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# Integrated Field, Laboratory, and Modeling Studies to Determine the Effects of Linked Microbial and Physical Spatial Heterogeneity on Engineered Vadose Zone Bioremediation

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## Executive Summary

While numerous techniques exist for remediation of contaminant plumes in groundwater or near the soil surface, remediation methods in the deep vadose zone are less established due to complex transport dynamics and sparse microbial populations. There is a lack of knowledge on how physical and hydrologic features of the vadose zone control microbial growth and colonization in response to nutrient delivery during bioremediation. Yet pollution in the vadose zone poses a serious threat to the groundwater resources lying deeper in the sediment. While the contaminants may be slowly degraded by native microbial communities, microbial degradation rates rarely keep pace with the spread of the pollutant. It is crucial to increase indigenous microbial degradation in the vadose zone to combat groundwater contamination.

The overall objective of the project was to increase knowledge of the feasibility of engineered bioremediation in the deep vadose zone, particularly at arid western sites where microbial populations and activities are low. Project accomplishments include:

- Development and use of a laboratory chamber to simultaneously study in real time how sediment physical properties, water and contaminant movement, and microbial processes interact. Novel processes and interactions were observed. From this laboratory research, a vadose zone reactive transport model was developed that more accurately describes interacting hydrologic and microbial processes, and incorporates microbial colonization. The resulting model can also be used for laboratory and field studies. This work resulted in two Ph.D. dissertations at Oregon State University.

- For the first time, numeric values have been defined for microbial colonization under non-flowing unsaturated conditions. How sand particle size and water content controls these values was also determined. Motile bacteria were able to rapidly migrate in response to nutrient addition. The work showed that aqueous nutrient delivery to vadose zones with patchy and low density microbial populations appears to be an effective technology for driving rapid microbial colonization in the field.
- Assistance to the U.S. Geological Survey Toxics Program, which was studying far-field migration of $^{14}C\text{-}CO_2$ near a shallow low-level radioactive and mixed waste burial site at the Amargosa Desert Research Site in Nevada. Microbial characterization was performed to evaluate where the $^{14}C\text{-}CO_2$ was being produced by microbes. The characterization led to the conclusion that degradation was occurring in or below the trench, and not near the far-field borehole.
- Microbial characterization of vadose zone sediments beneath the 216-Z-9 Trench at the DOE Hanford Site, where large amounts of carbon tetrachloride and radionuclides were disposed. The analysis showed that the highest carbon tetrachloride concentrations were co-located with the highest microbial populations and highest sediment moisture content. These sediments were further used in laboratory experiments to investigate the potential of the microbial community to utilize gaseous hydrocarbons and potentially transform CT under unsaturated conditions.
- Five hydrocarbons and a nitrogen and phosphorus that could be delivered to the vadose zone during engineered bioremediation via vapor-phase transport were tested. 80% of the sediments degraded at least one of the hydrocarbons. 20-45% of the sediment samples showed the ability to biodegrade a specific hydrocarbon. Greater

than 3 hydrocarbons were degraded in 70% of the sediments. Gaseous nitrogen and phosphorus did not stimulate removal of gaseous C sources compared to no addition of N and P, however, the microbial community appeared to have adequate levels of sediment-associated N and P to convert large amounts of hydrocarbon utilization to cell mass. Use of gaseous hydrocarbons to increase microbial populations in situ has great promise in Hanford deep vadose zone sediments. The ability to successfully deliver the gases to the in situ subsurface microsites where microbes exist should be tested in intact Hanford deep vadose zone cores.

- Sediment samples that displayed the ability to use gaseous hydrocarbons were tested for carbon tetrachloride-degrading ability. CT degradation occurred in only 5% of the bottles and only 50-60% was degraded after a 10 month period. It is likely CT degradation would occur more readily in the subsurface vadose zone where porosity and oxygen diffusion rates are lower and anoxic sites would be more readily formed as a result of aerobic degradation of gaseous hydrocarbons. Similar studies to those conducted here, but conducted in closed columns, are warranted to further investigate the potential for degradation of CT and other DOE organic contaminants in the vadose zone using gaseous hydrocarbon-promoted bioremediation.
- A colorimetric readout method was developed for real-time monitoring of $CO_2$ movement (as a proxy for tracking movement of gaseous microbial nutrients) through unsaturated 2-d chambers. This work is being extended to track movement of gaseous microbial nutrients in the 2-d chambers, to investigate relationships between hydraulic processes, gaseous nutrient delivery to microorganisms, and microbiological processes.

The project was a collaboration between Pacific Northwest National Laboratory and Oregon State University. Project objectives were addressed by the two groups, and twice-annual meetings provided important communication between the groups that resulted in excellent technical advice for ongoing research and significant changes in research directions.

Relevance, impact, and technology transfer: We believe that numerous individuals and institutions will utilize outputs of this project, including: (i) improved numerical models to simulate and better understand interacting vadose zone processes, (ii) numerical values describing microbial movement and colonization in the vadose zone, and (iii) the knowledge that (even though microbial populations are low in these environments) gaseous hydrocarbons can be effectively used to grow biomass in Hanford sediments and likely in sediments from other arid environments. These results will be used in the near-term by applied scientists and engineers for advancing the modeling and study of vadoze zone processes.

## RESEARCH OBJECTIVES

While numerous techniques exist for remediation of contaminant plumes in groundwater or near the soil surface, remediation methods in the deep vadose zone are less established due to complex transport dynamics and sparse microbial populations. Yet pollution in the vadose zone poses a serious threat to the groundwater resources lying deeper in the sediment. While the contaminant may be slowly degraded by native microbial communities; microbial degradation rates rarely keep pace with the spread of the pollutant.

Hydrologic and microbiological properties of the zone, and their interactions, are fundamentally different from the saturated zone: the vadose zone has an additional phase (air), higher levels of oxygen, and contaminant transport and water movement is predominantly perpendicular to geologic strata and occurs in water films. In addition, microbial populations in the vadose zone are sparse and spatially discontinuous, especially in arid climates.

At the Department of Energy's Hanford site in Richland, WA, numerous recalcitrant organic compounds were disposed of in the vadose zone, and now are continual sources of groundwater pollution. Among the most problematic of these is a plume of carbon tetrachloride (CT), a common solvent, the majority of which still resides in the vadose zone despite the presence of microbes that can degrade it and its byproduct chloroform. Gaseous nutrients can in principle be used to stimulate the native degrading population and has shown some promise in isolated field cases. However, there is a lack of knowledge on how physical and hydrologic features of the vadose zone control the spatial distribution of microbes, and the extent that microbes can colonize the vadose zone in response to nutrient delivery during bioremediation.

The overall objective of the project was to increase knowledge of the feasibility of engineered bioremediation in the deep vadose zone, particularly at arid western sites where microbial populations and activities are low.

Specific objectives were to:

1) Conduct laboratory studies of how physical and hydrologic features of the vadose zone control the spatial distribution of microbial growth and the ability of microorganisms to colonize microbially sparse or "empty" regions of the vadose zone.
2) Characterize microbiological properties of a carbon tetrachloride-contaminated deep vadose zone site at the DOE Hanford Site.
3) Evaluate the potential for gas phase feeding of carbon, nitrogen, and phosphorus to deep vadose zone microbial communities.
4) Use field and laboratory data generated from the project to parameterize an unsaturated zone transport model with microbial growth, colonization, and biotransformation kinetics and conduct reactive transport simulations.

Pacific Northwest National Lab (PNNL) and Oregon State University (OSU) jointly addressed objectives 1, 3, and 4. PNNL addressed objective 2. For objective 4, laboratory data was simulated during the project; field data was not used in modeling and

simulation due to the late initiation of the field study and the small number (n=24) of samples studied.

## METHODS AND RESULTS

Methods and Results are presented in this section of the report for the above 4 project objectives. For purposes of clarity, objective 4 activities are discussed as part of objectives 1 and 3.

### Objective 1. Conduct laboratory studies of how physical and hydrologic features of the vadose zone control the spatial distribution of microbial growth and the ability of microorganisms to colonize microbially sparse or "empty" regions of the vadose zone.

#### I. Two-dimensional studies of microbial growth and colonization under dynamic unsaturated flow.

The following studies used light transmission chambers packed with sand to mimic the vadose zone environment. These chambers are unique in that they employ backlighting, *lux*-gene induced microbial glow, and sophisticated camera imagery to portray real-time, *in situ* relationships between microbial growth, hydrodynamics, and introduced substrates. Studies were performed in a chamber with a single sand material (homogenous physical system) and with two sands of differing size, where one sand formed an engineered feature within the other sand (heterogenous physical system). The latter system serves as a model for features that exist in the subsurface and cause changes in water and contaminant transport and microbiological processes.

