking water as well.

Materials and Methods

Sediment and water samples were collected from the first layer (up to 49 m deep) of a Pleistocene, sandy, gravelly aquifer near Bocholt (Lower Rhine region, Germany). The regional location of the test field, as well as the composition of the aquifer, has been previously described by Köbel-Boelke et al. The positions of the seven bore sites-PES 5, DFG 1, DFG 2, DFG 3, DFG 4, DFG 5 and DFG 7-are drawn in Figure 2. Bore sites DFG 1 and 3, as well as DFG 2 and 4, were approximately 5 m apart from each other. Uncontaminated and undisturbed sediment samples were obtained by special pipe borings ("Schlauchkernbohrungen") without using drilling fluids. Multilevel wells were pumped to obtain water samples. Samples were collected and processed as described by Köbel-Boelke et al.

For each sample, cell numbers were determined. Water samples were processed directly, whereas sediment samples first had to be treated to release the microorganisms from the sediment particles. This was conducted by suspending sediment in sterile tap water and shaking it with glass beads. Viable cell counts were determined as colony forming units on P-agar (modified medium of Wolters and Schwartz: 0.1% Witte peptone, 0.01% glucose, 0.01% K2HPO4; 3H2O, 0.002% FeSO4; 7H2O, 1.3% agar, tap water, adjusted to pH 7.2) by either using spread plates or by filtering samples through sterile membranes and laying these on agar plates. Colonies were counted after 21 days of growth at 14°C. Total cell numbers were determined by the Amidoblock 10B stain. For isolating strains, about 40-60 colonies per sample were randomly picked from P-agar plates and used for viable cell counts. Each strain was characterized with regard to 155 features based on colony and cell morphology, as well as physiology. In order to physiologically characterize groundwater bacteria, many standard physiological test procedures were varied to obtain as many positive test results as possible with these bacteria. All tests were carried out with the microtiter plate method as described by Köbel-Boelke et al. Characters of all strains were evaluated by similarity analysis using the simple matching coefficient and complete linkage clustering. Division into clusters and subclusters was carried out with similarity values of 80% and higher.

Table 1 summarizes origin, type, and numbers of samples that were obtained from eight borings between 1983 and 1985. All samples that were collected from the same sampling site and at the same sampling date but from different depths were
given a similar bore number and were processed separately. This bore number (in the first column) indicates the type of sample (1 = water, 2 = sediment). The number behind the hyphen is the serial number of the bore.

Results

Altogether, 101 water and sediment samples were obtained by eight bores from seven different bore sites and were analyzed for cell numbers. From these samples, 60 were selected for examinations of their bacterial compositions. About 2700 strains were isolated, characterized and compared by similarity analysis (Table 1).

**Cell numbers.** In water samples, viable cell counts decreased rapidly from zero to 5 m. Below 5 m, there were usually less than 100 colony forming units (CFU) per ml, and in many cases even less than 50. Sediment samples generally yielded 10- to 100-fold higher viable cell counts, mostly between $10^2$ and $10^3$ CFU/gdw. Viable cell counts varied strongly from sample to sample probably due to heterogeneities in sediment composition and, therefore, the existence of different microhabitats, but they did not decrease with depth. Particles rich in organic carbon were much more colonized than mineral particles. Vertical profiles of CFU numbers have been published by Köbel-Böckele et al. Total cell counts mostly yielded 2-3 orders of magnitude higher values than viable cell counts. In water samples, total cell counts mainly ranged between $10^3$ and $10^5$ cells/ml and in sediment samples between $10^4$ and $10^6$ cells/gdw. In water samples, profiles of cell numbers resembled those profiles obtained for concentrations of oxygen, nitrate and organic carbon. For sediment samples such comparisons were not available.

**Community structures:** 60 samples, 23 from water and 37 from sediment, were selected for examinations of community structures. About 72% of all 2700 isolates stained Gram-negative and 28% Gram-positive or -variable. The major group of isolates, about 52%, belonged to the Gram-negative straight rods, followed by coryneform Arthrobacter-like bacteria with 17%. More than 50% of isolates showed cellular movement, mostly by flagella.

**Bacterial communities in water and sediment samples from the different bore sites:** Table 2 presents a comparison of the morphotype compositions of the eight communities isolated from the seven bore sites investigated. The term "community of a bore site" comprises all strains that had been isolated from the various samples collected from this site at the same sampling date; usually 250 -350 strains. In this table, distinct differences could be recognized between water and sediment communities. Water communities contained low proportions of Gram-positive bacteria, with exception of bore 1-03, whereas in sediment communities the proportions of Gram-positive isolates were, with one exception (2-04), much more higher. This was confirmed by comparing community structures of sediment and water samples which had been obtained from corresponding depths of bore site DFG 5 but with a time delay of eight months. Water samples (1-09) contained 6% Gram-positive isolates whereas in corresponding sediment samples (2-05) 43% of the isolates belonged to this group. Water communities were characterized by higher proportions of Gram-negative straight and curved rods whereas sediment communities contained more Arthrobacter-
like organisms, more Gram-negative gliding filaments of the *Cytophaga/Flexibacter* group, and more Gram-negative bacteria of variable cell forms. Water communities contained more motile bacteria, sediment communities more pigmented isolates. Despite these general similarities between water communities on the one hand and sediment communities on the other hand partly great differences in morphotype composition could be observed between these eight communities. Even communities isolated from neighboring bore sites, 5 m apart, as well as water communities 1-03 and 1-08 and sediment communities 2-02 and 2-04, were composed very differently. In reality, the microscopically recognized and for similarity analysis recorded differences between these eight communities were much greater than it could be expressed by this roughly differentiated morphotype listing.

Determinations of physiological capabilities of the isolates also revealed some differences between sediment and water bacteria. Sediment bacteria especially preferred macromolecular substances with the exception of polysaccharides, whereas water bacteria easily degraded monosaccharides as well as some macromolecules. Furthermore, glucose and lactose fermenters occurred almost exclusively in sediment communities, whereas denitrifying bacteria were more often isolated from water samples. Nevertheless, comparing the degradation capabilities of the eight different communities showed that each community possessed its own preferences for specific substrates or substrate classes. There was no special set of substrates that was preferred by all sediment or all water communities.

On the basis of 155 determined properties, all strains isolated from one bore site were compared by similarity analysis. Figure 1 presents the cluster matrix of sediment community 2-02, which was evaluated by clustering of 268 strains. This matrix could be divided into a great number of clusters and subclusters. This fact indicated very clearly the high diversity of this community, which had already been expected from the results of cell morphology and physiology but which now could be confirmed by the way of similarity analysis. Generally, sediment communities provided cluster matrices that were characterized by a great number of smaller clusters with only a few subclusters. Matrices of water communities often contained one or two big clusters with several subclusters as well as lots of smaller clusters. Generally, sediment as well as water communities were composed very diversely of a great number of different bacterial morpho- and physiotypes.

Another goal of the present investigation was to examine if different bore sites in the test field were colonized by different bacteria, or if some, or even many bacteria occur at several or all bore sites. From the results of cell morphology and physiology it was very obvious that partly strong differences existed. However, these could not be evaluated. For this purpose, all eight communities were clustered one with another to obtain information about the distribution of bacteria in the various bore sites. Figure 2 presents the results of these pair clusterings. In this figure, circles with community designations were arranged corresponding to the positioning of the bore sites in the test field that these communities were obtained from. Cluster matrices were searched for strains that gave similarities of 94% and higher with strains from another bore site.
Communities possessing similar strains were connected with lines. These strains corresponded in many positive characters and therefore seemed to be taxonomically similar. On the other hand, communities were connected by dotted lines, if the taxonomic similarity of the particular strains was uncertain, because those strains corresponded in many negative characters. The numbers beside the lines indicate the percentage proportions of very similar strains that could be observed within the connected communities. Figure 2 demonstrates very clearly that similarities between communities from different bore sites were very restricted or totally absent. Even communities from neighboring bore sites, as in the case of those from bore 2-02 and 2-04 or those from 1-03 and 1-08, were composed very differently. This was also true for the comparison of the sediment community 2-05 and the water community 1-09, both isolated from corresponding depths of bore site DFG 5, but with a time delay of eight months. Sediment communities as well as water communities, one with another, showed no higher similarities than did sediment with water communities. Furthermore, the direction of groundwater flow did not influence or create a higher similarity of community compositions.

**Bacterial communities in different depths or sediment materials of the same bore site:** Community structures did not only differ from one bore site to another, but also could differ from depth to depth or between different sediment materials. The term "community of a depth or sediment material" comprises all strains that had been isolated from one particular sample, usually 40-50 strains. Table 3 demonstrates clear differences in morphotype composition between communities, which had been isolated from neighboring depths of the same bore site or from different sediment materials obtained from the same bore core. A similar observation was made for water samples obtained from neighboring depths of well DFG 1. Table 3 also presents the surprising result that, morphologically, very different communities obtained from the same bore site could exhibit very similar preferences for particular substrates.

Cluster analysis demonstrated that at every bore site there regularly occurred a lot of bacteria whose presence was restricted to only one or two of the samples examined. Otherwise, there were often some bacteria which could be isolated from several or, in a very few cases, even from all samples. Cluster analyses indicated very clearly that the diversity of communities could vary from one depth to another, but did not decrease with increasing depth. This was also true for the physiological potentials of communities isolated from different depths of the same bore site.

**Discussion**

Viable cell counts in water samples agreed well with those reported by Wolters & Schwartz\(^\text{11}\) or Stetzenbach et al.\(^\text{10}\). In sediment samples, CFU numbers varied irregularly from depth to depth, which has been observed by Hoos & Schweisfurth\(^\text{5}\) and by Balkwill.\(^\text{1}\) These differences are probably due to the heterogeneous mineral composition of the aquifer environment and, thus, to the presence of microhabitats.

Bacterial communities isolated from different bore sites or different depths of the same bore site were composed very diversely of a great number of bacterial
morpho- and physiotypes. These results agreed very well with those of other investigators\(^1^,\)\(^3^,\)\(^4\) There was no decrease in community diversity or physiological potential with increasing depth. Similar results were reported by Balkwill\(^1\) for bacterial communities in deep aquifers whereas, in contrary, Balkwill and Ghiorse\(^2\) observed a decrease of diversity with depth in shallow aquifers.

Morphological and physiological characterization indicated strong differences between communities isolated from different bore sites and from different depths or sediment materials of the same bore site, which were probably due to differences in the prevailing hydrogeochemical conditions in the sites and layers.

Water and sediment communities, even from the same bore site, were composed very differently, which was, with regards to Gram-stain, pigmentation and particular physiological capabilities, also observed in another German test site (Segeberg Forest site).\(^7\) With regard to the Gram-stain, community structures of most water communities agreed well with those examined by Stetzenbach et al.\(^10\)

Sediment communities from the Bocholt site contained 35-43% Gram-positive isolates. These proportions of Gram-positives in sediment were much lower than those reported by Balkwill and Ghiorse\(^2\) in shallow aquifers on the basis of transmission electron microscopy of thin sectioned bacteria that had been released from the sediment. On the other hand, the values were higher than those observed by Balkwill\(^1\) in deep aquifers.

**Acknowledgements**

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References


Tables

Table 1. Collected water and sediment samples for the determination of bacterial cell numbers and the isolation of strains.

<table>
<thead>
<tr>
<th>No. of bore</th>
<th>Date of sampling month/year</th>
<th>Name of well</th>
<th>Kind of sample</th>
<th>Samples for direct and plate counts (depth in meters)</th>
<th>Samples for isolation of bacteria (depth in meters)</th>
<th>Number of strains isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-01</td>
<td>7/1983</td>
<td>PES 5</td>
<td>W</td>
<td>5;7;9;11;13;15;17;9;21;23</td>
<td>7;11;15;19;23</td>
<td>246</td>
</tr>
<tr>
<td>2-02</td>
<td>12/1983</td>
<td>DFG 2</td>
<td>S</td>
<td>3;7;9;14;15;21;30</td>
<td>7;9;14;15;21;30</td>
<td>268</td>
</tr>
<tr>
<td>1-03</td>
<td>8/1984</td>
<td>DFG 1</td>
<td>W</td>
<td>3;4;5;7;9;11;13;15;17;19;21;23;25;26;29;33</td>
<td>3;4;5;15;21;29</td>
<td>352</td>
</tr>
<tr>
<td>2-04</td>
<td>11/1984</td>
<td>DFG 4</td>
<td>S</td>
<td>3;7;9;14;15;21</td>
<td>3;7;9;14;15;21</td>
<td>341</td>
</tr>
<tr>
<td>2-05</td>
<td>12/1984</td>
<td>DFG 5</td>
<td>S</td>
<td>3;7;9;14;15;21;21;23;25;26;29;33</td>
<td>3;7;9;14;15;21</td>
<td>717</td>
</tr>
<tr>
<td>2-06</td>
<td>6/1985</td>
<td>DFG 7</td>
<td>S</td>
<td>4;5;6sand;6clay;6peat;7;9;12;14;16;23;27;31;31wood;33;35wood;40;45;47;49clay</td>
<td>4;6sand;6clay;6peat</td>
<td>266</td>
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<tr>
<td>1-08</td>
<td>8/1985</td>
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<td>W</td>
<td>3;4;5;15;19</td>
<td>4;5;15;19</td>
<td>181</td>
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<tr>
<td>1-09</td>
<td>8/1985</td>
<td>DFG 5</td>
<td>W</td>
<td>2;5;3;5;4;5;5;5;6;6;5;5;10;5;12;5;14;5;16;5;18;5;20;5;22;5;24;5;26;5;28;5;30;5</td>
<td>2;5;3;5;4;5;5;6;6;6;5;5;10;5;12;5</td>
<td>341</td>
</tr>
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Table 2. Morphological compositions of water and sediment communities isolated from different bore sites in the test field.

<table>
<thead>
<tr>
<th>Name of well Sampling No.</th>
<th>Water communities</th>
<th>Sediment communities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PES 5 1-01</td>
<td>DFG 1 1-03</td>
</tr>
<tr>
<td></td>
<td>DFG 3 1-08</td>
<td>DFG 5 1-09</td>
</tr>
<tr>
<td>% Gram-positive/variable isolates</td>
<td>11</td>
<td>57</td>
</tr>
<tr>
<td>% Gram-negative isolates</td>
<td>89</td>
<td>43</td>
</tr>
<tr>
<td>Gram-positive/variable isolates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Rods</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>% Coccolid rods/cocci</td>
<td>0</td>
<td>47</td>
</tr>
<tr>
<td>% Coryneform/pleomorph</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>% Branched filaments (actinomycetes)</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Gram-negative</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Straight rods</td>
<td>81</td>
<td>35</td>
</tr>
<tr>
<td>% Curved rods</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>% Unbranched filaments</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>% Coccolid rods/cocci</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>% Cells of variable forms</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>% Isolates with movement by flagella</td>
<td>69</td>
<td>33</td>
</tr>
<tr>
<td>% Isolates with gliding movement</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>% Pigmented isolates</td>
<td>6</td>
<td>20</td>
</tr>
<tr>
<td>% Isolates with stalks (Caulobacter)</td>
<td>0</td>
<td>4</td>
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</tbody>
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Table 3. Morphological compositions and most frequently occurred physiological degradation properties of bacterial communities isolated from two neighboring depths of bore site DFG 2 and from different sediment materials obtained from the same bore core (DFG 7, 6 m core).

<table>
<thead>
<tr>
<th>Name of well</th>
<th>DFG 2</th>
<th>DFG 7</th>
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</thead>
<tbody>
<tr>
<td>Sampling No.</td>
<td>2-02</td>
<td>2-06</td>
</tr>
<tr>
<td>Sample (depth, depth/material)</td>
<td>14 m</td>
<td>5 m</td>
</tr>
<tr>
<td>% Gram-positive/variable isolates</td>
<td>16</td>
<td>76</td>
</tr>
<tr>
<td>% Gram-negative isolates</td>
<td>84</td>
<td>24</td>
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<tr>
<td><strong>Gram-positive/variable isolates</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Rods</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>% Coccoid rods/cocci</td>
<td>0</td>
<td>16</td>
</tr>
<tr>
<td>% Coryneform/pleomorph</td>
<td>16</td>
<td>60</td>
</tr>
<tr>
<td>% Branched filaments (actinomycetes)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Gram-negative</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Straight rods</td>
<td>30</td>
<td>24</td>
</tr>
<tr>
<td>% Curved rods</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>% Unbranched filaments</td>
<td>51</td>
<td>0</td>
</tr>
<tr>
<td>% Coccoid rods/cocci</td>
<td>0</td>
<td>0</td>
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<tr>
<td>% Cells of variable forms</td>
<td>3</td>
<td>0</td>
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<tr>
<td>% Isolates with movement by flagella</td>
<td>17</td>
<td>4</td>
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<td>% Isolates with gliding movement</td>
<td>48</td>
<td>0</td>
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<td>% Pigmented isolates</td>
<td>67</td>
<td>25</td>
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<tr>
<td>% Isolates with stalks (Caulobacter)</td>
<td>0</td>
<td>0</td>
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</tbody>
</table>

Physiological degradation properties which occurred with highest frequencies (percentage of positive isolates)

- **AMD** (81)  GLU-O (80)  PO4 (100)  T40 (95)  DNA (64)
- GLU-O (81)  DNA (76)  DNA (100)  T20 (91)  PO4 (57)
- ONPG (79)  ASC (64)  LEC (100)  T60 (86)  T40 (57)
- PO4 (79)  PO4 (60)  T20 (100)  LEC (86)  MLO (55)
- DNA (79)  AMD (52)  T40 (100)  DNA (84)  T60 (52)
- ARA (76)  ONPG (52)  T60 (100)  PO4 (83)  ESC (50)

*AMD = starch, GLU-O = glucose oxidative, ONPG = β-galactosidase, PO4 = phosphatase, DNA = deoxyribonucleic acid, ARA = arabinose, ASC = esculin, LEC = lecithin, T20 = Tween 20, T40 = Tween 40, T60 = Tween 60, MLO = malonate.
Figure 1. Cluster matrix of sediment community 2-02 (well DFG 2) with clusters and subclusters.
Figure 2. Percentage proportions of very similar strains (similarity $\geq 94\%$) among communities from different bore sites.
Q and A

_S. Niemela:_ I was struck by this rather typical great diversity between samples and sediment deposits. Did you ever have a chance to study two samples that could be considered as parallel samples or a replication of the same sample.

_J. Köelbel-Boelke:_ No we didn't.

_S. Niemela:_ That's a pity.

_J. Köelbel-Boelke:_ There is such a great number of samples to examine that we didn't do parallel samples.

_B. Meglen:_ Of the 100, I assume that for your cluster analysis, you used 155 or so morphological variables. Can you tell me how well these were coded. Were they plus/minus, or yes/no? What sort of a scale were they?

_J. Köelbel-Boelke:_ They were coded yes/no.

_B. Meglen:_ Did you use any physiological variables in your cluster analysis?

_J. Köelbel-Boelke:_ Pardon?

_B. Meglen:_ Did you use any physiological variables, any chemical measurements at all when you did the cluster analysis?

_J. Köelbel-Boelke:_ There was no water chemistry.
The Distribution of Strict and Facultative Anaerobic Microorganisms in the Terrestrial Subsurface

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Abstract

The distribution of anaerobic microorganisms in both aerobic and anaerobic aquifers was measured. As expected, methanogens and sulfate-reducing bacteria were more numerically dominant in an anaerobic aquifer. However, the relative distribution of these organisms within the aquifer was in part influenced by the availability of sulfate as a terminal electron acceptor. Strict and facultative anaerobic organisms could also be cultured from highly oxidizing aquifers. The number of methanogens ranged from undetectable to greater than $10^5$/gdw. Similarly, sulfate-reducing bacteria ranged from less than detectable to greater than $10^5$/gdw, while coliforms and acetogens were detectable in numbers which exceeded $10^3$/gdw in some strata. In general, these organisms were more frequently isolated from transmissive layers, while clay layers harbored the fewest cells. Quality control efforts using serendipitous tracer organisms showed that the types of anaerobes cultured in our experiments could not be mere contaminants encountered during the drilling procedure. The data suggest that anaerobes are a normal part of the subsurface microflora in aquifers, and their presence is independent of the predominant redox availability of electron acceptors, the transmissivity of the formation, the presence of endogenous, fermentable carbon, and inorganic nutrients. Further, the data implies that contaminant bioremediation strategies that require an anaerobic processing step will probably not be strictly dependent on inoculation efforts for success.

'Oral presentation
Introduction

My laboratory has been interested in the anaerobic microbial ecology of subsurface environments. Over the recent years, I have been involved with and have had the opportunity to examine several different types of subsurface environments for the existence of strict and facultative anaerobes. I will discuss three of those investigations and emphasize some of the ecological factors that are involved in governing, or helping to govern the density and distribution of anaerobes in several very different regions of the subsurface.

Study locations included areas in Oklahoma, South Carolina, and Kansas (slide). At most locations, the number of sites that were examined were multiple: two in Oklahoma, four in South Carolina, and only one in Kansas. The depths ranged from very shallow to quite deep. Characteristics included an anoxic aquifer polluted by municipal landfill leachate in Oklahoma, the oxidizing Atlantic coastal plain sediments below the Savannah River complex, and a consolidated rock formation from a discovery oil well in Kansas.

These investigations were not designed to be comparative, but they did share several common objectives (slide). That first objective was to determine the presence and abundance of selective groups of strict and facultative anaerobes in the terrestrial subsurface. Second, some of those factors which control density and distribution were also determined. The third objective dealt with determining whether or not the organisms were indeed indigenous to the subsurface.

Discussion

Work began with a landfill in Norman, OK (slide) right on the banks of the Canadian River. Underlying the landfill was a shallow aquifer which flowed in a southerly direction toward the Canadian River most times of the year. Within this aquifer, two major sampling sites have been identified: one with a predominant flow of carbon and energy sources that goes through sulfate reduction, and another where they seem to lead to methanogenesis. They were very close in physical proximity, only about a 100 m apart. These sites were characterized on the basis of field observations, in situ measurements of methane production from the sites, chemical analysis of groundwater, various types of microbial enumerations, and activity profiles, along with the isolation of physiologically important anaerobes.

I will talk about some of the microbial enumerations in order to provide some feel for the groundwater chemistry, because it did impact the density and distribution of anaerobes during the year long sampling. The two sampling sites, a methanogenic and sulfate-reducing site, differed in terms of their dissolved inorganic carbon and chloride content. The methanogenic site was at least an order of magnitude greater in dissolved organic carbon and chloride than water samples from the sulfate-reducing site. Nitrate and oxygen were undetected at either site at least by field oxygen probes. Sulfate was generally undetectable in the methanogenic site, but it was transiently present, since it can be detected at certain times of the year. In the sulfate-reducing site, however, sulfate was always present and sometimes in concentrations up to 20 m levels.
The numbers of organisms, as determined by Acridine Orange Direct Counts (AODC), were compared with the numbers of sulfate-reducing bacteria and methanogens at these sites. The total number of organisms, as measured by direct microscopy at the sulfate-reducing site, did not really change very much over the course of time (slide). However, when the numbers of sulfate-reducing bacteria and methanogens that were utilizing the substrates acetate, formate, and hydrogen were assayed in the areas that are high in sulfate, the density of the sulfate-reducing bacteria tended to be larger at most times of the year than the methanogens (slide). Types of methanogens that utilized methanol and trimethylamine were also detectable. When comparing the sulfate-reducing site with the methanogenic site (slide), the sulfate-reducing bacterial numbers were dominant at most times of the year. Contrasting that with the methanogenic site, total numbers did not vary significantly throughout the year and they did not really vary from one site to another. However, when using the same electron donors, like acetate, formate and hydrogen, the methanogen became at least as dominant or even more dominant than the sulfate-reducing bacteria. Furthermore, the numbers of methanol and trimethylamine-utilizing methanogens were greater at the methanogenic site than they were at the sulfate-reducing site.

To summarize the results from the Norman landfill, the sulfate reducers and methanogens were at both sites, but their relative distribution seemed to be influenced by at least a few factors. Such factors were the chemistry of the groundwater and the availability of the terminal electron acceptor, in which this case was sulfate. Through subsequent studies, there were other factors that also influenced the density and distribution of the microorganisms, but these two appeared to be the predominant ones.

A different picture emerged when the density and distribution of anaerobes were investigated and samples were obtained from the subsurface formations underlying the Savannah River complex. After the three borings were completed, a fourth boring, C10, was drilled further down-dip from the other three. What was generic about the obtained samples from the Savannah River was that when they were sampled by the methods that Dr. Phelps described in Plenary Session 2, they were shipped on ice in plastic bags inside of mason jars and then immediately placed inside a glove box for anaerobic analyses. Generally, the analysis focused on the following two things: the enumeration of interesting bacteria and the various types of metabolic activity assays. In terms of our rationale for the enumerations, the C10 borehole was assayed for acetogenic bacteria for the breakdown of organic materials under anaerobic conditions, for short-chained fatty acids, for sulfate-reducing and methanogenic bacteria, for terminal hydrogen-consuming organisms, and of course, in the removal of fatty acids.

An interesting group of organisms, the coliforms, were also assayed. They are facultative anaerobes, nutritionally diverse, and their abundance in terrestrial subsurface is unknown. The techniques for the assaying were very well established and they ended up being fortuitous tracer organisms for monitoring the movement of organisms from the drilling mads into the formations.
Prior to that, a multi-investigator study was done in order to observe aerobacterial counts on a given sample using a dilute PTYG medium. The aerobic counts agreed fairly well from laboratory to laboratory. Using the same media, samples were incubated in an anaerobic glove box, and certain formations showed that anaerobes could be grown even in the dilute PTYG medium. This suggested that anaerobic heterotrophs were present. After picking some of the colonies (approximately 30-40), it was found that most of them were facultative organisms, as one might expect with this sort of an assay. Several isolates were strict anaerobes. So again, the strict types of anaerobes were in the formation.

Results of an enumeration for the sulfate-reducing bacteria per gram dry weight of sediments showed that the organisms were present in many strata from the various boreholes (slide). P28 showed the greatest number of sulfate-reducing bacteria, while P24 showed the fewest. In P28, the sulfate-reducing bacteria were detected throughout the vertical columns, except for a strata at 125 m in a consolidated clay layer; so indeed, throughout the geological column they are nondetected. The P29 site also harbored sulfate-reducing bacteria, except for three strata in or contiguous with the Ellenton/Williamsburg formations. The P24 site contained an uneven vertical distribution with eight of 16 strata containing no viable sulfate-reducing bacteria that could be detected. The organisms were found primarily in the transmissive zones and the numbers were inversely correlated with the clay content. The distribution of sulfate-reducing bacteria did not correlate with the groundwater sulfate content at any of the sites. Due to the greater depths of C10, sediment samples from the transmissive zones were much more difficult to obtain and thus comprised a smaller percentage of the sample. Nevertheless, the sulfate-reducing bacteria showed the same distribution pattern as in the other three boreholes, with the higher numbers occurring in sediments with the lowest clay content. In the C10 hole, the range of sulfate-reducing bacteria was similar to that found in the other boreholes (slide). Some sediments harbored as many as $10^5$ sulfate-reducing bacteria per gram, even in the deeper sediments.

Coliform bacteria, which are known to be common in a variety of surface environments, are an interesting group of organisms. The distribution of these organisms in the subsurface has generally been unappreciated and thus, were assayed in the Savannah River Site boreholes. The coliforms were present in sediments from all boreholes, but the majority were detected in the upper formations. The only exceptions to this were two samples, one from a lower Middendorf formation in P28 and the equipment formations in P24.

The question then became, "What kinds of coliforms are present?" A cursory identification procedure indicated that we were dealing with the Acetobacter Citrobacter types, and not dealing with fecal organisms. In fact, all tests for fecal and Streptococci at the elevated temperature tests, which indicate recent fecal origins, were all negative. Since coliforms are fairly vigorous organisms, the drilling muds were assayed for these organisms, as well as the dry and unused drilling muds, the water used to slurry the muds, and the slurries immediately after they were made. All were free of coliforms. After drilling started, however, it was noticed that high
numbers of coliforms could still be detected in the drilling mud reservoirs. In fact, the recirculated mud samples often contained numbers of coliforms that were many times greater than the numbers found in the sediments themselves. These organisms were used as tracers to monitor the drilling mud penetration into the core. Therefore, this group of organisms was intensively followed when the C10 hole was drilled.

Basically, drilling fluids at the surface of the hole were pumped down the drill stem to the bottom of the hole while drill tailings were carried out at the top of the hole. At the top of the hole, tailings were filtered and the mud was recirculated back into the drilling fluid; therefore, it was a cycle of drilling fluid. Throughout the course of the drilling, the number of coliforms were monitored by sampling the return line of the recirculating fluids. Cores were pared in the field, so both the pairings along with the interior of the core were monitored for these types of organisms.

Once again, the starting mud slurries, just after they were made, contained no coliforms. However, just before drilling commenced, the coliforms were noticed in the mud reservoir (slide). Before the drilling muds were used, they had over 10^6 organisms/gdw, which suggested that perhaps contamination came from the surface, which contained about 10^2 organisms/gdw. Yet just after the muds were slurred, the slurries were free of coliforms. After the muds were contaminated, the reservoir muds contained coliforms throughout the drilling process, with numbers ranging from 10^3 to 10^7/gdw. The total numbers of bacteria in the drilling muds did not change appreciably over the drilling operation.

Comparing that to the coliform densities found in the interior of the core sample, as well as the pairings, showed that no coliforms could be detected. Yet when a positive control was used, that intentionally inoculated the samples with coliforms. It was found that the formation did not inhibit the growth of the coliforms. Some of the samples did, however, have detectable levels of coliform bacteria.

If one makes the nonconservative assumption that all of these organisms arose as contaminants and one understands the detection limits of the MPN assay, then one will have some quantitative basis for estimating the amount of microbial contamination in the sample. To obtain the estimates, the number of coliforms in the sample was divided by the number of coliforms present in the corresponding drilling muds, and that ratio was expressed as a percent. In some cases the level of contamination in the clay was 0.0001%. If one were to examine the data, one would see that the worse case of contamination assumptions were at the 1% level for the transmissive zones. Generally, the more transmissive sediment zones, as one might expect, had more of an opportunity for contamination; therefore, they generally tended to have the highest levels of contamination.

The limits of microbial contamination in the Savannah River samples could now be quantitatively evaluated, thanks to work provided by Bill Ghiorse and Dave Balkwill. By taking that limit of contamination and multiplying it by the total count, one could estimate the number of organisms that would actually be there due to contamination. The upper limit of contamination should have been 10^6/gdw, yet the viable count was 10^7/gdw. Generally, even the viable count estimates were at least
an order of magnitude higher than the upper limits of microbial contaminations. Therefore, quantitatively assuming that all the contamination came from the drilling fluids, it was not enough to quantitatively explain the numbers of organisms that were obtained (i.e., cultured relatively easy from the samples).

Some of the results from the C10 borehole can now be summarized with regards to the methanogens, acetogens, and sulfate-reducers that were included in the analyses. Even though the environment was aerobic, anaerobic organisms, along with their metabolic activity (Ralph Jones in Plenary Session 4), tended to occur together and be limited to the saturated transmissive zones. This was important because the anaerobic degradation of organic matter is often strictly dependent on the consortium of microorganisms, each possessing a variety of metabolic activities. These findings suggested a consortium necessary for the anaerobic cycling of organic matter may already be present even in environments that are largely oxidizing. This consortium was not present in all of the sediments, but they tended to be in the major transmissive zones.

To summarize the Savannah River story, the density of anaerobes were generally low at each site that was examined, but they were nonetheless present. Once again, the saturated transmissive zones harbored the microorganisms that were generally necessary for the cycling of organic matter. The distribution of coliform bacteria in the muds and core samples was used to estimate the upper limit of microbial contaminations obtained from the drilling procedures. This analysis suggested that the methods used to collect the samples were sufficient to prevent a large degree of bacterial contamination in most of the samples. The data were not arguing that there was no contamination in the samples, but that the level of contamination in most cases was quite small.

The last investigation to discuss is the one that is whimsically referred to as the "hard rock" microbiology study, in which the density and distribution of microorganisms in consolidated rock formations was studied. Through the efforts of a graduate student, a core was obtained from a discovery oil formation in Kansas at 211 m. Records do not indicate that the oil field was ever subjected to any sort of water intrusion by drilling operations. This particular drilling site was attractive due to the employment of pneumatic drilling procedures. However, it was subsequently discovered that such techniques often employ lubrication water. Since in this particular case a local cattle watering pond was used as lubrication water, both the core sample and the pond water were examined.

Samples were shipped on ice to the laboratory and processed within 48 hours of collection, thus providing an opportunity to examine a rather fresh core from an oil well site. The core was sectioned into four pieces, and each portion was processed differently. One portion was dry-heat sterilized and used as a method control. The other two portions were used for aerobic and anaerobic microbiological assays, respectively. The fourth piece was used to characterize the core in terms of its physical and chemical properties. The logic in processing the core was to only take the interior portion. A sterile hammer and chisel was used to fraction the core and
to break it open, and then a sterile drill bit was used to gently drill into the interior. From the center of the core, the ground rock material was collected in a sterile mortar and used for microbiological analyses. Characteristics of a consolidated sample were as follows: it was a siltstone with very low porosity and very low permeability; it had less than one micro Darcy; pH was 7.8; nitrate was detectable; there was a trace of sulfate; and the in situ temperature was approximately 18-20°C.

The core material, the pond water, and the methodology control samples were all subjected to the same sort of microbiological analysis (slide), which included direct microscopic count and assaying for aerobic organisms, including aerobic heterotrophs on standard plate-count media, half-strength nutrient broth, and the coliforms. Anaerobic assays included an assay for heterotrophs, nitrate- and sulfate-reducing bacteria, and methanogens. The total numbers of organisms were very low. In fact, they were below the detection levels; therefore, little could be said about the total numbers of organisms in this core.

The viable plating assays indicated that there was some evidence of low numbers of organisms (aerobic heterotrophs) in the core and that there were no coliforms. However, the lubrication fluid contained all of these types of organisms; therefore, a large background population of these organisms did exist on the exterior of the core and in the water that the core was bathed in. The methodology control showed no growth of any of these types of organisms. Anaerobic assays were quite different in that none of the indicated types of anaerobes could be cultured from the core material, although lubrication waters contained all of these organisms, except for the methanogens.

The consistent finding in both types of assays was that the lubrication fluid contained high numbers of organisms, while the core itself harbored only low numbers. Furthermore, despite the quality control procedures, the question still arose as to whether the bacteria which were detected in the core were truly indigenous to the core and to the rock formation, particularly during drilling operations since the lubrication of water was subjected to pressures of up to 400 psi for 90 minutes.

Could such pressures play a role in forcing bacteria to the core material? This was tested by trying to simulate down-hole conditions using a pressure vessel in which a portion of the core was placed. Additionally, a conduit for the passage of microorganisms was intentionally created by fracturing the rock with sterile tools. It was found that pressurization had little effect on the migration of bacteria into the case material. On the positive control, on the face where the fracture was created in the core, the microorganisms could be detected. However, in the core sample itself, these organisms could not be detected.

**Conclusion**

To summarize, the low permeability of the formation, the pressurization experiment, the comparison of the microflora, and the lubrication fluids in the core sample all suggested that the contamination of the core with nonindigenous organisms was unlikely. The consolidated rock formation contained up to $10^4$ cells/gdw. It seemed to be largely aerobic heterotrophs, and no anaerobes could be found. It did
highlight the need for paring the core material in consolidated formations and to be careful not to contaminate the environment with undesirable organisms. This in particular refers to the oil industry, which is interested in eliminating or controlling the effect of sulfate-reducing bacteria. This study, as well as the Savannah River samples, have taught us that core paring procedures are really essential for obtaining or having a chance of obtaining subsurface samples that tend to be microbiologically uncompromised. Thank you very much.
D. Boone: It was not clear to me when you were explaining how you made your calculations on contamination of the samples at Savannah River Site whether you calculated percent contamination by coliform bacteria or whether you used that number to calculate the percent contamination by total bacteria from the drilling fluids.

J. Sufliita: First of all, the coliform organisms were used. It was presumed that none of what was seen was due to contamination alone. That gave us handle on contamination; however, multiplying the percent contamination times the total number of organisms would give us another number. That number was then compared with the viable organisms. So the bottom line is that a serendipitous tracer was being used, one that was an actual microorganism instead of a chemical analog.

D. Boone: It still was not exactly clear to me. There were, for instance, two or three logs higher counts of total bacteria in the drilling fluids than coliforms. Did your calculations take that into account and calculate the total number of bacteria that contaminated your sample or did you calculate the number of coliforms that contaminated the sample?

J. Sufliita: The percent contamination was calculated. The percent contamination was determined by the coliform analysis, and then by taking that analysis and multiplying it by the total numbers.

J. Wilson: Can you rationalize aerobic organisms under an anaerobic petroleum deposit? What were they doing there?

J. Sufliita: I am always asked, "What are the organisms doing there?" I do not know what they were doing there. They could have been laid down with the formation or they could have, perhaps, been seeking out a living on petroleum products. It is hard to know exactly what they were doing there.

W. Ghiorse: It is very interesting to know if there were microfractures, especially when the permeability of this stuff is very low. What I am thinking about is the pore throat size; for instance, was it big to accommodate 0.2 micrometer diameter cell?

J. Sufliita: Yes. The porosity was quite tight, less than one micro Darcy. I do not know if the pore throat sizes were a calculated value or not. We have not specifically done so, but maybe we can back that out of the data. It was interesting that some of the organisms that were isolated were not very small organisms. Using the quality control procedures that were involved, were there microfractures? Yes, there were, and we tried to avoid the larger fractures. An attempt was made to avoid those that could be seen.
J. Wilson: What is the possibility that you were counting oxygen-tolerant fermentative-type organisms in the Kansas discovery part? What was your culture media?

J. Suflita: The culture media was a Standard Plate Count Agar for those organisms. Other than enumerating the samples, no characterization has been done at all, so it is possible.

S. Kellogg: Your coliform data was set at the upper limit of contamination. Is that not being rather optimistic? Could that not be the lower limit of contamination? There is already a significant body of literature showing that E. coli does not transport like spore forms or other types of bacteria. If that is true, then the coliform concentration would be the lower limit. In question two, in terms of calculating concentration, I would really like to know whether you reduced the level by the coliform count or by the total count in the drilling fluid.

J. Suflita: The question is pertinent because there is more known about coliform organisms and how they move in the subsurface than other types of organisms. They are also a good class of organisms, or group of organisms, that can be easily enumerated. So for a tracer, those are advantages.

S. Kellogg: Yes, but you set it as the upper limit.

J. Suflita: Yes. It can always be argued that other cells move differently, but it is a start. It is not a chemical tracer. What is also clear is that bacteria do not move like viruses and bacteria do not move as bromide. They do not even move as microspheres, although they are both about the same size and shape.
Eukaryotic Microorganisms in Subsurface Environments

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Abstract

Eukaryotic microorganisms were examined by enrichment culture techniques in four pristine and two contaminated sites at different geographical locations and in different geological materials. Samples were obtained from shallow to deep sediments. In a shallow, uncontaminated aquifer in the floodplain of a small river in Oklahoma and a deep, uncontaminated aquifer in coastal plain sediments of South Carolina, sparse population densities of protozoa, fungi, and algae were found. Protozoa and algae tended to be distributed in sandy or gravelly sediments, whereas fungi were found in nearly all sediments. Protozoan and fungal numbers and types did not appear to be affected by depth up to 436 m at the South Carolina site. Small numbers of protozoa were found in sediments from four boreholes from as deep as 85 m in an unpolluted, glacial-buried valley aquifer in Kansas. Fungi were not found in any sample from this site. Only fungi were found in a dry, uncontaminated volcanic tuff from as deep as 53 m at Los Alamos, NM. Typically, 100 or fewer eukaryotes/gdw were found in the sediments of any of the uncontaminated sites. At a jet fuel contaminated site at Traverse City, MI, large numbers of protozoa were found in the unsaturated zone where contaminant vapors and oxygen mixed. Very large numbers of protozoa were found during biorestanation of an aviation gasoline contaminated site at Traverse City, where indigenous bacteria were stimulated to degrade gasoline by the addition of nutrients and hydrogen peroxide as an oxygen source. Therefore, although eukaryotes were not abundant enough to have an important ecological impact at pristine sites, the increased availability of carbon sources and bacterial populations at a site contaminated with organic pollutants allowed protozoan populations to increase to significant levels.
Introduction

Bacteria have been demonstrated to be common inhabitants of subsurface environments in recent investigations. Work on bacterial occurrence, distribution, and activities in the subsurface was reviewed by Ghirose and Wilson. Bacteria, in other environments such as surface soil, are accompanied by other types of microorganisms including eukaryotes, and thus, the question arises if eukaryotes are also found in the subsurface. Investigators addressing this matter have included Federle et al. who reported finding extractable phospholipids, including polyenoic fatty acid as indicators characteristic of eukaryotes in subsurface sediments of several different soils in Alabama. The presence of protozoa in the unsaturated subsurface has been recognized since early in this century, and more recently protozoa and fungi have been reported in groundwater. Harvey and George found protozoa in an aquifer contaminated with treated sewage.

Although eukaryotes have been found to occur in the subsurface, information is lacking on how widespread their occurrence is and also what factors influence their density and distribution. This paper will discuss the occurrence and distribution of eukaryotes found at four uncontaminated sites and one contaminated site and will discuss factors influencing the distribution of eukaryotes at these sites.

Materials and Methods

Site descriptions. The Lula, OK site was at the edge of the floodplain of a small river and is described by Wilson et al. This site was an uncontaminated shallow aquifer site and samples were obtained from a maximum depth of 8 m.

In South Carolina, three boreholes (P24, P28, and P29) were drilled in the Savannah River Plant, and a fourth hole (C10) was drilled near Allendale, SC. These sites are described in Sargent and Fliermans and Beeman and Suflita. The holes were drilled in uncontaminated areas to maximum depths of 213.4 to 525.8 m.

Four boreholes were drilled along the axis of a glacial, buried-valley aquifer in northeastern Kansas. This site is described in Denne et al. and Sinclair et al. The sediments were complex layerings of coarse to fine materials deposited by glaciers. Samples were taken from a maximum depth of 85.6 m and no environmental pollution had occurred in this area.

One borehole was drilled to a depth of 53.3 m at the Pajarito Plateau near Los Alamos, NM (Hersman, L., W. Purymun, and J. Sinclair, Abstract Annu. meeting Amer. Soc. Microbiol. N49). The sediments were volcanic tuff and all samples were from the unsaturated zone with little or no water from the surface ever having penetrated these sediments. This site was uncontaminated.

Two contaminated areas of a site at Traverse City, Michigan were investigated. This site is described in Wilson et al. and Twenter et al. The contaminated areas were spills of jet fuel and aviation gasoline at a Coast Guard base. The aviation, gasoline contaminated area was being cleaned with bioremediation by injecting hydrogen peroxide as an oxygen source and nitrogen and phosphorus as nutrients. The sediments were uniform sand with little variation in texture. Samples were taken to a maximum depth of 5 m.
Sample processing and enumeration of microorganisms. All samples were from parts of sediment cores that were free of microbial contamination and were handled under aseptic conditions. In all cases, the outer part of the core was removed and the inner, uncontaminated part of the core was used for microbiological analyses. The procedures for acquiring uncontaminated samples are described in Wilson et al.\textsuperscript{21} for the Lula, OK site, Phelps et al.\textsuperscript{12} for the South Carolina sites, Sinclair et al.\textsuperscript{17} for the Kansas sites, Hersman et al. (Hersman, L., W. Purdy, and J. Sinclair. Abstr. Annu. Meet. Am. Soc. Microbiol, 1988, N49) for the Los Alamos, NM site, and Wilson et al.\textsuperscript{22} for the Traverse City, MI site. Samples were shipped from the field sites to the laboratory via an overnight delivery service. Upon receipt of samples they were stored at 4°C until processed, which usually occurred at or before 72 hours after delivery to the laboratory.

Microorganisms were enumerated by enrichment culture techniques. Protozoan numbers were estimated by a modification of the Singh\textsuperscript{18} most-probable-number technique, which is described in Sinclair and Ghiose.\textsuperscript{15} Enterobacter aerogenes was supplied as a food source for the protozoa except for the Traverse City samples where Escherichia coli was used. Fungi were enumerated by plating on a 1/20-strength, potato dextrose medium.\textsuperscript{16} Algae were counted by plating on a mineral-salts medium as is discussed in Sinclair and Ghiose.\textsuperscript{16}

Results

At the Lula site, protozoan numbers decreased from densities of 1.9 x 10^6 to 6.4 x 10^5/gdw in the surface soil to 2.9 x 10^3/gdw at 0.25 m deep and 4.3 X 10^2/gdw at 0.5 m deep.\textsuperscript{15} From 0.5 m deep to the bottom of the unsaturated zone at 3.0 m deep, protozoa were found at densities of 50 or fewer/gdw in all samples examined.\textsuperscript{15} Thus, protozoa appeared to be continuously present in the unsaturated zone. In the saturated zone, protozoa had a discontinuous distribution with small numbers being found in two discrete layers. The first layer was near the top of the water table at 3.15 m-3.35 m deep in one borehole, where a single species of amoeba was found at densities of 44/gdw in the upper portion of this layer. This same population of amoebae was found at a density of 111/gdw near the top of the water table (2.74 m deep) in a second borehole, 21.7 m from the first borehole. The second layer where protozoa were found was one meter thick zone consisting of gravelly sand at a depth of 7-8 m. In this layer, there was a small population of mixed species of flagellates and amoebae. Four protozoa/gdw were found at 7.5 m deep in one borehole and six protozoa/gdw were found at 7.4 m deep in a second borehole, 1.9 m from the first borehole. The presence of protozoa in this layer appeared to be related to sediment texture since protozoa were not found in shallower or deeper samples which had finer textures.\textsuperscript{15} Protozoa were not found in samples from the saturated zone which contained more than 30% clay. In all samples from deeper than 0.5 m, only flagellates and amoebae were found with no ciliates being present.\textsuperscript{15}

Fungi were found in all samples regardless of texture and were even found in the basement clay at 8.0 m depth where no protozoa and few bacteria were found.\textsuperscript{17}
Fungal numbers were not determined. Only one type of fungus was found, which was identified as *Cylindrocarpon didymum*.

Algae and cyanobacteria were found in a sample from the 7.5 m deep gravelly layer. Although phototrophs were not enumerated, they were present at a density of approximately one cell/gdw of sediment.

At the South Carolina sites, the density and distribution of eukaryotes followed the same trends observed at the Lula site, although samples were taken from depths of as much as 40 times deeper. The bacterial and eukaryotic microbial populations were lower at sites P24 and C10 than sites P28 or P29 (Table 1). At all sites, 56,000-350,000 protozoa and 103,100-171,000 fungal propagules/gdw were found in the surface soil. In the subsurface at sites P24 and C10, typically 10 or fewer eukaryotes/gdw were found in samples where they were present. At sites P28 and P29, protozoan numbers were higher and algae were found (Table 1). Protozoa tended to occur in sediments which had coarser textures and were frequently not found in sediments with finer textures. As illustrated in Table 1, 0.2-3.9 protozoa/gdw were found in sediments having 72-90% sand and 5-11% clay from 34.4 to 262.1 m deep at site P24. The presence of protozoa was related to bacterial density in the samples. Protozoa were found in 34 samples from sites P24, P28, P29 and C10. Of the samples where protozoa were found, six contained fewer than 10^2 bacteria/gdw, three contained 10^3-10^6 bacteria/gdw, and 25 contained more than 10^7 bacteria/gdw as determined by plate counts on dilute PTYG medium (J.L. Sinclair and W.C. Ghiorse, unpublished). Only flagellates and amoebae were found in the four South Carolina boreholes.

Fungi were found at densities of 0.6-40 propagules/gdw in samples from all South Carolina sites. Fungi were not detected (detection limit 0.2/gdw) in the Middendorf formation of sites P24, P28 and P29 at 236.8, 204.2, and 181.1 m depths which contained 67, 38, and 40% clay, respectively. Fungi were found in almost all other samples regardless of texture. Unlike the Lula site, different types of fungi were found in different samples.

No phototrophic microorganisms were found in any sample from site P24 or C10, however, eukaryotic unicellular and filamentous algae were found with cyanobacteria at low numbers in the sandy sediments of site P28. These microorganisms, plus diatoms, were found in the sandy sediments of site P29. When present, numbers of phototrophs were typically low with numbers ranging from 1.2 to 49/gdw in sediments from as deep as 214.0 m at site P28 and between 0.6 and 267/gdw for sediments from as deep as 213.4 m at site P29. As was noted for protozoa, phototrophs were not found in sediments with high clay contents. Different types of phototrophs were found in different samples. The presence of phototrophs in sites P28 and P29 was accompanied by higher numbers of bacteria and protozoa than are often found in other pristine sites.

Eukaryotic microorganisms did not appear to be influenced by depth at sites P24, 28, or 29, and at least in the upper 436.2 m of the C10 borehole. A sample from the Middendorf formation of site C10 from 411.5 m deep contained 0.2 protozoa and
seven fungi/gdw, and a sample from the 436.2 m depth, also from site C10, contained 2.3 protozoa and 40 fungi/gdw. Protozoa and fungi were not found in deeper samples from the C10 borehole at 463.0 and 525.8 m deep, but it is not certain whether the absence of these microorganisms was due to depth or the same geological and hydrological factors which affect microbial distribution at any depth in the subsurface. Algae were not found at any depth in the C10 borehole, but they were found at maximum depths of 214.0 and 213.4 m in the P28 and P29 boreholes, respectively, which was at or near the greatest depth to which these boreholes were drilled.16

The Kansas glacial-buried-valley aquifer site was unusual in that fungi were not found in any sample from the four boreholes that were examined. The reason for the absence of fungi is not clear, but one feature of these sediments that may have influenced fungal presence was their high pH. Sediment pH in the four boreholes examined at this site ranged from 8.2 to 9.6.17 Phototrophic microorganisms were also not detected at this site. Protozoa were present in all four boreholes at densities typical of uncontaminated sites. When protozoa were found, their densities ranged from the detection limit of 0.2 to as many as 33/gdw. As at the Oklahoma and South Carolina sites, protozoan density was related to sediment texture and protozoa were not found in sediments with more than 28% clay.17 As was reported for the South Carolina sites, the presence of protozoa in sediments was related to bacterial density. Plate counts of at least 10^5 bacteria/gdw on dilute PTYG medium were required for protozoa to be present.17 Only flagellates and amoebae were found.

Fewer eukaryotic microorganisms were found at the New Mexico site than were found at the other sites. The sediments of this site contained less than 5% moisture. No protozoa or algae were found in any sample from this site. Three and one fungi/gdw were found at 15.2 and 30.5 m deep, respectively. No fungi were found at 45.7 or 53.3 m deep. Yeast were found at 15.2, 45.7 and 53.3 m depths at densities of 20-25 cells/gdw (Hersman, L., W. Purtymun, and J. Sinclair, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, N49).

At the Traverse City site, fungi and algae were not enumerated. Numbers of protozoa are given in Table 2 for the jet fuel contaminated area. Greater than 16,500 protozoa/gdw were present in sediments from 7.8 to 8.2 m deep in the unsaturated zone where jet fuel vapors and oxygen mixed. Few or no protozoa were found in the contaminated saturated zone or at the water table where contaminants were concentrated. Protozoa again appeared in the saturated zone where levels of contaminants diminished and were less toxic. Table 3 shows the number of protozoa found at an aviation gasoline contaminated area of the Traverse City site. All samples from this area were taken from slightly below the water table at depths of 4.70-5.26 m. The pristine area had 3.93 x 10^2 protozoa/gdw, which is slightly high but still within the range found at uncontaminated sites. At the contaminated but untreated anaerobic site, protozoa were not found. Large numbers of protozoa were found in the biological treatment zone where the nutrients and hydrogen peroxide were injected. A population almost exclusively consisting of one species of an amoeba was found 2.4 m from the hydrogen peroxide injection point, where
hydrogen peroxide levels may have been toxic. The density of this amoeba (Table 2) was similar to protozoan levels typically found in surface soil. The density of protozoa increased to 2.66 x 10^3/gdw at 9.4 m from the injection point of the hydrogen peroxide, at which point the hydrogen peroxide had broken down and was no longer toxic.

**Discussion**

At least one type of eukaryote was found as part of the geological profile at each site. These findings are in agreement with other investigators who have also reported finding protozoa, fungi, or algae in the subsurface.\textsuperscript{6,10} (Eleuterio, this volume). Eukaryotes must therefore be considered to be common inhabitants of the subsurface. Similarities were found between the Oklahoma, Kansas, and South Carolina sites in factors that affected the distribution of eukaryotes. One of the most important of these factors was sediment texture. The presence of both protozoa and algae was affected by sediment texture with both appearing more frequently in coarse textured sediments. Bacterial abundance has similarly been observed to be related to sediment texture.\textsuperscript{7} It is therefore not surprising that bacterial numbers also were found to be related to whether or not protozoa would be present at the Oklahoma, Kansas, and South Carolina sites. Fungi were less affected by texture than other subsurface microorganisms. Depth was expected to be a factor which influenced microbial populations; however, no effect of depth was observed for eukaryote populations in any of the boreholes of this study. Other factors which affected eukaryote presence and abundance appeared to be specific to individual sites. It is not clear why the Kansas site was found to have no fungi, although the high sediment pH may be a possible reason. The extreme dryness of the New Mexico site resulted in the absence of eukaryotes other than fungi and yeast. The differences found between eukaryote populations found at different sites are not unexpected since variations between sites have also been found for bacterial populations.\textsuperscript{7,11}

**Conclusion**

At all of the uncontaminated sites, the numbers of eukaryotes found were small. Typically, eukaryotes were not found at densities greater than 10^3/gdw. The densities of protozoa found in these subsurface environments were similar to those reported by Waksman,\textsuperscript{20} who examined protozoan abundance below the root zone. At these population levels, eukaryotes would not be expected to be important members of the subsurface microbial community. The factor limiting subsurface microbial populations at uncontaminated sites is generally regarded to be utilizable carbon.\textsuperscript{8}

Protozoa were found to be abundant at the Traverse City site, where spills of jet fuel and aviation gasoline provided abundant supplies of utilizable carbon in the subsurface to support bacterial growth. Large numbers of protozoa were noted in the unsaturated zone of the jet fuel spill area where the vapors of the jet fuel mixed with oxygen. Additionally, large numbers of protozoa were found in the aviation gasoline spill area where bioremediation was taking place. The protozoan populations in these samples would be capable of carrying out the same functions that protozoa would in
surface soil or aquatic environments. These functions include regulation of bacterial populations or mineralization of nutrients from bacterial biomass. Thus, although protozoan populations are small in uncontaminated sites, their numbers can increase to significant levels when conditions become favorable for their growth as was observed at the Traverse City site.

This work was supported in part by DOE grant DE-FG02-86ER6047. I would like to thank John Wilson, Guy Sewell, and Stephen Hutchins for providing samples from the Traverse City Site.
References


### Table 1. Range of Eukaryote Densities at Uncontaminated Sites.

<table>
<thead>
<tr>
<th>Site&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Depths (m)</th>
<th>Range of Protozoan Densities /gdw</th>
<th>Range of Fungal Densities /gdw</th>
<th>Range of Algal Densities /gdw</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>OK</td>
<td>3.0-8.0</td>
<td>&lt;0.2-111</td>
<td>P&lt;sup&gt;c&lt;/sup&gt;</td>
<td>P&lt;sup&gt;c&lt;/sup&gt;</td>
<td>14</td>
</tr>
<tr>
<td>P24, SC</td>
<td>34.4-262.1</td>
<td>&lt;0.2-3.9</td>
<td>&lt;0.5-10.1</td>
<td>0.5</td>
<td>15</td>
</tr>
<tr>
<td>P28, SC</td>
<td>14.3-216.1</td>
<td>&lt;0.2-1189</td>
<td>&lt;0.5-35</td>
<td>&lt;0.5-48.9</td>
<td>15</td>
</tr>
<tr>
<td>P29, SC</td>
<td>7.6-213.4</td>
<td>&lt;0.2-208</td>
<td>&lt;0.5-10.5</td>
<td>&lt;0.5-267</td>
<td>15</td>
</tr>
<tr>
<td>C10, SC</td>
<td>20.4-436.5</td>
<td>&lt;0.2-2.3</td>
<td>&lt;0.5-40</td>
<td>&lt;0.5</td>
<td>unpublished</td>
</tr>
<tr>
<td>KS</td>
<td>18.9-85.6</td>
<td>&lt;0.2-33</td>
<td>&lt;0.5</td>
<td>&lt;0.5</td>
<td>16</td>
</tr>
<tr>
<td>NM</td>
<td>15.2-53.3</td>
<td>&lt;0.2</td>
<td>&lt;0.5-3.0</td>
<td>&lt;0.5</td>
<td>(d)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Range of eukaryote densities for all samples from the depths listed.

<sup>b</sup> OK=Lula, OK, SC= South Carolina, KS=Kansas, NM= Los Alamos, NM

<sup>c</sup> P=present, but not enumerated.


### Table 2. Protozoan numbers found at the jet fuel contaminated area of the Traverse City, Michigan site.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Depth (m)</th>
<th>Description</th>
<th>Protozoa/gdw</th>
</tr>
</thead>
<tbody>
<tr>
<td>51AG3</td>
<td>2.38-2.50</td>
<td>Unsaturated, clean</td>
<td>&gt;16,500</td>
</tr>
<tr>
<td>51AG10</td>
<td>3.32-3.41</td>
<td>Unsaturated, contaminated</td>
<td>&lt;2</td>
</tr>
<tr>
<td>51AG26</td>
<td>4.57-4.69</td>
<td>Water table, contaminated</td>
<td>&lt;2</td>
</tr>
<tr>
<td>51AG24</td>
<td>4.82-4.91</td>
<td>Saturated, contaminated</td>
<td>1170</td>
</tr>
<tr>
<td>51AG27</td>
<td>5.15-5.27</td>
<td>Saturated, clean</td>
<td>210</td>
</tr>
</tbody>
</table>

### Table 3. Protozoan numbers found at the aviation gasoline contaminated area of the Traverse City, MI site undergoing biorestoration.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Depth (m)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Description</th>
<th>Protozoa/gdw</th>
</tr>
</thead>
<tbody>
<tr>
<td>50BF6</td>
<td>4.80-4.82</td>
<td>Untreated, pristine</td>
<td>393</td>
</tr>
<tr>
<td>50AT3</td>
<td>4.70-5.26</td>
<td>Untreated, anaerobic</td>
<td>&lt;2</td>
</tr>
<tr>
<td>50AX7</td>
<td>4.72-4.78</td>
<td>2.4 m from H₂O₂ injection</td>
<td>59,400</td>
</tr>
<tr>
<td>50BC6</td>
<td>4.83-4.85</td>
<td>9.5 m from H₂O₂ injection</td>
<td>266,100</td>
</tr>
</tbody>
</table>

<sup>a</sup> All samples were taken from slightly below the water table.
Q and A

C. Curds: I would like to ask two technical questions, both concerned particularly with the techniques that you are using. You said that you used an MPN method using bacteria as a food supply. Could you tell me if they were presented on an agar plate?

J. Sinclair: This was just a modification of the Singh-glass ring method, where glass rings were embedded in an agar medium and then nongrowing bacteria were introduced into each ring. Then a typical, most probable number count was done. At each dilution level, there were five replicate rings. For pristine sites, one can usually get away with dilution of about $10^0$-$10^3$. In contaminated sites, you have to have dilution as high as $10^7$ to cover everything.

C. Curds: So the normal liquid fill would be a bacterial food supply?

J. Sinclair: That is right. It is not a great one.

C. Curds: What worries me is that you may not find ciliates for that very reason. Were you using the same technique on each of the sites?

J. Sinclair: Yes. The ciliates were on the surface soil and we always saw them. Also, we did see them in the contaminated site, the last one. This is something that I did study when I was a graduate student – methods of optimizing the technique to pick up a broad range of protozoa, and when you have some water over the agar, you do pick up ciliates.

C. Curds: Are you trying to distinguish between encysted and trophic amoeba by using the same techniques?

J. Sinclair: Yes. I have done that at the site in Lula, Oklahoma and at the Savannah River Site in South Carolina. As you are well aware, it does take a lot of work, so you can only do it in certain selected samples. However, what I found was that the encysted counts were just about equal with the total count within the range of variation of the MPN method. It would seem that most protozoa are present in an encysted form. I have seen numbers that seem to suggest that this same situation occurs in surface soil too, which may not be entirely unexpected.

Z. Filip: Do you have any evidence that the fungal growth on your plates originated from spores or from material fragments? I ask this question because nothing is easier than getting a contamination by fungal spores, and then considering your plate count being less than 0.2 per gram of sediments. I would be more happy if you were able to show that you were able to identify at least some fungal-hyphal material microscopically in the particle of sediment.
J. Sinclair: We never did any sort of count to distinguish the mycelia from the spores. The one thing that we did do was to sprinkle the sediment material over the plate in some of the lowest dilution, and you could always see that the fungal-hyphal were growing out from the particles. You are right. It is very difficult to exclude fungal contamination from plates. However, as we saw a fungus growing from the edge of the plate for instance, something that might be defused along the edges of the plate, we did not count that. Generally, we felt most satisfied that we could actually see the fungi growing out from the sediment material.

P. Strom: In bacterial enumerations, there is of course a problem with the method you choose; it ends up influencing the results at the end. What about things like anaerobic protozoa, protozoa that have specific food requirement. It is possible that we are missing a group of protozoa?

J. Sinclair: Yes. This was always a factor and I initially did a study to try to optimize the number of protozoa that I obtained. I used different variations of this technique and I went with the one that seemed to give the highest density estimates. I think Aaron Mills has done a study on factors influencing MPN counts of protozoa. This was always a problem and there were always protozoa that may or may not have been favored by one such type of bacteria that uses a particular food source. I have never found a protozoa under anaerobic conditions and its entirely possible that they could be present. However, it is something I would like to investigate in the future.

N. Monir: Why did you find some algae at such depths?

J. Sinclair: Well, this is a good question. We speculated that there may have been some sort of connection to the surface. For instance, a lot of material could have been coming down through the seepage basin or there could have been very transmissive layers that were somehow connected with the surface. Or there could have been various propagules that could maintain their viability for long periods of time without nutrients. We also did a study where one of these algae were isolated in an axenic culture. We grew these on a medium that contained acetate as a food source and they were able to grow well, so it seemed conceivable that there might have been some heterotrophic growth possible. Microflagellates were known to supplement their nutrition by eating bacteria.

C. Litchfield: Two questions on your Traverse City work. One, could you find a reduced number of bacteria at the 9.4 m site, and two, have you calculated the bacteria population that you would need for that huge protozoan population?

J. Sinclair: No. I discussed these numbers. They are pretty much hot off the press, so I have not done any calculations of sort. However, from the direct counts that have been done on samples from this site, there were extremely large numbers of bacteria
present. They were over $10^6$ per gram. I am not sure how much depression of bacterial numbers that the protozoa would cause, but I am sure that without the protozoa they would keep building up until there were more and more.

*J. Wilson:* That material consumes about 100 mg of oxygen per liter of water per day, so there is a significant bacterial production.

*R. Harvey:* Do you have a correlation between protozoa abundance and bacterial abundance for a heavily contaminated site?

*J. Sinclair:* I have not done those correlations. The only correlations I have done are those for the pristine site and for the plate count. The correlation is $R=0.53$, and for AODC, it is $R=0.57$.

*R. Harvey:* Thank you. Now my second question. At your uncontaminated sites, the ratio of protozoa to the bacteria is about $1:10^8$. It seems that in surface waters there is a ratio of protozoa to bacteria of about $1:10^3$. Do you have any feelings as to why there are so few protozoa, considering the number of bacteria that are present?

*J. Sinclair:* For soil, this is actually pretty typical. In some of the work done by Alexander, one could see that the protozoa seemed to have difficulty finding nutrition below approximately $10^6$ or $10^7$ bacteria per gram of soil. It has been hypothesized that the distance between adjacent bacteria, when the protozoa are trying to cover these areas, is so great that they have to spend a great deal of energy moving around between the particles to find enough bacteria to support them.
Microorganisms in Deep Cretaceous Sediments of the Atlantic Coastal Plain: Vertical Variations and Sampling Considerations

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¹Pacific Northwest Laboratory, Richland, WA and ²Florida State University, Tallahassee, FL

Abstract

A series of subsurface sediment cores were obtained from the Middendorf (364-411 m) and Cape Fear (460-466 m) formations, which underlie the coastal plain in South Carolina, to determine the diversity and distribution of bacterial populations within single subsurface geological formations. Population of total heterotrophic bacteria, as enumerated on 1% PTYG, ranged from 10³ colony forming units (CFU)/g to >10⁶ CFU/g within the Middendorf and from below detection to >10⁵ CFU/g within the Cape Fear formation. Dentifirifiers ranged from 100 MPN/g to >10⁴ MPN/g in the Middendorf. In the Cape Fear, they were consistently lower, ranging from below detection to >10⁴ MPN/g. Sulfate-reducing bacteria were detected in two out of 12 core samples from the Middendorf and were not detected in the Cape Fear cores. Despite similarities in sediment textural properties, the hydraulic conductivities of the Cape Fear core samples were 2-3 orders of magnitude lower than in the Middendorf and were believed to be a key property in controlling microbial population density. Individual heterotroph isolates were analyzed for physiological properties using commercially-available, multiple test systems, and then used to determine the variation in the distribution of heterotroph populations within a formation and to assess the potential intrusion of bacteria from drill muds into sediment samples during coring. Transport experiments were conducted with intact cores and a Middendorf sand fraction using drilling mud solutions that contained a mix of bacterial isolates from recirculating drill muds and Br. In these studies, the bacteria comigrated with the bentonite clay and were retarded by a factor of 1.3 in relation to Br. The results from the physiologic analysis of core and drill mud bacteria demonstrate that there is a high degree of bacterial diversity within both formations and that the drill mud bacteria were physiologically distinct from the core isolates. These experiments indicate that the build up of filter cake can effectively limit the migration of bacteria associated with drill muds into core samples and therefore, contamination of pore water with recirculating drilling muds based on a conservative solute tracer can not be used as an indicator of microbial intrusion contamination of cores.

¹Oral presentation
Introduction

The emphasis for this study came from some of the earlier work in David Balkwill’s laboratory and our laboratory, some of which was presented earlier. The data showed a considerable diversity of bacterial heterotrophs, mainly the aerobes within Savannah River sediments.

One of the questions that has been asked in the laboratory is, "What are the relationships that the organisms have with the sediments themselves, based on both physical and chemical properties; and, what is the diversity on a finer scale?" Looking at multiple core samples within a particular formation, which was the major objective in this study, meant looking at the variability and trying to determine the variability in the microbial populations within a defined stratigraphic unit. This also meant determining whether or not the diversity that had been seen throughout the formations held true within a formation and also, attempting to identify hydrophysical and geochemical factors that were responsible for some of the variation that was seen. Another objective, one which I will not address to any great extent, was to assess the effects of drilling on the quality of the microbiological and chemical properties of the core samples that were obtained.

Materials and Methods

The methods that were used for these studies will now be discussed. First of all, pores were subsectioned and shipped from the Allendale C10 site to Pacific Northwest Laboratories (PNL) by an overnight express carrier, as were the previously described samples. A tremendous effort was put forth by Tom Phelps and Brent Russell and their crew in making these samples available to us. A lot of microbial characterizations, pore water expression, and physical analyses were done on subsections of the core samples. The predominant aerobic, chemoheterotrophic colony types were isolated and purified both at our laboratory and at Dave Balkwill's laboratory at Florida State University. The analyses of the isolates, as before, were done using the API-NFT multiple test strips. However, the Biolog, Gram-negative multiple test system, which basically consists of a microtitre plate with 96 different organic substrates, was also used in our laboratory. This test allows one to analyze the metabolic potential for a particular substrate by a particular colony or strain of bacteria.

The location of the C10 borehole was somewhat down-dip from the previous three boreholes that were located on the Savannah River Site (P24, 28 and 29). The C10 borehole was actually located offsite and down-dip, yet not necessarily down a hydraulic gradient. The flow paths from the recharge area are apparently a bit longer at the C10 borehole compared to the previous three boreholes.

The strata that was concentrated on for this particular study was in the Middendorf and the Cape Fear formations, two of the deeper formations that were sampled (slide). These two formations differed somewhat in their properties. The well log, which showed the resistivity, provided an indication of the permeability of the Middendorf formation and the ability of that formation to transmit water (slide). The Middendorf formation was directly overlying the Cape Fear, which was tighter, had a denser formation, and did not transmit water as well as the Middendorf. The Middendorf core samples were taken at 150 feet intervals, whereas the Cape Fear core samples
were taken over a much shorter interval of approximately 30 feet. Lignite, which is
determined visually during the core logging, was quite evident in some of the middle
Middendorf aquifer core samples that were obtained. In fact, relatively large pieces
of intact lignite were actually seen in a number of samples.

When the core segments were received, they were divided up for some of our
different analyses. In one of the core segments, some of the inner core material was
aseptically sampled through the microbiological, pore water chemical and total
chemical analyses. Another core portion was sampled for particle size, particle
density, and clay mineralogy analyses. An undisturbed section was also used to do
bulk density and moisture content measurements. Finally, a small, intact coarse
segment was saved for doing hydrological, microbial, and clay transport experi-
ments.

Results

A range of properties for the core samples from both the Middendorf and the
Cape Fear formations were obtained from in-depth analyses of approximately 12
core samples from those formations (slide). The percent clay content ranged from 3
to 8% for the Middendorf formation and a few of the core samples for the Cape Fear
had slightly higher clay content. The pH range was quite broad within the
Middendorf, ranging anywhere from 3.4 up to approximately 9, whereas the Cape
Fear was consistently higher, pH around 9-10. Total sulfur and total organic carbon
were also measured on these samples. In the Middendorf, total sulfur ranged from
a low of the detection limit all the way up to about 4%. So there were fairly high levels
of fixed sulfur in some of the samples. The organic carbon content also ranged from
relatively low concentrations all the way up to 0.5% in the Cape Fear sample, and all
the way up to 0.7% in the Middendorf formation, with the exception of one sample
that was basically of pure lignite material.

