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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 methodology. Sediments to a depth of 550 m were collected from boreholes at three sites on the Savannah River Site near Aiken, South Carolina, using techniques to insure a minimum of surface contamination. Clusters of wells screened at discreet intervals were established at each site. Bacterial densities in sediment were higher, by both direct and viable count, than in groundwater samples. Differences between direct and viable counts were much greater for groundwater samples than for sediment samples. Densities of bacteria in sediment ranged from less than 1.00×10^6 bacteria/g dry weight (gdw) up to 5.01×10^8 bacteria/gdw for direct counts, while viable counts were less than 1.00×10^3 CFU/gdw to 4.07×10^7 CFU/gdw. Bacterial densities in groundwater were 1.00×10^3–6.31×10^4 bacteria/ml and 5.75–4.57×10^2 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 suggest that oligotrophic aquifer sediments have unique and dense bacterial communities that are attached and not reflected in groundwater found in the strata. Effective in situ bioremediation of contamination in these aquifers may require sampling and characterization of sediment communities.

Introduction

Organic xenobiotic chemical contamination of groundwater has become the most important pollution problem of industrialized nations of the world. More

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than 15% of community drinking water supplies in the United States are contaminated with carcinogenic, chlorinated hydrocarbons [7, 23]. Identification of previously unknown waste disposal sites that are impacting groundwater occurs almost daily. Thus, the extent of the problem is undoubtedly greater than 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. [7, 23]. 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, ways have been sought to cleanup, i.e., remediate, contaminated groundwater. Because many aquifers are quite deep and contain tremendous volumes of water with slow turnover times, in situ bioremediation is very attractive and in some cases may be the only recourse.

In situ bioremediation has been practiced for more than 30 years by petroleum industries, e.g., petroleum land farming [4]. The initial process of inorganic nutrient infiltration of groundwater to stimulate biodegradation by indigenous bacteria in groundwater contaminated with petroleum was even patented by R. L. Raymond in 1974 (U.S. Patent 3,846,290). However, neither the scientific community nor industry has seen widespread application of this technology due to limited successes. This was in part due to the paucity of knowledge concerning the microbial ecology of subsurface sediments (see Ghiorse and Wilson [13] for a review of microbial ecology of the terrestrial subsurface). During the 1980s several laboratories, including ours, began studying the biogeochemistry of subsurface sediments in order to understand and control biodegradation process in groundwater.

The United States Department of Energy, Office of Health and Energy Research, began a comprehensive program to study subsurface microbiology in 1985 [9]. During this program four sites were chosen at the DOE Savannah River Site near Aiken, South Carolina. Sediments were sampled using special recovery techniques from the surface to bedrock, 550 m in the deepest borehole [24]. (For a comprehensive description of these studies see Fliermans and Balkwill [9] and *Geomicrobiology Journal* volume 7[1/2].) At three of the sites, a series of 6–8 new wells was 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 in situ bioremediation.

Materials and Methods

Study Sites

Sediment samples were taken by aseptic coring from three sites (P24, P28, P29) at the Savannah River Site, near Aiken, South Carolina. (For a thorough description of the geology and hydrology of these sites and this area of South Carolina, see Sargent and Fliermans [27]. For a general description of the U.S. Department of Energy Microbiology of the Deep Subsurface Project findings, see Fliermans and Balkwill [9] and *Geomicrobiology Journal* volume 7[1/2].) Water samples were

Table 1. Physical and chemical descriptions of cluster wells

Well units	Screen* (m)	Geological formation	Temp (°C)	pH	Cond (μmhos)	Redox (mv)	DO (mg/liter)
P28A	101–104	Pee Dee	20.6	6.0	44	0.102	3.8
P28TE	123–127	Black Creek	20.1	6.3	42	0.122	8.1
P28TD	151–154	Black Creek	24.9	7.1	118	0.127	0.3
P28TC	169–172	Middendorf	21.7	9.9	281	−0.023	0.5
P28TB	188–195	Middendorf	22.0	5.5	30	0.125	3.2
P28TA	228–238	Middendorf	21.9	5.7	50	0.161	4.2
P24D	14–20	Tobacco Road	21.0	6.8	55	0.145	9.1
P24C	40–46	Dry Branch	26.6	6.7	45	0.327	8.3
P24B	67–70	McBean	21.1	9.5	149	0.198	8.3
P24A	93–96	Congaree	23.1	10.7	187	N.D.	6.0
P24TD	149–152	Pee Dee	21.6	6.8	55	−0.043	1.0
P24TC	178–181	Pee Dee	21.8	5.9	97	−0.008	0.1
P24TB	242–245	Middendorf	22.2	6.2	60	0.120	3.2
P24TA	290–296	Middendorf	21.7	10.9	98	N.D.	2.8
P29D	28–34	Dry Branch	19.1	5.8	40	0.183	7.4
P29C	40–43	Congaree	20.0	5.6	103	0.102	2.5
P29B	55–58	Ellenton	20.8	6.4	39	0.141	8.3
P29A	93–96	Pee Dee	22.3	6.1	76	0.112	0.2
P29TD	124–131	Black Creek	21.3	5.3	60	0.057	5.7
P29TC	149–155	Middendorf	21.7	6.6	86	0.067	5.2
P29TA	204–210	Middendorf	21.7	5.3	29	0.143	6.7

