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Ecofunctional enzymes of microbial communities in ground water

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Abstract

Biolog™ technology was initially developed as a rapid, broad spectrum method for the biochemical identification of clinical microorganisms. Demand and creative application of this technology has resulted in the development of Biolog™ plates for Gram-negative and Gram-positive bacteria, for yeast and *Lactobacillus* sp. Microbial ecologists have extended the use of these plates from the identification of pure culture isolates to a tool for quantifying the metabolic patterns of mixed cultures, consortia and entire microbial communities. Patterns that develop on Biolog™ microplates are a result of the oxidation of the substrates by microorganisms in the inoculum and the subsequent reduction of the tetrazolium dye to form a color in response to detectable reactions. Depending upon the functional enzymes present in the isolate or community one of a possible 4×10^{28} patterns can be expressed. The patterns were used to distinguish the physiological ecology of various microbial communities present in remediated groundwater. The data indicate that one can observe differences in the microbial community among treatments of bioventing, 1% and 4% methane injection, and pulse injection of air, methane and nutrients both between and among wells. The investigation indicates that Biolog™ technology is a useful parameter to measure the physiological response of the microbial community to perturbation and allows one to design enhancement techniques to further the degradation of selected recalcitrant and toxic chemicals. Further it allows one to evaluate the recovery of the microbial subsurface ecosystem after the perturbations have ceased. We propose the term 'ecofunctional enzymes' (EFE) as the most descriptive and useful term for the Biolog™ plate patterns generated by microbial communities. We offer this designation and provide ecological application in an attempt to standardize the terminology for this relatively new and unique technology.

Keywords: Biolog; Microbial ecology; Groundwater; Enzyme; Bioremediation; Biodiversity

Contents

1. Introduction

As a result of various human activities, particularly those involving land disposal of chemical waste, the quality of groundwater has become increasingly threatened. The total annual world production of

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synthetic organic chemicals is over 300 million tons, and more than 1000 new compounds are marketed each year [1]. It has been estimated that over 57 million metric tons of hazardous industrial waste were produced in the US during 1980 alone [2]. For these reasons, pollution of groundwater due to hazardous waste is becoming a very serious problem with more than 10 million Americans using tap water with contaminants that exceed EPA standard levels [3].

Bacteria capable of degrading toxic organic chemicals have been isolated from aquatic systems, deep wells, and terrestrial subsurface sites [4–7]. These microbial consortia represent resources that are physiologically diverse and economically important for genomic stability and the enhancement of bioremediation activities in the terrestrial subsurface. Procedures previously used to determine the microbial diversity of subsurface environments and their ability to degrade or mineralize toxic contaminants required repeated enrichments and exposure of organisms to various concentrations of toxic substances for weeks or months [8,9]. Because the use of heterotrophic aerobic bacteria in the bioremediation of contaminated sites is increasing [10,11], the capacity to quickly characterize potential degraders, access remediation strategies, and evaluate subsurface communities and their recovery during and after perturbations will enhance bioremediation technologies.

The Savannah River Site is a 320 square mile facility owned by the U.S. Department of Energy and operated under contract DE-AC09-89R180035 by the Westinghouse Savannah River Company. The site is near Aiken, South Carolina (Fig. 1) and has been operated as a nuclear production facility for DOE since 1950. The 300-M Area operations of SRS were used to fabricate fuel and target elements that were later irradiated in SRS reactors. During these operations the elements were degreased at several stages in the process. These degreasing operation