In the distribution of microbial populations that were observed, as well as a
number of different groups, one was the aerobic chemoheterotrophs that were
determined on PTYG, as was done earlier on some of the other Savannah River
studies (slide). As for denitrifiers, the populations included both true denitrifiers as
well as organisms that could assimilate nitrate to ammonia. They tended to be the
predominant type of denitrifiers that were found in these samples. One of the main
observations that was made was the relationship of microorganisms to some of the
physical properties in the sediments, in that microbial populations were apparently
tied to the transmissivity of the sediments. This had been stated before and it was
consistent in our study.

One of the things that we wanted to do was to see if these population levels were
actually tied into specific measurable properties. In one particular case, the hydraulic
conductivity was measured. Hydraulic conductivity could not be done on all of the
samples, but was done on several of them. In the Middendorf formation, hydraulic
conductivities were considerably higher than for the Cape Fear. In fact, in some of
the Cape Fear samples barely measurable levels were obtained. The aerobic
heterotroph populations ranged several log units higher for the Middendorf than
those for the Cape Fear, which had relatively tight formations because of the dispersed clays. Therefore, in those samples, relatively low population levels were seen.

There are two ways to interpret this data: (1) the pore spaces were limiting for the organisms, therefore limiting the supply of nutrients because water flow is restricted through these sediments; or (2) the Middendorf samples were conducive to contamination by drilling muds and therefore, we may have been looking at organisms that basically came from the drilling muds. In the tighter Cape Fear formation, one would expect less penetration by the drilling muds. So this was obviously one question that needed to be to addressed.

It is suffice to say, and it will be discussed later in the proceedings, that solute tracers were present and did indicate pore water contamination, bromide being the particular one. Basically, all the core samples that were obtained from the drilling muds had bromide present, indicating that pore water was contaminated with drill mud water. However, it was concluded that the contamination by drill mud bacteria and clays was apparently minimal, and this conclusion came from a number of different observations. Samples collected from the drill mud pit indicated that these bacteria were physiologically, as well genetically distinct from the core bacteria that were obtained. Some analyses were conducted on the clays, the bentonite clays, and the drill muds, as well as the clays that were present in the core samples. Furthermore, the clays were analyzed by X-ray diffraction using pixy analysis. Those analyses also indicated that the clays, while they were indeed similar, were also distinct. Finally, some transport experiments were conducted in which the migration of drill mud bacteria into some of the intact core pieces was observed. It was discovered that the bacteria and the clay comigrated and were filtered out by the filter cake formation. Dr. Phelps mentioned in Plenary Session 2 about drill muds being the "kiss of death", and in some cases this is true. However, in this particular situation, it may have actually been the muds present in the material that saved the microbiology by acting as a filter by the build-up of the filter cake.

The Biolog plates were, as I mentioned earlier, a system that had 96 different substrates that one could test for utilization by a single organism. A total of 12 isolates were taken from the drill muds, along with isolates that were obtained from a number of different Cape Fear core samples, and a one-on-one comparison was done. In other words, one physiological profile for one organism from the Biolog was compared with that of another. That constituted the one-on-one comparison. The substrate utilization patterns were then studied to see how many total numbers of mismatches existed between them. A zero-to-ten range mismatch was used as the lower level, because in some cases the results could be a bit questionable with a Biolog. In the percent of the comparisons, there was relatively few mismatches (0-10). When the stringency was loosened to 11-20 mismatches out of the total 96, there was some overlap. However, if one would do a total comparison, then the drill mud bacteria, for the most part, would compare less well with the other core organisms than the core organisms did amongst themselves. This, along with some of the other data, indicated that very distinct organisms were being observed.
Some of the drill mud bacteria that were similar to our core bacteria were used for DNA hybridization. This test was even more stringent and in fact, it ruled out the possibility that all but one pair that was tested in the whole series could actually be the same organism. Therefore, although the evidence found in the study was a little different, it was very consistent with what Joseph Sulfita found on the use of some of the coliform bacteria as serendipitous drill mud tracers.

Now on to heterotrophic diversity, which was based on distinct colony types. Included with the diversity was a number of distinct API types. Consistent with what was found between the different formations, a large degree of diversity was found within these core samples that were quite similar in their chemical and physical properties (slide). Also, the lowest populations were all in the lignite and the sand that was immediately surrounding the lignite, which was really quite dark (slide). Populations were low, yet there was quite a bit of diversity based on the colony types and on the physiological analyses (slide). Although the populations of total aerobic, heterotrophic populations were consistently lower in the Cape Fear formation, in some cases there was no growth at all. However, there was still a reasonable amount of distinct colony types and distinct physiological types, ranging anywhere from two as the real low densities up to 10. When looking at how frequent the particular API types occurred in some of the different core segments, it was noticed that a total of 138 out of greater than 150 of the isolates occurred in only one particular core sample. Ten occurred in two of the core samples and it dropped off from there. Only two isolates occurred in six of the core samples, but none occurred in greater than six core samples. The greatest diversity, as one would expect, was in the Middendorf formation, which overall had higher populations of bacteria when compared to the Cape Fear (slide). Overall, there was an overlap of only six types between the Middendorf and the Cape Fear formation.

One of the problems that consistently had to be dealt with was that when one called an organism distinct based on this particular test, one had to understand that there could have been some fluctuation in some of the tests, approximately 22 different biochemical or substrate stimulation tests. Therefore, a difference of one may not have really been a good indicator of actual true strain type. Thus, with a distinct type based on either 1,2 or 3 differences in the physiological patterns in the API tests, the stringency was relaxed. For the most part, when the stringency was relaxed, there was really no large drop off in what was called the distinct isolate numbers. They did drop off by perhaps one or two, but for the most part, we were truly looking at what appeared to be physiologically distinct bacteria. If the same thing was done for the Cape Fear formation, the same types of results were seen. A big shift in the reduction of physiological types was not really noticed when this was done, which indicates that multiple tests such as these may have actually been acting as an initial step for a reasonable approach to obtaining a physiological profile on some of these bacteria.

As Dave Balkwill mentioned earlier, the API-NFT strip was really not meant to identify environmental isolates; it was more for Gram-negative, nonfermentative
bacteria that are of clinical origin. However, an attempt was made to go through these isolates and to determine how many, if any, actually keyed out taxonomically using the API-NFT strips. As would be expected, a vast majority of isolates that were obtained were really unacceptable for their identifications by the API strips (slide). Out of approximately 140 isolates, relatively few actually fell out into some type of bacterial classification using the API-NFT system. Almost the same results were seen for the Cape Fear formation. In fact, there was an even lower percentage that fell out into any type of acceptable identification pattern.

Looking at the distribution of genera using the API (either with a good or better identification pattern), the vast majority, approximately 2/3, fell into Pseudomonadales category, which was not surprising since that is a pretty broad group of organisms. It is interesting to note that some of the preliminary genetic analysis seemed to indicate that the organisms did fall into the Pseudomonas genera. It is also interesting to note that if one is looking at a potential application for bioremediation using these organisms, then there would be a reasonable distribution of Pseudomonad-type bacteria because of their tendency to have a broad assimilatory capacity and group capacity for degradation of chemicals that might be of interest.

Some of the total positive test results using the API strips were obtained from some select core samples (slide). The first three core samples, 24A, 24C and the lignite were from the Middendorf formation, while 28D and 28G were from the Cape Fear. A few things really stood out in the data: the fermentation of glucose was basically absent from any of the isolates that were examined, and the aerobic utilization of glucose was relatively high, except for some samples. One might expect this since glucose was a component in some of the isolation media. There were some differences, such as arginine dihydrogenase, where none of that activity or relatively low activity was seen in the Middendorf bacteria. Once again, in the Cape Fear formation, there were considerably higher totals (approximately 29% vs. the 2% in the Middendorf). Another thing to note was the utilization of the compounds such as citrates. Within the Cape Fear samples, utilization was anywhere from 11 to basically all of the strains within a particular core sample. So there were some similarities, yet differences, between the organisms that were obtained from the Middendorf formation and those from the Cape Fear.

Some interesting patterns were noticed when looking at the isolates from the Cape Fear formation using the Biolog Gram-negative substrates. The highest percentage of substrates that were utilized by some of the bacteria included glutamate and Tween 40. Interestingly enough, if one happened to look at some of the common compounds used by some of the bacteria that were studied in an earlier study from Germany (Kolbel-Boelke, this session), Tween 40 tended to be a commonly metabolized substrate as well. Beta hydroxy butyrate was also one, suggesting that these organisms may have the capability to actually store and utilize at a later time some type of storage polymer. Also, a high percentage of the isolated organisms utilized a lot of low molecular weight, amino and organic acids, acetate, and succinate. Alanine and proline were commonly metabolized compounds. Glucose,
some of the simple sugars, glucose and lactate, and other potential storage polymers such as glycogen and dextrin were commonly metabolized compounds. At the other end, there was a lower percentage of isolates utilizing substrates (between 25-50%), with more complex sugars such as fructose compounds and inositol, formate, glycerol, and histidine. With complex sugars, there were relatively few organisms that utilized these compounds to any great extent (e.g., galactose, mannose, and lactose).

**Conclusion**

In conclusion, physical factors that apparently continue to control the microbial populations in some of the Southeast Coastal Plain sediments also appear to be factors that control the water movement, such as hydraulic conductivity, clay content, and porosity. In addition, the inability to really get a good handle on what the chemistry of some of these sediments, specifically those associated with the samples that are obtained for microbial analyses, can be frustrating. It is important that we, as microbiologists, do not remain satisfied with just being able to get good samples for the microbial analysis. One has to look at it in a broader perspective and try to use better sampling techniques that will allow for better pore water chemistry data that can be used for direct comparisons with microbial populations. At this point, one cannot really say that a relationship does not exist between the pore water chemistry and the microbial populations, because no one is certain that there is a good, valid data set that can allow for those types of comparisons.

Another conclusion is that there appears to be continued, extensive diversity of heterotrophic bacteria within and between cores within a given formation. In other words, that diversity transcends not only from a formation level, but also down to a finer level in what appears to be very distinct types of organisms that are present in continuous core samples. That diversity transcends all the way down to what appears to be a relatively small scale.

Finally, a conclusion which is not really a part of this paper, but is important enough to make again. In spite of contamination with drill mud solutes, and the key word is solute, several lines of evidence indicated that core bacteria in this particular study were not, for the most part, drill mud contaminates. This evidence included direct comparisons with heterotrophic bacteria from drill mud bacteria and core sediment bacteria. The clay analysis, using X-ray diffraction and pixy analysis, indicated again that the clays in the bentonite-based drill muds were indeed distinct from those that were found in the core samples. Finally, in some of the mechanistic studies that have been done but were not reported here, both bacteria and clay did appear to be removed by filter cake formation. It was hypothesized that this was a mechanism for limiting bacterial contamination of the core samples, yet allowing for solute contamination.
Q and A

F. Chapelle: I think your data and the data shown earlier by David Balkwill is very important. It shows that there are differences between some of these hydrologic units. It is entirely consistent with our data, particularly with the sulfate reducers being present in the Middendorf but not in Cape Fear. One additional thing that I think is very important to consider in these studies is the depositional environment of the sediments. The Cape Fear and the Middendorf were deposited in very different sedimentary environments. The Middendorf, with its marginal, marine-type systems, was characterized by periodic intrusions of brackish water. The Cape Fear was entirely continental and was deposited entirely by fluvial-type sediments. That may have been one of the reasons for the distribution of sulfate-reducing bacteria. I guess my point is that depositional parameters should have been considered. My question is, and this is speculative, to what degree do you think this diversity between the sediments was attributable to their initial composition at deposition?

J. Fredrickson: Sounds like another why are they there question. I guess I can also offer some speculation. It is a question that I think we have been dealing with in the Savannah River work for quite a while. What you are trying to allude to is whether or not we are looking at organisms that were basically laid down in that sedimentary environment versus one that could have potentially migrated over long periods of time and therefore, ended up in some of our samples. At Savannah River, one is looking at a relatively complex question, and I am not sure I can fully answer it. From my own perspective, I would say that a majority of the organisms that are actually present there could be basically ancestors, descendents, or whatever, and represent what may have been part of the original depositional environment. We know that there was never any intense type of heat or any type of physical or chemical activities that would have actually tended to sterilize some of these sediments or have an impact on their viability over a long period of time. I suspect that indeed the organisms that are present there, in fact some of the diversity, may be due to the original hydrogeological environment and some of the complexities that happened during that depositional environment. From what I understand, there were a lot of complex things happening, especially around the Savannah River Site, in regard to how these types of sediments were laid down in barrier islands. I certainly would suspect that such occurrences could have lead to the diversity.

G. Mathees: You have shown us one slide where you connected permeability with microbiological data. I recalculated the highest permeability you had and it came out that the highest permeability was $10^8$ m/sec. It means, in a technical sense, that it was impermeable. Therefore, if one assumes that this was impermeable rock, then one must consider mechanisms other than Darcy flow, which is transporting nutrients or bacteria. It means one must consider diffusion as a transport mechanism. The second point, which had been raised by Bill Ghirose, is whether or not you considered microfractures? I think for the transport of bacteria and nutrients, particularly in these materials, microfractures may be the main pathways for transporting nutrients,
bacteria, and other microorganisms, which are not measured when the particle size is determined.

_J. Fredrickson_: Let me see if I can address the second question first, which was in regards to potential for fractures in this material to aid in the transport. One of the problems that has been encountered, as one well knows, was the tendency to characterize core samples that had undergone considerable physical disturbance during sampling and transport. However, in some of our core samples, it was noted that there did exist what appeared to be a fractured type of flow, even in the Cape Fear samples. I guess my answer to your question, if I understand it, is that yes, I believe that fractured flow can definitely be important in some of the samples. However, I also think that sometimes it is a difficult thing to get a handle on when one is attempting to obtain samples, as was done in this investigation. Your first question, as I recall, dealt with diffusion with respect to the supply of nutrients and the transport of bacteria for the transport of organisms and nutrients?

_G. Matheiss_: Well, mainly for nutrients.

_J. Fredrickson_: That is also a good question and one that I really have not given too much thought. However, I think that yesterday one of the papers from the USGS presented a model of diffusion out of some of the more tightly confined layers, which could have been a potential way for supplying electron acceptors to some of the bacteria in the more transmissive zone. I certainly do not know why the reverse of that could not be true when one has diffusion nutrients in, for example, some of the more transmissive zones in some of these types of confining layers.

_J. Tiedge_: My question relates to the interpretation of API and Biolog data. Generally, when this data is used, one tends to assume that it relates to genetic differences. However, it can also relate to subtle changes in expression which are not as significant in relation to genetic information. Do you have a comment relative to that in your experiences?

_J. Fredrickson_: I think that is an excellent point. That is one thing we have tried to address. It is one of the reasons why an attempt was made to relax some stringencies, to see if indeed they really were different. I recognize that even when one does that, there can still be a problem with expression. If one goes out to two or three different test references, or however many one wants to do, one can still have organisms that may be quite similar. I think one of the things that has been done recently in the lab at Florida State University is that they have attempted to observe some of these isolates using some of the restriction fragment analysis patterns as well as some ribosomal sequencing. As far as I can tell at this point, it appears that the API strips have been providing a pretty good indication of genetic diversity as well, although there were cases where there was only one or two differences when they appeared.
to be two of the same type. In other words, one needs to couple some of those genetic analyses along with the physiological analyses.

*T. Hazen:* In our poster presentation, several identical API-matched organisms were compared with DNA melts, and they did not match to type culture. This was supposed to be an excellent identification. I think there was probably a lot more diversity there than what the API test was showing. A cross-hybridization was also done and a couple of them were very good matches, but none of them really matched the type culture. There was not even 20% homology at the most. The DNA melts were not even close and it was probably *Pseudomonas*, which is what you identified also.

*J. Tiedge:* From what I saw, there were two kinds of characters that were important. One was the true heritage of the organism, which the genetic techniques would bring out, and the other was the physiological traits, which tended to be more ephemeral. I think there were two kinds of things and maybe it was not always wise to mix those together. From a practical point of view, you were really interested in physiological traits. You could have had those in very different kinds of backgrounds and still have had the physiological function. If one is interested in evolutionary characters, then it is really the genetic traits that one wants to have.

*G. Toranzos:* I want to express almost exactly the same thing. I am a little confused about two of your slides. When you were showing the API results, you had 0% for the strains that utilized glucose. However, when you used the Biolog, 50-70% of them utilized glucose, unless I misunderstood your slides. I think we should all try to standardize our techniques and maybe use the Biolog, because the Biolog is dependent upon the protection of NADH, while the API is only dependent on the collection of sulfate. Therefore, not only does a sensitivity problem exist, but there is also the possibility that there may be some strains that like glucose or a substrate that may utilize it all the time.

*J. Fredrickson:* I cannot provide the exact numbers, but I think that the glucose utilization between the two really matched up well. What one has to recognize is that one of those tests was for a fermentation utilization of glucose. However, I think that the numbers really matched pretty well between the two tests, somewhere between 1/2-2/3 of the isolates using both techniques.

*B. Reeves:* I would like to agree with what has been said before. Some of our results indicate that API's are not very good for looking at phylogenetic relationships. On the other hand, a group of organisms were observed that had identical API numbers at three different layers and they all were identical.

*M. McInerny:* I have one question on your one-to-one comparison between the drilling mud bacteria and the bacteria of the Cape Fear. Did you do a similar analysis with the other aquifers?
J. Fredrickson: No, that has not been done yet. One of the problems that was encountered was that we did not obtain a good drill mud sample that was comparative with the Middendorf sample. Therefore, we did not really have the bacteria to do that comparison. We also wanted to focus on the Cape Fear because there were relatively fewer numbers of organisms in total population; therefore, it was felt that the ones that were actually picked off of the plates would be more representative. In one case for the Middendorf, one is looking at perhaps 20 different isolates out of a total population that may be 10 million bacteria. It is not known how many of those were the same. In the Cape Fear, perhaps the same number was being observed, but in quite a bit smaller total population. Therefore, it was believed that there could have been a better chance of finding the contaminant, if indeed it was present.
PHYSIOLOGICAL ACTIVITY

Conveners: J. M. Sullita and M. J. McInerney

Included within:
Contributed Papers 2, Posters
Contributed Papers 2, Oral
Round Table 2
Abstract

Core samples of deep Coastal Plain sediments were obtained from depths up to 500 m in boreholes at four sites at the Savannah River Plant in Aiken, South Carolina. The potential for anaerobic mineralization of exogenous and endogenous carbon was present in almost all samples from four boreholes, with the broadest range of activities found in sediments from the saturated, transmissive zones. Methanogens and sulfate-reducing bacteria (SRB) were distributed in various strata throughout the vertical profile of each borehole. However, the rates of production of $^{14}$CH$_4$ and $^{35}$S from radiolabelled substrates suggest that anaerobic metabolism is limited in situ. A comparison of metabolic activities in sediments from various depths in the four boreholes suggest that the factors limiting anaerobic metabolic activity vary with site location and depth. Acetate accumulation and methane production in sediment slurries without exogenous carbon amendments suggest that some areas of the deep subsurface contain reserves of fermentable carbon and that anaerobic metabolism in those zones may be limited by other environmental factors. The anaerobic degradation of benzoate was observed, but was limited in some strata by the absence of acetoclastic methanogens. Phenolic compounds were not degraded in any of the subsurface sediments. Anaerobic O-demethylation of substituted aromatic compounds was observed in sediments from the transmissive zones in one borehole. Coliform organisms, indigenous to the surface and inadvertently circulated with the drilling fluids, proved to be useful indicators of microbial contamination in the deeper samples. The enumeration of these organisms in muds, core shavings, and core samples indicated that microbial contamination of the deeper samples due to drilling fluids was generally minimal or absent.

*Oral presentation.*
Introduction

In Plenary Session 3, Dr. Suflita presented data on the numerical distribution of anaerobic microorganisms in subsurface sediments and showed that deep sediments, or even sediments that may be predominantly aerobic, can harbor relatively large numbers of viable anaerobic bacteria. However, as Dr. McInerney previously pointed out, that is not enough. Simply demonstrating that the organisms are there does not adequately describe their metabolic potential. It is equally important to demonstrate the presence of the metabolic activities which are essential for the cycling of carbon and energy. So for this reason, assaying was also done for a variety of metabolic activities, to determine the metabolic fate of indigenous and exogenous substrates, and to monitor methane and sulfide production in sediments from the Savannah River boreholes. Our study had a number of objectives that would contribute to the understanding of the subsurface microbial activities at the Savannah River Site. They included the following: to detect activities of the anaerobes essential for the anaerobic cycling of carbon and energy, to evaluate the potential for the anaerobic biodegradation of model substrates, and to develop a baseline of microbiological information that could be useful for comparison with other sites.

In the stratigraphy of the boreholes, the important reference points are the confining layers, which separate the four major hydrologic zones: the upper vadose zone, which overlies the more saturated zone in the McBean and Congaree Formations; and then there are two lower aquifer zones, one in the Williamsburg/Ellenton Formation and then a much thicker zone in the Pee Dee, Black Creek, and Middendorf Formation (slide). A number of graphics emphasize the separation between the hydraulic units, because the distribution of anaerobic activity did correlate with the transmissivity and saturation of the zones.

Materials and Methods

Samples were obtained from various depths in these boreholes, processed according to the methods described by Dr. Phelps in Plenary Session 2, and shipped to us by express mail. Upon arrival, samples were placed in an anaerobic chamber for processing and storage. Many of the sediments were slurried with an anaerobic, mineral salts solution that lacked an inorganic electron acceptor, and then they were amended with various substances that are key intermittents in the anaerobic metabolism of organic material. Lactate, formate, and acetate were chosen as model substrates because they are fermentative intermediates, with acetate being an important end-product of fermentation and a substrate for methanogenesis. Benzoate and phenol comprised the major group of aeromatic monomers in the degradation of ligneous material, as well as a number of xenobiotic compounds. For this reason, the metabolic fate of benzoate, phenol, resorcinol and catechol was determined and a number of polyaeromatic compounds were also selected since they were potential intermediates in lignin degradation. Estimates of in situ rates of methanogenesis and sulfate reduction were also obtained by monitoring the production of labelled methane and sulfide from $^{14}$C-acetate and $^{35}$SO$_4$. Activities in slurries, both with and without nitrogen and phosphorous amendments were also compared to determine whether the nutrients were factors that limit in situ metabolism in the subsurface.
The rationale behind these particular assays can be made more apparent by reviewing the flow of carbon electrons in the major metabolic event that is involved in organic carbon and energy cycling (slide). Polymeric organic carbon is broken down into its constituent monomers at the first step, and in the case of lignaceous material, these are primarily benzoate and phenol derivatives. The monomers are then further broken down by acetogens and other fermenters into volatile fatty acids, alcohol, hydrogen and CO₂. The fermentative intermediates are then further acted upon by sulfate-reducing bacteria and proton-reducing organisms to produce hydrogen and CO₂. The hydrogen and CO₂ can then act directly as a substrate for methanogens or sulfate reducers, or it may be converted to acetate by homoacetogens. Acetate can also be used as a substrate for methanogenesis or sulfate reduction, with the final products being methane, CO₂, hydrogen sulfide, and water.

Results

As stated earlier, slurries were assayed for the ability to degrade fermentative intermediates, and lactate, formate, and acetate were chosen as representatives of this group. These slurries were amended with the above substrates at levels of 20 ppm carbon each, and then substrate disappearance and methane production were monitored. Lactate and formate were metabolized in almost all of the slurries, with lactate and formate having been metabolized at approximately the same rate. The rates of disappearance of lactate may also be considered as representative of the rates of formate disappearance (slide). The fastest rates of lactate and formate metabolism were seen in the P28 borehole, and complete disappearance of these compounds was usually seen within the first 30 days of incubation. Slurries from the P24 and P29 boreholes generally required longer periods of time, up to 2-8 months for complete disappearance of lactate and formate. In no case was the disappearance of lactate and formate accompanied by methanogenesis, which suggests that metabolism was coupled with an inorganic electron acceptor. However, in many cases, the disappearance of lactate and formate was accompanied by an increase in the levels of amended acetate in the samples.

Acetate mineralization plays a key role in anaerobic metabolism of organic compounds and acetate metabolism did vary in the different sediment slurries. However, there were basically four types or patterns of acetate metabolism observed. The first pattern was a simple lack of acetate metabolism. In those cases, the levels of amended acetate neither increased nor decreased over the course of an 11 month incubation period. A second pattern of acetate metabolism involved a slow disappearance of acetate over a 2-8 month incubation period, without methane production. Once again, this suggests that metabolism was coupled to an inorganic electron acceptor. However, any correlation between this pattern of acetate metabolism and the availability of inorganic ions, such as iron, sulfate, and nitrate in pore waters, could not be determined. A third pattern of acetate metabolism was involved in acetogenesis, or an accumulation of acetate, beginning early on in the incubations and often continuing for several months. However, there was no subsequent
disappearance of the acetate and no production of methane. This pattern was most often seen in the more transmissive zones, but this is one pattern that was also occasionally seen in some of the strata that had a higher clay content. A fourth pattern of acetate metabolism also involved acetogenesis early in the incubation, but it was subsequently followed by acetate mineralization and the production of methane.

The stoichiometry between acetogenesis and methanogenesis suggests that acetoclastic methanogenesis accounted for virtually all of the acetate mineralization in the sediments and a substantial proportion of the methane produced. An average of 1.6 nmol of methane was produced per mole of acetate. If one assumes a 1:1 stoichiometry, which is one mole of acetate producing one mole of methane, then approximately 63% of the methane derived in the slurries was derived from acetoclastic methanogenesis. Acetoclastic methanogenesis was not the only thing seen in samples that received a carbon amendment; acetogenesis and methane production were also seen in samples or slurries that received no carbon amendment. This suggests that in at least in some of the sediments, there were in situ reserves of fermentable carbon that were subsequently mineralized after the addition of a reduced mineral salt solution. Furthermore, this suggests that in some areas of the subsurface, microbial metabolism may not have been carbon limited, as was often assumed to be the case. Environmental factors or inorganic nutrients may have limited metabolism in some areas.

The distribution of the strata in which methanogenesis was observed compared to the numbers of sulfate-reducing bacteria, which were estimated in the MPN assays for sulfate reducers. The reason for comparing the data in this way is because a high correlation was seen between the methanogenesis and the strata that also harbored sulfate-reducing bacteria. In other words, they tended to occur in the same strata. The methane that was produced was easily detected and comprised of several percent of the final headspace volume in the incubation. In some sediments, methane production was less than 10% of the predicted amount based on carbon levels. Methanogenesis was easily detected in surface sediments from all three sites, but in the deeper sediments, the slurries from P28 displayed the most active methanogenic activity. In this particular borehole there was a definite correlation between the strata that harbored sulfate-reducing bacteria and that which produced methane. This pattern was also seen in the C10 borehole.

Assaying was also done for the ability to degrade aeroelastic compounds such as benzoate. In the slurries, benzoate was amended to the slurries in concentrations of 50 ppm carbon, or approximately 600 μM/l, and benzoate degradation and methane production were monitored. There was a strict correlation between benzoate metabolism and methanogenesis, with benzoate degradation occurring only in those sediments which produced methane. Also, a transient accumulation of acetate always accompanied benzoate degradation. Benzoate and acetate disappeared at the same time concomitantly with methane production. This suggests that the dependence of benzoate metabolism on methanogenesis was specifically dependent upon
acetoclastic methanogenesis. This observation was consistent with the known energetics of benzoate metabolism, which states that under standard conditions, the conversion of benzoate to acetate is thermodynamically unfavorable with a positive free-energy change of about 62 K joules per mole. However, when it is accompanied by hydrogen removal and acetoclastic methanogenesis, the complete mineralization of benzoate becomes thermodynamically favorable with a negative free-energy change of about 125 K joules per mole.

The degradation of phenols is also dependent upon the removal of intermediates, and the organisms responsible for the removal of these intermedients were found to be active in phenol amended slurries. Therefore, one might predict that there was successful degradation of phenols in the sediments. However, this was not the case. There was no degradation of phenol, resorcinol, or catechol in any of the slurry. There was methane production in many of the phenol amended slurries, but this production occurred without a corresponding decrease in the phenol. It may have been that the lack of phenol degradation was simply a lack of the appropriate phenol-degrading organisms. As was mentioned earlier, methanogenesis and acetogenesis in unamended samples suggest that in some sediments there were in situ reserves of fermentable carbon. This also implies that other nutrients might have limited metabolism.

Since nitrogen and phosphorous are common limiting substances in other environments, their effect on the production of labelled methane from $^{14}$C-acetate was tested. Sediments were slurried with distilled water and a sodium sulfide reductant, and then divided into three sets. The first set, which was the treated set, received nitrogen and phosphorous amendment. A second, untreated set received no amendment, and the third set comprised the autoclaved control. The slurries were then amended with $^{14}$C-acetate in concentrations of approximately 600 nM/l and then the production of $^{14}$C-methane was monitored. Results showed no production of labelled methane in the autoclaved control (slide). With the notable exception of the samples from the surface and from 194 m, it was apparent that the addition of nitrogen and phosphorous did somewhat stimulate the production of labelled methane in most of the slurries. If nitrogen and phosphorous were in fact limiting in situ metabolism in the lower sediments, then that could help explain the presence of reserves of fermentable carbon, even though it was demonstrated that there were organisms present that could otherwise deplete carbon reserves in a fairly short period of time.

The results of the MPN assays for sulfate-reducing bacteria showed that their numbers did not significantly correlate with the amount of sulfate in the sediments. This raised the question as to whether the sulfate-reducing bacteria were in fact metabolically active in the sediments. For this reason, an attempt was made to obtain an estimate of in situ rates of sulfate reduction by amending the slurries with $^{35}$SO$_4$ and then following the production of labelled sulfide. Recovery of $^{35}$S-sulfide after a 7-10 month incubation period showed that in most of the slurries, recovery of the labelled sulfide was less than five percent. This suggests that very little sulfate
reduction occurred (slide). However, the production of labelled sulfide in slurries from the surface, and from 21, 121, 406, and 411 m at least qualitatively suggests that in those sediments, in situ sulfate reduction may have been occurring at a very slow rate.

One of the first steps in the transformation of aromatic monomers to benzoate and phenol derivatives was the removal of o-methyl groups, which were presumably derived from lignaceous material. A number of methoxylated compounds were tested and it was found that o-demethylation activity was very easily detected in several of the strata. In most cases, the hydroxylated intermediates were recalcitrant and no further degradation of these phenolic compounds were seen over the course of the incubation. However, in the case of anisic acid, which was transformed to p-hydroxybenzoate on demethylation, complete degradation of the anisic acid was found. This suggests that at least in some strata, some phenolic compounds could be degraded.

Discussion

Many activity studies were performed on the C10 borehole samples and o-demethylation activities were observed (slide). This demethylation activity was observed in slurries from the surface, from the McBean Formation in the upper aquifer and in the Middendorf Formation in the lower aquifer. Acetogenesis was observed in all of the saturated transmissive zones, and as in previous boreholes, there was a fairly close correlation between methanogenic activity and the occurrence of sulfate-reducing bacteria. In selected strata, SRB were found to be present using MPN assays. There were countable, viable sulfate reducers in these sediments (slide). Differences appeared between those strata, and the ones in which in situ sulfate reduction was demonstrated using labelled $^{35}$S. These data underscored the need for assaying both microbial numbers and activities when assessing the microbial metabolic potential. Of course, one of the patterns that was seen in all of the boreholes was a variety of activities occurring within the same strata.

The anaerobic degradation of organic material depends upon the cooperation of a consortia of microorganisms with a variety of metabolic functions. Therefore, one of the important patterns that was seen in many of these strata was the necessary consortia for the full mineralization of organic compounds. The importance of this distribution can be made more clear by reviewing the flow of carbon electrons (slide). From the flow chart identical to the one discussed earlier, the following activities were seen: the degradation of monomeric organic carbon in the form of benzoate and sugars with the production of CO$_2$, methane, and fatty acids; acetogenic activity; the degradation of fermentation intermediates; and the presence of sulfate reducers and methanogens. The stoichiometric removal of acetate-producing methane was also investigated, as well as the nonmethanogenic mineralization of acetate. Therefore, it appears that in some areas, or in many areas of the saturated transmissive zones, the consortia necessary for the mineralization of organic carbon were present and intact.
Conclusion

In conclusion, *in situ* rates of sulfate reduction and methanogenesis are apparently fairly low at each of the Savannah River Sites, but nonetheless, they are present. The anaerobic metabolism of lactate and formate was readily observed, but the reductive degradation of benzoic acid was dependent upon acetoclastic methanogenesis. This was in contrast to phenols, which tended to be recalcitrant.

The transformation of lignaceous monitors was readily observed in the transmissive zone, with the initial step involving o-demethylation reaction. Acetogenesis, methanogenesis, and the turnover of radioactive tracer molecules in samples not amended with carbon suggest that fermentable carbon reserves do exist, at least in some areas of the subsurface. These reserves may not be measured during typical dissolved organic carbon analysis. Stimulation of *in situ* metabolic activity by the addition of nitrogen and phosphorous suggest that the availability of these compounds may limit anaerobic metabolism, at least in some areas of the subsurface. Finally, the saturated transmissive zones do seem to harbor the viable microorganisms necessary for the terminal steps in the anaerobic mineralization of organic matter, and these organisms can respond and proliferate under the proper conditions.
Q and A

M. Ivanov: First, did you use radioactive CO$_2$ for measuring this process. And second, if not, why not?

R. Jones: In the study that was shown with the $^{14}$C-acetate, the production of radioactive CO$_2$ was measured. It was recovered in sodium hydroxide and the activity was then counted. In most of the studies, methane production was monitored and the appropriate stoichiometry between CO$_2$ and methane was assumed. In most cases, radioactive CO$_2$ was not measured.

M. Ivanov: You obtained a lot of data about methane production in groundwater and samples from sediment cores. In many, many cases, over 95% of the methane was formed from CO$_2$, not from acetate.

R. Jones: All I can say is that what was observed here was a very good stoichiometry between acetate and methane production. Certainly, this study could be followed up. If more samples were obtained, radioactive studies could be done, which might elucidate the stoichiometry better. However, this pattern was pretty consistent at approximately 60% or so. When the acetate disappeared, stoichiometric production of methane was observed.

D. Lovley: It was clear that you had methanogenesis and sulfate reduction in your anaerobic incubations. However, it was not clear to me how you obtained the conclusion for in situ rates of sulfate reduction and methanogenesis, especially from my understanding that these environments were aerobic. I think the potential was there, but not at those in situ rates.

R. Jones: I guess the caveat has to be given that all these data really represent potential activities. The term in situ may have been inappropriately applied; however, those samples were reduced. They were slurried with distilled water and reduced. Then the label substrate was added, and from this, it was inferred that if there were reduced zones in this subsurface, then this in situ metabolism could be occurring. It was more of a qualitative than quantitative assessment.

T. Phelps: I would like to substantiate what you said. I also looked for radiolabelled sulfate reduction and radiolabelled methane formation. In our time course studies, where the sediments were not reduced, isotopes were added at the site and the experiments ended within hours. Radiolabelled methane, which was analyzed by gas proportional counting, was not detected from either radiolabelled bicarbonate or radiolabelled acetate in less than 21 days. It was estimated that the limits of our detection were in the nmole/kg per day range. This means the bacteria were there, but their activities were extremely low. Similar with sulfate reduction, our rates were less than one nmole/kg/day. Thus activity was seen within 16 hours of isotope incubation, but the rates were extremely low.
N. Monir: I do not agree that the electron donors were only organics.

R. Jones: That was certainly the case. We were operating under some very specific conditions, highly reduced conditions (e.g., ~400 mev potential). Although we were looking specifically at methanogenic and sulfate-reducing processes, that in no way discounts the possibility that iron reduction, nitrate reduction, and so forth, could be occurring. In fact, substrates were seen to disappear without methane production, which in many of the cases, indicated that something may have been happening.
Rates of Microbial Metabolism in Deep Subsurface Environments
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Abstract

A wide diversity of microorganisms have now been recovered from the deep subsurface and laboratory studies have demonstrated that some of these microorganisms have the potential to be metabolically active in situ. However, the in situ rates of microbial metabolism in deep subsurface environments have received little attention. Therefore, the rates of microbial metabolism in deep aquifers located in the Atlantic Coastal Plain of South Carolina were investigated with a variety of microbiological and geochemical techniques. Sediments were sampled with a new coring technique which increased recovery of coarse-grained sands and gravels and restricted the penetration of drilling fluid into the sediments. Sediment core integrity was monitored with one of two novel tracer techniques that are highly sensitive to sediment contamination with drilling fluids. The aquifers were anaerobic with either Fe(III) reduction (Florence Site) or sulfate reduction (Myrtle Beach Site) as the terminal electron-accepting process. At the Myrtle Beach site, rate measurements with [2-14C]-acetate gave estimates of CO2 production from organic matter oxidation of 10^-1-10^-2 mmol CO2/l/year for the Black Creek aquifer and 10^-4 mmol CO2/l/year for the Cape Fear aquifer. Similar acetate turnover rates were measured in the same aquifers at the Florence site. In general, the rates of CO2 production as estimated with the radiotracer technique were approximately four orders of magnitude lower than estimates obtained by measuring the accumulation of CO2 in the sediments over time in laboratory incubations. In contrast, modelling of groundwater chemistry along the flow path of these aquifers indicated that the rates of CO2 production were only 8 x 10^-5 and 2 x 10^-5 mmol CO2/l/year for the Black Creek and Cape Fear aquifers, respectively. Considerations of the sediment age (approximately 80 million years) and organic carbon content suggest that the rates of CO2 production can be no more than 10^-4 mmol CO2/l/year. Thus, the results from geochemical modelling are considered to be more closely approximate in situ rates than the rate estimates obtained from either of the sediment incubation methods. These results demonstrate that laboratory studies with deep subsurface sediments may greatly overestimate the in situ metabolic potential of microorganisms in the deep subsurface. When the rate of CO2 production estimates for these and other deep aquifers are compared with the metabolic rates of such oligotrophic environments as deep ocean waters, it is apparent that deep aquifer systems are among the most oligotrophic environments that still contain biological activity.

Oral presentation.
Introduction

There is interest in trying to develop techniques to assess microbial activity in deep aquifers because microbial metabolism has a number of important effects on the geochemistry and water quality of these important water supplies. Furthermore, if techniques can be developed by which the in situ rates of microbial metabolism in deep aquifers can be effectively estimated, then such techniques might also be useful in assessing the ability of the aquifer population to respond to perturbations, such as the introduction of organic contaminants.

Our studies focused on several deep aquifers located in South Carolina, as did many of the Department of Energy sponsored studies that are discussed in this volume. The difference is that the DOE sponsored studies were conducted theoretically in the recharge area, or near the recharge area, and thus could be considered to be somewhat hydrologically atypical. The groundwater flow rates were relatively fast and the groundwater was aerobic and relatively young. Most of the aerial extent of the aquifers was, of course, downgradient from the recharge area and in one particular downgradient area, the groundwater was typically anaerobic and the groundwater flow rates were relatively slow. Two boreholes will be discussed in this report, one located near Florence, South Carolina, and the other was located further downgradient near Myrtle Beach, South Carolina (slide). Various groundwater wells were used for the geochemical analysis.

Discussion

Microbial activity was assessed in the sandy sediments of the Black Creek, Middendorf, and Cape Fear aquifers, as well as in the clay sediments of associated confining zones. As was discussed earlier in this volume, iron reduction was a predominant, terminal electron-accepting process in the aquifer sediments at the Florence Site. At the Myrtle Beach Site, sulfate reduction was the predominant, terminal electron-accepting process in all three aquifers. Although the concentrations of dissolved sulfate were relatively low in the aquifers, concentrations of sulfate in the confining beds were very high. The flux of sulfate from the confining bed into the aquifers is what fueled sulfate reduction.

The sediment cores were obtained with a Christian Rotary type core barrel, using drilling techniques very similar to those that were described by Dr. Phelps in Plenary Session 2. As Dr. Phelps emphasized, the important aspect of this coring apparatus is that the inner core barrel must extend out in front of the rest of the drill bit. This enhances the recovery of coarse grain sand and it also prevents potential contamination of the sediment core with drilling fluids.

The sediment cores were screened for potential contamination by drilling fluid by one of two techniques. Barium was included in the drilling fluid mud at the Myrtle Beach Site; therefore, the concentrations of dissolved barium in the drilling fluid were about 10 mg/l. In contrast, the concentrations of barium in the native groundwater were at least 1000-fold less. Therefore, dissolved barium was a very sensitive indicator for the presence of drilling fluid contamination. A typical result for a core that was not contaminated with drilling fluids showed that although barium was readily detected on the exterior of the core, typically within one half of a
centimeter into the core, no barium was detected (slide). This lack of contamination of these sediment cores by a dissolved constituent in the drilling fluid appeared to be much different than what was described for the cores at the Savannah River Plant, where dissolved constituents from the drilling fluids did contaminate the sediment cores. Although it could be considered that cores contaminated by dissolved constituents may not affect the analysis of microbial populations in the sediments, this type of contamination is unacceptable when trying to make measurements of microbial activity. Results from one of the few cores that was contaminated by drilling fluid at the Myrtle Beach Site, showed that barium was detectable in the center of the core (slide). With cores like this, one would just start over.

Barite was not included in the drilling fluids at the Florence Site; therefore, fluorescent microspheres, one micrometer in diameter, were added to the drilling fluids. The sediment cores were then screened for the presence of the fluorescent microspheres with fluorescent microscopy. This turned out to be a very useful technique, because with a microscope set up at the drilling site, the sediment cores could be checked for contamination within half an hour of collection. An example of a core not contaminated with drilling fluid showed that one could detect microspheres at the very exterior of the core, but within one half of a centimeter into the core, fluorescent microsphere could not be found (slide). In a contaminated core, the fluorescent microspheres could be detected to the center of the core.

Some cores of the sediments were incubated under anaerobic conditions in serum bottles where tracer quantities of uniformly labelled $^{14}$C-glucose or $^{2-14}$C-acetate were injected into the sediments. At the Myrtle Beach Site, the accumulation of radiolabelled CO$_2$ over time could be seen when uniformly labelled glucose was injected into sandy aquifer sediments from the Black Creek aquifer (slide). There was initially a linear rate of CO$_2$ production from glucose oxidization. However, there was no production of $^{14}$CO$_2$ when the microorganisms in the sediment were killed prior to the injection of the radiolabelled glucose. In a similar manner, $^{2-14}$C-acetate was oxidized to $^{14}$CO$_2$ over time, but no $^{14}$CO$_2$ was produced in the sterile controls.

In all the sediments that were examined, production of radiolabelled CO$_2$ from radiolabelled glucose was detected. This was true in both the sandy aquifer sediments and the clay sediments of the confining zones. In a pattern consistent to that which has been previously discussed in this volume, it appeared from the glucose turnover rate, that the rates of microbial activity were faster in the aquifer sediment than in the clay sediments. Production of radiolabelled CO$_2$ from acetate was also detected in many of these sandy aquifer sediments. However, in the same aquifer sediments, as well as in all of the clayey confining bed sediments, the rates of acetate metabolism were so slow that production of radiolabelled CO$_2$ could not reliably be detected, even after incubation periods of 77 days.

The acetate concentrations in the aquifers were determined by sampling the groundwater wells that were tapping each aquifer. Acetate concentrations at the Florence Site ranged from 0.5 to 1.0 and at the Myrtle Beach Site they ranged from
1.4 to 1.8 µM. With the acetate concentration and the first order rate constant for acetate oxidation to CO₂, the rate of acetate oxidation to CO₂ in the aquifer sediments could be calculated and estimated. The rate estimates ranged from the undetectable levels in those sediments in which ^14CO₂ production could not be detected, to levels as high as 2.4 x 10⁻¹ mmol of CO₂ produced per liter of groundwater per year.

As was mentioned earlier, iron reduction was a terminal electron-accepting process at the Florence Site and sulfate reduction was the terminal electron-accepting process at the Myrtle Beach Site. Therefore, in both sediment types at each site, oxidation of acetate to CO₂ accounted for approximately half of the total CO₂ production from organic matter oxidation. The question then is, "How can we evaluate these rates that we determined with the radiotracer technique"?

Another technique for estimating the rate of CO₂ production in groundwater is geochemical modeling. In this technique, one can calculate the amount of organic matter oxidation to CO₂ by accounting for the various sources of dissolved inorganic carbon (DIC) along a groundwater flow path. There are a few important reactions that must be considered for the geochemical modeling. An important reaction, of course, is microbial oxidation of the organic matter to CO₂. Furthermore, it must be considered that the CO₂ produced from organic matter oxidation can react with the calcium magnesium, carbonate shell material in the sediments. In this reaction the shell material is dissolved with a release of dissolved calcium and magnesium and a further increase in DIC. The calcium and magnesium that is released from the dissolution of the carbonate can then exchange with the sodium cation. In the reaction, calcium and magnesium are removed from the groundwater and there is increase in dissolved sodium concentration.

These important geochemical reactions can be expressed mathematically. For example, the change in DIC along a given groundwater sediment flow path can be expressed as the sum of the amount of CO₂ produced from organic matter oxidation, plus the amount of carbonate shell material that dissolved. In the aquifers under study, the carbonate shell material was comprised of 98% calcium carbonate and 2% magnesium carbonate. Therefore, the changes in dissolved calcium along the groundwater flow path could be defined as 0.98 times the amount of shell material dissolved, minus the amount of calcium that exchanged with the sodium in the clay. In a similar manner, the changes in dissolved magnesium along the groundwater flow path could be expressed as 0.02 times the amount of carbonate dissolution, minus the amount of magnesium that exchanged with sodium. One can also calculate or derive an expression with a change in dissolved sodium along the groundwater flow path. This change is equal to the amount of any mixture of salt or seawater with the fresh groundwater, plus two times the amount of calcium and magnesium that exchange with sodium. One can calculate the amount of the seawater that is mixing with fresh groundwater from the change in dissolved chloride along the groundwater flow path segment.
These five mathematical expressions can be solved simultaneously for a given set of groundwater geochemistry data with a specialized computer program that calculates the amount of CO₂ produced from organic matter oxidation for any given groundwater flowpath segment. The rates of groundwater flow can also be calculated using Darcy's equation, \( R = \frac{k(th/dl)}{p} \), where \( R \) is the rate of groundwater flow, \( k \) is the hydraulic conductivity, \( th/dl \) is the hydraulic gradient, and \( p \) is the porosity. Once the amount of the CO₂ produced for that flow path segment is known, along with the rate of groundwater flow, then the overall rate of organic matter and oxidation to CO₂ for that flow path can be calculated. The rate of CO₂ production is equal to the amount of CO₂ produced, times the groundwater flow rate, divided by the length of the groundwater flow path segment.

Results for the various oxidations that went into the geochemical modelling effort, and the various flow path segments for the three aquifers under study, are summarized and provided (slide). Once the groundwater flow rate and the amount of CO₂ produced is known, a rate of CO₂ production for all of the flow paths can be calculated. All of the rate estimates were grouped around \( 10^{-5} \) mmol of CO₂ produced per liter of groundwater per year. The rate estimates from the radiotracer acetate technique for the Black Creek and Middendorf aquifers were about three orders of magnitude higher than the rate estimates from geochemical modelling. At both sites in the Cape Fear aquifer, the rates of acetate metabolism were so slow that \(^{14} \text{CO}_2\) production could not be measured; therefore, only an upper limit for the fastest rate of acetate oxidation in the aquifer sediments could be set. In one particular case the upper limit did agree, falling within the range that was estimated for clay geochemical modelling.

Results of a parallel study, which was conducted by McMahon, Williams, and Morris, and was in a recent issue of *Groundwater Journal* (January, 1990). In this study, sediments from the same intervals that were used for the radiotracer technique in our study were slurried under anaerobic conditions. The anaerobic slurry was then incubated under anaerobic conditions, and over time, the accumulation of DIC was measured as an indication of the rate of CO₂ production. Therefore, these rate estimates were orders of magnitude higher than those estimated by the radiotracer.

The obvious question then is why the large discrepancy of values and which technique most accurately estimates what is going on *in situ*. Geologists tell us that these sediments were deposited between 70-80 million years ago. Therefore, it can be calculated that even if these sediments had as much as 5% organic carbon at the deposition, a CO₂ production rate of 0.2 nmol of CO₂/l/year would consume all of the organic carbon in the sediments in less than the 70 million years that the sediments have been there. Given that these sediments still contain some organic matter, it seems likely that a maximum, theoretical upper limit for CO₂ production in the sediments can be no higher than 0.1 nmol of CO₂/l/year. All of the estimates from the geochemical modelling fell below this maximum theoretical upper limit, as did the upper estimates for the Cape Fear aquifer. However, these rate estimates from
the radiotracer technique for the Black Creek and Middendorf aquifers are about two orders of magnitude higher than the estimated, maximum upper limit. The estimates for the Black Creek and the Middendorf must have been over estimated. If the rates were correct, then all of the organic matter that is now present in the sediments would have been consumed on the order of 1000-5000 years.

Given that these sediments are about 80 million years old and they still contain organic matter, it seems highly unlikely they are going to burn through what organic matter they have left in just another 1000 years. If the rate estimates from the slurry incubation were right, then all the organic matter that is now in the sediments would be consumed in less than 10 years. This seems like an even less likely occurrence. Therefore, it is concluded that the geochemical modelling most accurately assesses the rates of microbial metabolism in the deep aquifers. Similar geochemical modelling efforts for other deep aquifer systems yielded rates comparable to those that were reported for this aquifer system. The rate estimates were orders of magnitude lower than the rate estimates for organic carbon oxidation, even in such an oligotrophic sediment as sediments from the deep sea. The fact is that the rates of metabolism for the deep aquifers were so low that they were as low or lower than the lowest rate estimates for organic carbon oxidation in deep sea waters (slide). From this comparison it is readily apparent that deep aquifers are among the most oligotrophic aquatic environments.

**Conclusion**

In conclusion then, geochemical modelling indicates that the rates of microbial metabolism in deep aquifers of the Atlantic Coastal Plains are extremely slow and are on the order of $10^{-4}$-$10^{-6}$ nmol of CO$_2$/l/year. The radiotracer techniques, as well as many of the other studies reported in this volume, indicate that the rates of microbial metabolism in the clayey sediments are even slower. Thus, it is apparent that subsurface microorganisms can remain metabolically active for long periods of time while metabolizing organic matter at very slow rates. In two-thirds of the systems that were studied, the radiotracer technique significantly overestimated the in situ rates of organic matter metabolism. In all cases, measurements of CO$_2$ production in laboratory incubations of sediment slurries significantly overestimated what could possibly be, theoretically, the highest limits for in situ metabolism. These results indicate that extreme caution must be exercised in extrapolating from laboratory-derived rates to rates that are taking place in situ. This probably has the most important implications in attempting to assess the laboratory incubations and the ability of deep aquifer populations to respond to perturbation. In fact, it is like a "catch-22"situation with laboratory studies. If one measures a rate of microbial metabolism in the laboratory incubation, it is almost certain that the rate being measured is higher than the rate of in situ metabolism. If one is smart enough to set up a system which accurately mimics the in situ environments, so that one will have in situ rates, then the rates of metabolism are so slow that they can not be measured on a reasonable time scale. Given this consideration, efforts are now being focused on indirect techniques such as the hydrogen gas technique, which discusses how to assess microbial metabolism in deep aquifers (Frank Chapelle, this volume). Thank you.
Q and A

A. Konopka: Do you have any insights as to why the acetate, when used as a radiotracer, was giving such a large overestimate?

D. Lovley: There is probably a couple of reasons. I think just the physical disturbance of the sediment cores and sampling had something to do with it. There was a general problem in trying to estimate the in situ of metabolism and sediments using something like radiolabelled acetate. It has shown up very commonly in marine sediments. The rate estimated by acetate metabolism, for example, in a sulfate-reducing system, was much higher than other rate estimates that used sulfate measurements in sulfate reduction. There were probably two things responsible for this; there was just some problem involving acetate measurements and sediments in general, and there was probably the sampling effects as well.

A. Konopka: Have you effectively changed the acetate concentration with the C14 that you have added?

D. Lovley: No. The pool was increased by no more than two-fold, yet we were in the first order rate of metabolism.

J. Wilson: If the rate of CO2 production was so slow, what would be the rate of hydrogen consumption?

D. Lovley: It was slow. However, it was pointed out by Frank Chapelle that the hydrogen technique was not dependent upon either the rate of production or consumption. So that is why it was so ideal for a wide variety of environments. It could be used even in an oligotrophic environment.

J. Wilson: One quick question; how long does it take water to move from the Fall Line to the coast?

D. Lovley: At least 50,000 years. I would ask Frank.

J. Wilson: He said 20-30,000.

D. Lovley: Oh, ok, he is the hydrogeologist.
Bacterial Growth and Activity in Porous Subsurface Materials

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Abstract

Efficient and economical treatment of contaminated subterranean formations using in situ bioremediation processes requires a thorough understanding of the activity and mechanisms of movement of microorganisms through porous media. The growth of Escherichia coli strains through porous medium, in nutrient saturated, sand-packed cores (permeability 7 to 7.5 Darcies) was determined under nitrate-reducing conditions. The chemotactic, motile strain RW262 had a growth rate two times slower in the core compared to that observed in the broth under similar growth conditions, indicating that microbial growth in porous medium was restricted. The strain grew and moved throughout the entire pore volume of cores in a band-like fashion at a penetration rate of 0.4 cm/hour. However, the mechanism of movement of nonchemotactic, motile strain RP5232 resembled a random process (diffusion). This suggests that the chemotactic strain sensed the nutrient-rich environment in all three directions and thus moved in a “nonrandom” process. In contrast, since the nonchemotactic strain was unable to sense the nutrient gradients, the route of the fastest moving cells was less tortuous than that of chemotactic cells, resulting in a “random” process of movement. After 41.5 hours of incubation, 4.8 mM galactose was consumed and 7.4 mM acetate was produced by strain RW262 inside the core showing that, though the microbial growth was restricted by smaller pore spaces, the metabolism of galactose was not altered since a similar stoichiometry for acetate production was observed in the broth culture. Until the breakthrough of cells from the core, the galactose consumption and acetate production was observed only in the front sections of the core showing that the cell propagation preceded the depletion of the substrate or accumulation of large amounts of products. From a practical standpoint, the information generated in the present study may help to evaluate and construct models for in situ bioremediation of contaminated subterranean forma-

No paper submitted.
Q and A

A. Konopka: I think I might have an objection to your third conclusion where you seem to be suggesting that you are getting movement before utilization. I was looking at your slides and one does not really see utilization of glucose until one gets to cell levels of $10^8$, or something like that. What I am suggesting is that you may have been getting metabolism but because the amount of cells was so low you just could not detect it. I do not know what technique you were using for looking at the formation of acetate. There may have been acetate formed, but it was just below your level of detection and similarly, you obtained such a high load of galactose.

P. Sharma: I can agree on the fact that there could have been a dilution indication for the lack of acetate. However the appearance of acetate was also observed, only in the top sections. That is the reason I assumed that propagation had proceeded significantly.

A. Konopka: What is the minimum concentration of acetate that you could detect? What type of assay were you using?

P. Sharma: It was being done by GC.

S. Kellogg: Well, I think you were right; there was probably some utilization of the substrates, although undetected. Obliviously, there was no growth. It is not that you were generating a tremendous change in the concentration of the substrates throughout the core, therefore, it was not like they had to utilize all the substrates before they could run.

Audience: Were the cells pregrown in galactose?

P. Sharma: Yes. We maintained the cells on slants, and then they were transferred to an anaerobic medium.

G. Toranzos: Your detection of acetate at the farthest fractions may be simply because acetate was used in the first fraction. Therefore, your detection of acetate did not necessarily mean that acetate was really produced at that point and time; it could have been produced before and then migrated.

T. Hazen: Did you ever try any experiments where you put galactose at one end of the column to see how fast the bugs might move with a point source of galactose?

P. Sharma: Two concentrations of galactose as a point source were compared and it did not make any appreciable difference. The localized point source was 10 cm away from the source of inoculation, and that did not make any appreciable difference.
T. Hazen: What was the origin of these isolates?

P. Sharma: E. coli K-12 laboratory strain.

T. Hazen: We had some subsurface bacteria that seemed to show quite different chemotactic patterns when we looked at actual chemotaxis using the Palleroni technique. It actually showed a bimodal response in that it was repelled by high concentrations of nutrients and attracted to very low concentrations. Some of this could affect how these bacteria move through the systems. It can be significant.
The Activity of Microbiological Processes in Stratal Waters of Oil Fields

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Abstract

Studies were done on the microbiological processes that are in progress in oil fields of the Middle-Volga region and western Syberia, which are exploited with flooding. The depth of deposition of the oil strata is 1500-1850 m and the salinity of stratal liquids ranges from 2 to 280 g/l. Microbial communities of these ecosystems were represented by various aerobic and anaerobic microflora, capable of both the direct destruction of oil hydrocarbons and the subsequent transformation of oil biodegradation products with CO₂, CH₄, CH₃COOH, and H₂S formation. Enrichments and pure cultures of aerobic bacteria, oil-oxidizing bacteria on media with 20% NaCl, and strictly anaerobic cultures of methanogenes that formed CH₄ on media with 15% NaCl, were isolated as well.

The radioisotopic methods showed the development of the modern sulfate reduction and methanogenesis processes in stratal waters with salinity up to 280 g/l. The rate of processes in brines was 0.2-1.2 mg/kg H₂S l/day and 0.04-0.24 mg/kg of CH₄/l/day, respectively. The rate of methanogenesis in low-sulfate and fresh-salt stratal waters was one to two orders of magnitude higher. Formate and methylamines, as well as H₂, CO₂, and acetate were determined to be substrates for methanogenes in stratal waters.

The maximum number of microorganisms and the highest rates of microbiological processes were observed in near-bottom zones of wells, which have been injected with fresh surface waters. The development of modern microbiological processes in oil fields has resulted in changing the carbon-isotope composition of dissolved carbonates, and in a number of cases, of methane carbon.
Introduction

The study of microbiological activity in deep, underground ecosystems is connected, first of all, with the understanding of the role of microorganisms in the formation, transformation, and degradation processes of organic compounds of oil, gas, and other deposits. For the last decade, these works have received a new development in connection with the fuel-energy problems, which arose in the world economy because of the insufficiency and the nonrecoverability of natural resources of combustible fossil fuels. The study of microbiological processes in underground waters have acquired a great importance because of increased anthropogenic load on biosphere and aggravation of ecological problems.

The purpose of this paper was to study the distribution and geochemical activity of microorganisms in working oil fields and to characterize briefly the isolated microorganisms.

Materials and Methods

The samples of stratal liquids were taken from producing and injecting wells in sterile vessels and gassed with N₂ with no air contact. The chemical composition of water was determined by standard methods.

The enumeration of microorganisms was made by serial dilutions in liquid media. The number of hydrocarbon- and oil-oxidizing bacteria was determined using the Raymond media. Identification of methanogenic organisms was carried out according to the substrate specificity and mineralization. Sulfate-reducing bacteria were isolated using the Postgate "B" media with lactate. Oligocarbophilic bacteria were enumerated using solid media that contained peptone (10 mg/l). The number of saprophytes was determined using meat-peptone agar (MPA). The salinity of media was adjusted to match the salinity of the studied waters.

The rates of bacterial methane oxidation, sulfate reduction, and methanogenesis were determined by the radioisotopic method, using labelled compounds ¹⁴CH₄, Na₂³⁵SO₄, ¹⁴CH₃COONa, and NaH¹⁴CO₃, respectively. The stratal water samples were incubated with labelled substrate for up to 48 hours at the temperature of the stratum.

The isotopic composition of methane, carbon, and carbonates obtained from stratal liquids was measured using a mass spectrometer CH-7 ("Varian-Mat") by the two-beam compensating method, using CO₂ as a working gas.

For electron-microscopic investigations, a JEM-100C electron and scanning microscopes were used.

Objectives

The Bondyuzskoe oil field, situated on the right bank of the Kama River in the northeastern part of the Tatar Republic, has been the object of field investigation for many years. Sandstones and aulorites of the upper Devonian formation serve as oil collectors. Sand collectors are also prevalent. The depth of the oil-bearing deposits ranged from 1500 to 1700 m, and the temperature of stratum varied from 30° to 40°C. The oils of the Bondyuzskoe field are of high sulfur content and they contain paraffins. The relative oil density is 0.871-0.876.
Natural stratal waters of the Terrigenic layer of the Devonian formation belong to the chlorine-calcium type and they have a total salinity up to 330 g/l. They are characterized by low sulfate (0.5-100.8 mg/l) and carbonate (0.6-42.7 mg/l) content, and the value of pH is approximately 5.0.

The field has been working for more than 30 years. The flooding of the oil stratum with fresh surface waters, or waters with different degrees of mineralization, has been applied to maintain the stratal pressure.

Undoubtedly, the injection of fresh waters in the stratum led to the change of chemical composition of high-saline stratal liquids. With the decreasing mineralization of stratal brines, the concentration of cations, Ca\(^{+2}\) and Mg\(^{+2}\), in the water decreased and the hydrocarbonate-ion content increased. The pH value changed from low-acidic to neutral. The values of Eh for the stratal waters corresponded to the slightly reduced conditions. As a whole, the flooding of fields with fresh waters resulted in the formation of a wide spectrum of ecological niches that varied in salinity from 1 to 300 g/l and had different chemical composition.

Another important property of injected surface waters is the fact that they serve as a source for different microorganisms entering the stratum.

Results and Discussion

The microbiological characteristic of waters injected into the field is given in Table 1. A rather high total bacterial number, 10\(^7\) cells/ml of water, was obtained. Aerobic bacteria, able to oxidize oil and individual hydrocarbons, \(C_{16}H_{34}\) in particular, were present in the waters. It was found that sulfate-reducing bacteria, and even such strict anaerobes as methanogenic bacteria, were present. It is possible that viable cells of methanogens survived in the injected, oxygen-containing waters, having been absorbed on sediments or inside of solid particles.

The studies of microbiological processes occurring at the bottom zone of injecting wells was of great interest, because only in these areas of the field did the surface waters and stratal waters, with different composition and properties, come in contact. The data given in Table 2 show that when oil strata were flooded, dissolved oxygen was introduced into the stratum together with water. In the near-bottom zone, oxygen was quickly consumed, which resulted in a sharp decrease of Eh. Judging from everything, it appears that oxygen was consumed for the microbiological oxidation of residual oil in the near-bottom zone, and for the oxidation of methane and other hydrocarbons as well. This can be confirmed by the increase in oil-oxidizing bacteria, the development of the active processes of methane oxidation, and an increase in the content of organic compounds that were dissolved in water, acetate in particular. The activation of an anaerobic process, methanogenesis in particular, was the consequence of the decrease of the oxidizing-reducing potential and the increase of organic carbon content.

The enumeration of stratal-liquid microorganisms showed that the total number of bacteria and the counts of some groups of aerobic and anaerobic bacteria in stratal waters were considerably lower than those in injected waters. This could be explained by the fact that not all of the microorganisms survived. Some of them died
under the rather strict stratum conditions. Undoubtedly, some of the bacteria were transformed to the immobilized state, in that way decreasing the viable number of bacteria in the liquid phase. Viable cells of aerobic and anaerobic microorganisms were found in all the samples of stratal water with a salinity range of 6-272 g/l (Table 3). The higher number of saprophites and oligocarboophilic bacteria was noted in highly freshened waters. In this case, the number of oligotrophs, as a rule, was higher than the saprophites, which were grown on protein rich media. The number of aerobic, hydrocarbon-oxidizing bacteria, anaerobic methanogenic, and sulfate-reducing bacteria varied from $10^3$ to $10^4$ cells. They did not exhibit a well pronounced correlation with the degree of stratal brine desalination, and therefore, with the number of microorganisms introduced with the fresh surface waters. For an explanation of the microbial distribution, the idea about the existence of aboriginal halophilic microflora may be useful.

Perhaps the explanation is more simple than it appears. Inspite of special measures, some microorganisms may have gotten into the samples from the systems of columns and pipelines, causing the observed results. On the other hand, the distribution of microorganisms in a multiphase system (water, oil, and rock) is not simple, and it depends on the total mineralization of the stratal liquids. The illustration for this form of distribution is presented in the Table 4, which shows that at separation of the stratal liquids in oil and water, the number of saprophitic bacteria was higher in oil than in water.

The data obtained by radioisotope methods (Table 5) served as confirmation of the fact that microorganisms found in stratal waters were active.

The modern processes of sulfate reduction and methanogenesis occurred in a wide range of stratal water with salinity from 6-273 g/l. However, their activity was one or two orders lower than that in the near-bottom zone of the injection well. The highest values of methanogenesis of magnitude were within the range of water salinity of 50-150 g/l. In the same salinity range, the acetate mineralization increased considerably with the process of bacterial methane generation.

Table 6 gives the data on isotope composition of carbonates, CO$_2$, and methane of stratal waters from an oil field and injected surface waters. A considerable interval in values of $^{13}$C of carbonates (-9.15 to 17.11%) in comparison with those of $^{13}$C of methane (-50.0 to -52.9%) was observed. It is known that isotope composition of carbonates from stratal waters is formed with the participation of carbonates from water-bearing rocks, relic brines, and injected surface waters. A considerable contribution is also made by microbiological processes, such as organic oil matter oxidized to CO$_2$, carbonic acid reduced to methane, etc. The integration of all these processes provides an explanation for the observed values of $^{13}$C for the carbonates of the stratal waters.

Isotope composition of methane and carbon, dissolved in stratal waters, varied insignificantly. The correlation between $%^{13}$C of methane and stratal water mineralization was also not observed. There was no such correlation with the activity of
modern bacterial methanogenesis, which can probably be explained by a small portion of methane formed in the total content of relic, gaseous hydrocarbons.

At the same time, a five year study on the bacterial methanogenesis in the experimental area of an oil field indicated that the rate of methane formation increased by 1-2 orders of magnitude within four years of observation (Table 7).

In the course of microbiological investigations of oil fields, some cultures of hydrocarbon-oxidizing and methanogenic bacteria were isolated and studied. Microorganisms were isolated from stratal liquids that were characterized by high salinity. A brief characteristic of two hydrocarbon-oxidizing bacteria is described below. Strain 367-2 is an aerobic organism with a Gram-positive cell wall. The cells are rod-shaped (0.6-0.8 x 10-12 μm), splitting to small sphere-like pieces (0.8 x 1.0 μm) during cell division (Figure 1). They utilize, as a source of carbon and energy, the following substrates: glucose, formate, acetate, propionate, buterate, octane, decane, and oil. Ammonium salts as well as nitrate salts serve as nitrogen sources. The growth was observed at temperatures that varied from 6 to 37°C and in the pH range of 5.2-9.2, with an optimum of 5.7-6.0. The organism utilized acetate and glucose in media that contained up to 20% NaCl. The maximum growth rate as determined by the incorporation of labelled acetate into biomass was observed at 15% NaCl. According to morphological and physiological-biochemical properties, the organism would be identified as *Rhodococcus longus*, except the G+C DNA content was too low (30.1 mol%). The literature describes the representatives of the genus *Rhodococcus* with a G+C of 59-73 mol%.

*Micrococcus* sp. (strain 367-3), an aerobic organism with a Gram-positive cell wall, has sphere-shaped cells with a diameter from 0.5 to 3.5 μm which forms aggregates (Figure 2). The organism utilized glucose, acetate, propionate, buterate, and n-alkanes (C₁₂-C₃₀). It utilized ammonium and nitrate as a source of nitrogen. The growth was observed in a wide range of pH (4.7-9.2) and at temperatures varying from 6 to 37°C. The optimum value of pH was 6.6 while the maximum growth rate was observed on acetate with 20% NaCl. The G+C content was 61.7 mol %.

Both organisms required yeast extract and vitamins when growth took place on media with a NaCl content of more than 10%. The pH and temperature optima for growth were close to the values of the stratal waters. Both organisms did not grow without NaCl. At NaCl concentration of less than 1 g/l, 70-80% of the cells lysed.

Besides methanogenic bacteria, the following are also of special interest. The halotolerant organism, *Methanosarcina* strain 47, was isolated in slightly saline stratal waters. It was attributed to the *Methanosarcina* genus on the basis of its specific morphology (Figure 4). Substrates utilized for growth and methanogenesis were acetate, methanol, and methylamines. The organism could not use CO₂ or H₂ as carbon or energy sources, respectively. CH₄ formation was observed in the presence of NaCl, up to 100 g/l (Figure 5). The optimal NaCl concentration is 1.0-20.0 g/l. The growth temperature ranged from 10° to 50°C. Optimal growth temperature was 37°C. The pH range was 5.4-8.4, with an optimum of 6.5-7.5. The
G+C content of the DNA was 48.2 mol%. It is believed that strain 47 can be an independent strain among known mesophilic strains of *Methanosarcina mazei*.

Halophilic, methanogenic bacterium, *Methanococoides euhalobius*, was isolated from the stratal waters of an oil deposit with a salinity of 140 g/l and contained high concentrations of Ca$^{2+}$ and Mg$^{2+}$. The cells were irregular cocci, varying in size from 1-3 μm. Significant polymorphism was seen in the electron micrographs (Figures 6a and 6b). Multi-angular cells were prevalent. Binary fission was the most common method of cell division. Methanol, along with tri-, di-, and monomethylamines, were substrates for growth and methanogenesis. The organism required biotin for growth. The optimum growth temperature was near 37°C while the optimum pH level for growth was from 6.8-7.3. The G+C content of the DNA was 43.0 mol%. The organism had high requirements for Ca$^{2+}$ and Mg$^{2+}$, since in the absence of these ions, the organism could not grow. Growth and methanogenesis were possible at the Ca$^{2+}$ range 7-140 mM. The optimal NaCl concentration in the medium was 60-70 g/l (Figure 7).

