IMMUNOLOGICAL TECHNIQUES AS TOOLS TO CHARACTERIZE THE SUBSURFACE MICROBIAL COMMUNITY AT A TRICHLOROETHYLENE-CONTAMINATED SITE

C. B. Fliermans, J. M. Dougherty, M. M. Franck, P. C. McKinzey, J. E. Wear, and T. C. Hazen

ABSTRACT

Effective in situ bioremediation strategies require an understanding of the effects pollutants and remediation techniques have on subsurface microbial communities. Therefore, detailed characterization of a site's microbial community is important. Groundwater samples were collected from a trichloroethylene (TCE)-contaminated site before and after in situ air stripping, bioventing, and methane injection bioremediation techniques were used. Subsamples were processed for heterotrophic plate counts, acridine orange direct counts (AODC), community diversity, direct fluorescent antibody (DFA) enumeration for selected bacteria, and Biolog® evaluation of enzyme activity. The presented data describe the use of specific techniques to evaluate bacterial communities in groundwater from both a synecologically and an autecologically perspective with regard to the subsurface treatments during bioremediation. AODCs were orders of magnitude higher than plate counts and remained relatively constant with depth except for slight increases at the surface depths and the capillary fringe of the vadose zone. Nitrogen-transforming bacteria, as measured by serospecific DFA, were significantly affected both by the in situ air stripping and the methane injections. Microbial utilization of selected organic compounds was measured by Biolog® technology, and differed among the wells in relationship to their stimulation both by air and methane. The complexity of subsurface systems makes the use of selective monitoring tools essential.

BACKGROUND

The Savannah River Site (SRS) [illegible]

extensive research projects. The Gas Research Institute (GRI) in collaboration with Savannah River Technology Center (SRTC) has been funding research and development of a methanotrophic treatment process for TCE-contaminated groundwater for the past 4 years. During one such activity, the Integrated Demonstration Project, indigenous microorganisms were stimulated through the use of dual horizontal wells to degrade trichloroethylene (TCE), tetrachloroethylene (PCE), and their daughter products in situ by the addition of gaseous nutrients to the contaminated zone. Biodegradation is a highly attractive remediation strategy because contaminants are destroyed, not simply transferred to another location or immobilized. Bioremediation has been shown to be among the most cost effective technologies where applicable (Radian 1989; Legrand 1993). The application of horizontal well technology to bioremediation has formed the foundation of the SRS Integrated Demonstration Project and provided significant advantages over vertical wells and conventional bioremediation techniques. The increased surface area provided by the horizontal wells has allowed better delivery of nutrients and a more efficient recovery of gas and water, while minimizing formation plugging (Looney & Kaback 1991).

Because air/methane mixtures have been shown to stimulate selected members of the indigenous microbial community that have the capability to degrade TCE (Little et al. 1988; Vogel & McCarty 1985; Wilson & Wilson 1985), the principal nutrients supplied via the horizontal wells were methane (1 to 4%) and nitrogen in air. Although the lower horizontal well provided an efficient delivery of gas throughout the contaminated region, a vacuum was applied to the upper well located in the vadose zone. Such a combination of wells encouraged air/methane movement through the upper saturated and lower vadose zones while inhibiting the spreading of the organic plume (Looney & Kaback 1991). An extensive monitoring program using existing monitoring wells and soil borings has served to determine the biological response in the soil, sediment, and groundwater systems following the injection of air/methane. Data from Phase I (air injections), Phase II (1% and 4% methane injections), and Phase III (pulsing of 4% methane) of the Integrated Demonstration Project have illustrated the effectiveness of in situ bioventing and methane injections for the bioremediation of TCE and PCE (Hazen 1992).

This manuscript describes an affect of the injection perturbations on selected microbiological components in the groundwater influenced by the horizontal wells. The data are derived from the use of species-specific and serospecific fluorescent antibodies to detect and enumerate selected microbial populations. Additionally, the analyses included the use of Biolog® technology to determine the metabolic capability of the microbial populations present in the groundwater with respect to 95 different carbon and energy sources.

MATERIALS AND METHODS

Soil, sediment, and groundwater samples were collected aseptically as previously described (Fliermans & Balkwell 1989; Fliermans & Hazen 1990; Phelps

et al. 1989). Groundwater samples were collected from 12 monitoring wells on a bimonthly schedule over a 12-month period. Wells were pumped according to established protocol (WSRC 1991) in order to stabilize chemical, physical, and biological parameters before a 4 liter samples were collected. Viable bacterial densities were determined by plating samples on laboratory medium as described by Balkwell and Ghiorse (1985). Total bacterial densities in groundwater samples were measured by direct epifluorescence microscopy using AODC techniques of Balkwell & Ghiorse (1985).

A modified direct immunofluorescent technique using serospecific polyvalent fluorescent antibodies (Fliermans et al. 1992) was employed to measure selected bacterial strains in groundwater samples which were concentrated 250-fold by centrifugation. These antibodies were prepared as described by Bohlool & Schmidt (1968) and Fliermans et al. (1974). Aliquots (10 µL) of the groundwater samples were heat-fixed onto toxoplasmosis slides, layered with blocking fluid, stained with the specific antibodies, washed in phosphate buffered saline, and viewed by epifluorescence microscopy.

Selected bacterial strains were chosen because they represented some of the microbial structural components of the ecosystem being investigated. Species-specific direct fluorescent antibodies were prepared against the major microbial communities involved in nitrogen transformation in soils and groundwater, i.e., *Azotobacter, Nitrosolobus, Nitrosomonas, Nitrobacter*, and *Bradyrhizobium*, as well as those species of *Nitrosomonas* involved in the production of enzymes capable of degrading TCE; a bacterium that is pathogenic, but widely distributed in nature (*Legionella pneumophila*); a widely distributed bacterium associated with our systems and involved in iron transformations (*Thiobacillus ferrooxidans*); and two organisms that have been involved in the destruction of TCE as well as transformation of methane in the laboratory (*SRL-MIIF, Methanobacterium*). Each of these organisms have been isolated from SRS habitats except for *Bradyrhizobium japonicum*.