**Homogenous system.**

Methods

The microbial growth and colonization studies were conducted in a 2-D light transmission chamber which was also utilized in later experiments. This chamber is constructed of two plate glass sheets, each 51.0 cm wide by 61.0 cm tall by 1.3 cm thick, which are separated by a 1.0-cm-thick U-shaped aluminum spacer and sealed with fluorocarbon rubber O-ring stock (Viton; Dupont Dow Elastomers, Wilmington, Delaware). Nine port holes were drilled into the front glass sheet in a cross configuration and sealed with high-temperature silicon sealer in order to allow inoculation or sampling. Larger ports were also drilled into the glass (Figure 1) to allow the placement of sparging stones for gas entry and exit. A drain port at the lower side of the aluminum spacer controls the relative proportions of saturated and unsaturated sand within the chamber, in conjunction with an integral manifold covered with a 10-µm-nominal-pore-size Twilled Dutch Weave stainless steel wire screen (Screen Technologies Group, Inc., Washougal, Washington).

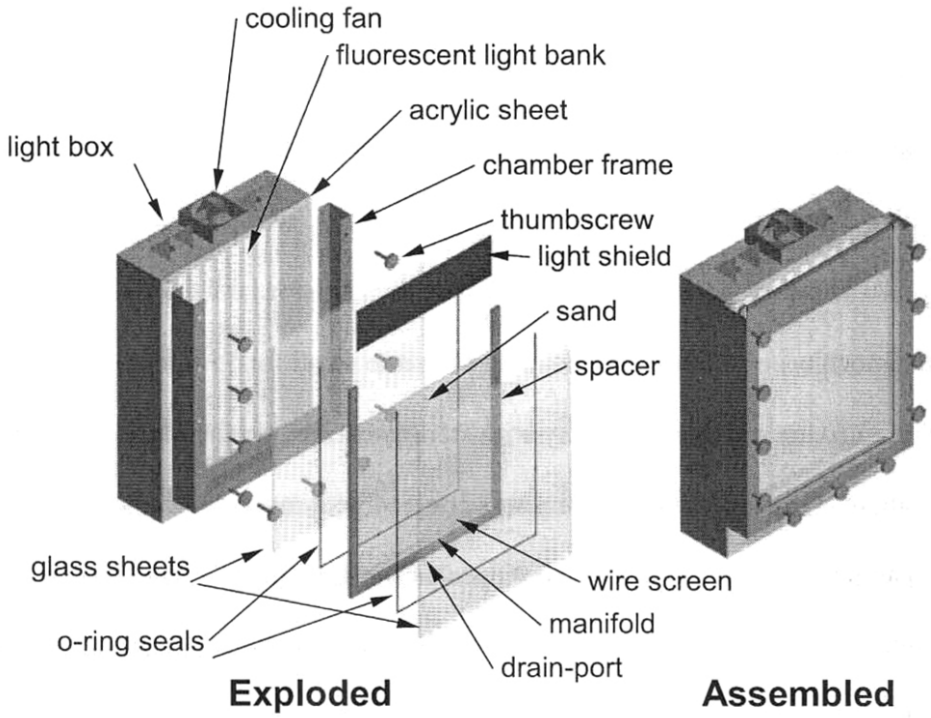



**Figure 1.** Major components of the light-transmission chamber (left), and the chamber's appearance when assembled (right).

The chamber is clamped within an aluminum frame, which is then mounted in front of a light bank of 10 fluorescent tubes housed in a fan-ventilated sheet metal container. This light bank is used in the creation of light transmission images which indicate water content of the sand.

In order to be used for microbial experiments, once assembled, the chamber must be autoclaved and all surfaces were sterilized by UV light. The system is then packed in the laminar flow hood (Yarwood et al 2002) with Accusand (Unimin Corp., Le Sueur, Minnesota) silica sand, the properties of which are summarized by (Schroth, Ahearn et al. 1996). The chamber is initially purged with carbon dioxide to remove trapped air and then saturated and drained to a partially saturated state, at which point it can be inoculated.

This chamber was used to noninvasively monitor glucose-dependent growth of the *luxCDABE* reporter bacterium *Pseudomonas fluorescens* HK44 in quartz sand under unsaturated flow conditions. HK44 has been genetically engineered to carry a naphthalene degradation promoter fused to a *lux* gene. As a result, the organism can emit high levels of light upon naphthalene or salicylate degradation, allowing its presence and response to stimuli to be monitored in real time.

In this case, the spatial and temporal development of growth was mapped daily over seven days by quantifying salicylate-induced bioluminescence (Rockhold, Yarwood et al. 2002). These methods were used for continuous observation of microbial development while simultaneously monitoring hydrodynamics in porous media in a two-dimensional system under unsaturated flowing conditions. Bioluminescence was used to monitor the temporal and spatial development of colonization. Water contents and hydraulic flow

paths were determined by measuring the transmission of light through the system. Greater detail in Yarwood et al, manuscript in preparation.

Results

A nonlinear model relating the rate of increase in light-emission after salicylate exposure to microbial density successfully predicted growth over four orders of magnitude ($R^2 = 0.95$). Simulated biomass distributions are compared to bacterial density predicted by bioluminescence patterns in Figure 2. Because the bioluminescence reaction used to predict bacterial density is dependent on a variety of environmental factors, such as DO concentrations, a direct comparison between experimental and model images is not possible. However, total model-predicted growth agreed with growth calculated from the mass balance of the system using previously established growth parameters of HK44 (predicted, $1.2 \times 10^{12}$ cells; calculated, $1.7 \times 10^{12}$ cells). Colonization expanded in all directions from the inoculation region, including upward migration against the liquid flow. Both the daily rate of expansion of the colonized zone, and the population density of the first day's growth in each newly colonized region remained relatively constant throughout the experiment. Nonetheless, substantial growth continued to occur on subsequent days in the older regions of the colonized zone. The proportion of daily potential growth that remained within the chamber declined progressively between day 2 and day 7 (97 to 13%). A densely populated, anoxic region developed in the interior of the colonized zone even though the sand was unsaturated and fresh growth medium continued to flow through the colonized zone. These data illustrate the potential of a light transmission chamber, bioluminescent bacteria, and sensitive digital camera technology to non-invasively study real time hydrology-microbiology interactions associated with unsaturated flow in porous media (Rockhold, Yarwood et al. 2002)

Bacterial growth and accumulation over the one-week course of the experiment had a significant impact on the hydraulic properties of the otherwise homogeneous media. Microbial colonization caused localized drying within the colonized zone; decreases in saturation approached 50%, and the capillary fringe height was lowered by 5 cm. The dewatering effect can be seen in Figure 3, in which areas of dense colonization correspond to regions of low water content. Observed effects of growth on water content were fairly accurately predicted by the model (Figure 4). Most of the fluid was diverted around the colonized zone, and flow within that zone was retarded significantly. The solute velocity through the colonized region was reduced from 0.39 cm $min^{-1}$ ($R^2 = 0.99$) to 0.25 cm $min^{-1}$ ($R^2 = 0.99$) by the sixth day of the experiment. Effects of the colonized region on a conservative dye tracer, Bromophenol Blue, can be seen in Figure 5, as the dye plumes spread laterally around the colonized region. In turn, diverted flow probably contributed to large changes observed in the extent of colonization over the course of the experiment. We hypothesize that the distribution of cells was not determined by water flow alone, but rather by a dynamic interaction between water flow and microbial growth (Yarwood et al, manuscript in preparation).