It was found that Ca$^{2+}$ ions, vital for *M. euhalobius* development, influenced the rigidity and structure of the cell wall. An hour after Ca$^{2+}$ was omitted from the medium, changes in cell shape were observed. The cells lost their native shape and became spherical cocci. Such a culture was not viable and lysed.

A new halophilic methanogen, strain GF 283, was isolated from stratal waters. The cells of this organism were rod-shaped, single, in pairs, or in long filaments surrounded by a transparent sheath. The rods were motile and had flagella (Figures 8a and 8b). The organism used trimethylamine as a substrate for methanogenesis. The culture was able to grow using H$_2$ as the electron donor, but only in situations where H$_2$ did not exceed 10% of the gas phase. The culture did not use methanol, acetate, formate, propionate, glucose, or yeast extract. It could not reduce sulfate or elementary sulfur and it could not produce acetate as well. Optimal temperature for growth was 45°C. The growth was possible in the 10-20% NaCl range with an optimum at 15%. The novelty of the organism was based on its unique morphology. Halophilic, methylotrophic methanogens of the *Methanococoides* and *Methanococcus* genera are irregular, nonmotile cocci. There are no representatives of the described species of these genera, which are rod-shaped and have a sheath.

**Conclusion**

Microbial associations of the worked oil field are represented by diverse aerobic and anaerobic microflora, which carry out the direct destruction of oil hydrocarbons as well as the subsequent biotransformation of oil into CO$_2$, CH$_3$COOH, CH$_4$, and H$_2$S as final products. The development of the modern processes of sulfate reduction and methanogenesis in stratal liquids with salinity up to 270 g/l was shown by radioisotopic methodology.

The maximum numbers of microorganisms and the highest rates of microbiological processes were noted in near-bottom zones of the wells, which were injected
with fresh surface waters.

Several pure cultures of hydrocarbon-oxidizing and methanogenic bacteria from highly mineralized stratal waters of oil fields were isolated and studied. The isolated organisms were characterized by high halotolerance and halophilicity. Some of the isolates are representatives of new taxa.
References

Table 1. Microflora of injected waters of the studied oil field (1979-1988).

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<th>Well Number</th>
<th>Salinity, (g/l)</th>
<th>Hydrocarbon oxidizers (cells/ml)</th>
<th>Methanogens (cells/l)</th>
<th>Sulfate reducers (cells/ml)</th>
<th>Total number cell/ml x10^6</th>
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Table 2: Microbiological processes in the zone contacted between injected and shield waters of the oil field (366 well).
Table 3. The number of microorganisms in stratal water of the Bondyuzhskoe oil field (cells/ml; 1987-1989)

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<thead>
<tr>
<th>Well Number</th>
<th>Salinity (g/l)</th>
<th>Sapro-</th>
<th>Oligo-</th>
<th>Hydrocarbon-oxidizers</th>
<th>Methanogens</th>
<th>Sulfate</th>
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ND = No data.
Table 4. The number of saprophic bacteria (cells/ml) grown on MPA in stratal liquids (water, oil) of the Bondyuzskoe oil field (1987-1988).

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Table 5. Rate of microbiological processes in stratal waters of oil deposits.

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<th>Well Number</th>
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ND No date
Table 6. Isotope composition of carbonate and methane from stratal water of the Bondyuzhskoe oil field.

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ND  No data.
Table 7. Rate of methanogenesis in the stratal water of the oil deposit during field experiment (ml CH$_4$/10$^6$/1/day).

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ND  No date.
Figures

Figure 1. The cells of the isolate 367-2 (magnified x 60,000); NaCl content in the medium is 10%.

Figure 2. The cells of the Micrococcus sp., strain 367-3 (x 50,000); NaCl content in the medium is 10%.
Figure 3. The culture growth on the medium with low NaCl content (0.03\%):
   a. The slicing of the cell envelopes of the isolate 367-3 (x 11,000).
   b. The lysed cells of the Microacoccus sp. strain 367-3 (x 40,000).

Figure 4. The cells of *Methanosarcina*, strain 47: ultrathin section (x 18,000).
Figure 5. Development of *Methanosarcina*, strain 47, on media with different NaCl concentrations.

Figure 6. The cells of *Methanococcoides euhalobius*:
  a. Scanning electron microscope (x 20,000).
  b. Ultrathin sections (x 12,000).
Figure 7. Development of *Methanococcoides euhalobius* on media with different NaCl concentrations.

Figure 8. The cells of the new isolate GF 283:
- a. Cells in the sheath (x 16,000).
- b. A single cell (x 16,000).
Biochemical Markers for *In Situ* Microbial Community Structure

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Abstract

Signature biomarker techniques based on phospholipid esterlinked fatty acid pattern analysis (PLFA) provide data on the total viable or potentially viable communities without the necessity of quantitative recovery from the sediments or the ability to culture the organisms. The technique provides evidence for the nutritional status (starvation or unbalanced growth) *in situ*. To perform these analyses on deep subsurface sediments, increasing the sensitivity of the analysis was required. Inverse sequential extraction was coupled with selected ion monitoring, which together provided the necessary sensitivity. Recoveries of PLFA significantly above background and the patterns of PLFA proportions indicated insignificant drilling mud contamination. Ratios of total cell counts to the extractable PLFA, show three degrees of cultural recoverability relative to potential viability based on the presence of PLFA. The degree of cultural recoverability correlates with the permeability of the sediment. The similarities of the total community structure of the deep subsurface show clustering of the most similar PLFA patterns in the permeable sedimentary horizons with distinct differences in the surface soil and impermeable clay aquicludes. The PLFA patterns of the microbiota in recovered drilling muds show no similarity with those from the sediments. Preliminary indications show the PLFA clustering of isolates from the Subsurface Microbiology Culture Collection (SMCC) into four major classes that correspond to distinct fatty acid biosynthetic pathways. The four major types of organisms with specific PLFA patterns are not uniformly distributed throughout the sedimentary column.
Introduction

The Department of Energy's Microbiology of the Deep Subsurface Program had, as its original goal, the determination of whether microbial life existed in deep subsurface sediments, and if microbiota were present, was there a potential for manipulation of the community for bioremediation purposes? To unequivocally establish that deep subsurface sediments contained microbiota required clear evidence that the microorganisms recovered from the sediments were indigenous and not contaminants of the recovery process. The extraordinary care in sample handling and tracer-recovery experiments in this volume show that the likelihood of contamination of the microbiota was extremely unlikely. The determination of the biomass, community structure, and nutritional status of microbiota from environmental samples without the bias of cultural selection or a requirement for quantitative recovery of microorganisms from the soil particulates by utilizing signature phospholipid biomarkers, has been established in many systems including shallow subsurface sediments.\textsuperscript{1,5,6} The application of these signature biomarker methods for determinations of \textit{in situ} microbial biomass, community structure and nutritional status is reported.

Materials and Methods

Samples were recovered from the subsurface, frozen, and shipped overnight to the laboratory.\textsuperscript{2} At the laboratory, samples were lyophilized, extracted, fractionated, derivatized, and analyzed by gas chromatography/mass spectrometry (GC/MS) as previously described.\textsuperscript{4}

Results

\textit{Increasing PLFA sensitivity.} Some of the sediments recovered from deep subsurface samplings contained <10\textsuperscript{2} bacteria/gdw. This required the PLFA analysis to be done at sub-femtomolar (10\textsuperscript{-15} molar) sensitivities. A major problem was the long chain fatty acid contamination of ultra-pure solvents required for extraction of the lipids. When the same volume of ultra-pure solvents was used on a series of sediments from a specific sample, the results showed significantly lower contamination. Comparison of a single extraction of 25 g of Pee Dee Clay sediment with 28 ml of the Bligh and Dyer extractant (chloroform: methanol: water) to the triple extraction of 61 g of the same sediment showed major differences. The single Bligh and Dyer extraction recovered 12 fatty acids with 20:0, 22:0, and 24:0 (number of carbon atoms:number of double bonds) composition. These fatty acids comprised 83\% of a sample of Pee Dee Clay and had a concentration of 22 pmol/gdw. These long chain fatty acids are common contaminants of ultra-pure solvents. Three serial extractions from a total of 61 g of this sediment with the same extractant produced a pattern of 25 PLFAs with the following proportions: 20:0, decreasing from 57\% to 1.5\% (the major PLFA of most bacteria); and 16:0, increasing from <0.1\% to 28\% of the total PLFAs with a total concentration of 17 pmol/gdw. The effectiveness of the "inverse serial extraction" together with the micro column fractionation, derivatization, and selective ion GC/MS analysis,\textsuperscript{4} allowed for the detection of total PLFA of one to two orders of magnitude greater.
than the level of 16:0 in the blanks in all sediments. The background 16:0 averaged 110 fmoles/gdw. The 16:0, in all but three cases, was >1.0 pmole/gdw and represented about 10% of the total PLFA.

Community structure of the deep subsurface microbiota. The data in Table 1 show that the subsurface samples from the C10 hole exhibited three classes of microbiota. In samples from the surface, and subsurface (G-5, G-9, and G-16), the ratio of PLFA to viable cell counts was 0.04 femtomoles/cell. This was expected based on conversion factors derived with subsurface isolates grown under nutrient limiting conditions with 4 x 10^11 cells/gdw. These sediments had clay contents between zero and 45%, but with a permeability of >7 Darcy's. Where the sediments were essentially impermeable and the clay content was between 12 and 55%, there were between 17 and 50 fmoles/cell. This indicates that the cells were present and potentially viable, but not culturable using the diluted media in the Balkwill and Pheips techniques. The ratio of PLFA-containing cells to culturable cells in these sediments was roughly 100:1. Two permeable samples, one from the bedrock surface (G-22) and the other from a shallow aquifer (G-3), showed intermediate values for the PLFA/culturable cell of roughly one in 10 being nonculturable.

If one submits the total PLFA analyses from each sample to a hierarchical cluster analysis, the relatedness of specific microbial communities can be estimated (Table 2). The lower the cluster factor, the more closely the microbial communities resemble each other. Sediments G-5, G-9, and G-22 all had high permeability and showed the greatest similarity, with a rescaled cluster factor of 4. The microbiota in these three samples came from highly permeable strata defined as a "sand", although G-5 contains 45% clay, and the fractured bedrock. These samples contain relatively healthy cells based on the PLFA/viable cell ratios (Table 1). The microbiota of highly permeable sands (13% clay) of G-3 and the anomalous, inactive, nonculturable, but preserved microbiota in impermeable clay of G-10, form a second cluster group (rescaled value of 6) which have a similarity value of eight with the first three samples. The microbiota of the highly permeable sand (5% clay) in G-16 are different, healthy, culturable, and have patterns related to the other two groups with a cluster value of 14. The microbiota of G-16 can be clustered with the microbiota from the other subsurface sites to comprise the major group of subsurface bacteria from this site. The subsurface group is related to the surface microbiota by a rescaled cluster factor of 16. The clear differences between PLFA patterns of subsurface sediments and the surface soils have been observed repeatedly. The highly active microorganisms from the permeable surface, together with the subsurface microbiota, are clearly different from the two impermeable clays, G-13 and G-20 (rescaled cluster factor of 22). The impermeable clays and the surface and subsurface microbiota PLFA patterns are the most different from the microbiota of the three drilling muds DM-10, DM-11, and DM-12 (rescaled cluster factor of 25). The drilling muds form a cluster based on the PLFA of the microbiota (rescaled cluster factor of 14) that is clearly distinct from any patterns found in the sediment samples.
Community nutritional status indicators. PLFA patterns can be indicators of metabolic stress. Specific PLFA accumulate under conditions of starvation or electron donor/acceptor shortage.\textsuperscript{5,6} The accumulation of cyclopropane rings in monoenoic fatty acids (cy17:0, cy19:0) or in monoenoic PLFA with the trans configuration occur in stressed monocolonies or biofilms. High proportions of these markers can be detected in the PLFA patterns found for the impermeable sediments G-10 at 239 m, G-13 at 303 m, and G-20 at 437 m (Figure 1). These "clays" have low permeability and a very high ratio of PLFA/culturable cells (Table 1).

PLFA analysis of deep probe isolates. Isolates recovered from the fourth hole at various depths have been characterized by physiological and morphological tests and maintained in the SMCC by David Balkwill. An initial group of 19 strains, which were chosen by D. Balkwill to represent various colonial morphologies and depth localizations, were cultured in 1% PTYG medium for PLFA analysis. PLFA data were pooled and a hierarchical cluster analysis revealed four major classes of bacteria. Cluster Groups 1 and 2 were more closely related than the other two cluster groups. Cluster Group 2 was split into two subgroups. None of the strains gave an identical PLFA pattern, although Cluster Group 2 showed two highly similar subgroups. PLFA found in significantly greater proportions in a particular cluster group were also found to be in significantly higher proportions by ANOVA and multiple comparison of means of the arcsine x the square root transformed mole percent PLFA profiles. The PLFA patterns of each cluster group showed dominance of specific biosynthetic groups. Cluster Group 1 showed a predominance of 18:1w7c (eighteen carbon alkyl chain with one unsaturation in the cis configuration seven carbons from the non-polar terminus). Cluster Group 3 was characterized by the branched fatty acid biosynthetic pathway with predominance of i14:0, a15:0, i16:0, and a17:0 (prefixes i and a refer to iso and anteiso methyl branching). Cluster Group 4 showed predominance of the aerobic desaturase pathway of microeukaryotes and some prokaryotes with 18:1w9c together with the prokaryotic odd chain PLFA, 17:0.

Localization of isolates in the sedimentary column. With the resolution of the isolates into four major cluster groups, which reflect the end products of different microbial biosynthetic pathways, the depth distribution of each of these groups of isolates could be defined (Figure 2). Organisms from Cluster Group 1 (anaerobic desaturase, 18:1w7c predominating) were found from the surface to 300 m. Cluster Group 2 (anaerobic desaturase, 16:1w7c predominating with 16:0) were recovered from virtually all depths. Cluster Group 3 (branched, saturated with i15:0 and a15:0 predominating) was isolated from the surface and between 400-500 m. Cluster Group 4 (microorganisms with the aerobic desaturase producing 18:1w9c and the odd chain 17:0 PLFA) were recovered from depths below 350 m.

Discussion

Improved sensitivity of PLFA analysis. The pattern of the PLFA recovered from the serial extractions was easily rationalized from known microbial biosynthetic pathways and was similar to those recovered from other subsurface sediments that contained a thousand times more bacteria. When the volume of the extractant
solvents was decreased and the number of the serially extracted sample subsets increased, the number of typical bacterial PLFA patterns increased dramatically at the expense of the contamination patterns. Utilization of several key ions from the mass spectrometric detection system with the "extracted ion chromatography program" increased the sensitivity from $10^{-9}$ to $10^{-12}$ molar, so each sample could be run with the GC/MS directly without a preliminary GC analysis. The sensitivity was increased to $10^{-15}$ molar with the application of smaller extraction volumes and chemical ionization mass spectrometry as the detection system.

*Polar lipid analysis.* Polar lipids are a part of every cellular membrane. In bacteria and nearly all living cells, the major polar lipids are phospholipids. These polar lipids actively turnover and with cell death they are transformed into neutral lipids. In other words, petroleum contains no phospholipids. Consequently, phospholipids are a measure of the viable or potentially viable cells in a sample. Phospholipids are an excellent measure of the biomass of the subsurface microbiota. The estimate of bacterial numbers and biomass from acridine orange direct cell counts (AODC), muramic acid (a unique component of the bacterial cell wall), the polar lipids, and the cellular adenosine triphosphate of a subsurface sediment gave identical values.1

*Community structure distribution.* The permeability, rather than the proportions of clay, sand, and silt, correlated best with the ability of the bacteria to be grown on artificial medium (Table 1) and the similarity of PLFA patterns (Table 2). The hierarchical analysis of PLFA from the subsurface illustrated in Table 2 is independent of isolating or culturing microorganisms from the sediments or the drilling muds, or of artificial groupings of fatty acids as defined by the investigators. The clear differentiation of the microbiota from the surface and all the subsurface sediments from the drilling muds is powerful evidence that there was essentially no significant contamination of the samples by the drilling muds used in sample recovery. The sample that was most suspect of contamination by drilling muds by various investigators was the highly permeable sandstone found in G-16. G-16 clearly contained a microbiota more like the rest of the subsurface active microbiota than the drilling muds. Cluster analysis of the data indicated that the microbiota in G-16 was more closely related to the surface sands that were collected with a flamed shovel, than any drilling muds (Table 2).

*Community nutritional status.* The parallel detection of high ratios of PLFA to culturable cells and the *in situ* detection of high proportions of cyclopropene or trans monoenoic PLFA in the least permeable sedimentary horizons, clearly indicates these are areas of high stress to the resident microbiota.

*PLFA of isolated microbiota from the deep subsurface.* The pooled PLFA from 17 isolates collected from different sedimentary horizons were three factored into four major groups by hierarchical cluster analysis. Cluster Group 1 (major PLFA, 18:1ω7c) represents a pathway of anaerobic desaturase unique to eubacteria, which forms both this PLFA and 16:1ω7c. Cluster Group 2 shows the other predominant product of the anaerobic desaturase (16:1ω7c), along with the saturated analogue (16:0). Palmitic acid (16:0) is the most common PLFA found in Gram-negative rods.
like *Pseudomonas*. The anaerobic desaturase is functional in both aerobes and anaerobes. With nutritional stresses, the microorganisms in these biosynthetic groups form cyclopropane PLFA from the monounsaturates; cy19:0, w7,8 from 18:1ω7c and cy17:0, w7,8 from 16:1ω7c. These organisms also form the trans isomers 16:1ω7t and 18:1ω7t in the PLFA under conditions of starvation and microcell formation. Cluster Group 3 shows predominantly short-branched, saturated PLFA. This pathway is characteristic, but not limited to Gram-positive bacteria such as *Bacillus* and the Gram-positive, high G+C, DNA-containing group of *Arthrobacter*. Cluster Group 4 consists of a group of organisms with a desaturase typical of the aerobic pathway found in eukaryotes and some bacteria that have 18:1ω9c as the primary monounsaturated PLFA. The isolates in this group also show a predominance of the unusual PLFA, 17:0. Eukaryotes show PLFA patterns with 18:1ω9c and polyunsaturated fatty acids (PUFA), whereas the prokaryotes do not contain PUFA. The horizons containing culturable fungi also contained the highest proportions of PUFA (data not shown).

**Conclusion**

The recoverable organisms from the DOE’s subsurface collection showed, at least in the preliminary analysis, specificity as to depth distribution. It is clear that the microbial community structure of the deep subsurface sediments shows definite differences between geologic horizons, with the best correlation being the permeability of the formation (Tables 1 and 2; Figure 1). The community structure differences in the organisms that can be isolated from different geologic horizons are clear even in a small subset of the total collection (Figure 2).

**Acknowledgements**

This work was supported by contract DE-FG05-88ER60643 from the Office of Health and Environmental Science, United States Department of Energy, the Interdisciplinary Subsurface Science Program initiated and managed by Dr. Frank Wobber at DOE, Drs. Carl Fliermans and Jack Corey at the Savannah River Laboratory. The VG tandem mass spectrometer was purchased with funds from ARO 27187-LS-RI Army Research Office, 2-4-01018DEG Department of Education, and DE-FG05-88ER75379 from the DOE URIP programs.
References  
Table 1. Relationship between total PLFA/viable cell count ratio and permeability. The relationship used was \(4 \times 10^{11}\) cells/gdw and 100 umoles lipid/gdw; groundwater cells therefore contained \(0.04 \times 10^{-15}\) mole PLFA/cell.

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\(^a\) Depth in meters.
\(^b\) Permeability in Darcy's.
\(^c\) Femtomole PLFA/Cell (Plate count and fungal cell count from previous report).
Table 2. Hierarchical Cluster Analysis of Deep Subsurface Field Samples by PLFA Profiles

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\textsuperscript{a} Depth in meters.

\textsuperscript{b} Permeability in Darcy's.
Figure 1. Deep Sub-Surface Field Lipid Stress Indices.

Figure 2. Distribution of PLFA Cluster Groups of Isolates.
**J. Wiegel:** How fast do you get the hydrolysis of the substrate?

**D. C. White:** When they die?

**J. Wiegel:** Yes. What kind of stage of death do you get?

**D. C. White:** It is hard to define death, because one must irradiate them so they will not grow. One can detect the lipids of hydrolysis in an hour or two. That was done a long time ago, and it is very fast. The turnover of phosphatidylyl glycero1, for example, is a very good measure of the bacterial growth rate. As the phosphate is lost in phosphate turnover, there are some lipids such as the phosphatidylyl choline that do not turn over very fast. A lot of them, however, do turnover. With the growth rate, they are very active. In growing organisms, if one does a pulse-chase of $^{32}$P, one can get a 20-minute half-life. A 10-minute half-life is obtained for phosphate.

**J. Wiegel:** What happens to those organisms that are down there? Do they really grow down there or are they just remnants? What kind of rate of hydrolysis do you get then? Do you compare those two different stages of being alive?

**D. C. White:** This has only been done with marine sediments. This is an unfair way to do it, but a whole system was used. I think the question is a very good one. Let's put a little $^{33}$P down the well and see.

**N. Tonso:** Bacteria change their cell walls with their environment. Can you tell me how much you think that growing them on laboratory media will change them from environmental surface stresses?

**D. C. White:** This happens with every grant I write, in that comments are made to the effect that one can not tell anything about a bacteria by its fatty acids. If one takes a monoculture and puts it in a chemostat, one can really change it. Taking it from its optimum temperature and moving it up 20 degrees can really make it change. One can make some bacteria change, but some will not change at all. What one has to do is look at the conditions in which they exist in nature. For instance, we had some experience, sort of by mistake, with the organism that causes tularaemia. That organism makes a characteristic pattern of ester-linked fatty acid, which we found in soil, in infected rabbits, in pus from humans, and also in pieces of lung. It is obvious that the organism was there; therefore, it does not change. It can not grow under conditions that would change its fatty acid. Now there are organisms that, certainly Lactobacilli, for example, can pick up whatever is in the media. Treponema pallidum, as far as we know, picks up human fatty acids. With that one, there is a problem. Every time (in at least eight times) that methane has been added with air to the soil, a Type II methane oxidizer has always been obtained. So its absolutely
unique, fatty acid signature, which is an ester-linked 18:1w8c as a major fatty acid, always appears. Under conditions that are stable, the organism always makes the same thing. If it can exist in competition, then it apparently can do that. The same thing has been true with sulfate reducers and sulfur oxidizers, the various kinds that we have looked at to see if they work. Sure, they can change, but the question is do they change under the conditions that one is studying them, where they have to compete with other organisms? If you move them away from their optimum growth conditions, they have to be able to grow better than other organisms that have optimum conditions.
PHYSIOLOGICAL ACTIVITY

Contributed Papers 2
Posters
Characterization of Phototrophic Microorganisms from a Deep Terrestrial Subsurface
Marianne Eleuterio
Biology Department, West Chester University, West Chester, PA 19383

Abstract
A selective technique was used to isolate phototrophic bacteria and a green alga from a rock and two sediment cores from the C10 borehole drilled near Allendale, South Carolina sponsored by the United States Department of Energy’s Microbiology of the Deep Subsurface Program. An enrichment medium, light, and anaerobic conditions were used to isolate reddish-brown to purple colonies of motile, Gram-negative bacteria from all three samples. Winogradsky columns were used to enrich for phototrophs in the cores. Algae grew out in the GEM medium that was covering the rock after exposure to air. The phototrophs could grow slowly heterotrophically in the dark, but without pigment production. The absorption spectra and characteristics of these isolates were those of purple non-sulfur bacteria. The anoxygenic phototrophs and algae that were found add to the diversity previously described in the microbial community of the coastal plain subsurface.
Introduction

The Microbiology of the Deep Subsurface Program provided evidence of bacteria in cores at a depth of 1700 feet below the surface. Although the presence of photosynthetic bacteria had not been reported in the cores from the Savannah River sites, these bacteria are of special interest because they are often regarded as transitional forms from chemosynthetic to photosynthetic organisms. Photosynthetic bacteria are widely spread in nature, found in stagnant lake waters and ponds below the surface algal layer, in soils, and in oil pools. In 1933-35, purple bacteria were discovered by Isachenko in Russia in the formation waters of spouting oil wells operating at a depth of 1300-1700 m. Because of the amount of pink water produced (1000 tons per day) and the duration of spouting (8-10 months), it was believed that the bacteria came from the boundary between the oil deposits and water and evolved in the geological period (the Precambrian period) in which the plant biomass and microbial forms were buried. One isolate from the oil well was a purple, non-sulfur species (Rhodopseudomonas issatchenkoi). This organism could form pigments when grown on organic acids in the light and could also grow, albeit very slowly, in the dark on organic compounds such as oil and naphthenic acids.

By utilizing enrichment methods designed to favor anoxicogenic, photosynthetic, N₂-fixing bacteria, Gest et al., isolated strains from soils, coastal waters, and from a 55°C water sample from Thermopolis Hot Spring in Wyoming. This organism produces desiccation-resistant cysts when grown aerobically in darkness with butyrate as the sole carbon source. These studies illustrate the diversity of sites, including the great depths in which phototrophic bacteria have been found. By utilizing the enrichment techniques of Gest et al., photosynthetic bacteria, most probably purple non-sulfur species, were isolated from two cores from the C10 borehole near Allendale, South Carolina, G-13 (303 m) and G-20 (437 m), and a highly consolidated sediment sample from 528.5 m.

Materials and Methods

The medium used in this investigation, GEM, has been previously described. The pH was adjusted to 6.8 and alternate reducing agents ascorbic acid (GEM A), Na₂S₂, (GEM B) or sodium thioglycollate (GEM C) were added after autoclaving. PY medium contained 3 g/l of peptone, 3 g/l of tryptone, and 3 g/l of yeast extract (Difco). YPTG contained 10 g/l of yeast extract, 5 g/l peptone, 5 g/l of tryptone, and 10 g/l of glucose. After autoclaving, 2 ml of 1 M MgSO₄ and 2 ml of 1 M CaCl₂ were added to the PY and YPTG media. The RCV medium contained malic acid and thiamine as the sole carbon and vitamin sources at pH 6.8. The BG11 medium contained per liter, 1.5 g/l of NaNO₃; 0.4 g K₂HPO₄·3H₂O; 0.75 g/l of MgSO₄·7H₂O; 0.36 g/l of CaCl₂·2H₂O; 0.006 g/l of citric acid; 0.006 g/l of Fe(NH₃) citrate; 0.001 g/l of EDTA; 0.02 g/l of Na₂CO₃; and 1 ml of trace elements. Agar (15%) was added to each medium for plates and 0.6% agar was added to PY or YPTG for stab cultures. Deionized water was used for all solutions. Reagent grade chemicals, obtained from Fisher Scientific Company or VWR., were used where possible.
Two sediment cores, G-13 and G-20, and a highly consolidated sediment sample from a depth of 528.5 m were collected from the C10 borehole near Allendale, South Carolina, by methods described by Phelps et al.9

Isolation of Microorganisms From The Highly Consolidated Sediment Sample. The highly consolidated sediment sample was aseptically transferred from the shipping bag to a sterile beaker filled with sterile GEM medium and sealed to exclude air. It was illuminated with 60-watt, incandescent lamps and held at 25-28°C for one week. An aliquot was removed, diluted 1 x 10⁻⁵ and 1 x 10⁻⁶ in sterile saline (0.85% NaCl), and plated on PY and RCV plates, and incubated in anaerobic jars surrounded by lamps for one week. Reddish-brown colonies were transferred by sterile toothpicks to RCV and PY plates and incubated anaerobically. After a second purification, the colonies were transferred into 2 ml screw-cap vials that were completely filled with GEM A medium, and incubated in the light until red pigmented growth was evident (4-5 days).

Isolation Of An Alga From The Highly Consolidated Sediment Sample. Incubation of the highly consolidated sediment sample immersed in GEM medium in the light was continued after an aliquot was removed. After two weeks, the medium gradually turned bright green. Aliquots of the medium were removed, diluted 1:4 into BG11 tubes, and incubated aerobically with fluorescent light. Isolated colonies of a green alga were isolated by further dilution and plating on BG11 plates under fluorescent light.

Isolation Of Phototrophs From Sediment Cores. Aliquots of G-13 and G-20 samples were transferred into Winogradsky columns, inside a N₂ filled glove bag. The columns were filled with sterile water to a few centimeters above the packed samples, sealed with plastic wrap, removed from the glove bag, and incubated at 22-28°C between 60-watt lamps for two weeks. When a reddish or orange pigmented band appeared in the column, the mud above it was scooped out and discarded. Aliquots of the pigmented band were transferred to 20 ml screw-cap glass tubes filled with GEM B medium and incubated in the light for 10-14 days. Serial dilutions in sterile saline were plated on PY plates. The plates were incubated anaerobically in the light for 7-14 days or until colonies were visible.

Samples from the G-20 Winogradsky column were diluted into 50 ml Hypovials (Pierce Chemical Company) containing 30 ml of GEM A, B, and C Medium, flushed with N₂ for two minutes, incubated in the light until pigment developed, and then diluted and plated on 5% YPTG, RCV, and GEM plates. Two colonies each, isolated from GEM A, B, and C, were purified on GEM A plates, grown in GEM A with 0.2% YPTG, and flushed with N₂. Isolates were stored in 30% glycerol at -70°C. Aerobic cultures of the photosynthetic bacteria were grown in PYTG tubes at 25-28°C on a tissue culture roller drum, or 50 ml of YPTG in 250 ml Erlenmeyer flasks on a LabLine Model 3528-5 orbital shaker.

Absorption Spectra Determination. Phototrophic isolates, TSG 23, 24, 131, 142, and 144, from the highly consolidated sediment sample, and isolates G13-2, 5, and 6 from the G-13 core, were all grown anaerobically in screw-cap tubes of GEM
A medium with N₂ for four days. Two milliliters of GEM A cultures were centrifuged (1200 rpm) for five minutes in a Microfuge, and the pellets were resuspended in 1 ml of 30% bovine albumin. The absorption spectra were measured in a Perkin Elmer lambda 9 or lambda 4B spectrophotometer.

Results

Photosynthetic bacteria and a photosynthetic alga were isolated from the highly consolidated sediment sample from a depth of 528.5 m, and photosynthetic bacteria were isolated from sediment cores at 303 and 437 m. Forty-two isolates were taken from the rock, seven from G-13 and nine from G-20.

All of the bacteria appeared to be heterotrophic and grew slowly and aerobically in the dark on rich media (i.e., YPTG). Although the isolates (GEM and RCV) grew photosynthetically under anaerobic conditions on defined media, they grew very slowly and did not form large colonies. The majority formed colonies of only 1-2 mm under all conditions. Some of the isolates at least grew with NaS or Na thiglycollate as a reductant, but all utilized ascorbate. The absorption spectra of eight isolates showed peaks characteristic of bacteriochlorophyll a.

Aerobically, the phototrophs did not produce pigment. Colonies grown anaerobically sometimes continued to enlarge slightly in the presence of air, but the borders were white. The phototrophic bacteria were highly motile Gram-negative rods. Most of them formed aggregates and grew mostly as a reddish sediment in the bottom of tubes incubated in the light.

A green alga was also isolated from the highly consolidated sediment sample after the enrichment medium became exposed to the air. It was isolated in pure culture and morphologically resembled a Chlamydomonas species. It had a red eyespot and two polar flagella.

When the selection plates were removed from anaerobic jars and exposed to air, a variety of colonies, white, tan, yellow, gold, and pink appeared. Most of them were Gram-negative rods; one Gram-positive coccus and two yeasts were isolated. Most of the Gram-negative rods grew rapidly on nutrient agar or PY to form colonies in 24 hours at 22-28°C. They were unable to grow in anaerobic jars.

Discussion

All the photosynthetic bacterial isolates appeared to be heterotrophic as well, and thus, were probably purple, nonsulfur bacteria. The number of photosynthetic and nonphotosynthetic isolates was larger from the highly consolidated sediment sample than from either of the sediment cores. This difference may be due to slight differences in the procedures and media used for isolation or to the composition of the samples. The highly consolidated sediment sample was composed mostly of skeletal remains of marine organisms, molluscs, and oyster shells (40-60%), together with a small amount of clay (3-8%) and sand. G-20, from which more phototrophs but only a few aerobes were isolated, contained silt from the Lower Middendorf formation at the Cape Fear clay interface. G-13 consisted of Upper Black Creek clay. Pigment was produced by the phototrophs only in anaerobic
culture. Reddish-brown to purple colonies on YPTG plates exposed to oxygen for one week or more may have enlarged slightly, but the borders were usually white. The absorption spectra of eight isolates were determined for intact cells suspended in 30% bovine albumin. The absorption maxima were characteristic for bacteriochlorophyll $a$ (375, 590, 805, 803-890 nm).

The conditions of low sulfate concentration in GEM A medium were selective for nonsulfur, purple bacteria; thus, the isolation of bacteria-most of which were like *Rhodospirillaceae*-may have been the result of the isolation procedures and not necessarily due to the absence of other phototrophic bacteria. The type of enrichment media used selects between the major groups of phototrophs. The salinity and mineral composition of the medium is important in isolating marine species. In some experiments, the GEM A cultures were spread on GEM plates with 5 g/l NaCl, but growth of the phototrophs on the salt plates was poor. Although the highly consolidated sediment sample appeared to have originally come from a marine environment, the bacteria isolated from it did not appear to be marine forms.

Microscopically, the isolated algae resembled *Chlamydomonas* species. In the fluid, there were also aggregates of four round, thick, walled cells with pale green contents. It is possible that the algae can form cysts and therefore were present in a dormant form, growing vegetatively only when released into the enrichment medium and exposed to oxygen and light.

A variety of rapid-growing, nonphotosynthetic bacteria were also present in all three samples. Under conditions which did not enrich for anoxicogenic photosynthetic bacteria, these would undoubtedly overgrow the phototrophs.

**Conclusion**

Although a site 300-500 m below the earth's surface seems unfavorable for growth of photosynthesizing bacteria, the purple, nonsulfur bacteria are versatile organisms and can utilize a variety of organic compounds for aerobic growth and, under some conditions, can even grow fermentatively in the dark. The isolation of purple bacteria from the spouting waters of an oil well in Russia three fold deeper than the Savannah River boreholes, and from hot springs, illustrated the extremes in which they can be found. A further physiological characterization of the isolates from the C10 borehole and genetic analyses will determine whether they are new or unique species, how they are related to known species, and the role they play in the ecology of the subsurface.

**Acknowledgements**

I thank Richard M. Busch, West Chester University Department of Geology and Astronomy, for geologic analysis of the highly consolidated sediment sample; and Ruth Patrick, Academy of Natural History, Philadelphia, PA, for identification of the alga. This research was supported by a DOE grant, subcontract No. ORA3120.01, Independent Project Student, West Chester University.
References


Isolation of Bacteriophage from Deep Subsurface Sediments  
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Abstract

The occurrence of bacteriophage in deep subsurface sediments is unknown, despite their potential importance in regulating the number and types of bacteria present in this environment. Sediment samples from zero to 1725 feet were tested for bacteriophage. Soil extracts were either assayed directly or after the use of enrichment techniques. A variety of bacterial hosts were tested, including natural soil isolates from the sediments and known phage hosts including laboratory strains of Escherichia coli, Streptomyces griseus, Bacillus subtilis, Arthrobacter globiformis, and Pseudomonas fluorescens. No bacteriophage were isolated from sediments using the laboratory strains. A variety of tailed and nontailed bacteriophage were isolated from sediment samples taken from depths of zero, 392, 697, and 1330 feet. Phage densities ranged from zero to approximately $2.8 \times 10^3$ PFU/g soil. Bacteriophage isolates appeared to be specific for certain bacterial hosts. Most bacteriophage tested did not infect bacterial isolates from other sediment depths. The bacteriophage isolated from sediment samples may be unique to the microenvironments present at certain depths.

No paper submitted.
Tracing Contaminating Microorganisms in Groundwater Using Intracellular Granules of INT-Formazan and Gene Probes
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Abstract

Currently used chemical methods for tracing microorganisms in groundwater (e.g., halogen salts or fluorescein dyes), aside from being toxic, do not necessarily reflect bacterial movement. Living cells reduce 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) to INT-formazan, which accumulates as intracellular granules. The granules can be used as markers for genetically engineered microorganisms (GEMs) as well as naturally occurring pathogenic microorganisms. The use of genetic probes has been shown to be a very sensitive, although cumbersome technique for the detection of organisms under a variety of conditions. In this study, a strain of formaldehyde-fixed, INT-formazan granule-containing GEMs were seeded into sand columns. The adsorption rate and travel time of the GEMs were determined at varying times by concentrating granule-containing cells onto cellulose acetate membranes; four milliliters of a solvent passed through the membranes and the resulting extract read spectrophotometrically. Radioactively-labeled probes were also used to detect these very same cells. Viable cells did not behave differently from non-viable cells in the sand columns. However, the travel times of the two GEMs were different. These results indicate that the adsorption rates as well as possible travel distances in the subsoil will have to be determined for each individual microorganism. In addition, these results show that both methods can be used alone or conjointly to trace any microorganism through the subsoil in a very rapid and safe manner.
Introduction

Very little is known about the incidence of biological contamination of groundwater in Puerto Rico. Due to the importance of contaminated groundwater as a possible vector of etiological agents of enteric disease (e.g., viruses, bacteria and parasites), it is imperative to determine the exact prevalence of biological pollution of groundwater. Most reported outbreaks of waterborne disease in the United States have been linked to groundwater contamination.\(^4\)\(^5\) Thus, we can extrapolate to those communities in Puerto Rico which are dependent on groundwater as a source of drinking water and as such are in danger of being exposed to enteric diseases if the groundwater is contaminated. Studies by Gerba et al.\(^7\) indicated that movement of microbial contaminants in soils is dependent on a variety of factors. Some of these factors are type of soil, cation exchange capacity, specific surface area, degree of unsaturation of the soil matrix, and pH of the soil environment.

Fifty percent of all reported outbreaks of gastroenteritis in the United States have been linked to groundwater contamination.\(^1\) In Puerto Rico, groundwater totals 24% of the daily water consumption (Quifiones and Alicea-Ortiz, United States Geological Survey Open-File Report 85-642). Thus, the possibility of waterborne outbreaks on the island is increased as more people become dependent on the use of groundwater.

The movement of microorganisms and toxic substances which cause groundwater pollution can only be assessed through in situ studies. However, as a result of the variations in soil composition, and several physicochemical differences, studies at one site do not necessarily indicate the behavior of microorganisms at other sites. Therefore, soil columns have been used extensively to mimic in situ conditions. The soil columns allow for an easy and convenient way to conduct preliminary studies on the interactions between microorganisms and the soil matrix.

These interactions are usually studied with tracers. The use of biological tracers is extremely important in order to investigate the possible fate of pathogens and other contaminants in areas such as those that use On Site Sewage Disposal Systems (OSDS), such as septic tanks. Therefore, the development of methods which allow us to realistically study the interactions between the soil matrix and the microorganisms is imperative.

To date, the most widely used tracers have been chemical compounds (e.g., fluorescein dyes or halogen salts), which can be easily detected and quantified. The biggest drawback of using these tracers is that they only indicate the movement of chemicals in the groundwater or the flow of water, and not necessarily the movement of any contaminating microorganisms.\(^8\) In addition, some of these chemicals have been found to be potential carcinogens, and thus, limited utility. There is a great need to develop techniques which indicate or predict the movement of microbiological pollutants through the subsoils. The use of bacterial tracers have several advantages over chemical tracers. The most obvious is the fact that bacterial tracers would very accurately predict the movement of bacterial contaminants in groundwater. Bacterial tracers, unlike chemical tracers, are not carcinogenic.
Fecal coliform bacteria (e.g., *Escherichia coli*) have been extensively used as tracers of bacterial movement. However, it is difficult to differentiate the organisms being used as tracers from those derived from the suspected source. Thus, to circumvent this problem, antibiotic-resistant strains of *E. coli* were used by Hagedorn and co-workers, Rahe et al., and by McCoy and Hagedorn. However, there is increasing concern about using antibiotic-resistance markers present in bacteria due to the possibility of genetic transfer to resident microflora or pathogens present in sewage. The latter concerns can be minimized by using the antibiotic resistance that resides in the chromosomal DNA rather than in plasmid DNA. Antibiotic resistance, which is coded for in the chromosome, cannot be readily transferred to other bacteria. Several other strains of bacteria and yeasts have also been used as tracers. However, there exist intrinsic problems with all bacteria used to date; in fact, one of the most widely used genus of bacteria (Serratia spp.) has been recognized as a potential pathogen. Regrowth of the microorganisms being used and the inability to differentiate between resident flora and the tracers being used are also potential problems faced when doing tracer studies.

For the last few months, experiments have been conducted in our laboratory using bacteria, containing intracellular granules of 2-(para-iodophenyl)-3-(para-nitrophenyl)-5-phenyl-tetrazolium chloride-Formazan (INT-formazan) as possible tracers of biological contamination. A tetrazolium salt, INT, is a colorless compound and a water-soluble redox indicator. Upon reduction, it is converted to water-insoluble, red-formazan derivatives. Jeffrey and Paul described the solubilization of INT-formazan granules in either methanol or ethanol; the solubilized product can then be read in a spectrophotometer.

There are many promising applications of GEMS in agriculture, industry, and medicine. Lately, there have been concerns raised over the possibility of releasing GEMS into the environment. The solubilization technique had been used conjointly with hybridization techniques to trace the movement of GEMS through a soil matrix. The simultaneous use of both techniques allows for a thorough comparison in terms of the feasibility and sensitivity of both methods. In addition, the use of GEMS allows one to have a genetic probe which hybridizes very specifically with the DNA inserted in concentrated, seeded bacteria.

The purpose of this study was to determine the feasibility of using solubilization of intracellular INT-formazan granules conjointly with genetic probes to trace groundwater bacterial contamination.

**Materials and Methods**

*Bacterial Strains.* Strains used in this study were *Escherichia coli* (ATCC 15597), *Salmonella typhimurium* (ATCC 7823), and two GEMS labelled with INT-formazan were used for the tracer studies. The GEMS (kindly supplied by Dr. G. Candelas) were an *E. coli* DH1 strain containing a derivative of the pBR322 plasmid (this plasmid derivative contains a 437 bp fragment cloned into the PvuII - Avai site of pBR322), and *E. coli* JM103 strain containing pUC9 plasmid with a 147 bp fragment cloned at the polylinker region.
**INT-Formazan Labelling.** All bacteria were grown in Luria's Broth (LB) at 37°C to mid-log phase. At this stage, 2-(para-iodophenyl)-3-(para-nitrophenyl)-5-phenyl-tetrazolium chloride (INT) was added to the cultures to a final concentration of 0.02%. Cells were fixed by adding Formalin solution (0.1% v/v) and washed twice in physiological saline to get rid of culture media and remaining Formalin.

**Granule Solubilization.** Cells labelled with granules were concentrated onto a 0.45 μm pore diameter, cellulose acetate membrane filters (Millipore Corp., Bedford, Mass.) The membranes were allowed to dry at 45°C for 20 minutes. A five-ml volume of 95% ethanol was passed through the membranes containing the labeled bacteria and the filtrate was measured spectrophotometrically (Beckman Model 35, Irvin, Ca.) at a wavelength of 485 nm. Negative controls consisting of aliquots of unlabelled bacteria were treated in the same manner as the bacteria containing intracellular granules and the resulting solutions used as a blank in spectrophotometric readings, and they were always included in each experimental run.

**Column Studies.** All column experiments were conducted using column apparati as described by Bales et al. Three and ten grams of soil (sand) from Vega Baja, Puerto Rico were loaded into the columns and saturated with distilled water. Washed, labelled bacteria were loaded into the column and allowed to migrate. Flow rate was approximately 1.0 ml per minute. Aliquots were obtained at different times, bacteria were concentrated onto membrane filters as described above, and granules were solubilized. The resulting filtrate was read spectrophotometrically.

**Hybridization Analyses.** The GEMs were grown, marked with INT-formazan, and run through soil columns as described above. The movement of these GEMs was monitored using a radiactively labelled genetic probe. The preparation of the probe was done using the random-primed extension technique of Feinberg and Vogelstein, as follows: plasmid DNA was purified as indicated in Maniatis et al., digested with PvuII and Aval, and run in a 1% low-melting agarose gel. The band corresponding to the insert was excised and diluted, and the eluted insert was labelled using the Klenow fragment of DNA polymerase I in the presence of 32P-labelled (3000 Ci/mmole; 10 uCi/ul), random primers and unlabelled deoxynucleotides. All labelling reactions were carried out as indicated by Sanchez and Toranzos.

Any bacteria concentrated on the filters were lysed as indicated for colony hybridizations. The DNA was fixed onto the membranes in a vacuum oven and prepared for hybridization. Hybridizations were analysed by autoradiography using Kodak X-Omat AR film at -70°C.

**Results and Discussion**

Experiments have been conducted in which the optimal conditions to carry out the solubilization with the highest detection limits have been determined. *E. coli* cells were observed to reduce INT in approximately 30 minutes at log phase of growth (Figure 1). At this time period, 100% of the cells contained granules. Figure 2 shows that a 24-hour period of reduction of INT is required for a maximum incorporation of intracellular granules.
Unpublished results (Toranzos, et. al.) indicated that granule-containing, fixed bacteria retain their granules, and their numbers decrease only slightly even after a four month period. Samples of the INT-formazan labeled bacteria were inoculated into distilled, unsterile water and kept at room temperature and at refrigerated temperatures over a period of one year. The slight decrease has also been observed in high bacterial concentration. This suggests that our labeled bacteria will not be destroyed due to interactions with the resident microflora and that travel time, autolysis, and concentration of the resident microflora, will not be limiting factors in the resiliency of our labeled bacteria.

Columns studies with three and ten grams of soil (sand) demonstrated that the INT, granule-containing bacteria can be used for tracing studies in soils (Figures 3 and 4). Results are indicative of the interactions between the soil particles and bacterial cells. Tracing studies showed that bacteria can in fact be concentrated onto membranes and the intracellular granules solubilized with ethanol. Studies were also conducted by lysing the bacteria on membrane filters prior to ethanol solubilization, but in several trials the efficiency was not improved.

The data in Figure 5 shows that fixed, granule-containing bacteria do indeed mimic the movement of live bacteria through the soil. The results from these experiments show that fixed bacteria moved at the same rate as live pathogenic bacteria. Thus, the data reported here suggests that the use of easily identifiable, killed pathogenic bacteria may circumvent the possible concerns over injecting live bacteria in the subsoil. In addition, it may be possible to directly and accurately assess the movement and travel distance of pathogens through the subsoil, and thus, it may be possible to determine sources of groundwater pollution. However, the travel times of the two GEMs were different (Figure 6). These results indicate that the absorption rates, as well as possible travel distances in the subsoil, will have to be determined for each individual microorganism in order to accurately assess their movement through the subsoil.

Currently, the detection limit for the solubilization technique is approximately 10^6 bacteria (Figure 7). Work is currently being done on the possible use of other analytical techniques to improve the detection limit of the solubilization technique. Also, radioactively-labelled gene probes were used to detect the very same cells, and results demonstrate that gene probes can successfully be used to trace bacteria in the subsoil (unpublished results). However, the detection limit of this technique has not been determined. Nonetheless, preliminary work showed an order of magnitude more sensitive than the solubilization technique. A disadvantage with the use of gene probes is that techniques, laboratory facilities, and training of personnel necessary to use this technique are not within reach of most laboratories.

**Conclusion**

It should be noted that all the techniques being proposed complement each other in terms of their rapidity, and simplicity (INT-formazan, granule-containing cells) and sensitivity (use of genetic probes, and gene amplification). In addition, even though the solubilization of the granules could be done at the testing site, the
very same membranes used to concentrate the bacteria can be shipped to a laboratory for amplification and hybridization techniques. The solubilization technique has proven to be useful to trace any bacteria, since all bacteria are capable of reducing INT to INT-formazan. Also, the technique is inexpensive and can be easily done with the minimum of laboratory facilities. Tracer studies can be performed in areas not readily accessible by motorized vehicles and microorganisms can be concentrated onto membrane filters and then shipped to laboratories that can solubilize the granules and determine migration of microorganisms very rapidly. In addition, these results show that both methods, solubilization of granules and gene probes, can be used alone or conjointly to trace the movement of any microorganism through the subsoil in a very rapid and safe manner.

Acknowledgements

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References


Figures

Figure 1. Indicates the time required for the intracellular accumulation of INT-Formazan granules.

Figure 2. Optimal time required for a maximum incorporation of intracellular granules.
Figure 3. Results from tracing experiments in a 3.0 g soil column with *E. coli* DH1.

Figure 4. Results from tracing experiments in a 10.0 g soil column with *E. coli* DH1.
Figure 5. Results from tracing experiments in a 10.0 g soil column with *E. coli* DH1 viable and nonviable.

Figure 6. Results from tracing experiments in a 10.0 g soil column with *E. coli* DH1 and *E. coli* JM 103.
Figure 7. Detection limit of the solubilization technique.
Properties of Aerobic Heterotrophic Bacterial Isolates from the C10 Borehole Near Allendale, South Carolina
R. S. Karunaratne,1 J. L. Sinclair,2 and W. C. Ghirose1
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2NSI Technology Services Corporation, Ada, OK.

Abstract

More than 400 bacterial isolates from uncontaminated samples were selected randomly from aerobic and heterotrophic enumeration plates of surface soil and core samples to a depth of 526 m. The goal was to test the isolates for properties relevant to the subsurface sediments from which they originated. One group was screened for colonial and cellular morphology, pigment production, motility, storage polymers, utilization of organic acids as sole carbon-energy sources, fermentative ability, nitrate reduction potential, catalase and oxidase activities, and microaerophilic growth. A second group was screened for Gram-reaction, catalase activity, and growth on a complex medium under strictly anaerobic conditions. Isolates from surface soil showed greater variety of colony types and other morphological variations than subsurface isolates, but most subsurface samples showed greater colonial diversity relative to population density. Gram-negative, PHB-depositing bacteria were more common in high sand than in high clay samples; Gram-positive bacteria were more abundant in high clay samples. Most other properties did not correlate strongly with texture or other physicochemical characteristics of the sediments; however, some physiological properties did show depth-related trends. For example, more fermentative bacteria were isolated from samples obtained above a clay layer at 238 m than from below it. Also, more isolates from below the clay layer were microaerophilic and more were catalase-negative than isolates from above the clay layer. The depth-related trends suggested that the deepest aquifer formations (Black Creek, Middendorf, Cape Fear) typically contained non-fermentative, microaerophilic bacteria. The physiological properties of isolates from the C10 borehole indicated that low dissolved oxygen concentration may be a dominant environmental factor regulating microbial activity in the deep aquifer formations in this region of the Atlantic Coastal Plain.

No paper submitted.
Biodegradation of Synthetic Chelates in Subsurface Sediments
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Abstract

Synthetic chelating agents (e.g., EDTA, DTPA, NTA) have been used for nuclear decontamination and waste processing because they form stable watersoluble complexes with radionuclides and heavy metal ions. Co-disposal of synthetic chelating agents and radionuclides at several Department of Energy sites has resulted in increased radionuclide transport in the subsurface environment. Microbial transformation of synthetic chelates in subsurface sediments is currently unknown, but could influence the persistence of chelates and thus, alter the subsurface transport of chelated radionuclides and metals. Subsurface sediments were collected from five formations (surface to 376 m) near DOE's Savannah River Site in South Carolina. Sediment slurries (1 g/10 ml) were incubated with $^{14}$C labelled EDTA, DTPA, and NTA at $10^{-5}$ M concentrations and evolved $^{14}$CO$_2$ was determined over time. Aerobic biodegradation of chelates occurred in select sediments indicating that subsurface microorganisms have the potential to alter chelate persistence. The extent of mineralization of these compounds after 115 days in other sediments was limited, indicating chelates may be stable in some strata. The relative order of chelate persistence was EDTA, DPTA, and NTA with the maximum amount mineralized at 15, 26 and 43%, respectively. Maximum mineralization of all three chelates did not occur in the same sediment sample, indicating that different microbial populations are responsible for the biodegradation of each chelate. These compounds were all stable under denitrifying conditions. The presence of additional soluble carbon (acetate, citrate, glutamate and succinate) reduced aerobic chelate biodegradation to low levels, demonstrating degradation via cometabolism was unlikely and that chelate degradation may be controlled by catabolite repression.
PHYSIOLOGICAL ACTIVITY

Contributed Papers 2
Oral
Bacteria and Surfaces in the Groundwater Environment
Hans-Jorgen Albrechtsen
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Abstract

This study investigated the influence of different groundwater sediment particle sizes on bacteria and their activity. Samples of groundwater sediments were fractionated into different groups according to size by successively filtering a sediment suspension through progressively smaller mesh-sized filters (500, 100, 55, 20, 1.2 and 0.2 μm). The numbers of bacteria in each of the sediment-size fractions were much higher (up to 5200 times) than the numbers in unfractionated sediments (4.2-59.8 x 10^4 CFU/gdw). In all the samples, 69-87% of the total number of bacteria in the fractionated sediment was found in the 20-100 μm fraction, 13-31% in 1.2-20 μm fraction, but only 0.01-0.04% in the 0.2-1.2 μm fraction.

The microbial activity (measured as the 14CO2-evolution from acetate) showed the same pattern. After two days of incubation, 51-77% of the total amount of CO2 evolved from the 20-100 μm fraction, 17-28% from the 1.2-20 μm fraction, but only 0.2-5.4% from the 0.1-1.2 μm fraction. The activity per gram dry weight was much higher (35-553 times) in each fraction size than in the unfractionated sediment. The results showed that most of the bacteria and their activity were associated with small particles and thus, not free-living in the pore water. Even though the abundance of the more coarse particles were much higher, the bacteria were associated with small particles which might be explained by the large surface area of these. The activity measurements showed that at least some of the adsorbed bacteria were active and were not resting or inactivated at the particle surfaces.

No paper submitted.
Q and A

*R. Jones:* How were your measurements standardized, according to the gram's dry weight or gram's wet weight? Particle size was increasing and you had a decreasing surface-to-volume ratio; therefore, I just wondered if the decrease in colony forming units, or the uptake of acetate, might be a reflection of this surface-to-volume ratio rather than the actual decrease in the number of bacteria that were associated with those particles.

*Hans-Jorgen Albrechtsen:* I am not sure I understand the question.

*R. Jones:* If one is standardizing according to gram dry weight of material and larger particles are obtained, then one will have a smaller surface-to-weight ratio. Therefore, if you are standardizing according to the weight, I just wondered if there may actually be the same number of bacteria per square centimeter of particle material on the larger particle. Could that tailing off on the 50 and 500 micrometer particle be a function of surface-to-volume ratio?

*Hans-Jorgen Albrechtsen:* I think it is a function of the surface area. We are planning to make an accurate measurement of the total internal surface in the sediments, and to get a closer answer to your question. However, all the counting of the microbacteria was normalized to that amount of volume.

*R. Harvey:* What effects did your fractionation procedure have upon the plateability of the samples?

*Hans-Jorgen Albrechtsen:* It is hard to check that one. However, another bacteria per gram dry weight in the different fractions was compared to the original sediments which were not fractionated. A much higher amount of bacteria in the fractionated sediments was obtained. If one makes some sort of recovery by adding all the fractions and bacteria together, one will obtain between 30-90% recovery.
Microaerophilic Bacteria from Subsurface Sediments

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Abstract

The hypothesis that deep subsurface sediments provide habitats which favor the growth of microaerophilic bacteria was tested. Bacterial isolates obtained from oligotrophic plate counts and MPN procedures on DSMS (Dilute Substrate-Mineral Salts) medium were screened using surface soil and subsurface sediments from 406, 416 and 463 m depths from the C10 site of the Department of Energy's Deep Subsurface Science program. Bacteria were isolated from enumeration experiments initiated on-site and from laboratory experiments using 463 m depth sediments. A semi-solid medium (DSMS medium with 0.15% purified agar) was used to screen for microaerophiles, which produced a characteristic turbid cell band. The isolated bacteria tolerated atmospheric oxygen, but grew faster at 1-10% oxygen, and some isolates were more sensitive to oxygen partial pressure than others. On aerobic agar plates, these microaerophiles grew as translucent spreading colonies on and beneath the agar surface. Colony diameter exceeded one centimeter and visual detection of colonies relied upon the light scattering characteristics of the biofilm. Most of the isolates were short Gram-negative rods or coccobacilli, and demonstrated rapid motility. Microaerophilic bacteria were isolated from the three deep formations examined and accounted for greater than 10% of the bacteria from the 463 m depth. The motility characteristics of microae rophiles may promote colonization of subsurface habitats. Microaerophiles were also detected in subsurface soil from the 31 m depth of the coastal plain in Virginia.
Introduction

Microaerophiles are organisms for which oxygen is both beneficial and deleterious. In this study, microaerophiles are defined as organisms with the following characteristics: capable of oxygen-dependent growth and able to use oxygen as a terminal electron acceptor; they are incapable of growth under strict anaerobic conditions with the exception of anaerobic respiration; and, they grow optimally in oxygen partial pressures of less than one atmosphere but may grow poorly in a 21% oxygen atmosphere. The preference of these organisms for low oxygen concentrations as an electron acceptor is what distinguishes them from oxygen-tolerant anaerobes or facultative anaerobes. The use of a semi-solid agar assay to screen these organisms from deep subsurface sediments was a conservative test because some microorganisms may meet the above definition but lack the ability to form the characteristic band of cells in semi-solid agar.

Some of the microaerophiles which have been described include: Spirillum volutans, Gallionella, and many methane-oxidizing bacteria. Beggiaota, and Aquaspirillum magnetotacticum, are examples of microaerophiles which use chemotactic responses to optimize habitat position. Ferrara-Guerrero and Bianchi recently reported that microaerophiles were a significant part of the bacterial population at the interface of a water-sediment marine system.

The use of controlled drilling technology employed in the Microbiology of the Deep Subsurface Program at the Savannah River Site permitted microbiologists to examine deep subsurface sediments. Some of the characteristics of deep subsurface sediments were unexpected (e.g., the generally oxidative redox state of the habitat), and the microbial diversity and biomass of some aquifers was closer to that of typical surface soils than abiotic sediments. It was hypothesized that evolutionary processes in the deep subsurface favored the survival of organisms which had an efficient aerobic metabolism in an oligotrophic habitat and conservatively managed to metabolize a scarce supply of oxygen. Given the sedimentary history of the subsurface formations examined in this study, microaerophilic microorganisms were a possible product of this process. This study is the first report of microaerophiles in the deep subsurface. An abstract of their preliminary work was published.

Methods

Techniques used to obtain soils from the Allendale site of the Microbiology of the Deep Subsurface Program are described elsewhere in this volume. Procedures used to obtain soil from the 31 m depth of the coastal plain at Dunfries, Virginia have been previously reported.

Culture Isolation from Subsurface Soil. The spread-plate technique used by Balkwill et al. to isolate bacteria from Savannah River subsurface sediments was used in this study, except the isolation medium contained a lower nutrient concentration. A DSMS (Dilute Substrate-Mineral Salts) medium was used because the substrate concentration more closely resembled that of many pristine subsurface habitats. The DSMS medium contained: NaNO₃, 9 mg; NH₄Cl, 55 mg; MgSO₄·7H₂O, 50 mg; Na₂HPO₄·12H₂O, 180 mg; KH₂PO₄, 68 mg; CaCl₂·2H₂O, 5 mg; MOPS buffer (Sigma), 100 mg; micronutrient solution, 3 ml; vitamin solution,
1 ml; peptone (Difco), 10 mg; trypticase (Difco), 10 mg; yeast extract (Difco), 10 mg; glucose (Difco), 10 mg; and distilled water, 1000 ml. The pH of the medium was 6.5 after autoclaving. Fifteen grams per liter of noble agar (Difco) was used for routine plates while semi-solid agar DSMS medium was prepared by adding 1.5 g/l of noble agar.

The trace element solution contained: nitritotriacetic acid, 1.5 g; FeSO₄·7H₂O, 100 mg; MnCl₂·4H₂O, 100 mg; CoCl₂·6H₂O, 170 mg; CaCl₂·2H₂O, 100 mg; ZnCl₂, 10 mg; CuCl₂·2H₂O, 2 mg; H₃BO₃, 10 mg; Na molybdate, 10 mg; NaCl, 1000 mg; Na₂SeO₃, 17 mg; NiCl₂·6H₂O, 26 mg; Na tungstate, 29 mg; and distilled water, 1000 ml. The nitritotriacetic acid was added to 200 ml of water and the pH adjusted to 6.5 with KOH. This solution was added to 600 ml of water and each component dissolved in order. The final volume was adjusted to 1000 ml. The vitamin solution contained: biotin, 20 mg; folic acid, 20 mg; pyridoxine HCl, 100 mg; thiamine HCl, 50 mg; riboflavin, 50 mg; nicotinic acid, 50 mg; pantothenic acid, 50 mg; cyanocobalamin, 1 mg; PABA, 50 mg; lipoic acid, 50 mg; and distilled water, 1000 ml. The solution was stored under nitrogen in a cold room.

DSMS medium spread plates inoculated with subsurface soil were incubated for two weeks at 25°C and colony growth was checked daily. The plates were incubated as long as three months before discarding. Bacteria from the Dunfries, VA subsurface sediments were isolated on soil extract agar (e.g., 100 ml autoclaved subsurface soil extract, 900 ml distilled water, and 15 g agar). The spread-plate procedure was used. The plates were incubated in air for a minimum of two weeks at 25°C.

Virginia groundwater samples were obtained by aseptic procedures and spread plated on nutrient agar (Difco). The plates were incubated in air at 28°C for one week.

Isolation of Microaerophilic Bacteria. Microaerophilic bacteria from deep subsurface sediments were isolated by two techniques: bacteria growth on DSMS medium spread plates of subsurface sediments and isolation from turbid MPN (Most Probable Number) tubes of DSMS broth medium after five months of incubation at 25°C. The MPN tubes and the spread plates were inoculated on-site immediately after removal from the bore hole. Stored subsurface sediment samples were used to repeat the spread plate procedure in the laboratory. Some subsurface samples exhibited less than a 10% loss of microbial colony forming units after one year of storage.

The stab-culture technique was used throughout to inoculate 15 ml of DSMS semi-solid medium in regular screw-cap culture tubes. A thin disc of growth was visible halfway between the top and bottom of the medium, and parallel to the surface of the medium after 24-48 hours of incubation at 25°C. The disc of cells moved through the agar (about 0.5 cm/day) and became dispersed when it reached the bottom. The cultures were viable for weeks after the disc dispersed. The movement of the cell disc may be a scavenging response to substrate levels because the cell disc had a greater density and remained near the top of the medium when the substrate
concentration of the DSMS medium was increased. Some isolates required a longer incubation time to form a disc. The cultures were streaked to purity on hard agar plates of DSMS medium. Other complex media were used to ascertain culture purity. Facultative cultures grew the entire length of the stab, whereas strict aerobes grew near the surface.

Microaerophiles from the Virginia subsurface soils were isolated by streaking and transferring cultures from the soil extract medium spread plates into semi-solid nutrient broth (Difco-10% concentration) culture tubes with 0.2% agar. The method of Ghiorse and Balkwill,6 was used for the acridine orange direct bacterial counts.

Microaerophiles from the Virginia groundwater samples were isolated by transferring each colony type from the nutrient agar plates to semi-solid nutrient broth with 0.2% agar.

**Results**

Motile microaerophiles were detected by their characteristic thin-spreader morphology on the DSMS spread plates after incubation in air. These colonies were several centimeters in diameter 4-6 days after inoculation, and spread completely over a moist plate after 10 or more days of incubation. These translucent colonies had a hydrocarbon-like surface sheen which could be detected with careful observation of the biofilm with reflected light. The presence of bacteria in the biofilm was confirmed by Gram stains and transfer of biofilm material with sterile picks to fresh plates of DSMS medium.

The data showing the presence of microaerophiles in the turbid MPN tubes of DSMS medium are shown in Table 1. High dilution MPN tubes without turbidity were used as a negative control. These data favor the hypothesis that microaerophiles are present in the deep formations at the Allendale site as a significant part of the microflora. Only the thin translucent colonies growing on the DSMS medium were motile microaerophiles using the semi-solid agar assay. When resazurin was added to the semi-solid, DSMS agar medium, resazurin was oxidized above and reduced below the cell band.

Sediment spread plates of Cape Fear sand (463 m), which had been prepared from deep subsurface dilution water blanks at the Allendale Site, were examined for microaerophiles after five months of incubation. This sediment sample was used because the plates were not contaminated and they had not dried out during incubation. Eighteen percent of the opaque colonies on the highest dilution countable plates produced the mobile-disc response in semi-solid agar. When pure cultures were isolated from the semi-solid media culture tubes, bacteri of the same translucent colony type as in MPN tubes were observed. It was concluded that some of the opaque colonies growing on the Cape Fear sand DSMS agar plates were mixed cultures. Furthermore, the microaerophiles isolated from the MPN tubes and agar spread plates of the Cape Fear sand had similar phenotypic characteristics. The isolates were Gram-negative rods.

Microaerophilic isolation experiments were repeated with culture media inoculated at the drill site and archived samples. After the plates were inoculated,
they were observed on a daily basis and the growth of different colony types was monitored for several weeks. The data are shown in Table 2. After purifying many microaerophilic strains medium, the correlation between the spreader colony morphology and microaerophilic growth characteristic was established. The DSMS medium recovered microorganisms from subsurface sediments, but it was a difficult medium on which to differentiate colony types. When the substrate concentration was increased in the isolation medium, the spreading characteristic of the microaerophiles was decreased and their colonies were more opaque. Decreasing the amount of oxygen in the incubation environment of the DSMS medium spread plates did not increase the recovery of microaerophiles from the subsurface sediments.

Microaerophiles have been detected in the Middendorf and Cape Fear subsurface formations by independent investigations (Sinclair and Ghirose, this volume). In their study, microaerophiles were more abundant in the deep Middendorf and Cape Fear sediments than formations nearer the surface. The medium used in our study was more oligotrophic than the medium used by Sinclair and Ghirose. Otherwise, the techniques were similar.

The data in Table 3 were collected from a site on the coastal plain in Virginia. The soil at the unsaturated 3 m depth had a low quantity of bacteria as CFU/g soil and no detectable microaerophiles. Microaerophiles in the saturated subsurface soil samples were a significant part of the viable bacterial population. Facultative anaerobes and microaerophiles were the dominant physiological types at the 31 m depth. The oxygen level at the 31 m depth was less than 0.2 mg/l. The low concentration of oxygen in this nearly anaerobic subsurface sediment did not prevent the growth of microaerophilic bacteria.

A survey of microaerophiles in three groundwater samples of the coastal plain of Virginia was conducted. Fifteen of the 94 bacterial isolates which were isolated on nutrient agar were microaerophiles.

Discussion

Microaerophilic bacteria comprised a significant portion of the microflora of some deep subsurface sediments at the Allendale drill site of the DOE's Microbiology of the Deep Subsurface Program. They were also detected in Virginia well water and some shallow subsurface sediments as a significant portion of the plateable microbiota. If there were many nonmotile microaerophiles in subsurface sediments, then the assay used in this study may have underestimated the total microaerophilic population.

The niche that microaerophiles occupy in the subsurface is unknown. If their characteristic spreader qualities on agar plates and their capacity to form a motile disc in culture tubes are indicative of how these organisms respond in situ, then they may use their chemotactic potential to sense quantitative differences in the spacial distribution of oxygen and substrates in aquifers. Jannasch used chemostat techniques to show that various freshwater and marine strains of spirilla behaved as microaerophiles in heavily aerated natural water containing a growth-limiting
substrate. Ferrara-Guerrero and Bianchi observed that media containing low quantities of substrate were advantageous for the isolation of microaerophiles from sediment. In this study, oligotrophic media were useful in the isolation of motile microaerophiles. Furthermore, the microaerophiles isolated in this study could only be detected on oligotrophic semi-solid agar, otherwise, the growth pattern of these isolates in the semi-solid agar medium was similar to aerobes growing near the top of the tube.

A current theory of microaerophilic response to oxygen assumes that electron acceptor taxis does not result from sensing by a specific chemoreceptor for oxygen (or another electron acceptor), but rather is mediated by the electron transport system and by perturbation in the proton-motive force.$^{11}$ The capacity of some deep subsurface bacteria to show a sensitive chemotactic response to substrate has been demonstrated (T. Hazen et al., this volume). The chemotactic responses of deep subsurface, motile microaerophiles to oxygen in low substrate semi-solid agar culture was consistent with the proton-motive force response model. The geological formations sampled were millions of years old, with formation waters thousands of years old. Mechanisms for the survival of microorganisms in the extremely oligotrophic, low oxygen concentration, deep subsurface environments are not understood.

We thank N. R. Krieg for introducing us to the subject of microaerophiles and helping us learn some of their secrets. One of us (REB) thanks David White for his hospitality at the Institute for Applied Microbiology. We thank Andrew Arrange for his technical assistance. Portions of this work were supported by grant DE-FG05-88ER60702 from the United States Department of Energy, with Dr. Wobber serving as the program manager.
References


### Table 1. Evidence for the presence of microaerophiles in deep subsurface sediments from the DOE's Subsurface Microbiology Program. Data are expressed as the number of positive microaerophilic responses/MPN tube showing cell turbidity.

<table>
<thead>
<tr>
<th>Formation name</th>
<th>Subsurface depth (m)</th>
<th>Subsurface sediment MPN dilution $10^5$</th>
<th>$10^6$</th>
<th>$10^7$</th>
<th>$10^8$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ellenton Sand</td>
<td>213</td>
<td>_</td>
<td>_</td>
<td>1/4</td>
<td>_</td>
</tr>
<tr>
<td>Middendorf Sand</td>
<td>416</td>
<td>_</td>
<td>4/4</td>
<td>3/4</td>
<td>1/4</td>
</tr>
<tr>
<td>Cape Fear Sand</td>
<td>463</td>
<td>_</td>
<td>3/4</td>
<td>2/5</td>
<td>1/1</td>
</tr>
</tbody>
</table>

### Table 2. Presence of microaerophiles in deep subsurface sediments as measured by different colony types with special emphasis on spreader-type microaerophilic colonies.