More defined characterization for the bacteria are as follows:

- Azotobacter chroococcum is a free-living heterotrophic, nitrogen-fixer that lives under aerobic conditions.
- *Nitrosolobus multiformis* and *Nitrosomonas europea* are morphologically distinct chemolithotrophs that obtain their energy from oxidizing ammonia to nitrite while fixing CO_2 as their sole carbon source.
- *Nitrobacter agilis* and *Nitrobacter winogradskyi* are chemolithotrophs that secure their energy by oxidizing nitrite to nitrate while fixing CO_2 as their sole carbon source.
- *SRL-MIIF* is a Type II methanotroph isolated from the SRS with trichloroethylene (TCE)-degrading capabilities.
- *Methanobacterium formicicum* is a heterotrophic, strict anaerobe that produces CH_4 from organic compounds such as acetate and cysteine.
- *Thiobacillus ferrooxidans* grows as a chemolithotroph that gets its energy by oxidizing ferrous iron to ferric iron while fixing CO_2 as its carbon source.

- *Legionella pneumophila* Serogroup 1 is a human pathogen and the major etiologic agent of Legionnaires' Disease, as well as an aquatic and terrestrial bacterium.
- *Bradyrhizobium japonicum* is a symbiotic nitrogen-fixer that is present in association with soybean nodulation and occupies ecological niches near the soil surface.

Unconcentrated groundwater samples were pipetted (150 µL/well) into each microtiter well of triplicate GN Biolog® plates and incubated at the in situ groundwater temperature of 23°C. The optical density of each well was read at 590 nm after 3 weeks of incubation, and the reduced color in each of the wells was recorded. After incubation each plate was read using a Biolog® plate reader, and the optical density of the tetrazolium dye was recorded. The Biolog® automated plate reader was programmed to zero the instrument based on the optical density color of the reference well (A-1) in the Biolog® plate. Therefore, optical density readings determined at each inoculum pattern were significantly above the threshold level. The data were expressed as optical density for each of the 95 organic compounds tested, placed into reactive groups as previously described (Gorden et al., in press), and evaluated. The enzyme activity associated with the utilization of each of the compounds was expressed in terms of the location of the sampled well along with the in situ perturbations occurring at the time of sampling.

Data were analyzed by analysis of variance using JMP, version 2 (SAS Institute Inc., Cary, NC). Heterogeneity of variances was reduced using Log(Y+1) transformation. Pairwise comparisons were made using the Tukey method because it provided narrower confidence limits than methods used for general contrasts. The Kramer adjustment allowed for the unequal sample sizes tested. For all tests a probability level of 0.05 was assumed to be the critical level of significance.

RESULTS

The locations of the monitoring wells used in the Integrated Demonstration Project and sampled during this investigation are shown in Figure 1. Generally the lower numbered wells, MHT-1 through MHT-7, are more affected by the changes being incorporated into the horizontal wells, whereas the higher numbered wells, MHT-8 through MHT-11, are less affected. Moreover these higher numbered wells are affected at a latter time period as it took longer for the perturbation to reach these wells. Much of the physical/chemical data collected and analyzed from MHT-7 suggests that this well is clearly an outlier. The microbiological data also reflect this assessment. This may be a result of the way the well was physically developed rather than reflective of the groundwater at that location.

Data from the groundwater sampling reported here were acquired using the DFA and Biolog® techniques to assess selected and general bacterial dynamics in the groundwater, respectively. The DFA technique provided the collection of autecological data necessary to determine the population densities of selected nitrogen-cycling bacteria in the subsurface as well as other selected microbial