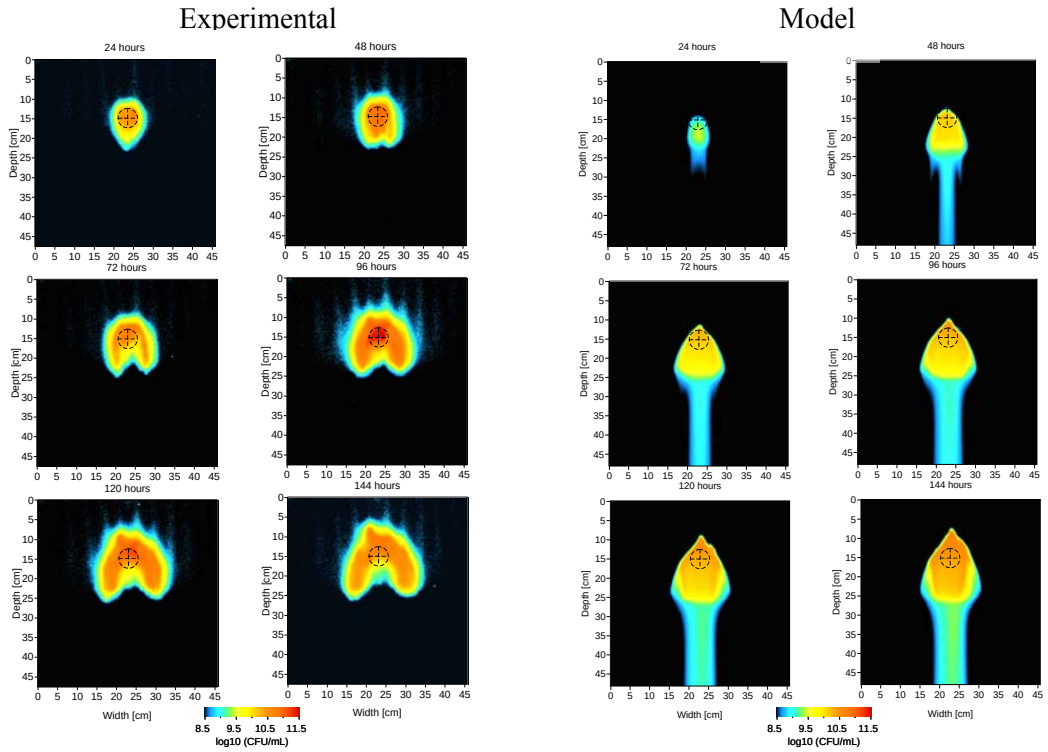



Figure 2: Biomass distributions as calculated from bioluminescence (left) and as predicted by the model (right). Note that the additional areas of microbial density predicted by the model may not be shown by bioluminescence, as it is subject to substrate- or oxygen- limtations. Post-experiment measurements of bacterial density are more closely correlated with the simulated images.

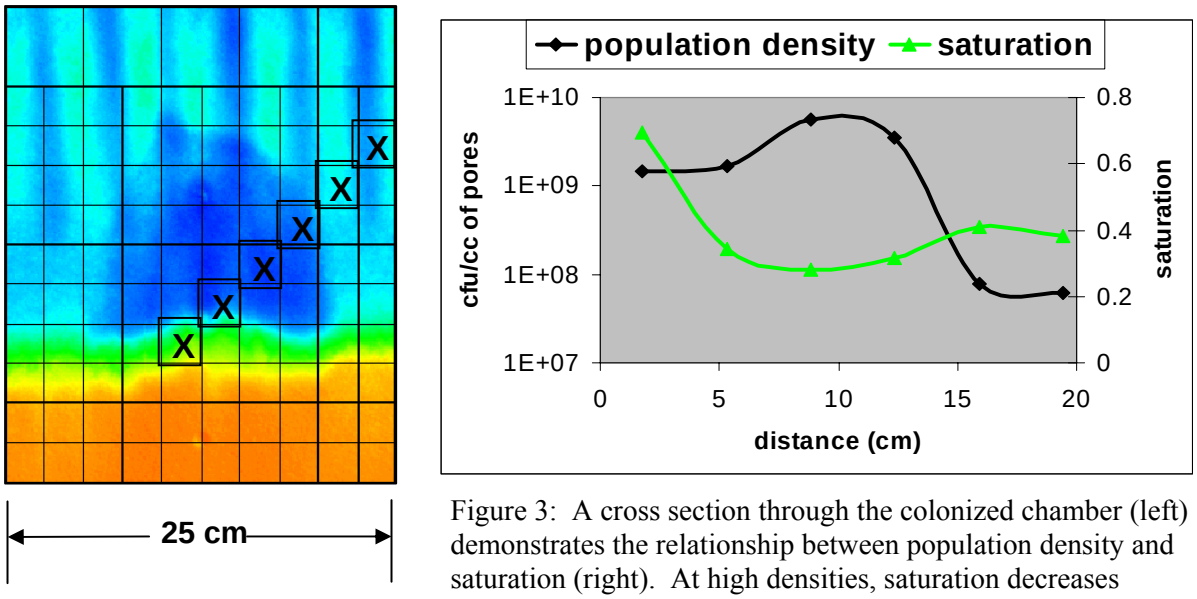



Figure 3: A cross section through the colonized chamber (left) demonstrates the relationship between population density and saturation (right). At high densities, saturation decreases sharply, whereas saturation rises as microbial density falls.

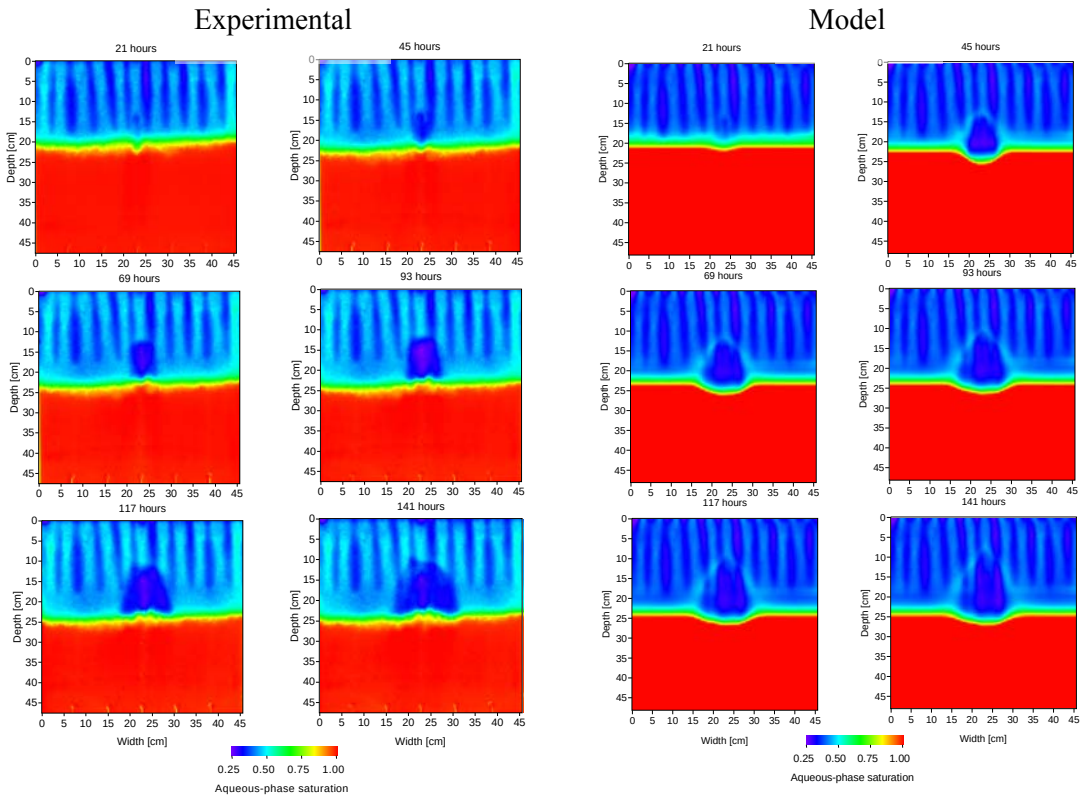



Figure 4: Saturation distributions as calculated from light transmission (left) and predicted by the model (right).