<table>
<thead>
<tr>
<th>Formation &amp; depth</th>
<th>Log CFU/g soil</th>
<th>% Spreader-type</th>
<th>% Opaque-microaerophilic microaerophiles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Middendorf Sand</td>
<td>6.3</td>
<td>12</td>
<td>18</td>
</tr>
<tr>
<td>416 m</td>
<td></td>
<td>(19/160 colonies)</td>
<td></td>
</tr>
<tr>
<td>Cape Fear Sand</td>
<td>5.2</td>
<td>5</td>
<td>11</td>
</tr>
<tr>
<td>463 m</td>
<td></td>
<td>(11/210 colonies)</td>
<td></td>
</tr>
</tbody>
</table>
Table 3. Characteristics of subsurface sediments from a Coastal Plain Site in Virginia. The water table was located at 14 m.

<table>
<thead>
<tr>
<th>Depth (m) and soil texture</th>
<th>CFU/g soil</th>
<th>Direct count/g soil</th>
<th>Dissolved oxygen (mg/l)</th>
<th>Soil dilution where microaerophiles were detected</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 clay</td>
<td>&lt;10^3</td>
<td>5.4 x 10^6</td>
<td>ND*</td>
<td>&lt;10^3</td>
</tr>
<tr>
<td>9 sand</td>
<td>5.6 x 10^5</td>
<td>2.9 x 10^6</td>
<td>ND*</td>
<td>10^3</td>
</tr>
<tr>
<td>16 sand</td>
<td>5.2 x 10^6</td>
<td>8.0 x 10^6</td>
<td>ND*</td>
<td>10^6</td>
</tr>
<tr>
<td>31 sand</td>
<td>1.1 x 10^5</td>
<td>3.5 x 10^6</td>
<td>0.2</td>
<td>10^5</td>
</tr>
</tbody>
</table>

*ND = not determined.
J. Wiegel: Did you check for any microaerophilic nitrogen fixers?

R. Benoit: No, we did not. That is one of the things that has to be looked at. I guess we started with the premise that there would not be a lot of them down there, and thus we decided not to go that route.

J. Tedge: I was unclear as to the conditions when you obtained spreaders, both microaerophilic and low nutrient incubation conditions.

R. Benoit: The lower the concentration of the substrate, the more likely it is to be a spreader under aerated conditions. As the oxygen is increased, one is more likely to get a colony. If one increases the substrate level, one will more likely get a colony.

W. Ghiorse: With the plating medium that we use, there is 2% agar to control the spreading. Is that what Tom Phelps was using in his media? We have been aware of spreaders for quite a long time. When we take our colony off the plates, we are very careful to look for that very thin sheen of spreaders that you described. I think now that you have explained to us what those are, I will be much more excited to get them out.

R. Benoit: Because we have had to go back and forth from plates to semi-solid in several rotations, we have seen colonies that we thought were not contaminated, but were when put on the semi-solid medium. In fact, one can often get double or triple bands. In other words, one should plate right off the sediment isolation plate. One can sometimes obtain five or six bands. This alerted us to the fact that we frequently had a mixed culture. Therefore, we had to go back and do this.

W. Ghiorse: I know that some of the isolates that we have are morphologically uniform, but are quite distinct physiologically. I would not be at all surprised to find that is very true. I am not sure that very many of us who worked with a lot of isolates from these samples have actually taken that into account yet. Dave Balkwill may have a big problem on his hands.

R. Benoit: If one has not had experience with microaerophiles, then take note. The reason is that my colleague, Noel Craig, looked at this and said something is terribly wrong. If one is used to working with Campylobacter, then one knows what happens in an organism when it is inoculated; the band forms in the middle and moves up as the cells grow. With these organisms, in very dilute oxygen concentrations, one can obtain a very small band that moves to scavenge what is left. When one puts resazurin in the medium as a redox indicator, there is oxygen above the band. We have not mentioned the oxygen that you have, but we find that when using resazurin, there is aeration above the band, but it is anaerobic below the band, and the organisms move. As the substrates are exhausted, the band disappears. If one puts substrate back, the band reappears.
The Deep Subsurface of Mars: Possible Habitat for Extant or Recently Extinct Microbial Life

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Abstract

The question of extant or recently extinct microbial life on Mars is being recommended in light of the most recent information about the planet and known subsurface microbial ecologies on Earth. Subsurface cryptic niches could conceivably support or have supported life in the past. New knowledge of the planet Mars gained from the Viking mission data has allowed us to identify the conditions that would be the most challenging to terrestrial lifeforms and would presumably have to be overcome by any Martian organisms as well. Various niches protected from the harsh Martian environment have been suggested as abodes of microbial life. Earth analogs to these niches include the bottoms of permanently ice-covered lakes in the Antarctic, within rocks of the polar deserts, deep oceanic hot vents and cold seepage areas, and perhaps most relevant for Mars, in deep aquifers to depths of several kilometers below the surface of the earth.

Adaptation of terrestrial, anaerobic organism assemblages to Mars-like conditions in laboratory-scale simulations are being investigated using a "whole microbial community" approach. Initially, concentration is primarily on sulfur-cycle bacteria. Sulfur has been suggested as the possible basis for a Mars microbiota. Knowledge gained from simulation studies will enable planning of Mars sampling strategies and instruments for future robotic and human-piloted missions to that planet.

No paper submitted.
Water Potential and Starvation Stress in Deep Subsurface Microorganisms
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Abstract

Nine intact core samples, collected aseptically from depths of 10-436 m near the Savannah River Plant in South Carolina, were tested for water potential, microbial numbers, and microbial activity. Although all samples were collected from below the water table, two samples (a Pee Dee clay from 238 m and a Middendorf clay from 324 m) showed unsaturated conditions (-2.7 and -2.1 MPa, respectively). Both of these samples had very low numbers of culturable cells, low microbial biomass (ATP assay), and low microbial activities (measured as respiration), suggesting that low matric water potentials in these strata are limiting factors to microorganisms. An Acinetobacter sp. isolated from the 324 m depth was found to maintain viability under starvation conditions in sterilized aquifer material, even when subjected to severe desiccation (-22 MPa). A Pseudomonas sp., with the ability to oxidize thiosulfate to sulfate, was isolated from the 378 m Middendorf clay sample. This organism survived nutrient deprivation reasonably well; however, the presence of thiosulfate appeared to interfere with its normal ability to maintain viability by endogenous metabolism. Cells cultured in the presence of thiosulfate did not undergo dwarfing and cell viability declines. These are two examples of indigenous subsurface microorganisms, each with different adaptations for long-term survival under conditions of desiccation and/or starvation.
Introduction

Concerns regarding the safety of groundwater supplies have fostered an intense interest in the microbiology of subsurface terrestrial environments. Early studies of relatively shallow groundwater sediments (10-30 m) clearly demonstrated that microbial life extends well below surface soil horizons.\textsuperscript{4,5,7,9,14,16,17,30} These studies have been followed more recently by far deeper probes of subsurface environments, and these have also unearthed a diverse microflora.\textsuperscript{2,3,11,13,15,24} The existence of indigenous communities of microorganisms in these groundwater sediments suggests a potential for \textit{in situ} microbiological degradation of organic pollutants in contaminated groundwater supplies. With the development of bioremediation as an eventual goal, a number of basic studies have been undertaken to determine the presence and abundance of microorganisms in deep subsurface environments and to assess their potential for survival and metabolic activity under the limiting environmental conditions of the subsurface.

As in most natural environments, the numbers of microorganisms which can be directly observed by microscopy exceed by one or more orders of magnitude those which can be quantified by culture methods (e.g., standard plate counts).\textsuperscript{7,9,14,17,24} Examples of non-culturable microorganisms include: (1) cells which are dead or dying, (2) cells which are fastidious (i.e., requiring growth factors that are not provided by standard plate count media), and (3) cells which are starving or injured. The latter two groups comprise viable nonculturable cells, which require non-culture methods for assessing their potential for metabolic activity. Given the low concentrations of organic carbon typically found in these subsurface sediments, it is to be expected that a high proportion of microorganisms are in starvation-survival mode. In addition to nutrient stress, reduced water availability may be a factor which is limiting to microbial activity, particularly in the vadose zone, but possibly in regions below the water table as well. In the present study, subsurface samples from near the Savannah River Plant (SRP) in South Carolina were characterized in terms of water potential and microbiological parameters. An effort was made to use nonculture methods which have been developed for soil microbiology, along with standard plate counts, to determine biomass, rates of \textit{in situ} metabolic activity, and the physiological status of deep subsurface microorganisms. Desiccation and starvation responses of individual isolates from the Middendorf formation were also examined.

\textbf{Materials and Methods}

\textit{Sample collection.} Subsurface sediment samples were collected aseptically from borehole C10 as part of the United States Department of Energy's Deep Subsurface Microbiology Program. The C10 site is located approximately 40 km east of the Savannah River Plant. Samples were collected from August through October 1988, from depths of 10-436 m. The aseptic sampling technique consisted of a subcoring procedure described by Phelps et al.\textsuperscript{23} Portions of the intact subcores were sent by overnight express to New Mexico Tech for immediate microbiological testing.

\textit{Sample characterization.} Moisture content was determined gravimetrically after 24 hours of desiccation at 105°C. Water potentials were measured by thermocouple psychrometry (Decagon Devices, model SC-10, Pullman, WA).
**Standard plate counts.** Initial ten-fold dilutions were made in 0.1% Na₄P₂O₇·10H₂O (pH 7.0). Further serial 10-fold dilutions were made in phosphate-buffered saline. Diluted sediment suspensions were spread plated in triplicate onto PTYG and 1%PTYG agar. Colonies were counted after eight days of incubation at 22°C.

**Basal respiration.** One-gram (wet weight) sediment samples were placed in 8-ml serum vials, sealed, and incubated at 22°C. Carbon dioxide was measured by gas chromatography immediately after sealing and after 24 hours. Immediately prior to gas sampling, the vials were vortexed for five seconds to mix headspace and sediment gases. Three replicates of each sediment sample were tested. Controls poisoned with 0.5% NaN₃ were used to quantify abiotic CO₂ production and the resulting values were subtracted from those of the live treatments.

**Substrate-induced respiration.** Biomass carbon was estimated by the SIR assay as modified by West and Sparling. One-gram (wet weight) sediment samples, along with 30 mg glucose, were placed in 8-ml serum vials, sealed, and incubated at 22°C. Carbon dioxide in the headspace was measured by gas chromatography at 0.5 and 2.5 hours after the vials were sealed. Immediately prior to gas sampling, the vials were vortexed for five seconds to mix headspace and sediment gases. Biomass was calculated using the following relationship:

\[ y = 40.04x + 0.37 \]

where \( y = \text{biomass C (μg 100/gdw)} \) and \( x = \text{respiration rate (ml CO₂ 100/g of sediment (h⁻¹))} \). Five replicates of each sample were tested.

**ATP and adenylate energy charge.** Adenylates were extracted by a modification of the method of Vaden et al. A 10-ml solution of extractant containing 2 N H₃PO₄, 0.02 M ethylenediamine tetraacetic acid (EDTA), 2 M urea, and 0.24 M dimethylsulfoxide (DMSO) was added to one-gram (wet weight) sediment samples. The samples were sonicated on ice for five minutes at a constant output of 75 watts, centrifuged at 12,100 x g for 10 minutes at 5°C, and decanted. The resulting extract was stored at -80°C prior to analysis. The efficiency of extraction was determined by amending the extractant to contain 0.5 μM each of ATP, ADP, and AMP (Sigma Chemical Co., St Louis, MO). The amended extractant (10 ml) was added to a one-gram sample of the same sediment and sonicated and centrifuged as described above. Adenosine triphosphate standards were made up in the extractant, and the plain extractant served as the blank. Prior to analysis, the pH of standards, blanks, and sediment extracts were adjusted to 7.70 - 7.90 with 0.1 M tricine buffer (pH 10.0) and 5 M ethanolamine. Adenylates were measured as ATP by a luciferin-luciferase assay. Adenosine triphosphate was measured directly. Adenosine diphosphate and AMP were measured as ATP following enzymatic conversion as described by Martens. Adenylate energy charge (AEC) was calculated according to the formula: AEC = (([ATP] + 1/2[ADP])/([ATP] + [ADP] + [AMP]).

**Isolation and characterization of a starvation-tolerant microorganism.** Although initial plate counts yielded no viable microorganisms from the 324 m depth sample in the Middendorf formation, plate counts performed using 1%PTYG agar after several weeks storage of the core material at 5°C showed the presence of 1.9 x 10² Colony Forming Units (CFU)/g of a single colony type. One colony was selected and
identified by morphological and biochemical tests. This isolate was tested for its ability to survive in the core material under conditions of nutrient limitation and desiccation. Core material (G-14) was crushed, mixed, placed in 1.0 g aliquots in 8-ml serum vials, and sterilized by Tyndallization. The success of the Tyndallization was tested by plating onto 1%PTYG agar. The isolate was cultured in 1%PTYG broth at room temperature on a shaker (200 rpm). When the culture reached a late logarithmic stage of growth, cells were centrifuged (5,000 x g, 10 minutes) and resuspended in a sterile starvation medium (inorganic constituents only from 1%PTYG). This cell suspension, containing 9.6 x 10^6 cells/ml, was then added in 300 µl aliquots to each serum vial. Sediments were mixed in the vials on a vortex mixer, then subjected to varying degrees of desiccation by exposing them for varying lengths of time to the flow of air in front of a HEPA filter in a sterile transfer hood (Environmental Air Control, model TT-4830, Hagerstown, MD). After varying periods of desiccation, the vials were sealed with sterile septa and incubated at 22°C. Numbers of surviving cells were quantified immediately, and after 13, 26, and 53 days by standard plate counts on 1%PTYG agar as described above. Final water potentials and water contents of the desiccated samples were measured as described above after 53 days of incubation.

Isolation and characterization of a heterotrophic thiosulfate-oxidizer. A bacterial strain isolated from the Middendorf formation (378 m) on ATCC Medium 290, containing thiosulfate and solidified with 15 g/l agar, was characterized by standard morphological and biochemical tests and by the use of a Biolog GN plate (Biolog, Inc., Hayward, CA).

This organism was tested for thiosulfate oxidation in medium 290 and amended with 0.10 g of glucose, 0.1 g of yeast extract, 0.05 g of peptone, and 0.05 g of tryptone per liter. The medium was inoculated with a starter culture which had been incubated for four days in the same medium on an orbital shaker (200 rpm) at 26°C. Cultures were incubated at 26°C and shaken at 200 rpm. Concentrations of sulfate were measured by barium sulfate precipitation; thiosulfate, tetrationate, and trithionate concentrations were measured by a cyanolysis method. Cell numbers and cell sizes were monitored by phase contrast microscopy.

Results

Physical and biological characterization of core samples. The majority of the core samples showed saturated conditions; however, two samples (G-10 and G-14) had low water potentials (-2.70 and -2.07, respectively) indicative of unsaturated conditions (Table 1). Standard plate counts yielded no viable cells from four of the nine samples; the others had values ranging from 5.0 x 10^2 to 4.0 x 10^4 CFU/g. Respiration rates ranged from below detection (actually appearing to be negative due to slightly higher CO₂ production in the poisoned control) to 1.08 µg CO₂/gw/h. Microbial biomass was detected by the SIR method in eight of the nine samples, whereas ATP was detected in only five samples. Five of the eight samples for which the AEC could be calculated had values below 0.5. Respiration rates were
correlated with ATP values \( r = 0.77 \) and adenylate energy charge values \( r = 0.86 \).

**Starvation/desiccation survival.** The strain isolated from sample G-14 was identified as an *Acinetobacter* based on its being a Gram-negative, strictly aerobic, oxidase negative, catalase positive, nonmotile rod. It proved to be remarkably tolerant of nutrient deprivation, even under conditions of severe desiccation (-21.9 MPa) (Figure 1). Only those exposed to the most severe desiccations (-7.7 and -21.9 MPa) declined in numbers and even these declined by a factor of less than two during 53 days incubation.

**Heterotrophic sulfur oxidizer.** The bacterium isolated from sample G-15 on a thiosulfate-containing medium was identified as a *Pseudomonas* sp. based on its being a Gram negative, strictly respiratory, oxidase positive, catalase positive, motile rod. It was identified as *P. testosteroni* by the Biolog test. This identification was supported by the finding that the isolate could degrade testosterone as a sole carbon source in minimal medium. In thiosulfate-containing growth medium amended with organic substrates, the isolate was shown to oxidize thiosulfate slowly to sulfate, with the production of tetramionate and trithionate as intermediates (Figure 2a). Growth and thiosulfate oxidation in medium containing only thiosulfate was very poor. Oxidation of thiosulfate did not appear to enhance the growth rate of *P. testosteroni* in organic media; generation times were approximately 0.5 hours in both media (Figure 3). However, the presence of thiosulfate in the growth medium had a profound effect on cell size (Figure 2b) and viability. Cells grown in a heterotrophic medium underwent dwarfing beginning in the stationary phase of growth, whereas cells cultured with thiosulfate grew to an average length of 3.3 μm and width of 1.5 μm after 25 hours and diminished in size only slightly after prolonged incubation. Long-term survival of cells cultured with thiosulfate was much lower than those cultured in plain organic medium. Percent viability, calculated as \((\text{viable counts/direct counts}) \times 100\), declined steadily to 0.6% during 31 days incubation with thiosulfate, while 77.5% of cells cultured without thiosulfate remained viable after the same incubation period.

**Discussion**

The application of assays borrowed from soil microbiology has the advantage that the methods are intended for microbial populations which are frequently in a relatively inactive state. Also, as in soil systems, many subsurface microbial populations are undoubtedly forced to subsist on diets of recalcitrant polymeric compounds such as humic and fulvic acids. The respiration- and adenylate-based assays used in this study are appropriate for the viable nonculturable cells which comprise large proportions of the microbial communities in soils and in subsurface sediments. The disadvantage of these methods for subsurface applications lies in their sensitivity. Biomass estimates and rates of activity of microorganisms in subsurface environments are typically several orders of magnitude lower than those in surface soils, and so they are often below detection. Nonetheless, the values obtained here provide some insights into life in the deep subsurface.

Adenosine triphosphate concentrations have been shown to be good predictors
of contaminant biodegradation rates in shallow aquifer material. In the SRP samples, AEC was even more strongly correlated than ATP with overall microbial respiration rates, suggesting that AEC may be a more accurate predictor than ATP of potential for contaminant biodegradation.

Ratios of microbial biomass carbon to total organic carbon (TOC) have been used in soil studies as indicators of the degree of development of microbial communities, with high values corresponding to relatively "new" or recently disturbed communities, and low values occurring in later successional stages. These ratios also reflect the forms of available carbon, in that "older" microbial communities are coincident with higher proportions of stabilized, recalcitrant compounds, such as humic and fulvic acids. Total organic carbon measurements were available for four of the nine sediment samples received, and of these four, three had biomass levels which were detectable by the SIR method. The TOC's for samples G-5, G-6, and G-20 were 0.52, 2.2, and 11.0 mg of carbon/gdw, respectively. Combining TOC's with the SIR biomass values as ratios yields values of 11.0, 5.18, and 3.68 mg biomass-C/ mg organic carbon for the three samples. These ratios are considerably lower than those typically found in surface soils and, therefore, are in agreement with what we already know or can surmise about these communities (i.e., that they are extremely "old," stable, and surviving amid predominantly stable, recalcitrant organic carbon).

The biological characterization of these deep subsurface samples indicates relatively inactive, severely nutrient-stressed microbial communities, a conclusion which is consistent with the data of other subsurface investigations. Most samples showed some biological activity, as evidenced by the respiration-based biomass and activity assays; however, not all of these samples showed viable cells in the standard plate count assays. This suggests the presence of viable non-culturable cells which may be starved, injured, or nutritionally fastidious. As an example, cells could not be cultured from sample G-14 in the upper Middendorf formation until after a prolonged period of storage at 5°C, a step which may resuscitate starving or injured cells. Indications of highly stressed cells in this upper Middendorf region have also been found in the phospholipid analyses. The AEC values for most of the strata suggest inactive and physiologically stressed cells. Adenylate energy charge values in the range of 0.50-0.75 are considered to represent dormant or inactive cells; values below 0.4 are thought to indicate dead or dying cells. In at least two of the samples from the Pee Dee and Middendorf formations, desiccation may have contributed additional stress to microorganisms. Desiccation in porous media inhibits microbial metabolism directly by loss of water and indirectly by the reduction of solute diffusion.

The finding of a starvation-tolerant Acinetobacter in the deep subsurface is perhaps not surprising since members of this genus are nearly ubiquitous in other natural environments, including soil and aquatic environments. Acinetobacters are found in high concentrations in groundwaters; they are common isolates from the SRP boreholes; and they have been found in subterranean volcanic tuff at the
Nevada Test Site (Penny Amy, personal communication). The extraordinary starvation tolerance as well as desiccation tolerance of this bacterium is reminiscent of the frequently studied soil bacteria of the genus Arthrobacter. In fact, Acinetobacter might be considered a Gram-negative subsurface counterpart of soil arthrobacters. The actual physiological basis of this starvation tolerance has yet to be studied, but one may assume that it includes a limiting of the rates of respiration and endogenous metabolism as has been found in Arthrobacter crystallopoietes and numerous other starvation-tolerant bacteria.10

Conclusion

Evidence of heterotrophic sulfur oxidation has been found by other deep subsurface investigators (J. K. Fredrickson, personal communication). Thiosulfate oxidation by strictly heterotrophic bacteria has been found in bacteria from soil and marine habitats and has been the subject of sporadic study for decades.22,25-27 The selective advantage, if it exists for this phenomenon, has remained a mystery. Most investigators have failed to demonstrate an increased growth rate when thiosulfate is present. One might hypothesize an advantage in long-term survival during organic nutrient deprivation. However, the data of the present study fail to support this hypothesis and, in fact, indicate a distinct disadvantage of thiosulfate oxidation in this particular strain of P. testosteroni. Thiosulfate appears to interfere with the normal dwarfing response in this isolate. This is perplexing in that thiosulfate is actually present in the porewater of the Middendorf formation; and so presumably these microorganisms are similarly disadvantaged in their natural environments. We have also tested the starvation/desiccation responses of this P. testosteroni isolate by the system described above for Acinetobacter in this system, P. testosteroni remains viable only under moist conditions (data not shown).

Starvation survival appears to be a requisite trait for existence in subsurface systems. Desiccation tolerance may be advantageous in selected groundwater microhabitats, and it may also indicate that at least some groundwater microorganisms are derived from more water-stressed environments such as surface soils and unsaturated subsolos.

Acknowledgements

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References


Table 1. Characteristics of subsurface samples from Borehole C10 near the Savannah River Plant in South Carolina.
Figure 1. Starvation survival of an *Acinetobacter* sp. inoculated into sterilized Middendorf clay from SRP and incubated under varied degrees of desiccation stress.
Figure 2. Growth of *Pseudomonas testosterone* isolated from a Middendorf clay. (a) Concentrations of thiosulfate and its oxidation products in the polythionate pathway in an organic medium amended with thiosulfate. (b) Average cell sizes when cultured in organic broth with and without thiosulfate amendment.
Figure 3. Growth of *Pseudomonas testosteroni* (direct microscopic counts) cultured in organic broth with and without thiosulfate amendment.
The Potential of Gas Metabolism in the Sediments of Eutrophic and Acidified Lakes. Application in the Treatment of Anaerobic System

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Abstract

Extremely different metabolic types were revealed during the comparative studies on the methanogenic metabolism in sediments of eutrophic and acidified lakes. In the eutrophic Plussee lake, the gas bubble analysis revealed minimal CO₂ content in contrast to the 60:40 ratio of CH₄:CO₂ in the genuine gases produced in the sediments. This indicated that the CO₂ formed in the sediments was not leaving the lake via the bubbles but was involved in equilibrium and conversion reactions. In Tonteich, with no gas ebullition, there was a shift from a balanced CH₄:CO₂ production to almost only CO₂ formation, CH₄ being negligible. This deviation from "standard" conditions was a reflection of the situation arising from the acidification of the water. In the acidified lake, the water was devoid of a carbonate/bicarbonate system and the photosynthetic maximum of the almost predominating Dinoflagellate, *Peridinium pusillum*, was shifted downwards at the sediment/water interface. The CO₂ needed for photosynthesis was fluxed upwards directly from the sediments as no CO₂ was diffusing from the atmosphere into the water column.

The methanogenic activities could be initiated by amending some methane precursors in both sediment types which would show, however, different precursor preferences. The principal of stimulating methanogenesis by amending organic additives has found its successful application in the treatment of anaerobic systems like sludge and waste water, landfills, manure, etc. The influence was not a mere synergism, but a real acceleration and degradation of recalcitrant organics which otherwise would pass undegraded using known conventional treatment techniques.
Introduction

The metabolic regulations in sediments of aquatic ecosystems are mostly oriented to changes in the environmental conditions and reflected in the end products. The present comparative investigations on the gas sediments metabolically produced in the eutrophic lake and acidified pond illustrated such regulative mechanisms. The fate of the produced gases in the sediments was defined in their solubility in the interstitial waters and, in case of super-saturation, in the formation of gas bubbles. The bubbles were partly trapped in sediment "pockets", partly released by ebullition into the atmosphere, and partly fluxed by diffusion into the water column. The dissolved gases were subjected to equilibrium and conversion reactions (e.g., in case of CO₂: carbonate/bicarbonate system).

The present studies are concentrated on two sediment gases: CH₄ and CO₂ in an eutrophic lake (Plussee lake) and an acidified pond "Tonteich". The gases were analyzed in collected gas bubbles, in sediment cores, and from incubation experiments. Plussee lake lies in the vicinity of the Max-Planck-Institute in Ploen and is one of the best investigated lakes in Europe. The lake is a humus-forest lake, has a surface area of 14.3 hectares and has a remarkable funnel-shaped bottom topography. The nearest acidic water body is "Tonteich", a pond in the neighborhood of Hamburg in northern Germany. The present pond is a water-filled clay-dredged depression. It has an area of 1.5 hectares and has a maximum depth of 3.2 m. The acidity of the water, which had a pH between 3.3 and 3.8 during the investigation period (1985/86), was due to the formation of free sulfuric acid resulting from the oxidation of the pyrite in the mica clay and from the hydrolysis of iron sulfate. The water was consequently free of carbonate and bicarbonate. The pond was also fish-free and it showed a plankton bloom of the dinoflagellate, Peridinium pusillum, dependent in its density upon the presence of the predator rotifer, Brachionus urceolaris f. sericus. The photosynthetic intensities showed a peculiar pattern in that they increased with depth, reaching a maximum of 200 mg C/m³/d in the surface sediments compared to 50 mg in the surface waters.

In view of the antagonistic characteristics of the water of these two aquatic ecosystems, the "gas metabolism" was correspondingly adjusted and reflected two different metabolic patterns. Moreover, the mechanisms found and the manipulations adopted in the context of mobilizing the methanogenic potential in the sediments were principally applicable in the treatment of anaerobic systems exemplified in the treatment of sludge.

Materials and Methods

Sampling Sites, Coring and Processing. Triple SCUBA-diver cores were obtained from the eutrophic Plussee lake at its deepest point from the middle of the "funnel," and from the acidic Tonteich at two sampling sites. It should be mentioned that manual coring produced intact cores, while gravity cores, especially in the organic-rich Plussee lake, became ruptured due to violent gas escape.

pH and Redox Potential. One of the cores was used directly for pH and redox potential measurements, using micro electrodes and computerized meters. Measurements were taken every 0.5 cm, beginning downwards from the overlying water. The electrodes were thoroughly washed and blotted after each measurement.
The other two cores were processed in the laboratory using special techniques for the estimation of the vertical distribution of genuine gases and for incubation experiments.

*Vertical Profiles of Gases.* Isolated sediment samples from the second cores were degassed and equilibrated by vigorous agitation with helium in the head spaces of the used syringes. Gases were then withdrawn from the head space by 100 µl, Hamilton gas-tight syringes for analysis with gas chromatography.\(^{11}\)

*Incubation Experiments.* The third core was processed likewise for inoculation and incubation experiments. The sliced sediment samples were introduced in prepared standard vials, flushed with helium, and sealed with gas-tight screw seals. Inoculations were carried out by injections. The vials were arranged in special boxes, flushed with helium, and tightly covered with silicon and rubber sheets. The boxes were incubated at 28°C. Gas analyses were carried out periodically by direct withdrawal of samples with 100 µl, gas-tight syringes from the head spaces of the vials through the rubber seal without exposing the individual vials. No attempt was undertaken to correct for the prevailing pH.

The inocula used were some chosen methane precursors (i.e., acetate and methanol) in end concentrations of 50 mM, and CO\(_2\)/H\(_2\) (2:3 v/v) in the presence and absence of 1% peptone solution. The influence of protein hydrolysates on the stimulation of the methanogenic conversion of the precursors has been elucidated in previous works.\(^{8,10,12}\)

*Gas Bubble Collection.* Gas bubbles were collected in inverted funnels approximately one meter in diameter and installed at different depths in the water column of the Plussee lake. No gas ebullition was ever observed from the Tonteich. Each funnel was supplied with a 2l-gas sampling cylinder for quantitative measurements of the ebullition. Collected gases were analyzed after equilibration with atmospheric pressure.

*Gas and Chemical Analyses.* Gas and chemical analyses were performed as described in previous investigations.\(^{11}\)

**Results**

*pH and Redox Potential.* The pH of the sediments of Plussee lake (approximately 6.5) did not differ widely from that of the overlying water. The sediments were reduced with a redox potential of −280 mV in the upper layers and gradually decreased downwards. The pH of the sediments of Tonteich was subjected to acidic overlying waters with an approximate pH level of three. However, the pH was rapidly neutralized in the upper 4 cm of the sediments to a level of six. The redox profile is almost a mirror image of the pH curve, becoming negative in the upper 3 cm. pH redox and vertical profiles in the Tonteich are illustrated in Figure 1.

The physico chemical conditions were thus favorable for methanogenesis, assuming that the basic requirements of the degradation chain were not functionally interrupted or limited.

*Vertical Profiles of CH\(_4\) and CO\(_2\).* The genuine distribution of CH\(_4\) and CO\(_2\) in vertical profiles of the sediments of the Plussee lake and the Tonteich are shown in
Figure 2 and Figure 3, respectively. It is clear that the interstitial CH₄ and CO₂ had a balanced average ratio of 60:40 in the Plussee sediments. In the sediments of the Tonteich, on the other hand, the CH₄ part was almost negligible and the average ratio for CH₄:CO₂ was approximately 95:5.

Gas Bubble Analysis. The gas bubbles that were collected were analyzed for CH₄, CO₂ and N₂, and are shown in Figure 4. The major constituent is represented by CH₄, whereas CO₂ is minimal.

Incubation Experiments. The maximal CH₄ and CO₂ production from the amended sediments in the upper and lower 5 cm of two representative cores from the Plussee lake and the Tonteich are represented in the column diagrams (Figure 5 and Figure 6). From this, the following was clear:

1. The methanogenic activities could be induced in different magnitudes in both types of sediments, especially in the surface sediments which were the most active metabolic sites.

2. The CH₄ and CO₂ showed a balanced ratio of approximately 60:40 in the eutrophic Plussee sediments. This ratio was strongly shifted to the CO₂ production in the acidic Tonteich.

3. The precursor preference in the Plussee sediments was in the following order: methanol, acetate, and CO₂/H₂, with methanogenesis being more intensive in the presence of peptone. In the Tonteich sediments, the precursor preference lay in the reduction of CO₂, especially clearer in the second 5 cm of the investigated core.

Discussion

In the acidified Tonteich, the acidity of the overlying water was rapidly neutralized in the upper 4-5 cm. The redox vertical profiles revealed that reduced conditions were already manifested in the upper 4 cm downwards. The prevailing physical conditions would apparently favor methanogenesis in subsurface layers. Similar conditions have been reported for other acidic aquatic ecosystems. The observed neutralization has been attributed to bacterial sulfate reduction.

Organic matter decomposition rates in Lake 223 sediments appeared unaffected by lake acidification. However, concern has been expressed that lake acidification will slow down the recycling processes in sediments, leading to extreme cases of oligotrophication. The reasons for the pH neutralization in Tonteich was not examined and needs further investigation. As for the eutrophic Plussee lake, conditions were favorable for sediment gas metabolism, which in fact, was functioning with different intensities depending upon the seasonal fermentative autochthonous and allochthonous input. This was reflected in the gas ebullition symptomatic for eutrophic sediments, but not for oligotrophic sediments.

The genuine distribution of gas sediments in vertical profiles, analyzed in vertical cores, revealed a balanced CH₄:CO₂ ratio of 60:40 in the eutrophic sediments. In the sediments of the acidified pond, however, this ratio was totally distorted to 5:95. The same trend was found in the gases from the incubation experiments. The reasons for this phenomena are speculative. Environmental adaptation including changes in the substrate uptake kinetics, have been recognized among aquatic bacteria.
The collected gas bubbles from the Plussee lake resulted in a 77:2% ratio of CH₄ to CO₂, which was totally different from the genuine gases produced. This is a clear indication that the CO₂ that was formed in the sediments was not leaving the lake via ebullition, but was involved in equilibrium and transformation reactions.

In both sediment types, the methanogenic potential could be mobilized by amending some methane precursors (e.g., acetate, methanol, CO₂/H₂, etc.). Apart from the different metabolic intensities, a clear methane precursor preference was visible and different in both sediment types. Obviously, this was dependent upon the nature of the available organic carbon rather than on its quantity. The precursor preference was methanol in the case of the Plussee sediments and CO₂/H₂ in case of the Tonteich. Methanogenic methanol preference was manifested in sediments with a considerable content of pectins. The degradation of pectins, in which galacturonic acid was esterified with methanol, lead to the liberation of methanol and the enrichment of the corresponding microorganisms.

One of the interesting phenomena was the shift of the CH₄:CO₂ ratio in the acidic Tonteich towards the formation of relatively higher CO₂, with CH₄ being negligible even though the methanogenic potential was present and could be stimulated. The produced CO₂ was made available to directly feed the deeper-located, photosynthetic center in the sediment/water interface. Moreover, an intensive heterotrophic CO₂ fixation (e.g., PEP-carboxylation) was manifested to ensure an optimal utilization of produced CO₂. These were obviously reflections of changes encountered in the environmental conditions, as illustrated in the acidity of the Tonteich.

**Conclusion**

From this and other investigations, it could be postulated that the stimulation of methanogenesis is not a mere conversion of the organic additives into methane, but is rather a real acceleration and intensification of the biodegradation.

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Figure 1. pH and Redox verticle profiles. Redox in mV corrected for reference electrode (Ag/AgCl=210, 5mV at 20°C).
Figure 2. CH$_4$ and CO$_2$ verticle profile (Plussee lake, August 1987).
Figure 3. CH$_4$ and CO$_2$ verticle profile. 2 sampling sites 1 & 2 (Tonteich, Sept. 1987).
Figure 4. Analysis of funnel gasses from Plussee lake (Dec. 1988).

Figure 5. Maximal CH₄ and CO₂ produced in sediment incubations.
Figure 6. Maximal CH$_4$ and CO$_2$ produced in sediment incubations.
Activities of Microorganisms in Deep, Unconsolidated Eastern Coastal Plain Sediments

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Abstract

Activities of microorganisms residing in deep Eastern Coastal Plain sediments were examined from more than 100 sediment samples from eight boreholes. Radiolabeled time-course experiments assessing in situ microbial activities were initiated at the site within 30 minutes of core recovery. \([^{14}\text{C}-\text{C} }]\)acetate incorporation into lipids, \([\text{methyl}^{3}\text{H}-\text{H}]\)hymidine incorporation into DNA, \([^{14}\text{C}-\text{C} }]\)acetate and \([^{14}\text{C}-\text{U}-\text{U}]\)glucose mineralization, and microbial enrichment and enumeration studies were examined in surface and subsurface sediments. Surface soils contained the greatest biomass and activities followed by the shallow aquifer zones. Water-saturated sands exhibited orders of magnitude greater activity and culturable microorganisms than the low permeable dense clay zones. Subsurface sediments in association with older groundwater typically exhibited less biomass and activity. Sediments severely contaminated with trichloroethylene exhibited negligible activity and biomass, whereas water-saturated sediments beneath the plume exhibited high levels of activity. Regardless of depth, sediments which contained greater than 20% clay particles exhibited low activities and lower culturable microorganisms.
Introduction

Studies have shown a variety of microbial communities present in subsurface environments. These communities include aerobic and anaerobic heterotrophs, methanogens, and sulfate reducers. It was also reported that subsurface microorganisms could impact groundwater quality by producing carbon dioxide. Little is known about the activities of microorganisms residing in subsurface sediments. Highly contaminated subsurface sediments were examined at the Savannah River Plant (SRP) where microbial activities were detected in numerous sediments but not in a zone severely contaminated with trichloroethylene. Beneath the contaminated plume were sediments which exhibited substantial metabolic activities as demonstrated by radiolabeled acetate incorporation into lipids and thymidine incorporation into DNA. From these highly active sediments, cultures were enriched which could degrade trichloroethylene at 50 mg/l. These results suggested a significant role for subsurface microorganisms and a potential for in situ bioremediation.

Examination of the ecology, physiological potentials, in situ carbon and electron flow, growth and nutrient status, are all prerequisites to satisfactory bioremediation of subsurface environments. This study examined the activities of microorganisms using radioisotope time-course experiments initiated within minutes of sediment recovery.

Materials and Methods

Description of Experimental Site. The boreholes were located within the Upper Atlantic Coastal Plain on the Aiken Plateau adjacent to the Savannah River. Unconsolidated sediments extended to depths of 400 m and were underlain by crystalline metamorphic rock or consolidated mudstone. Geologic formations beneath the SRP are discussed elsewhere. The two major confining clays were the Pee Dee and Middendorf, through which very little water flows. Groundwater recharge occurred approximately 40 km west and northwest of SRP.

Field Studies. Boreholes were drilled with a rotary bit and continuously flushed with recirculated sodium-bentonite, viscousifying drilling fluid (Quik-gel, NL Baroid/ NL Industries, IN Houston, TX). More detailed descriptions of sampling techniques, use of conservative drilling fluid tracers, and quality assurance are published elsewhere (Phelps et al., this volume). Retrieved materials were removed from the sampler and the core liner was immediately carried into the Mobile Microbial Ecology Laboratory (MMEL). As sediments were extracted, they automatically entered a N₂ flushed glove bag (Coy, Ann Arbor MI). Within thirty minutes of sample recovery, subsurface samples were used for initiation of on-site activity measurements.

Gases, Chemicals and Isotopes. Nitrogen and N₂:CO₂ (90:10%) were greater than 99.9% pure. [¹⁴C-1]-acetate (56 mCi/mmol), [³H]-acetate (3.3 Ci/ mmol), and [³⁵S]-sulfate (481 mCi/mmol) were purchased from New England Nuclear Corp. (Boston, MA). [¹⁴C-2]-acetate (56 mCi/mmol) and [¹⁴C-U-] glucose (2.8 mCi/mmol) were obtained from Amersham (Arlington Heights, IL).
**Time Course Experiments.** Sediment aliquots were inoculated for aerobic and anaerobic activity experiments on-site within 30 minutes of core extrusion. All isotope solutions (1-50 μCi) were frozen prior to use, thawed, and transferred with gas-tight syringes (Hamilton Company, Reno, NV). Time-course experiments were performed in duplicate, using sterile polypropylene centrifuge tubes for aerobic isotope incorporation experiments and anaerobic crimp top tubes (Bellco Glass Company, Vineland, NJ) for anaerobic experiments. All incubations were at ambient temperature, which was similar to the *in situ* temperature of 20-25°C.

Acetate incorporation experiments contained 2.0-gram sediment, 5.0 μCi of radiolabeled acetate, and 1.0 ml of sterile distilled water. At $t_0$ and appropriate time points, duplicate incorporations were inhibited with 3.0 ml of a phosphate-buffered, chloroform-methanol solution. Sulfate reduction experiments utilized 2 μCi of isotopes and time points were inhibited with 0.5 ml of 2.0 M anaerobic, sodium hydroxide. Mineralization time-course experiments also contained 2.0 μCi of isotopes and were inhibited with sodium hydroxide.

**Analytical Procedures.** In the laboratory, acetate incorporation experiments were thawed and sediments extracted by a modification of the single phase, chloroform-methanol method. The lipid extraction was evaporated to dryness and portions were counted by scintillation counting to determine the amount of radioactivity incorporated into microbial lipids. The earliest time points yielding measurable results were used to calculate a linear rate which was then extrapolated to dpm/day.

Radioactive $^{14}$C-carbon dioxide and $^{14}$C-methane from mineralization and time course experiments, respectively, were examined by gas chromatography-gas proportional counting. A Packard 417 gas chromatograph (GC), equipped with a thermal conductivity detector, was connected to a Packard 894 gas proportional counter. One hour before analysis, tubes were acidified with 0.5 mL of 6 M hydrochloric acid. Radioactive sulfide, from sulfate reduction experiments, was recovered after acidification into zinc acetate and counted via scintillation counting.

**Results**

In all boreholes examined, surface samples exhibited the greatest culturable counts and activities followed by the aquifers. Lowest activities and biomass were associated with confining clay zones. Depth did not appear to determine the subsurface biomass or radiotracer incorporation rates. Instead, there were zones deep beneath the surface which exhibited large and active microbial communities. This report focuses on nutrient limitations, sulfate reduction, and the influence of geology and hydrology on microbial biomass and activities.

The results in Table 1 show the effects that nutrient additions on subsurface water-producing sands. Five μCi of carrier-free $^{3}$H-acetate was added to replicate 3-g sediment aliquots which contained 1.0 ml sterile nanopure water. The water contained either no additions, 10 μM or 500 μM of sodium nitrate, sodium phosphate, sodium sulfate, or glucose. Two additional treatments included a supplemental mineral solution or varying the water content to 0.25, 0.5, 1.0 and 2.0
ml. Treatments were tested in duplicate at t = 0, 2, 6, and 16 hours. All results were compared to the 1.0 ml water treatment, which was the amount generally used to assess anabolic activities of subsurface microorganisms. In the upper Pee Dee sands, all treatments resulted in stimulation of activities within six hours of incubation. Surprisingly, the addition of 2.0 ml of water led to stimulatory effects over the generally used 1.0 ml water. Lower Pee Dee sands showed stimulation by phosphate and water. In all sands examined, the addition of 10 μM phosphate resulted in anabolic stimulation as did the addition of 2 ml of water. In three sands, the addition of sulfate resulted in stimulation. Glucose, nitrate, and mineral additions were stimulatory in only one sand formation examined.

The data in Table 2 show the effect of nutrient stimulation for confining clay layers. As was found with the sands, the addition of water was most stimulatory followed by the addition of phosphates. These results suggested that the mixing of available nutrients was increased by water additions and vortexing, whereas glucose rarely resulted in anabolic stimulation within the 16 hour time courses. As in many freshwater environments, phosphates could be limiting in the deep subsurface even though abundant sulfate could be available.

The data in Table 3 show the differential incorporation of [3H]- and [14C]-acetate into lipids. Five μCi of isotope was added to each tube in the time-course experiments. Results show the log dpm, radiolabeled acetate incorporation into microbial lipids for sandy samples. In three of the four aquifer sands, considerably more acetate was incorporated when the lower specific activity [14C]-acetate was used. In thirteen of fifteen samples examined with both isotopes, more label was incorporated when the lower specific activity [14C]-acetate was available. For all samples there was an average six fold increase in incorporation with [14C]-acetate versus [3H]-acetate. It appeared that as specific activity of the isotope decreased, and additions of the label corresponded to μM increases in pool size. Such stimulation was noted within 16 hours of sample incubations.

Sulfate-reducing activities are shown in Table 4. Thirteen samples were examined over time-course experiments to quantify radiolabeled sulfate conversion to sulfide. Even though 1.0 μCi of carrier-free sulfate was added to each tube, no sediment exhibited greater than 26,000 cpm of sulfide production per day. Sulfate pool sizes varied markedly, but all values greater than 100 mg/Kg are considered to include sulfate contamination from drilling fluids. Waters recovered from wells rarely exceed 30 mg/kg. The fourth column in Table 4 shows an approximation of the sulfate-reducing activity. Note that values greater than 3 μmol/kg of sulfate reduction per day are viewed to be overestimates from the 10-day incubations. This overestimate occurred because clays were stimulated by the addition of water, sulfate was added from drilling fluids, or general stimulation occurred over the long time course.

Evidence suggests that particle size and moisture content may be major determinants of subsurface microbial ecology. Sediments were separated into three groups based upon sediment lithology and measures of permeability and hydraulic...
conductivities without regard to depth (Table 5). Sediment lithologies were of two major types of particle distributions, either greater than 20% clays or greater than 70% sands. Sandy samples were considered in two sub groups, unsaturated or with D<10 (which also meant that hydraulic conductivity < 200 μm/sec) or saturated with D>10 and hydraulic conductivity(K) > 200 μm/sec. Nineteen samples contained greater than 20% clays, 17 samples were unsaturated or low permeability sands, and 25 samples were water-producing sands. The data in Table 5 show the relationship between sediment particle size and moisture content with microbial activities and culturable biomass.

Clays consistently exhibited lower activities and biomass than sands regardless of depth. Culturable biomass in sands were similar regardless of permeability. Interestingly, microbial activities between the two sand groups were dramatically different. Unsaturated or low permeability sands exhibited activities similar to the clay samples, while activities from water-producing sands averaged 100 times more activity than low permeability samples. These results suggest that although many sandy sediments may contain similar populations, those with abundant water availability may exhibit far greater activities.

**Discussion**

Presence and diversity of subsurface microorganisms is well established.\(^\text{1,3,8-10}\) Investigators agree that water-producing sands exhibit greater colony forming units than do confining clays. Factors controlling activities and biomass are less understood. This work reports evidence that water may be the single most important factor controlling subsurface microbial activities.

The addition of water or nutrients led to stimulation of microorganisms residing in subsurface sediments within 16 hours of collection. Two of six sediments were stimulated by additions of glucose, suggesting that carbon may not be the most limiting factor. Physical evidence\(^\text{10}\) from lithological logs indicated that lignite and even wood was present in several of the subsurface formations, again suggesting that carbon may not be the most limiting nutrient. Mineral solutions were not stimulatory to most sediments, which agreed with the pore water chemistries that trace elements were present in subsurface sediments and pore waters. Nitrate and sulfate, which are alternative electron acceptors for respiration, led to stimulation in a couple formations, but evidence suggests that these electron acceptors are not major participants in subsurface carbon and electron flow in these sediments. Phosphate additions of 10 μM led to stimulation of microbial activities in five of six samples examined. Pore water chemistry data agreed that phosphates were below 1 mg/Kg and a likely limiting nutrient. The only sample not stimulated by phosphate was a dense clay layer in the Pee Dee formation. This formation exhibited few colony forming units and negligible activity.

The only addition which caused high levels of stimulation in all sediments examined was the addition of 2.0 ml of water. Water stimulation of clays was expected since nutrients were likely sedimented or bound in the confining layers. Water stimulation of sands was unexpected. Additions of 0.5 - 1.0 ml of water did
not result in stepwise stimulation, rather the addition of 2.0 ml water caused increased activity over the lesser water additions. Reasons for water stimulation of aquifer sands are not understood but they could be related to vigorous vortexing and mixing of existing nutrients, thereby making them more accessible to the resident microflora.

Additions of glucose did not result in widespread stimulation of microbial activities, but additions of low, specific activity $^{14}$C-acetate led to greater accumulations than did higher, specific activity $^{3}$H-acetate. Generally, additions of $\mu$M pool sizes of acetate are not considered likely to cause stimulation within hours. However, in these subsurface sediments, additions of $\mu$M concentration acetate resulted in an average six-fold increase in the accumulation of acetate into lipids.

**Conclusion**

Although previous subsurface studies reported the presence of microorganisms, it was only recently suggested that microorganisms may alter groundwater chemistry.\(^3\) Harvey et al.\(^5\) demonstrated that microorganisms could be transported through subsurface materials, thus providing a source for inocula. This and collaborating works have demonstrated an abundant microflora deep beneath the surface. Surprisingly, this work showed that subsurface microorganisms are metabolically active and this activity can be stimulated by additions of water, phosphates, and sometimes micronutrients, exogenous carbon, or electron acceptors.
References


Tables

Table 1. Evaluation of nutrient limitations in water producing subsurface sediments.

<table>
<thead>
<tr>
<th>Formation (Depth m)</th>
<th>Nutrient</th>
<th>Stimulation&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Nutrient</th>
<th>Stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pee Dee Sands (256 m)</td>
<td>NO&lt;sub&gt;3&lt;/sub&gt;</td>
<td>+</td>
<td>Glu</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>PO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>±</td>
<td>Min&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>SO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>+</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;O&lt;sup&gt;c&lt;/sup&gt;</td>
<td>±</td>
</tr>
<tr>
<td>Pee Dee Sands (290 m)</td>
<td>NO&lt;sub&gt;3&lt;/sub&gt;</td>
<td>-</td>
<td>Glu</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>PO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>±</td>
<td>Min</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>SO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>-</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>±</td>
</tr>
<tr>
<td>Middendorf Sands (406 m)</td>
<td>NO&lt;sub&gt;3&lt;/sub&gt;</td>
<td>-</td>
<td>Glu</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>PO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>±</td>
<td>Min</td>
<td>-</td>
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<tr>
<td></td>
<td>SO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>±</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>+</td>
</tr>
<tr>
<td>Cape Fear Sands (463 m)</td>
<td>NO&lt;sub&gt;3&lt;/sub&gt;</td>
<td>±</td>
<td>Glu</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>PO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>±</td>
<td>Min</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>SO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>+</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>++</td>
</tr>
</tbody>
</table>

<sup>a</sup> Stimulation of 1.5 - 3 over control = ±, >3 = +, and >6 = ++. Tests were no additions, 10 μM, or 500 μM.

<sup>b</sup> Min. was a complex mineral solution at conc. used in defined media.

<sup>c</sup> H<sub>2</sub>O was total water added to sediments which was 0.25, 0.5, 1.0 and 2.0 ml, respectively.

Experimental procedures. An inhibited control and pairs of duplicate tubes containing 3-gram sediments and 1.0 aqueous phase (except H<sub>2</sub>O tubes) were inhibited after two or six-hour incubations and radioactive lipids extracted and counted. Aqueous phases contained 5.0 μCi of [methyl<sup>3</sup>H] acetate and appropriate additions. Results were compared to the 1.0 ml H<sub>2</sub>O experiment. Additions were sodium nitrate, sodium phosphate, sodium sulfate, glucose, and a stock mineral solution.
Table 2. Evaluation of nutrient limitations within subsurface confining layers.

<table>
<thead>
<tr>
<th>Formation</th>
<th>Nutrient</th>
<th>Stimulation</th>
<th>Nutrient</th>
<th>Stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ellenton Clay</td>
<td>NO₃</td>
<td>-</td>
<td>Glu</td>
<td>+</td>
</tr>
<tr>
<td>(194 m)</td>
<td>PO₄</td>
<td>+++</td>
<td>Minᵇ</td>
<td>±</td>
</tr>
<tr>
<td></td>
<td>SO₄</td>
<td>-</td>
<td>H₂Oᶜ</td>
<td>++</td>
</tr>
<tr>
<td>Pee Dee Clay</td>
<td>NO₃</td>
<td>-</td>
<td>Glu</td>
<td>-</td>
</tr>
<tr>
<td>(239 m)</td>
<td>PO₄</td>
<td>-</td>
<td>Min</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>SO₄</td>
<td>-</td>
<td>H₂O</td>
<td>+++</td>
</tr>
</tbody>
</table>

ᵃ Stimulation of 1.5 - 3 over control = ±, >3 = +, >6 = ++, and >10 = +++ . Treatments were no additions, 10 μM, or 500 μM.
ᵇ Min. was a complex mineral solution at conc. used in defined media.
ᶜ H₂O was total water added to sediments which was 0.25, 0.5, 1.0 and 2.0 ml, respectively.

Experimental procedures: An inhibited control and pairs of duplicate tubes containing 3-gram sediments and 1.0 aqueous phase (except H₂O tubes) were inhibited after six or 16 hour incubations and radioactive lipids extracted and counted. Aqueous phases contained 5.0 μCi of [methyl ³H] acetate and appropriate additions. Results were compared to the 1.0 ml H₂O experiment. Additions were sodium nitrate, sodium phosphate, sodium sulfate, glucose, and a stock mineral solution.

Table 3. Differential incorporation of [¹⁴C]- and [³H]-acetate into lipids of microorganisms from subsurface aquifers

<table>
<thead>
<tr>
<th>Depth (m)</th>
<th>Acetate incorporation into lipids (log dpm per day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[¹⁴C]-acetate</td>
</tr>
<tr>
<td>256</td>
<td>4.7</td>
</tr>
<tr>
<td>290</td>
<td>4.7</td>
</tr>
<tr>
<td>406</td>
<td>5.2</td>
</tr>
<tr>
<td>463</td>
<td>3.4</td>
</tr>
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</table>
Table 4. Sulfate reducing activity observed from subsurface sediments.

<table>
<thead>
<tr>
<th>Formation</th>
<th>Depth (m)</th>
<th>Activity (35S-HS-cpm x 10^3/day)</th>
<th>SO4 conc (mg/kg)</th>
<th>Approx. SO4 reduction rate (umol/kg per day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ellenton clay</td>
<td>194</td>
<td>11</td>
<td>82</td>
<td>50&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ellenton sand</td>
<td>213</td>
<td>25&lt;sup&gt;a&lt;/sup&gt;</td>
<td>26</td>
<td>34&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Pee Dee clay</td>
<td>239</td>
<td>0</td>
<td>1.2</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>Pee Dee sands</td>
<td>256</td>
<td>7.2</td>
<td>31</td>
<td>1.3</td>
</tr>
<tr>
<td>Pee Dee sands</td>
<td>260</td>
<td>0</td>
<td>14</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Pee Dee sands</td>
<td>290</td>
<td>&lt;1</td>
<td>340&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt;8&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Black Creek clay</td>
<td>303</td>
<td>&lt;1</td>
<td>6</td>
<td>&lt;1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Middendorf clay</td>
<td>325</td>
<td>6.2</td>
<td>2.6</td>
<td>0.8&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Middendorf clay</td>
<td>378</td>
<td>8.1</td>
<td>19</td>
<td>8&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
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<td>Middendorf sands</td>
<td>406</td>
<td>26&lt;sup&gt;a&lt;/sup&gt;</td>
<td>328&lt;sup&gt;b&lt;/sup&gt;</td>
<td>440&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>Middendorf sands</td>
<td>416</td>
<td>0</td>
<td>286&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt;2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cape Fear clay</td>
<td>437</td>
<td>&lt;1</td>
<td>25</td>
<td>&lt;0.3</td>
</tr>
<tr>
<td>Cape Fear</td>
<td>463</td>
<td>9.2</td>
<td>6</td>
<td>2.9</td>
</tr>
</tbody>
</table>

<sup>a</sup> Results reflect stimulation of activities; six hour values were 0.0.
<sup>b</sup> High sulfate concentration likely from drilling fluids.
<sup>c</sup> Results are overestimates from a or b above or because clays do not transport water and results are from stimulatory effects.

One μCi 35S-SO4 plus 3-gram sediments were incubated in duplicate sets for 0 - 240 hours. Sulfide was trapped, collected, and counted for radioactivity by scintillation counting.
Table 5. Influence of geological and hydrological properties on microbial biomass and activity.

<table>
<thead>
<tr>
<th>Sediment characteristic</th>
<th>Colony forming units (log)</th>
<th>Activity (logs) as $^{14}$C-acetate incorp. into lipids</th>
<th>Number of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt; 20% clay</td>
<td>2.1</td>
<td>2.2</td>
<td>19</td>
</tr>
<tr>
<td>&gt;70 % sands unsaturated or D&lt;10, (K&lt;200 um/s)</td>
<td>4.2</td>
<td>2.3</td>
<td>17</td>
</tr>
<tr>
<td>&gt;70 % sands saturated and D&gt;10, (K&gt;200 um/s)</td>
<td>4.2</td>
<td>4.3</td>
<td>25</td>
</tr>
</tbody>
</table>

Results from 61 sediment samples from 7 to 500 m depths examined from four core holes over a four year period.
PHYSIOLOGICAL ACTIVITY

Round Table 2
How Do We Determine Density and Activity in the Subsurface?
Convener: David C. White

NO ABSTRACTS
HYDROGEOLOGY IN RELATIONSHIP TO MICROORGANISMS

Conveners: H. Bledsoe and F. H. Chapelle

Plenary Session

Included within:
Round Table 3
Hydrogeology in Relationship to Microorganisms
Dr. John Zachara, Battelle Pacific Northwest Laboratories, Richland, WA.

Abstract
No abstract provided.

Introduction
I was asked to give this presentation and it was not until I began to put it together that I realized how difficult it really would be to talk about this topic, mainly because there is not a whole lot of information on it. I will try to focus on the influence of geohydrologic factors on subsurface microbiological populations. I will talk about the potential influence of different factors on subsurface microbiological populations and summarize the general status of knowledge in this regard. I think one will see that there is in fact a very limited body of knowledge and limited correlations on the influence of many of these factors on microorganisms and I will try to discuss some of the reasons for the correlations. I will present some documented interrelationships between geohydrology and microbiological populations that have been developed. I will conclude by discussing or at least proposing some strategies for future studies, given the limitations and our knowledge based on this subject.

Discussion
There are some very good reasons why one should strive to understand the relationship between microbiology and geohydrology, and why one should strive to understand these interrelationships. The first reason is that it would allow us to estimate viable microbial populations in the subsurface given some knowledge of methodology and geological stratigraphy, water composition, and measured hydrologic properties. This is not saying that one wants to replace the direct characterization of microbial communities in the subsurface, but rather we want to have an ability to be able to extrapolate known characterization data to other locations such as a formation or an aquifer, so one can estimate what is there. This information is also leading to an understanding and an estimation of metabolic functions and activities in the subsurface and an understanding of the relationship between microbiology and geohydrology, which will allow us to further understand aquifer processes—in particular, the biogeochemical evolution of these systems. Certainly, when aquifer processes are understood, at least from the standpoint of biogeochemical processes, one will then be able to predict and improve bioremediation efficiencies and restoration procedures.

There are some general reasons why there exists such an interest in this topic and why it is important (slide). In the most general sense, the importance of hydrology and chemistry to microbiology can be summarized as follows: hydrology and chemistry influence the colonization and transport of organisms in subsurface environments. Hydrology and chemistry are important in nutrient flux and dynamic; therefore, they are important in the substance and activity of subsurface microorganisms. Additionally, the linkage of these things shows that these factors are also important in evolution and diversity of the subsurface microflora community (slide).

Oral presentation.
When microbiologists, geochemists, or hydrogeologists go out into the field, certain situations are typically encountered. This is a highly schematic subsurface system, but I think it will serve to point out some of the important things that need to be considered from the standpoint of the influence of hydrology on microorganisms (slide). In many of the systems that are dealt with, they contain a vadoze zone, an unsaturated region overlying some type of unconfined aquifer, and often there is a confined aquifer underneath. These situations are typically very complex and in most cases, the coarse media is extremely heterogeneous with stratified layers. These layers have different methodology, different textures, different chemistry, conceptually varied cross-bedding, lenses of sand or clay in certain layers, and generally, they give a very heterogeneous distribution to the materials. Within a given unconfined aquifer, there may be a wide range of water velocities reflecting the different porosity and hydraulic conductivity. In the layers, there are often inclusions of clay-size materials in the sand and the flow fields in fact divert around these areas (slide). When one proceeds from the unconfined zones to the confined aquifers, often there are major lithological changes. So under those conditions, the chemistry of the waters and the chemistry of the porous media may change dramatically.

As microbiologists, we are often asked questions about the microbiology of these systems. Often times, one probes the system by taking sediment cores (slide). The questions that arise after one enumerates the existing bacteria and organisms and finds out what is there are usually as follows: Why are these organisms there? Why do we get the distributions that we see with depth? What are the factors that really cause such distributions? Often, more than one core is taken and then additional questions arise from that. What is the variability and how do things change as one proceeds laterally along an aquifer system? Can the changes that occur be interpreted?

The changes and the distribution of organisms within the systems are very much controlled by the geohydrologic properties of the system, which is what I would like to discuss in more detail. The potential factors that really influence microbial distributions and activity in subsurface systems can be summarized in the following manner. The porosity and core diameter of the sediments is important in controlling the accessibility of the sediments to microorganisms, which then effect the colonization of the materials by microflora and also the filtration of organisms as water flows through the formations. Hydraulic conductivity and the pore water velocity are also important in that they control the flux and transport velocity of water through the system. Soil flux is obviously important to organisms since the flux of nutrients that pass the organisms will in fact be strongly controlled by these factors. Nutrient transport is very important simply because one can have differential transport through layers of different conductivity that may be very close to one another. This differential transport allows for nutrient gradients over very short distances and also has a major impact on colonization of these materials. Moisture potential can be important in some of the dense clay zones that may not be saturated. Pore water
composition is important simply because it relates to the nutrients that are present in the organisms, as well as the availability, concentration, and nature of electron acceptors. Also, in terms of water contamination, contaminants are a major concern to us, since in many cases the contaminants are nutrients to many of the organisms. Lithology is of clear importance simply because it governs the solid-state composition in the chemistry of the porous media and also in the surface chemistry. The transport of many of these organisms are in fact influenced strongly by certain chemistries; therefore, lithology may serve as an influence on distributions that are seen in certain subsurface systems.

Let us go back now to the subsurface system that I previously mentioned and speculate a little bit on what kind of differences one may expect to see and why one should see differences across two closely associated layers in coarse media that may have different physical properties. Take, for instance, two layers that are closely connected in coarse media that may have different porosities, textures, and possibly bulk densities that give rise to different hydraulic conductivities. It is envisioned that the flow rates through the systems under a constant head gradient have flow rates in a lower system with a high conductivity zone that exceeds the lower conductivity zone (slide).

Some of the variables may be different between these two layers due to the physical and chemical nature that is associated with such layering. In the high conductivity zone, a younger water may be anticipated simply because the flow rates are in fact quicker through these zones. One may expect higher dissolved oxygen because the flow rates are quicker and these waters are younger and possibly more closely connected to the recharge zone. Depending upon the actual conductivities of this high conductivity zone, the coarse base may be less restricted to the entrance of organisms. As a result, organism filtration and movement through that zone clearly would be significantly less than the low conductivity zone. Because of the rapid water velocity, the mineral water contact times may be significantly less, thus giving rise to differences of pore water composition. The dissolved organic carbon (DOC) in this level, may also be significantly different because of its more youthful age and closer conductivity to the recharge zone. Also, in this type of conductivity zone, the solid surfaces may be significantly less reactive simply because its base is coarser, dominated by silica and felspars. They may also be lower in clay minerals and oxide content. Furthermore, these high conductivity zones may be lower in a particular organic carbon, because particulate organic carbon is often found to be associated with a finer material.

By analogy, a comparison can be drawn of what one may expect in a lower conductivity zone. The water may be older; dissolved oxygen will be lower; it may be anoxic, simply because of the travel time; there will be a limited coarse base, with accessibility depending upon its actual size; clearly, a lot higher potential for organism filtration will exist; there will be a longer mineral water contact time; and, a different pore water composition may exist. Additionally, the organic carbon may be more refractory and unsorted simply because the travel is contacting surfaces that
are more reactive, and the surfaces in there may be significantly more reactive to microorganisms because of their surface area and electric chemical properties. Also, in these higher conductivity zones, one may see a higher particulate organic carbon, simply because carbon does have a tendency to associate with oxides. Therefore, if this system is looked at conceptually, one may expect to see some significant differences between present organisms within short, vertical distances just due to the physical and chemical configurations of this system.

What has in fact been found with regards to a situation between geohydrochemical variables and subsurface microbiological populations has been summarized. In a little aquifer in Oklahoma, microbial populations, at least at one site, were shown to be greatest in a coarse texture gravel zone. A low viable to total count ratio was also found there in a clay zone as was a high viable to total count ratio in the sandy zone. In Cretaceous sediments in the South Carolina Coastal Plain, some similar findings occurred. In looking at a large transect of formations in the southeastern coastal plain, the percent clay in the sediments was found to be the predictor of heterotrophic populations. Additionally, and similar to Oklahoma, it was found that the ratio of viable to total cell counts was also lower in clay layers and that ratio was higher in sandy layers. In Alberta, Canada there was not a correlation of viable cell counts with dissolved organic carbon.

Finally, work by Ron Harvey demonstrated that the populations of free-swimming organisms were very spatial with depth in the aquifer, and were found to correlate with the electrical conductivity and DOC of the system. In the contaminated system (slide), the populations and the nature of the microbial community were shown to reflect the nature of the carbon source in that system. Therefore, these rather simple examples showed that in fact some agreement or some correlation has been observed between factors relating to hydrology and chemistry in subsurface systems (slide). However, there have not been a lot of very good correlations reported between hydrologic and geochemical factors and microbiological populations. I think a general observation concerning this lack of well-reported correlations is that microbial data cannot be as readily rationalized as the hydrologic and hydrochemical data. This is a generalization, but certainly there are cases where there has been rationalization between the two.

Nonetheless, let us try to summarize some of the reasons why the agreement between hydrology and the microorganisms has not been obvious, and why correlations have not been quantitative. I think there are a lot of reasons for such obscurity. First of all, measurements have often been performed on different types of samples. For instance, people looked at microorganisms in one sample, then performed characterizations on an associated, but possibly different sample. Sometimes microbial data are generated on cores used for chemistry analyses. I also think that there has been a lack of appropriate measurements, and often times, it is difficult to really understand and identify what the appropriate measurements are. This is getting back to a point that will be discussed at the end of this paper concerning the need in this area for a more intense, more interdisciplinary evaluation of the problems, and
some sort of collective interdisciplinary assessment of what actually are some of the most appropriate measurements to make on these systems. One can make many observations and measurements, but many of those may not be at all relevant to answering the questions regarding the microbial community.

I think one has also seen that there can be major sampling effects. Sampling effects have in fact minimized our ability to make quantitative correlations in many cases. The sampling effect that I am referring to is sediment compaction. When one takes cores of subsurface materials, often times the core materials are compacted. When an attempt is made to make measurements of hydraulic conductivity, porosity, and density, one may find gradients in a core sample suggesting that compaction has occurred. If an attempt is made to measure parameters on that sample, one will find that correlations between those parameters, which in fact are partially artificial in the microbial community, cannot be made.

There are also contamination effects, arising from drill mud and other things associated with the drilling operations. In many cases, these drilling effects have compromised at least the integrity of pore water chemistry and water quality data that are generated on subsurface material. If the data are compromised, then one cannot really understand the geochemical factors that are most important to the organisms.

Also in the collection of core samples, materials may sometimes drain. They drain because the air entry potential with the subsurface sediments is such that water can drain out just due to the nature of the hanging column of water. Drainage can have a significant impact simply because when something drains, air or some other material has to fill that pore space. This can lead primarily to oxidations, but sampling effects can be related to both oxidation and reduction.

Another reason why good correlations have not really surfaced is because a complex multibarrier problem is being dealt with. In other words, the organisms are reflecting a number of sensors. These include both factors associated with the lithology (lithology being the mineralogy and chemical composition) and the influence of physical and hydrologic factors associated with the placement of the coarse media. These are clearly reflected in geochemistry. Therefore, this is a system that has many things impacting the microbial community, and identifying any of these that dominates becomes fairly difficult.

In some recent sampling that was sponsored by the Department of Energy at the Savannah River Site, there was an opportunity to look at some core samples from a couple of different formations within the South Carolina Coastal Plain sediments. While recognizing some of the limitations, an attempt was made to look at two formations with some degree of detail. The microbial community was observed and an attempt was made to rationalize the microbial distributions in the formations that with some of the hydrologic and chemical factors in the sediments (slide). An attempt was made to recognize some of the limitations that had been involved and to sample some of the geological units fairly closely to see if the microbial distribution could be rationalized with selected properties of the subsurface sediments.
A brief word or two needs to be said about the placement of the sample from a certain schematic, which represents the borehole that was taken near the Savannah River Site at Allendale, SC (slide). It was the fourth hole sponsored by DOE in that area, and core samples were taken from two different formations, the Middendorf and the Cape Fear. These formations are at depths ranging from 1150 to 1550 feet below the land surface. Approximately 25 core samples were taken from the Middendorf and Cape Fear formations (slide). A number of hydrologic measurements and a few chemical and microbiological measurements were performed on these core samples.

A couple of things about these formations should be brought to your attention. The Middendorf formation is a water-producing unit; it is an aquifer. In contrast, the Cape Fear formation is not considered to be a water-producing unit (slide). It is a fairly tight formation, which was reflected in the resistivity survey.

In this case, an attempt was made to obtain a series of samples from the two distinct formations, to look at them in some detail, and to then determine if the microbial populations could be rationalized with chemical and hydrologic variables.

Some of the properties of those two units have been summarized just to bring their differences into focus. The Middendorf formation has a pH range of 4-8, which was the pH of pore water that was displaced from cores (slide). The major cation in these materials was calcium. The major ions were sulfate and bicarbonate. The DOC range was 3-6 mg/l, and particular organic carbon ranged up to almost 1%. Viable microbial counts for selected microbial groups showed that chemoheterotrophs ranged between log 3.1 and 6.4/gdw in the Middendorf and denitrifiers ranged between log 1.7 and 5.7. The Cape Fear formation is of high pH, ranging from between 9 and 10 at least in terms of the pore water. The dominant ion was sodium bicarbonate. The DOC was high and similar to that found in the Middendorf. The organisms ranged from no growth at all in the enumerations to levels that were slightly below or significantly below those found in the Middendorf.