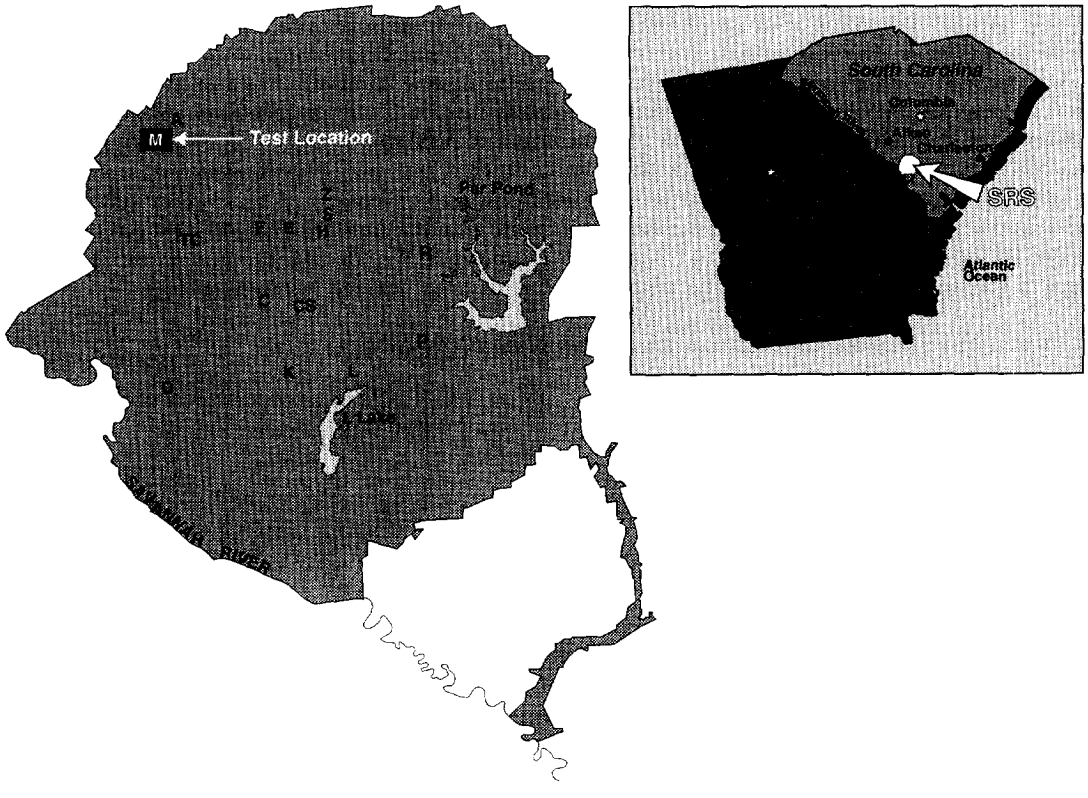
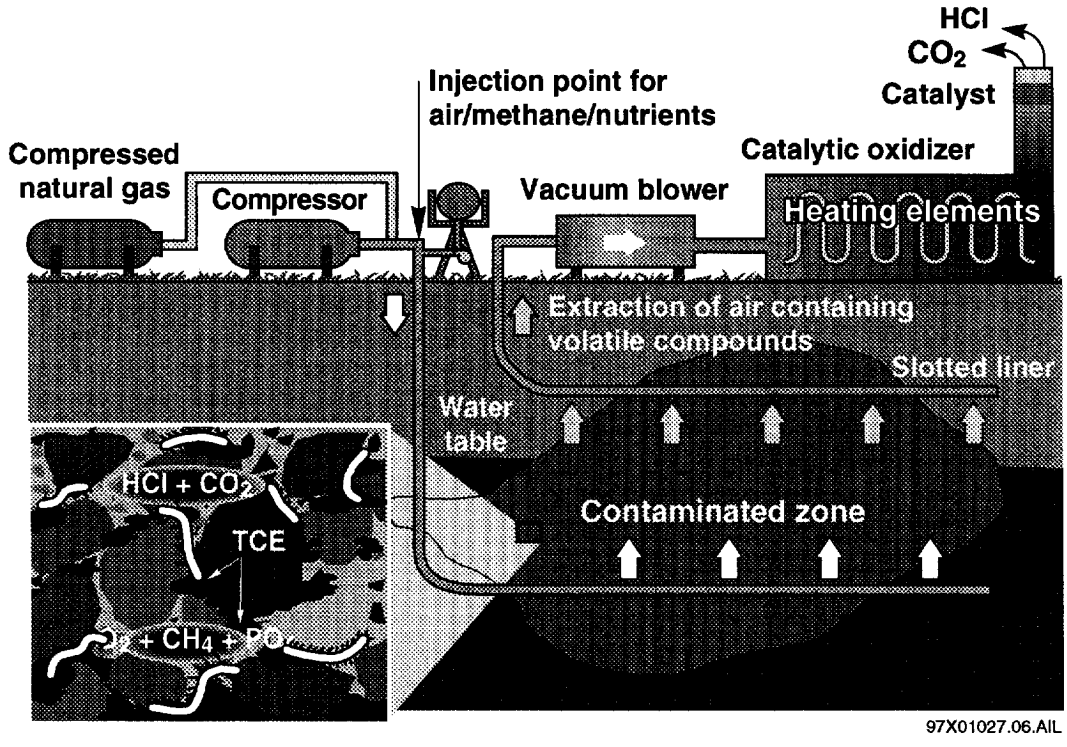


Fig. 1. Location of the Savannah River Site near Aiken, SC, USA.