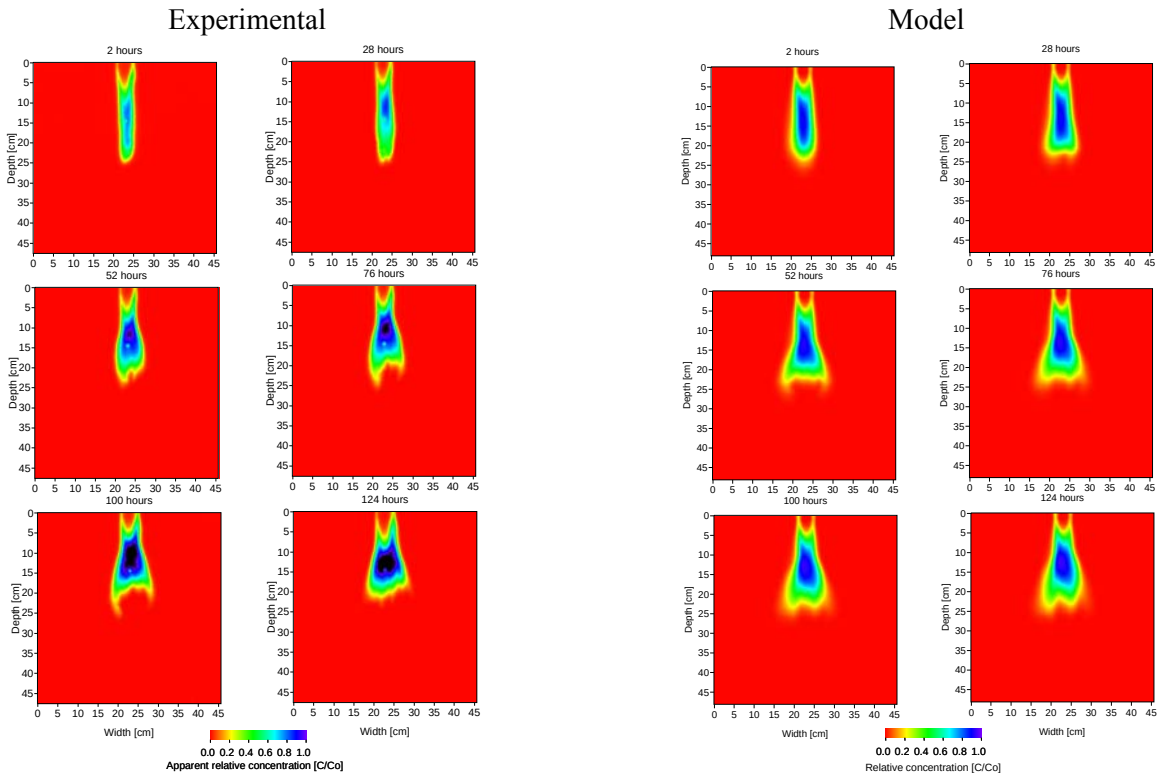



Figure 5: Distribution of an aqueous-phase dye as calculated from light transmission (using attenuation)(left), and predicted distribution of a non-reactive, conservative tracer.

## Heterogenous system

The light transmission chamber was assembled as described above, but packed with a wedge of 12/20 mesh size sand embedded in a matrix of 30/40 mesh size sand. Packing proceeded in a series of steps at the end of which excess sand would be vacumed out according to the desired geometry. The final effect is shown in Figure 6.

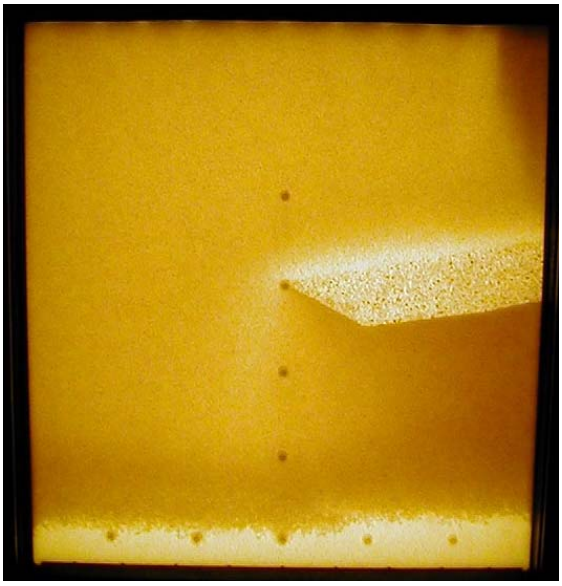
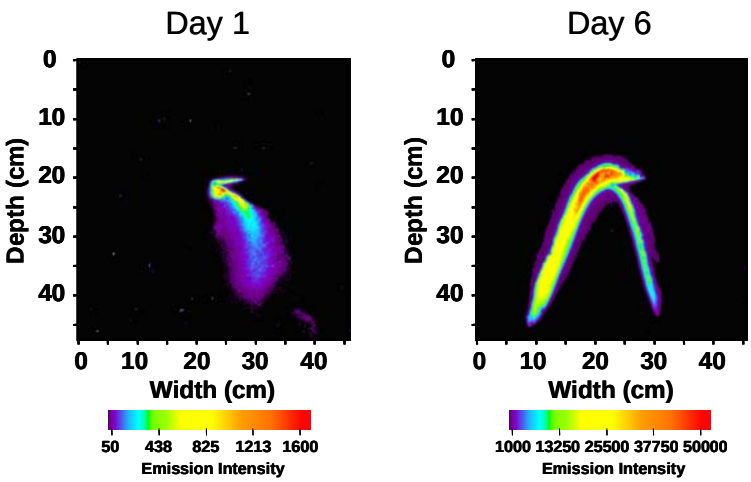


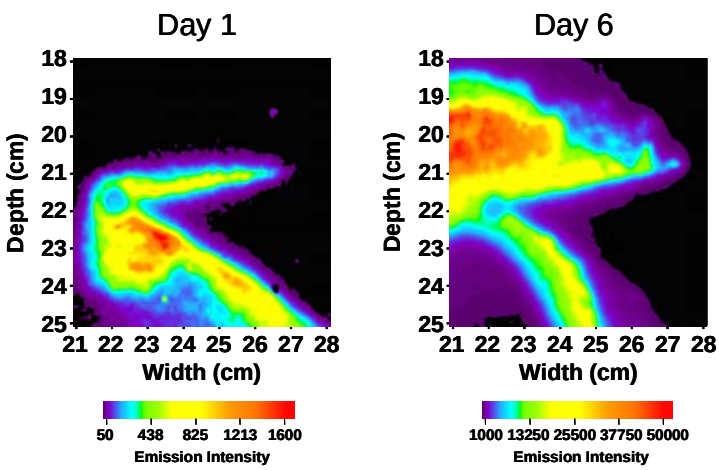
Figure 6: Heterogeneous geometry with a wedge-shaped inclusion.

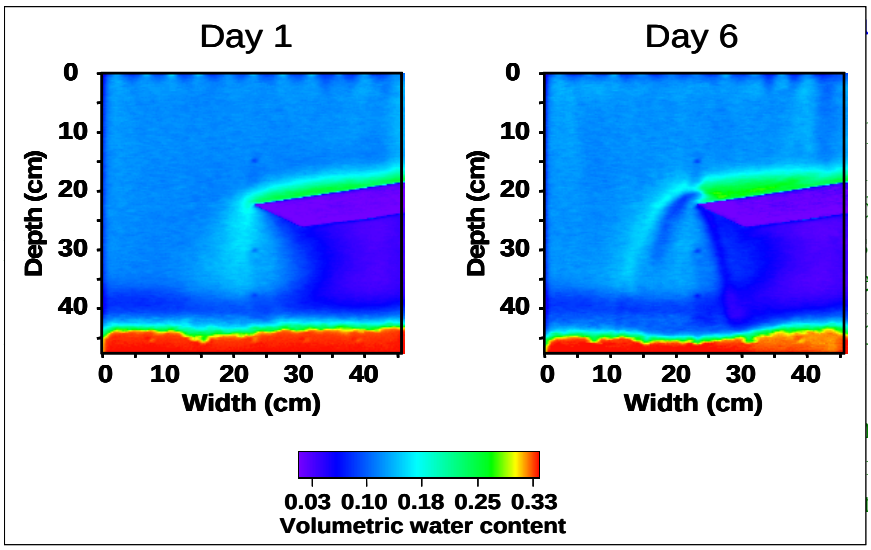
Microbial inoculation occurred at the inlet seen at the tip of the wedge. An influent dripper unit at the top of the chamber allowed water to be applied uniformly at a rate of 7.4 L/h through 10 of the 11 drippers (light-colored spots at top, Figure 6), with the right-most dripper secreting glucose solution (dark region in top right, Figure 6) at the same rate. Microbial colonization and water content were monitored over a period of 6 days via *lux* luminescence and light transmission, respectively.