The saturated conductivities were measured on intact core samples while horizontal saturated conductivity was measured through the core samples. Therefore, it was apparent that significant conductivity differences occurred between certain zones (slide). It is primarily a reflection of the fact that the Cape Fear formation is a sodium-saturated system. The clay minerals in it are almost fully dispersed and the conductivity is very low.

General appearance of the data showed that there are high bacterial populations in certain areas of the Middendorf, a couple areas where the populations decrease significantly. In the Cape Fear formation, the populations are significantly less (slide). So some fairly simple questions are being asked as to what was controlling these populations fluxes within these two units and furthermore, could this be rationalized based on some properties that were measured on the intact core samples.

Before I summarize, let me just interject something. In the course of looking at the core samples, certain observations led us to suspect that the chemical and possibly the microbiological integrities of these samples had been compromised. As a result
of that, a number of extensive analyses were performed to determine and document that the samples were in fact not contaminated. A number of surface chemical analyses were done in order to look at the nature of clay minerals in these materials and at both the mineralogy and surface chemistry of the clays. A number of microbiological characterizations were done, including DNA hybridization, and certain metabolic patterns of the organisms in these zones were observed. The conclusion that was reached was that in fact the microbiology was not reflective of any contamination that could be discerned, but that the pore water chemistry was in fact significantly impacted by the drilling fluids from the drilling procedure.

These two factors then lead us to the following observation concerning the significant population differences between the units. Poor correlations were seen between the heterotrophic populations and most physical and chemical properties. I think that the poor correlations that were observed between the pore water chemical properties were a reflection of the fact that the pore water chemistry was not being observed as it actually existed in the formation per se. Unlike past studies, where texture or percent sand and clay had been an adequate predictor of microbial populations, texture by itself was inadequate in this case because the subsurface systems had a different ion balance and their hydraulic conductivities were strongly influenced by the chemistry rather than by the texture of the formation.

Some select correlations were observed between certain properties of the sediments and the microorganisms. However, it was found that the lowest bacterial populations in both of the units were clearly associated with identifiable lithologic events or lithologic properties in the core materials. For instance, the lowest bacterial populations were associated with lignite bands in the Middendorf. The lowest bacterial populations in the Cape Fear formation were associated with very high bulk density and low porosity zones. The overall differences in the bacterial populations between these formations were ascribed to hydraulic conductivity and pH.

Now let me give an example of the correlations that we did find between measured parameters and microorganisms. Recall that there were not many correlations that could be observed. A simple correlation was found between denitrifiers in the Middendorf and Cape Fear formations and the porosity (slide). In other words, this correlation came from pulling all the data from both formations. What was found was essentially very little or no correlation between denitrifiers and porosity. However, if observations were made on a formation-by-formation basis, then some significant correlations between certain properties were seen. This is one of about three different, good correlations between microbial populations and hydraulic or chemical properties. For instance, a good correlation between denitrifiers and porosity was obtained in the Cape Fear formation, where porosity in one particular case ranged from a low of approximately 0.35 to almost 0.55 in terms of porosity. To summarize the results of this example, it can be seen that some correlations could be identified between the organisms and both chemical and physical properties only when the data was observed on a unit-by-unit basis.
Conclusion

To conclude, I would like to summarize a couple of points and then move on to a statement of some future strategies for these types of studies. I think everyone would agree that hydrologic factors clearly must influence subsurface microorganism population. I think that we can look at the situation and collectively speculate as to what effects these factors may have. Surprisingly, however, good correlations are limited, and they are limited for a number of reasons that were summarized earlier. There has been an absence of definitive studies, and sampling is often driven by opportunity. This is not a criticism, it is just a reality. Also, the influence of contamination and disturbance is a critical one, and I think it contributed to the lack of really observable, good correlations. It appears that a general finding of many studies is that greater organism populations and higher viable counts are associated with core transmissive zones. When trying to rationalize microbial populations between different formations with different lithologies and chemistry, the multibarrier nature of the interactions really becomes problematic for us in order to pin down the priority causes for the microbial distributions. One can do statistical analyses to look at the causal factors, but one gets to the point where there are so many interactions that are occurring that it becomes difficult to have a lot of confidence in understanding what the primary factors are.

It seems that the mechanisms of interaction between hydrology, geochemistry, and the organisms remain unclear. For instance, as I have tried to express, there are a number of correlations between texture and microbial populations. Therefore, one would ask the question, "Are these correlations a reflection of the hydraulic conductivity, pore diameter, or surface area?" I believe that these factors are all related and when it comes right down to it, texture is just a measurement that is a reflection of many of these other properties.

I will get back to that in just a moment. Another important question deals with whether the microorganism distributions in the subsurface systems are transport or nutrient flux controlled. This raises a final question. To what extent is pore water composition of any significance? Clearly we know that the microorganisms influence the pore water composition, but in terms of governing their distributions, one may wonder how important that really is. An attempt has been made to show some relationships although nothing very good has been seen. I just want to reiterate one point and try to show why answering questions such as these on the mechanisms of interaction is in fact difficult. What is intuitively obvious to all of us is that many of these properties are in fact co-related. For instance, hydraulic conductivity is influenced by the particle size distribution (or its texture), by the bulk density, and so on down the list. I think one can see that particle size distribution is related to just about all of these properties, and so is bulk density. Therefore, when one measures some of these easily accessible parameters, trying to tie them to what is really a causal effect is difficult at best.

Now for a statement as to where we go from here. It seems a greater emphasis is needed when looking at intact core materials, where one can characterize the organisms, yet one can also be able to look in some detail at the lithology and
hydraulic properties of these materials on a scale in between the microscopic and macroscopic levels. I think it is important to look at the spacial distribution of organisms with respect to lithologic properties in order to get a better feel for what really controls their distributions. It is important to measure, and to continue to measure, priority in situ hydrophysical characteristics such as vertical and horizontal hydraulic conductivity, porosity, and maybe pore diameter. These may be important characteristics, but until one really goes out on a limb and measures these things and tries to relate them to the microorganism, we really do not know how important they are.

I think all of us are striving to develop better sampling procedures to eliminate drill mud contamination and sample disturbances. This is an ongoing thing, and as more people become interested in this subject area, these improvements will necessarily develop. It is a priority area. More analyses are needed on these systems pertinent to the microorganisms, including the composition of dissolved organic carbon fraction.

The important thing that is needed in these cases is more interaction between the microbiologists, the chemists, and the hydrologists. Often times, these interactions are difficult to foster, not because people do not want to; it is just typical not to discuss or talk across disciplines. I think to really understand these subsurface systems, this is what is required.

I would propose that maybe there is more need to focus on distinct formations. This is because often times within a distinct formation one can at least hold certain variables constant, such as the age of the water, maybe carbon, and other things. If certain variables can be held constant, one may be able to look more intently at the control that the physical hydrology, or even geochemical variations, have over the microscopic scale on the microbial distributions. If one looks at a distinct formation, one can then focus more intently on downgradient evolution both in the microbial populations and diversity as well as the chemistry. Thank you.
Q and A

P. Long: I think you have done an excellent job with a difficult topic, John. I would like to comment on one additional area that might be important in terms of why this strong correlation is not seen when it is expected, and that is the effective heterogeneity. For example, your comparison of high conductivity and low conductivity strata assumes that there was some relative homogeneity up to the point of discharge, that one had a constant head for both cases, and the velocity and so forth would be controlled by the physical properties of this porous medium. In reality, one may have situations where there are high conductivity zones when one drills through and samples the microbial populations that are totally surrounded by low conductivity. In that case, one would not anticipate the types of correlation that have been predicted. I think that is consistent with what you were suggesting in terms of looking into detail at individual units. I think heterogeneity is critical to those interpretations.

J. Zachara: Given that comment, one could have almost asked the question of whether it is really possible in many cases to make sense out of microbial distribution (given the complexity of the hydrogeology with respect to heterogeneity), because one could envision coring through many isolated zones like the ones that are simply reflections of the microscopic environment.

P. Long: Yes I would agree it makes it extremely difficult. However, I think that as long as one understands what kinds of geometry are being dealt with, or attempt to understand them, then we have a chance of doing something there.

J. Tiedge: There are a couple of other biological factors that I think are important when considering whether organisms reflect their environment or not. An important one is really the growth rate in the environment, because it takes a lot of growth for the selection effect to show itself and maybe there just is not enough growth. That is one factor. The second thing is the genetic plasticity of these organisms. They may just be adapted to many of the different heterogenous environments that are down there, particularly if they originated in their heritage. They lived on the soil surface at sometime. From the surface soils, we know that these correlations are not very common either. I think it is because they have adapted to the wide ranging environmental conditions that they experience over seasons and over time. So if they have that heritage, one would expect them to also exist in a lot of different environments and therefore, not show specific correlations with specific niches. So both the selection component and the plasticity component, I think, are important in evaluating for this relationship.

J. Zachara: Well then, let me say this. Your comment would seem to deemphasize the importance of the transport phenomenon to the distribution microorganisms in subsurface environments.
J. Tiedge: I am not sure that it would de-emphasize it. The way I see it, the microorganisms could arrive at a site by transport and that still could happen. However, they still were able to grow in that environment to show that they dominate in that particular niche. If they do not grow enough, then you would not see that particular correlation.

B. Russell: John, just a comment in relationship to the data from the C10. I think that we probably had some very inherent biases in the sample that was obtained for you as well. However, the technique that we used was better for recovery of material in one formation than other types of formations, whether there were more clays or silts or what have you. I just have a comment to reinforce what you said. When our future field operations begin, we do not want to be thinking about having just a single technique for every purpose. We may want to have selected tools that are biased toward providing us better geochemical samples. They may not be the same tools that are necessarily used for the microbiological samples. Then we will have to struggle with the question of how to deal with samples from maybe two different intervals that are simply close together.
Evaluation of Core Segment Pore Water Contamination from Tracers, Well Water Sampling, and Sediment Extractions
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Abstract

Contamination of the pore waters in core segments sampled from the fourth hole drilled for the Subsurface Science Program in South Carolina with soluble constituents from the drilling fluids was most reliably measured from added known levels of KBr during makeup of the drilling fluids and analysis of Br in the pore water. With a knowledge of both the concentration levels from waters sampled after development of screened intervals at the depth of core removal and the concentration levels in drilling muds in contact with the core, poorly-reactive species such as Si, Na, Cl, and F also gave similar drilling fluid intrusion levels in the Middendorf sand interval.

A second source of "contamination", specifically the initiation of oxidation of trace-reduced components in the sediments, is more difficult to quantify. Oxidation results in major increases in total soluble iron (ferrous), sulfate, calcium, magnesium, potassium and acidity. Well waters from screened intervals installed in the Middendorf at P24, 28, and 29 two years ago are still showing high levels of these oxidation products.

The ability to obtain contamination-free pore fluids at depths below 200 m will require significant modifications of existing drilling methodologies.

†Oral presentation.
Introduction

The goal of this paper is to show that by using the best available drilling techniques, the pore water that was extruded from the core samples was in fact contaminated from two sources. The data indicated that there was both drill mud filtrate and oxidation products which were initiated during the time of drilling. I would like to make clear that the contamination that I am talking about is primarily the soluble components of the pore water. Secondly, I would like to make it clear that the contamination was not really the fault of any of the drillers or anybody distributing samples or from any taking of the samples. It really was a consequence of our existing growing technology.

Discussion

The bromide tracer did in fact provide the level of drilling mud filtrate that was in the flow water that was extruded (slide). However, if concentrations are also known in the mud and a developed well at the same interval that pore or core samples are taken, then chloride, fluoride, and sodium will also provide a fairly decent showing of the level of filtering. The evidence that we see with oxidation is a little more difficult to evaluate; however, it is initially present and it does increase with time (slide).

Finally, I would like to provide some recommendations of how to handle this problem in the future. Samples were collected from boreholes P24, 28, 29 and C10 in the South Carolina Coastal Plain. In the general characterization scheme that has been used for all the core segments, KBr and other tracers were added to the drilling mud. Once the core was collected, a segment of the core was brought to water saturation. This was done for one purpose only: to have pore water data on all the core segments. The water was initially extruded with an admissible solvent. Many of the samples that were taken, especially in the high clay samples, would probably not have had sufficient pore water to do the analysis.

A secondary technique was also used, which had a hot water extract. The hot water extract provided a measure of materials in the sediment that were more closely related to what might be available without solubility controls, etc. Samples were incubated for 72 hours and expressed through an ultrafilter. The samples were immediately analyzed onsite for pH, conductivity, and the sensitive ion components. In other areas, it took two days for the inorganic carbon, organic carbon, sulphur species, all the anions, and the elemental analyses. Tracer analyses were sent out for special analyses. Particle size distribution was determined on the incubated sample.

In a plot of drilling mud concentrations (slide), the variation of sodium during the time of drilling could be seen. The sodium concentrations varied very little, around 600 mg/l. Variation in the bromide concentrations also occurred during the drilling process (slide). There were major concentration fluctuations because the bromide tracer had to be continually added as the drilling muds were used down the hole. However, if one were to utilize the data from the muds, one could make a reasonable calculation for the level of contamination.

One particular set of data came only from the Middendorf levels at P24, 28, and 29. In the well waters, concentrations of bromide were nondetectable and the concentration of sodium was relatively low. The assumption was made that the data
in the well waters actually showed the level that should have been seen in the pore waters. In the pore waters, the bromide was not measured and tracer was not added to the first three holes, but it was added to the last one. The concentrations of sodium that were seen in the pore waters from the Middendorf were shown (slide). Calculation for the percent contamination for bromide in C10 resulted in 34% and the sodium was at 38%, respectively. Similar calculations for sodium, with the concentration of sodium being about the same in the drilling muds of the first three holes, resulted in contamination levels in these core samples of 30-84%. Similar calculated contamination levels were found among bromide, fluoride, chloride, and sodium.

More results were gathered from a single core, the C10 Middendorf core at 416 m (slide). The well screen was slightly different from where the core was taken (at 427 m), and 433 m was the closest microbiological sample that we actually had. The core values for bromide, fluoride, chloride, and sodium were all less than they were in the mud, and much higher than they were in the well. The calculated concentrations of the percent contamination were all about the same. This was not the case for most of the other constituents. Looking at organic carbon, sulfate, bisulfate, calcium, iron, and manganese in the same sample showed that in the core sample, the concentrations were generally higher than they were in the mud, but much higher than they were in the well.

The calculated percent of the mud infiltrate is essentially a value that is essentially meaningless, other than just for the magnitude. The reasons for this, we believe, comes from two sources, the first being oxidation. As the sample was taken, oxidation was initiated. What resulted was an oxidation product of disulfate. The increase in iron, and the increase in manganese, sulfate, and organic carbon, was primarily due to the processes that were initiated by oxidation. The calcium, as well as the magnesium that was in the barium, were probably there because of exchange reactions on the muds or exchange sites within the sample. Data were obtained from a particular sample (from P24 at 657 feet) and it displayed the full extent of what can happen (slide). The sample was stored in the refrigerator at 4°C in a sealed container for approximately a year. The pH from the original pore water chemistry had a value of 7.32. The water was reexpressed after about a year and the pH decreased to 1.14. Massive increases with dissolved organic carbon, as well as Fe²⁺, calcium, manganese, sulfate, and even ammonia were evident. There were also increases in other constituents like cobalt and tracer elements. Thank you.
Fe\textsuperscript{3+}-Reducing Bacteria in Deep Coastal Plain Aquifers: A Mechanism for the Origin of High Iron Concentration in Groundwater

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Abstract

Viable Fe\textsuperscript{3+}-reducing bacteria are present in aquifers (10-230 m) that underlie Florence, SC, where ferrous iron concentrations in groundwater typically exceed 2 mg/l. Acetate-oxidizing, Fe\textsuperscript{3+} hydroxides were recovered from deep subsurface sediments using a defined culture media containing synthetic, poorly crystalline Fe\textsuperscript{3+} hydroxide as the sole electron acceptor. The presence of viable Fe\textsuperscript{3+} reducers in these ancient (Late Cretaceous) sediments, the observed linear correlation of dissolved inorganic carbon (DIC) to dissolved iron concentrations in groundwater, and \textsuperscript{13}C values of DIC that are similar (approximately -26 to -22 per mil) to the isotopic composition of sedimentary organic material present in these aquifers, suggest that bacterially-mediated iron reduction coupled with the oxidation of sedimentary organic material is an important process leading to the accumulation of dissolved iron in groundwater.

Sulfate-reducing bacteria are also present in these aquifers near Florence. However, because ferric oxyhydroxides are readily available in these nonmarine sediments, Fe\textsuperscript{3+} reduction appears to be the predominant terminal electron-accepting process. Downgradient of Florence, the sediments are predominantly marine in origin, ferric oxyhydroxides are much less abundant, and sulfate reduction appears to be the predominant electron-accepting process. This change in bacterial processes from Fe\textsuperscript{3+} reduction to sulfate reduction is the most likely cause of the observed downgradient decrease in ferrous iron concentrations.

\textsuperscript{1}Oral presentation.
Introduction

In the mid-80s, when I first became involved in microbiology, not surprisingly, I made a number of mistakes. One of them was asking a microbiologist from the University of Maryland what she thought the difference was between microbiology and geology. She looked me right in the eye and said it is the difference between right and wrong. In fact, microbiology as a discipline is very different in outlook from geology and hydrogeology. Microbiology is largely laboratory-based deductive science. This is because the kinds of problems that interest microbiologists are meaningfully addressed in the laboratory. Many of the problems we consider in geology, however, are simply not suited for laboratory investigations. One cannot, for example, go into the lab and design an experiment to either prove or disprove plate tectonics. For that reason, geology is typically much more inductive. A process is proposed and then accepted or rejected based on the weight of evidence that can be brought to bear on one side or the other. This is one reason why geologists get into interminable arguments.

Discussion

This paper discusses a bacterial origin for high iron concentrations in groundwater. The following two things must be accomplished to do so: (1) to show that the weight of evidence clearly demonstrates that this is a commonly observed record of the activity of microorganisms; and (2) just as importantly, to illustrate that this conclusion can really only be arrived at inductively. The necessities of making such inductive reasoning, I think, is very important to be understood so that geologists and microbiologists can come to some sort of cooperation. The problem can be illustrated in a number of ways (slide). Coastal plain aquifers are typically characterized by having very high iron groundwaters that occur in bands parallel to where the aquifers are recharged. In looking at this sort of data, one really cannot come to any sort of conclusion about what causes the increase in dissolved iron, which is typically on the order of 1-5 ppm. Certainly there is nothing in this which would make one think that it is biological in origin.

Historically, high iron concentrations in groundwater have been interpreted on the basis of thermodynamic relationships. Where there is a reducing condition, the ferrous iron is soluble and one has mobilization of ferrous iron. One thing that is difficult to explain, for example, is why one does obtain these bands. Why would one get it all the way down in this flow system? This high iron, which occurs in the Middendorf aquifer of South Carolina, occurs throughout the coastal plain of the United States (slide).

Some data were received from the Patuxent aquifer in Maryland, which crops out between Baltimore and Washington (slide). The first thing the data showed was dissolved oxygen at 10 ppm. At the recharge area, concentrations of dissolved oxygen were on the order of 10 ppm. Six miles downgradient in the system, the dissolved oxygen had been completely consumed. From this part onward, one would be dealing with an anoxic aquifer. One of the things that was immediately noticeable was that the sulfate data was basically a mirror image of the dissolved oxygen data. In the outcrop area, the sulfate concentrations were very low, 1 ppm or less. As one went downgradient, oxygen was consumed and sulfate concentrations increased.
Obviously, sulfide oxidation was being dealt with. I was suitably impressed with that conclusion.

There were, however, two things that were inexplicable to me at the time. One is why, for example, when the water became anoxic was there really no down turn in sulfate? One would expect that as the aquifer became anoxic, sulfate reduction would have initiated to decrease the sulfate concentration. This was not observed in this part of the aquifer. The second thing at six miles downgradient, where the dissolved oxygen is essentially consumed, high concentrations of iron began to develop. Similarly, at that same part of the flow system, concentrations of DIC, expressed as alkalinity, increased in a parallel fashion with the dissolved iron.

At the time (1981), there was really no particular way to explain this. The correlation between the DIC and iron could be expressed very nicely in the anoxic zone in the Patuxent aquifer. Fairly nice correlations of dissolved iron (in micromoles) and DIC (in millimoles) were obtained, making the slope appear a little bit steeper. I was aware of the correlation and I knew it was occurring, but there was really no framework within which to propose a mechanism to explain the data. It was just an inorganic reaction. I did not see any reason why one would expect this kind of correlation.

From the Middendorf aquifer of South Carolina, essentially the same sort of thing was obtained (1985). In this case, with the Middendorf aquifer being in a high iron zone, there was a cross-section through it where one could see the high iron concentrations rising and then decreasing once you were downgradient in the furthermost downgradient part of the flow system. Again, DIC concentrations correlated very nicely with dissolved iron concentrations.

About 1985, Derek Lovley of the United States Geological Survey was doing some work with iron-reducing bacteria, and he proposed a mechanism whereby organic matter could be completely mineralized with the reduction of ferric iron. The thing that struck me immediately was the final process: ferrous iron in solution and DIC. However, there was a framework within which I could begin to think about what was causing these high dissolved iron concentrations. According to Lovley's model, if one was obtaining the DIC from oxidation of organic matter as opposed to other possible sources of DIC, then one should be able to see a similarity between the DIC of the water and the $\Delta^{13}C$ of the DIC. In fact, in the Middendorf aquifer, this is exactly what one sees. The DIC is very light isotopically, and this is in very sharp contrast to the downgradient anaerobic part of the aquifer which is low in iron.

So far a number of different threads have been obtained, which are sort of nudging us in the direction of a microbial process. If you are a groundwater chemist at this point in the game, you sort of have a choice. You can agree that what you have is it and quit, or you can tell yourself that the only way that this mechanism can be considered as plausible is to show that microorganisms capable of this type of metabolic activity are in fact present in the aquifer.
Lovley developed a defined media with acetate as the electron donor and carbon source and synthetic ferric iron hydroxide as the sole electron acceptor. When the medium is inoculated with organisms, it is very easy to see the reduction of ferric by the iron-reducing organisms. If one does not inoculate the defined media with the organism, the ferric iron will essentially remain stable, at least on the order of years. Therefore, the development of this particular media made it possible for us to go out into the field and to actually screen some of the sediments for the presence of this particular metabolic activity or function.

A hole was cored near the town of Indianhead in Maryland that penetrated the Patuxent aquifer, for which we had some data (slide). The Arundel clay in the Patapsco aquifer was also cored, and again, some water chemistry data had already been gathered concerning this aquifer. Observations were made for two particular types of acetate-oxidizing bacteria: the sulfate reducers, since they are easy to do, and of course, the iron reducers. In the upper part, the first 50 m of the section, the water was aerobic. However, down in the Patapsco aquifer, which is the principle aquifer there, and down into the Patuxent aquifer, each of the core sediments that were looked at actually had the presence of ferric iron-reducing bacteria. In Maryland, as in South Carolina, the same results were obtained. Furthermore, with the core hole that was done in Florence, which is right smack dab in the middle of a high iron zone, it was again found that it is easy to culture iron reducers in the Black Creek, Middendorf, and in the Cape Fear aquifer. Again, this is sort of inductive, considering the process is occurring and the belief that bacteria ought to be there and the bacteria are evidently there.

One other very interesting piece of evidence has to do with what happens when the actual sediments from these aquifers are inoculated with the iron-reducing organisms. Lovley showed that if one has dissolved iron on one axis and if it is incubated for a number of days as a sterile control, then there is really no change in the dissolved iron and there is no change in the color of the sediment. Initially, the sediment is red. If it is inoculated with the Fe$^{3+}$ reducer (in this case, GS-15 was used), then there is a very pronounced increase. This is what one would expect from dissolved ferrous iron; however, the color of the sediment also changes. In the Cape Fear aquifer of South Carolina, at the 675-foot depth, much of the sediment is a greenish, reduced-looking color. However, it is very typical of this part of the system to obtain these mottled, red-green zones. These green zones transect primary depositional features such as crossbeds and lamenii. They are clearly diagenetic. The obtained color change is very similar to what one obtains when the red sediment is in a laboratory.

One other piece of information that was considered when coring this hole was that when one takes a plug of sediment from the red material that is entirely red and inoculates it with the media, no growth is ever obtained. A similar textured material was taken, but it was red and no iron reducers grew. However, if this sediment sample was taken at either the interface of the red-green boundary or from the more reduced looking areas, then growth of this particular organism could be found, although not in every case.
Conclusion

So what we have is a classic geologic inductive agreement. I would argue that the weight of evidence that was obtained is strongly in favor of the following observed phenomena: The higher iron concentrations are in fact related to the metabolism of ferric iron reducers. However, it does not prove it because a controlled experiment has not really been done in the field. One can argue that this is not even possible to do. On the other hand, if one is going to argue that ferric iron reduction in the system is in fact an abiological process, it is then incumbent, I think, to come up with similar arguments of which I know of none to support that contention. Thank you.
E. Bouwer: Can you finish the story and tell us why further downgradient you did not see the iron? Is it because they ran out of food source or because carbon source was limited, or was there a lack of iron-free minerals in that area?

F. Chapelle: There are several possibilities. In the Middendorf aquifer in the upper gradient areas where the high iron concentrations were, the sediments were entirely continental in origin. There was no carbonate material shown. Those continental origin sediments were characterized by a high abundance of ferric iron coating the grains. Down-dip in this particular basin, the sediments were largely marine in origin and marine sediments typically do not have those kind of grain-coating, ferric iron reduction minerals that were present upgradient. So one factor was simply the presence of reducible iron. There is a number of factors that could have happened. One is that downgradient sulfate reduction becomes a much more important microbial process. If there is any dissolved iron that is produced, it is going to get tied up with the sulfide and you are not going see it. We also know that secondary calcite cements that are downgradient are in fact ferrous calcite, where Fe$^{2+}$ substitutes for calcium. So that was probably another thing that occurred. My own bias is that the difference in upgradient and downgradient deals more with the availability of ferric iron as an available electron acceptor.

W. Ghiorse: Frank, if you are seeing iron reduction, what about manganese? Could manganese be going along with this? Iron and manganese minerals are always present together.

F. Chapelle: First of all, in many sediments such as river sediments, one does see that. In these aquifers, manganese is a real small percentage. Pyrolusite is a real small percentage of what one sees as well. One does not see a lot of manganese mineralization. I have actually toyed around with looking at manganese in relation to this and it is just not clear. That may be due to the concentrations, which are very low. Certainly, one would expect that if iron reduction is an important process, then manganese could be an important process.
Attached and Unattached Bacterial Populations in Deep Aquifer Sediments from a Site in South Carolina

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Abstract

Deep aquifer sediment samples (depths from 120 to 500 m) were obtained aseptically at the United States Department of Energy's Savannah River Site in Aiken, SC. Viable bacterial populations in these samples ranged from $10^4$ to $10^6$ cells/gdw of sediment (triplicate spread plating on concentrated and dilute media). Attachment of the aquifer bacterial populations to sediment particles was studied with a low-speed centrifugal washing procedure used in conjunction with plate counting. The majority of the bacteria in all samples appeared to be firmly attached to the sediments. That is to say they were not removed by extensive centrifugal washing, but the extent to which the microflora was attached varied widely (from 55% to >99%) from one sediment to another. The attached and apparently unattached (easily removed by centrifugal washing) populations were isolated by plating on concentrated and dilute media and then compared. An analysis of colony morphologies (color, type of edge, surface characteristics, etc.) and the responses of each isolate to 21 selected physiological tests, including ability to use 12 different compounds as sole sources of carbon, indicated that the attached and unattached microbial populations in all samples were composed largely of distinct microbial strains. Relatively few, usually less than three, of the morphological and physiological types isolated from each sediment sample could be found in both the attached and unattached populations. The attached and unattached populations from each sample also differed in their group physiological characteristics, as indicated by the percent of isolates in each group that displayed a positive response to each physiological test. The physiological differences between the attached and unattached portions of the microflora were not consistent from one aquifer to another, however.

*Oral presentation.
as compared to the other two, either in the number of cells that were attached or in
the degree of detaching them with the centrifugal washing. Given the limited
number of sediments that were observed, no obvious conclusion could be drawn in
terms of the physical characteristics of these samples or their chemistry that could
explain the difference. Obviously, the data indicate that more samples will need to
be observed in order to obtain that sort of information, because as one knows,
aerobic, chemoheterotrophic populations in these samples tend to be fairly diverse.

Another point of interest dealt with the effect of the washing procedures on the
diversity in each of the fractions. Starting with the usual approach, the colony
morphological characteristics were observed on the plates for the different frac-
tions. In the McBean and the Ellenton samples, a reasonable number of different
colony types did show up in the first three supernatant washings. Thus, there was
a reasonable diversity of organisms that were easy to detach or were unattached to
begin with. Also, a reasonable diversity of organisms was left in the final pellet.
There seemed to be a lower number of organisms that showed up in the later
washings. Of course, the Middendorf picture was very different because very few
of the organisms washed out initially. There were some technical problems that
prevented us from obtaining the data on the final pellet in this particular case.

Because the McBean and Ellenton samples had both attached and unattached
organisms, our concern centered on whether these organisms were the same or if
they were distinctly different strains that tended to be both attached and unattached.
If one noted just the colony morphological characteristics, then the McBean aquifer
seemed to have a pretty sharp difference on both media. The colony characteristics
of the attached isolates were quite different from those of the presumably unattached
isolates. In the Ellenton aquifer, it was not quite as sharp of a break. For example,
on the PTYG, four of the six attached isolates were quite different morphologically
from the unattached ones. Two of them, however, appeared to be identical.
Therefore, there may have been some overlap. There may have been some strains
that showed up both in the attached and unattached fractions, yet there were some
obvious differences between the two groups.

In hopes of getting a little more detailed information, API-NFT tests were done
to see if these organisms continued to appear different in terms of their physiological
traits. The results for the McBean aquifer were similar to those seen with the colony
analysis. In the McBean aquifer, a couple of the attached isolates turned out to be
virtually identical to the unattached ones in their physiological traits.

A similar picture could be seen in the Ellenton aquifer. The attached and
unattached portions of the microflora consisted largely of distinct strains that could
at least be distinguished on the basis of their colony morphology and their
physiological traits.

Physiological studies were also done in order to look at group responses to the
test and the system. The PTYG and 1% PTYG isolates from the McBean and the
Ellenton were lumped together for analysis. The attached and unattached groups
responded quite differently to some of the tests, and that was true for both
formations. However, the attached isolates in the McBean did not differ from the
unattached in the same way that the Ellenton attached differed from their unattached counterparts. It is apparent that more sediment samples need to be observed and obviously, more physiological tests must be included, perhaps switching to something like the Biolog system or one of the other systems that includes a greater number of characteristics. However, there did seem to be some physiological differences between the two groups and it is an interesting subject that could be pursued somewhat further.

One last experiment that was completed, just for the sake of curiosity, was the addition of some nutrients to one of the samples to see what would happen to the attachment picture. A sediment sample from the Pee Dee formation at a depth of 256 m was chosen because the sample volume that was required for the analysis. A small amount of sodium acetate was mixed with this sample, approximately 0.2 mg/g of sediment. The material was incubated aerobically for 35 days at 23°C, and periodically, 5 g of material were removed. It was then subjected to the centrifugal washing procedure that was discussed earlier, and then the sample was plated on 5% PYTG. This was sort of a compromise medium when only one medium was used. The total number of viable cells in the samples at each stage were determined, followed by the estimate of the percentage of cells that seemed to be attached.

The results were expressed in "days" after the addition of the nutrients. Initially, about 67% of the organisms were attached to the extent that they could not be removed in the first three washings. After the nutrient addition, the numbers increased approximately two orders of magnitude in the first week. Then the total numbers of viable cells remained constant throughout the rest of the experiment. The percentage of attached organisms dropped rather rapidly after the addition of nutrients and slowly began to come back again throughout the rest of the experiment. Of course, in a situation like that, one would tend to think that the cells that do appear as a result of nutrient addition, the new cells, are not attached or are not attaching very rapidly; therefore, they are very easy to remove from the sediment material in the washing procedure. Later on they may begin to start attaching to the surface as the nutrients run out. Such an assumption may have some interesting implications if that is really what is occurring. Of course, we do not know that for sure. However, it may have some implications as to the types of things that can happen if one throws nutrients into an aquifer in an attempt to stimulate the population to degrade organics. There could be a shift in terms of where the organisms are, whether they are on the surfaces or not.

**Conclusion**

As was previously mentioned, this was just a preliminary investigation leading to some preliminary results. It is not an area that is likely to be pursued much further in our lab because there are too many other things going on that are now a higher priority. However, I wanted to shed some light on this material in hopes that the topic could be pursued further, especially in labs where they have a little more expertise in dealing with things like attachment. I would very much like to see some of this pursued further. Thank you.
Q and A

* A. Mills: David, are the isolates you sent me among the isolates you talked about today?

* D. Balkwill: Yes they are.

* A. Mills: We are going to be doing some studies of cell-surface properties, like hydrophobicity and so forth, when we do some attachment studies with these organisms. Perhaps in a little while we will have some more information to add to your data collection.

* D. Balkwill: Good.

* J. Wilson: Dave, what is the benefit of attachment?

* D. Balkwill: Well, much of the standard dogma is that if the nutrients are low, the organisms are attached to the surface, and there may be a concentration of nutrients in the layers that are close to the surface. There seems to be some correlation from these experiment. Often if there is a lot of organic nutrient around. I think there is less attachment, so that might be part of it.
Analysis of Environmental Factors Affecting Abundance and Distribution of Bacteria, Fungi and Protozoa in Subsurface Sediments of the Upper Atlantic Coastal Plain, USA

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Abstract

Exploratory statistical analyses of microbiological, hydrological, and geochemical data for samples from four boreholes drilled into the Upper Atlantic Coastal Plain sediments near the Savannah River Site in Aiken, SC showed highly significant correlations between bacterial abundance (AODC and CFU) and hydraulic conductivity (K). Sediment texture variables % sand (S), % silt, % clay (C), and S/C were strongly interrelated with K and therefore, also correlated with bacterial abundance. AODC did not correlate with the concentrations of dissolved inorganic nitrogen (DIN) or dissolved organic carbon (DOC) in pore water. CFU also did not correlate with DIN, but a negative relationship was found between the CFU and DOC for sandy sediments, suggesting that microbial activity may control pore water DOC concentration. In some, but not all boreholes, AODC and CFU correlated negatively with the pore water concentrations of metals and positively with pH. Protozoan abundance correlated strongly with AODC and CFU in the two boreholes closest to the recharge areas for their major aquifers. It also correlated with sediment texture variables, but not with K. Fungal abundance did not correlate with the abundance of other microbial types when data from individual boreholes were considered; however, it did correlate with both bacterial and protozoan abundance when data from all four boreholes were combined. There was no relationship between fungal abundance and either K or sediment texture. We conclude from this study that hydraulic conductivity and sediment texture are closely related master variables that predict bacterial population density in these subsurface sediments. Metal concentrations and pH are less consistent in their predictive value. Neither nitrogen nor carbon appeared to be growth limiting factors. Protozoan abundance was at least partly controlled by factors regulating bacterial populations, but the environmental factors regulating fungal populations are not clear.
Introduction

Until recently the terrestrial subsurface was not recognized as a habitat for microorganisms, much less an ecosystem. Instead, it was generally assumed that little, if any, life existed in subsurface regions beneath the root zone. Petroleum and mineral exploration industries had reported microbes in deep geological formations bearing petroleum and sulfur, but because aseptic sampling techniques were not routinely employed, these reports were not given much credence by microbial ecologists. In the early 1980s, concerns about groundwater pollution led to renewed interest in investigating the potential for biological activity at depth in terrestrial systems. Subsequently, several research groups obtained subsurface sediments using aseptic coring techniques. The results of these earlier studies showed clearly that diverse and active communities of bacteria, and even fungi and protozoa, can be found in aquifer sediments, even at depths of several hundred meters. Thus, the ground work was laid for a new subfield of ecology focused on subsurface microbial processes.

The objectives of subsurface microbial ecology are similar to those of general ecology: (1) describe the abundance, distribution and activity of resident populations, in this case bacteria, fungi and protozoa, and (2) identify the environmental controls on these populations. Considerable progress has been made towards describing where and in what densities microorganisms are found in aquifer systems, although many more sites must be examined before broad generalizations can be made. By contrast, little research has been directed towards the more difficult task of identifying environmental controls.

There are two basic approaches used by ecologists to identify controlling environmental factors. First, samples can be collected from the environment under study, and their biological, chemical and physical characteristics quantified. The data then can be analyzed statistically for correlations between populations densities and environmental factors. Secondly, samples can be studied in laboratory or field experiments in which a single environmental variable is manipulated while others remain constant. The response to a single variable will reveal whether it is a controlling factor. The two approaches are best used in concert, as each has its own strengths and weaknesses. Correlation analysis suffers from its inability to establish causality. Often it is unclear which of two related variables is independent. Worse, two variables may correlate not because they influence one another, but because they are both related to a third independent factor. Through stringent controls, laboratory experiments may provide reasonable proof of cause and effect. However, experimental conditions often differ so much from those in situ that extrapolation of laboratory results to the environment is risky.

The most intensive study of deep subsurface microbiology to date has been the Deep Probe Project of the United States Department of Energy (DOE). In 1986, three boreholes were drilled through Upper Atlantic Coastal Plain sediments on DOE’s Savannah River Site (SRS) in Aiken, SC to depths between 210-280 m. Two of these boreholes, P28 and P29, were drilled within 3 km of one another, while P24 was drilled approximately 10 km to the southeast and down-dip relative to the prominent
aquifers. A fourth hole, C10, was drilled in 1988 and penetrated the ground to a depth of 530 m. It was located outside the SRS, approximately 20 km southeast of P24. In this paper, we have used statistical correlation analyses to explore the relationships between microbial population densities and the physical and chemical properties of samples from the four boreholes. Despite evidence that drilling fluids penetrated some samples, and may have compromised their pore water chemistry, the pore water chemistry data gathered during the Deep Probe study are included in our analyses. We believed it was better to seek correlations in less than perfect data than to ignore these data all together.

**Materials and Methods**

The population density measurements included in the analysis were those measured in our laboratory at Cornell University, using methods described previously: viable protozoa and fungi (from most probable number techniques), total bacteria (acridine orange direct counts, AODC), and viable bacteria (aerobic spread plate counts, CFU, on a dilute Peptone-Tryptone-Yeast extract-Glucose medium, PTYG). In addition, we calculated the ratio of log CFU/log AODC as an index of potential for heterotrophic growth. We did not include in our bacteriological analysis data from any sample for which there was evidence of contamination by drilling fluid bacteria.

We included the following physical and chemical measurements in the analysis: the sediment texture variables, which included % sand, % silt, % clay, sand/clay ratio, and log S/C (for both International and American units), hydraulic conductivity, pore water pH, and the pore water concentrations of dissolved inorganic nitrogen, dissolved organic carbon, Al, Cd, Cu, Fe²⁺, Fe³⁺, Ni, and Zn. Some variables of interest, such as dissolved phosphorus concentration, were not included in the correlation analysis because too many of their measured values, over half, were below the detection limit. Non-numeric data, such as those <0.01, cannot be used in correlation analysis.

Hydraulic conductivity estimates made on cores using a permeameter, were obtained from the South Carolina Water Resources Commission. K. Sargent (Furman Univ., SC) provided additional estimates of K for sandy samples, based on grain size distribution. Data on pore water chemistry and sediment texture were provided by Pacific Northwest Laboratory (methods described in Fredrickson et al.).

During part of the Deep Probe sampling program, ethanol was used for cleaning. In our analysis, we did not include carbon data from samples shown to be contaminated with ethanol during microbiological subsampling.

Scatter plots were made for each pair of variables, and the standard correlation statistic, the Pearson Product Moment Coefficient (r), was calculated employing the Data Desk computer program (Odesta Corporation). The data on metal concentrations, hydraulic conductivity, and biological densities were transformed to logarithmic numbers before statistical analysis to compensate for wide ranges in magnitude and lognormal distributions. Chemical concentrations and microbial densities that fell
below the limit of detection for the assays employed were assumed to be at the limit of detection. Standard tables were used to determine the statistical significance (p < 0.05) of each r value based on the number of data pairs available for analysis. Variables for individual borehole profiles and for individual geological formations across the four boreholes were compared using two-tailed T-tests (p < 0.05).

Results

**Microbial Distribution and Abundance.** Visual inspection of the vertical profiles for distribution of bacteria in each of the four boreholes (Figure 1) suggested that bacterial density does not decrease progressively with depth. Correlation analysis supported this conclusion: when data from all four boreholes were considered, the r values for both log AODC/gdw vs. depth (r = -0.21) and log CFU/gdw vs. depth (r = -0.05) were below the threshold for statistical significance (r < -0.25). Moreover, correlation between our index of potential for heterotrophic growth, log CFU/log AODC, and depth was very close to zero (r = -0.01). Although fungi and protozoa were present in lower numbers than bacteria, their population densities also showed no decreasing trends with depth (log fungi vs. depth, r = -0.20; and log protozoa vs. depth, r = -0.26). The threshold value for significance here was r = -0.30 because the sample size was smaller than for other groups.

The variability within each vertical profile was substantial. AODC varied over two orders of magnitude and CFU over seven orders of magnitude (Figure 1). Some of this variability might be attributed to variations of environmental factors between geological formations. Therefore, we compared the mean densities of bacteria (AODC and CFU), fungi and protozoa, within each geological formation (Table 1). These results suggested that some formations are more biologically productive than others. In other words, some formations (e.g., the Congaree) have higher mean population densities horizontally over the four sample sites than others (e.g., Tobacco Road). Attractive as this suggestion may be, our T-tests did not support it. No pair of mean values for individual formations differed at a significance level of < 0.05. This lack of statistical support for the data in Table 1 was surprising and it may be explained by the small number of samples (3-17) obtained from each formation. Alternatively, facies changes within a geological formation, which represent changes in depositional environment over time or space, could cause substantial variability in environmental factors within a formation, and thus have led to the variation we observed in microbial densities.

The horizontal distribution of microorganisms was examined by comparing the mean values for biomass estimates in the vertical profiles of each borehole (Table 2). It was found that bacterial (AODC and CFU), fungal, and protozoan population densities all decreased along the transect between the P28-P29 cluster and C10 (Table 2). T-tests indicated that for bacteria, the differences among the profile means for P24, P28, and P29 which were separated by 3-10 km, were not statistically significant, while the mean for C10, which was 20-30 km downdip from the other boreholes, was significantly lower than the others. C10 was a much deeper borehole than the others (530 m vs. <300 m), but depth did not explain its overall lower
biomass. The bacterial densities in C10 samples from above 300 m depth were as low as, or in some cases, lower than those in samples from deeper than 300 m (Figure 1). For fungi, the boreholes fell into two groups. P28 and P29 contained higher fungal biomass overall than P24 and C10. Within these two groups, the borehole mean values for fungal biomass were not significantly different. The values for protozoan biomass were also lowest in the C10 borehole. Protozoan densities differed significantly in each borehole, except for the P24-P29 pair, whose means could not be distinguished statistically.

**Correlations between bacterial abundance and environmental factors.** As anticipated from previous work, both total (AODC/gdw) and viable (CFU/gdw) bacterial densities correlated with several sediment texture variables; negatively with % silt and % clay (C), and positively with % sand (S), S/C, and log S/C at a significance level of <0.05 (Table 3). In addition, significant relationships were found between both log AODC and log CFU and hydraulic conductivity (K). However, the magnitude of r for these last relationships was strongly influenced by a few samples with low K values; only seven of 42 K values reported in the Deep Probe study fell between 10⁻⁸ and 10⁴, whereas 35 were between 10⁻³ and 10⁻¹. Log K and the sediment texture variables were highly interrelated (r > 0.6 or < -0.7).

Neither AODC nor CFU correlated at a statistically significant level with the dissolved inorganic nitrogen (DIN) concentration in the pore water (Table 3), an indicator of nitrogen availability. Furthermore, despite the dominance of subsurface microbial communities by aerobic heterotrophic bacterial populations, bacterial biomass estimates did not correlate significantly with dissolved organic carbon (DOC) concentration. For CFU the weakness of the relationship to DOC may have been caused by a small group of clay-rich samples from which few bacteria were cultured. When only sandy samples (sand ≥ 70%) were included in the analysis, a significant negative relationship (r = -0.48) was obtained for CFU vs. DOC. Here it is interesting to note that C10, which had a significantly lower bacterial biomass than the other boreholes, also had the greatest mean DOC concentration, 8 mg/l, compared with means of 2.5, and 3 mg/l in boreholes P24, P28, and P29, respectively (all these means were significantly different at a level of 0.05).

Analysis of the combined data set for the four boreholes showed no correlations between bacterial biomass (AODC or CFU) and pH (Table 3); however, significant relationships were found when data were examined borehole by borehole. Pore water pH values correlated positively with AODC in boreholes P28 and P29, and with CFU in P24, P29 and C10. Lack of correlation for the overall data set may be explained by the range of pH values represented. Neither very high ambient pH nor very low ambient pH would favor prolific microbial growth on the neutral pH plate-count medium; an optimum environmental pH would be expected to occur nearer to neutrality. The lack of correlation might also be explained by nonlinearity of the relationship between microbial biomass and pH. For non-linear relationships, the correlation analysis we employed is not appropriate.
In borehole P29, AODC and CFU correlated significantly (and negatively) with the pore water concentrations of several metals: Al, Cd, Cu, Fe cations (Fe$^{2+}$ + Fe$^{3+}$), Ni, and Zn. Fewer of such relationships were found in P28. AODC correlated negatively with Al and Zn, and CFU correlated only with Zn. No such correlations were found for P24 and C10. Strong relationships between biomass and metals in P29 (-9.0 < r < -7.0) biased the significance of relationships within the overall data set. AODC correlated with Zn, and CFU with Al, Ni, Cu and Zn (Table 3).

**Correlations between microeucaryote abundance and environmental factors.** Relationships between fungal density and environmental factors changed from borehole to borehole. Furthermore, the combined data set for all boreholes yielded a different set of relationships than was found for the individual boreholes. For borehole P28, none of the measured environmental factors were related to fungal biomass. At P29, CFU, K, % sand, S/C, pH, and the concentrations of DIN, Al, Ni, and Cd all correlated positively with biomass, while Fe cations and % clay correlated negatively. For borehole P24, there was a negative correlation with Zn, and for C10, a positive correlation with Fe cations. The overall data set indicated positive relationships between fungi and all three of the other biological parameters (AODC, CFU, and protozoa), negative relationships with DOC and pH, and a positive relationship with DIN concentration (Table 3). It showed no relationship between fungal density and either the sediment texture variables or K.

Protozoan density correlated strongly with AODC and CFU (r > 0.7) at the two up-dip sites (P28 and P29), but not at the lower sites (P24 and C10). No relationship between protozoan abundance and pore water chemistry was indicated for boreholes P28 and P29. At P24 and C10 significant correlations were found between protozoan biomass and the concentrations of Fe cations (negative) and Ni, respectively. Protozoan density correlated with K only in borehole P29. It correlated with sediment texture variables both in this borehole and in P28. Using the entire data set (Table 3), we found significant relationships between protozoa and each of the following: AODC, CFU, fungi, and negatively with sediment texture variables of % sand, % clay, log S/C, fungi, and Cd.

**Discussion**

This study provides statistical support for the now common observation that below the root zone and down to several hundred meters, microbial abundance does not diminish progressively with depth. However, this observation should not be interpreted to imply that microbial biomass is uniform within subsurface sediments. To the contrary, the microbial distribution and abundance data set examined in this work showed considerable spatial variability. Over vertical profiles, bacterial, fungal, and protozoan densities fluctuated over at least two orders of magnitude, and boreholes 3-30 km apart had significantly different profile means for microbial population densities (Table 2).

We hypothesize that underlying the observed spatial variability in microbial abundance are physical and chemical factors that control growth and that reflect
environmental heterogeneity. Our long term objective is to identify the factors
controlling growth. This would enable us to predict the influence of environmental
factors on the function of subsurface ecosystems.

Several Deep Probe investigators\textsuperscript{2,5,9,11,17} have already reported relationships
between bacterial biomass or activity, and sediment clay content or S/C ratio. We
confirmed these relationships, and also found that other sediment texture variables
(\% S, log S/C) correlated with abundance of microorganisms (Table 3). Sediment
texture may simultaneously affect many other environmental factors important to
microorganisms, such as hydraulic conductivity (K), pore size, and water availabil-
ity. Thus it would be desirable to examine the influence of these sediment-texture
related factors independently.

In this work, we were able to examine the influence of K. Our statistical analyses
showed that bacterial density, both AODC and CFU, correlated with K at a level of
statistical significance similar to that of the density-sediment texture relationship
(Table 3). In addition, K and the sediment texture variables were all highly
interrelated. Thus, it appears the impact of sediment texture on distribution and
abundance of microbial biomass may be linked to its affect on K. In turn, the
influence of K is likely to be linked to its influence on nutrient and electron acceptor
supply rates.

The productivity of most ecosystems is nutrient limited, usually by nitrogen in
forests and coastal oceans, or by phosphorus in freshwater. Unequivocal evidence
for nutrient limitation usually is achieved through experimentation. However,
exploratory statistical analyses such as those presented in this paper can provide
some clues about the regulatory roles of nutrients. In the Deep Probe sediments,
neither carbon nor nitrogen appeared to be growth limiting, as indicated by lack of
strong positive correlations between microbial abundance and the DOC and DIN
concentrations of pore water. However, these estimators of carbon and nitrogen
availability neglect nutrients associated with particles (either adsorbed on sediments
or temporarily stored in the microorganisms), and they do not adequately reflect
nutrient levels in through-flow water. DOC and DIN values can be taken as measures
of standing stock, but they are really residual quantities which reflect an instantan-
eous balance between pore water nutrient sources (mineralization, desorption,
inflow, etc.) and sinks (microbial uptake, adsorption, outflow, etc.).

Nevertheless, nutrient concentrations can provide valuable information about
relationships between environmental supplies and microbial demand. When S>>D,
the dissolved concentration of a nutrient is controlled by abiotic processes which is
to say that microbial uptake would be a small flux relative to other fluxes. This seems
to have been the case for nitrogen in the Deep Probe studies in that neither the AODC
nor the CFU of bacteria correlated with pore water DIN. Furthermore, many
sediment samples showed relatively high pore water DIN concentrations (mg/l)
when compared to the concentrations in N-limited pelagic ecosystems, typically 1
\(\mu g/l\).\textsuperscript{10,18} Drilling fluid intrusion could not be blamed for the high concentrations in
pore waters. In most instances, the DIN concentration in drilling fluids was below
the detection limit.
Microbial processes become important in controlling the dissolved concentrations of nutrients as the magnitude of supply approaches that of demand (see Levine for details).\textsuperscript{18} Provided that \( S > D \), an inverse relationship between biological density and nutrient pool size should develop. Such a relationship was found for CFU and DOC concentration in the Deep Probe samples. Drilling fluid intrusion could not have produced this negative relationship since both microbial densities and DOC concentration were greater in drilling fluid than in most of the sediments.

For a growth-limiting nutrient, \( S = D \) at steady state. Equality is maintained by microbial populations, whose demands rise and fall with supply. The dissolved pools of limiting nutrients are as fully depleted as is physiologically possible at all microbial densities. None of the nutrients which we examined in the Deep Probe data set showed the characteristics of a limiting nutrient. However, we did not attempt to correlate bacterial density with phosphorus concentration, for the pragmatic reason that total phosphorus was undetectable in almost every pore water sample. Although low pore water phosphorus concentrations might be attributable in part, or wholly to abiotic processes such as phosphate precipitation and adsorption onto sediment particles, it is also possible that they reflect exhaustion of phosphorus resources by microorganisms. Evidence supporting the latter hypothesis was presented by Phelps and White,\textsuperscript{5} who found that phosphate additions to subsurface sediments consistently stimulated \(^{14}\text{C}-\text{acetate} \text{ incorporation into lipids, whereas nitrogen and sulfur amendments only occasionally had this effect.}"

**Conclusion**

The exploratory statistical analysis reported in this paper sets the stage for experimental work to test hypotheses concerning nutrient limitations on microbial abundance in oligotrophic subsurface sediments. Such experimental work should also lead to a better understanding of the physical and chemical factors affecting microbial activity in these sediments.
References


### Table 1. Mean microbial densities for different geological formations. Only formations from which three or more samples were obtained are included here.

<table>
<thead>
<tr>
<th>Formation</th>
<th>Logarithm of numbers per gram dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AODC</td>
</tr>
<tr>
<td>Tobacco Road</td>
<td>6.62</td>
</tr>
<tr>
<td>Dry Branch</td>
<td>6.54</td>
</tr>
<tr>
<td>Griffins Landing</td>
<td>6.80</td>
</tr>
<tr>
<td>Congaree</td>
<td>7.51</td>
</tr>
<tr>
<td>Ellenton</td>
<td>6.90</td>
</tr>
<tr>
<td>Pee Dee</td>
<td>6.81</td>
</tr>
<tr>
<td>Black Creek</td>
<td>6.97</td>
</tr>
<tr>
<td>Middendorf</td>
<td>6.89</td>
</tr>
</tbody>
</table>

### Table 2. Vertical profile means for microbial biomass within the four boreholes at the Savannah River Site.

<table>
<thead>
<tr>
<th>Borehole</th>
<th>Logarithm of numbers per gram dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AODC</td>
</tr>
<tr>
<td>P24</td>
<td>6.87</td>
</tr>
<tr>
<td>P28</td>
<td>7.07</td>
</tr>
<tr>
<td>P29</td>
<td>7.02</td>
</tr>
<tr>
<td>C10</td>
<td>6.40</td>
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</table>
Table 3. Correlation coefficients (r) for relationships between microbial density in SRS sediments and geochemical and physical factors

<table>
<thead>
<tr>
<th>Physical Variables</th>
<th>Log AODC</th>
<th>Log CFU</th>
<th>Log Fungi</th>
<th>Log Protozoa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sediment Texture</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>International Units</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Sand (S)</td>
<td>0.57*</td>
<td>0.59*</td>
<td>0.14</td>
<td>0.39*</td>
</tr>
<tr>
<td>% Silt</td>
<td>-0.45*</td>
<td>-0.52*</td>
<td>-0.24</td>
<td>-0.36</td>
</tr>
<tr>
<td>% Clay (C)</td>
<td>-0.56*</td>
<td>-0.57*</td>
<td>-0.09</td>
<td>-0.36*</td>
</tr>
<tr>
<td>S/C</td>
<td>0.58*</td>
<td>0.53*</td>
<td>0.17</td>
<td>0.44*</td>
</tr>
<tr>
<td>log S/C</td>
<td>0.58*</td>
<td>0.58*</td>
<td>0.12</td>
<td>0.39*</td>
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<tr>
<td>American Units</td>
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</tr>
<tr>
<td>% Sand (S)</td>
<td>0.50*</td>
<td>0.62*</td>
<td>0.11</td>
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</tr>
<tr>
<td>Log K</td>
<td>0.54*</td>
<td>0.53*</td>
<td>0.07</td>
<td>0.18</td>
</tr>
<tr>
<td>Chemical Variables</td>
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<td></td>
</tr>
<tr>
<td>DOC</td>
<td>-0.10</td>
<td>-0.23</td>
<td>-0.29*</td>
<td>-0.16</td>
</tr>
<tr>
<td>DIN</td>
<td>0.12</td>
<td>0.23</td>
<td>0.33*</td>
<td>0.11</td>
</tr>
<tr>
<td>pH</td>
<td>-0.03</td>
<td>0.17</td>
<td>-0.40*</td>
<td>-0.28</td>
</tr>
<tr>
<td>Metallic Cations</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>log Al</td>
<td>-0.23</td>
<td>-0.28*</td>
<td>-0.11</td>
<td>-0.06</td>
</tr>
<tr>
<td>log Cd</td>
<td>-0.18</td>
<td>-0.24</td>
<td>-0.13</td>
<td>-0.50*</td>
</tr>
<tr>
<td>log Cu</td>
<td>-0.11</td>
<td>-0.26*</td>
<td>0.21</td>
<td>0.54*</td>
</tr>
<tr>
<td>log Fe^{2+} + Fe^{3+}</td>
<td>0.03</td>
<td>-0.19</td>
<td>0.05</td>
<td>0.17</td>
</tr>
<tr>
<td>log Ni</td>
<td>-0.12</td>
<td>-0.32*</td>
<td>-0.11</td>
<td>-0.13</td>
</tr>
<tr>
<td>log Zn</td>
<td>-0.27*</td>
<td>-0.39*</td>
<td>-0.01</td>
<td>0.06</td>
</tr>
</tbody>
</table>

Data from all four boreholes are combined. Statistical significance (p < 0.05, *) was indicated for bacteria (N=60) at r > 0.25 or < -0.25, for fungi (N=56) at r > 0.26 or < -0.26, and for protozoa (N=44) at r > 0.30 or < -0.30. The r for individual boreholes are available from the authors at request.
Figures

Vertical Distribution of Bacteria

Figure 1. Vertical profiles for AODC and CFU densities within four boreholes at the Savannah River Site in Aiken, SC.
Q and A

B. Benoit: Very little has been heard at this meeting about autotrophs. Is there any evidence when you go through the information that these organisms are present at any point, specifically, mixotrophs? Would there be any advantage in using iron or ammonia, or are we excluding this as a possibility?

S. Levine: Autotrophs were looked at to some extent and algae was present in the upper sites. I did not include them in the correlation analysis because we have some reservations of how long the populations have been there because there has been a lot of drilling in that area, and they may have been newly introduced.

J. Fredrickson: A quick comment on that last question. We did look for autotrophs in the first three boreholes. In fact there is some information that is published in the Geomicrobiology Journal on our findings. For the most part, we found relatively low abundances of lithotrophic bacteria, like ammonium-oxidizing bacteria or the iron-oxidizing bacteria. In most instances, they were basically at or below the detection limits, but there were indications of positive activity in certain samples.

T. Hazen: Since C10 was the last hole and had lower densities than some of the other holes, whereas the first holes had the higher densities and there were changes in tools and sampling procedures, how confident are we that reduction in density is not due to reduction and contamination or to changes in tools and that sort of thing?

S. Levine: I will defer that question to someone else, to Bill, because he actually was in charge of the count.

W. Ghiorse: It really depends on what you mean by contamination, Terry. As far as our counting procedures go, we are very confident that we have good procedures and we know how to count with the direct count and the plate counts. In terms of contamination, I have always seen P28 and 29 as areas of the Savannah River Site that perhaps have a lot of contamination problems on the surface that might have somehow gotten into the aquifers that we sampled. I am not saying that the samples were contaminated when they were taken, but the sediments being closer to recharge areas or infiltration areas may have been influenced by surface activities. I have always looked especially at the P28 Congaree sample as an outstanding enrichment of bacteria.

T. Phelps: I would like to rephrase Terry's question in a different way, especially in light of Bill's comment about the Congaree being an excellent aquifer. We sampled more clays than aquifers at C10. The ratio of clay to aquifer samples was far greater in part because of drilling fluid contamination. I am sure Bill would agree that he did not receive the sample of C10 Congaree aquifers because of its large aquarium
gravel, for which drilling muds had completely penetrated, and thus we could have never obtain samples with any degree of confidence. So I would suggest that the reason C10 counts were lower may have been a result of higher clay and more clay samples taken, and subsequently fewer aquifer samples taken.
HYDROGEOLOGY IN RELATIONSHIP TO MICROORGANISMS

Round Table 3
Cooperation Between Microbiologists, Hydrologists,
Geologists and Drillers and Modelers
Conveners: C. B. Fliermans and K. A. Sargent
Importance of Site Liaison to Successful Completion of Large-Scale Multidiscipline Drilling and Sampling Programs

H. W. Bledsoe, Westinghouse Savannah River Company, Aiken, SC.

Abstract

On large-scale projects where investigators with differing objectives and requirements are involved, the timely and successful completion of the program rests on effective communication and cooperation between the different disciplines. Given the imitations imposed by schedule, budget, and the goals of the program, the role of the site liaison is to assimilate the different requests of the investigators and evaluate those requests as to the feasibility for successful completion. This would include the evaluation of different sampling tools or changes in the drilling method in order to obtain an optimum sample. A geologic sample that is perfectly adequate for stratigraphic and hydrogeologic analysis may be totally unacceptable for microbiological purposes, due to excessive contamination by drilling fluids. The site liaison has to make the decision as to when the direction of the drilling program should be changed, modified, or stopped in order to meet the overall needs and requirements of the program. Of primary importance is the control of the field program budget. If drilling and sampling activities have necessitated modifications or additions, the liaison has to be prepared to document and justify the deviations and may have to pursue additional funding if the program is to achieve the intended results. This may require renegotiation of contracts, especially if costly drilling problems have been experienced on the project.

No paper submitted.
Interdisciplinary Collaborations for Successful Subsurface Science Field Operations

T. J. Phelps, University of Tennessee, Knoxville, TN.

Abstract

Successful field operations rely upon constructive collaborations between interdisciplinary scientists, technical personnel and contractors. Each scientific discipline should be involved in planning and implementation. The bidding specifications represent an opportunity to require experienced contractors, to establish goals, expectations, lines of communication, and to serve as a major determinant of onsite collaborations. Each site participant should be orientated to a single goal and cognizant of their responsibility. Job descriptions for each participant are essential. Description of functions required within the group of collaborators is important and may require alterations as needs change. Availability of managers upon demand and regular meetings between all parties at the site are required. Independent space for each group at the site is desirable. Fluidity must be built into the system because program success will hinge upon problem solving capabilities.

No paper submitted.
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T. J. Phelps, University of Tennessee, Knoxville, TN.

Abstract

Successful field operations rely upon constructive collaborations between interdisciplinary scientists, technical personnel and contractors. Each scientific discipline should be involved in planning and implementation. The bidding specifications represent an opportunity to require experienced contractors, to establish goals, expectations, lines of communication, and to serve as a major determinant of onsite collaborations. Each site participant should be orientated to a single goal and cognizant of their responsibility. Job descriptions for each participant are essential. Description of functions required within the group of collaborators is important and may require alterations as needs change. Availability of managers upon demand and regular meetings between all parties at the site are required. Independent space for each group at the site is desirable. Fluidity must be built into the system because program success will hinge upon problem solving capabilities.

No paper submitted.
Abstract

Obtaining aseptic core samples at great depths is crucial for microbiological technology. This paper outlines how to achieve successful specialized drilling projects. The project completion is dependent upon client-contractor relationships, contract specifications, and prompt efficient field changeorders. The fabrication of specialized tools in the field is essential for project completion. Using an experienced drilling crew is extremely helpful in situations where new techniques and equipment are employed.

The onsite field coordinator (microbiologist, hydrologist and geologist) must have complete knowledge of equipment and crew capacity. This knowledge, combined with good client-contractor understanding of what is expected of each party, will ensure a timely completion.

The final completion of the project requires site evacuation which meets client expectations. Finalizing the billing discrepancies and how to deal with the contractor are important in maintaining a good working relationship for future projects.
Introduction
Graves Environmental and Technical Services Company is a diversified drilling company specializing in environmental drilling. Graves' 40-plus years of experience and proficiency as a leading water well contractor made us a "natural" for groundwater monitoring programs. Graves initially became affiliated with the Department of Energy (DOE) when we purchased Professional Services, Inc. (PSI) in 1987. The people employed with PSI had worked with DOE on the Savannah River Plant before 1987 on similar projects, even aseptic sampling projects. Through this relationship, Graves was invited to submit a proposal in May 1988.

The request for proposal outlined three major objectives for this project. These objectives were as follows:

1. Observation wells needed to obtain geohydrologic data in order to define the stratigraphy and groundwater conditions.

2. Data that previously recovered microorganisms were not a result of drilling operations.

3. Predictability of microbial biomass, community structure, nutritional status, and metabolic activities serve as a function of groundwater chemistry, hydrology, and sediment structure.

These project objectives translated into the following objectives for Graves in order to make the project a success:

1. Collection of continuous geologic samples from the first well to identify stratigraphy and selected microbial samples.

2. Collection of 300 feet of selected (aseptic) samples from the second well.

3. Leave high-quality observation wells for long-term monitoring of water quality and quantity.

Discussion
In June of 1988, Graves was awarded the contract to perform this project. The contract had many similarities and differences when compared to typical drilling contracts. The contract outlined objectives, specifications, equipment, requirements, pricing, schedule, insurance requirements, and general provisions of the projects. This format was typical of most drilling contracts. However, the major differences were the warranty requirement, payment schedule, and the unique project scope.

After executing the contract, project operations began. The key components of successful operations were project planning, qualified personnel, capable and reliable equipment, coordinated work schedule, and communications between the contractor and the client.
Project planning began by developing a sequence of operations and requirements for personnel, equipment, and materials to meet the project specifications. Developing a project plan was imperative. This plan, although ever changing, is used as a guide to operations and must be communicated to field crews and clients alike.

In a project of such unique requirements, qualified people are crucial. Even the best developed plans will fail if not conducted by experienced and knowledgeable personnel. This experience and knowledge must be a basic understanding of drilling techniques and methods along with the ability to visualize abstract concepts.

Equipment of the proper size is almost as important as qualified personnel. Requirements outlined in the specifications must be well within the range of the equipment used. Equipment must be in good working condition to reduce downtime and poor quality samples caused by mechanical failure. If equipment does break down, the contractor should have qualified personnel available to immediately correct the problems.

Crew fatigue and exhaustion were bound to surface on a project which required working twenty-four hours a day, seven days a week. Two crews are a minimum requirement to operate this schedule. A relief crew is the logical and preferred solution to only two crews. This additional crew allows one of the other crews time to rest. A schedule with these three crews may be fourteen twelve-hour shifts working and seven days off. Exhaustion and fatigue can be minimized by alternating crews. The project Graves recently completed used only two crews. Our crew’s involvement lessened the effects of exhaustion and fatigue. Constant interaction between the crew and the client’s field coordinator creates this involvement. The crew must be recognized for their efforts, ideas and contributions. The crew thrived on positive reinforcement, and this recognition drives them to develop more innovative ideas, ignore the long hours and adapt to the constant changes. Constant communication and feedback from the crew attributed to the project’s success.

The communication between scientist and drilling contractor is the single most important ingredient to a successful project. By spending time with the contractor evaluating procedures, objectives and discussing difficulties that arise will allow the scientist to minimize changeorders and field problems, and to improve quality data.

Once the contractor is selected, daily communication concerning schedules, equipment, site conditions, data, etc., will ensure proper initiation of the drilling project. Communication is extremely crucial

- during preparation of the original contract
- during contract negotiations
- before mobilization of equipment
- on site while operations are progressing
• during any change orders and/or addendums to the original contract scope or pricing

• before finalizing the project

The contractor should be involved in as much project discussion as possible. Even when some discussions may not address the operation as it directly affects the contractor, they can provide valuable insight into the client's needs.

Communication between contractor and client is one of many crucial interactions. Communication with vendors, the public, and others are just as crucial. An example of this type of communication problem was provided with the wire-line sampling equipment. Wire-line coring equipment had been specified and aseptic samples were the client's project goal. The manufacturer of the sampling equipment failed to understand the primary objective of acquiring samples in an aseptic state. Mechanisms were incorporated into "assembly-line" equipment, theoretically enabling uncontaminated samples to be collected. However, the equipment's primary objective was to enable manufacturers to recover the maximum core sample in the shortest time possible; therefore, reducing the overall cost per foot. On this particular project, 100% recovery of a contaminated sample was worthless. The initial design concepts were good, but the concessions made to improve recovery defeated the primary project goal. However, once this was understood by client, contractor, and vendor, an effective sampling system was developed. We accomplished this by utilizing a team effort of onsite and offsite fabrication, mixed with constant modifications and learning from success and failure.

Recognizing and accepting variations in the contract scope is also very important. This allows the project objectives to be met while also allowing the drilling contractor to maintain acceptable profit margins. It is impossible to anticipate or expect all problems which may occur when dealing with subsurface conditions. Contractor and client must be willing to negotiate change orders and addendums. These items should not be perceived as negative, but as a positive tool which allows the operation to be more efficient and flexible. Without proper communication however, a change order and possibly the project will not succeed.

Regardless of project objectives, a project is truly successful when properly finalized. When the site disposition meets the client's expectations and all billing discrepancies are resolved, only then is the project completed. The best way to ensure proper project completion is effective communication between the client and the contractor. In order to minimize problems at the end of the project, the following tools should be considered:

• daily quantity sheets agreed upon daily by the client and the contractor,

• daily project logs by the client and the contractor, and
- a punch list of items to be completed or corrected before removing equipment and personnel.

Other items can be used as long as the goal is to improve and maintain a good working relationship for future projects.
Role of the Geologist in the Deep Subsurface Program

K. A. Sargent, Department of Geology, Furman University, Greenville, SC.

Abstract

Geological input is desirable in all phases of a program such as the Microbiology of the Deep Subsurface, and it may necessitate more than one geologist to fulfill all of the requirements for successful completion of the program. The contributions provided by the project geologist(s) fall into four categories: (1) assistance in planning the strategies for the drilling and sampling program; (2) recordkeeping and troubleshooting during the drilling and sampling program; (3) providing guidance to the investigators concerning the subsurface geology, history, and hydrology of the materials sampled; and (4) specific investigations of the sampled materials that might impact the interpretation of the microbiological data. The geologist selected for tasks one, two and three should have specific knowledge of the subsurface geology and hydrology of the sampling area, and be familiar with the conditions and potential problems of drilling in the subsurface at the sampling site. The efforts involved in task four may in some cases be handled quite well by the same person selected for the first three tasks. However, some of the desired investigations of the sampled materials may be outside of the geologist's expertise, and thus make it necessary to bring other geologists into the research group.

No paper submitted.
Role of the Site Geologist in a Subsurface Microbiological Sampling Program

W. Timothy Griffin, Golder Associates, Inc., Richland, WA.

Abstract

The role of the site geologist in a subsurface microbiological sampling program is two-fold. First, the site geologist is responsible for providing geological support to the project, including core sample descriptions and detailed documentation of drilling and sampling procedures. The geologist must have knowledge of the local stratigraphy, drilling and sampling procedures best suited to that stratigraphy, and a clear understanding of the sampling objectives of the microbiologists.