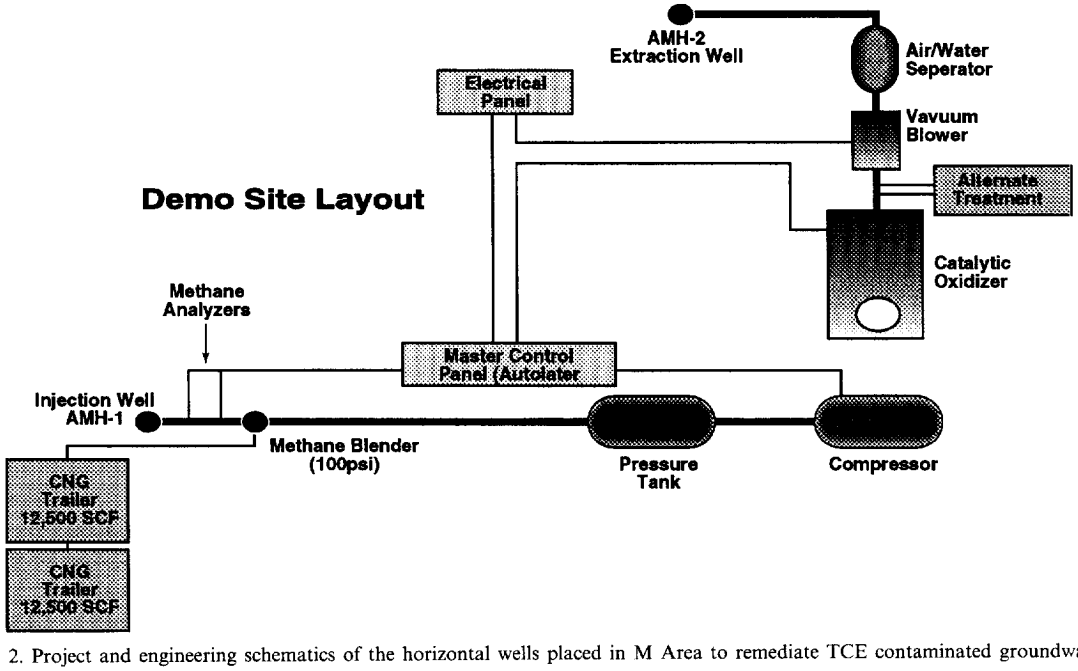


Fig. 2. Project and engineering schematics of the horizontal wells placed in M Area to remediate TCE contaminated groundwater.

generated large amounts of metal-degreasing solvent wastes. From 1952 to 1982, M Area used an estimated 13 million pounds of chlorinated degreasing solvents [33]. Evaporation alone accounted for 50–95% loss, while the remainder went to the M Area process sewer system. Marine and Bledsoe [12] estimate that as much as 2 million pounds may have been released to the sewer that leads to the M Area Settling Basin; another 1.5 million pounds went directly to the A-14 outfall at Tims Branch. The discharges to the M Area Settling Basin consisted primarily of trichloroethylene (TCE: 317 000 lb), tetrachloroethylene (PCE: 1 800 000 lb), and 1,1,1-trichloroethane (TCA: 19 000 lb) [33]. The solvents discharged into the settling basin spread through the vadose zone and entered the groundwater below the basin. The leaking process sewer line used to convey these wastes to the basin also released large quantities of the solvents into the surrounding vadose zone sediments. A conventional groundwater extraction and treatment system (air stripper) has been in operation since 1984.

Groundwater and sediment contamination in M Area is extensive [32]. Subsurface soils and water adjacent to an abandoned process sewer line at the SRS have elevated levels of TCE. The residual solvents in the vadose zone associated with the abandoned process sewer line and the settling basin continue to leach into the groundwater contaminating more than 1 square mile. This area of subsurface and groundwater contamination was the focus of an integrated demonstration of new remediation technologies utilizing horizontal wells. Horizontal wells used in bioremediation have enhanced the recovery of groundwater contaminants for bioreactor conversions from deep or inaccessible areas (e.g., under buildings) and to enhance the distribution of nutrient or microbial concentrations in an in situ bioremediation.