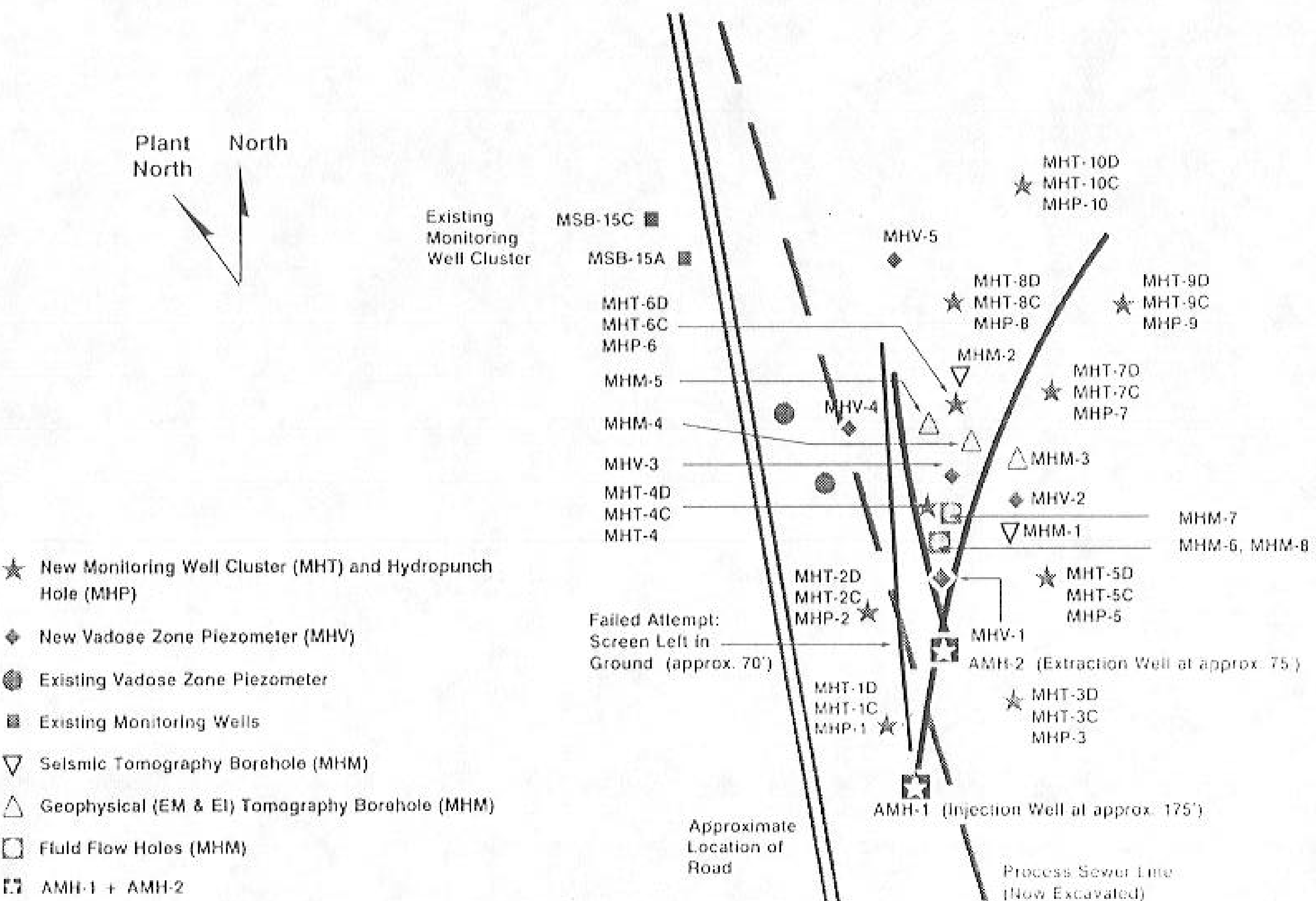

FIGURE 1. Location of horizontal and monitoring wells at the M-area Integrated Demonstration Project at Savannah River Site.

populations that were affected by the in situ bioventing and methane injection perturbations. Moreover, the Biolog® technique provided synecological data on the metabolic capability of the groundwater microbial community without regard to a particular organism.

The data in Figure 2 show the statistical analyses of DFA concentrations of *Azotobacter chroococcum* for all 12 wells during the perturbation regimes. Densities of *Azotobacter chroococcum* were significantly higher ($p<0.05$) during air and 1% methane injection than at any other time. The data indicate that in wells most affected by the perturbations the densities of *A. chroococcum* were greatest just after the beginning of the air injection and fell off rather dramatically after the start of the methane injections. Although the *Azotobacter* populations were highly variable, bioventing (which consisted of air injection in the bottom horizontal at 200 scfm along with vacuum extraction in the upper horizontal well at 240 scfm) provided a nitrogen source that appeared to encourage the growth of the free-living nitrogen-fixers. Such a stimulation is consistent with the DFA data. Subsequently the addition of 4% methane significantly reduced the densities of the *A. chroococcum* population, and those populations have been able to recover to the pretreatment levels during the pulsing regime of 4% methane and air.

The data in Figure 3 show the statistical analyses of DFA concentrations of *Nitrosomonas europea* for all 12 of the wells during the perturbation regimes. Densities of *Nitrosomonas europea* were significantly lower during injection of 4% methane than during other pretreatment or air injection. Once nitrogen is reduced during nitrogen fixation, the first step in the nitrification process is the oxidation of ammonia to nitrite. Thus if the population densities of the free-living nitrogen-fixers were to increase, it is reasonable to assume that the bacterial populations of the chemoautotrophic nitrifiers might increase as well. The data for the 12 sampled wells indicated that the densities of *N. europea* were greatest just after the start of air injection and fell off dramatically after the start of the

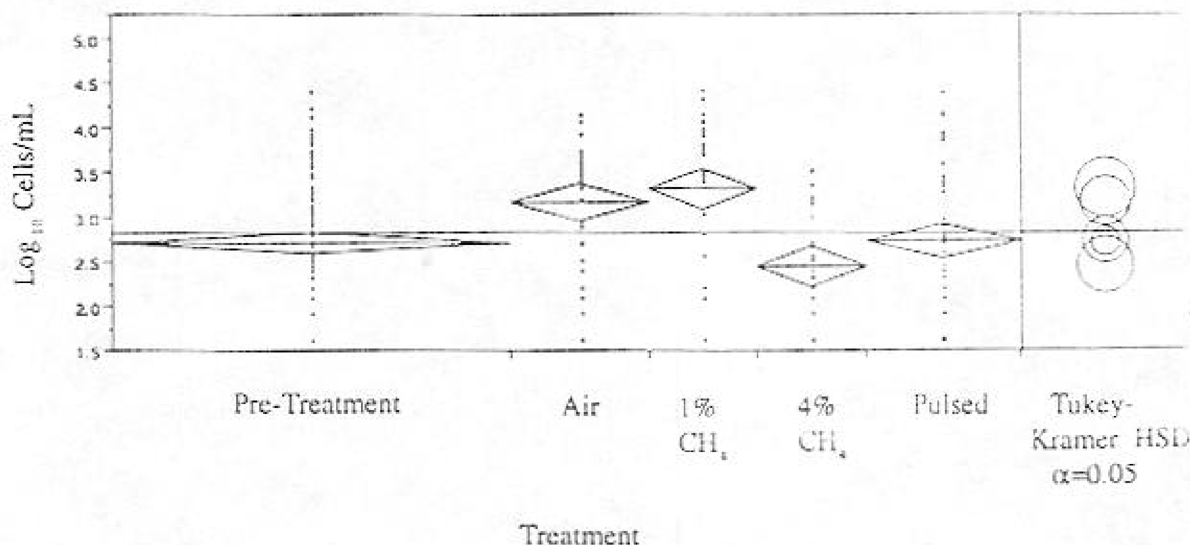

FIGURE 2. Density and statistical analyses of *Azotobacter chroococcum* in groundwater samples from 12 wells during in situ remedial perturbations.