Results

Microbes preferentially colonized the top tip of the wedge, moving against flow from their original inoculation point. Figures 7 and 8 demonstrate observed emission intensity, a function of microbial density, on the first and sixth days. While the colonization center appears proximal to the inoculation point on the first day, over time the center spread out to encompass a wider region around the tip of the wedge. By the sixth day, colonization was greatest (red region) in front of and on top of the wedge tip, and emission intensity increased thirty-fold. This concentration of microbes appears to have caused ponding above the textural interface between the fine-grained matrix sand and the coarser inclusion sand. Figure 9 shows the increase in volumetric water content on the top of the wedge (green region) between the first and sixth days. The microbial colonies are visible as a slightly dewatered region (darker blue), a hydraulic phenomenon described above.


Day 1
Day 6
Depth (cm)
Width (cm)
0 10 20 30 40
50 438 825 1213 1600
1000 13250 25500 37750 50000
Emission Intensity


Day 1
Day 6
Depth (cm)
Width (cm)
18 19 20 21 22 23 24 25
21 22 23 24 25 26 27 28
50 438 825 1213 1600
1000 13250 25500 37750 50000
Emission Intensity


Day 1
Day 6
Depth (cm)
Width (cm)
0 10 20 30 40
0.03 0.10 0.18 0.25 0.33
Volumetric water content

## II. One-dimensional studies of microbial growth and colonization under "static" unsaturated conditions.

The above studies mimic unsaturated flow conditions in high recharge and/or near-surface vadose zones where large water fluxes associated with storm events and high annual precipitation events cause large inputs of water. In arid climates, or in high precipitation climates where porous media experiences flow bypass due to stratigraphic or geological features, annual recharge can be less than one millimeter or one centimeter. In addition, care must be taken during aqueous-based delivery of nutrients in engineered bioremediation to minimize spreading of the contaminant plume. This means nutrients must be added slowly and in relatively small volumes.

The great majority of subsurface contaminants at DOE sites exist in deep vadose zones in arid portions of the US. Bioremediation has the potential to biodegrade or immobilize these contaminants, however microbial populations and activities are low in arid deep vadose zones and other vadose zones with low annual recharge relative to high recharge vadose zones and the capillary fringe. In addition, the distribution of microbes is very patchy at scales below 1-10 centimeters. For bioremediation to be successful in these environments, microbial populations must be increased by several orders of magnitude and must be able to colonize a large fraction of the pore spaces.

Given the above considerations, the objective of the one-dimensional studies reported in this section was to study microbial colonization under conditions with little hydrodynamic flux. Studies were designed to systematically examine how microbial colonization is controlled by water content and particle size.

Methods

Experiments were run with 4 different sand sizes, each at 4 different water contents, with a carbon source (see below)either present of absent, yielding a 4x4x2 experimental design. The following grades of accusand, a well sorted near-spherical quartz sand, were used: 20/30 (0.71 mm diameter), 30/40 (0.53 mm diameter), 40/50 (0.36 mm diameter) and 50/70 (0.21 mm diameter). Volumetric water contents (vWC) ranging from 5% to 20% were selected to provide for different degrees of pore-scale water connectivity and water film thicknesses. Select experiments were also run using a 12/20 accusand (1.10 mm diameter), and 1.3% vWC.

Columns were constructed by removing the tip end of a 10 ml sterile plastic syringe, adding 1.0 $cm^3$ oven-dry sand to the bottom of the column to form the inoculation zone, adding 2 $x10^7$ washed cells to the dry sand in the appropriate volume of mineral salts to achieve the desired water content, adding sand pre-wetted to the desired water content sand in small increments to the remainder of the column to form the colonization zone (7.7 $cm^3$), removing air voids as sand increments were added by applying minimal pressure with a packing implement, and sealing the syringe with a rubber stopper (Figure 10). Stocks of accusand were prepared by washing 3 times to remove fines. To provide for equal water content throughout the colonization zone, accusand was prepared for each experiment by mixing (15% and 20% vWC) or equilibrium centrifugation followed by mixing (5% and 10% vWC). The liquid used in the column experiments was minimal media containing nitrate, phosphate, and micronutrients. Experiments were run with

either acetate (3.5 mM) present in both the inoculation and colonization zones of the column, or absent in both zones. Each experiment used duplicate columns with 2 to 4 treatments.

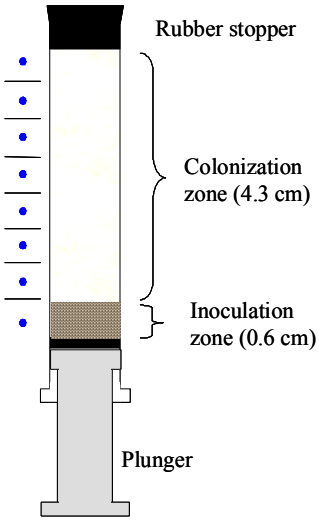



Figure 10. Experimental system for investigating microbial colonization in accusands.

The motile organism *Pseudomonas stutzeri* strain KC was used. It is the only known bacterium able to degrade carbon tetrachloride (CT) under denitrification conditions without production of chloroform, and was selected so colonization data could be used for future studies addressing CT degradation. A limited number of experiments used a second motile microbe, *Pseudomonas fluorescens* strain HK44. Cells were grown and prepared in exactly the same manner for each experiment, using the identical archived cell preparation as inoculum and with special attention to time intervals in the protocols, to minimize differences in cell biochemistry and behavior. Cells were grown in minimal media (identical to that used in the column experiments) to a density of between $6x10^8$ to $8x10^8$ per ml, washed twice to remove residual acetate, and quantitated by microscopy to enable the same number of cells to be added to the inoculation zone in each experiment.

Columns were stored at room temperature in racks in the position depicted in Figure 10 for various lengths of time, the porous media extruded in 1.1 $cm^3$ increments, and plate counts performed to determine the number of microbes in each section. In some experiments quantitative polymerase chain reaction (Q-PCR) was run to compare culture-independent and culture-based quantitation. Both methods gave nearly identical values at 10,15, and 20% vWC; culture-based counts were typically 3-5 fold lower than Q-PCR at 5% vWC. Culturing was used because the detection level is orders of magnitude lower than (less time-consuming) protein or chemical biomass measurements.

Additional experiments were run in larger columns with inoculation and colonization zones of 1.0 cm and 29.0 cm long, respectively, with a volume of 72 $cm^3$. Columns were constructed in flexible tygon tubing (15/16 and 11/16 inch o.d. and i.d. respectively) to enable facile sectioning of the column. Tubing was inserted into 1 inch i.d. PVC pipe for stability, and the annulus packed with 50/70 sand to prevent fracturing of the sand column within the tygon tubing during experimental setup. Sand was loaded into the

stabilized column in a manner similar to that described for the short columns, except the inoculation zone was added last. Columns were incubated in a horizontal position to minimize vertical water redistribution.

To enable modeling of microbial movement, independent experiments were conducted to determine microbial growth kinetics and the extent of water redistribution in the columns. Water redistribution was characterized in all 16 primary treatments (4 sands x 4 vWCs) and water content in the 1.1 $cm^3$ sections determined gravimetrically. The kinetics of cell growth and acetate utilization were determined in batch cultures to provide substrate utilization parameters for modeling microbial growth in the columns. Cells were cultured and processed in an identical fashion to the column experiments (as described above), diluted, and transferred to large volume culture flasks to ensure oxygen was maintained at non-limiting conditions. Acetate and biomass was monitored over time by high pressure liquid chromatography and plate counts.

Water redistribution, solute transport, gas diffusion, and bacterial colonization dynamics were simulated using a numerical finite-difference model. Solute and bacterial transport were modeled using advection-dispersion equations, with reaction rate source/sink terms to account for bacterial growth and substrate utilization, represented using dual Monod-type kinetics. Oxygen transport and diffusion was modeled accounting for equilibrium partitioning between the aqueous and gas phases. The movement of bacteria was modeled adopting the approach of Barton and Ford (1995) for saturated systems, which uses equations developed for modeling molecular diffusion to describe bacterial movement. The term *Dm* is a coefficient representing random motility. The term 'random' is used to distinguish this motility from chemotactic motility towards an attractant. For application to our unsaturated system, we modified this approach by using an equation for modeling molecular diffusion in unsaturated conditions (Olesen et al. 1996, *Soil Science*). Effective diffusion coefficients, *Deff*, were calculated for each experimental condition using the equation,

$$D_{eff} = D_m 0.45\theta\left(\frac{\theta - \theta_c}{\theta_s - \theta_c}\right)$$

where *Dm* is the random motility coefficient, $\theta$ is the volumetric water content, $\theta c$ is a critical water content for transition from connected to non-connected water films, and $\theta s$ is the saturated water content or porosity. The random motility coefficient was estimated by optimization to experimental data. The flow and transport equations used in this work are based on a continuum assumption. Hence the parameters in these equations represent effective properties at a macroscopic scale, rather than microscopic (e.g. pore or molecular) scales.