The second role of the geologist is to oversee site operations, including integration of the microbiological/geoscientific sampling objectives with the requirements and constraints of the drilling operation, site decontamination and safety, drilling and sampling quality assurance, and documentation of all site operations. Communication is a key element to the success of the sampling project, and as a result, the site geologist must ensure formal and informal communication between the driller and scientists. Formal communication should begin with a pre-construction or "pre-spud" meeting at which all participants are introduced, chain-of-command is clarified, and the geology and sampling objectives are presented. Time-frames, drilling constraints, and deadlines, as well as site safety, should also be emphasized. Daily morning briefings should be held to discuss sampling objectives and procedures, potential problems and solutions, site safety, and decontamination procedures. Finally, weekly meetings should be conducted with both field and office personnel. These meetings provide an opportunity to make procedural changes with management approval and to ensure that management is providing sufficient logistical support to the field.
Introduction

A successful subsurface microbiological sampling program requires that the microbiologist, driller, and geologist clearly understand the project sampling objectives, the procedures to be followed to attain those objectives, and the constraints placed on those objectives by both the geology and drilling procedures. The site geologist should ensure that this understanding exists by maintaining communication between the sampling team members on both a formal and an informal basis. When the site geologist successfully carries out this responsibility, the microbiologists and the drillers can focus on their respective tasks, and the sampling program will benefit accordingly both in sampling efficiency and sample quality.

Discussion

*Geological Expertise.* In order to provide adequate field expertise to the project, the site geologist must be familiar with the local stratigraphy. When possible, data from nearby offset wells should be incorporated and made available to all the members of the sampling team. The site geologist is responsible for describing all core or cuttings as they are retrieved, maintaining a daily log showing rock type and descriptions, field formation picks, drilling data (mud weights, viscosities, pump pressures, bits used, etc.), and any other pertinent operational information, and should properly package and store all archival samples. With the descriptive data, the site geologist should then be better able to predict the depth and rock type of selected sample intervals.

In addition to knowing the local stratigraphy, the site geologist should be familiar with sampling tools and procedures and the rock types to which they are best suited. The site geologist should also be aware of the microbiologist's sampling requirements (sands, clays, fractures, contacts, etc.) and objectives, and should work with the microbiologist to best determine how those requirements and objectives can be met. Coupled with knowledge of the local stratigraphy, the sampling techniques and procedures that will optimize sample recovery can then be selected for each upcoming sample horizon. After each sample run, the site geologist - along with the other members of the field sampling team - should evaluate the effectiveness of the procedures employed and make any necessary changes that might enhance sample recovery. These data should be fully documented for future reference. This documentation is mandatory on projects conducted under NQA-1 requirements.

*Operations Coordinator.* The primary responsibilities of the site geologist as operations coordinator are to integrate the sampling objectives of the microbiologists and geoscientists with the requirements and constraints of the drillers, to oversee site decontamination procedures and site safety, to ensure that drilling and sampling quality assurance procedures and schedules are adhered to, and to document all site activities and drilling and sampling procedures. In order to accomplish these objectives, the site geologist should encourage communication between all members of the sampling team on both a formal and informal basis.

*Informal Communication.* Informally, the site geologist should serve as a liaison between all the sampling team members. The driller should be kept informed by the site geologist of the stratigraphy, upcoming sample horizons and procedures,
recovery data from previous runs, site decontamination, and safety requirements. The site geologist should also solicit the driller's input on proposed sampling procedures and potential sampling problems. This same information should then be relayed to the field microbiologist, along with drilling progress and problems. Sampling procedures and procedural changes should be continually evaluated with both the microbiologist and the driller. The site geologist should also keep the drilling superintendent or foreman informed of the project objectives and progress, as well as the relative success of specific sampling tools and procedures. Finally, the site geologist is responsible for contacting project managers daily and briefing them on all site operations.

*Formal Communication.* Communication on a formal basis should begin before mobilization of the sampling team to the drill site with a pre-construction or "pre-spud" meeting. The "pre-spud" meeting should include all the members of the field sampling team as well as office support personnel and project managers. All the members of the team should be introduced and their respective roles defined, including chains-of-command and respective decision making authority. Overall project objectives should be outlined along with project deadlines. The geology, sampling objectives, and potential drilling problems should also be discussed, and site decontamination and safety procedures should be emphasized.

After the sampling begins the site geologist should hold daily morning briefings to discuss upcoming sampling intervals and procedures as well as potential problems and solutions. Here again, site decontamination and site safety should be stressed.

Weekly or bi-weekly meetings should be conducted by the site geologist that should include both field personnel and project managers. These meetings provide management an opportunity for first-hand observation of site operations on a periodic basis. This first-hand observation enhances communication and allows management to offer immediate solutions to procedural and logistical problems that may have developed. Field personnel, in turn, can demonstrate to management where and why logistical support from the office can be improved.

**Conclusion**

The site geologist must perform the dual role of both providing geological expertise to the project and coordinating and supervising all site operations. To provide geological expertise, the site geologist must know the local stratigraphy and the sampling tools and techniques best suited to the stratigraphy. As project coordinator, the site geologist must integrate the sampling needs of the microbiologists with the requirements and constraints of the drillers. To successfully fulfill this dual responsibility, the site geologist must encourage communication between all the members of the sampling team on both a formal and informal basis. Good communication will result in greater sampling efficiency and sample quality.
Acknowledgments

The author wishes to acknowledge the valuable field experience gained while working on projects conducted under the auspices of Golder Associates Inc., the Department of Energy, The Environmental Science Section of the Savannah River Laboratory, and the South Carolina Water Resources Commission.
Information Extraction and Collaborator Interaction in Interdisciplinary Studies

R. R. Meglen, Center for Environmental Sciences, Laboratory for Chemometrics, University of Colorado at Denver.

Abstract

The diversity of scientific disciplines represented in studies of the subsurface environment reflects the complexity of the system under study. The strength of these studies comes from the collaborative effort of hydrologists, geologist, chemists and microbiologists. However, the experimental designs for these studies tend to focus on the sampling and measurement process. The majority of resources are focused on instrumentation and acquiring data, and too little effort is given to designing the experiment for optimal information extraction. When large multivariable databases are generated and when interdisciplinary collaboration is required, a formal data analysis plan becomes a crucial element of experimental design. The answers will not just flow out the measurements. The database is a domain that requires probes and tools to extract relevant information. Instruments of reasoning to probe the data are as important to information extraction as measurement instruments are to data acquisition. Even the most experienced investigator may not have access to some of the sophisticated mathematical and statistical tools that may be necessary access to some of the sophisticated mathematical and statistical tools that may be necessary to fully exploit the data. Furthermore, project success depends on a sustained collaborative interaction among the investigators. Past experience indicates that investigator collaborative interaction is at a maximum in the sampling and initial data gathering phases of most interdisciplinary efforts. However, in later phases, individuals tend to narrow their focus to their specific experimental objectives. Collaboration often is induced only by the external stimulus of report writing deadlines. A formal mechanism for effecting investigator interaction and information exchange should be incorporated into the data analysis plan. Most projects could be strengthened by the participation of a data analysis specialist. The data specialist provides technical expertise needed to critically evaluate data, functions as a conduit for its transfer, and acts as a catalyst for collaborative interaction.

No paper submitted.
Field Experiments with In Situ Bioremediation
Carol Litchfield, Du Pont Environmental Services, Aston, PA.

Abstract
No abstract provided.

Introduction
I would like to introduce the topic that is not quite deep subsurface microbiology, but is subsurface microbiology. To date, there has been no actual bioremediation efforts at depths below approximately 150-200 feet. Therefore, my task is to review the principles of in situ bioremediation, where it has been accomplished and what its possibilities and limitations are. Before I begin I would like to thank the numerous people who have helped in the various field studies I will be describing: Mike Lead and Dick Raymond, Sr., both of whom are with Du Pont Remediation Services; Lee Belcher and Gene Bessicks, who are with Du Pont Haskell Laboratory; Herb Ward and Virginia Gordy from Rice University, and their coworkers who have been involved in some laboratory work confirming some of the field work we have been doing; Ed Salizar and Jack Whitcomb, who are from the Du Pont Victoria Plant, which I will be describing as the drill site; Nancy Frank, who is with the Texas Water Commission; Ron Gibson and Mike Wheeler, who have performed a lot of our field construction and provided innovative ideas on how to deliver nutrients; Calvin Chin from Du Pont Engineering, who has been the modeler involved in a couple of these projects; and Bob Serbert, who is a geochemist with Conoco.

Discussion
Most of our projects have all involved multidisciplines and in many cases multiple institutions. This diagram depicts a generic idealized type of spill (slide), where a fuel storage tank had leaked and the liquid had come through the vadoze zone. If the contaminant is a light substance, it will float on the surface of the water table while dissolving some of its components into the aquifer. If it is a sinkard, of course, it will hit the bottom of the water table. In either case, one usually has at least three types of remediation to consider: the vapors, the dissolved phase, and the product phase.

What are the remediation options? To do nothing is something that has fallen out of favor, but in some cases it should be seriously considered because a drinking water aquifer or an aquifer that would have industrial use would not be impacted. An attempt should always be made to recover the free product, thus providing several types of remediating options. One of these options is containment, where one would use the slurry wall or biocurtain or precipitation to stabilize the contaminant in some manner. Probably the most expensive option is to excavate and incinerate. One could also landfill if one has materials that are not land-banned or biotreat. Then one could use pump-and-treat systems. The more traditional treatments are airstripping, carbon absorption (which is becoming very expensive), membrane filtration, and chemical oxidization, using ozone, UV, or peroxide. Again,

*Oral presentation.
biotreatment can be combined with air-stripping, carbon absorption, or membrane filtration. Finally, one could use in situ treatments such as soil venting, thermal or microwave treatments, soil washing and then treatment of the liquid either by carbon absorption or biotreatment, and then in situ biotreatment (ISB). It is this last aspect upon which I will concentrate.

Some say why bother to treat the soil and why not just concentrate on the liquid phase. A slide from John Wilson showed where 400 pore volumes of water were passed over a sand column that was contaminated with dyed petroleum hydrocarbon (slide). A great deal of the liquid hydrocarbon spill was retained within that sand column. Typically, one would expect somewhere between 40 and 70% of a contaminant, particularly in petroleum hydrocarbons, to be retained in the soil. Therefore, it is essential that the soils be remediated as well as the aqueous phase. With some other compounds, one may find from 10-20% retained. There is always a fair amount of residual material that seems to be trapped in the soil particles. In the broadest sense, bioremediation is the breakdown by biological systems of any material or compound into another form.

In this presentation, mineralization, production of CO₂, and transformation from a toxic to a less toxic or nontoxic component will be discussed. What are some of the principles under which bioremediation is believed to be working? As it was amply demonstrated throughout the symposium, bacteria occur naturally in subsurface soils and water. They have adapted to these contaminants and in general, microbial activity is limited by environmental or nutrient factors. When these limiting factors are corrected, the organisms can be forced to metabolize and mineralize the contaminants. When this is done aerobically, it is done so with the production of CO₂, water, biomass, and appropriate salts. One of the things that was attempted in our real world experiences was to control microbial growth by prior testing in the laboratory, along with the determination of the balance between growth and metabolism. Uncontrolled growth of the microorganisms will inevitably lead to complete plugging of the formation. That is not particularly desired either by the regulators or your client. Aerobic metabolisms are preferred because they are usually faster when complete mineralization is obtained.

There are several environmental factors that are important in bioremediation. One is pH, which can be controlled to some extent. Redox potential, of course, can be controlled by aerobic processes. Water activity is something that may or may not be of importance, depending upon where one is trying to work in the vadose or the saturated zone. Nutrients are obviously components that can be controlled and altered. In general, some of the preferable electron acceptors for in situ bioremediation are compressed air, the addition of ozone, liquid oxygen, and the addition of hydrogen peroxide (slide). More recently, the Environmental Protection Agency has been testing nitrate in Traverse City, MI.

It was found that with a contaminant, the things one wants to have are oxygen, nutrients, and microbial activity. How can one optimize these factors? It was found early on that the amount of colony growth per gram of soil was dependent upon the
available oxygen, with 40 ppm being the critical breakover point. That level can be reached only with liquid oxygen, ozone, or the hydrogen peroxide. In fact, with hydrogen peroxide, up to 250-500 ppm of oxygen have been achieved. This is important because if one has air-saturated water, only 8 mg/l are being obtained. The minimum number of gallons of water required to remediate the 1000 gallons of fuel is approximately $3 \times 10^8$ if one is working with air-saturated water. It drops to $6 \times 10^7$ with oxygen-saturated water, and drops down to around $1 \times 10^7$ with peroxide. It is obviously going to decrease the amount of time required to complete the bioremediation. In the commercial arena, time is money, so an attempt is made to go for the optimal environmental change.

When looking for microbial population indicators, the first thing to look for is a depressed, dissolved oxygen concentration in a contaminated zone. One should look for an increased number of viable colonies recovered on selected media when compared to an upgradient well. Sometimes one must look for increased levels of ATP, and microcosms are also employed for transformation products.

What then are the actual steps in preparing for a real world ISB? The first thing to do after one obtains the contract is a screening study, and then a nutrient optimization study is done. A degradation rate study is attempted just to provide some idea of whether the program will take four or 40 years, recognizing the limitations of laboratory rates evaluation. Additionally, the nutrient package and soil interaction are observed to be sure that they will not clog the aquifer. Finally, the GCMS analysis of the soil is observed to provide a good definition of the problem. One must be sure that what is produced is something that is less toxic or complete mineralization.

The next phase is design. With this phase, a customer needs hydrology and computer models. In the design phase, the hydrologist is probably as important, if not more so, than the microbiologist. One really needs to understand the geochemistry of the environment.

The next phase is construction or implementation, where there is initial start-up with heavy and very intense sampling to determine nutrient breakthrough and to see how good one's computer model is. Then there is routine maintenance testing to see if everything is going along as expected. This includes interim soil boring. Finally, one hopes to get to closure. This is usually achieved with chemical analysis of soil borings and groundwater to show that one is at the targeted level, followed by quarterly or annual groundwater monitoring, depending upon regulatory requirements.

The first step is collecting aseptic samples in the field using hollow-stem augers. The outside layer of the core is broken off, and then the internal core parts are used to determine the actual microbial numbers. The materials are brought back and a screening test is run, which is usually a groundwater soil slurry incubated in a small bottle. After 2-4 weeks of incubation, the biomass is monitored and the loss of the contaminants is assessed. At the same time, one can also test various nutrient combinations and try to obtain as much information as possible out of such a study.
If increases are seen in the biomass and decreases in the contaminants, one should go on to an optimization study.

I will now talk about the fluorobenzene site. I had expected to be able to talk totally about the fluorobenzene site, but the vagaries of client and regulatory restrictions have meant that the pumping of the nutrients into the site will not occur until about two weeks from now. Biodegradation of fluorobenzene was evident from the initial screening study, and in one particular case there was very good loss of fluorobenzene (slide). This encouraged us to look at various nutrient combinations, everything from ammonium sulfate, ammonia chloride, various phosphate combinations, and other micro nutrients. It was then on to the optimization test, where $^{14}$C label fluorobenzene and incubation periods of 6, 10, and 24 hours were used. The results of fluorobenzene in soils were plotted, showing that within 48 hours there was a 30% conversion to $^{14}$CO$_2$, which leveled off after five days to approximately 50-57% $^{14}$CO$_2$ produced in each of the flasks. With that, we went into the field for the start-up of drilling and the installation of wells.

I want to talk about a particular site that is the last of the actual field demonstrations. The site is a perchloroethylene (PCE) and trichloroethylene (TCE) site on which we had a field demonstration. The contaminated aquifer starts at 80 feet, providing a fairly long string of PVC pipe. Our system included an injection well, a recovery well, flow meters (one for each recovery well), a filtering system to remove debris, and a nutrient system. An oxygen delivery system using liquid-oxygen rather than peroxide was established since our concentrations of PCE were less than 10 mg/l. After the system was installed, samples were periodically collected to determine the groundwater level as part of our standard routine testing.

Certain types of analyses were done in the field, including dissolved oxygen, pH, temperature, and conductivity. The concentration of ammonia, chloride, and phosphate were routinely monitored in the laboratory to determine whether the nutrients were indeed getting through the wells as expected.

I will now talk about three specific sites. The first site is in the state of New York (slide). It is in the sandy aquifer and it has an area of free product. At this site, a gas station had leaked an undetermined amount of gasoline during the previous several years. Approximately 33,000 gallons of free product were recovered. This became an extremely political issue, and the company that owned the gas station ended up buying over 20 homes. Thus, it became an extremely sensitive area in which to do some work. As a result, this area had probably more wells than anyone would have wanted or desired, but politically there are times when one will put in wells just to keep regulators and neighbors happy.

The design was to recover that 33,000 gallons of free product. An infiltration gallery was installed slightly upgradient and across two major highways, and four recovery wells, two of which were in the center of the plume, were also installed. After the system was in operation, fluoride concentrations showed that we did indeed have control of the water and at no time had we ever recovered any free product or dissolved products from well DW-4, which was not that far away from the original station. There were wells as far away as one mile.
After operating for about two years, a small pocket of contamination was still evident right under the middle of the highway. The direction of flow was changed and injection began on one of the previous recovery wells. The site has actually been closed and annual groundwater monitoring is currently being conducted. Soil borings have shown no contamination with BTX's. In studies similar to what has just been presented, the number of hydrocarbon-degrading bacteria increased as the amount of gasoline that was contaminating the aquifer decreased.

The Du Pont plant in Victoria, Texas is the site that Du Pont Environmental Remediation Services has been working on jointly with Herb Ward's group at Rice University and Conoco, and the Texas Water Commission (slide). The site is fairly well populated, except for a portion where a landfill was used back in the 1950s and 1960s. It has been closed as a landfill for approximately 20 years. Unfortunately, during the time it was a landfill, they deposited 55 gallon drums of PCE, TCE, and benzene. The bottom of the landfill is two feet into the perched upper-aquifer. Below this eight-foot-thick perched aquifer, there is approximately 20-40 feet of clay, and below that is the real water-bearing zone (slide). In this zone, the field demonstrations are being done.

From a map of the plume, one can view the following: the Guadeloupe River Canal in one direction; zones of 10 ppm, 5 ppm, 1 ppm, and 0.1 ppm; and total chlorinated aliphatics. A small segment in the 1-5 ppm region was used for our field demonstration. There were a total of 250 wells throughout the whole plant. Upgradient from us was a series of wells that could be used as evidence of what might be coming into the area of our demonstration site. Flowlines were calculated and it was decided to run the demonstration on the 60-foot square area of the huge site. It was originally run for only six months.

In our test, we had an injection well, two monitoring wells and a recovery well (slide). In order to control flow, we had two sets of peripheral wells (injection, recovery, injection, recovery) that maintained the flow of nutrients through the area to prevent too much exterior water from intruding into the test region. Interim borings were done at three months and the chloride concentrations rose up and stabilized somewhere between 600-1000 hours, depending on the wells. The three wells were 10 feet downgradient from the injection site, 40 feet downgradient, and 60 feet downgradient (slide). The dissolved oxygen concentrations (remember, liquid oxygen was being used in this case) had only about 1-3 ppm oxygen when this system was started and it had stabilized at approximately 25-30 ppm, depending upon the well. As for oxygen breakthrough, it took approximately 1200 hours before the oxygen really began to reliably increase.

The results of interest, after three months, were recorded (slide). This system began at approximately 5 ppm of PCE and this decreased to 1 ppm and has been fluctuating a little bit back and forth since then. TCE also decreased and interestingly enough, the dichloroethylene (DCE) increased, but it has not gone any higher. We are hypothesizing that the method of degradation in this case was through dichloroethylene and that was the rate limiting step. No vinyl chloride was seen. At
well 129, the same pattern was seen, except the PCE went up very markedly soon after the loss of benzene (slide). Benzene was found in nondetectable levels. Additional compounds are being added to try to reinduce the metabolism of PCE and TCE. Finally, at the furthest recovery well, it was observed that benzene had been slower to degrade and a fair amount of loss of PCE and TCE was still being obtained. There was an accumulation of DCE (slide), but it did not accumulate too high. It just went up to a certain level and stayed there. In all, approximately 75% of the PCE and TCE was lost in the three wells, and in the comparison test wells at either corner of our test area, only about a 20-25% degradation was observed.

**Conclusion**

I cannot emphasize too much how the success of any bioreclamation project depends upon the hydrology, the microbiology, and the geochemistry of a site study. If one ignores any one of these three, one does it at their peril and the peril of the aquifer.

In conclusion, *in situ* bioremediation is already frequently occurring. Enhanced *in situ* biotreatment is a technology to clean both the groundwater and the subsurface soils; it is a total cleanup. In our interim borings for the PCE and TCE site, nondetectable levels of all three of the contaminants were found in the soils three months after initiation of the biodegradation. *In situ* biotreatment is rapid and it is as predictable as any remediation technique.

Frequently, a traditional geologist will say, "Well, you cannot control things down there." However, if you ask them how sure they are that they can control anything in an air-stripper, they are not real sure that they know how it works, even though they feel more comfortable with it. Something that needs to be emphasized is that when *in situ* biotreatment is accepted as a remediating technology, it is very cost competitive. *In situ* biotreatment treatment moves with the plume, and one of the nice things that has been shown time and time again is that when enhanced *in situ* biotreatment stops, when the nutrients are depleted, one will find that the aquifer will return to normal. The bacterial numbers will return to normal, and one is not left with a deteriorated aquifer. Instead, one is left with an aquifer that is not quite pristine, but very close. Thank you.
Q and A

A. Konopka: I wonder if you could clarify a figure that you showed very early in your talk, which was a plot of colony-forming numbers of gasoline degraders as a function of oxygen concentration. Do you get a saturation curve when you put it on a log scale?

C. Litchfield: Yes.

A. Konopka: It is not clear to me if the colony-forming numbers were a function of how much oxygen was present when you were plating the organism, or were the soils exposed to different levels of oxygen under those conditions?

C. Litchfield: The soils were exposed to different levels of oxygen. This was a laboratory experiment where the soils were exposed to different levels of oxygen, not while the plates were being incubated, but while the tests were going on.

A. Konopka: The soil was incubated rather than the plates?

C. Litchfield: Right.

B. Russell: On one of the slides, you talked about the importance of the hydrology and the geochemistry. Could you elaborate on the specific aspects of the hydrology in addition to the flow fields? That seemed to be what you emphasized in the presentation. Do you spend time looking at the pore size and looking at the geochemistry of what is in the pore waters? I guess I am grasping to find out what level of detail you get into with the hydrology.

C. Litchfield: This would partly depend upon the complexity of the site. Also, it sometimes depends upon your client, whether they are willing to pay for the necessary studies. In general, what we do is look at the expressed pore water. The whole core and water will be observed to see what sort of interactive precipitation might occur. In terms of the geology, it is very important to know what your transmissivity is (e.g., if you have clay lenses in sand where there is gravel). All of that sort of information feeds into the final design as to where you place your wells, how many wells you use, infiltration gallery versus the injection system, and so on. The geologist, the geochemist, and the microbiologist work very closely together in any of the designs.

J. Wilson: Would you comment on ISB as an add-on treatment?

C. Litchfield: It is an excellent process. In fact, ISB is frequently sold on that basis. There has been airstripping in place for the last 10-15 years and a plateau has been reached. The wells are already in place, and frequently, even the injection system
is there. Therefore, a nutrient-addition system is put in, allowing us to, if you will, polish off a project that someone has calculated may take 40 years. Microorganisms will get rid of that material in just a few years.

A. Mills: In what kind of situation would you say ISB is not going to work right at the beginning?

C. Litchfield: In cases where there is not an acclimated population, where the water cannot be moved through the aquifer in what is considered a reasonable time period. Even there, however, we are considering the fact that if one is looking at a 50-year process for airtreppering, why not at least try in situ bioremediation. We are starting to look at some of the lower transmissivity soils, but in general we tend to get a little uncomfortable if it is something below 5 or 6 x 10^7. The other area which we will obviously not try to do bioremediation is where there are heavy metals, asbestos, or radionuclides. We are looking at whether we should try complex mixtures right now, things like coal-tars and 50-year old refineries. One wonders just how much can be done. Laboratory work is now being done in those areas, but no one has gone into the field successfully with them.

T. Kieft: You mentioned early on the need for cleaning up the vadose zone. Did you say anything about bioremediation efforts directed specifically for the vadose zone?

C. Litchfield: Through the infiltration gallery, frequent attempts are made to add nutrients through the vadose zone so that one covers the area that was primarily contaminated with the flow paths of the contaminant. A perched fresh water table can also be added and the vadose zone can be resaturated if it is not too thick. There are various hydrogeological techniques one can use in order to get the nutrients into that vadose zone. Attempts are made to make sure that the vadose zone is cleaned up as well as the capillary and the saturated zones.

M. Nelson: Carol, your results indicate an aerobic degradation of PCE. Would you care to speculate on the mechanisms of that?

C. Litchfield: Other than saying that DCE is the rate limiting step at this point, this is a field demo that is still going on until the 21st of February and I do not have all the data. I definitely cannot go much further than that at this point. The results are very intriguing.

M. Nelson: Have you planned any lab experiments to get at that?

C. Litchfield: Yes. Herb Ward's group has done some 14C-PCE work and they found up to 10-12% 14CO2 produced and we found about 15% 14CO2 produced from labelled PCE. We cannot, however, seem to get that any higher, so there is obviously
some other material. We are looking now at what the transformation product is, because there is a lot of transformation. I forget the exact concentrations. Herb, do you remember what the percentage was of transformation? At this point, the final product is not known.

Z. Filip: I have two remarks concerning your final evaluation of the effectiveness of these treatments. I think in practice, total mineralization never occurs. We had the experience in Germany and one can create a lot of by-products from these bioremediations. This can perhaps be undesirable for the groundwater quality, not only from the chemical point of view, but also from the biological point of view. You mentioned that the microbial count just returned to the normal state when the treatment had been finished. What are these populations composed of? We know from one case in Germany that these remediations resulted in almost pure cultures, but a very rich culture of *Pseudomonas aruginosa* that can be highly undesirable from the bacterial point of view of the groundwater quality.

C. Litchfield: We have never seen anything going to pure culture. We do not identify the organisms. Obviously, that is an area that we would like to do, but we just do not have the time to do such. However, I have never seen things go to a pure culture after any of the remediation efforts.

Z. Filip: The population is put under selected pressure by adding nutrients, by adding source of oxygen, and so on, and it is well known that many populations which use the pollutant belong to the *Pseudomonas*. It can happen; it happened in West Berlin in one case. The remediation resulted in almost pure culture of *Pseudomonas*.

C. Litchfield: That should have been predictable. One of the things that we have found is that where we have had the opportunity to do studies of that type, the organisms that were present tended to be not only *Pseudomonads* (obviously, they are the predominant one), but a fair number of *Nocardia* and coryneform-type bacteria were also present. These die off very quickly when the nutrients are removed and they will go back to maybe 1% of the population. Those studies, however, are not routinely run.

Z. Filip: I will mention that the evaluation of the activity of the organisms, when doing appearance or disappearance of the pollutant is very critical for one’s director. However, one has to be very careful from the view of the health quality of the groundwater.
W. Ghiorse: Carol, we have been concerned about whether or not highly aerobic conditions select against microaerophiles, which in some cases might be the population that actually degrades. I wonder if you have had any experience with that, given that you have injected a lot of oxygen and that you do not always see every compound going away at the same rate?

C. Litchfield: No. In fact, as petroleum carbons go, the regulators are interested in the loss of BTX. That is really what you are after. You are not going to remove all of the aeromatics and many of the longer chained compounds. I think that is a very valid question that you asked me about the microaerophiles. In fact, I have been puzzled on the PCE business as to whether we have added too much oxygen and should back off from that.

W. Ghiorse: In some cases in microcosms, we have found just recently that phenol degraders do better under microaerobic conditions than under fully aerobic conditions. So there may be a real problem there that could be worked out quite easily.

S. Kellogg: What happened in response to the contaminants when bioremediation was not done? Do you have data for those two specific wells based over a long period of time? Many times, one sees the kind of data that you showed without perturbing the system.

C. Litchfield: The systems there are constantly being perturbed because they are pumping 250 gallons/day to an airstripper. However, the upgradient wells have shown the elevated level of PCE, TCE, and DCE that we started out with. Our peripheral wells used for controlling the flowlines showed only a 20-25% loss of the contaminant. Therefore, what is being seen is a major change over those levels. As I said, they have been airstripping there for almost 10 years, so they have a lot of data.

J. Corey: Carol, I am a soil physicist, and years ago Carl kept saying that nutrients were going to be pumped down to this hole and that the contaminant was going to be degraded. As a soil physicist, I said there has got to be a better way to do that. I can not get nutrients from here to there because my flow line will not let me do that. Therefore, we developed a horizontal technique for the drilling company so one could intercept a whole set of flow lines and one bubbles gas with nutrients in it. I would love to see you tackle some of those because you deal with point sources and sinks; therefore, your flow lines look like bananas, rather than linear. Have you ever considered injecting your nutrients with a gas like ammonia? It is a little hard to do with phosphorous, unless you use phosgene or something like that.
C. Litchfield: There are a few problems with ammonia, too. Some places do not like having liquid ammonia sitting around; they consider it a safety hazard. However, the idea of injection through the lateral system or horizontal system is certainly very appealing because obviously, more area would be covered and one would not have to worry about long nutrient losses. I did not show the ammonia data, but it took almost a week for it to break through to the 60-foot level on the ammonia. That meant a lot of ammonia was lost just to the soil particles, ammonia that was not being used by any of the organisms. It would be nice to avoid that.
In Situ Biodegradation of Trichloroethylene

Michael Nelson and John Kinsella, ECOVA Corporation, Redmond, WA.

Abstract

No abstract provided.

Introduction

This paper will, in a way, continue the lines of thought that Carol Litchfield has initiated, which is to look at the potential for bioremediation from the standpoint of actual applications in the field. This work was done by myself and John Kinsella, who is a hydrologist. I would like to say that the way that I am going to present this is slightly different from the way the previous paper was done. I am going to first present a short perspective from a cleanup standpoint on the importance of bioremediation, followed by a fair amount of information on the actual development of the methods that were utilized in the field. Therefore, a fair amount of laboratory data will be provided, which will be followed by the actual results of the very recent in situ pilot demonstration that was done with PCE cleanup.

As Carol has already alluded to, with respect to chlorinated compounds in contaminated groundwater, the technology that has been applicable until recently has been a mechanism by which one does a pump-and-treat, bringing the contaminated water up to the surface for treatment. Then there is treatment involving some sort of physical transfer of the contaminant, via either airstripping or some sort of carbon absorption mechanism in which the contaminant is then transferred to the solid carbon matrix. In either case, one simply transfers the contaminant from one physical condition to another, for example, from liquid to air or from liquid to solid, and the contaminant is still present.

Up until recently, there has not been any applicable, on-site constructive technology available for these chlorinated solvents. This is a system in place at a contaminated site where the groundwater had been contaminated from a solvent storage warehouse (slide). In this particular instance, there was a number of contaminants present, including alcohols and ketones, as well as a variety of chlorinated solvents. The way in which the problem was attacked was to utilize a biological system for the alcohols and ketones that were readily degradable. However, to attack the chlorinated compounds, an air-stripping unit was used up front to remove the contaminants.

There are several options that would provide improvements on the system. One improvement would be some sort of destructive mechanism. Being microbiologists, our preference is to come up with a biological system that can treat the chlorinated compounds on the surface and destroy the compounds, thus avoiding the need for the physical transfer systems that are presently being used. A further improvement upon that system, previously indicated by Dr. Litchfield, would be a method by which one could stimulate the destruction of the compound in the ground so that the amount that

Oral presentation.
has to be pumped to the surface would actually be reduced. She also indicated that a simple washing-type mechanism frequently, and probably almost always, would not provide a true cleanup system. This is due to the adherence of the contaminants to the soil particles, meaning that if one would stop treatment and check it later, then one would find that indeed contaminants do show back up because of their adherence to the soil.

Discussion

My first discussion will involve the laboratory development of the system that was recently tested in the field. I would like to comment that a lot of the work was done at the Environmental Protection Agency laboratory in Gulf Breeze, Florida, where I worked prior to ECOVA.

The plans were to find some sort of microbial mechanism for the biotransformation and biodegradation of trichloroethylene (TCE). The project was initiated by screening a variety of environmental samples for biological decreases of TCE as compared to a parallel scale control. From a number of samples that were tried, one was found from an industrial waste treatment facility that demonstrated a substantial decrease in TCE as compared to the sterile controls. Unsure of exactly what was found, subcultures of the material were taken and the results were produced. What was found was that whenever the attempt was made to take a subculture from the original samples, the TCE-degrading activity was lost. Subsequent to that, an addition of filter-sterilized water from the site was tried. It was discovered that indeed, if one added back-sterile water from the site from which the isolate was obtained, then one could subculture the activity and transfer it serially.

A GC analysis of the water indicated an organic constituent that could be used for TCE treatments by the microbial consortium. Subsequently, the material was identified as phenol. In addition to phenol, a number of other aromatic compounds were tested without TCE degradation. Several other compounds were found that could support biological degradation of TCE. In addition to phenols, toluene, ortho cresole, and meta cresole supported the biological transformation of TCE. It has been seen under certain circumstances, that paracresole and benzene will also support this activity. At this point, work was no longer being done with a consortium. However, an organism designated as strain G-4 was isolated, and it has subsequently been identified as a strain of Pseudomonas cepacia.

Radioisotopic studies were done and the results indicated that approximately 60% of the carbon that was present in TCE was converted to CO₂. An inorganic chloride analysis was also done to determine the amount of chloride produced from TCE, and within experimental error, approximately three chlorides were produced. This indicated that a complete transformation and dechlorination of this molecule by strain G-4 had occurred.

A number of other aerobic degraders were tested for their ability to degrade TCE. Of the organisms that were tested at the time, it was found that two strains of P. putida, which were toluene degraders, also degraded TCE. Another strain of P.
*putida* that degraded TCE by the TOL mechanism, a somewhat different mechanism, was found not to have significant activity on TCE.

There are a number of different aerobic, aerobic pathways for toluene degradation. The common pathway, the TOL pathway, was the one that did not indicate that it was operational on TCE degradation. However, three other pathways were active. One was that of *P. putida*, which degraded toluene by initial attack, by toluene dioxygenase. Strain G-4, which evidently degrades toluene by an initial monooxygenase attack, represents a newly found pathway for toluene degradation as well as TCE degradation. Another system is a para-monooxygenation of toluene, which is followed by subsequent oxidation. There are then, at a minimum, three different toluene pathways that are able to cometabolize TCE.

Work that I was involved with initially and continues at Gulf Breeze by a number of people, as well as some work that has been done at the Grays Freshwater Institute, has shown that the initial oxygenases are the ones that are responsible for the attack on TCE. Before moving to ECOVA, I tried to look at some potential applications for TCE degradation. Certain methods were discovered that could maintain the activity of strain G-4 without the addition of the aromatic compounds. I have mentioned this so that we can come up with a method of maintaining the TCE degradation activity, without the addition of compounds, that would be somewhat nonconducive to getting regulatory support.

Subsequent to these findings, a simple experiment was tried in the laboratory wherein a simple, simulated aquifer was made in an aquarium. The system was set up as such because the proper pumps and plumbing were already established. Therefore, an artificial gradient of groundwater flow could be passed through a soil matrix within the aquarium (slide). There was an upgradient monitoring well and a downgradient monitoring well. Upgradient and downgradient of the recharged area, in which strain G-4, nutrients, and oxygen were injected. This was required in order to maintain its activity, in hopes of making a biocurtain that would consume the TCE that was passing through the system. The results were encouraging. Prior to and slightly after additions of strain G-4 in the nutrients, there was a very slight removal of TCE in the downgradient well versus the upgradient well. After a few days, however, and on into several days thereafter, there were significant levels of decreases of TCE in the simulated aquifer.

Following these findings, a client was found who was interested in trying something innovative in the field, and thus a pilot system was put together to demonstrate whether or not the found activity could in fact occur in a true field scenario. At the site used for the pilot system, the conditions which were present at the particular contaminated area were some of the more desirable, although not necessarily absolutely required conditions. Since the system being studied was aerobic, the fact that the aquifer was aerobic to begin with was a good indication. As was already indicated, one of the biggest problems and usually the great limiting step in any *in situ* treatment is the delivery of oxygen for an aerobic system. Part of the reason for the aerobic system, in this particular situation, is the fact of a very low total
organic carbon present in the site. In addition, there was evidence of a fairly rapid recharge from surface waters into the groundwater matrix. Along with that was a highly permeable strata. High permeability allows transport of both nutrients and microorganisms. It also allows for rapid communication between injections and monitoring wells such that one can see the responses fairly quick. In fact, the responses were seen much more quickly than what was initially expected.

At the site, there were two aquifers, the upper-aquifer zone and a lower-aquifer zone, separated by a clay layer (slide). The upper-aquifer zone was also somewhat separated by clay or sandy clay into a shallow, upper aquifer and a deep, upper aquifer. It was the deep, upper-aquifer area in which the actual degradation experiment was focused. Work was done at a 50-118 foot range. The wells that were put in place were screened such that the injection was from approximately 95-115 feet. The site was very old and TCE had not been present at this particular plant for over 20 years; therefore, the age of the plume was at least 20 years (slide). The higher concentrations ranged from 3000 to 5000 ppb. The plume extended over approximately a 600-foot length and was impinging upon some municipal wells and production wells. Therefore, there was some concern about taking care of the problem.

The pilot test was done in a fairly confined part of the plume. An area was chosen that contained the highest recorded levels of TCE, which was around 3000-5000 ppb. The well cluster was then installed slightly upgradient of an actual plant production well.

As I previously mentioned, this particular demonstration was quite small in area. The well cluster was located about 75 feet upgradient. The center well was the injection well (slide). The EU1 well was an upgradient monitoring well (slide). The M1A well was an off-gradient, referred to as such because it was off-angled from directly downgradient. The ED1 well was the directly downgradient monitoring well. The distance in between all of the wells was only about 10 feet.

The tests were divided into two phases, the tracer injection phase and the biotreatment phase. In the tracer phase, the influent recharge water was injected at the same rate that was intended to be used for the biotreatment phase with a conservative tracer such as sodium chloride. This provided some information about the communication between the injection and the monitoring wells and also, some information regarding what the possible dilution effects could have been from just injection of the water itself. A schematic of the system that was used shows how fairly straightforward the system was (slide). Subsequent to TCE removal from a commercial carbon unit that was on site, a line was tapped from the plant production well and oxygen was then added by oxidation with 100% O₂. Then the required nutrients and microorganisms were added to the recharge line. The various monitoring wells for the site were then observed.

As I indicated, when the tracer tests were done, the flow rates were the same; however, the only thing added was sodium chloride. As for the results of the tracer tests (slide), what was looked for in the conductivity versus TCE test was some
indication of an inverse relationship. If in fact one was having a significant dilution effect on the TCE concentrations in the wells from the simple injection of the water, then as the conductivity from that injected water went up, TCE concentrations should have gone down. In fact, in one of the tests on the upgradient and off-gradient wells, there was no indication of such a relationship. Some sort of relationship, however, was seen in the directly downgradient well. Based on the flow rates that were calculated and the injection rate that was used at the site, it was calculated that approximately 30-40% dilution could be expected in the directly downgradient well. Therefore, the summary that was obtained from the tracer test was that there was good communication between the injection well and the monitoring well, as was expected. Communication was within hours for the downgradient and the off-gradient well, and within 24 hours for the upgradient well.

As I indicated, the rapid-flow velocities could be measured in feet per hour. It was estimated that 1-3 feet per hour was the natural flow in this particular aquifer. Keep in mind the effect of being 75 feet upgradient from a plant production well, which is mainly responsible for high flow rates. It was mentioned earlier that there was a dilution effect in the ED1 well, which was estimated to be approximately 30-40%. Therefore, something higher than this was needed in order to indicate that bioremediation was occurring. Significant dilution effects were not seen in the EU1 and the 1A wells, the upgradient and off-gradient wells, respectively.

Results from the upgradient and downgradient wells were obtained for the first nine days of the test (slide). Time zero was when the flow of water was initiated. However, it was not until 24 hours after initiation of the water flow that feed of the nutrients and the strain G-4 was initiated. I should also comment that the average concentration of TCE during the tracer tests for the upgradient wells was 2800 ppb. For the off-gradient well, it was 2500 ppb. For the directly downgradient well, the average was 1800 ppb. Therefore, 2000-3000 ppb was the mean concentration in the wells during the salt tests. Initial concentrations were approximately 3000 ppb in the upgradient well and approximately 2500 ppb in the downgradient well prior to start of treatment, but subsequent to initiation of water feed. Over the first few days, a slight decrease in the upgradient concentration was obtained; however, a substantial decrease was obtained in TCE concentration during the first few days (from 2500 ppb down to 140 ppb). The concentration in the off-gradient well did not drop as dramatically or radically as did the directly downgradient well, as was expected; however, the concentration did go down to approximately 500 ppb after 10-12 days. The downgradient well decreased and stayed below 100 ppb with a mean concentration of 80 ppb from Day 9 to Day 19. Therefore, a substantial decrease in TCE concentrations after initiation of the treatment did occur.

The presence of organisms was looked for in the wells, specifically the presence of strain G-4. A semi-conservative marker, which is the natural resistance of strain G-4 to ampicillin, was used for screening to see whether or not the organism was present. There were low levels of strain G-4-like organisms already present at the site. Subsequent to initiation of treatment, no significant increases in concentration
occurred until after the sixth day, which was the last day of treatment. At this point in time, a significant 1-2 order of magnitude increase in strain G-4-like organisms was seen. Another sample was taken out at Day 22 and no detectable levels of strain G-4-like microorganisms were found in any of the test wells.

**Conclusion**

The method in which this particular system made its way to the field was initially from a fairly basic research standpoint. An effort was made to see if anything could be found that would show degradative activity toward TCE. Subsequently, an organism was identified and some fairly detailed work was done concerning the metabolic pathways by which this organism and other organisms did degrade the compound. This information allowed for the development of some field-applicable methods for TCE degradation. From that point, a laboratory simulation was tried to determine what the plausibility was, and then a field pilot was used to perform a field validation of this particular system. Thank you.
Q and A

T. Hazen: Did you have any PCE at the site, and did you monitor and detect any DCE or vinyl chloride?

M. Nelson: No detectable PCE was at the site. The EPA Method 601 was being used for monitoring the TCE, so if any significant levels of vinyl chloride or DCE did come up, they would have been spotted, and they were not.

G. Matthey: You have observed a very strange observation. You appeared to have no dilution effect when you injected water and when you treated the plume. At this point, you had to have a fluid phase of TCE when you were flushing by diluted water, yet you had no kind of concentration change when you flushed.

M. Nelson: No change in concentration when we flushed?

G. Matthey: Yes. You showed the slide and you had no change. You always had the same level only in two of your observation wells.

Nelson: That is correct. However, it is a matter of dilution, the relative amount of the injected water that reaches those two wells and mixes with the plume. The water gradient that was coming by was what would determine the relative decrease simply by injection of the water. The rate of flow of the injected water that you are putting down represents only a low percentage of the total flow of the aquifer through that area.

G. Matthey: May I ask another question? Did you have a liquid phase of your TCE? Did you consider it, that this may be biodegraded as a liquid phase?

M. Nelson: Free products is what you are discussing?

G. Matthey: Yes.

M. Nelson: All indications at this particular site were that there was no free-phase product present. What exactly the effects of the organism on a free-phase product would be, I am not sure. My guess would be that, as I indicated earlier in the talk, the fact that when one is treating down in the ground, the organism is not going to grow in free-phase TCE. However, if one gets down in the ground in the proximity of that free phase, then one may facilitate the dissolution of the free phase and cause it to be removed much more rapidly than a pump-and-treat scenario would allow.

S. Garbedium: I want to make a couple of comments, and then I have a question for both you and the other speaker. The first observation is that the in situ bioreclamation is obviously a very promising technique for removing some of these xenobiotic compounds. However, the comment that I want to make is that removal occurs down
to the part per billion range, yet there is still a trace amount that remains. This trace amount is an order of magnitude above the regulatory limits. So the aquifers in question after being treated are still contaminated, heavily contaminated in most situations with respect to drinking water limits. Given the problems of xenobiotic adsorption on the soil and aquifer materials, sometimes very slow desorption processes occur, either diffusion control or nonequilibrium processes of one sort or another. What sorts of possibilities are there as far as ISB in removing trace-level amounts of these xenobiotics, again given the problem that one is an order of magnitude above the drinking water limits?

_M. Nelson_: I have some comments first, then I think Carol will probably want to respond also. With regard to the remaining trace level, which in this instance was approximately an order of magnitude above 5 ppb regulations for drinking water. The downgradient well ended up at approximately 20 ppb. However, let me comment that this was a very small pilot scale test. The intention was not to clean up the site. The actual test did not last over six days and actually, all that was looked for was a statistically significant decrease in the TCE concentrations to indicate that the biotreatment did have some potential for application. Studies have been done in the laboratory which have shown that in fact TCE concentrations can be obtained with this system down to below GC detection. So from that standpoint, I do not think there is any problem as far as the ability of this particular system to reach the levels that are necessary. When one is working in the ground, there are obviously a lot of complications involved. However, I do not think the ability to reach regulatory levels is a wall that cannot be broken through. Carol?

_C. Litchfield_: I certainly concur with what Mike has said. In sites that were closed in California, as well as the site in New York that I mentioned, we were at or below the detection limits for the BTX’s. I really think what one is seeing here is some of the leading edge work in ISB. The system has really not been pushed, and in fact, an attempt is being made right now in the field to see how far the system can be pushed. The client was sufficiently interested in what was obtained in regards to PCE/TCE degradation and told us to see how low we could possibly get. That is where we are, on the cutting edge of trying to apply ISB. None of us working with the xenobiotics really expects to have it come in full bloom the first time, but I think significant progress is being made and an attempt is being made to see just how low we can go.

_C. Fliermans_: Very interesting Mike. Do you believe that the G-4 organisms were doing the degradation or do you think it was the organisms that were already there, the ones that have been subjected to that impact for over 20 years?
M. Nelson: That is a very good question. From the organisms that have been adapted to that plume for 20 years, and as far as the mechanisms that were being invoked (the cometabolic activity), there would be no definite selective pressure for the microorganisms that were in the ground. However, as I indicated, it has been shown that a number of organisms are capable of this cometabolic activity. In fact, Gulf Breeze Lab has gone on to screen other organisms and has shown that a wide variety of genera are capable of this type of activity. Under the proper conditions, the natural microflora are quite capable of doing this particular activity. Whether or not it was G-4 or the natural flora, I can not say for sure. The only thing I know is that what was apparently some survival of strain G-4 was observed in the aquifer, at least for a limited period of time. This would suggest that it did contribute to the activity. However, I would strongly suspect that the natural flora were also contributors to the activity.

C. Fliermans: I would like to make one other comment. I think we sometimes look at two different aspects of rates in these kinds of systems. We talk about in situ rate and it being very slow. Then we do our shake-flask cultures and refer to that as optimization or the maximum. What is actually occurring with adaptive organisms stimulated in situ may well be the maximum as opposed to what one tries to take into the laboratory and make the maximum. Therefore, the thing that one sees in the laboratory may in fact be slower than what one can accomplish under ISD conditions.

M. Nelson: I would concur with that.

F. Brockman: Am I right Mike that you were actually injecting an inducer with the organisms into the groundwater or was the inducer strictly on the surface?

M. Nelson: I cannot go into the detail of exactly what was done. The metabolic pathway that was being maintained as active, if you will, was the cometabolic system that was initially identified as the phenol degradation system.

F. Brockman: Were you able to see whether the organisms that were coming out at your monitoring well were induced? Were you able to do that in this particular case?

M. Nelson: In this particular instance, no attempt was made at trying to show whether or not these were induced or noninduced organisms. That would be a little tricky in the field.

F. Brockman: I have one other question. I was just wondering if you went back after this test was ended, say a week or a month or something, and looked at TCE concentrations to see if they would have rebounded or if they would have stayed down?
M. Nelson: It was expected that they would rebound, but they did not. This causes a problem because we want to go back and redo the tests, but the concentrations are too low.

Z. Filip: Let me please recall two numbers from your laboratory results. Please correct me if I make any mistakes in interpretation. You said that 59% of the carbon of the TCE could be released as a CO₂, and then you mentioned something in your laboratory experiment about 96% of the TCE having been removed. What could have happened to the remaining 37% of TCE?

M. Nelson: In an aerobic system, when a compound is being degraded, one typically obtains 50 or 60% conversion to CO₂ with the remainder being converted to cell mass. In this particular situation, of the 40% that remained unaccounted for by the ¹⁴CO₂, it was observed that there was between 60 and 80% that could be recovered as cell-associated material. No evidence of the formation of any significant levels of products was seen, such as DCE or vinyl chloride. From this evidence, I would conclude that some of the material does not go directly to DCE. One can draw out some very involved and interesting and confusing mechanisms by which TCE is going to CO₂, both by the initial biological oxidative attack, followed by subsequent chemical rearrangements. The bottom line is that something in the form of chloroacetic acid and things of that nature are probably being obtained, which are subsequently metabolized into a cell material and/or CO₂.

B. Russell: One of the things that I always struggle with is the selection of well locations. Could you share with us how you select your well locations and what assumptions you make as far as the formation characteristics in that selection process?

M. Nelson: That is easy for me, I ask the hydrogeologist. The main reason for selecting the locations where we did was because of the rapid groundwater flow that resulted from the downgradient production well. In addition, a fairly small area was desired for the test because we wanted to be able to see fairly quickly whether or not any activity was going to be obtained.

C. Litchfield: That is the sort of thing where you bring in your hydrogeologists. However, one of the things we were looking for was an area that would not be too terribly impacted by other plant activity. If one is in an active plant site, then the wells cannot be put in the middle of the road. One does not want to be in the middle of a drainage field where there are other ditches that contain flowing water. Things of that sort determine where one's test area will be. In addition, in the Victoria plant, the area that was selected was done so because there was an upgradient well already present. Therefore, it could be used for our controls.
T. Hazen: In areas that have been contaminated with petroleum hydrocarbons, soil-gas surveys have been used. In these surveys, there was no hydrology to look at. Thus, the amounts of methane coming up through the soil were used to indicate that there might have been some contaminant in the areas. That actually helped pinpoint where to put wells in one case of a leaking underground storage tank.
Biodegradation of Nitrogen-Containing Aromatic Compounds in Deep Subsurface Sediments

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Abstract

The aerobic biodegradation of $^{14}$C-labeled aniline, quinoline, and pyridine in deep (176-463 m) subsurface sediments obtained from four different boreholes underlying the southeast coastal plain in South Carolina was investigated. Aniline mineralization generally occurred more frequently in the various samples and was more extensively mineralized than quinoline, which in turn was more readily degraded than pyridine. Biodegradation did not correlate with pH, clay content, or populations of aerobic, heterotrophic bacteria. Observations of significant mineralization (>10% released as $^{14}$CO$_2$) were not consistent between formations, between sediments from the same formation but sampled from different boreholes, or between sediments from the same formation sampled at different depths in the same borehole. These findings indicate considerable vertical and horizontal spatial variability of microbial populations able to metabolize these compounds in deep subsurface sediments. Relatively deep geological formations of the southeast coastal plain are habitats to diverse microbial populations with the potential to degrade a variety of energy-derived aromatic compounds.
Introduction  Nitrogen-containing homocyclic and heterocyclic aromatic compounds are often found in waste products of energy production and in creosote-contaminated sites, and some groundwaters have been found to be contaminated by these compounds or their transformation products. For example, groundwater from an aquifer underlying a wood-treatment facility in Pensacola, Florida, contained a number of nitrogen-containing heterocycles, including several isomers of quinoline, acridine, and carbazole. A wide variety of other nitrogen-containing homocyclic and heterocyclic compounds, including alkylpyridines, aromatic amines, and nitroaromatics, have been identified as groundwater contaminants. Groundwater contamination by these nitrogen-containing homocyclic and heterocyclic compounds is of concern because they are toxic and/or carcinogenic.

Microbial transformation of nitrogen-containing homocyclic and heterocyclic compounds can be an important factor controlling the fate and behavior of these compounds in the environment. The degradation of compounds such as quinoline, pyridine, aminonaphthalene, nitrophenols, and isomers of aniline has been demonstrated in soil and aquatic environments. In addition, microbial transformation of alkylpyridines, isomers of quinoline, and carbazole has been shown to occur in some shallow (< 50 m) groundwater aquifers. However, information regarding the transformation of nitrogen-containing homocyclic and heterocyclic compounds in relatively deep (> 50 m) subsurface environments is limited.

Recent work has demonstrated the presence of a viable, active microbial population in deep southeast coastal plain subsurface sediments (Special Issue on Deep Subsurface Microbiology, Geomicrobiology J., volume 7 and 8). Populations of total culturable aerobic heterotrophic bacteria ranged from below detection to greater than 10^6 colony forming units (CFU)/g sediment, and acetate incorporation into lipids, a measure of microbial activity, ranged from zero to greater than 10^4 dpm/day. In general, activities and numbers were higher in sandy, transmissive formations than in unsaturated sediments with high clay contents. Although the majority of sediment samples were obtained from depths greater than 50 m, aerobic, heterotrophic populations were consistently higher than anaerobic populations, and mineralization of a variety of organic substrates was considerably greater under aerobic than under anaerobic conditions. The current study was undertaken to (1) investigate the potential for aerobic microbial metabolism of the nitrogen-heterocycles pyridine, quinoline, and aniline in deep subsurface sediments, and (2) determine if the capacity of deep subsurface microflora to degrade these compounds is distributed uniformly within a given southeast-coastal-plain subsurface formation.

Materials and Methods  Subsurface Sample Collection. Subsurface sediment samples were obtained from four boreholes, three located on the Savannah River Site (SRS), designated P24, P28, and P29, and one offsite borehole, C10, located near the town of Allendale, SC (Figure 1). The samples were collected from the Black Creek, Middendorf, and...
Cape Fear formations that underlie the southeast coastal plain (Figure 2). The Cape Fear formation is the basal sedimentary unit in the plain and is associated with an upper-delta-plain depositional environment. The Cape Fear is generally low in hydraulic conductivity and is not considered an aquifer. The Middendorf formation, lying above the Cape Fear, is relatively high in hydraulic conductivity and is an important regional aquifer. Like those of Cape Fear, Middendorf sediments were deposited in an upper-delta-plain environment. The Black Creek formation, lying above the Middendorf, represents a lower-delta-plain depositional environment. The Black Creek formation is considered a confining layer between the Middendorf and overlying Pee Dee formations, but it has some sandy, transmissive layers. All three formations are associated with the upper Cretaceous period.

The core samples ranged in depth from approximately 170 to 470 m below the surface (Table 1). Borehole C10 is located geologically down-dip from boreholes P24, P28, and P29 (Figure 1). Because the sedimentary sequence thickens considerably between boreholes P24 and C10, samples from C10 from the same formation were from greater depths than those from the SRS boreholes (Table 1). Sediment cores were collected and processed using methods described by Phelps et al. Briefly, cores were pared to remove outer, potentially contaminated core material. The sediments were then aseptically mixed and placed in sterile Whirl-Pak bags, which were placed in canning jars flushed with nitrogen. The jars were sealed and shipped on ice by overnight-express carrier to Pacific Northwest Laboratory in Richland, Washington.

Subsurface sediment characteristics. Particle-size analysis to obtain relative fractions of clay-, sand- and, silt-sized particles was done using standard methods. Chemical analyses of the subsurface samples were performed as described elsewhere. Aerobic heterotrophic bacteria from each subsurface sample were enumerated by viable-plate-count methods on 1% PTYG (Peptone - Tryptone - Yeast extract - Glucose) agar. Sediment samples were shaken in sterile 0.85% NaCl for 20 min, diluted in sterile saline, and plated on 1% PTYG. Plates were enumerated after 7- and 14-day incubation periods at 22°C.

Chemicals. [U-14C]Aniline (13.6 mCi/mmol), quinoline (8.5 mCi/mmol), and pyridine (18.2 mCi/mmol), were purchased from Sigma (St. Louis, MO). Compound purity was greater than 98%. All other chemicals and reagents were of reagent grade or higher.

Biodegradation. Approximately 5 g of subsurface sediment from each sample was used to inoculate modified soil-respiration flasks containing a 47.5 ml medium. Triplicate samples were amended with the specific compound to obtain a final slurry concentration of 10 ppm carbon with a total activity of 0.1 μCi. Poisoned controls consisted of the above with HgCl2 (100 μg/ml). Flasks were incubated for 28 days at 24°C, the approximate in situ temperature of water in the Middendorf formation.

Biodegradation was monitored by trapping the evolved 14CO2 in 2 ml 0.3 M KOH. At regular intervals, the KOH was removed and replaced with fresh solution.
KOH removed from the traps was added to vials containing 15 ml of Ready-Solv EP scintillation cocktail (Beckman Instruments, Inc., Fullerton, CA). Radioactivity was measured by LSC (model LS7000; Beckman Instruments, Inc.). Biodegradation is expressed as a percentage of the added radioactivity released as $^{14}$CO$_2$, and the values are the averages of triplicate flasks. Corrections were made for the abiotic loss of radioactivity by subtracting values obtained from the HgCl$_2$-poisoned control.

**Results**

*Microbiological and physicochemical characteristics.* Selected microbiological and physicochemical characteristics of the subsurface sediment samples used in this study are given in Table 1. Total heterotrophic-bacterial population densities varied from 1.9 log CFU/g in the 262 m sample from P24 (P24-262) to 6.5 log CFU/g in sample P28-203. The total viable-plate count for aerobic heterotrophs tended to be lower for samples from the C10 borehole, regardless of depth or formation, than from the three boreholes on the Savannah River Site. The pH values of the sediment pore water varied considerably between samples and even between samples within a single geological formation. Clay contents of the various samples ranged widely, from 5 to 63%. The low-clay samples generally were from transmissive aquifer zones, and the high-clay samples were from confining interbeds or non-transmissive formations.

*Biodegradation.* Of the compounds evaluated, the relative order of degradability was aniline > quinoline > pyridine (Table 2). In several of the sediments, a greater percentage of the labeled quinoline than the aniline was mineralized during the 21-day incubation. Significant mineralization (>10% released as $^{14}$CO$_2$) of aniline was observed in seven of the 13 subsurface samples, while quinoline was mineralized in four samples and pyridine in only two. There appeared to be limited relation between the extent of mineralization of the various compounds and viable heterotroph populations, pH, or % clay, (Table 1). The highest correlation between any of these factors ($R^2 = 0.4$) was that between the extent of aniline mineralization after 28 days and log CFU/g of aerobic heterotrophs.

In general, the formation with the greatest activity toward the labeled compounds was the Middendorf, although in several of these samples (e.g., P28, 180 m and C10, 416 m) none of the compounds were significantly mineralized. The biodegradation of these compounds did not appear to diminish in the same strata geologically down-dip, as evidenced by the significant mineralization of all three compounds in some of the C10 core samples.

The rate of degradation of aniline, quinoline, and pyridine in the 176-m sample from P29 is shown in Figure 3. Although they were mineralized to different extents, the relative rates at which aniline and quinoline were degraded were similar. A lag period of approximately 15 days preceded pyridine mineralization. The percent mineralization data are presented for incubations up to 28 days since no significant increase was observed beyond this time.

**Discussion**

The aerobic biodegradation of aniline, quinoline, and pyridine in several of the subsurface sediments indicates that deep subsurface microbial populations, present
in sediments underlying the southeast coastal plain, have the capacity to degrade some nitrogen-heterocycle and homocyclic compounds. Previous findings had demonstrated the ability of deep subsurface microbial populations from the SRS to degrade less-refractory compounds such as acetate, phenol, and 4-methoxybenzoate. A quinoline-degrading bacterium isolated from these sediments was shown to degrade a number of other organic compounds, including several benzoate derivatives (e.g., benzoate, salicylic acid, protocatechuic acid), pyridine derivatives (e.g., picolinic and nicotinic acid), naphthalene, biphenyl, and benzene. Recent studies have indicated a relatively high incidence of plasmids in these bacteria, some of which have shared homologous regions with the archetype catabolic plasmid, TOL. Brockman et al. found four plasmids ranging from 50 to 320 kb in a bacterium isolated from subsurface sediments that grew on quinoline as a sole carbon and energy source. Whether or not the high frequency of large plasmids in SRS isolates is responsible for the increased biodegradative abilities of these populations remains to be tested.

One potential explanation for bacteria in deep southeast coastal plain sediments being able to catabolize aromatics is that the indigenous microbial populations have evolved or retained the capacity in order to exploit the relatively recalcitrant carbon (e.g., lignite) that could provide a major energy source for heterotrophic bacteria in formations like the Black Creek and Middendorf. Lignite, a condensed polyaromatic structure derived from the decomposition of plant material, would be a source of aromatic compounds in these formations, although this would require either an abiotic or biotic depolymerization (e.g., peroxidase-type activity) to make them available for bacterial metabolism.

Previous studies have shown that microbial populations from pristine shallow aquifers also have the ability to degrade a variety of organic compounds. In the deep sediment samples in which we observed biodegradation of the aromatic compounds, the rate of biodegradation was equivalent to or higher than the rates observed in shallow aquifers. For example, Aelion et al. found that aniline was degraded at a rate ranging from 0.06-0.17%/day. In comparison, aniline mineralization rates in sediment from the Middendorf formation were from 1.68-2.63%/day (Figure 3). These findings, coupled with previous findings that deep subsurface, coastal plain sediments harbor populations of chemoheterotrophs as high as or higher than shallow aquifers, suggest that different environmental influences operate and that the bacterial composition of deep southeast coastal plain sediments is different from those of relatively shallow aquifers.

The patterns of mineralization in the various subsurface samples indicates considerable spatial heterogeneity, even within a single geological formation, in the metabolic capabilities of microorganisms inhabiting deep southeast coastal plain sediments. There often was as much or more of a difference in the extent of mineralization of the three compounds in multiple samples from one formation from the same borehole as there was between samples from different formations (Table 2). This observation is consistent with the results of Balkwill et al., who showed that
there was considerable physiological diversity among chemoheterotrophic bacteria isolated from sediments of the various formations from the SRS boreholes. They determined that organisms from different formations differed considerably in their responses to a number of physiological tests, the majority of which were substrate-assimilation tests. The same was true of isolates from the same formation but from different core samples or different boreholes, albeit to a lesser extent.

No significant correlational relationships between properties such as pH and % clay and the extent of mineralization of any of the three compounds was observed. Neither was there any apparent relationship between the heterotroph population, as determined by viable plate count, and mineralization. Earlier studies indicated that clay content was the most dominant factor controlling the size of the heterotroph populations in the various formations within a given borehole and that % clay, pH, and heterotrophic populations all significantly correlated with the mineralization of acetate and phenol. The likely explanation for the apparent lack of correlation with these same factors in this study is the difference in the relative degradability of the organic compounds between the two studies. Acetate and phenol are readily metabolized by a variety of aerobic bacteria, whereas aniline, quinoline, and pyridine require more specific ring-cleavage enzymes to be metabolized. Hickman and Novak observed high positive correlations between the rate of biodegradation of methanol and phenol and bacterial densities of surface and subsoils, determined by aerobic plate-count methods. However, for the more recalcitrant tert-butyl alcohol, the correlation coefficients between microbial populations and degradation rate tended to be lower. Thus, it appears that for more recalcitrant organic compounds, measures of general microbial populations may not be indicative of biodegradation.

The two-ring N-heterocyclic compound quinoline was degraded more rapidly and to a greater extent under aerobic conditions than was its single-ring N-heterocycle counterpart, pyridine (Figure 3). This difference likely is due to the relative aromatic stability of these two compounds. Several reports have shown that bacteria able to degrade quinoline under aerobic conditions are incapable of degrading pyridine, although the initial hydroxylation of quinoline occurs on the N-heterocycle ring, forming two-hydroxyquinoline. The electron density of the pyridine ring of quinoline is higher than that of pyridine alone, therefore the susceptibility of the pyridine ring of quinoline to electrophilic-substitution-type reactions would be less than it would be for pyridine. Overall, aniline was more readily degraded and was mineralized to a greater extent than was either quinoline or pyridine. The amino group of aniline tends to withdraw electrons from the ring, thereby increasing the stability of the ring and making it susceptible to electrophilic substitution reactions.

Conclusion

These results indicate that some aerobic heterotrophic bacteria in deep subsurface sediments underlying the southeast coast have the ability to aerobically degrade a variety of aromatic compounds. However, there is considerable spatial variation, even within a geologically defined formation, in the capacities of the indigenous
microflora to metabolize these compounds. These studies also indicate that microbial populations inhabiting deep subsurface environments provide a mechanism for removing organic contaminants from deep aquifers. However, limited information is currently available on the nature of these organisms, the biochemical mechanisms by which they degrade these compounds, and the genetic regulation of these processes. These are important topics for future studies.

Acknowledgments

This work was supported by the United States Department of Energy, the Office of Health and Environmental Research, and the Ecological Research Division under Contract DE-AC06-76RLO 1830 as part of OHER’s Subsurface Science Program.
References


## Tables

Table 1. Aerobic heterotroph populations, pH, and clay content of subsurface sediments.

<table>
<thead>
<tr>
<th>Formation</th>
<th>Borehole</th>
<th>Depth (m)</th>
<th>Aerobic heterotrophs (log CFU/g)</th>
<th>pH</th>
<th>% clay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Black Creek</td>
<td>P24</td>
<td>200</td>
<td>5.2</td>
<td>7.3</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>C10</td>
<td>303</td>
<td>3.1</td>
<td>8.0</td>
<td>16</td>
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<tr>
<td></td>
<td>C10</td>
<td>325</td>
<td>2.5</td>
<td>8.7</td>
<td>26</td>
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<tr>
<td>Middendorf</td>
<td>P24</td>
<td>262</td>
<td>1.9</td>
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<tr>
<td></td>
<td>P28</td>
<td>182</td>
<td>3.7</td>
<td>4.7</td>
<td>63</td>
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<tr>
<td></td>
<td>P28</td>
<td>203</td>
<td>6.5</td>
<td>4.9</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>P29</td>
<td>176</td>
<td>5.6</td>
<td>5.8</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>P29</td>
<td>193</td>
<td>4.5</td>
<td>6.1</td>
<td>10</td>
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<tr>
<td></td>
<td>C10</td>
<td>378</td>
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<tr>
<td></td>
<td>C10</td>
<td>416</td>
<td>3.5</td>
<td>7.3</td>
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</tr>
<tr>
<td></td>
<td>C10</td>
<td>407</td>
<td>3.2</td>
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<td>6</td>
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<tr>
<td>Cape Fear</td>
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<td>2.6</td>
<td>7.6</td>
<td>47</td>
</tr>
<tr>
<td></td>
<td>C10</td>
<td>463</td>
<td>3.3</td>
<td>10.5</td>
<td>10</td>
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</table>
Table 2. Aerobic mineralization of select nitrogen-containing aromatic compounds in southeast coastal plain subsurface sediments after 28 days.

<table>
<thead>
<tr>
<th>Formation</th>
<th>Borehole</th>
<th>Depth (m)</th>
<th>Mineralization</th>
<th></th>
<th></th>
</tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Aniline (％ released as $^{14}$CO$_2$)</td>
<td>Quinoline</td>
<td>Pyridine</td>
</tr>
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<td>200</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
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<td></td>
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<td>C10</td>
<td>325</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Middendorf</td>
<td>P24</td>
<td>262</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td></td>
<td>P28</td>
<td>180</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
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<td>P28</td>
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<td>53</td>
<td>37</td>
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<td>C10</td>
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<td>407</td>
<td>18</td>
<td>43</td>
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<td></td>
<td>C10</td>
<td>463</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>
Figure 1. Relative location of deep subsurface microbiology boreholes in South Carolina.
Figure 2. General stratigraphy of deep subsurface sediments in the southeast coastal plain on the Savannah River Site and in the vicinity thereof.
Figure 3. Aerobic mineralization of aniline, quinoline, and pyridine in P29-176 sediment microcosms.
Q and A  

C. Fliermans: Fred, as you are aware, there is a subsurface microbiological culture collection from the Savannah River Site. Are these microorganisms part of that?

F. Brockman: I believe they are and Dave Balkwill would be the first one that would know, but I am quite sure they are. (Jim Fredrickson shook his head yes.)

J. Fredrickson: If Dave's laboratory has not managed to kill them off, I think we did send them off so they should be in the culture collection now. One of the things I do not recall is what they were designated as in that culture collection. I think they have the 3N3A designation.

A. Konopka: You mentioned it takes about 8-16 hours to do total degradation. As a reference, what is the generation time on quinoline alone?

F. Brockman: Of the cells?

A. Konopka: Yes, of the cells.

F. Brockman: A specific look at mineral salts media has not been done but rich media is very fast. Growth is obtained overnight, so I would suspect it is in the 4-5 hour range for generation time. We do have data on it; however, I never calculated the generation times. It is relatively fast.

A. Konopka: The next question relates to the data looking at the kinetics of quinoline mineralization over a range of concentrations. At a variety of concentrations you seem to have a similar rate of mineralization plotted as the percent of carbon mineralized. When I look at that, I see you are going over three orders of magnitude difference in quinoline concentration and 40% of 2 ppb of quinoline. That is a mass of quinoline which is quite different than 20% of other nutrients. Could you plot those in the absolute concentration per hour?

F. Brockman: Yes. When the Vmax is taken into consideration, what one is doing is taking the slope of the initial removal. Vmax statistics basically take into consideration the differences in concentrations. Yes, and it was similar. It was the same from 2 ppb up to 20 ppm.

W. Ghiorse: Have you checked the American Type Culture Collection to test the Pseudomonas cepacia strain for similarities to yours?