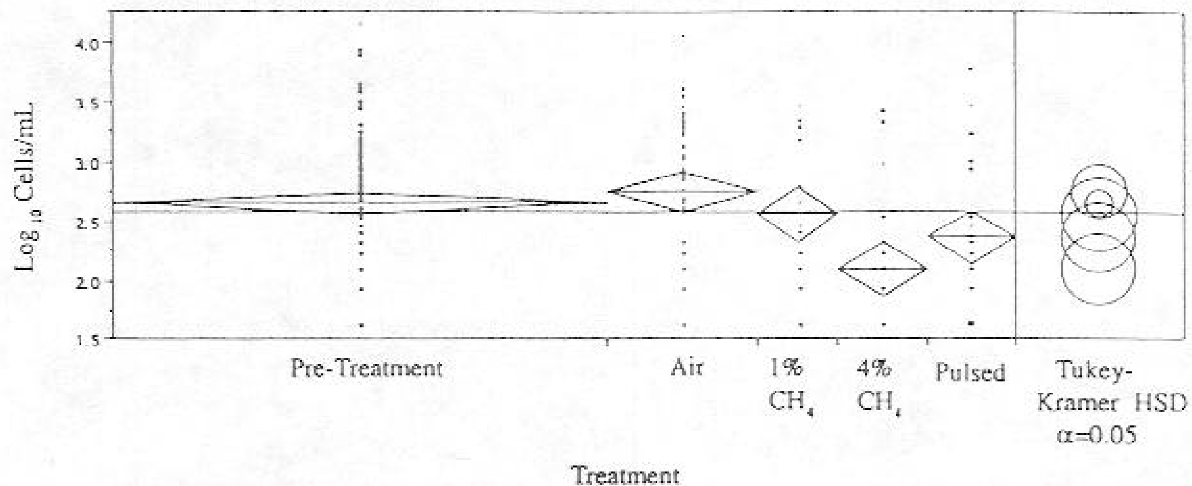

FIGURE 3. Density and statistical analyses of *Nitrosomonas europea* in groundwater samples from 12 wells during in situ remedial perturbations.

methane injections. The populations have as yet not been able to recover to the pretreatment levels, even with the pulsing regime.

Nitrosolobus multiformis, a lesser studied nitrifier, was never detected in very large concentrations in any of the samples and appears to play a rather minor role, numerically speaking, in these habitats. Densities of *N. multiformis* did not differ significantly between injection regimes.

The data in Figure 4 show the statistical analyses of DFA concentrations of *Nitrobacter agilis* and *Nitrobacter winogradskyi* for all 12 of the wells during the perturbation regimes. The DFA for these two bacteria were combined for the analyses, since they are so very much alike and may in fact be serotypes of *N. winogradskyi*. The DFA concentrations of *Nitrobacter agilis* and *Nitrobacter*

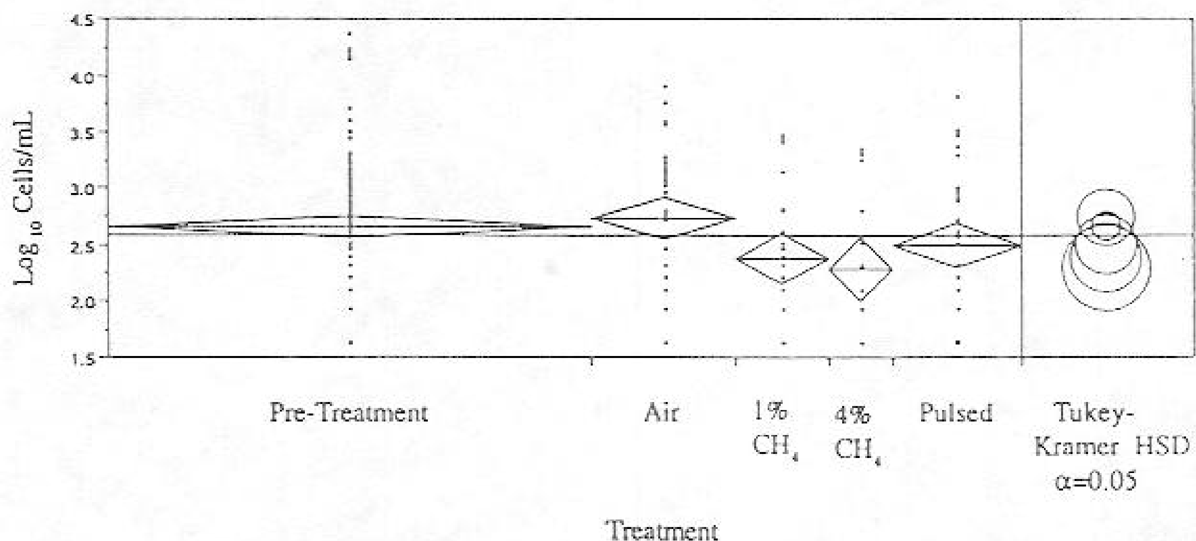

FIGURE 4. Density and statistical analyses of *Nitrobacter agilis* and *Nitrobacter winogradskyi* in groundwater samples from 12 wells during in situ remedial perturbations.

winogradskyi appear to follow a similar pattern as the ammonia-oxidizing bacteria. Once the methane injections began, the densities of the nitrite-oxidizing bacteria in these wells declined dramatically to the point where the concentrations of 70% of the samples were below detectable limits for *N. agilis* and *N. winogradskyi*. Densities of *N. agilis* and *N. winogradskyi* did not differ significantly between injection regimes when all the wells were grouped together for analyses at the $p<0.05$ level.

The data in Figure 5 show the statistical analyses of DFA concentrations of *SRL-MIIF* in each of the 12 wells. The data indicate that the bacteria were observed throughout the sampling perturbation period. Of the 12 samples collected before methane injection, only 25% of the samples showed detectable levels of the bacterium whereas 36% showed its presence after the methane injections had begun. Densities of *SRL-MIIF* were not significantly different between injection regimes when all the wells were grouped together for analyses at the $p<0.05$ level. In the overall analyses of the 12 wells it appears that this particular organism was not actively stimulated by the injection of methane or any of the perturbation regimes.

The data in Figure 6 show the statistical analyses of DFA concentrations of *Methanobacterium formicicum* in all 12 of the wells. Densities of *Methanobacterium formicicum* were significantly higher ($p<0.05$) prior to treatment and during air injection than during pulsed injection but were not significantly different from densities during 1% or 4% methane injection. There is a trend in the data that indicates that the injection of 1 and 4% methane decreased the densities of this bacterium. Statistically the densities of *Methanobacterium formicicum* were significantly lower ($p<0.05$) during pulsed injection of 4% methane than at any other time.