Results

*Non-biological (i.e., physical-chemical) movement of bacteria.* In initial experiments with 12/20 Accusand, substantial movement of bacteria occurred in 24 hours in 10%, 15%, and 20% volumetric water content (vWC) columns lacking acetate. The movement was typically half of the movement seen in experiments at 15% and 20% vWC in the presence of acetate. While some movement was expected as a result of bacterial motility using endogenous energy reserves, the amount of movement was greater than expected.

To understand this phenomena repeat experiments were run for 12, 6, 3, and 1 hours. Surprisingly, the 24 hour and 3 hour columns were very similar. Experiments were then repeated at 60 min, 30 min, and 10 minutes with the same results: nearly all of the bacterial movement observed at 24 hours occurred in the first 10 minutes. To understand this phenomena, fluorescent microspheres and bacteria were added together to columns run for 10 minutes; both microspheres and bacteria moved to the same extent. Experiments with bromide and bacteria showed the solute and the microbes moved to a similar degree.

It was visually noted that, even in the 10 minute experiments, there was more water present in the bottom of the column than in the top of the column. To determine if there was a temporal correlation between macroscopic drainage and the rapid movement of bacteria, 10 minute and 24 hour experiments were repeated without bacteria and the distribution of water determined by oven drying of the column sections. Both 12/20 and 50/70 sands were used. The results showed that water drainage came to equilibrium in both sands by 10 minutes, indicating a temporal correlation between drainage and rapid bacterial movement.

To investigate if there might be other causes for the rapid movement of bacteria, we tested whether the emplacement of the colonization zone on top of the inoculation zone was also contributing to movement of bacteria. Half-cell experiments were conducted where the inoculation zone was loaded into one end of a syringe and the colonization zone was loaded into the end of another syringe, and the two syringes were clamped together to form the column. This experiment was conducted in 12/20 and 40/50 Accusands at 15% and 20% vWC. There was no significance difference between half-cell experiments and the standard protocol, indicating the colonization zone emplacement method was not causing the movement.

To test whether extrusion of the sand during sectioning was contributing to movement of bacteria, we used a heated razor to quickly slice/melt through the plastic and section the column. This method did not did not change the resulting bacterial distribution, indicating the sand extrusion method was not significantly contributing to bacterial movement.

Drainage would not be expected to transport large numbers of bacteria centimeters up the column because draining water moves to the bottom of the column. Since we were unable to identify any artifact produced by our experimental protocols, we hypothesize that a Marangoni-like effect (Adamson and Gast, 1997) characterized by microscopic hydrological or chemical turbulence is occurring at the gas-liquid interfaces of the water films, and this convective process is responsible for the rapid spreading and rafting of bacteria along these surfaces. The fact that there is a temporal correlation between drainage and rapid bacterial movement suggests the former causes microscopic turbulence that is responsible for the rapid initial movement of bacteria.

*Microbial colonization via motility.* To minimize the above problems the 12/20 sand was not used in subsequent experiments. Experiments were run comparing bacterial movement at 10 minutes (i.e., capturing the physical/chemical process) and 24 hours, for each sand-vWC combination. Differences in bacterial movement at the time points were indistinguishable for sands at 5% and 10% vWC. At 15% and 20% vWC the profiles showed movement was greater at 24 hours than at 10 minutes, providing evidence for

motility-assisted bacterial colonization. At a given volumetric water content, bacteria traveled further with increasing sand size (Figure 11). Although the bacteria moved slightly further at 10% than at 5% vWC, there was no significant difference in bacterial movement as a function of sand size at either vWC. The profiles in Figure 11 represent both the physical/chemical and biological motility processes, however, modeling efforts (below) used the 10 minute profile as the t=0 distribution of bacteria.

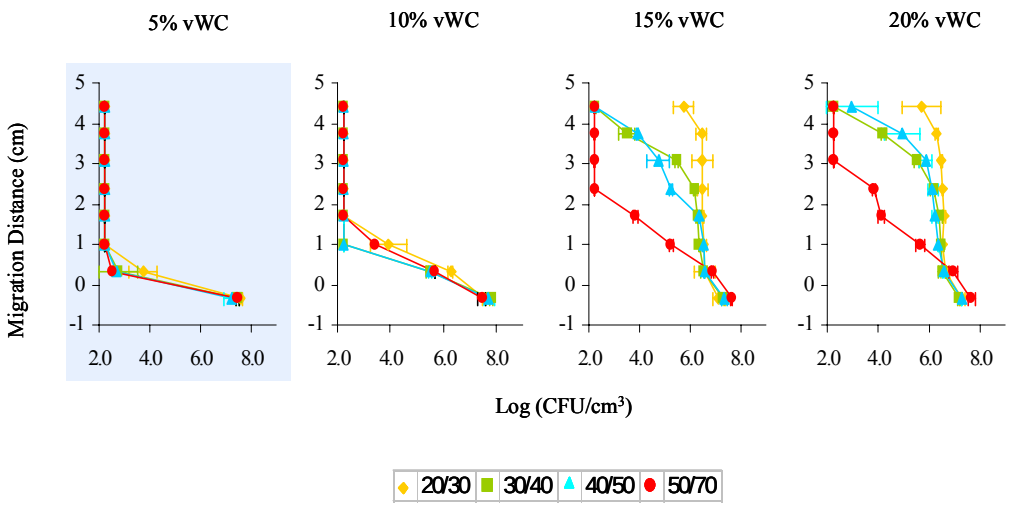



Figure 11. Effect of sand size and vWC on bacterial movement after 24 hr in the absence of acetate as carbon source. Each panel shows bacterial profiles for the 4 sands. 20/30 is the coarsest sand, 50/70 the finest sand.

In the presence of acetate in the columns, at a given sand size the bacteria traveled further with increasing volumetric water content (Figure 12). After 24 hours, bacteria were present at high density throughout the 4 cm long column at the higher volumetric water contents and larger sand sizes. When looking at a given vWC across the 4 panels of Figure 12, there was little difference in the colonization profiles at 5% vWC, and the same was true at 10% vWC. In contrast, the colonization profiles were shortened at 15% and 20% vWC in the finer sands. Bacterial movement was not detected at 5% volumetric water content in the two smallest sands. Colonization in the presence of acetate was also not detected in 12/20 sand at 1.3% vWC after 24 hours or in much longer 12 day experiments (data not shown).

To determine if colonization would continue in the columns over longer periods of time, select experiments were conducted in 30-cm long columns and run for 7 days. Rates of acetate-driven colonization observed in 24 hr experiments under the same sand and vWC conditions (Fig. 12) were maintained over a 7 day period (Figure 13).

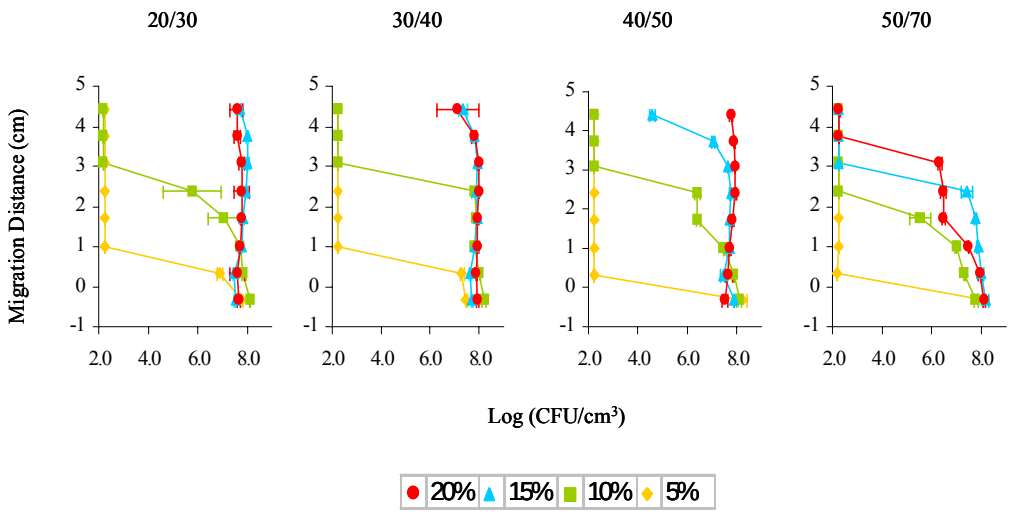



Figure 12. Effect of vWC and sand size on colonization after 24 hr in the presence of acetate as carbon source. Each panel shows CFU bacterial profiles for the 4 vWC's. 20/30 is the coarsest sand, 50/70 the finest sand.