F. Brockman: No, we have not.
W. Ghirose: *P. cepacia* is a very common isolate and there is probably lots and lots of strains of them out there. I am thinking about similarities, whether anyone has really looked at *P. cepacia* strains, because they really do degrade a great variety of different substrates and they may have already been described in the past.
Deep Surface Bacteria Responses to Contaminants

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Abstract

Deep subsurface communities have some unique characteristics that will make bioremediation more difficult in some cases and easier in others. One serious drawback of subsurface remediation will be our inability to readily identify bacteria present and the necessity for deep sediment sampling. Sediment samples, when compared with groundwater from the same geological zone, have higher densities, physiological activities, and species diversity. DNA analysis of sediment samples and bacteria isolates reveal that these communities are unlike anything on the surface. However, these same methods have demonstrated that these communities have great intrinsic potential for use in bioremediation. A high frequency of plasmid-containing isolates has been found and most isolates show a much broader range of carbon assimilation capabilities than surface bacteria. They also have a unique bimodal chemotactic response to nutrients and very strong attraction to trichloroethylene (TCE), even at concentrations that are toxic. The deep subsurface has hinted that it can be bioremediated, but with problems that must be addressed carefully.

*Oral presentation.*
Introduction

This presentation looks at the potentials and the problems with doing bioremediation beneath the surface, as well as some of the specific data in response to contaminants. Some of the other work that has been presented in these proceedings will be reviewed.

Discussion

To obtain an idea of what the problem is in the United States, it would be appropriate then to first discuss the problems. The Office of Technology Assessment has recorded that 1-6% of all groundwater in the United States is contaminated and this number is constantly being revised upwards. Transient groundwater withdrawals have been well documented to be increasing exponentially for domestic, industrial, and agricultural uses, and a reliance of the groundwater as a drinking water source has also increased dramatically. This illustrates the urgency of the problem and also, as the shallower aquifers become more and more contaminated, our reliance upon deeper aquifers is going to increase.

Take, for example, underground storage tanks. The United States Environmental Protection Agency (EPA) estimates that there are more than 5 million underground storage tanks in the U.S. and at least 1.1 million are going to be subject to regulation. In one study with 12,000 tanks, it was revealed that 30% were leaking. Through extrapolation, it was evident that almost 500,000 tanks could be leaking now or will be shortly, and 50,000-100,000 of these sites are expected to require mandatory action. This number is constantly increasing and it does not include landfills and other waste sites, only leaking underground storage tanks.

McCormick (1985) estimated that it is going to cost at least $100 billion to clean up the 93,000 dumping sites that are known to exist, and this number is increasing daily. Therefore, the urgency in finding methods that are going to save money is real. This is also why some of the extreme activity that is occurring in bioremediation has been seen.

Surveys done by the EPA (reported by McCormick in 1984) provide an idea of some of the compounds that involve the United States. One reason a lot of field work is being done with TCE and chlorinated hydrocarbons is that it is one of the most common compounds found in groundwater. It has been detected in 35% of 25 communities samples. Of course, these compounds are all either experimental carcinogens or known human carcinogens. Furthermore, the Resource Conservation and Recovery Act (RCRA) prohibits land disposal of many hazardous substances and it, as well as other laws, mandates that these sites be cleaned up. Contaminates have gotten into groundwater environments and there are a large number of possibilities as to how these contaminates infiltrated the groundwater (slide). Infiltration sometimes occurs even by direct well injection.

At the Savannah River Site, all of these problems exist, including some instances in which chlorinated hydrocarbons were directly dumped into clay-lined seepage basins. They have since penetrated into some deep aquifers, though most of it is confined to the shallow areas because of the large amount of clay in the soils. A lot of soil, microbial survival, and groundwater factors have been identified that
may affect contaminate removal. Knowing that bacteria do exist below the surface sheds light on the doctrine of infallibility. People sometimes do not like the strength of this statement, but it is of course one of the selling points of bioremediation. Simply put, there is no compound, man-made or natural, that bacteria cannot degrade given the right conditions.

**Bioremediation of the deep subsurface.** While aquifers continue to become contaminated, they are also becoming a major source of water for many industrial processes and agricultural water. Because of the depth, slow turnover times and large volumes involved, *in situ* bioremediation, in many cases, will be the only possibility for some sites. *In situ* bioremediation has been around for quite a while, and the degradation of hydrocarbon was actually first established by Dick Ramond in the early 1970s. A full adoption of this technique was not seen until the early 1980s, but a very dramatic increase can be seen now. It is probably more realistic to look at bioremediation, not in terms of biorestoration, or taking a toxic site and changing it into a rain forest. Most of the time, the best one can hope for is removal or transformation of some of the toxic components and achieving a less toxic environment. Bioremediation companies will quite often try to sell the biorestoration scenario.

In the Deep Subsurface Science Program, boreholes have been drilled at the Savannah River Site on the border of South Carolina and Georgia (slide). They were deep holes, with the last one going down to a depth of over 1700 feet. David Balkwill, Bill Ghirose, and others have recorded that there were many organisms in the sediment profile and they represented quite a number of different types (slide). The number of colonies or biotypes and the number of API-NFT were identified by Dr. Balkwill, as well as the PTYG counts versus the number of viable counts on 1% PTYG (in borehole P29). The numbers were fairly similar, quite surprisingly, and the number of types (colony diversity) was also quite high (slide). The same thing occurred in P24 and P28. A large number of types existed, either by counting or colony diversity. There was also a large number of organisms with really no decrease as one went down the borehole.

There was also a strong interest in how good the identifications were and how different the microorganisms really were. They may have been identified by API-NFT at the 99% confidence interval, but were they really that particular species since the database that was used to identify the microorganisms was not clinical in origin? Therefore, in light of this question, actual DNA melts were done. For those of you not familiar with this, it is a way to separate bacteria, at least exclusionary. Of course, this technique does not look at the DNA structure, but instead at the amount of G+C in the particular bacterium. Therefore, it can only be used as an exclusionary technique because if the measurements are different, the bacteria have to be different. If they are the same, it does not mean that the same bacteria has been obtained.

However, using this type of technique along with the G+C and the API identification code, the data showed that all of these organisms (in the slide) from
the subsurface had identical phenotypes. There was a large range of % G+C, so even though they had identical phenotypes, the genotype was not the same. Thus, they could not have been the same bacterium. What should be emphasized here is the high diversity that was evident by the API-NFT tests and by the other types of physiological measurements. Furthermore, colony morphology was probably even much greater.

These bacteria were quite different and DNA homologies verified this point. From the data, it was assumed that 70% homology was the same organism. The probe used in these analyses was ATCC _P. putida_. However, using that probe and cross matching showed that none of these bacteria were similar even though they were API-NFT identification.

A number of probes were also made against deep subsurface bacteria and DNA was extracted from the bacteria. A probe was made by attaching the bacteria to a filter, and the similarity between bacteria was measured. Some of the data collected by one of the graduate students, Luis Jimenez, showed that only the homologous system proved a match at one site. None of the data indicated that even the same species were present. In a few cases, they may have been similar, but there was actually quite a difference among all of the bacteria even at one site.

The frequency of plasmids, in general, seems to increase with depth, which suggests that there is greater possibilities for remediation. Furthermore, as the environment becomes more recalcitrant, the bacteria pick up a higher plasmid load, which allows them to degrade a wider variety of substances. In general, the studies that have been run, along with the information David Balkwill has provided, have shown that the plasticity of the bacteria in terms of their ability to assimilate different compounds, also increases as depth increases. This certainly provides hope that there is quite a bit of potential at depth for any type of bioremediation of toxic compounds.

Carl Fliermans and Tommy Phelps have also shown that in surface and subsurface environments contaminated with TCE, there is a lot of activity in these environments that is contaminated and that the microorganism there can degrade the compound. At very high concentrations of TCE, very little activity can be seen, possibly due to toxicity. However, in one particular zone of activity, the ability to mineralize both above and below the plume was observed. This provided more evidence that a site could be remediated, especially at the Savannah River Site. Fliermans et al. also reported that one could stimulate bacteria from the Savannah River Site to degrade TCE cometabolically and they presented a number of different electron donors like propane, methane, and methanol to stimulate TCE degradation.

As a part of that work, a link was made with the Gas Research Institute, and their interest in methane is obvious. They were interested in how we might be able to use a methanotrophic bioreactor or use methane in remediation, using the methanotrophic organism's ability to degrade TCE. The fatal flaw analysis has been done by Radian Corporation for the Gas Research Institute. They also did a cost analysis which showed that the technology was possible and feasible. The cost analysis was very
helpful as well because an effort was made to put this into a real world situation to determine if it was really better than the conventional technology. The analyses showed that the conventional technology centers on direct carbon sorption from the water and stripping of the air stream by granulated activated carbon. If one looks at the capital cost for a variety of the same scenarios, including either 100 or 1000 ppm TCE in the groundwater and flow rates of either one or ten million gallons per day, then one can see that the costs are lower for the methanotrophic system than both the carbon sorption system and the air-stripping system (slide). The major cost is the carbon. However, the methanotrophic system was 40-60% cheaper just for capital costs, except at the very highest flow rates and at the very highest TCE concentrations (slide). At those rates and concentrations, a much larger system would have been needed, one that exceeded the costs of the airstripping and granulated activated carbon. When the operating costs of these systems were actually looked at based upon a conservative analogy from three years ago (because the rates of degradation of TCE that are now being found are much higher than what was used in this particular model), a 40-60% difference in costs was once again found between the methanotrophic system and any type of airstripping or carbon sorption system. Furthermore, the air-stripping system prevailed as well, whether the water ratio of 85:1 or 300:1 was used (slide). Therefore, it appears that it will really be a cost effective way to remediate TCE.

We went back to some bioremediation bioreactors that have actually run to see if we were in a real world situation, since the methanotrophic system was hypothetical. In 1988, Nyer published data on a toullic acid bioreactor, and from it one can see that the off-site disposal cost was at 20 cents per gallon and activated carbon cost was 80 cents per gallon. However, when it went to a bioreactor, the cost was less than one cent per gallon. This was a very substantial savings for the site; therefore, development began for a methanotrophic bioreactor system. This system is being developed with a research program between the Savannah River Laboratory and the Gas Research Institute. It is a four-year program.

Drs. Phelps and White have taken Savannah River Site bacteria and put them into an expanded fluidized bed design bioreactor. They put the Savannah River consortium of bacteria into the reactor system and added either propane or methane. There was a significant reduction in the amount of TCE when the methane was added, and with propane there was an even better degradation rate. Two different consortia were used to ensure the results of the technique could work. We are in the process of scaling up with a trickle filter system, but we are going to have to overcome the mass transfer problems that we might have with the fluidized, expanded bed bioreactor for the pump-and-treat system.

The work done by Dr. Phelps shows some of the most interesting things that might apply to pump-and-treat systems. Dr. Phelps put very contaminated Oak Ridge water through his system and looked at the controls versus the two microbial consortia from the Savannah River Site. The consortium actually degraded everything else that was in there, including benzene, toluene, and xylene. In some cases, it
actually degraded the benzene, toluene, and xylene a little bit better than it did the TCE. Therefore, an added bonus was obtained. Not only were all of the volatile compounds being removed, but so were a lot of the less volatile compounds. This suggests that these bioreactors may be really useful for direct treatment of water streams in a pump-and-treat scenario. A methanotrophic system may operate in a similar manner, with interceptor wells pulling up a contaminated plume, mixing with methane in air, putting it into your methanotrophic bioreactor, and then recharging it back into the subsurface aquifer.

One of the problems that will be encountered with any type of pump-and-treat scenario is that contaminant recovery will have a diminishing return as one tends to encounter more clay and more subsurface pockets. Once a certain depth is reached, fewer returns will be seen. This may be a good area to concentrate on in situ bioremediation so that the microorganism can be delivered to those areas, or filtrate nutrients into those sites and degrade the compounds that are already there in situ. Therefore, the pump-and-treat scenario may work best for uniform sand and gravel aquifers, especially in areas like the site at Traverse City, Michigan. However, it may not work as well in areas that have a lot of clay lenses.

Jack Corey indicated earlier that some horizontal wells have been installed as a method of extraction and in situ aStriping. This method is an adaption from technology used by oil companies. Our wells have been placed at approximately 125 feet deep, where the water table is at 100 feet and the upper horizontal well is at 75 feet. Injection of air is expected to begin within the next two months. Air will be injected in the lower well and a vacuum will be applied to the upper well. Hopefully, movement of air through the system will be obtained, which will provide actual in situ aStriping. It will probably affect microbial activity as well, and it will be measured. The single horizontal well may also be a way to reduce the cost associated with deep aquifer remediation. With the single horizontal well, only a single hole is needed, allowing further expansion in the plume (instead of drilling a variety of vertical holes just to get into the contaminant plume). Therefore, this may be a way to get around some of the problems that are encountered with deep subsurface systems.

At the Savannah River Site, there were a large number of cluster wells at each of the boreholes that were used to sample bacteria in the sediment. Therefore, I had the opportunity to go in and sample at very discreet interval and to compare that to the sediment that was found in another site. This other site, which was originally thought to have been very contaminated, was found to have all of its contamination on the soil surface. The bulk water from very discreet screen zone regions was sampled and compared in terms of numbers and types of bacteria with the sediment sample that was found. This comparison provided some very interesting results (slide). The numbers were fairly consistent between AODC's and viable counts, indicating fairly high activities (except in some of the clay areas). A different picture was seen with the groundwater. Densities from the AODC and PTYG were three to four orders of magnitude lower, and the differences between the AODC and the
PTYG were also much greater. This indicated that these bacteria were under a much greater stress. Some of the other measurements that were made would indicate the same thing. Measurements of diversity and G+C, and a variety of other measurements, showed that what was in the water was quite different from what was in the sediments. These results showed quite well that these bacteria are fairly strongly attached, especially as one gets deeper and deeper into the subsurface. A comparison was also made between the groundwater and sediment. Again, there was a five orders-of-magnitude difference in some cases and barely detectable concentrations in others.

Typically, groundwater is used to monitor biodegradation rates and microbial population changes. However, this may produce erroneous results, especially in the deep subsurface, where indigenous microorganisms are strongly attached to the sediment. This strong attachment allows only stressed, dead, or maladapted organisms to be observed in the groundwater, which is quite different from what is actually present in the sediments. In addition, pore water microorganisms are often associated with these wells, which can provide false indications as to what is occurring in bioremediation.

How bacteria move, that is to say their ability to transport between wells and their ability to move themselves, is of particular interest. Chemotraction, which is movement of a bacteria toward a nutrient gradient, is one way that they can move. A capillary tube technique was used to observe the chemotaxis to a variety of amino acids and sugars (slide). This work was done principally by one of my other graduate students, Geryalyne Lopez. She developed 10 different sugars and 10 different amino acids, along with TCE for chemotaxic responses over a wide range of concentrations. The results showed what one would typically see in a chemotaxis experiment with chemotactically positive substrates. As the concentration increases, a threshold is reached, which triggers a positive movement from the bacterium. As the concentration continues to increase, the movement of the bacterium is always toward the attractant, never away (slide).

When deep subsurface bacteria were observed in our study, something quite unusual was found. In general, a negative chemotaxis or repellent response in high concentrations was seen with most of the bacteria. A fairly strong negative chemotaxis was observed at some high concentrations and a fairly strong positive chemotaxis, sometimes double that amount, was observed over very low concentrations of substrates (slide). Motility controls were run to make sure it was not just random motility due to increased activity of the substrate. The response range was a bit wider than what is normally seen for bacteria for similar substrates. However, I have never observed this bimodal phenomenon with surface strains and various other bacteria that have been checked.

Some of the adaptations that these microorganisms have to low nutrient environments, very oligotrophic environments, are reflected in their response to some of the amino acids. There were a couple of differences with respect to the type of bacteria. However, very different responses were seen for TCE. As the concentration of TCE was diluted to $10^{-10}$ Molar, no negative responses were ob-
erved, only positive chemotaxis. Again, however, some of the highest chemotaxis indexes that have ever been recorded were four to five times greater than water alone. This was a very unusual response.

It is believed that this response was due in part to the fact that TCE affects the chemo-receptors, which are methylated proteins. Of course, this TCE response needs to be studied a lot more. It is believed that there is actually a binding of the TCE to conformation changes in the receptor proteins by turning them on quite nonspecifically even if the concentrations were toxic for the bacterium. This was evident when observing the bacterium even with acridine orange-stained bugs. The bacteria go screaming into the TCE in a response which is called the "fetal attraction", but it may have some significant implications. This was not universal, but it was common in almost all of the bacterial screens that were tested, and it may have some applications to the terms of bioremediation and the controlling bioremediation processes. It may provide some idea of what could happen in a TCE-contaminated aquifer if the attracting of microorganisms does not specifically destroy them. We have actually filed for a patent on some of these observations.

To see if this technique was applicable to the real world, a crude model was developed that used sediment samples from the Myrtle Beach Site and the Middendorf aquifer at the C10 borehole. A water reservoir was placed at one end of the column and then the whole system was autoclaved. A small number of bacteria were introduced at one end and then observations were made to see how long it took for the bacteria to break into the reservoir. When a very small amount of TCE was put into the reservoir, the bacteria literally went through the sediments twice as fast. Sterile and killed controls were done and no breakthrough was observed over the 3-4 week period. It was a crude system, but it provided an idea of what might happen in the real world.

A few calculations were also done in regard to this model. With the distance between the preliminary hole and the C10 hole being 190 feet, it would take 49 days for a bacteria that was introduced in the preliminary hole to get to the investigators hole, that is assuming it was traveling at the fastest rate. At the lowest rate, it would take 444 days. We were on the site 49 days, so if there was something strongly attractive being introduced into the C10 hole, it is possible that we could have seen some of the bacteria breaking through the investigators hole from the preliminary hole.

**Conclusion**

From the various presentations of investigators in these proceedings and from what I have discussed, many of the problems concerning bioremediation of the subsurface have been brought forth. One problem could be the unique microflora that exists in the deep subsurfaces. The subsurface microbial community is largely prokaryotic. The monitoring of groundwater provides little information about changes in activity and numbers that are very relevant for *in situ* bioremediation. Concentration of nutrients, slow water flow, slow recharge rates, and a recalcitrant nature of deep subsurface environments may make conventional nutrient manipula-
tions fairly difficult. Perhaps things such as the horizontal well will help overcome these problems, but it certainly is going to be difficult. The epilithic nature of these environments will make any strategic infusion of nutrients more difficult.

On the positive side, the good news is that the microflora is Gram-negative. There are a lot of plasmids present, and it looks like they can degrade many compounds. This has already been seen from work done in various laboratories for these particular bacteria at the Savannah River Site. They have a lot of plasmids, which leads to a lot of potential. Deep subsurface microorganisms also show great diversity in the types of organics that they can assimilate. Large proportions of plasmids in the communities suggest a high potential for plasmid degradation of contaminants. This may be fairly important.

Some studies have been recently published. In *Biotechnology Journal*, Amgen Corp. reported the extraction of genes from a *Pseudomonas* strain that coded for the degradation of TCE and put it into *E. coli* K12. In doing so, they uncoupled it from the cometabolic process, allowing the bacteria to degrade TCE in a very simple cultured system. The TCE was degraded from 40,000 ppb down to less than 10 ppb in four hours. This finding raises all sorts of possibilities for genetically engineered microorganisms and for introducing them into contaminated environments. However, one must first look at the transferability of genetic elements and some of the problems that might be associated with it. In addition, chemotaxic behavior in subsurface bacteria may be used to help control or enhance remediation. Probably the best thing to say right now is do not pollute. Thank you.
J. Bauld: You and other people have stressed the probable epilithic nature of the communities present downhole. Do you think that it is probable that when you crank up the nutrient levels down in the hole when you are attempting bioremediation, that there may be significant changes in the distribution of organisms between the epilithic and planktonic populations and in fact, that it may be a significant problem in bioremediation?

T. Hazen: That certainly could be a problem. David Balkwill presented some data in Plenary Session 5 that showed such a problem with nutrient addition. With the deepest sediment, they did not seem to switch that much, and after the nutrient was withdrawn, they switched back fairly quickly. Is that a fair synopsis David? That is probably some of the best evidence that we have that such a shift may not occur in deep subsurface.

D. Balkwill: I think it is a little hasty to say whether they shift back swiftly. I think Ron Harvey has some very interesting information in terms of distribution of the organism being attached to those kinds of nutrient levels.

B. Russell: I want to say that I really appreciate seeing the diagrams that showed the information characteristics of breakthrough curves. Could you elaborate on the conditions that were associated with running the experiment in a test tube? Did you create any sort of hydraulic head associated with the experiments? Did you look at any variations of flow velocity through that material? Then, did you try varying formation characteristics just for the heck of it to see what happens when you introduce clays or other sorts of things, or do you plan to do that if you have not already?

T. Hazen: I have not done that yet and it was a very crude experiment. I am waiting for the next graduate to come along to continue some of that work. We also are building a chamber system that Carl and I have worked out where sediment and water can actually be taken from the subsurface and it can be pumped into sterile Middendorf sediment to see if the communities can be reestablished there and observed that way. This appears to be a better way to examine some of those problems. Those are very good things to look at and that is why I kept qualifying that this was a crude experiment, because I do not know what some of those parameters would have done. However, since the differences were great enough, they were mentioned.

B. Benoit: I want to compliment you on the chemotactic experiment. The question I have is do you think the organisms were responding to factors besides substrates? The reason I ask the question is the microaerophiles we have isolated in the Middendorf are responding to oxygen and substrates. If you look at the literature, there is also evidence that some of the microaerophiles are quite sensitive when you increase the phosphate concentration.
T. Hazen: Clearly they responded to things other than their substrates, because TCE is not a substrate. I am sure that they were responding both for avoidance and for attraction to various compounds, which may have been metabolic by-products or things like that. It has been well shown that certain fatty acids or similar compounds can be an attractant or a repellant depending on what that bacteria likes. How significant chemotaxis is in subsurface environments is pretty speculative right now. It could be fairly important and the unusual thing is the bimodal response that was seen on the subsurface bacterium negative and positive for the same substrate. What a great strategy for a bacteria that was adapted to a fairly oligotrophic environment like you have seen presented by a lot of other people.

C. Litchfield: I would like to make one comment on the epiphytic versus planktonic nature of the bacteria. This may well have something to do with substrate as well as sediment type. From the studies that we have done on the chlorobenzene-degrading organisms, we have found more chlorobenzene degraders in the groundwater that we recovered from our monitoring wells than we did from adjacent boreholes. This was not true with hydrocarbons; we found more organisms in the soil than we found in the groundwater. Therefore, we may have to be careful about how this is interpreted depending upon substrate and soil types and not just look a for a simple, single answer.

R. Dietz: Given what we have seen about the techniques for cleaning up the subsurface environment, whether it be biochemical or stripping, or whatever, and the diversity of contaminated subsurface environments that probably exist, and the cost of cleanup with the exception of removing free-standing pollutants, has anyone really thought about assessing whether or not the direction that we seem to be heading in is the most viable direction? Namely, cleaning up all the subsurface environments down to drinking water qualities, rather than just getting rid of free-standing subsurface contamination and then cleaning up using a lot of these technologies? Rather than cleaning the water to drinking water standards? Rather than cleaning up 99.9% of what will never be extracted? Where do we stand in terms of the legal aspects? Has the scientific community responded in this direction?

T. Hazen: I have talked to some EPA personnel about the same thing. They said that sooner or later we are going to realize that the United States Government cannot afford to absolutely clean all the sites. We are going to have to start thinking more, at some sites, in terms of containment, preventing it from spreading rather than absolute total cleanup. This probably will become more realistic as the study moves along. Right now, with the regulatory environment such as it is, there is a lot of contamination to get rid of. That is probably unrealistic given the monetary resources that even the United States has.
G. Matthess: I think you are right. The point is very important if you consider the whole cost of the cleaning procedure and the containment procedure. These indeed are very expensive. I think from the standpoint of the geochemist, I will say that we have to try to define acceptable levels of emissions into the environment. If one does not accept any emission, it is not possible. Even if there is containment, there are emissions—that is a natural law. So we have to define what is acceptable for the environment with respect to chemicals; they are not natural products. One could say that they do not want any man-made chemicals in the environment. This is a very poor approach here in the United States.

C. Fliermans: I think the concept in aquatic microbiology may have applicability here and that is assimilative capacity of streams. When one gets into subsurface environments, is there such a thing as assimilative capacity that will allow one to, as was already mentioned, remove free products so that there are organisms that are capable of an assimilative capacity that can be utilized? I think assimilative capacity is a concept that needs to be part of our ongoing research.

P. Strom: It seems as though there may be some contradiction in thinking of organisms as both epiphytic and motile, at least at the same time. In fact, may not motility be a response to contamination?

T. Hazen: I have difficulty dealing with that too. These organisms in the deep subsurface are uniquely different than anything that has been on the surface. Another comment I would like to make concerning the horizontal wells is that in our next phase this coming year, we are planning to inject methane directly through one of the horizontal wells, trying to encourage methanotrophs. We think that is a plausible way to remediate a site in the near surface. Furthermore, using methane to try to stimulate quite specific components of the community might allow us to avoid some of the plugging problems and things that may be encountered with a general nutrient addition, since the methanotrophs are a fairly small component of the system. By adding methane, the increases of every bacterium under the sun that might be seen with the addition of phosphates, nitrates, and types of hydrogen peroxide will never be seen. We have also shown, from direct DNA extraction of sediments of the C10 hole, that TOL 1 plasma bacteria are present. Probing can actually be done with the TOL 1 plasma, which indicates a hit in some of the lower sediments that have been directly extracted. Thus, it is in high enough concentration that it can be detected. This has provided even more evidence that there is a significant potential in there for degradation for a lot of compounds such as TCE.

M. Fletcher: I want to comment on the previous question about the apparent paradox between attachment and the ability to be chemotactic. I think if any microorganism is going to be successful, it must contain some options. It is not going to go down the evolutionary trail that will trap it into a particular habitat. It is always living on
particular sites on the surface. It may be untrue to speculate here, but it may in fact be that chemotaxis is more important to the organism that is adapted to the epilithic mode of growth, because it is not in an environment where it can move about by water flow.
Microorganism of Groundwater and Decomposing Refuse as Viewed by the Transmission Electron Microscopy

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Abstract

The chief problem in obtaining an objective view in the native microbial communities is the lack of suitable experimental techniques. In our laboratory, a simple preparative method was used in order to make electron microscopy investigations of indigenous microorganisms from groundwater and decomposing municipal refuse possible. This method included the following: (1) suspension of the complex microflora from the refuse sample in a buffered water solution or a direct use of groundwater samples; (2) embedding of microorganisms from the suspension on the surface of collodion filmed and carbon coated electron microscopy grids by direct centrifugation in the Beckman Airfuge; and (3) one-step staining with 1.5% of phosphotungstic acid at a pH value of 6.8 in centrifuge rotor chambers. The main advantage of our method is based on a supporting effect of the centrifugal power towards the concentration and adhesion of microbial cells at the surface of electron microscopy grids. Electron photomicrographs of microorganisms from groundwater and decomposing municipal refuse, which were obtained using this technique or a standard method, are presented.
Introduction

In addition to culture techniques in environmental microbiology, it is also of interest to obtain insight in the morphology of the specific indigenous microflora. Light microscopy may be useful in part, but its magnification and resolution powers are rather limited. In addition, changes in the cell morphology are often caused by fairly crude fixation and staining techniques. In this paper, a simple technique is described which allows a direct examination of the indigenous microorganisms from decomposing municipal refuse and groundwater using the transmission electron microscope.

Materials and Methods

Sample preparation. Samples of municipal refuse were collected from a large controlled dumping site located in Braunschweig, Germany. A fresh sample was suspended in sterile distilled water and ten-fold serial dilutions up to $10^{-3}$ were prepared. Groundwater samples were obtained from a deep pristine aquifer (polluted with leakage water) located in Braunschweig, Germany. Some of groundwater samples were used without any pretreatment, others were enriched in culture media containing starch or mannitol as the main carbon source.

Transmission electron microscopy (TEM). Conventional TEM grids (Cu/Rh) with 200 mesh were filmed with collodion and carbon coated by sputtering (Balzers union SCD 20). The grids were put into chambers of the EM 90-rotor from the Bechman Airfuge with the coated side on top, and they became fixed to the wall of the chamber adhesion. One-tenth of a milliliter of the suspended sample was filled into the individual chambers and centrifuged at 40,000 x g for five minutes. After centrifugation, the supernatant was replaced in the chambers with 1.5% (w/v) solution of phosphotungstic acid at pH 6.8, and the centrifugation was repeated for two minutes. This one-step staining procedure resulted in a significant contrast enhancement as already documented for viruses. The stained grids with microorganisms collected on their surface were dried under an infrared lamp (Balzers Union 03105-T) for 30 seconds at a distance of 30 cm from the light source. The preparations were viewed in the Zeiss EM9 transmission electron microscope.

Results and Discussion

Microorganisms were almost non-detectable in native, diluted samples when a simple sedimentation technique was used. Sample dilution, however, is imperative when both microbial cells and different noncellular materials, which are often abundant in refuse or groundwater, are to be separated from one another. Otherwise, amorphous organic material and different inorganic particles would outnumber and optically overlap cells in samples.

Different, well distinguishable microorganisms, mainly rods, are shown in Figures 1-5. These figures demonstrate that by using the described preparation technique, a high cell resolution can be obtained for TEM (Figure 2). Noncellular materials can also still be recognized, but probably due to the sufficient dilution, these materials do not disturb the electron microscopic reflection of microbial cells (Figures 1 and 3).
If the concentration of bacteria in samples for electron microscopy is high enough and noncellular material does not substantially interfere, standard preparation techniques, which do not involve an additional concentration step, may also result in reasonable electron photomicrographs (Figure 5).

As in the studies published by Bae et al.,¹ and Bone and Balkwill,² no attempt was made to make an identification of the microbial species based on the photomicrographs. However, in comparison to these authors, who dealt in their studies with the transmission electron microscopy of different soils and sediments, the preparation technique described in this paper is simpler and less time consuming. It also allows at least a crude systematic determination of the microorganisms (Figures 4 and 5).

Conclusion

In conclusion, our simple, short-term technique for preparation of native samples from groundwater and municipal refuse for transmission electron microscopy reveals an undisturbed view of the morphological structure of indigenous microflora in heterogeneous environments.

Acknowledgements

For the TEM photographs, the facility of the laboratory for Electron Microscopy of the Paul-Ehrlich-Institute at Langen was used. The authors thank Dr. H.A. Fischer for his support. This study is a part of the project Fi 268/4-4 granted by the Deutsche Forschungsgemeinschaft in Bonn, Germany.
References


Figures

Figure 1. Bacteria from municipal refuse masked by amorphous organic material and inorganic particles. TEM magnification: 3400 x.

Figure 2. A slightly curved rod bacterium from groundwater. TEM magnification: 3400 x.
Figure 3. An aggregate of bacteria and abiotic materials from a groundwater aquifer. TEM magnification: 21,000 x.

Figure 4. A mycoplasma bacterium from a pristine groundwater enriched in starch medium. TEM magnification: 21,000 x.
Figure 5. A caulobacter from a polluted groundwater sample after enrichment in mannitol containing medium. TEM magnification: 7500 x.
Resistance of Groundwater Bacteria to Organic Chemicals
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Abstract

Microorganisms from a pristine groundwater aquifer were enriched in the laboratory and the biomass, consisting mainly of the species of Arthrobacter, Pseudomonas, Alcaligenes and Flavobacterium, was freeze dried. More than 60 organic chemicals, which are known as groundwater pollutants, were tested for their possible effects on the groundwater microflora. The tests used were ATP estimation, dehydrogenase activity, and oxygen uptake measurements.

At concentrations near the full water saturation capacity, aromatic compounds exerted only weak inhibitory effects, whereas chlorinated and nitrous aromatic compounds were toxic to the microflora. Similar toxic effects, even at far lower concentrations were observed for chlorinated aliphatic compounds. Anilines showed a distinct toxicity between 100-1000 ppm, whereas polycyclic aromatic hydrocarbons (PAHs) and softeners did not exert toxic effects. Ethers showed toxic effects only near the full water saturation; phenols were bactericidal at concentrations of 1 ppm or lower. In long-term tests, an adaptation of the groundwater microflora to the organic chemicals was often observed.
Introduction

Groundwater represents more than 90% of the world's freshwater resources. Since it plays a principal role as a source of drinking water in many countries (e.g., approximately 50% in the United States and more than 70% in the "Federal Republic of Germany" (now part of the United Germany)), groundwater deserves a high degree of protection. Nevertheless, organic chemicals and other pollutants may contaminate wells and aquifers with detrimental effects on the groundwater quality. In the "FRG", more than 50,000 sites exist that have already been identified as having contaminated groundwater and soil, which may create a hazard for human health, and thus, have a high priority for remediation. The decontamination of polluted groundwater aquifers by utilizing the metabolic activity of autochthonous microflora offers many advantages in comparison with other treatments. However, to reclaim an aquifer, the microorganisms must not be inactivated by the chemical pollutants. In this research, the resistance of groundwater bacteria to a wide spectrum of organic chemicals which have repeatedly been identified as groundwater pollutants was determined.

Materials and Methods

Microorganisms. A sample of groundwater was taken from a deep well (31.8 m) in a pristine aquifer near Dieburg (Hessen, "FRG"). A long-term enrichment culture in PYGV-Solution yielded a biomass composed of bacteria that belonged to the Arthrobacter spp., Pseudomonas spp., Flavobacterium spp., Alcaligenes spp. (Figures 1 and 2). The biomass was freeze-dried and aliquots were used for the individual tests.

Test used. The resistance of groundwater bacteria to organic chemicals was determined in short-term (16-24 hours) and long-term (six weeks) tests at 10°C. The short-term tests included the estimation of ATP concentrations, dehydrogenase activity, and oxygen uptake by the Warburg method. In long-term tests, only the ATP concentrations were estimated. The chemical compounds tested included nonhalogenated aromatics, chlorinated and nitrated aromatics, chlorinated aliphatics, polycyclic aromatic hydrocarbons, ethers, anilines, phenols, and softeners. Individual compounds were applied in five concentrations relative to their water solubility. For short-term tests, a minimum effect concentration (MEC) was estimated, which represented the lowest concentration at which a significant decrease of bacterial biomass or activity was observed. For long-term tests, a minimum inhibition concentration (MIC) was estimated, which represented the lowest concentration at which complete inhibition of bacteria occurred. The validity of the results was tested statistically using Student's T-test.

Results and Discussion

Mean minimum effective concentrations of the chemical from the individual tests are presented in Tables 1, 2 and 3. The results for the main groups of chemicals that were tested have been summarized.

Nonhalogenated Aromatic Compounds. Negative effects on groundwater bacteria were observed at concentrations near water saturation (Table 1).

Chlorinated and Nitrate Aromatic Compounds. Negative effects on groundwater bacteria were observed at concentrations near water saturation; however, those
concentrations were considerably lower than the ones for nonhalogenated aromatics. Strong toxic effects were found for nitrated aromatic compounds (Table 1).

*Chlorinated and Nitrated Aromatic Compounds.* Negative effects on groundwater bacteria were observed at concentrations near water saturation; however, those concentrations were considerably lower than for nonhalogenated aromatics. Strong toxic effects were found for nitrated aromatic compounds (Table 1).

*Chlorinated Aliphatic Compounds.* Volatile compounds of this category belong to the most common groundwater pollutants. With the exception of trichloromethane, trichloroethene, and dichlorophane, which exerted negative effects at low concentrations, these compounds caused inhibition only at concentrations near water saturation (Table 2).

*Aniline, PAH’s, Softeners.* Even though PAH’s and softeners did not inhibit the bacteria tested, the anilines were highly toxic between 100-1000 ppm (Table 3).

*Ethers.* Ethers negatively affected groundwater bacteria mainly at concentrations near water saturation. Methoxybenzene, however, caused inhibition at low concentrations (Table 3).

*Phenols.* Chlorinated phenols were especially toxic to groundwater bacteria, even at concentrations below 1 ppm. However, some adaptation of bacteria to these compounds has been observed in long-term tests.

**Conclusion**

The results indicate that organic chemicals with well known toxicities such as anilines, nitrobenzenes, chlorophenols, and methoxybenzene may cause a strong inhibition of groundwater bacteria at low concentrations. Most of the 60 organic chemicals involved in this investigation, however, caused negative effects on bacteria (ATP), their respiration ($O_2$ uptake), and enzymatic (dehydrogenase) activity, only at concentrations near water saturation capacity. These findings indicate that an acute toxicity should be expected primarily at or near the point of the contamination source. In conclusion, utilization of groundwater bacteria for biological remediation of chemically polluted aquifers can be successful, if microbial adaptation, selection of resistant strains, enhancement of nutrients, and site conditions are considered.

**Acknowledgements**

TEM photomicrographs were kindly made by Dr. Raija Smed-Hildmann using the facility for electron microscopy of the Paul-Ehrlich-Institute at Langen.
References


Table 1. Concentrations of some nonhalogenated, chlorinated, and nitrated aromatic hydrocarbons that affected groundwater bacteria (conc. as ppm).

<table>
<thead>
<tr>
<th>Substances</th>
<th>Solubility in water</th>
<th>MEC* Short-term tests</th>
<th>MIC* Long-term tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzene</td>
<td>1170</td>
<td>1000</td>
<td>1000</td>
</tr>
<tr>
<td>Toluene</td>
<td>470</td>
<td>&gt;300</td>
<td>sat.¹</td>
</tr>
<tr>
<td>o-Xylene</td>
<td>175</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Chlorobenzene</td>
<td>500</td>
<td>300</td>
<td>300</td>
</tr>
<tr>
<td>1,2-Dichlorobenzene</td>
<td>100</td>
<td>60</td>
<td>SAT</td>
</tr>
<tr>
<td>Hexachlorobenzene</td>
<td>0.006</td>
<td>NE</td>
<td>NE</td>
</tr>
<tr>
<td>Nitrobenzene</td>
<td>1900</td>
<td>10</td>
<td>30</td>
</tr>
</tbody>
</table>

*See Material and Methods.
SAT=Saturated.
NE=No effect.

Table 2. Concentrations of some chlorinated aliphatic hydrocarbons affecting groundwater bacteria (conc. as ppm).

<table>
<thead>
<tr>
<th>Substances</th>
<th>Solubility in water</th>
<th>MEC* Short-term tests</th>
<th>MIC* Long-term tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trichloromethane</td>
<td>9000</td>
<td>300</td>
<td>1000</td>
</tr>
<tr>
<td>1, 1, 1-Trichloroethane</td>
<td>500</td>
<td>SAT</td>
<td>SAT</td>
</tr>
<tr>
<td>1,1, 2-Trichlorotrifluoroethane</td>
<td>170</td>
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<td>NE</td>
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<tr>
<td>1,2-trans-Dichloroethene</td>
<td>600</td>
<td>375</td>
<td>NE</td>
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<td>Trichloroethene</td>
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<tr>
<td>Tetrachloroethene</td>
<td>150</td>
<td>SAT</td>
<td>SAT</td>
</tr>
<tr>
<td>1,3-Dichloropropane</td>
<td>2750</td>
<td>100</td>
<td>100</td>
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*See Material and Methods.
SAT=Saturated.
NE=No effect.
Table 3. Concentrations of different organic chemicals affecting groundwater bacteria (conc. as ppm).

<table>
<thead>
<tr>
<th>Substances</th>
<th>Solubility in water</th>
<th>MEC* Short-term tests</th>
<th>MIC* Long-term tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenanthrene</td>
<td>0.6</td>
<td>NE</td>
<td>NE</td>
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<td>Tributylphosphate</td>
<td>400</td>
<td>NE</td>
<td>NE</td>
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<td>N-Methylamine</td>
<td>30000</td>
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<td>30</td>
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<tr>
<td>2,4,5-Trichlorophenol</td>
<td>2000</td>
<td>0.3</td>
<td>&gt; 3</td>
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<tr>
<td>Phentachlorophenol</td>
<td>2000</td>
<td>0.3</td>
<td>&gt; 3</td>
</tr>
<tr>
<td>Methoxybenzene</td>
<td>1500</td>
<td>300</td>
<td>ND</td>
</tr>
</tbody>
</table>

*See Material and Methods.
NE=No effect.
ND=Not determined.
Figures

Figure 1. *Arthrobacter sp.* isolated from the groundwater enrichment culture.

Figure 2. *Pseudomonas sp.* isolated from the groundwater enrichment culture. Note the polar flagellum.
Abstract

Declining production capacities in water wells have often been linked to occlusive biofilm formation (plugging) at the well screen slot, the gravel pack, or at adjacent aquifer locations. The nature of the plug frequently precludes long-term success by shock chlorination or acidization, with or without the use of penetrants. The Blended Chemical Heat Treatment (BCHT) process incorporates the application of heat, along with a sequential application of disinfectants, pH amendments, and customized penetrants to first apply shock, then to disrupt, and finally, to disperse the plug to again allow the unimpeded flow of water to the well. Of the 38 wells treated to date, 36 (95%) have been returned sufficiently to meet the production criteria established by the user. Monitoring systems are employed to determine the status of the microbial vectors being discharged from the water well during the diagnostic and restoration process.
Introduction

The subject of this paper is subsurface biofouling and the use of the Blended Chemical Heat Treatment (BCHT) process for restoration of biofouled wells. A BCHT process model aquifer was developed by Dr. Roy Cullimore and his staff at the University of Regina to observe bacterial activity under dynamic situations that one may find in a pumped well (slide). This model provides an idea on what is occurring in the aquifer with the bacteria, how they will plug and actually constrict the well to the point where it will longer produce.

Discussion

The current model in use allows a 360° observation of the well with a 2 inch screen down the middle. This unit can be operated under both aerobic and anaerobic conditions. In this model, volumes can be checked, observations of well operation can be considered, and a CAT scan can also be done. The biomass in the dynamic system can develop in a number of different matrices, colors, and textures. Everything from the soft, fluffy types to the slippery and slimy can develop, draining into almost a tubercle-type situation involving manganese, iron and beginning signs of sulfide deposition. What has been observed in these models is similar to what Dr. Costerton covered earlier in this Session as to how the attachment normally works on the gravel patch and how the bacteria begin to develop with the biomass eventually plugging the well.

This model was used to demonstrate the wicking principle (slide). When the water was dropped at approximately 10 cm below the surface, the biomass continued to wick the water up to the top and as it evaporated, and it would deposit the salt on the surfaces. The salt crystals were attached to the granular surface and the slime or biomass interface could be seen with the crystal.

When observing a real world situation of biofouling in groundwater systems, the different surfaces, colors, and textures of the slimy-type biomass can be seen. (The tubercle develops due to sulfate-reducing bacteria activity underneath the biomass.) The sulfate reducers end up corroding out the pipe in that particular surface. This happens not only to metal surfaces, but to PVC and teflon as well. The bacteria are not really choosy where they set up. It just takes a little longer for them to attach on to one surface or another.

From a study done two years ago, it was discovered that even on metal surfaces it did not matter what was used, be it titanium to arsenic. The bacteria would actually select and attach to any surface that was there for them. The biomass volume, which was observed for approximately 57 days, showed a marked increase in biomass volume until a pulse area was hit (slide). Through sluffing and compaction, it began to drop off. The same thing was seen with flow, having decreased to the point that a slumping action was obtained. If a recovery in flow would have been obtained, it would have usually been much higher than the original flow rate of the model in its pristine condition.
This same situation transfers very easily to the field in our studies using BCHT to restore a well. BCHT is a three step operation, consisting of a shock phase, a disruption phase, and a dispersion phase. The shock phase is a process where one goes into the well with a heated chlorine solution, along with a wetting agent so that damage can be done to the biomass in the closed area of the well.

In our study, it was discovered that the problem of fouling was not just at the core or just in the gravel. The fouling had actually graded back into the aquifer itself. In some work that was done about two years ago from the center line of the well, the biomass was traced approximately 50 m into the aquifer. Therefore, something had to be developed to soften it so that one could get into the consolidated biomass.

The disruption phase is usually followed (within 24 hours) with the treatment, which consists of the injection of acid, disinfectants, and polymer wetting agents that are under pressure with steam. The tools are set up down in the well and injection is started. The top of the well is sealed to enable further backing into the formation and to allow break-up of the biomass to begin. As the heat begins to build up, the interface for the cold water and the heat is obtained. The cold pulls the chemical and the heat back into the formation. An increase in activity occurs with the increased temperature and the uptake of the disinfectants is enhanced. The wetting agents facilitate penetration and a better flow rate is obtained.

Following a reasonable contact time, which can range anywhere from 24 to 48 hours, one can enter the well and begin to surge it using known well water development techniques such as air surging, a surge block, jetting, or whatever is available in the area. A combination of these has served very well. One can then begin to pull the biomass back into the well and pump it off with intermittent surging.

All of this was tested in the laboratory prior to taking it to the field. Fortunately, the United States Environmental Protection Agency (EPA) set us up with a Phase I and Phase II Small Business Innovative Research Grant in order to start development of this technique. The Army Corp of Engineers found out about this technique, and since they had such a problem with wells and drains on dam and levee structures, they also provided money to help develop the technique and sampling devices.

So the shock phase simply cleans and the disruption phase returns one to the formation in order to clean the gravel and the screen. During the subsequent dispersal phase, much of the biomass from the dead bacteria is removed from the well. This technology was transferred very easily to the field, although it took a lot of time, a lot of effort, and a lot of dedicated people willing to put up with long hours and a lot of heat and cold. On the pipe from the submersible pumping well, there is a lot of fouling diversity that one must contend with in the formation. Through work done with the Army Corp of Engineers when studying the development of biomass that was moving toward the pump well, observations were made of the consortia changing over time as the well was pumped. The bacteria began to build more biomass and became tighter and tighter as they moved into the well. Eventually, the bacteria built a plug in the well itself.
Following the laboratory studies, a number of wells were treated in the last few years. One particular well was done in Armstrong, Ontario, which is approximately 200 miles north of Thunder Bay, and it is definitely bush (slide). Six-hundred people lived there in the summer and approximately 200 people in the winter (slide). This well had actually reached the point of zero production. When the pump was turned on, it would immediately draw down, break suction, and it would take anywhere from 10 to 15 minutes to recover to the point where they could pump it again. It was originally designed to pump 16.5 l/second, yet they were only able to get about 13 l/second when everything was working right. This deteriorated over a two-year period to the point where they could not use the well at all.

Following the treatment of the well in our study, the flow was brought back to approximately 11.5 l/second which was acceptable to the engineer on the site (slide). The well, when completed, was at 15.5 l/second. It was starting to degrade, then it recovered, then it degraded again, and then it spiked. Roy Cullimore termed the spikes as facilitated flow, which lead to a direct match in the laboratory between these spikes and biomass development. Each time, the spikes were greater than the original, normal flow (slide). A three-day averaging chart showed spikes that were a little more consistent. The downgradient time became progressively worse as work was done out through the system. The recoveries were always much greater, than the norm, throughout the system, and this continued on through the entire plugging cycle of the well. Over time, the plugging cycle continues to get closer and closer together until the well will not perform anymore.

Our study was continued by placing a discharge line on the well so that acids and chlorine could be added to the well from time to time and furthermore, so the well could be surged using the pump in order to keep the well going. A surge block was put into the well, and during this short period of time, the biomass has not had a chance to really tighten up. It was surged for approximately 15-20 minutes, and it went right back to the 11 l/second delivery. Thank you.
Q and A

S. Kellogg: Do you have any idea what the chemistry of the water was in this particular situation that would provide such nice growth? What do they do in Armstrong? Mining, timber?

G. Alford: No, absolutely nothing. It is a timber area, and is the primary means of employment. It was originally a train stop on the Trans-Canadian rail line, a water stop for the trains. This particular site was an old military base, so the town just grew up around it. The actual total organic carbon level in the well was unknown. The subsurface is a straight glacial till to several meters below the well. Prior to treatment, this particular well had been run from time to time in order to observe the metals, primarily iron and manganese, which exceeded the MCL. ICP analysis indicated total iron at 10,000 ppm, copper was 9.7 ppm, aluminum was 55 ppm, manganese was 24 ppm, and zinc was 9.7 ppm. All of this was accumulated by the bacteria in the biomass in less than two years. If one wants to treat heavy metals, go to Armstrong to get the microorganisms.
Studies on the Utilization of a Floating Intercedent Device (FID) in the Biological Activity Test System (BATS) to Identify the Presence and Activity of Various Bacterial Groups in Groundwater

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Abstract

The recent realization of the various roles that different groups of bacteria can perform in the ongoing functioning of a water well or groundwater system has accelerated the need to be able to conveniently identify which bacterial groups are present within a given water system. Three of the groups have been generally identified as the iron-related bacteria (IRB), the slime-forming bacteria not involving iron, and the hydrogen sulfide that produces sulfate-reducing bacteria (SRB). By utilizing different selective media and a floating intercedent device (FID) to stratify aerobic and anaerobic zones, the complex reaction patterns have allowed the development of a semi-qualitative and semi-quantitative method for monitoring these groups of bacteria. The sensitivity of the method (patent pending) is similar to the extinction-dilution, spread-plate count technique with positive detection of bacteria in water occurring at population levels as low as one and 30 colony forming units/ml.

¹Oral Presentation.
Introduction

Most of us working out in the field on a day-to-day basis do not have the luxury of coring every time we want to determine the level of biological activity. Therefore, this report deals with some test kits that have been developed, one in particular being the Biological Activity Reaction Test System (BART) that is used to monitor the level of activity for population size of the most important particular group of bacteria in groundwater systems. George Alford has already mentioned the importance of detecting these bacteria in groundwater systems. These field test kits were developed to detect or monitor planktonic population or detached populations of microorganisms. From some studies done about five years ago, it was found that detached populations will normally range an average of 10% of the attached population.

Discussion

Three different test kits can be used to test the following three important populations: the iron-related bacteria, the slime-forming bacteria, and the sulfate-reducing bacteria. The BART test has a floating intercedent device, better known as a ball, that floats on top of the water. The ball restricts the amount of oxygen that can pass between the ball and the wall of the tube, which establishes an oxygen gradient. Aerobic conditions are established at the surface of the tube all the way down to reduce oxygen or anaerobic conditions at the bottom of the tube. There are also nutrients in the bottom of the tube that diffuse up. Once the water sample is put in, it diffuses upward; thus, one has an oxygen gradient coming down and a nutrient gradient going up. This allows the bacteria that is taken from an environmental sample to establish themselves or to grow where the conditions are best for them to grow.

Various reactions occur in the tubes due to microbial growth. With the iron-related bacteria, nine different reaction types occur, some of which correspond to environmental conditions that the bacteria normally grow under. If the bacteria grow predominantly at the surface, then they are aerobic organisms. If they grow on the bottom of the tube, they are anaerobic or microaerophilic.

Gas producers and some color reactions (i.e., yellow, red, green, dark green) can also occur. The color reactions correspond to the major populations. Extensive laboratory results from ATCC-type culture collections have provided more information concerning such color reactions in pure cultures. A red color is a *Kebsiella*, yellow is the *Citrobacter*, brown is an *Arthrobacter*, a dark green is *Pseudomonas*, and a light green is *Acinetobacter*.

Reactions for the slime BART, which are basically a slime-forming bacteria (extracellular polysaccharid producers), were obtained. They can show some of the environmental conditions that the bacteria grow under. They can also show fluorescence and one can obtain pigment generations from these tests. The sulfate-reducing bacteria either produce black or they do not produce black. The iron sulfide deposit blackens the ball, the bottom of the tube, or the entire length of the tube. Different reaction types can be obtained in each of the bacteria populations. In sulfate-reducing bacteria and iron-related bacteria, gas bubble formations develop showing some of the darker colors. They can also be used for extinction dilution
series to determine microbial populations.

These BARTS have already been used quite extensively in both well rehabilitation and other industrial or municipal type systems. The test really has been designed for use in the field because it is very easy to set up and it only requires a foot of water until the ball fills up to a certain level. While setting up the tests may be easy, reading the results is a different thing. When iron-related bacteria is present, some of the bacteria can actually form blocks where they will cement the balls to the wall of the tube. They can cement the balls to the wall of the tube and actually turn the tube upside down. This is important because in the water well industry, these bacteria cause a lot of problems. Approximately 80% of all problems associated with water wells are of bacterial origin, because there are a lot of the cementing agents, the extracellular polysaccharides, that act as a glue to hold everything else together. The hydraulic pressure necessary to break that ball away from the wall has been tested, and the results have shown that it takes a 30-100 mm head of water (depending on the organism) to break the seal.

A fluorescent BART called Fluorobart is also being developed, which will be used to detect fluorescent pseudomonads. The importance of fluorescent pseudomonads are obvious in the industry, and this detector should be ready to be field tested in the near future.

The BART name is a trademark name, and the BART is also patent pending. The patentable feature of the BART is the floating intercedent device, better known as the ball. The testers can provide a whole variety of information. The data that is taken from a reaction that occurs will depend upon the population size or the activity of the bacteria in the sample. Currently a 1-3 day reaction time is used to indicate that there are severe problems. A 4-6 day reaction time indicates moderate problems, and a 7-10 day reaction time indicates that minor problems may exist. Some data is now being gathered that can provide an actual range of microbial numbers that are needed for a reaction to occur.

Some field test results are also being obtained that will provide a correlation of comparison between the total results for a particular material and the days that it takes for a reaction to occur. It appears that a six day reaction time is considered important. If reaction time is below six days, then a severe problem, corresponding to about a 2 ppm particulate solution, is present. A comparison between the time it takes for that reaction to occur and microbial populations as determined on BHI medium has been obtained as well. It appears that if there is less than a six day reaction, then there will be greater than 500 colony forming units (CFU) per ml. On the other hand, if the reaction time is six days, then there will be less than 500 CFU/ml.

In the last three months, I have used approximately 1000 of these tests out in the field. One of the interesting things that was noted quite recently in Gainesville, Florida (from time-dated sampling) was that different dominant populations could be detected at different sampling times. Of those working in the industry, this is important if one is trying to use such information to better customize a treatment to rehabilitate any particular problem that one has. Thank you.
Q and A

G. Alfred: Neil, this system allows one to take an immediate sample out of the well itself, which would provide an idea of what is occurring in the water after it has a chance to set. Then the one-minute, five-minute, and 30-minute samples are taken to provide an idea of what is occurring back in the formation, as far as a broad general view. One can actually see the change and obtain a good idea of what the next move will be.

N. Mansuy: You are right. This system really offers an inexpensive tool that can be used by small water companies and the water industry in general. There is great value if a test like that can be utilized. Before any type of treatment is done, a positive reaction can be obtained in less than a day. If samples are set up monthly, every two months, or every three months, and if a reaction is obtained within 5-10 days, a treatment can be done before a more extensive treatment is done.

T. Hazen: How expensive are the BARTS?

N. Mansuy: They can be bought for less than $10 each.
Spatial Distribution of Microbial Biomass, Activity, Community Structure and Xenobiotic Biodegradation in the Subsurface

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Abstract

The vertical distribution of microbial biomass, activity, and community structure, as well as the mineralization of xenobiotic chemicals, was examined in two 20-m soil profiles in northern Wisconsin. One profile was impacted by infiltrating wastewater from a laundromat, while the other served as a control. Biomass and community structure were determined by measuring concentrations of phospholipid derived fatty acids (PLFA) and by acridine orange direct counts (AODC). Microbial activity was estimated by measuring fluorescein diacetate (FDA) hydrolysis, thymidine incorporation into DNA, and mixed amino acid (MMA) mineralization.

Mineralization kinetics of linear alkylbenzene sulfonate (LAS) and linear alcohol ethoxylate (LAE) were determined at each depth. Except for MAA mineralization rates, measures of microbial biomass and activity exhibited similar patterns with depth and correlated with each other. PLFA concentration and rates of FDA hydrolysis and thymidine incorporation decreased 10- to 100-fold below 3 m and then exhibited little variation with depth. Fungal fatty acid markers were found at all depths and represented from 1 to 15% of the total. The relative proportion of tuberculostearic acid (TBS), an actinomycete marker, declined with depth and was not detected in the saturated zone. The profile impacted by wastewater exhibited higher levels of PLFA, but a lower proportion of TBS than the control profile. This profile also exhibited faster rates of FDA hydrolysis and amino acid mineralization at most depths. LAS was mineralized in the upper 2 m of the saturated zone, but not in the vadose zone (2-14 m) of both profiles. LAE was mineralized at all depths in both profiles and the mineralization rate exhibited the same general pattern with depth as biomass and activity measurements.

1Oral presentation.
Introduction

Among the xenobiotic chemicals that are produced in the greatest volume and have received the most widespread use worldwide are in fact those which are ingredients of household cleaning products. Two of the most common are LAS and LAE. LAS is an anionic surfactant, and in 1987 alone, approximately 1.8 million metric tons were consumed worldwide. It is a major ingredient in just about every leading brand of laundry detergent. LAEs are non-ionic surfactants and their annual consumption is approximately 1/2 million metric tons. These cleaning compounds are discarded down the drain and removed by a combination of absorption and biodegradation at sewage treatment plants. However, they commonly enter subsurface environments through leach fields that serve home septic tanks and on some occasions, leach fields that serve rural laundromats. Therefore, in the work described in this paper, the four objectives were as follows: to determine the vertical distribution of microbial communities in soil profiles that were affected and unaffected by infiltrating waste water from a laundromat; (2) to examine the ability of subsurface communities to mineralize mixed amino acids, LAS, and LAE; (3) to compare biodegraded activity of subsurface soil and groundwater that was obtained from the same depths as impacted and nonattached sites; and (4) to establish whether biodegraded activities correlated with measures of microbial biomass activity.

Materials and Methods

The study site was located in northern Wisconsin in the town of Summit Lake. It consisted of a laundromat of 24 washing machines which discharged their wastewater through a cycling tank through a percolation field, through a sluce way, and then to a waste water pond. Monitoring wells were located upgradient and downgradient from the pond in the percolation field. In addition, soil samples were taken at various depths from two sites: the leach field site, which was located adjacent to the percolation field, and the control site, which was located upgradient. The depth to the water, to the underlying aquifer, was approximately 14.5 m.

Various measures of water quality to the upgradient and downgradient wells were gathered. Conductivity, alkalinity, and hardness, as well as the concentration of a variety of inorganic ions, including phosphate, were all elevated in the downgradient well. Notably, the water was oxygenated in both wells, and methylene-blue acid substances, including LAS, and cobalt thiocyanate substances, including LAE, were not detected in either well.

Soil samples were obtained by drilling through the desired depth with a hollow-stem auger and then driving in a coring device. The outer surfaces of the cores were pared using a device similar to that developed by the Ada Group to generate aseptically obtained subsurface material.

Various techniques were used for characterizing microbial communities. Microbial activity was estimated by measuring the rates of FITC-diacetate hydrolysis (FDA) and thymidine incorporation into DNA. FDA is a chromogenic substrate that is cleaved by a wide range of intercellular esterases and proteases. Bacterial numbers were estimated using the AOAC procedure and total biomass was estimated by measuring the concentration of phospholipid fatty acids. Community structure was...
assessed by looking at the relative abundance of surface fungal fatty acids, protozoan fatty acids, and fatty acids, which are unique markers for actinomycetes in mycobacterial.

**Results**

FDA hydrolysis rates, the leach field soil profiles, and the rate of thymidine incorporation, which is a function of depth in the control, were used to obtain activity measures (slide). Both activity measures exhibited a 10- to 1000-fold decline at a depth of 2-3 m. This drop occurred in both profiles and appeared to be independent of the presence of infiltrating waste water. FDA activity, but not the thymidine incorporation rate, was consistently higher in the leach field compared to the controlled soil profile. The bacterial numbers and phospholipid concentrations, as a function of depth, showed that the same discontinuity that was seen with the activity measures was also present with the measures of biomass. Bacterial numbers declined five-fold while phospholipid concentrations declined 10- to 100-fold, depending upon the profile. While bacterial numbers were the same in both profiles, phospholipid concentrations in the leach field profile were up to 10 times higher than what was observed in the controlled profile.

The relative abundance of fungi, actinomycete and protozoan fatty acid were shown as a function of depth in the soil (slide). Fungal fatty acids were present at every depth in both profiles. Their relative abundance tended to be a bit higher in the upper vadose zone compared to the lower vadose zone and the saturated zone. The greatest concentration was at the 3 m depth within the leach field zone. The relative abundance of actinomycetes fatty acids decreased with depth in both profiles and the rate of this decrease was faster in the leach field profile. None of these fatty acids were detected in the saturated zone of either site. Protozoan fatty acids were detected in the upper vadose zone and in the saturated zone of the control site. In general, the relative abundance of protozan fatty acids was a bit higher in the controlled site.

Bacterial numbers in groundwater and in soil from the upgradient and downgradient sites showed that the counts in the soil were similar. At the upgradient site, bacterial numbers in groundwater were about an order of magnitude less than in the soil. In the downgradient site, the difference was two orders of magnitude. Notably, groundwater from the upgradient site had approximately five times more bacteria than that from the downgradient site.

Mineralization or biodegradation was determined by incubating uniformly-labelled fatty acids \(^{14}\text{C-LAE}\) to \(^{14}\text{C ring labelled LAS}\) with groundwater in subsurface soil to follow the evolution of radiolabelled \(\text{CO}_2\) over time. First order rate constant \(K\) and the yield of \(\text{CO}_2\) was estimated from the data using nonlinear regression. The first order rate constants and the \(\text{CO}_2\) yield were used to describe the mineralization of amino acids in soil as a function of depth (slide). Amino acid mineralization did not exhibit a consistent pattern of depth when compared to other measures of microbial activity, and biomass actually varied a little with depth. In the control profile, rates were highest in the upper vadose zone and then they decreased. In the leach field profile, the rates were lowest in the upper vadose zone and then they
decreased. Contrasting the mineralization of amino acids with soil and water from the upgradient and downgradient sites showed that mineralization of amino acids in the soil was similar at both sites; however, while mineralization of amino acids in groundwater were comparable with soil in the upgradient site, the mineralization was reduced in the downgradient site, which was consistent with the lower bacterial counts at this site.

The rate and yield of CO₂ were used to describe the mineralization of LAE in soil. LAE was mineralized at every depth with a maximum lag of approximately five days. Once again, there was a sharp discontinuity in the rate of LAE degradation at the depth of 2-3 m. Interestingly enough, the rates of LAE mineralization tended to be higher in the control profile as compared to the leach field profile. Another look at water and soil from the saturated zone shows that LAE mineralization was faster and more extensive in soil compared to groundwater. In the upgradient site, mineralization was proceeded by a seven-day lag with groundwater. In the downgradient site, this lag was not apparent.

Unlike LAE and mixed amino acids, LAS mineralization did not occur at every depth in the soil. LAS was mineralized fairly rapidly in the upper vadose zone, then not mineralized again until the saturated zone where the rate was about 1/10 of that in the upper vadose zone. Both profiles pretty much exhibited the same general pattern. LAS mineralization in soil was extensive; however, no LAS was mineralized in the groundwater. Hence, it appears that LAS biodegraded activity is isolated in the aquifer solids rather than present in the groundwater. This is not surprising given the fact that LAS is a very sorptive material. Mineralization in soil from the upgradient site was proceeded by a seven-day lag. Again, this lag was not nearly as apparent in the downgradient site.

There were some correlations among the various parameter measures. Measures of biomass and activity tended to correlate pretty well with one another. The rate of LAS mineralization correlated somewhat, but not real well with phospholipid concentrations, the rate of FDA hydrolysis, and the rate of thymidine incorporation. There was no correlation with direct count. The rate of LAE mineralization correlated, although not really well, with thymidine incorporation and it actually correlated best with the rate of LAS mineralization.

**Conclusion**

In summary, there was a marked discontinuity in the vertical distribution of microbial biomass activity in the biodegradation of LAS and LAE below 2.5 m. The presence of infiltrating waste water affected the magnitude of discontinuity, but it did not affect its existence or depth. The soil profile that was impacted by infiltrating waste water was characterized by higher biomass and activity and also, by a lower diversity (particularly in the upper 5 m), which appeared to be the zone of maximum impact. Groundwater had lower bacterial numbers and exhibited less biodegraded activity than soil obtained from the same site and depth. LAE and amino acid biodegradative activity was present in groundwater and at every depth in the soil profile. In contrast, LAS biodegradative activity was limited to soil in the upper
vadose zone and the saturated zone. Finally, despite the patchiness of the distribution of biodegradative activity, neither of these compounds were detected in groundwater. Thank you.
Q and A

P. Strom: From the plot plans, it looks like that was an open pond. Is that correct?

T. Federle: Yes. In fact, this system has been there since 1962. It was a little tourist community, and the laudromat was built next to the road. In general, the wastewater was running out the back door and creating an ecosystem of suds in the pond.

P. Strom: Were there any kind of analysis of the pond itself?

T. Federle: Yes. In fact, an integrated study looking at fate processes in the surface and subsurface has been done.

P. Strom: And that was where it was degraded?

T. Federle: It is degraded in many different places, depending on the compound. A lot depended upon the availability of oxygen because some of the compounds required molecular oxygen for their initial degradation step.

A Konopka: I have a question about the thymidine incorporation. It seem like a number of investigators use thymidine incorporation in the sediments. It creates more problems than in planktonic samples because it starts getting incorporation into some macro molecules. Did you look at that at all?

T. Federle: Yes, in fact, the thymidine incorporation work was done by Roy Ventullo at the University of Dayton. He just had a paper in Microbial Ecology not too long ago, describing the methodologies he used, including data from this site. They used proteases and RNAses to look for labels in other components of biomass. There were steps in the extraction and the purification procedure to hydrolyze the RNA and so forth. Therefore, we have pretty good data that this is thymidine incorporation into the DNA and not just thymidine incorporation into TCA precipitable materials.

A Mills: I have a comment on the thymidine. We found that a lot of the anaerobes in the sediments take up thymidine and incorporate it at different rates. I do not think that it changes the shape of the curve too much, since it is probably a systematic error. However, you might want to take a look at it if you are going to publish productivity numbers.

J. Wilson: Why was the deeper, unsaturated zone not acclimated to remove LAS?

T. Federle: That is a good question.

J. Wilson: I will entertain speculations if you do not know.
T. Federle: I thought a lot about this. In the vadose zone, which is very sandy, did things just shoot through there very fast? Was the loading intermittent because it was based on a cycling tank that possibly you never really do select in that zone, but where water collects in the saturated zone we do select, we do see acclimation there.

J. Wilson: Did you look for any kind of chemical tracer that would indicate that the pond had actually drained past the particular point in the saturated zone from which you acquired your core, such as elevated or extractable phosphorous, iron leaching, or something like that?

T. Federle: In the groundwater, there was real good evidence that we were definitely in communication. In the soil profile, there were very high, elevated levels of organic carbon at the site where percolation was occurring. Some LAS determinations were done, but they were not very good. However, the levels were elevated relative to the blanks although the blanks were also high, yet we feel confident that we were in the perch zone with our samples.
Development of Microcosms Designed to Evaluate the Effects of Adding Microorganisms to Aquifers as Bioremediation Agents

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Abstract

Aquifer microcosms, designed to simulate a sand and gravel aquifer located on Cape Cod, Massachusetts, were used to evaluate the survival and activity of nonindigenous microorganisms that could be introduced into an aquifer for bioremediation purposes. *Pseudomonas* sp. strain B13 and its genetically modified derivative, *Pseudomonas* sp. strain B13 FR1(pFRC20P), were used as model pollutant-degrading microorganisms. The following approach was taken for the development of microcosms: (1) design simple microcosms that simulate the environment; (2) determine the survival of model microorganisms and their ability to degrade specific pollutants in the microcosms; (3) compare the microcosms to the actual aquifer by comparing biological, chemical, and physical properties; and (4) compare the survival of microorganisms introduced into the aquifer with survival in the microcosms. To date, the survival of the microorganisms and their ability to degrade pollutants in batch and flow-through column microcosms has been determined. The microorganisms survived for the ten-week study period in some microcosms, depending on the source of the aquifer sediment, and they degraded the specific pollutants in batch microcosms.
Introduction

Groundwater pollution has become a major concern for human health and economic reasons, primarily due to the diminishing supply of clean water that is available to an ever growing human population. To increase water supplies and to lessen health concerns, it is necessary to remediate existing, contaminated sources of groundwater. The potential for naturally occurring groundwater bacteria to degrade pollutants has been documented. Thus, one approach is to use aquifer microorganisms for in situ bioremediation. Such a method is both cost effective and environmentally acceptable. It is even possible to augment the natural biological activity of subsurface microorganisms by pumping essential nutrients and either oxygen or hydrogen peroxide as a source of oxygen into the contaminated aquifers.

If the microbial community of an aquifer does not express pollutant degrading activity, another possible approach is to add microorganisms which have been acclimated to degrade the pollutants. These can often be isolated from the environment, but natural populations of pollutant-degrading microorganisms do not always exist. To obtain microorganisms with appropriate biological activity, it may be possible to genetically modify bacteria to degrade otherwise recalcitrant compounds. However, the pollutant-degrading activity and fate of such genetically engineered microorganisms (GEMs), once introduced into the environment, cannot be predicted *a priori*. This report describes the initial design and use of aquifer microcosms to determine whether model pollutant-degrading GEMs have the potential to survive and express their catabolic phenotypes under simulated *in situ* conditions. Microcosms were chosen as appropriate tools based on their proven usefulness in demonstrating biotransformations of pollutants by natural microbial communities and in predicting the fate of specific pollutants in aquifers.

The microorganisms used were *Pseudomonas* sp. strain B13 (B13) and its genetically modified derivative, *Pseudomonas* sp. strain B13 FR1(pFRC20P)(FR120). B13 uses 3-chlorobenzoate (3CB) as its sole substrate by metabolism through an ortho-cleavage degradation pathway. This pathway was modified by the incorporation into B13 of genes coding for enzymes from the TOL plasmid of *Pseudomonas putida* (pWWO) and from *Alcaligenes eutrophus* JMP134. The derivative, FR120, was thereby able to simultaneously degrade 3CB and 4-methylbenzoate (4MB) through a modified ortho cleavage pathway. In nature, the simultaneous presence of both of these substrates, together with microorganisms that are able to degrade one but not the other, leads to the formation of degradative intermediates that are toxic and/or dead-end products. Thus, FR120 serves as a model GEM that was constructed to degrade environmental contaminants in situations where naturally occurring microorganisms are unable to do so.