* Screen = screened interval of well below surface; Temp = temperature; Cond = conductivity; DO = Dissolved oxygen; N.D. = not done

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 analyses were taken from Balkwill [1] and Sinclair and Ghiorse [28]. (For a detailed description of sediment sampling techniques see Phelps et al. [24].)

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 1 hour. Sterile, one-liter bottles were filled with water for bacteria counts. 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 diameter, HA type, membrane filters (Millipore Corp., Bedford, MA). Filters were placed on 1% peptone-tryptone-yeast extract-glucose (PTYG) medium [1] and incubated at 23°C for 2 weeks. The media, temperature and time of incubation were all demonstrated to be optimal for subsurface enumeration of bacteria at this site and others

by Balkwill and coworkers [1–3, 5]. Bacterial colonies were counted with the aid of a stereomicroscope. Total cell counts were determined by direct count (AODC) methods using acridine orange [17, 22]. The activity index was estimated by dividing the log density of AO direct counts by the log density of viable counts on 1% PTYG.

Bacterial 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 from Florida State University, Tallahassee, FL. (For further details on the isolation of deep subsurface bacteria from sediment and the media used see Balkwill et al. [3] and Balkwill and Ghiorse [2].) Water isolates were obtained by 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. [3] and Bone and Balkwill [5].

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 [31]. Any probability less than or equal to 0.05 was considered significant.

Results

Direct enumeration by AODC of bacteria in sediments revealed from less than 1.00×10^6 bacteria/gdw up to 5.01×10^8 bacteria/gdw (Table 2). Viable counts using 1% PTYG medium varied from no detectable growth, i.e., less than 1.00×10^3 CFU/gdw, up to 4.07×10^7 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 AODC were two orders of magnitude higher than viable counts (Table 2). (For further analysis of sediment enumeration techniques and geochemistry see Balkwill [1], and Sinclair and Ghiorse [28].)

Groundwater AODC measurements ranged from 1.00×10^3 to 6.31×10^4 bacteria/ml (Table 3), whereas bacterial densities as measured by viable counts on 1% PTYG ranged from 5.75 to 4.57×10^2 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 ranged from 2 to 3 orders of magnitude.

Comparison of viable counts from the sediment and water demonstrated

Table 2. Sediment direct vs viable counts for selected sediment samples

Formation	P28		P24		P29	
	AODC[a]	PTYG[b]	AODC[a]	PTYG[b]	AODC[a]	PTYG[b]
Surface soil	8.4	6.38	8.6	6.49	8.7	6.59
Tobacco Road	6.6	2.88	6.9	4.09	7.2	5.67
Dry Branch	7.6	5.58	6.4	6.37	6.7	6.20
McBean	—	—	6.6	4.81	—	—
Congaree	7.7	7.01	7.2	6.48	7.6	6.72
Williamsburg	7.3	3.84	—	—	—	—
Ellenton	—	—	7.1	5.76	6.5	NG
	—	—	7.1	3.22	—	—
Pee Dee	7.8	7.29	6.0	3.24	7.0	4.74
	6.6	NG	7.2	6.65	—	—
Black Creek	7.6	5.16	7.2	4.97	6.6	NG
	7.3	3.27	7.0	4.98	7.2	5.54
	—	—	6.5	2.12	—	—
Middendorf	6.7	5.72	7.4	4.40	7.2	6.56
	<6.0	3.68	6.8	NG	7.2	6.28
	7.5	7.16	7.4	6.46	6.7	2.25
	7.7	7.61	7.4	2.48	7.4	5.90
	6.3	6.40	—	—	7.0	5.20
	<6.0	3.90	—	—	7.2	5.93
	—	—	—	—	7.8	6.89
Mean	7.14	5.26	7.05	4.72	7.20	5.37

All values are $\log_{10}$ densities, per gram dry weight soil
[a] From Sinclair and Ghiorse [28]
[b] From Balkwill [1]
NG = No growth on lowest dilution (10^{-3}); — = no sample

very significant differences (Table 4; $F = 85$; df = 1,36; $P < 0.0001$). Differences ranged from 2 to 5 orders of magnitude, with an average of 3. 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 3 to 5 orders of magnitude (average = 4) higher than adjacent groundwater densities.