The data in Figure 7 demonstrate the statistical analyses of DFA concentrations of *Thiobacillus ferrooxidans* in the sampled wells. The data indicate that the densities

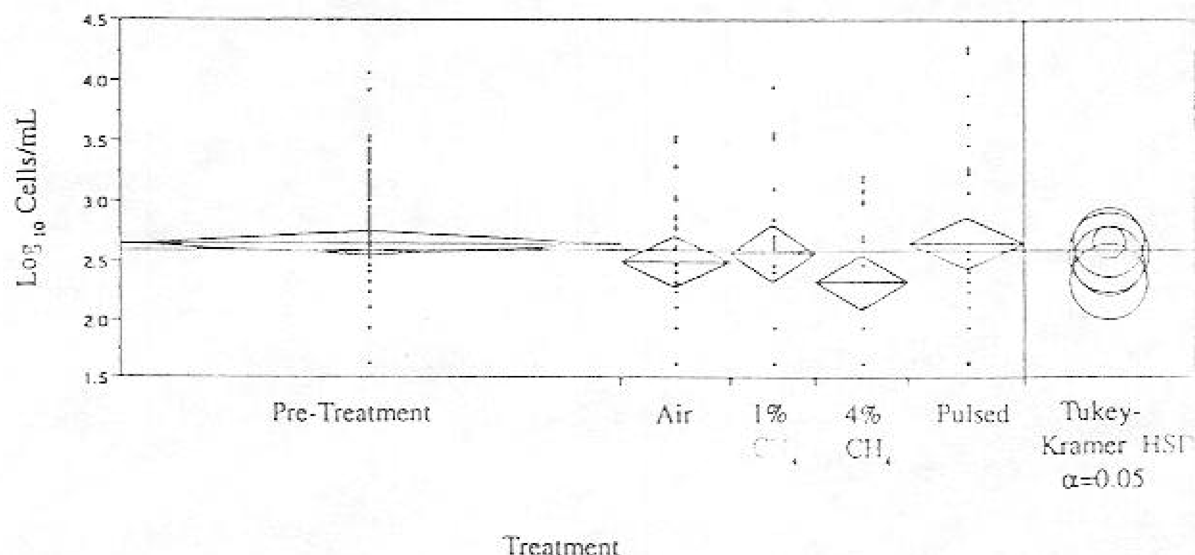

FIGURE 5. Density and statistical analyses of *SRL-MIFF* in groundwater samples from 12 wells during in situ remedial perturbations.

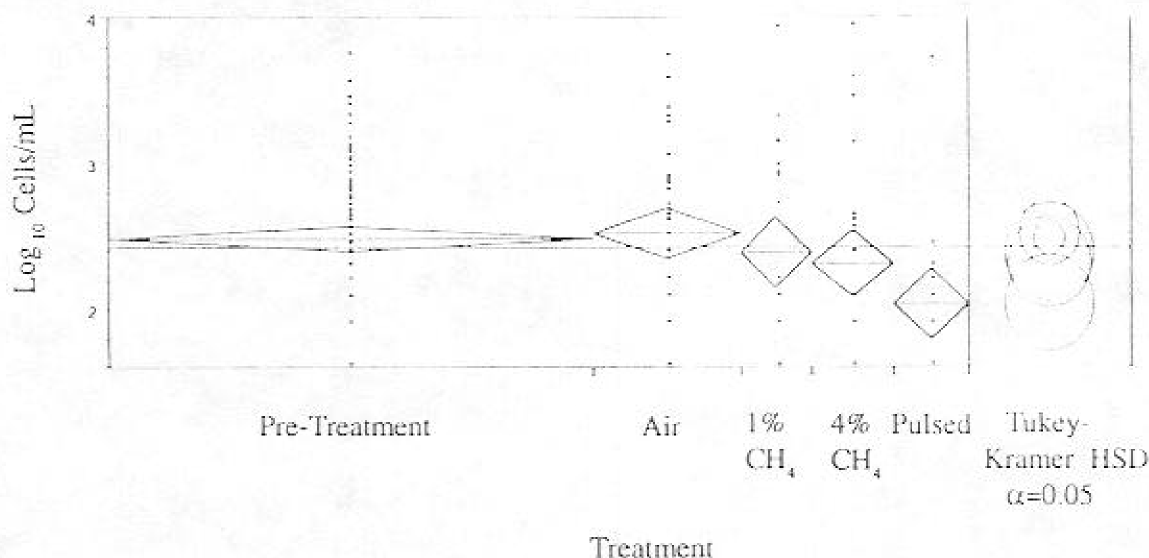

FIGURE 6. Density and statistical analyses of *Methanobacterium formicicum* in groundwater samples from 12 wells during in situ remedial perturbations.

of this particular strain of *T. ferrooxidans* were significantly higher ($p<0.05$) during 1% methane injection than at any other time. No significant difference was seen among the other treatments.

Finally, The data in Figure 8 show the statistical analyses of DFA concentrations of *Legionella pneumophila* Serogroup 1 in each of the 12 wells. The data indicate that the perturbations did not enhance the densities of *L. pneumophila* Serogroup 1, but infact the densities of *L. pneumophila* Serogroup 1 were significantly higher ($p<0.05$) prior to treatment than during 1% methane, 4% methane or pulsed perturbations.