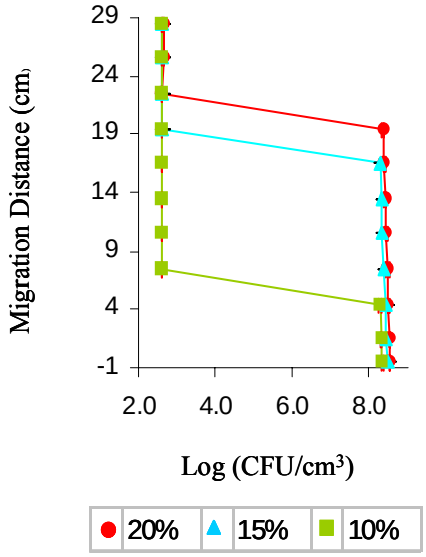



Figure 13. Bacterial profiles in 40/50 sand at 3 different vWCs after 7 days. Note the columns are 6 times longer than in Figures 11 and 12.

To determine the extent that microbial colonization would be impaired by a poorly-sorted non-spherical sand, short and long columns were run with sand collected from the Hanford site. The average particle size of the Hanford sand was similar to the 30/40

Accusand, but it has a broad particle size range, was much less spherical when viewed under a microscope, and had some secondary mineral coatings. The Hanford sand was 3-6% very coarse sand (>1.0 mm), 29-32% coarse sand (0.5 to 10.0 mm), 52-55% medium sand (0.25 to 0.50 mm), 8-10% fine sand (0.125-0.25 mm), and 1-3% <0.125 mm. The colonization profile in a 24 hour experiment at 20% vWC was intermediate to the 5% and 10% vWC profiles in the 30/40 Accusand (Figure 14; compare to Figure 12). In a separate experiment in a 30-cm long column, the rates of acetate-driven colonization over a 7 day period was greater than that observed after 24 hrs. Colonization profiles at 7 days were similar to those observed in the 40/50 Accusand (right panel in Figure 14; compare to Figure 13). Although the porosity was higher in the repacked sand column as compared to the in situ sediment, the data indicate substantial rates of colonization can occur in relatively coarse-grained "non-ideal" vadose zone porous media.

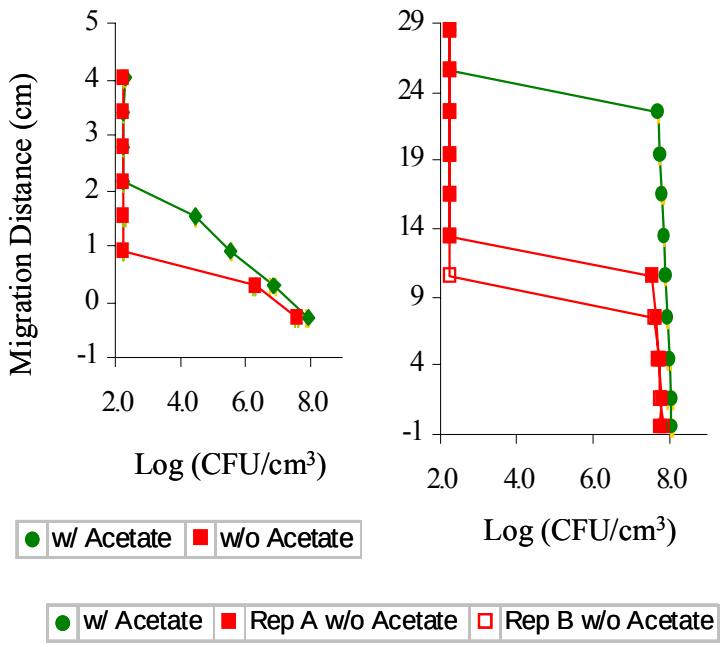



Figure 14. Effect of acetate on microbial distribution in a poorly-sorted non-spherical sand at 20% vWC. Panel A, 24 hr; panel B, 7 days.

*Water content at which bacteria would not be expected to move.* The theoretical water content at which bacteria would not be expected to move from pore to pore in our experiments was estimated by calculating the critical water content, $\theta c$, at which water films in pores transition from a funicular (connected) to a pendular (disconnected) state. This transition defines where microbial movement is not possible because microbes can not move in porous media by either flagella-mediated swimming or pili-mediated crawling in the absence of water films. The $\theta c$ was calculated from the geometries and volumes of pendular rings in porous media consisting of uniform, spherical particles (similar to the uniform grain size, highly spherical quartz sands we used) for two different types of packing: an offset close pack and cubic pack. The porosity of our packed columns was 0.426, which is in between the theoretical values for the offset close pack (porosity of 0.260, $\theta c$ of 0.063) and the cubic pack (porosity of 0.476, $\theta c$ of 0.087)

and lies close to the values of the cubic pack. By interpolation of the critical water contents determined for the two ideal packings, the $\theta c$ for our experiments was determined to be 0.081 or 8.1% vWC. Thus the 10% vWC columns would contain (mostly) connected water while the 5% vWC columns would be entirely disconnected. Our colonization profiles match the theory (see Figure 12).

*Microbial kinetics*. Substrate utilization and growth was measured as a function of time (Figure 15). This relationship, in combination with the biomass (colonization) profiles in the columns and other components of the model as described in the methods, enabled the random motility coefficients to be calculated.

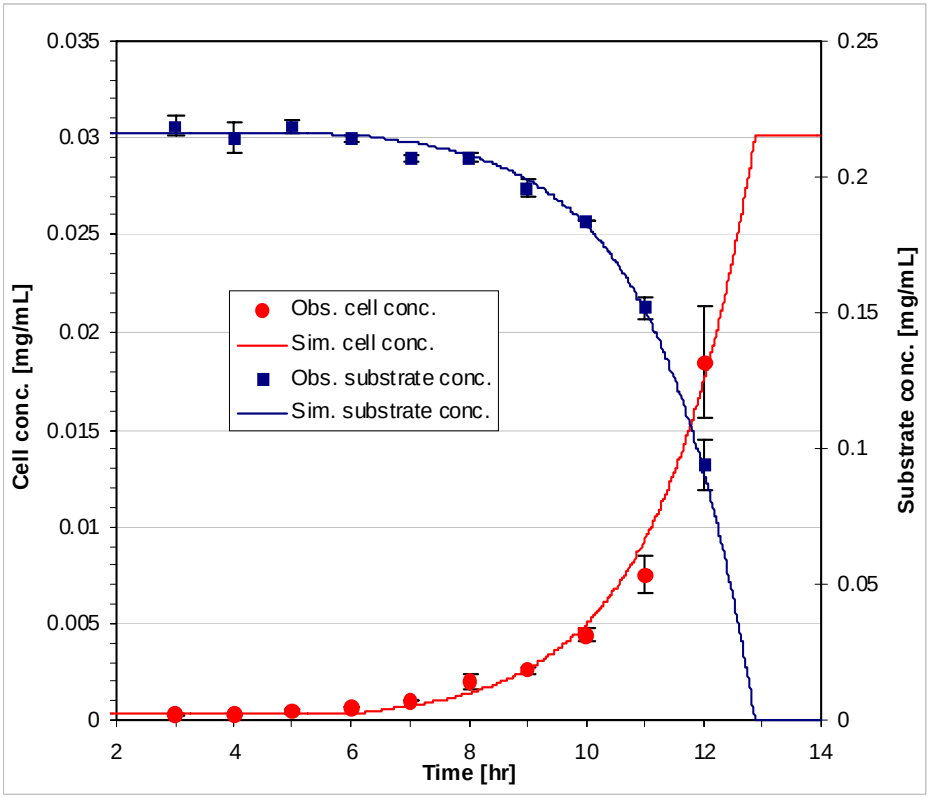



Figure 15. Acetate consumption and biomass production of *Pseudomonas stutzeri* strain KC in batch culture.