The project and engineering schematic are shown in Fig. 2. The horizontal wells were installed in 1988 with monitoring well clusters placed in 1990 and 1991. Air was extracted constantly from the upper, vadose zone, horizontal well, at 240 SCFM (range 239–246, average 245 SCFM). The extracted air was treated via an electrically heated catalytic oxidation system (halo hydrocarbon degradation catalyst, HDC, from Allied Signal Inc.). Rigorous sampling of both the influent gas to the catalytic oxidation system and the effluent gas revealed that the off-gas treatment system destroyed more than 94% of the total VOCs. Average daily emissions were less than 1.9 lb total VOCs. More than 108,206,345 scf of air and 1,392,774 scf of methane were injected during the demonstration.

The area has been extensively characterized in terms of its hydrology, geology and microbial ecology [11]. During a 14 month period (February 1992 to April 1993) the site was used to demonstrate in situ air stripping, bioventing, 1% and 4% methane injections, pulsed injections and post recovery via the horizontal wells. Each campaign was approximately 3 months in duration [34,35].

This paper describes one portion of the investigation where a rapid screening procedure using Biolog™ multiwell plates (Biolog™, Hayward, CA, USA) to test the enzymatic capabilities of bacterial consortia in groundwater samples. The response of these groundwater communities were evaluated during the bioremediation perturbations to determine their ability to degrade selected organic substrates during the in situ degradative process [15].

2. Materials and methods

The bioremediation demonstration project consisted of using two horizontal wells for injection and extraction at a site contaminated with chlorinated solvents (TCE/PCE) from a leaking process sewer line. The lower injection well (54 m depth) was installed below the water table (37 m) and the upper extraction well (25 m depth) was in the vadose zone above the water table. Air was injected into the lower well at a constant rate of 200 SCFM, while air was extracted from the upper well during all operating campaigns at 240 SCFM. Extracted air was treated by a thermal catalytic oxidizer. Air, water and sediment samples were taken before, during and after the demonstration as per the Test Plan for this demonstration [11]. The data presented here are results from groundwater samples collected from 11 different cluster wells located around the demonstration facility as shown in Fig. 3.

Ninety-five different carbon-source compounds amended with groundwater samples were used to

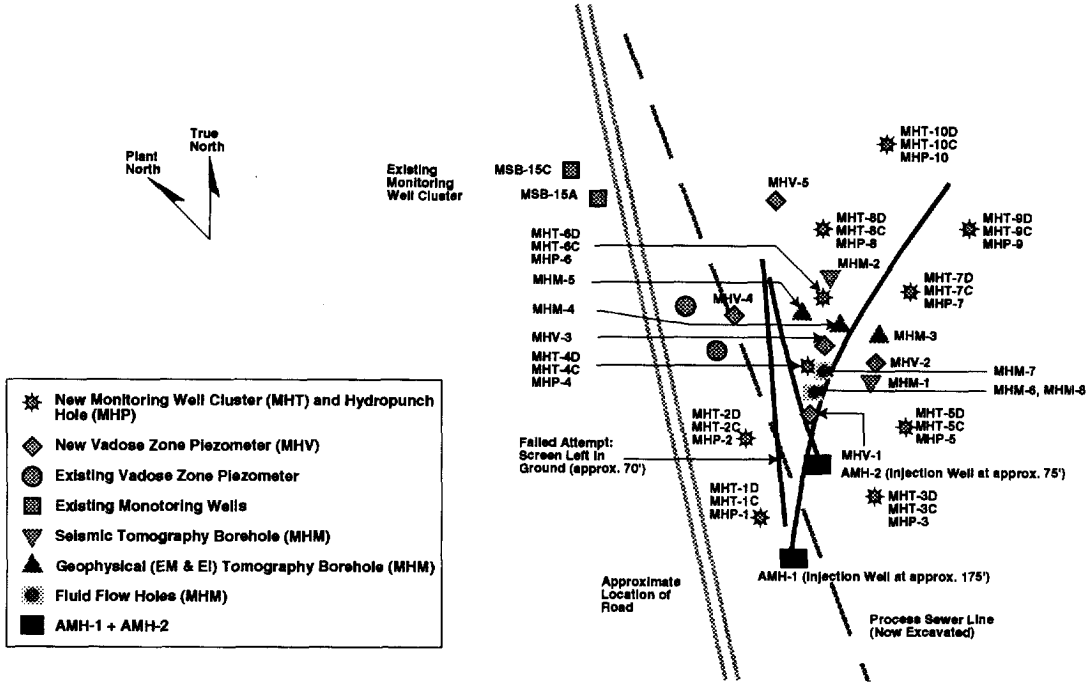


Fig. 3. Placement map for boreholes and wells used in the bioremediation studies.