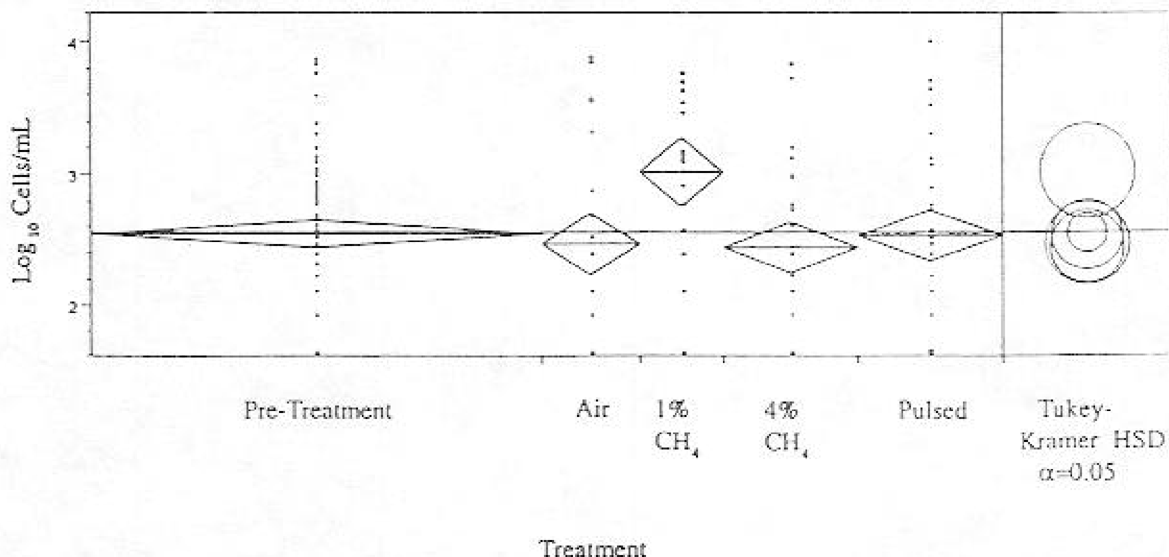

FIGURE 7. Density and statistical analyses of *Thiobacillus ferrooxidans* in groundwater samples from 12 wells during in situ remedial perturbations.

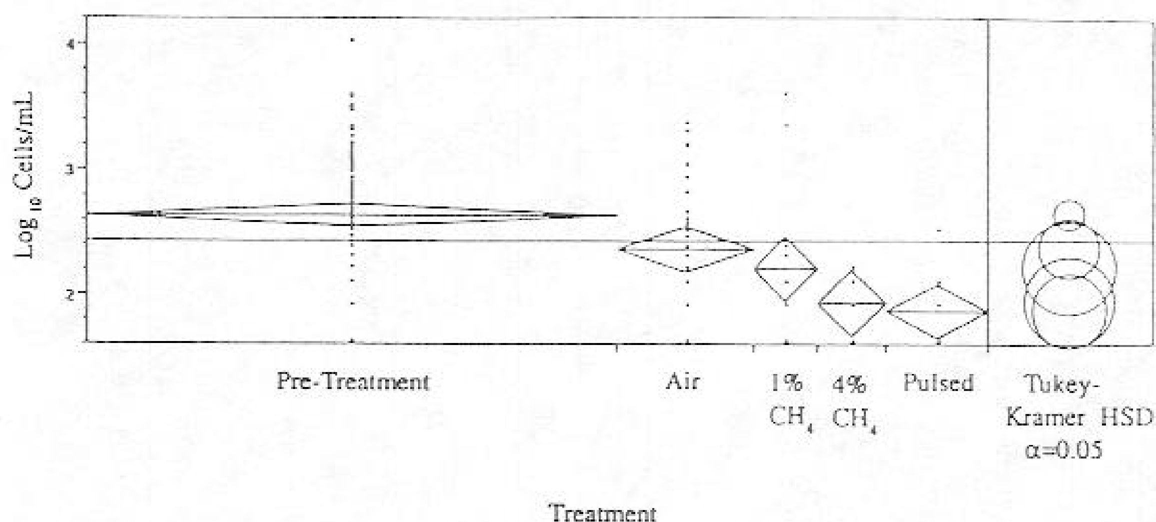

FIGURE 8. Density and statistical analyses of *Legionella pneumophila* serogroup 1 in groundwater samples from 12 wells during in situ remedial perturbations.

Bradyrhizobium japonicum was not found in any of the groundwater samples, although it was observed in the shallow soil samples from the site (data not shown).

Biolog® plates were used to determine the metabolic versatility and activity of the microbial consortia present in the groundwater samples collected from the wells. Groundwater samples from each sampling period were inoculated into triplicate GN Biolog® test plates. The optical density of the tetrazolium reactions, a indication of the ability of the groundwater microbial community to metabolize the 95 individual compounds listed in Table 1, were measured and plotted with respect to the perturbations made in the horizontal wells.

The tetrazolium reactions were grouped based on their chemical relationships and plotted. The data in Figure 9a and b show examples of the reaction of the microbial community in each of the 12 wells to the utilization of the tested carbohydrates (28) and phosphorylated compounds (3), respectively over the duration of the Integrated Demonstration Project. There is a clear difference in the ability of the microbial consortia among the 12 wells to use the various tested compounds. Each of the 95 individual compounds is currently being evaluated for significance of utilization both among and between wells and the various treatments.

DISCUSSION

Each of the two basic approaches to the study of microbial systems in nature, the synecological and the autecological approaches, has its merits and shortcomings. In the synecological approach, the entire microbial community is investigated in relationship to a given habitat. In this approach there is little concern about the type of microorganisms involved in the functioning of the habitat, but rather focus is given to the extent of microbial processing that occurs. This approach provides the investigator with process-oriented data and information

TABLE 1. Sole carbon sources present in Biolog® GN microtiter plates.

Carbohydrates	Carboxylic Acids	Amino Acids
N-Acetyl-D-galactosamine	Acetic acid	D-Alanine
N-Acetyl-D-glucosamine	*cis*-Aconitic acid	L-Alanine
Adonitol	Citric acid	L-Alanyl-glycine
L-Arabinose	Formic acid	L-Asparagine
D-Arabitol	D-Galactonic acid lactone	L-Aspartic acid
Cellobiose	D-Galacturonic acid	L-Glutamic acid
i-Erythritol	D-Gluconic acid	Glycyl-L-aspartic acid
D-Fructose	D-Glucosaminic acid	Glycyl-L-glutamic acid
L-Fucose	D-Glucoronic acid	L-Histidine
D-Galactose	α-Hydroxybutyric acid	Hydroxy-L-proline
Gentiobiose	β-Hydroxybutyric acid	L-Leucine
α-D-Glucose	γ-Hydroxybutyric acid	L-Ornithine
m-Inositol	p-Hydroxyphenylacetic acid	L-Phenylalanine
α-Lactose		L-Proline
Lactulose	Itaconic acid	L-Pyroglutamic acid
Maltose	α-Keto butyric acid	D-Serine
D-Mannitol	α-Keto glutaric acid	L-Serine
D-Mannose	α-Keto valeric acid	L-Threonine
D-Melibiose	D,L-Lactic acid	D,L-Carnitine
β-Methylglucoside	Malonic acid	γ-Aminobutyric acid
D-Psicose	Propionic acid	
D-Raffinose	Quinic acid	**Amides**
L-Rhamnose	D-Saccharic acid	Succinamic acid
D-Sorbitol	Sebacic acid	Glucuronamide
Sucrose	Succinic acid	Alaninamide
D-Trehalose		
Turanose	**Aromatic Chemicals**	**Amines**
Xylitol	Inosine	Phenylethylamine
	Urocanic acid	2-Aminoethanol
Esters	Thymidine	Putrescine
Mono-methylsuccinate	Uridine	
Methylpyruvate		**Alcohols**
	Polymers	2,3-Butanediol
Phosphorylated Chemicals	Glycogen	Glycerol
	α-Cyclodextrin	
D,L-α-Glycerol phosphate	Dextrin	**Brominated Chemicals**
Glucose-1-phosphate	Tween 80	Bromosuccinic acid
Glucose-6-phosphate	Tween 40	