**Materials and Methods**

*Microorganisms.* *Pseudomonas* sp. strain B13 (B13) and *Pseudomonas* sp. strain B13 FR1(pFRC20P) were routinely grown in standard M9 medium, supplemented with either 5mM 3-chlorobenzoate (3CB) for B13 or a mixture of 3CB and 4-methylbenzoate (4MB) (2.5 mM each) for FR120. Bacteria used in microcosm
studies were collected by centrifugation and washed twice in M9 medium that contained no substrates.

**Microcosm Design.** Material for constructing microcosms was obtained from the United States Geological Survey's Groundwater Contamination Study Site, Cape Cod, MA, as previously described. The site contains a plume of sewage contaminated groundwater. Aquifer sediment and groundwater were obtained from three different depths. Depth 1 was from 4.7 to 5.7 m and was located above the contaminant plume in a zone of relatively high oxygen concentration. Depth 2 was from 6.2 to 7.0 m and was located within the contaminant plume where measurable levels of oxygen still existed. Finally, Depth 3 was from 13.9 to 14.5 m and was also located within the contaminant plume, but at a depth where no oxygen could be detected. Sediment for batch microcosms was obtained in October, 1988, and for flow-through microcosms in June, 1989.

Batch microcosms consisted of aquifer sediment (20 g) and groundwater (2 ml) from the same depth and they were placed together in a 100 ml serum bottle which was sealed with a teflon coated silicon stopper. Oxygen was provided to all microcosms through the large air-filled headspace in the bottles. Flow-through microcosms consisted of aquifer sediment (40 g) placed inside a 15 mm x 300 mm plexiglass column and flanked with pea gravel; the columns were then stoppered on both ends. Groundwater was collected every three days in hospital infusion bags to minimize exposure to oxygen and was pumped into the bottom of the columns at a flow rate similar to the aquifer (0.4 m/day). Control microcosms consisted of sterile aquifer material that had been autoclaved twice for one hour on consecutive days. Both batch and flow-through microcosms were incubated at 12°C in the dark.

**Experimental.** Sediment for batch microcosms was mixed together with either B13 or FR120 (10⁶ cells/g sediment). A solution of 3CB was added to some of the microcosms containing B13 (final concentration of 50 nmol/g sediment), and 3CB and 4MB were added to some microcosms containing FR120 (25 nmol/g each). In both cases, this was done to determine whether the bacteria would degrade the substrate and whether this would affect bacterial survival. Control microcosms did not receive bacterial additions, but 3CB and 4MB were added to determine whether indigenous microorganisms could degrade substituted benzoates. B13 and FR120 were enumerated and the concentration of substituted benzoates was determined in triplicate microcosms at 0, 1, 4 and 10 weeks.

Flow-through microcosms contained sediment with either B13 or FR120 (10⁶ cells/g sediment), to which no microorganisms were added. Groundwater pumped through the microcosms contained 3CB and 4MB (100 μM each) or they had no addition of substrates. Microorganisms were enumerated in duplicate microcosms at 0, 1, 4 and 10 weeks.

**Bacterial Enumeration.** B13 and FR120 were extracted from samples of sediment (20 g) by shaking for 30 minutes with 50 ml of 0.1% Na₃P₂O₅ (pH 7.0). Extracts were serially diluted in the same buffer and 100 μl of the dilutions were spread onto a minimal agar medium that contained either 5 mM of 3CB or 2.5 mM each of 3CB and 4MB to select for B13 and FR120, respectively. No background
growth appeared on these plates; the detection limit for both microorganisms was 10^3/gdw sediment.

Analytical. Water samples were acidified to a pH level of 2 with conc. HCl and extracted twice by shaking for 30 minutes with 20 ml of ethyl acetate. The extract was collected, dried with Na_2SO_4 and evaporated to dryness. Samples were then resuspended in methanol and the concentration of 3CB and 4MB was determined using a Beckman System Gold HPLC with a Beckman C-18 column. The wavelength was set at 280 nm; the solvent contained 40% H_3PO_4 (0.01%) and 60% methanol. Effluent from flow-through microcosms was collected every three days and analyzed directly.

Results

Batch Microcosms. Survival of B13 in batch microcosms was related to the depth from which the aquifer sediment was obtained (Figure 1a). After one week of incubation, B13 was no longer detectable in microcosms containing sediment from Depth 1. However, in microcosms with sediment from Depth 2 or 3, the numbers of B13 did not decrease during the ten week incubation. B13 degraded 3CB, but only in microcosms containing Depth 2 sediment (Figure 1b). This degradation corresponded to an increase of two log units in the number of B13. No degradation was observed due to the presence of B13 in microcosms that contained sediment from Depth 1 or 3. Degradation of 3CB by indigenous microorganisms was not observed in microcosms that contained aquifer material from any depth and indigenous microorganisms able to use 3CB could not be isolated using selective medium.

The number of FR120 enumerated in the microcosms was similar to that of its parent, B13: no decrease in number was observed in microcosms that contained aquifer sediment from Depth 2 (Figure 2), whereas after one week, FR120 could not be detected in microcosms that contained sediment from Depth 1 (Results not shown). Both 3CB and 4MB were degraded in microcosms made with Depth 2 aquifer sediment that contained FR120, which corresponded to a two log unit increase in cell number.

Flow-Through Microcosms. B13 and FR120 survived for ten weeks in microcosms that contained both sterilized and nonsterilized aquifer sediment (Figure 3). There was no apparent degradation of 3CB in the flow-through microcosms with or without added FR120, which was probably due to the short retention time of the substrate (Results not shown). On the other hand, 4MB was rapidly degraded whether or not FR120 was present.

Discussion

Genetically engineered microorganisms (GEMs) have been suggested as potential agents for the bioremediation of polluted environments and a number of modified microorganisms have been constructed as model pollutant-degrading GEMs. These microorganisms contain broadened substrate ranges and express novel, well-regulated catabolic pathways. In order to elicit the degradation of environmental pollutants, it may be necessary to introduce such GEMs into contaminated environments. Unfortunately, little information exists concerning
either the ecological consequences of environmental introductions of GEMs or the ability of laboratory modified microorganisms to survive, and of their constructed pathways to function in environmental conditions. This study describes preliminary efforts to construct aquifer sediment microcosms to be used in evaluating the pollutant-degrading potential of GEMs and the ecological consequences of environmental introductions prior to field trials. The experiments were designed specifically to determine whether the GEMs could survive and then function as pollutant degraders under in situ aquifer conditions.

*Pseudomonas* sp. strain B13 and its derivative, *Pseudomonas* sp. strain B13 FR1(pFRC20P) (FR120), were used in batch microcosm studies both as a means of comparing the survival of a parent bacterium with that of its genetically modified derivative and to determine whether catabolic pathways constructed in the laboratory would function under in situ conditions. Both microorganisms survived equally well in microcosms that were constructed with sediment taken from the two depths of the aquifer that were located within the pollutant plume (Depths 2 and 3). This demonstrated that the genetic modifications did not present a “genetic load” that was able to lessen the competitiveness of the modified strain. Degradation of substituted benzoates occurred only in microcosms constructed with sediment obtained from Depth 2. Since *Pseudomonas* spp. are aerobic microorganisms and the degradation of 3CB by these microorganisms is an aerobic process, it was expected that the conditions most favorable for both survival and 3CB-degrading activity would occur in microcosms that contained sediment from Depth 1, where neither microorganism survived. This depth was outside of the pollutant plume and as such should contain fewer indigenous microorganisms that are able to compete with the added strains. Protozoan grazers may have contributed to the comparatively poor survival of FR120 and B13 in these microcosms, although this has not been verified.

Flow-through microcosms have the advantages of maintaining some of the structure of the aquifer sediment and of simulating water flow effects. In preliminary flow-through microcosm experiments, the number of B13 decreased by two log units, whereas in autoclaved controls B13 maintained a steady population level during the entire ten week study period. Thus, there was apparently some effect of competition or predation on B13 survival. The number of FR120 also decreased, with the decrease occurring most rapidly in microcosms which contained added 3CB and 4MB. The reason for this is not clear, but it may be due to the stimulation of competitors which have the ability to degrade 4MB.

**Conclusion**

These preliminary studies have demonstrated that GEMs have the potential to both survive and to effect degradation of environmental contaminants under in situ conditions, and furthermore, that microcosms can be useful tools in determining how environmental variables affect survival and activity. As the systems become more sophisticated, the quantity and complexity of the data relating to the microbial ecology of aquifer sediments will increase.
Acknowledgements

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References


Figure 1. Enumeration of B13 (row a) and analysis for substrate disappearance (row b) in batch aquifer microcosms. Microcosms were made with aquifer sediment which originated from three different depths in the aquifer (see text). Microcosms with B13 (row a) contained either 3CB (+3CB; 50 nmol/g wet weight) or were unamended (-3CB). Those used to determine substrate disappearance (row b) contained B13 (+B13) or had no addition of B13 (indigenous). CFUs and substrate values are averages of samples from triplicate microcosms. BDL = below detection limit.)
Figure 2. Enumeration of FR120 (2a) and analysis for substrate disappearance (2b) in batch aquifer microcosms that contained aquifer sediment from Depth 2. Microcosms with FR120 (2a) contained either a mixture of 3CB and 4MB (+ 3CB, 4MB; 25 nmol/g each) or were unamended (- 3CB, 4MB). Substrate disappearance (2b) was determined for the above corresponding microcosms in which FR120 was enumerated (2a). CFUs and substrate values are averages of samples from triplicate microcosms. (BDL = below detection limit.)

Figure 3. Enumeration of B13 (3a) and FR120 (3b) in flow-through aquifer microcosms that contained both aquifer sediment and groundwater from Depth 2. One-half of the flow-through microcosms with B13 (3a) were autoclaved to determine the effect of indigenous microorganisms on B13 survival. The groundwater pumped through the flow-through microcosms with FR120 (3b) contained either a mixture of 3CB and 4MB (+3CB, 4MB; 100 μM each) or was unamended to determine the effect of a selective substrate on FR120 survival. CFUs and substrate values are averages of samples from duplicate microcosms.
Q and A

T. Hazen: Were your microcosms initially sterile?

D. Dwyer: No. They were all present with indigenous microorganisms, but there were controls run that were sterile to see what happened as well.

T. Hazen: You selected media then for...

D. Dwyer: Enumeration of the microorganisms, yes.

T. Hazen: Did you check to see if there was any loss of the plasmid over time?

D. Dwyer: Not in this case. The reason why is because we have only been doing aquifer microcosms for a little while. However, microcosms have been done for other aquatic environments, and if one checks over long periods of months both in rich laboratory media and in these river sediment microcosms, as well as in microcosms taken from activated sludge, one will notice that plasmids were never seen.

T. Hazen: Did you ever check to see if the plasmid and the genetic modifications that you knew the bacteria represented gave a significant change in the metabolic flow?

D. Dwyer: That was one of the reasons why we always try to compare these systems with the survival of the parent microorganism (B13), and with the survival of the genetically engineered strains. There was never a change in number.

T. Hazen: Was it in culture?

D. Dwyer: This was in rich culture. There never appeared to be a load due to that.

T. Hazen: How large was the plasma?

D. Dwyer: It was approximately 55 Kdaltons.

J. Wilson: We had exactly the opposite experience with the TOL plasma in an indigenous organism that was isolated from the aquifer. Gary Sayler put the TOL into a bacteria that was renamed P. pleomorphus. When it was returned to the original ambient conditions in microcosms, it said, “I’m home.” The plasma was discorded and it refused to degrade toluene.

D. Dwyer: During the making of this particular strain, there were strains which lost the plasma. This one was selected for its ability to hold onto the plasma for a long time under the laboratory conditions that were set up. Another TOL plasma was worked with which had genetic modifications made to it. In this case, the plasma
tended to disseminate to the other microorganisms. It did not lose the plasma. Instead, it actually allowed the plasma to run wild through the whole population. So in contrast to this microorganism, which held onto the plasma, it had no mobilization function present.

_J. Wilson:_ Obviously there are aquifers and then there are aquifers. In our aquifer material, we had plenty of potential hosts for TOL, at least 10^6/gm. Therefore, Sayler had one of these radioactive markers for both host chromosome and plasma. Host chromosome was established at approximately 10^4 and the plasma was down below its detection limit at 10^2. Therefore, it neither passed on to a more willing host, nor did it survive the organism in which it was inoculated. This was a situation in which one case worked and another case did not.
Bacterial Transport in Heterogeneous Porous Media
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Abstract

Transport of bacteria through porous media was examined in columns of clean quartz sand (0.35-mm diameter). Preferred flow paths were constructed in some columns by embedding cylinders of relatively coarse sand (1.68-mm diameter) in a matrix of the finer material. A pulse of bacterial cells and NaCl (as a conservative tracer) was injected into each column under steady flow conditions. Samples (0.25 pore-volume each) were collected and analyzed for concentration of chloride and bacteria. Breakthrough curves were similar in shape for the bacteria and chloride. In homogeneous columns, the peak breakthrough occurred at one pore-volume. Numbers of bacteria breaking through were approximately two orders of magnitude lower than the theoretical prediction for a conservative substance. A long "tail" of bacteria subsequently flowed from the column over several pore volumes, a result that is inconsistent with the advection-dispersive theory. The "tailing" of the breakthrough is presumably a response to the release of filtered or adsorbed cells into the eluent flushing of the columns after the pulse injection. In columns with a structural heterogeneity, doubly peaked breakthrough curves were observed. The first peak represented transport through the preferred path, and the second peak represented a combination of transport through the matrix and release of chloride and bacteria "stored" in the matrix to the preferred paths. The recovery of cells in those columns was about 50% as compared to about 5% for the homogeneous case. A dual-porosity expansion of the advection-dispersion model was applied to the results of the heterogeneous flow experiments. While a "perfect" fit to the data was not achieved, the results suggest that such a model may be applied to the transport of bacteria in some heterogeneous flow situations.
Introduction

Questions of bacteria transport are relevant to several topics in subsurface microbiology. Basic ecological questions on origins and distribution of deep subsurface bacteria require consideration of transport phenomena for testing of appropriate hypotheses. Questions of bacterial transport are essential in consideration of public health issues such as contamination of drinking water supplies by bacteria (and viruses) from sewage disposal, septic fields and landfills. Finally consideration of bacterial transport is a critical element for any in situ biorestoration program, especially those which might involve introduction of microorganisms to contaminant plumes or lenses.

Porous media are supposed to be good filters for microorganisms. The number of septically contaminated wells and aquifers provides evidence that porous media are not perfect biological filters. For all of the aforementioned area of concern, a workable, predictive capability is essential. Recent exercises in modeling bacterial transport have resulted in the development of some complex and comprehensive theoretical models that include every conceivable factor germane to bacterial transport. Unfortunately, such models are not useful in the real world due to the large numbers of parameters for which estimates must be provided, and the difficulty in obtaining site-specific estimates for even a few of those. Furthermore, such models are formulated for homogeneous situations only. Much recent work indicates that bacteria move farther and faster than predicted by filtration theory-based models formulated for homogeneous media.

We are especially interested in appropriate simulation of transport in the presence of discrete flow paths such as fractures of macropores. We have begun to explore bacterial transport in simple laboratory models to allow development of theoretical models that account for the mechanics that are associated with the heterogeneous situation. The results of our initial studies suggest that a dual-porosity approach may be applicable in some heterogeneous situations.

Methods

Experiments explored the behavior of an Arthrobacter sp. isolated from a pristine aquifer by Baldwill and Chiorse. Column experiments were carried out by injecting the bacterial cells as a slug through a column packed with clean quartz sand. In these experiments, resting cells were used to allow appropriate mass balance calculations that would be precluded if the cells were permitted to grow. Details of the general methods for column preparations are published elsewhere (Scholl et al., submitted for publication). A 1-ml portion of a suspension of the bacterium was added to the liquid (approximately 2 ml) on top of the column. The injection contained about $10^8$ cells/ml; the actual concentration varied from experiment to experiment, but was determined for each experiment. Samples were collected at 0.25 pore-volume intervals in syringes mounted in a soil-water extractor.

In addition to the bacterial suspension, 1 ml of a 0.1 M NaCl solution was added to the slug as a conservative tracer. The chloride was added 15 minutes before the bacteria so that the ions would not affect the behavior of the bacteria. Appropriate volume corrections were made so that the chloride and bacterial breakthrough curves
could be plotted together against the pore volumes that were eluted. Samples eluted from the columns were split for analysis of chloride (by specific ion electrode) and for counts of bacteria by acridine orange direct counts (AODC).\textsuperscript{4}

The treatments that were examined dealt with a comparison of bacterial breakthrough under homogeneous vs. heterogeneous flow conditions. Transport in the homogeneous case was described by the one-dimensional advection-dispersion equation:

$$\frac{\partial C}{\partial t} = D \frac{\partial^2 C}{\partial Z^2} - V \frac{\partial C}{\partial Z} - R$$  \hspace{1cm} (1)

where $V$ represents the pore velocity, $D$ the dispersion coefficient, and $R$ is a reaction term that accounts for any physical, chemical, or biological interactions of the material being transported with the mineral medium. In packed sand columns, bacteria appeared to behave similarly to the chloride tracer (Figure 1), which means the peak for the breakthrough curve occurred at one pore volume. Mass balance calculations showed that all of the chloride tracer was recovered, but only about 5\% of the bacterial cells were recovered. Scholl et al. (submitted) suggested that the low recovery was due primarily to physical filtration (straining, pore clogging). Additionally, the breakthrough of chloride was complete by approximately two pore volumes, whereas elution of bacteria continued for as long as determinations were made (up to seven pore volumes in some experiments).

Given the results of experiments by School et al. (submitted) and the results reported here, the homogeneous case can be dealt with in a straightforward manner. This situation is ideal for experiments designed to sort out effects of chemical and biological factors on bacterial transport, but the homogeneous case is not particularly interesting hydrologically, nor is it a good analog for real groundwater flow situations.

Experiments in advective bacterial transport under heterogeneous flow conditions were formulated in a very simplistic manner. For these purposes, heterogeneity has been defined in two ways. Unstructured heterogeneity refers to variations in pore-throat diameters caused by the random distribution of particles of a range of sizes. Thus, any discrete flow paths created may not be continuous in the porous bed. Structured heterogeneities may be best defined by example. Fractures, coherent macropores, and other preferred flow pathways represent our concept of structured heterogeneity because they provide continuous, discrete flow paths for some major distance in an aquifer.

The physical formulation of structured heterogeneity consisted of a vein of porous material (coarse sand, 1.68-mm diameter) embedded in a column of less porous sand (fine sand, 0.35-mm diameter). Experimental columns were constructed by pouring a column of fine sand around a plastic soda straw (6-mm diameter). The straw was then filled with the coarse sand, whereupon the straw was removed (Figure 2).
Breakthrough of both bacteria and chloride in the heterogeneous case (Figure 3) differed greatly from that observed for the homogeneous case. Two peaks were observed, with the initial breakthrough at approximately 0.33 pore-volume based on total volume of column; that volume was approximately one pore-volume for the coarse-grain vein. The bacteria preceded the chemical tracer (as predicted by size-exclusion principles for heterogeneous transport), and a strong tailing was observed for both the bacteria and the chloride. The second peak occurred at approximately 1.5 pore-volumes for both the bacteria and the chloride, after which the chloride concentrations returned to background. Meanwhile, the bacteria continued to elute for the rest of the experiment, as was observed for the homogeneous case. Mass balance calculations showed that all of the chloride was recovered, and for several experiments, the recovery of bacteria ranged from 25% to 75%, with most values around 50%.

To mathematically model such behavior, the following dual-porosity extension of the advection-dispersion model was used:

\[
\frac{\partial C_A}{\partial t} = D_A \frac{\partial^2 C_A}{\partial Z^2} - V_A \frac{\partial C_A}{\partial Z} - a(C_A - C_B) / \theta
\]

\[
\frac{\partial C_B}{\partial t} = D_B \frac{\partial^2 C_B}{\partial Z^2} - V_B \frac{\partial C_B}{\partial Z} - a(C_B - C_A) / \theta
\]

Manipulation of this model represents a "first-cut" attempt to elucidate the mechanics of bacterial transport that led to the doubly peaked breakthrough curve always seen in these experiments.

The dual porosity model has three parameters for which estimates must be provided: \(V\), the pore velocity; \(D\), the dispersion coefficient; and \(a\), the exchange coefficient. Pore velocities (\(V\)'s) can be estimated as follows (this model is based on the concept of parallel flow in the two domains with Darcian velocities (\(u\) through each domain; Figure 3):

\[
u_A = K_A (dh / dz)
\]

\[
u_B = K_B (dh / dz)
\]

Thus,

\[
u_A / \nu_B = K_A / K_B = r
\]
Values for K are unavailable, but the Kozeny-Carmen relationship can be used to estimate the ratio of the Darcian velocities (r):

\[ r = \left( \frac{\phi_A}{\phi_B} \right)^2 \]  \hspace{1cm} (7)

where \( \phi \) represents the grain diameter in each domain. The continuity equation gives the following:

\[ u_A A_A + u_B A_B = Q \]  \hspace{1cm} (8)

where the A’s represent respective cross-sectional areas. Using Eq. (6) with Eq. (8) provides the following two equations:

\[ u_A = \frac{Q}{(A_A + A_B / r)} \]  \hspace{1cm} (9)

\[ u_B = \frac{Q}{(rA_A + A_B)} \]  \hspace{1cm} (10).

The pores velocities (V’s) are the Darcian velocities (u’s) divided by the porosities. Thus, the estimations of pore velocities requires the discharge (Q), the porosities (\( \phi \)’s), the grain diameters (\( \phi \)’s), and the diameters of the "straw" and of the column so that the areas (A’s) may be computed. Measurements or reasonable estimates of all of these are available.

The dispersion coefficients were not known, but values could be guessed at. Given that the column was 12 cm long and 2 cm in diameter, the dispersivities should have been on the order of a few tenths of a centimeter. Estimates could also be gained from fitting data from homogeneous columns of appropriately sized sand. The exchange coefficients were also unknown, but could eventually be determined empirically from the data.

To test the model, chloride data from an experimental run were compared with model output (to determine if the dual-porosity model was appropriate for the hydrologic properties of the physical model that was created). For the first run, D was set at 0.3 cm times the velocity, \( \alpha \) was set at zero (no exchange between domains), and the diameter of domain A was set at the diameter of the soda straw, 6 mm. For those conditions, the peak from the fine material occurred much too late (Figure 4A). Changing estimates of D or \( \alpha \) would not change the position of the peak; the problem was associated with the advective part of the transport.

The advective portion of the model could be changed by altering the diameter of the domains. The effective diameter of the heterogeneity might have been less than 6 mm due to the "caving in" that occurred when fines mixed into the vein as the straw was withdrawn. Figure 4B shows that a diameter of 0.4 cm gave the right timing and magnitude for the second peak. The first peak remained very high and
the simulated concentrations were well below the observed values in the interpeak time period. By giving the exchange coefficient ($\alpha$) a non-zero value, the interpeak concentrations were elevated, but the magnitude of the first peak was not greatly reduced (Figure 4C).

The model's simulation of the size of the first peak was not in good agreement with the observations from the experimental run. Adjustments to the model parameters did not result in better agreement. It was considered that the discrepancy might have been due to the fact that the experimental data represented averages made over 1/4 pore-volume intervals. The values from the previous run were averaged over a time interval corresponding to 0.25 pore-volume samples (Figure 4D). While a "good" fit was not achieved, the results did suggest that at least part of the discrepancy could be attributed to the effect of averaging.

**Conclusion**

The results of this study suggest that the dual-porosity approach to modeling bacterial transport in heterogeneous media may be an appropriate means to include the effect of discrete flow paths and exchanges between materials of differing transmissivities. The application of dual-porosity models to flow and transport of bacteria in the field, however, will require the resolution of a number of difficult problems. Flow in natural geological media can hardly be expected to be along straight, one-dimensional pathways. Extension of the model to solve transport in complex natural materials will require examination and testing of new conceptual models (e.g., networks of interconnected linear segments). The model will have to be adapted to deal with a statistically characterized network of preferred paths. A stochastic approach to stimulations aimed at assessing contaminated sites will also be needed. The incorporation of bacterial transport into the model will entail additional difficulties. The importance of various physical and chemical factors to a myriad of processes controlling transport of bacteria is largely unknown. Experiments aimed at resolving some of the vexed questions regarding the factors controlling bacterial transport in porous media are underway in our laboratory.

**Acknowledgements**

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References


Figure 1. Transport of bacteria and chloride through columns packed with clean quartz sand: the homogeneous case. (Note the continued elution of bacteria from the column after all the chloride has passed.)

"Structured" Heterogeneity (Dual-Porosity Model)

Figure 2. Structural heterogeneity. A and B refer to the flow domains established for the dual-porosity model. $u_A$ and $u_B$ refer to the Darcian velocities for those domains under the assumption of parallel flow.
Figure 3. Breakthrough of bacteria and chloride from columns containing a structured heterogeneity.
Figure 4. Comparison of output from the dual-porosity model with actual chloride concentrations obtained in an experiment with a column containing a structured heterogeneity.
A. Mills: There is one thing that was brought up earlier that I think is important to realize and that is the very large filtration effect that was obtained. These columns have been loaded with $10^8$ cells right up to the top of the column. Therefore, a lot of straining was occurring, which was one reason why such low natural recoveries were obtained.

S. Garabedian: That is a very nice piece of work and I compliment you on your efforts here. I did not catch in your talk though, how you came about calculating the velocities of each component, the fine sand and the coarse sand.

A Mills: You want me to go all the way through it or should we do it afterwards?

S. Garabedian: Maybe afterwards. That would be a good idea because obliviously, one can shift those velocities around and make a piece fit. So there must have been some effort in trying to calculate the velocity.

A. Mills: All of the estimation derivation for the velocity was skipped. I will be glad to talk to you later about it.
MICROBIAL TRANSPORT AND ADHESION IN SUBSURFACE ENVIRONMENTS

Round Table 4
Problems with Identification, Taxonomy and Phylogeny
Conveners: J. Fredrickson and D. Balkwill

NO ABSTRACTS
MICROBIAL TRANSPORT AND ADHESION IN SUBSURFACE ENVIRONMENTS

Round Table 5
Tracers
Conveners: C. B. Fliermans and R. W. Harvey
Abstract

Perfluorocarbon tracers (PFTs), a multitracer technology of seven chemical tracers, were used to tag the drilling muds used in the C10 Hole of the Microbiology of the Deep Subsurface investigations. PFTs are fully fluorinated, alkyl substituted cycloalkanes, which are inert, non-toxic, chemically non-reactive, and detectable in these applications to 1 picoliter/gram of sample by gas chromatographic electron capture detection (GC-ECD). PFTs were incorporated into the drilling muds at the 100,000 pl/g level by addition of a 1% PFT microemulsion in H2O during the periodic preparation of drilling muds. A total of four different PFTs were used to tag the drilling mud used in the stratigraphic core and sample boreholes; two PFTs were used in the stratigraphic and two further PFTs in the sample boreholes. After sampling, a small aliquot of sample was forwarded to the Tracer Technology Center (TTC) for PFT analysis along with samples of the tagged muds. The samples were centrifuged and headspace sampled for PFT. Results of the PFT analyses were then used to assess the degree of drilling mud contamination of the retrieved samples. The results indicated some contamination of the retrieved samples and some transport of fluids from the core borehole to the sampling borehole, which are quantitated by the measured PFT concentrations.
Introduction

The Tracer Technology Center (TTC) at Brookhaven National Laboratory has developed a perfluorocarbon tracer (PFT) technology over a fifteen year period which has been applied in various atmospheric, hydrological, and subsurface applications. PFTs are a class of perfluorinated, alkyl substituted cycloalkanes that are uniquely detectable at extremely low levels, down to $10^{-16}$ moles PFT/liter, and have been extensively used as tracers of gas and liquid flows competitive with radioactive tracers in terms of detectability. Traditionally, fluorescent dyes have been used as tracers of liquid flow; however, the PFT technology has some advantages compared to the fluorescent tracers. These advantages are as follows:

1. Completely non-toxic, stable, and inert (chemically and biologically).
2. Approximately four orders greater sensitivity for detection (i.e., circa $10^{-16}$ moles PFT/liter).\textsuperscript{1,5}
3. A multi-tracer technology, up to five or six PFTs may be used in an experiment, with the same deployment, sampling, and analysis system.
4. Relatively nonadsorptive in hydrological scenarios (i.e., conservative, in transport and in dispersion studies).\textsuperscript{3,4}
5. Colorless, odorless and tasteless for use in hydrological systems.

The disadvantages of using PFTs in hydrological scenarios is their low aqueous solubility, which requires special deployment techniques,\textsuperscript{3,7} and their volatility, which imposes some constraints on the sampling methods.\textsuperscript{4} The relatively nonadsorptive property of PFTs, which is relatively conservative as compared to fluorescent dyes can be improved by using multiple PFTs in place of a single PFT application. The observed PFT results can then be extrapolated to a totally "conservative tracer" result as a function of the physical property, causing a nonconservative nature such as physical or chemical adsorption of the tracer onto components present along the hydrological transport path.

The PFT technology is based on a GC-ECD method of analysis, which permits the high sensitivity of detection for the PFTs. This is further enhanced by a laboratory-based PFT analysis system (incorporating the GC-ECD) with a unique sample processing train, which removes the majority of all interferents in the analysis. Further details of the PFT technology and other applications are available in Dietz.\textsuperscript{1}

The PFT technology was applied in the C10 boreholes of the Microbiology of the Deep Subsurface project. Briefly, the goal of this project was to obtain subsurface samples for subsequent microbiological examination. Ideally, these samples must not be contaminated by external agents during their retrieval from depths up to 2000 feet below the surface. However, the press of obtaining these sample cores exposed them to the drilling muds that were used in the drilling of the sampling borehole. After retrieval, cores had their drilling mud, exposed surfaces pared away to remove any potential contamination. Consequently, PFTs were incorporated into the drilling mud so as to provide a quantitative measure of the residual degree of drilling mud
contamination remaining in the pred cores. In effect, PFTs were used as a quality assurance technique for the retrieved samples.

Since there were two boreholes drilled (approximately 200 feet apart), one for the purpose of determining the stratigraphic core (core borehole) and the other for obtaining the samples (the sample borehole), both the drilling mud of the core well and sample well were tagged with different PFTs. This allowed for further quantification of any residual contamination of the sample core from the drilling mud of either the core borehole or sample borehole. It is possible that there could have been subsurface transport of the drilling mud fluids from the core borehole bearing a different PFT; therefore, this would allow quantification of this source of potential contamination.

Materials and Methods

Four different PFTs were used in the C10 Hole, two in the core borehole and two in the sample borehole as shown in Table 1. The PFTs were prepared as 1% PFT microemulsion in water using Pluronic F68 as the surfactant, which was added during the preparation of the drilling mud, as made in 100 gallon lots. The 1% PFT microemulsion was proportionately added so as to form a nominal 1 ppm PFT concentration in the drilling mud, because the PFT is soluble in water at these concentration levels based on earlier work. It was expected that the PFT microemulsion would completely dissolve within tens of minutes. Most likely, this dissolution was enhanced by the turbulent mixing of the drilling mud during preparation. As stated earlier, the PFT is volatile and it was expected that some PFT would be lost from the open drilling mud pit to the atmosphere, consequently, aliquots of drilling mud were sampled and analyzed for the resulting PFT concentrations, so as to account for this loss. A total of eight gallons of 1% PFT microemulsion was used in the investigations.

Aliquots of drilling muds, core parings, and core samples were collected at the drilling site and sealed in 15 ml plastic centrifuge tubes with a tight sealing septum-like cap. Upon receipt of the tubes that were containing the samples, they were centrifuged to remove air pockets and pellet the samples. This was necessary since the samples ranged from liquids to slurries to solids, and sampling for PFT was performed by headspace sampling. Each sample was then weighed, the cap was pierced, and a 100 µl gaseous headspace sample was injected onto a CATS (Capillary Adsorbent Tracer Sampler), a carbonaceous adsorbent sampler for PFTs (Dietz, 1986) and submitted for PFT analysis in the laboratory-based PFT analysis system. Two headspace samples were withdrawn from each sample.

Results

The results of the PFT analysis of the samples received are given in Table 2. Table 2a through 2d are the results for the core parings and core samples, which Table 2e shows the results for the drilling muds and Table 2f shows the results for various washes of the drill rods, core samplers, etc. The columns across Table 3 are as follows: (1) CATS ID, which is the identification number of the sampler used in the analysis of the C10 samples. Two 100 µl aliquots of headspace air were withdrawn
from each sample and adsorbed onto two CATS samplers for analysis. Consequently, each C10 sample was analyzed twice, and thus, there are two rows of PFT concentrations for each sample (except for some washes, rinses, and other test blanks). (2) Sample description, which is copied directly from the received sample vials. In general "clean" or "good" refers to a core sample and "dirty" or "paring" refers to a discarded core paring. (3) The geological formation from the sample as recorded from the received shipping documentation. (4) The last four columns give the concentration of the PFT found in the sample according to tracer type in units of picograms (10^-12g) per gram of sample, as calculated from the weight of the sample received, the measured headspsce volume, and the measured PFT concentration. Tracer 4 contained approximately 0.5-1% of Tracer 1. Consequently, concentrations of Tracer 1 above 1% of Tracer 4 should be regarded as above background, otherwise it is derived from tracer 4. It was originally planned to use only two tracers, but due to drilling difficulties, four tracers were needed on short notice. They could not be obtained highly purified, and thus, the 1% Tracer 1 impurity in Tracer 4.

**Discussion**

The results of the PFT analysis of the drilling mud, as given Table 2e, indicates a PFT concentration in the drilling mud of 2-12% of the design 1 ppm (i.e., 1,000,000 picoliters PFT/gram of sample). The loss was due to PFT volatilization from the open mud pit and headspace sampling efficiency that was not quantified. Nonetheless, this efficiency is a constant throughout the experimental analyses.

A summary of the degree of drilling mud contamination as calculated from the PFT analyses of the core samples is given in Table 3. Samples above G-11/G-12 have drilling mud contamination from the stratigraphy core borehole at 0.02%-0.1% level, present in both the parings and the core samples. Below G-11/G-12 there is a measurable drilling mud contamination of all samples from the sampling borehole drilling mud. For many samples, two core samples were taken from different sections of the core and the measured degree of drilling mud contamination differs, especially in core samples from G-12, G-13, and G-18. However, only in sample G-17 though G-20 does the drilling mud contamination rise above 1%.

The full dynamic range of the PFT technology was not exploited. Approximately 2-3 orders of PFT detection was available, but the five orders of detectable dilution observed in these samples was quite sufficient.

**Conclusion**

In conclusion, the multitracer PFT technology has allowed assessment of the degree of contamination in the core samples from different sources of drilling muds used in the C10 Hole of the Microbiology of the Deep Subsurface Investigation. The microbiological investigators will have to objectively assess as to whether these levels of drilling mud contamination compromise their microbiological results with respect to drilling mud contamination, although it is unlikely that PFT's move as bacterial contaminants in sediment core material.

**Acknowledgments**

This work was in part sponsored by the Savannah River Plant, E. I. du Pont de Nemours and Co., under contract to the U.S. DOE, Contract DE-AC09-76SR0001, and by the U.S. DOE, Office of Energy Research.
References


Table 1. PFTs Used in the C10 Borehole.

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<tr>
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<tr>
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<td>perfluoromethylcyclohexane (PMCH)</td>
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<td>perfluorotrimethylcyclohexane (PTCH)</td>
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Table 2a. C10 Borehole Sample PFT Concentrations.

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*Samples in which PFT loss may have occurred during handling, especially for PMCP.
### Table 2b. C10 Borehole Sample PFT Concentrations.

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*Samples in which PFT loss may have occurred during handling, especially for PMCP.
Table 2c. C10 Borehole Sample PFT Concentrations.

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<td>Bag Cover</td>
<td>---</td>
<td>---</td>
<td>3.0</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>5182</td>
<td>Senum Glove</td>
<td>---</td>
<td>---</td>
<td>6.4</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>5607</td>
<td>Senum Pad</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>7470</td>
<td>Senum Glove Sleeve</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>1.6</td>
</tr>
<tr>
<td>1885</td>
<td>Senum Supernatant</td>
<td>---</td>
<td>---</td>
<td>2.1</td>
<td>732</td>
<td>---</td>
</tr>
</tbody>
</table>
Table 3. Drilling Mud Contamination in the Sample, as Derived from PFT Analyses

<table>
<thead>
<tr>
<th>Sample</th>
<th>Degree of Contamination</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-6</td>
<td>Contaminated by drilling mud from stratigraphy core borehole, circa 0.1% in the core and paring samples.</td>
</tr>
<tr>
<td>G-8</td>
<td>Contaminated by drilling mud from stratigraphy core borehole, circa 0.05% in core and paring samples.</td>
</tr>
<tr>
<td>G-9</td>
<td>Contaminated by drilling mud from stratigraphy core borehole, circa 0.1% in core and paring samples.</td>
</tr>
<tr>
<td>G-10</td>
<td>Contaminated by drilling mud from stratigraphy core borehole, circa 0.1% in core and paring samples.</td>
</tr>
<tr>
<td>G-11</td>
<td>Stratigraphy core well contamination = 0.02%. Sample core borehole mud contamination = 0.06%.</td>
</tr>
<tr>
<td>G-12</td>
<td>One core had 0.03% contamination, other core had 2.3% contamination.</td>
</tr>
<tr>
<td>G-13</td>
<td>One core had 0.07% contamination, other core had 0.7% contamination.</td>
</tr>
<tr>
<td>G-14</td>
<td>Less than 0.002% contamination.</td>
</tr>
<tr>
<td>G-15</td>
<td>Core samples had 0.009% drilling mud contamination.</td>
</tr>
<tr>
<td>G-16</td>
<td>Core samples had 0.2% drilling mud contamination.</td>
</tr>
<tr>
<td>G-17</td>
<td>Core samples had 2.9% drilling mud contamination.</td>
</tr>
<tr>
<td>G-18</td>
<td>One core sample had 3.7% drilling mud contamination, another core sample had 42% drilling mud contamination.</td>
</tr>
<tr>
<td>G-20</td>
<td>One core sample had 0.9% drilling mud contamination, another core sample had 2.6% drilling mud contamination.</td>
</tr>
<tr>
<td>G-21</td>
<td>Core samples had 0.14% drilling mud contamination.</td>
</tr>
<tr>
<td>G-22</td>
<td>Core sample had 0.5% drilling mud contamination.</td>
</tr>
<tr>
<td>G-23</td>
<td>Less than 0.002% drilling mud contamination.</td>
</tr>
</tbody>
</table>
Q and A

*T. Hazen:* How confident are you of your calculations of percent contamination that came from the drilling mud samples that you took from the core. I wonder how exact some of those comparisons were because of the circulation of the drilling mud.

*G. Senum:* When they took core they took a sample of drilling mud at the same time. Therefore, we had analyses of about 10 or 11 different drilling muds. The PFC concentration of the drilling mud ranged from approximately 2% up to 20% of the expected 1 ppm. So there was volitalization of the tracer from the drilling muds, but the calculation of chemical contamination was made from the drilling mud taken at the same time the core was pulled.

*T. Hazen:* Would Tom Phelps comment on how close the timing would be on that?

*T. Phelps:* With the three tracers (perfluorocarbon, bromide, and rhodamine), it was found was that the chemical contamination was highest with the bromide tracer and the perfluorocarbon was much closer to a level of contamination with rhodamine. The perfluorocarbon told us that every sample was contaminated, but it also told us approximately how much of it was contaminated. The goal was set at 10⁶. On several samples, the goal was reached, but the perfluorocarbon was always detected. However, the perfluorocarbon was always less than the bromide.
Evaluation of Particulate and Solute Tracers for Investigations of Bacterial Transport Behavior in Groundwater

Ronald W. Harvey, United States Geological Survey, WRD, Menlo Park, CA 94025.

Abstract

The utility of particulate and dissolved tracers in delineating abiotic aspects of bacterial transport behavior in groundwater was evaluated in forced- and natural-gradient experiments performed in different types of aquifers. In small-scale tracer experiments that involved coinjection of bacteria and bromide (assumed conservative), dissimilarity between the respective breakthrough curves increased with increasing degrees of secondary (preferred flow path) pore structure in the aquifer. Peak bacterial abundance occurred well in advance of the bromide peak in both a fractured-rock and a layered basalt aquifer, but was almost coincident with that of Bromide in a well-sorted sandy aquifer. Differences between bromide and bacterial breakthrough in the sandy aquifer (Cape Cod, Massachusetts) appeared to be due largely to sorptive interactions with surfaces, whereas differences in breakthrough among tracers in the layered basalt aquifer (Oahu, Hawaii) appeared to be due to a complicated pore structure.

At the Cape Cod site, a number of sizes and types of bacterial-sized microspheres were used to obtain abiotic information about bacterial transport behavior. The microspheres were useful in obtaining site-specific information about the optimal size for transport, the importance of sorptive (vs straining) filtration, and the effects of size and surface charge upon retardation and attenuation. However, their transport behavior differed substantively from that of bacteria in terms of attenuation, dispersion, and retardation. In general, the usefulness of particulate vs. dissolved tracers as constituents in bacterial transport experiments depended upon the information sought, the experimental design, and the pore structure of the aquifer.
Introduction

Recent interest in migration of bacteria through the subsurface is leading to efforts to model their transport more accurately through porous media. Transport of microbial pathogens to water supply wells from sources upgradient presently accounts for a substantial fraction of the reported cases of water-borne illness in the United States. On the other hand, cotransport of indigenous and "waste-adapted" bacteria with organic contaminants may result in enhanced degradation and in the "seeding" of areas downgradient from the contamination sources with acclimated bacterial populations that are capable of breaking down refractory compounds. Several theoretical models have been developed that describe bacterial movement through porous media.\(^1\)\(^2\)\(^9\) However, experimental data are scarce. It is clear that more field experiments performed in a variety of aquifers are needed to delineate the transport behavior of bacteria in groundwater. Well-defined tracers can be useful in accounting for abiotic processes in such studies, which are typically complicated by a number of geohydrological, chemical, and biological factors. This report discusses the use of well-defined solute and particulate tracers in recent published and unpublished in situ transport experiments with bacteria in groundwater.

Materials and Methods

The advantages and limitations of using dissolved, non-reactive and particulate tracers were examined in small-scale groundwater injection and recovery experiments. The experiments involved transport of bacteria, bacteria-sized microspheres, and halide tracers (assumed non-reactive) through a layered, sandy aquifer sediment in Cape Cod, MA and through a fractured, layered basalt aquifer in Oahu, HI. At Cape Cod, natural-gradient tests were run in October of 1986 with chloride and bacteria-sized microspheres\(^5\) and in October of 1987 with bromide and stained bacteria. Stained bacteria and bromide were also employed in the June 1988 Oahu experiment (Harvey, et al., unpublished data), which involved a small-scale (27 m travel distance), forced-gradient (convergent) test. Relative differences in transport behavior between tracers and bacteria were compared among these small-scale tests and to other experiments involving cotransport of bacteria and tracers.

For the Cape Cod experiment, a morphologically diverse bacterial population was collected from the aquifer as described by Harvey et al.\(^6\) The bacteria were then concentrated on-site from 600 l of groundwater into 1-2 l using a hollow-fiber tangential-flow filtration (TFF) device with periodic back-pulsing capability (Kuwabara and Harvey, in press). Recovered bacteria (0.2-1.4 μm, 0.6 μm average cell length) were stained with a DNA-specific fluorochrome, 4',6-diamidino-2-phenylindole (DAPI), using a previously described procedure.\(^5\) The stained bacteria were diluted with groundwater collected at the injection test site to a final volume of 90 l. The consequential dilution of the DAPI stain precluded the staining other bacteria in the aquifer. Stained bacteria or fluorescent, bacteria-sized microspheres of differing types and diameters and chloride or bromide, were added slowly (0.85 l/m) to the aquifer at 8.5 and 9.1 m below land surface and monitored as they moved with the natural flow of groundwater past a row of multilevel sampling devices set perpendicular to the direction of groundwater flow, 6.8 m downgradient. Ground-
water samples (500 ml) were collected daily from sampling ports located in the path of injectate travel.

For the Oahu forced-gradient experiment, an isolate was obtained from the aquifer, cultured in nutrient media, harvested by centrifugation, resuspended in 4 l of filter-sterilized water, and stained with DAPI. The DAPI-stained bacteria were then diluted to a final volume of 1 l and co-injected with bromide into a highly-conductive zone between two layers of fractured basalt. Groundwater samples were taken from the pumping well located 27 m downgradient and operating at approximately 10 million liters per day. In both the forced- and natural-gradient experiments, halide tracers were first measured in the field with a specific-ion electrode and later confirmed by ion chromatography (Waters ICP-A column with borate gluconate buffer at 1.2 ml/minute at 25°C). Preparations for enumeration of DAPI-stained bacteria or microspheres (Cape Cod experiment) were made with 100 to 200 ml of sample to obtain accurate counting statistics. The DAPI-stained bacteria were fluoresced under incident UV light (340 -380 nm excitation) and were enumerated on black polycarbonate membrane filters (0.2-μm pore size, 25-mm [millimeter] diameter) using a microscope that was fitted for epifluorescence, as described by Harvey.³

Results and Discussion

Retardation factors for bacteria in the natural- and forced-gradient tracer test (Cape Cod and Oahu) are listed in Table 1. These values are compared to the results of other groundwater tracer experiments in which transport of microorganisms relative to bromide were examined. For both zones in the natural-gradient experiment and for the closer sample in the forced-gradient experiment (Cape Cod), arrival time downgradient of the stained bacteria was nearly coincident with that of Bromide. In contrast, calculated retardation factors (ratio of time required to reach peak abundance for the microorganisms to time to peak concentration for Bromide) for all of the other injection tests employing microorganisms were substantially less than 1.0. Retardation factors substantially less than 1.0 indicate that transport of microorganisms not attenuated by the medium is, on the average, significantly faster than that of Bromide and, presumably, mean groundwater flow.

The apparent enhancement in transport velocity of the unattenuated microorganisms was greatest in the experiment that involved a fractured crystalline-rock aquifer. It is hypothesized that this phenomenon is caused by preferential transport of the microorganisms along preferred flow paths (large pores and channels), because they may be excluded from the smaller pores on the basis of size. The near absence of rapid transport of the indigenous bacteria relative to bromide at the small-scale, natural-gradient tests may be caused by the absence of secondary pore structure; this is consistent with the tightly packed, well-sorted nature of the aquifer sediments.

The dissimilarities in transport behavior between bromide and bacteria at the Oahu site differed significantly from what was observed at the Cape Cod site. Peak abundance of labelled bacteria appearing at the pumping (sampling) well downgradient...
in the 24-hour long, convergent tracer test in Oahu significantly preceded peak abundance of bromide. In the Cape Cod tests, a greater degree of "tailing" was observed for the concentration history of labelled bacteria than for bromide, due to a retardation of a portion of the bacterial population. However, in the Oahu test, a 90% decrease in concentration from peak breakthrough took approximately four times longer for bromide than for the bacteria. The data suggest that most of the bacteria appearing downgradient in the Oahu test (where much of the flow occurs within channels between the layers of basalt) had been transported within preferred flow paths.

The results of the various in situ transport experiments suggest that the usefulness of halide tracers in studying the transport behavior of bacteria depends upon the pore structure of the aquifer. At the Cape Cod site, the pattern of breakthrough for bacteria and bromide were similar. Breakthrough of bromide and stained bacteria each exhibited single peaks and followed similar temporal patterns. In this system, bromide was useful in the construction of the hydrological portion of the overall bacterial transport model. In contrast, the breakthrough patterns observed for bacteria in forced-gradient experiments performed in other types of aquifers were quite different from that of bromide. The earlier arrival of peak abundance and shorter duration of breakthrough for the bacteria relative to bromide suggests that there are significant differences in paths of travel between bacteria and dissolved species in fractured or fractured-layered rock aquifers. Therefore, a great deal of caution should be used in employing conservative tracers in bacterial transport tests in such systems.

The advantage of using bacteria-sized microspheres instead of halide tracers to delineate abiotic aspects of bacterial transport behavior is that the microspheres and bacteria should follow the same flow paths, even in aquifers with substantial preferred flow-path structure. One disadvantage of using microspheres is that the nature and intensity of interactions with solid surfaces may differ substantially from those experienced by bacteria. The differences in relative transport velocity and breakthrough between bromide or chloride and various types of bacteria-sized colloids in a small-scale, natural-gradient tests at Cape Cod are listed in Table 2. Differences in transport behavior between microspheres and bacteria can involve the magnitude of retardation; in the Cape Cod experiments, bacteria were not retarded (relative to a conservative tracer), whereas most of the microspheres were subject to substantial retardation. Only the neutral (uncharged) microspheres traveled at approximately the same rate as bromide or chloride. However, the degree of attenuation (immobilization at solid surfaces) for the neutral microspheres was several hundred fold higher than that observed for the bacteria in similar experiments. Although the polyacrolein microspheres exhibited rates of immobilization much closer to those of bacteria, they were significantly retarded, as were the carboxylated latex spheres.
Conclusion

In summary, none of the particulate or dissolved tracers considered in this study appeared to be ideally suited for investigations of bacterial transport behavior in groundwater. In tightly-packed, well-sorted aquifer sediments with little secondary pore (preferred flow path) structure, conservative (non-reactive) tracers such as bromide or chloride appear to be useful in delineating the hydrologic aspects of bacterial transport behavior. None of the bacteria-sized microspheres tested exhibited the same combination of attenuation, retardation, or apparent dispersion as bacteria. However, microspheres appear to be useful in the delineation of certain abiotic aspects of bacterial transport behavior.
References


### Table 1. Differences in apparent transport velocity between microorganisms and bromide in small-scale groundwater tracer experiments.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Microbe</th>
<th>Aquifer</th>
<th>Type of test</th>
<th>Distance (meters)</th>
<th>Retardation factor&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Champ and Schroeter, 1988</td>
<td><em>Escherichia coli</em></td>
<td>Fractured crystalline rock</td>
<td>forced-gradient</td>
<td>12.7</td>
<td>0.1</td>
</tr>
<tr>
<td>Harvey, Voss and Souza, unpublished</td>
<td>Indigenous isolate <em>(Bacillus sp.)</em></td>
<td>Layered basalt</td>
<td>forced-gradient</td>
<td>27</td>
<td>0.6</td>
</tr>
<tr>
<td>Wood and Ehrlich, 1978</td>
<td><em>Saccharomyces cerevisiae</em></td>
<td>Sand and Gravel (with clay and carbonate)</td>
<td>forced-gradient</td>
<td>1.5</td>
<td>0.7</td>
</tr>
<tr>
<td>Harvey et al., 1988</td>
<td>Indigenous bacterial population</td>
<td>Well-sorted sand and gravel</td>
<td>forced-gradient</td>
<td>1.7</td>
<td>1.0</td>
</tr>
<tr>
<td>Harvey and Garabedian, submitted</td>
<td>Indigenous bacterial population</td>
<td>Well-sorted sand and gravel</td>
<td>natural-gradient</td>
<td>6.7</td>
<td>1.0</td>
</tr>
</tbody>
</table>

<sup>1</sup>Calculated as the ratio of transport velocity (at peak abundances) of bromide to that of the microorganisms.

### Table 2. Differences in apparent transport velocity and magnitude of breakthrough for bacteria-sized colloids relative to bromideomideomide in small-scale, natural-gradient groundwater tracer experiments (Cape Cod, MA).

<table>
<thead>
<tr>
<th>Reference</th>
<th>Colloid</th>
<th>Type</th>
<th>Diameter (microns)</th>
<th>Retardation Factor&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Relative breakthrough&lt;sup&gt;2&lt;/sup&gt; (x 10 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Harvey and Garabedian, submitted</td>
<td>bacteria</td>
<td>indigenous population</td>
<td>0.6</td>
<td>1.0</td>
<td>15-21</td>
</tr>
<tr>
<td>Harvey et al., 1989</td>
<td>plain latex</td>
<td>uncharged</td>
<td>0.6</td>
<td>-1.0</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>polyacrolein surface gels.</td>
<td>carbonyl</td>
<td>0.8</td>
<td>1.3</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>carboxylated latex</td>
<td>carboxyl surface gels.</td>
<td>0.5</td>
<td>1.4</td>
<td>0.04</td>
</tr>
</tbody>
</table>

<sup>1</sup>Calculated as the ratio of transport velocity (at peak abundances) of bromide to that of the microorganisms.

<sup>2</sup>Calculated as the integral of the dimensionless concentration history normalized to that of bromideomideomide.
A Comparison of Results from Small- and Large-Scale Tracer Tests in a Heterogeneous Aquifer, Cape Cod, Massachusetts


Abstract

Recent research on the transport and fate of contaminants in groundwater at the United States Geological Survey's Toxic Waste Research site on Cape Cod, Massachusetts, has focused on the identification and quantification of processes that determine the rate of contaminant movement, spreading, and reactions in the subsurface environment. Twenty-seven tracer tests have been conducted during the past five years as part of this research effort at the site. These tests were controlled and in situ experiments were designed to test specific hypotheses for the processes under investigation. The tracers used in the experiments include inorganic (bromide, chloride, ammonia, nitrate, lithium, molybdate, chromate, selenate, nickel, zinc) and organic compounds (methane, chlorofluoromethane, EDTA), bacteria, and fluorescent microspheres. The tracer tests were conducted with forced- and natural-gradient flow fields, with travel distances of 2-280m. Although the forced-gradient tests are appealing because they have short durations, the natural-gradient experiments were found to be the most useful because they match the flow conditions in the aquifer.

The method of analysis for the natural-gradient experiments has depended on the scale of the test. Spatial-moments analysis, which is an integrated statistical approach, was used to calculate model parameters from the results of the single large-scale test conducted at the site. The approach for the small-scale tests has been to match the output of analytical models to the time-concentration breakthrough curves. Although the curve-matching approach is very useful for process-orientated research, problems can arise in uniquely identifying the model structure and parameter values. These problems arise especially when the aquifer properties are heterogeneous, as is the case for the Cape Cod site.
Introduction

The U.S. Geological Survey's Cape Cod Toxic Waste Research site is the focus of research on the geologic, hydrologic, chemical, and microbiological processes that affect the movement of contaminants in a plume of sewage-contaminated groundwater originating from Otis Air Base. In the early phase of research at the Cape Cod site, observed distributions of contaminants and bacteria were used to infer major processes that affect the transport and attenuation of contaminants in the plume, and hypotheses concerning transport of contaminants were proposed and tested. More recent research has focused on the use of tracer test experiments to quantify these processes. This paper compares the results of different types of tracer tests conducted at the site.

Materials and Methods

Site Description. The study area is in the northern part of Falmouth, Massachusetts, on a broad sand and gravel outwash plain that slopes southward to Nantucket sound. The top 30-40 m of the outwash is composed of glacially derived, stratified, and well-sorted sand and gravel. The sand and gravel overlies fine sand, silt, and, in some areas, a dense, sandy till. These sediments overlie a crystalline (granodiorite) bedrock surface, which generally slopes from west to east across the study area.

Groundwater in the sediments is unconfined. The water table slopes toward the south at approximately 1.5 m/km; water-table altitudes range from 15 to 9 m above sea level across the study area. Estimated recharge to the aquifer from precipitation is 0.5 m/year, which is approximately 45% of the total precipitation. Seasonal variations in recharge cause an annual water-table fluctuation of 0.3-0.9 m; the highest levels are in the spring and the lowest are in the fall. Estimated horizontal velocities of ground-water in the sand and gravel range from 0.2 to 0.6 m/day, based on the hydraulic gradient, with a range of hydraulic conductivity of 60-120 m/day, and a range of porosity of 30-40%.

Sewage Plume. LeBlanc described the extent of contamination in the aquifer caused by sewage disposal at Otis Air Base in the northern part of the study area. In 1979, the plume of contaminated groundwater, formed by the disposal of secondarily treated sewage onto rapid infiltration beds since 1936, was 0.8-1.1 km wide, 23 m thick, and more than 3.4 km long. The plume moves in the direction of ambient flow to the south and is overlain by up to 15 m of uncontaminated groundwater derived from local recharge from precipitation.

The plume of sewage-contaminated groundwater is characterized by elevated concentrations of dissolved solids, boron, chloride, sodium, phosphorus, ammonium, nitrate, and, in some locations, volatile organic compounds (VOC). Boron, chloride, and sodium appear to be moving conservatively and are attenuated primarily by hydrodynamic dispersion. Phosphorus movement is greatly retarded by colloidal precipitation and adsorption onto the sediments. Although a maximum nitrate concentration of 16 mg/l of N has been detected in the sewage effluent, the maximum observed concentration in the center of the plume is 3.2 mg/l. Within 1.5 km of the infiltration beds, the predominant nitrogen species in the plume is
ammonium. Farther than 1.8 km from the beds, the predominant nitrogen species is nitrate. It was hypothesized that this distribution of ammonium is caused, in part, by adsorption onto the aquifer sediments, which retards the movement of ammonium.

Within the sewage plume, detergent concentrations exceed 0.5 mg/l methylene-blue-active substances (MBAS) from 0.9 to 3.0 km downgradient from the sewage infiltration beds. This distribution of detergents reflects the use of nonbiodegradable detergents during 1946-64. Elevated VOC concentrations are present in two zones, one which is immediately downgradient from the infiltration beds and the other more than 1 km downgradient. VOC concentrations in the most downgradient zone exceed 50 μg/l, suggesting that the VOC are mobile and not readily degraded in the sandy aquifer.1

Bacterial populations are as large as 21 million/ml near the infiltration beds, decreasing to about 250,000/ml at 1 km from the beds (Harvey, personal communication). These numbers appear to correlate with the availability of degradable organic compounds; concentrations of dissolved organic carbon decrease from 4 mg/l to less than 1 mg/l over the same distance. The decrease in bacterial numbers with distance, particularly bacteria smaller than 0.4 μm in diameter, also suggests that both transport and adsorptive filtration of bacteria are occurring. Water and sediment assays were used to measure microbial activity rates in the plume6 because more than 90% of the bacteria were found to be attached to silt- and clay-sized particles.9 These measurements show that rates of microbially mediated denitrification are greatest in water and sediment samples collected from a 1- to 2-m-thick zone near the top of the plume.17

Results and Discussion

The following two factors limited adequate testing of the hypotheses developed in the early phases of research at the Cape Cod site: (1) the history of chemical composition of the sewage-plant effluent is largely unknown; and (2) methods to measure rates of dispersion and reactions in the aquifer were inadequate. Unknown source history and inadequate sampling are problems that face investigators at most toxic-waste sites. Therefore, current research at the Cape Cod site has focused on the development and use of field tracer experiments and specialized sampling methods to improve the understanding of transport processes.

Twenty-seven tracer tests were conducted at the Cape Cod site during 1984-89. These tests were designed to be controlled, in situ experiments for testing specific hypotheses related to the transport processes under investigation. The tracers used in the experiments include inorganic (bromide, chloride, ammonium, nitrate, lithium, molybdate, chromate, selenate, nickel, and zinc) and organic compounds (methane, chlorofluoromethane, and EDTA), bacteria, and fluorescent microspheres. The tests were conducted with forced- and natural-gradient flow fields, with travel distances of 2-280 m.

Forced-Gradient Tracer Tests. The forced-gradient tests included doublet-well tests, in which the rate of injection in one well and the rate of pumping in the other well were about the same. A convergent test was done, where the withdrawal rate
at the pumped well is large and the injection rate at the injection well is relatively small and of short duration. Divergent tests, in which the injection rate is high and the withdrawal rates (through ports on a multilevel sampling well) are small were done as well. Although forced-gradient tests are appealing because of their short duration, they are difficult to interpret when the flow field is nonideal. A nonideal flow field can be caused by partial penetration of the injection well into the aquifer, which creates flow that is neither radially cylindrical nor spherical, or by large contrasts in hydraulic conductivity (heterogeneity) around the injection site. For example, partial penetration in the divergent forced-gradient tests caused rapid vertical displacement of the solutes from the zone of injection.\textsuperscript{7} Heterogeneity of hydraulic conductivity can produce different velocities within the injection zone as evidenced by multiple-peaked breakthrough curves. An examination of the variability of hydraulic conductivity at the Cape Cod site has shown that lenses with similar hydraulic conductivity can be really extensive, with a horizontal correlation scale of 5 m,\textsuperscript{12} and yet, be typically less than 25 cm thick.

Examples of forced-gradient tests include a divergent test in which heat was used as a tracer to calculate aquifer porosity, longitudinal dispersivity for mass and temperature, bulk thermal conductivity, and apparent longitudinal thermal conductivity.\textsuperscript{2} Two other divergent tracer tests were conducted at the same site with ammonium, potassium, nitrate, and bromide as tracers in order to test the previously developed hypothesis that ammonium transport is retarded by adsorption. Results of these tests showed that potassium and ammonium, both cations, are retarded by ion exchange relative to the nonreactive anionic tracer, bromide, causing cations such as calcium, magnesium, and sodium to be released into solution.\textsuperscript{4} It was also observed that nitrate (an anion) was not retarded relative to bromide. In each of the above examples, a comparison of average or peak arrival times at various depths for reactive and nonreactive tracers were used to interpret the behavior of the process in the aquifer; however, in many of the forced-gradient tests, the appearance of multiple-peaked breakthrough curves made test interpretation difficult and ambiguous.

**Small-Scale Natural-Gradient Tracer Tests.** In addition to small-scale forced-gradient tests, small-scale natural-gradient tracer tests have been used in the study area to test hypotheses related to transport processes in the aquifer.\textsuperscript{5,10,11,17} Natural-gradient tests have been conducted by injecting tracers into the aquifer, in most cases over a short period of time, and sampling the cloud of tracer-labelled water downgradient from the injection well. The disadvantage of this type of test is that it requires longer durations than required for forced-gradient tests because the natural water velocity is much slower in natural-gradient tests than in forced-gradient tests. An advantage of natural-gradient tests is that they match the flow conditions that occur in the aquifer, which is particularly important when the chemical or microbiological processes under study are controlled by kinetics or contact time with the aquifer sediments. Natural-gradient tests are also somewhat easier to interpret than forced-gradient tests because it can be assumed in many cases that the velocity is unidirectional, thus eliminating problems caused by diverging or converging flow fields.
An example of a small-scale natural-gradient test is one in which $^{13}$C-labelled methane and a chlorofluoromethane were used as tracers\textsuperscript{15,17} to measure the potential for microbially mediated oxidation of methane under denitrifying conditions. By comparing time-breakthrough curves for the dissolved gases to chloride (a nonreactive anionic solute), it was found that the gases were transported without retardation. However, concentrations of methane apparently decreased because of oxidation to carbon dioxide. Methane oxidation occurred predominantly in a zone of denitrification; the rates of methane oxidation and denitrification (measured in sediment cores in the laboratory) were highest at the same depth. The results from this tracer test indicate that nitrate may be a possible electron acceptor for methane oxidation in groundwater systems.\textsuperscript{17}

Several natural-gradient tracer tests have been conducted to examine the effect of cell size and surface charge characteristics on the transport of bacteria.\textsuperscript{11} In one experiment, fluorescent, bacteria-sized microspheres of differing sizes and surface charges were injected.\textsuperscript{10} It was found that, for carboxylated microspheres with similar surface charge, the smaller particles were more greatly attenuated than the larger particles. For microspheres of similar size but differing surface charge, the uncharged, neutral (latex) particles arrived before the charged (carboxylated) particles.

In another test, DAPI-stained bacteria were injected along with bromide (R.W. Harvey, written communication). In this experiment, the bacteria moved at about the same rate as bromide through 7 m of the aquifer, but the relative bacteria concentrations were attenuated and breakthrough curves showed longer tails. The breakthrough curves for bromide were interpreted using a one-dimensional advective-dispersive model and the breakthrough curves for bacteria were interpreted using a colloid-filtration model. As part of this curve-matching process, it was necessary to superimpose model breakthroughs from two zones with different velocities in order to match the observed, multiple-peaked breakthrough curves.

A nonuniqueness problem can arise using this interpretive approach, because the effect of individual parameters on the model solution can be similar, resulting in sets of different parameter values that have nearly identical model solutions. Also, it is possible to create increasingly complex models by including additional velocity zones or operative processes (reactions) to improve the model fit to the observed breakthrough curve. In doing so, the nonuniqueness problem becomes more difficult as the number of parameters increases. Therefore, although the curve-matching approach is very useful for process-oriented research, problems can arise in uniquely identifying the model structure and parameter values. Field tracer tests are inherently affected by the physical transport processes, and any experiment designed to examine chemical or microbiological reactions has this built-in limitation. Therefore, a field experiment designed to isolate a single process has a distinct interpretive advantage over an experiment affected by many closely related processes that can produce similar effects on the observed breakthrough curve.
Large-Scale Natural-Gradient Tracer Test. Interpretation problems for tracer tests in heterogeneous aquifers are reduced when the size of the experiment is much larger than the scale of the heterogeneities. An example of a large-scale, natural-gradient tracer test is the experiment conducted at the Cape Cod site during 1985-87. This test was designed to measure dispersion in the aquifer and to determine geochemical controls on reactive transport in a heterogeneous aquifer. The movement and spreading of the tracers, bromide, lithium (a cation), and molybdate (an oxyanion of molybdenum) were monitored over time using a three-dimensional sampling network of 9600 sampling points located as far as 280 m downgradient from the injection wells. The complete spatial distribution of the tracers was sampled about every month for the first 16 months and then every six months afterward for the reactive tracers. Interpretation of the more than 100,000 tracer concentration values measured during the 3-year period was done using spatial moments. Spatial moments are an integration of the tracer concentration multiplied by the spatial coordinates raised to a designated power. If the coordinates are raised to the zero power, the resulting zero moment is the mass of tracer; if the power is one, the resulting first moment is the location of the center of mass; and if the power is two, the resulting second moment is the spatial variance of the tracer distribution.

Spatial moments analysis provides an accurate, averaged description of the characteristics of each tracer distribution at a given sampling time during an experiment. For example, the calculated total mass of bromide for the large-scale test for each sampling date varied from 86 to 105% of the total injected mass. The lack of any trend in the calculated mass over time confirmed the conservative transport of the bromide ion. The rate of horizontal displacement of the bromide center of mass was 0.43 m/day. This observed velocity very closely matched the predicted velocity, which was calculated using estimates of aquifer properties. In addition, a nonlinear, increasing trend in the bromide longitudinal variance was observed during the first 40 m of distance traveled, indicating that the dispersion process was non-Fickian in the early part of the test. After 40 m of travel, the longitudinal variance followed a linear trend and had apparently reached a Fickian limit. The corresponding longitudinal dispersivity, defined as one-half the change in variance with travel distance, is approximately 0.96 m. The observed dispersivity was significantly larger than the values that are typically found in laboratory column experiments (0.01-2 cm), demonstrating the strong influence of heterogeneity on the dispersive-mixing process in this aquifer.

The two reactive tracers used in the large-scale tracer test, lithium and molybdate, were significantly retarded relative to bromide. For lithium, adsorption occurs both on the mineral surfaces and, more significantly, inside small pores within the weathered feldspar grains. Adsorption occurring inside the feldspar grains is controlled by the chemical diffusion of solutes from the void space around the grains into the micro pores that may be lined with clay mineral alteration products inside the grains. The result is a skewed distribution of lithium induced by the diffusion-limited adsorption occurring inside the grains. It was found, using a spatial-moments
analysis, that the mass of lithium in solution decreased during the first 300 days of transport until approximately 10% of the injected mass remained in solution. The velocity of the lithium in solution was initially the same as bromide velocity (0.43 m/day) and then it decreased to approximately 0.05 m/day after 300 days. The average distribution coefficient for the lithium adsorption was estimated to be approximately 2.0 ml/g for the latter part of the test. It was also found that adsorption of molybdate is affected by pH and by the concentration of phosphate, another oxyanion which competes for adsorption sites, causing a vertical variation in the rate of movement for molybdate at this site.18,19

**Conclusion**

Forced- and natural-gradient tracer tests were used extensively at the Cape Cod site to test specific hypotheses related to the solute-transport processes in sewage-contaminated groundwater. These tests allow a degree of control over experimental conditions that does not exist in the sewage plume. Direct measurements of hydraulic conductivity and indirect evidence of heterogeneity from tracer tests showed that the variability in the rate of solute movement has a large influence on transport processes and interpretation of tracer tests. The natural-gradient experiments were the more useful of the two test types because they approximate flow conditions in the aquifer and were easier to interpret. A large-scale, natural-gradient tracer test produced the least ambiguous results, both because of the scale of the experiment and the large number of data generated during the experiment. However, small-scale experiments will continue to be conducted at the site because they provide useful data for process-oriented research and vital information for designing larger experiments with possible reductions in time and cost.
References


Evaluation of Core Segment Pore Water Contamination from Tracers, Well Water Sampling and Sediment Extractions

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Abstract

Contamination of the pore waters in core segments sampled from the C10 hole drilled for the Subsurface Science Program in South Carolina with soluble constituents from the drilling fluids, was not most reliably measured from added known levels of KBr during makeup of the drilling fluids and analysis of Br in the pore water. With a knowledge of both the concentration levels from waters sampled after development of screened intervals at the depth of core removal and the concentration levels in drilling muds in contact with the intrusion levels in the Middendorf sand interval.

A second source of “contamination,” specifically the initiation of oxidation of trace reduced components in the sediments, is more difficult to quantify. Oxidation results in major increases in total soluble iron (ferrous), sulfmate, calcium, magnesium, potassium and acidity. Well waters from screened intervals installed in the Middendorf at P24, 28 and 29 two years ago, are still showing high levels of these oxidation products.

The ability to obtain contamination-free pore fluids at depths below 200 m will require significant modifications of existing drilling methodologies.
Use of Rhodamine Dye as a Tracer for Drilling Muds in Deep Subsurface Investigations.

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