evaluate heterotrophic community-level physiology associated with the ongoing bioremediation project. Using dedicated submersible pumps, groundwater samples were collected aseptically on a bimonthly schedule from 11 monitoring well clusters influenced by the perturbations of a TCE and PCE bioremediation project [11]. Wells were pumped according to established protocol whereby four well volumes are pulled before samples are collected in order to stabilize chemical, physical, and biological parameters. Four liter samples were collected for a variety of microbiological and analytical analyses [35]. Each groundwater sample was inoculated, without concentration, into triplicate GN Biolog™ (Biolog™, Hayward, CA, USA) microtiter plates at a level of 150 μl per well. The specifics of the system and the selected carbon sources are given elsewhere [13,14]. The plates were enclosed in sterile Whirl-Pak bags to prevent moisture loss and incubated at in situ groundwater temperature, 18°C. After 21 days of incubation, plates were read on a microtiter plate reader (590 nm) used in conjunction with MicroLog 2, Release 2.01 software (Biolog™). The A-1 well of the microtiter plate serves as a control since it has no substrate, and thus no nutrient source with which to initiate a metabolic reaction Automated plate reader was programmed to sense a threshold optical density value for each plate based on the color of the A-1 reference control well. If the A-1 well (no nutrient source) had an optical density (OD) value of 0.40 or greater, then the data for the plate were discarded. Data were collected as OD values for each of the 96 wells of the microtiter plate and normalized against the control well. Thus, the OD readings which determined each inoculum pattern were significantly above the threshold level.

The community level approach is based on the direct inoculation of whole groundwater samples into the Biolog™ microtiter plates and the color production in the wells due to microbial respiration was evaluated. Time course evaluations for color development maximizes in these systems after a 3 week incubation period so that all readings were standardized for that time period.

Data were analyzed using prepared programs for the Macintosh, including Systat version 5.1 (Systat, Evanston, IL, USA) and JMP, version 2 (SAS Institute, Cary, NC, USA) and using statistical tests as