for an overall picture of the functioning of the habitat. Such data are highlighted by information on the cycling of nutrients through an ecosystem and the transformation of chemical components of the system by microorganisms intrinsic to the ecosystem without regard to the microorganisms involved. Such investigations often rely on the mineralization or transformation of particular compounds which have been characteristically labeled, thus providing a marker for trans-

FIGURE 9. (A) Activity and statistical analyses of microbial community populations in the groundwater of all 12 wells with respect to 28 carbohydrate compounds. (B) Activity and statistical analyses of microbial community populations in the groundwater of all 12 wells with respect to 3 phosphorylated compounds.

incubation, degradation, and mineralization to enable measurements of the breakdown products, intermediates, and rates.

Furthermore, the autecological approach focuses on a particular microorganism and asks questions with regard to that organism and how it functions in the studied habitat. The data and information gained from this perspective are exceedingly valuable, particularly when dealing with an organism that is potentially pathogenic or is greatly involved in a functional aspect of the ecosystem. Such an approach allows one to pursue questions about the organism, its particular functioning in a given ecological niche, and the potential control of the organism that could not otherwise be made. The disadvantage of the autecological approach is that it requires specialized techniques for the identification and assessment of the bacterium. Few, if any, bacteria can be reliably characterized by their morphological structure even at the genus level. Many bacteria are pleomorphic, depending on their nutritional status, and thus morphological considerations are not acceptable criteria for assessment.

The apparent lack of techniques for autecological investigations is overcome through the use of specific serological and genetic probes for identifying the bacterium to a particular species and subgroup without culturing the organism. Under appropriate conditions the DFA technique can evaluate a bacterium as to its density, distribution, viability, state of physiological health, and transformation of selected isotopically labeled compounds using the techniques of epifluorescent microscopy, cytochrome activity (Fliermans et al. 1981), fluorescent probes (Rodriguez et al. 1992), and microautoradiography (Fliermans & Schmidt 1975), respectively.

Our discussions here center on the autecological approach to the ecology of selected bacteria. Specific polyvalent and/or monoclonal fluorescent antibody probes have been developed to view, enumerate, and assess the role of these bacteria in complex ecological environments that are neither sterile, monoculture, nor easily sampled. Immunofluorescent techniques initially were used in diagnostic medical microbiology, and only in the last few decades have they been employed in the areas of microbial ecology for agricultural, terrestrial, and aquatic microbiology investigations. More recently, DFA has been used to define the ecology of microorganisms associated with the degradation of toxic and hazardous wastes.

As with any technique there are positive and negative features. The DFA technique is capable of visualizing a specific bacterium in very complex habitats such as soils, metal corrosion piping, sediments, vascular plant material, coal, sewage sludge, or virtually any habitat. If one employs the DFA technique for any of the defined bacteria, as little as a single specific cell per milliliter of liquid sample can be visualized. This detection limit could be pushed even further given a larger sample size and a greater concentration factor, but the value of such is not apparent. DFA prepared with serospecific polyvalent antibodies against whole cells or bacterial cell walls are the most useful tools to date for quantifying and visualizing a bacterium in its natural habitat. Using polyvalent antibodies, the DFA can be made specific enough to react with single species or serogroups of any genus without cross-reacting with nonhomologous organisms outside the serogroup or with other species or genera of bacteria.

The DFA approach also can be used with monoclonal antibodies, although monoclonal antibodies have relatively little use in an in situ setting. This lack of functionality is owed to the high specificity of the antibody allowing only a very limited number of organisms in one serotype to react with the DFA prepared from monoclonal antibodies. Although another serotype of the same species may be present in the habitat, if it is not the one reacting with the specific monoclonal antibody, it will not be detected and thus the number of a particular bacteria will be greatly underestimated. Monoclonal applications are valuable in the typing of pure cultures of specific groups of bacteria within the genus, species, and serogroup of the homologous strain that may have come from a selected environment and caused a specific clinical or environmental response. In these circumstances monoclonals can be used to accurately determine the similarity of the organisms in both diagnostic and forensic investigations.

A second limitation of the DFA technique is that the DFA conjugate stains intact organisms, whether alive or dead. If the organism being detected were lysed either by natural events in their habitat or through human-induced events (i.e., addition of biocides), then cell wall debris caused by such events will stain and be observed microscopically. While at first this may seem to be a problem, in the real world it is less of a problem because bacteria are an excellent source of nutrients being at the lowest end of the food chain and, once dead, they are no longer capable of keeping the "wolves" from the door and are readily lysed and serve as food for other microbial populations.

Thus, in nonsterile aquatic or terrestrial habitats, the dead bacterium, is not readily detected by the DFA technique because the organism does not appear to remain intact for an extended period of time. This phenomenon has been observed in sterile soils and sterile aquatic systems. Bohlool & Schmidt (1968) placed killed DFA-positive organisms into a sterile soil system, and the DFA-positive bacteria remained intact for extended periods of time (days). In nonsterile systems, the DFA-positive organisms were observed as debris within a few hours. Thus, it is likely that the intact bacteria observed in the habitat are viable, but may be nonculturable as Hussong et al. (1987) have demonstrated for *Legionella*.