Activity index (D/V, the ratio of direct to viable counts) 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 3 of the samples had a higher water D/V than the sediments and only 1 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 (n = 120) and sediment (n = 430) collected at the same sampling interval were compared in terms of their physiological abilities using API-Rapid NFT (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 assayed ($F = 3.32$; df = 1,22; $P < 0.01$). Eleven of 13 compounds were assimilated at a higher frequency by the sediment isolates.

Table 3. Water direct vs viable counts for selected water samples

Formation	P28		P24		P29	
	AODC	PTYG	AODC	PTYG	AODC	PTYG
Surface soil	—	—	—	—	—	—
Tobacco Road	—	—	3.5	2.39	—	—
Dry Branch	—	—	3.7	2.66	4.8	2.02
McBean	—	—	3.7	1.78	—	—
Congaree	—	—	3.4	0.88	3.6	2.63
Williamsburg	—	—	—	—	—	—
Ellenton	—	—	—	—	4.4	2.05
	—	—	—	—	—	—
Pee Dee	3.4	1.46	3.0	1.75	4.2	1.89
	—	—	3.1	2.26	—	—
Black Creek	3.8	1.40	—	—	4.2	2.33
	3.5	1.43	—	—	—	—
	—	—	—	—	—	—
Middendorf	3.4	1.43	4.3	0.87	—	—
	—	—	—	—	—	—
	3.3	1.48	—	—	—	—
	—	—	3.4	0.98	3.5	0.76
	—	—	—	—	—	—
	3.2	1.39	—	—	—	—
	—	—	—	—	3.4	0.81
Mean	3.43	1.43	3.51	1.70	4.01	1.78

All values are $\log_{10}$ densities per ml water
— = No sample

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 [24]. Sixteen separate lines of evidence using a variety of tracers and microbiological assays suggest 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 utilize rapidly 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-Rapid NFT assay. Related studies of these same isolates have also demonstrated their ability to degrade a variety of toxic compounds, e.g., trichloroethylene, quinoline, phenol, 4-methoxybenzoic acid [6, 10, 16].

Poindexter [25] suggested that bacteria adapted to oligotrophic conditions might be expected to have less specific uptake systems, having adapted to utilization of a broader range of substrates. Similar findings have been reported for oligotrophic bacteria in Antarctica [30]. The sediments and water in the present study were oligotrophic based on the low concentrations of P and N, although the dissolved organic carbon was 0.1–9.1 ppm [11]. Thus, although

Table 4. Water vs sediment samples using plate counts on 1% PTYG

	P28		P24		P29	
Formation	Water	Sediment[a]	Water	Sediment	Water	Sediment
Surface soil	—	—	—	—	—	—
Tobacco Road	—	—	2.39	4.09	—	—
Dry Branch	—	—	2.66	6.37	2.02	6.20
McBean	—	—	1.78	4.81	—	—
Congaree	—	—	0.88	6.48	2.63	6.72
Williamsburg	—	—	—	—	—	—
Ellenton	—	—	—	—	2.05	NG
	—	—	—	—	—	—
Pee Dee	1.46	7.29	1.75	3.24	1.89	4.74
	—	—	2.26	6.65	—	—
Black Creek	1.40	5.16	—	—	2.33	NG
	1.43	3.27	—	—	—	—
	—	—	—	—	—	—
Middendorf	1.43	5.72	0.87	4.40	—	—
	—	—	—	—	—	—
	1.48	7.16	—	—	—	—
	—	—	0.98	2.48	0.76	5.90
	—	—	—	—	—	—
	1.39	3.90	—	—	—	—
	—	—	—	—	0.81	6.89
Mean	1.43	5.42	1.70	4.81	1.78	5.21

All values are $\log_{10}$ densities per gram dry weight soil or per ml water
[a] From Balkwill [1]
NG = No growth on lowest dilution (10^{-3}); — = no sample

the sediment bacteria in our study may be living in an oligotrophic, recalcitrant environment, they were able to maintain high standing biomass that 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 4–5 orders of magnitude [18, 26]. This was further supported by Tunlid et al. [29] who showed that the phospholipid fatty acid profiles of bacteria in these sediments suggested that they were under nutrient stress. Balkwill [1] and others [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 [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 for the bacteria to increase their plasmid burden in order to increase the number of compounds available for degradation.