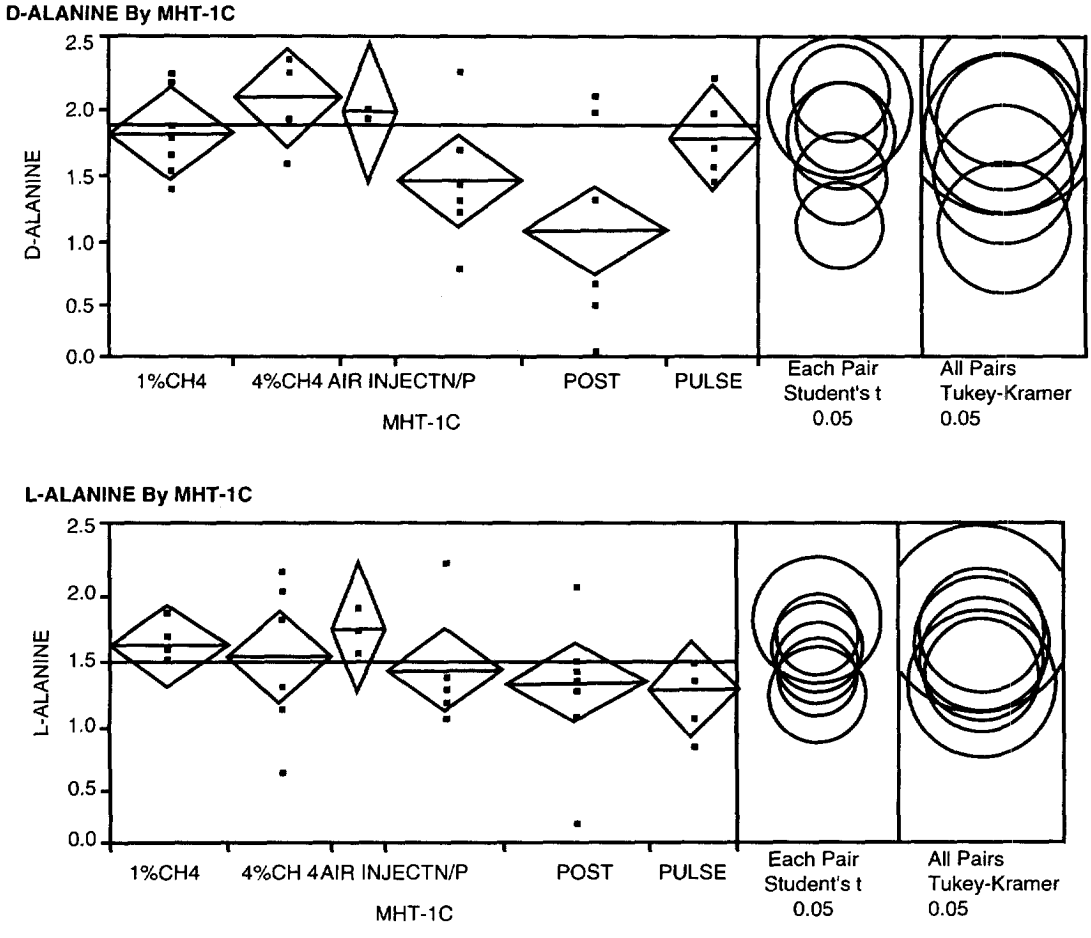


Fig. 4. Expression of ecofunctional enzyme activity for microbial communities capable of utilizing stereoisomers of alanine during various remediation treatments.

previously described [12]. The Tukey-Kramer test was used to test multiple comparisons of means. Any statistical probability equal to or less than 0.05 was considered significant.

Each remediation campaign, i.e., air injection, 1% methane injection, 4% methane injection and the pulsed nutrient injections, was 3 months in duration as described elsewhere [11]. Samples were collected every 2 weeks during each campaign.

3. Results and discussion

The basic procedures described here use Biolog™ GN plates which contain minimal nutritional factors along with various organic substrate in each of the 95 wells in a microtiter plate [13–15]. Unconcentrated groundwater samples from 11 of the Integrated Demonstration Wells were inoculated into each microtiter plate well. The plates were incubated at groundwater temperature for 3 weeks, and the color changes indicative of the characteristic metabolic patterns were recorded. Although the 95 different carbon sources in Biolog™ GN and GP plates were pre-selected specifically for characterizing and differentiating Gram-negative and Gram-positive aerobic bacteria, respectively, they are also useful in demonstrating metabolic patterns for mixed cultures. Carbon sources in the Biolog™ plates are dominated by 28 carbohydrates, 24 carboxylic acids,

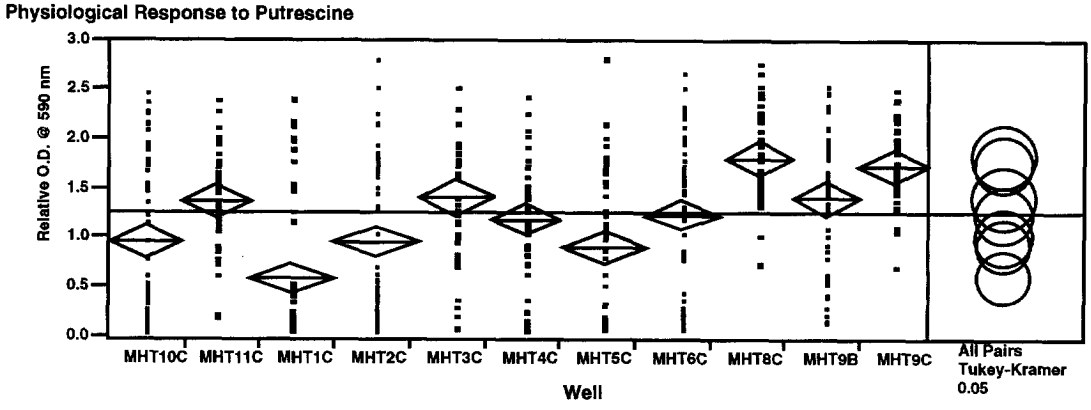


Fig. 5. Expression of ecofunctional enzyme activity for microbial communities capable of utilizing putrescine during the air injection campaign for all the tested well clusters.

and 20 amino acids plus various amides, aromatic chemicals, and other compounds as previously described [13].