The data from these autecological investigations associated with the Integrated Demonstration Project indicate that selected microbiological communities can be efficiently, effectively, and accurately monitored using immunofluorescent techniques. It is necessary to note that, although these immunofluorescent probes are specific for the microbial constituents, they may or may not be representative of the most numerous or metabolically active bacteria that are present in these subsurface environments. Only one organism, *Bradyrhizobium japonicum*, has not been isolated from the Integrated Demonstration Site or the SRS complex. The other bacteria studied in this investigation are present at the site, are serologically homologous, and are likely to be the bacteria associated with the various transformations, particularly for nitrogen cycling.

The data for each of the bacteria studied provide some interesting observations. The data in Figure 2 for *Azotobacter chroococcum* indicate that the bacterium was stimulated by both air and 1% methane during the course of injections into the subsurface but was rather stongly inhibited by the 4% methane. During the pulsed perturbations the densities of *A. chroococcum* nearly recover to the pretreatment stages but not to those levels observed during the purging of air and 1% methane to the subsurface. This is probably somewhat reasonable since the bacterium is a free-living nitrogen-fixer and air contains high levels of nitrogen. Only when the methane concentration levels were enhanced to a constant 4% was *Azotobacter chroococcum* inhibited.

The data in Figure 3 for *Nitrosomonas europea* indicates that the organism was present more often before the methane injections than after. *N. europaea* is an ammonia-oxidizin g, Gram-negative chemolithotroph that produces nitrite from ammonia through a monooxygenase involvement. Suzuki et al. (1976) demonstrated that in *N. europaea*, ammonia oxidation was inhibited by CO, methane, and methanol. Thus the lowering of *N. europaea* densities during 4% methane injections would be compatible with the known physiology of the organism. Additionally, Hyman et al. (1988); Vannelli et al. (1990) and Rasche et al. (1991) have demonstrated the physiological versatility of *N. europaea* with its degradation of halogenated-aliphatic compounds, including trichloroethylene (TCE). Thus *N. europaea* may well be involved in situ with the degradation of TCE, but inhibited by the increase concentrations of methane as demonstrated by the DFA technique.

The data in Figure 4 represents the density and statistical analyses for *Nitrobacter agilis* and *Nitrobacter winogradskyi* for all 12 of the wells during the perturbation regimes. *Nitrobacter* spp. are Gram-negative organisms that grow as chemolithotrophs using CO_2 as the sole source of carbon while oxidizing nitrite to nitrate as an energy source. They represent the terminal step in nitrification while depending on the end product of *Nitrosomonas*. Thus it is not surprising that when *Nitrosomonas* declined during the methane perturbation, the decline of *Nitrobacter* would follow.

The response of *SRL-MIIF* to the remedial perturbations was rather uninteresting. *SRL-MIIF* was part of a microbial consortia that has been shown to actively degrade TCE aerobically (Fliermans et al. 1988), yet there was no significant change in the density of this organisms with any of the perturbations (Figure 5).

The data in Figure 6 represents the density and statistical analyses for *Methanobacterium formicicum*, which is a Gram-positive to variable bacterium strict anaerobe that oxidizes hydrogen and utilizes ammonia as its nitrogen source. Once the subsurface perturbations began, the densities of this particular organism declined rather precipitously. Although these subsurface systems are aerobic, anaerobic niches are readily maintained by the biological activity of heterotrophic organisms or the low diffusivity of oxygen through tight geological formations. The perturbations had a major component of air and may well have lowered the niches where *M. formicicum* was able to survive.

The data in Figure 7 represent the density and statistical analyses for *Thiobacillus ferrooxidans*, which is a Gram-negative bacterium capable of mixotrophic growth. It is rather common member of the soil bacteriology community. Only

under perturbations of 1% methane did the densities of this organisms increase and then return to pretreatment levels after subsequent 4% and pulsed treatments.

Legionella pneumophila Serogroup 1 showed a considerable drop in cell density with all of the perturbations including air stripping alone. The data indicate that *L. pneumophila*, while part of the subsurface population, is not stimulated by any of the remediation techniques used in this investigation. Although *L. pneumophila* represents a very minor part of the pathogenic organisms in nature, it is necessary to establish that the in situ remediation techniques that were used do not stimulate this pathogenic portion of the microbial community.

The use of Biolog® GN plates during these remediation techniques provides the capability of evaluating microbial systems adapted to metabolizing selected chemicals as their sole carbon and/or energy source. In adapting the Biolog® system to groundwater sampling, the goal was not to provide a consistent level of microbial inoculum, but rather to provide a constant volume of groundwater with an indigenous bacterial density of ca. 10^4 to 10^5 cells of a variety of species to the Biolog® plates. Thus, the time of incubation of the Biolog® plates needs to be standardized for the particular groundwater, but generally it was 3 weeks at groundwater temperatures of 23°C.

Based on the results from the GN screening plates a variety of information was obtained. One is able to determine the best metabolite or group of metabolites that stimulate the microbial community present in the sampled groundwater. Because subsurface environments in a particular local are not microbiologically similar (Fliermans & Balkwell 1989), Biolog® information can be used to selectively stimulate the naturally occurring organisms at a particular location under the various perturbation regimes encountered.

Initial results from both the carbohydrate and phosphorylated compound utilization data demonstrated that each well had its own microbially active communities as measured by their utilization responses. The data from these two sets of compounds provide representation of the data currently being evaluated for each of the 95 compounds present in the GN Biolog® plates. Previous studies (Fliermans & Balkwell 1989; Fliermans & Hazen 1990) on the subsurface microbial populations at SRS have demonstrated the stimulation of microbial populations to nitrogen additions. This same phenomenon has been observed for selected amino acids, aromatics, esters, amides, amines, and polymers (in press).

Thus, these results suggest that Biolog® technology is a useful tool for screening bacterial isolates and consortia to determine their ability to survive, to metabolize, to degrade selected organic chemicals, and potentially to provide screening of groundwater systems for compounds that are useful in stimulating the microbial populations during bioremediation projects.

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