In contrast to sediment, 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 under greater stress because

Table 5. Water vs sediment sample using acridine orange direct counts

Formation	P28		P24		P29	
	Water	Sediment[a]	Water	Sedi-ment	Water	Sedi-ment
Surface soil	—	—	—	—	—	—
Tobacco Road	—	—	3.5	6.9	—	—
Dry Branch	—	—	3.7	6.4	4.8	6.7
McBean	—	—	3.7	6.6	—	—
Congaree	—	—	3.4	7.2	3.6	7.6
Williamsburg	—	—	—	—	—	—
Ellenton	—	—	—	—	4.4	6.5
	—	—	—	—	—	—
Pee Dee	3.4	7.8	3.0	6.0	4.2	7.0
	—	—	3.1	7.2	—	—
Black Creek	3.8	7.6	—	—	4.2	6.6
	3.5	7.3	—	—	—	—
	—	—	—	—	—	—
Middendorf	3.4	6.7	4.3	7.4	3.5	7.4
	—	—	—	—	—	—
	3.3	7.5	—	—	—	—
	—	—	3.4	7.3	—	—
	—	—	—	—	—	—
	3.2	<6.0	—	—	—	—
	—	—	—	—	3.4	7.8
Mean	3.43	7.15	3.51	6.88	4.01	7.09

All values are $\log_{10}$ densities per gram dry weight soil or per ml water
[a] From Sinclair and Ghiorse [28]
— = No sample

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-Rapid NFT compounds. Studies in Germany [20, 21] comparing sediment and water bacterial communities also found that viable count densities of bacteria in the sediments were 2–3 orders of magnitude higher than nearby groundwater. In these 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 comprise epilithic communities, i.e., the microbial communities of these aquifers are nearly all attached. It appears that the microorganisms free in the groundwater are either dead, stressed attached bacteria, propagules of 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 [26]. 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, do not show significant homology (<26%) with any of 23 groundwater isolates [19]. API-Rapid NFT assays could not be used for identification of sediment bacteria,

Table 6. Ratio of direct to viable counts (activity index) for selected water and sediment samples

Formation	P28		P24		P29	
	Water	Sediment[a]	Water	Sediment	Water	Sediment
Surface soil	—	—	—	—	—	—
Tobacco Road	—	—	1.46	1.69	—	—
Dry Branch	—	—	1.39	1.00	2.38	1.08
McBean	—	—	2.08	1.37	—	—
Congaree	—	—	3.86	1.11	1.37	1.13
Williamsburg	—	—	—	—	—	—
Ellenton	—	—	—	—	2.15	2.17
	—	—	—	—	—	—
Pee Dee	2.33	1.07	1.71	1.85	2.22	1.48
	—	—	1.37	1.08	—	—
Black Creek	2.71	1.47	—	—	1.80	2.20
	2.45	2.23	—	—	—	—
	—	—	—	—	—	—
Middendorf	2.38	1.17	4.94	1.68	4.61	1.25
	—	—	—	—	—	—
	2.23	1.05	—	—	—	—
	—	—	3.47	2.94	—	—
	—	—	—	—	—	—
	2.30	1.54	—	—	—	—
	—	—	—	—	4.20	1.13
Mean	2.40	1.42	2.54	1.59	2.67	1.49

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

[a] From Sinclair and Ghiorse [28] and Balkwill [1]

— = No sample

because isolates with identical phenotypes by these assays did not have similar DNA homologies or even similar mol% G+C composition of the DNA [19]. Identification comparison of sediment and water isolates will, therefore, not be possible until sediment phenotypes can be differentiated and further characterized.

The present work has demonstrated that deep oligotrophic aquifers have dense attached communities of bacteria that are not reflected in the groundwater from that aquifer. This has serious implications for the 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 microbes, the principal biologically active component of these aquifers. Harvey et al. [15] and Harvey and George [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, nec-

Table 7. Percent of bacterial isolates from sediments and groundwater that tested positive for API-Rapid NFT compounds

	Sediment	Water
Metabolic tests		
Nitrate reductase	58	51
Tryptophanase	<1	0
Arginine dihydrolase	5	15
Urease	42	16
Esculin hydrolysis	54	ND
Gelatinase	32	37
β-Galactosidase	56	44
Oxidase	39	37
Glucose fermentation	1	2
Aerobic assimilation tests		
D-Glucose	86	64
L-Arabinose	51	36
D-Mannose	56	36
D-Mannitol	61	47
N-Acetyl-D-glucosamine	35	45
Maltose	69	60
D-Gluconate	70	48
Caprate	15	20
Adipate	53	31
L-Malate	76	53
Citrate	44	42
Phenylacetate	36	11
Total number of isolates	430	120

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

essary 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 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.

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