Each of the 95 compounds were analyzed for each of the 11 groundwater wells for each time perturbation events. The description of the operational modes for the bioremediation tested during the 14 month demonstration are as follows:

Injection operations	Start date	End date
Air extraction and injection	2/26/92	4/20/92
1% methane	4/20/92	8/05/92
4% methane	8/05/92	10/23/92
Pulsed methane/air	10/23/92	1/25/93
Nutrients/pulse/air	1/25/93	4/30/93
Post characterization	4/30/93	ongoing

The data in Fig. 4 represent the variability in the microbial community present at each of the well clusters during the air injection campaign with regard to the utilization of the amine, putrescine. Data analyses for each of the carbon sources for each of the well clusters during each perturbation are reported elsewhere (Fliermans et al., in preparation). Water samples were collected from each of the cluster wells and the developed optical density read following incubation. The data are plotted in the JMP statistical package for the means and tests how these means differ. The upper and lower points of the means diamond span a 95% confidence interval computed from the sample values. The line drawn through the means diamond is the group average for the set of data. The mean for the entire data set is given by single horizontal line on the graph. A set of comparison circles displayed to the right of the plots provides a graphic test as to whether the mean data are statistically significant. Student's *t*-test is sized for individual pairwise comparisons for all combinations of group means and displays the comparisons with comparison circles. The Tukey-Kramer HSD (honestly significant difference) compares group means and provides a conservative test that is sized for all differences among the means [38].

The center of each circle is aligned with the mean of the group it represents. The diameter of each circle spans the 95% confidence interval for the data that it represents. Whenever the two circles overlap the confidence intervals of the means overlap and suggests that the means may not be significantly different from each other. If the two circles do not intersect then the represented group means are significantly different. Thus with regard to the data in Fig. 4, several of the groundwater samples are significantly different in that MHT1C metabolizes putrescine to a lesser extent than the microbial populations in MHT3C, MHT11C, MHT4C, MHT6C, MHT8C, MHT8B or MHT8C Fig. 5. It is interesting to note that MHT9C is screened at 49.2 m while MHT9B is screened at 54.9 m in the same aquifer and there is no significant difference with regard to the microbial utilization of putrescine during this portion of the investigation.

Because of the location of wells with respect to the injection well, each well did not receive the same

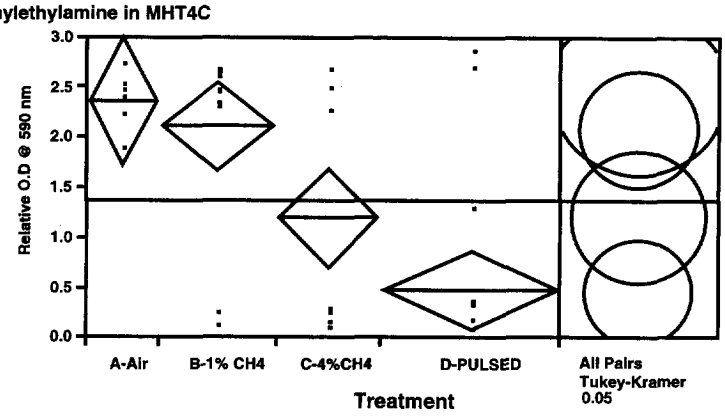


Fig. 6. Expression of ecofunctional enzyme activity for microbial communities associated with the MHT4C well cluster capable of utilizing phenylethylamine.

level of nutrients or perturbations. Well cluster MHT4C was more affected by the injection scheme than MHT1C as reflected in the results in Figs. 4 and 6. The data in Fig. 4 demonstrate that during the injection of air and 1% methane the level of phenylethylamine utilization was high and not significantly different. Once the levels of methane were elevated to 4% either continuously or pulsed, the levels of phenylethylamine utilization dramatically decreased. The data suggest that the microbial population responsible for phenylethylamine utilization was affected by the input of high concentrations of methane gas.

The data indicate that wells most influenced by the remediation techniques were the most variable in their response. The microbial community present in the groundwater changed their activity against specific groups of compounds (phenylethylamine) during changes in remediation parameters of air and methane for a given sampled well (Fig. 4). Furthermore, the metabolism of selected stereoisomers of amino acids varied with differences in the remediation strategies employed at the site (Fig. 6). These data provide information into the understanding and modification capabilities of the microbial ecology in subsurface environments during remediation. Additionally, such analyses provide a means to evaluate the recovery of the microbial systems after perturbations have ceased.

4. Ecofunctional enzymes

Biolog™ technology was initially developed as a rapid, broad spectrum method for the biochemical identification of clinical microorganisms. Demand and creative application of this technology has resulted in the development of Biolog™ plates for Gram-negative and Gram-positive bacteria, for yeast and *Lactobacillus* sp. The Biolog™ identification library now has more than 600 environmental species and strains. Microbial ecologists have extended the use of Biolog™ plates from the identification of pure culture isolates to a tool for quantifying the metabolic patterns of mixed cultures, consortia, and entire microbial communities. As the use of this technology has expanded authors have struggled to develop terminology to describe the Biolog™ plate pattern in terms of its usefulness for microbial ecology.

Patterns that develop on Biolog™ microplates are a result of the oxidation of the substrates by microorganisms in the inoculum and the subsequent reduction of the tetrazolium dye to form a color in response to detectable reactions. Depending upon the functional enzymes present in the isolate or community, one of a possible 4×10^{28} patterns can be expressed [14]. The patterns are distinctive for isolates of different species and are now being used to distinguish the physiological ecology of various microbial communities.

In the absence of standard terminology for the Biolog™ patterns, a plethora of descriptive terms has flooded the literature. The patterns have been referred to as 'phenotypic fingerprints and composite metabolic profiles' [18]; 'microbial community structure' [15]; 'microbial community classification' [19]; 'sole-carbon-source utilization' [20]; [19]; 'community metabolic potential' [21]; 'metabolic analysis' [22]; 'carbon substrate utilization profile analyses' [23]; 'metabolic similarity' [24]; 'Biolog™ profile and functional profile of community' [25]; 'patterns of carbon source utilization' [26]; 'Biolog™ community assay' [27]; 'overall physiological capabilities of the community for the strict utilization of 95 substrates' [28]; 'functional differences' [29]; 'microbial diversity' [30]; 'carbon source utilization patterns' [31]; and 'microbial community phenotype' (J. Vaun. MacArthur, personal communication).

Biolog™ technology offers a unique capacity to measure functional aspects of bacterial enzyme activity in a reproducible and quantifiable way. Biolog™ plate patterns develop due to enzyme activity in each positive well. The enzyme activity of pure cultures enables the identification of the isolates based on the phenological patterns. When water samples collected from natural aquatic systems are inoculated into Biolog™ plates, the resulting patterns are reflective of the enzymes expressed by the functioning microbial community and may be defined as ecologically functional or ecofunctional enzymes.

Ecofunctional enzymes (EFE) are enzymes which are being expressed or used by or within a microbial community in order to enable individuals or microbial populations to survive, maintain, or grow. Alternatively, EFE may be latent enzymes ready to be used or expressed under more favorable or opportunistic conditions. Such enzymes may be present in every microbial community; in fact, it is expected that the suite of EFE expressed at any moment by the microbial communities present in surface waters is a direct reflection of the dominant and contributing microbial populations of that community and of the environmental factors impacting the ecosystem at the time of sampling.

We propose the term 'ecofunctional enzymes' (EFE) as the most descriptive and useful term for the Biolog™ plate patterns generated by microbial communities. We offer this term in an attempt to standardize the terminology for this relatively new and unique technology. As the application of this technology in microbial ecology continues to grow and develop we urge the use of the acronym EFE, to avoid further confusion in the literature.

Biolog™ technology is the basic component of the rapid screening procedure and is predicated on tetrazolium dye reduction as an indicator of enzyme systems capable of sole carbon source utilization. While the technology does not depend on the isolation of microorganisms, it does require a physiological response in order to provide a recordable signal. To achieve such a signal the organisms must respond by using the organic substrates as electron donors to the tetrazolium chloride for the subsequent formation and deposition of formazan within the microbial cell. This transformation requires that the microorganisms supply enzymes capable of transporting and respiring the particular compound. The utilization of these compounds indicates that the microbial systems are capable of utilizing the compound under the experimental conditions. The use of the Biolog™ technology provides a rapid means for evaluating the synecological response of the microbial community to perturbations encountered during bioremediation. While it does not identify the microorganisms responsible for the observed transformations it does assess whether the microbial consortium is able to make transformations and how those transformations change with variety of perturbations. Additionally, the recovery of the microbial community can be assessed in a synecological approach so that one can determine whether the ecosystems have been significantly altered with respect to its community-level physiology.

While it has been argued that microorganisms in the environment may go through a viable but nonculturable phenomenon [36,37], this reported use of Biolog™ technology focuses on the capability of the microbial community under the experimental conditions and their response to bioremedial perturbations.

Although originally designed to identify clinical bacterial isolates using 95 different sole carbon sources, the technology has been extended to identify environmental isolates, MPN of soil and groundwater habitats [16], rapid screening of bacteria capa-

ble of degrading toxic organic compounds [17], the investigation of community diversity [19].

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