*Simulation.* The 10 minute curve was used as the initial distribution to account for the initial rapid physical-chemical movement of bacteria, and the model was used to simulate the growth of bacteria while fitting the observed points to determine the random motility coefficient. Simulations were run for 32 data sets: 4 sands at 4 vWC's with and without acetate. An example of the actual and simulated data is shown in Figure 16.

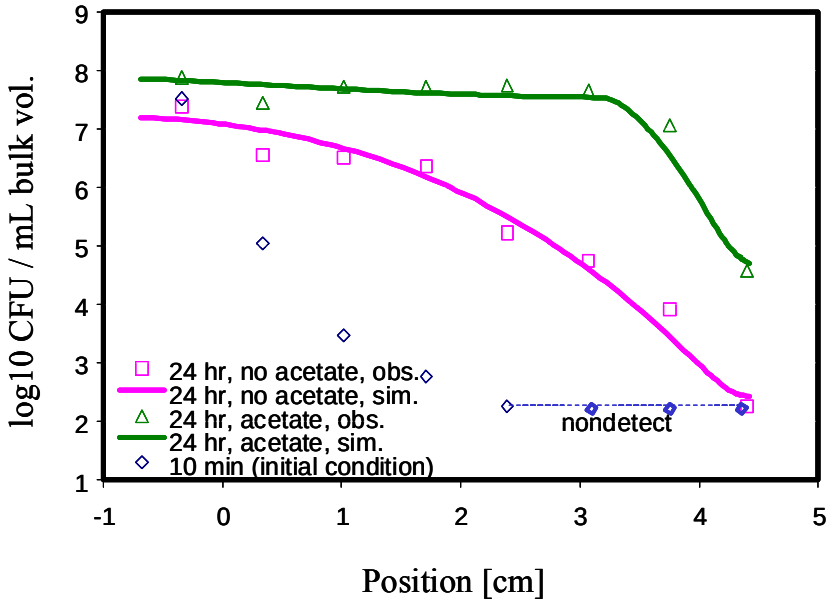



Figure 16. Example of observed and simulated bacterial distributions in the *presence* of acetate in 40/50 sand at 15% vWC.

Random motility coefficients are plotted in Figures 17 and 18. Values are not shown for cases where bacterial profiles were flat or pore water became unconnected; in both cases the model is unable to solve the equations. Decreasing values at smaller grain diameters are due to decreasing minimum water film thickness in the connected pore water, which restrict bacterial motility. The random motility coefficients (0.005 to 0.1 $cm^2$/hr, equal to 1.4 x $10^{-6}$ to 2. 8 x $10^{-5}$ $cm^2$/sec) are in the same range as those found for saturated systems (3.5 x $10^{-6}$ to 3.5 x $10^{-5}$ $cm^2$/sec). This is a surprising result given the presence of air in the pore spaces, which results in a longer path length for bacteria to travel. Instead of being slowed by the longer path in water films, it appears that the water films enhance and 'direct' bacteria as compared to a saturated pore space.
Some researchers have observed that air-water interfaces attract and 'trap' bacteria via surface tension effects; it is also likely that oxygen limitation in the experiment played a significant role in causing these results. Both modeling and analytical results (i.e., a decrease in nitrate concentration indicative of nitrate being used as an electron acceptor) showed oxygen limitation was not a significant issue in our experiments. Our results indicate that *Pseudomonas stutzeri* KC and *Pseudomonas fluorescens* HK44, in our experimental system (accusands lacking organic and secondary precipitate coatings), did not significantly partition to air-water interfaces. Some trapping may have occurred, however, a substantial fraction of the cells were able to avoid air-water interfaces through a passive or active mechanism.

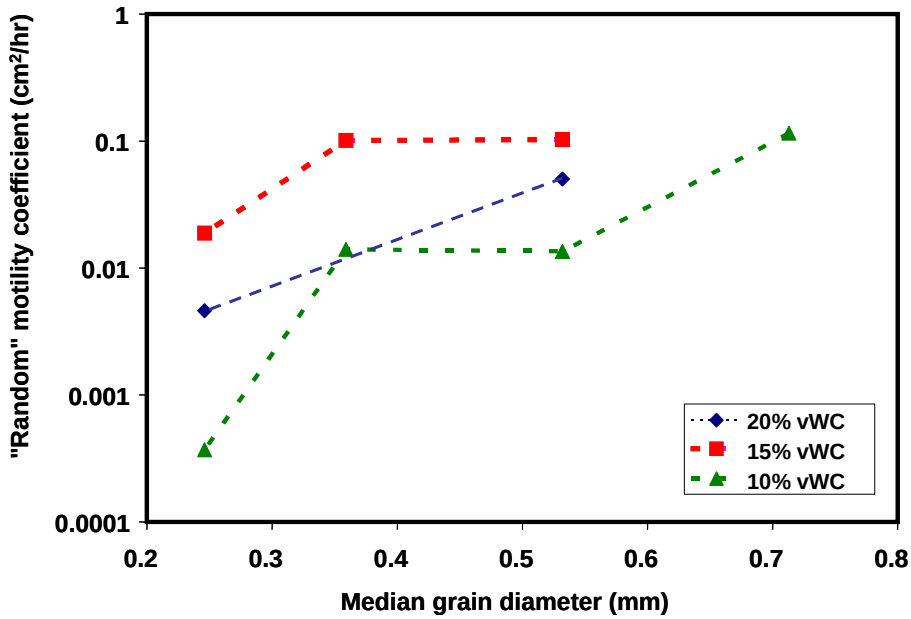



Figure 17. Random motility coefficients as a function of vWC and sand size in the *presence* of acetate.

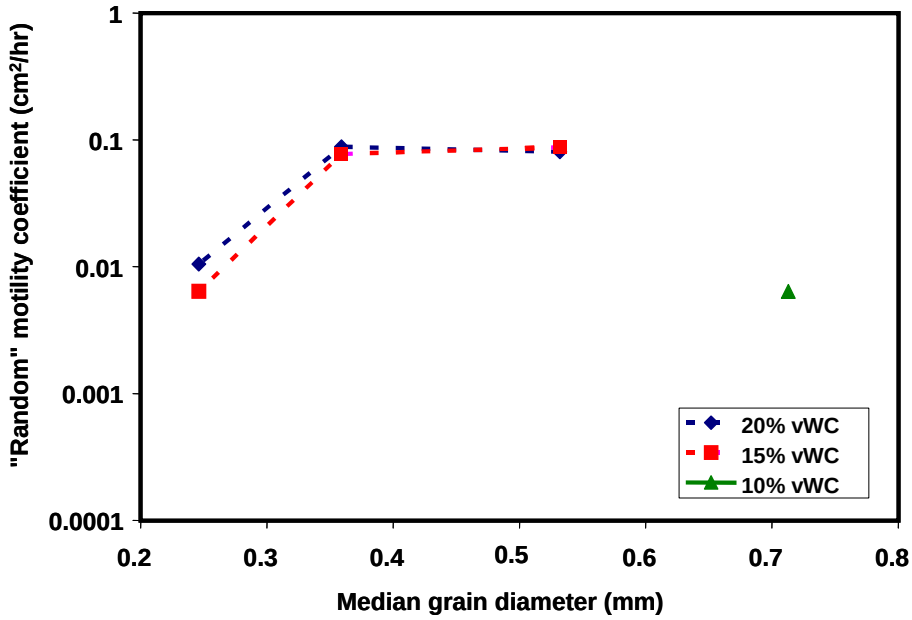



Figure 18. Random motility coefficients as a function of vWC and sand size in the *absence* of acetate.

Conclusions.

• Two mechanisms of movement were found in the columns. In the absence of an energy source, cells moved several centimeters in less than 10 minutes via a chemical-physical process. In the presence of an energy source throughout the column, this initial

movement was followed by a motility-driven colonization that proceeded at rates of approximately 10 micrometers per minute. Both mechanisms of movement were controlled by volumetric water content and size of the sand grains.

- This is the first time random motility coefficients of microbial movement have been defined for unsaturated porous media. The random motility coefficients we obtained are in the same range as those found for saturated systems. Given the longer travel paths in unsaturated versus saturated porous media, this observation indicates that water films apparently 'direct' bacteria and a substantial fraction of the cells avoid being trapped at air-water interfaces.
- The results, in a system without advective flow, show that motile bacteria were able to rapidly migrate in response to nutrient addition in well-sorted unsaturated sands of relatively high porosity, at volumetric water contents that occur in many sandy vadose zones. Migration would be expected to be decrease in sediments of lower porosity and in the presence of organic and mineral coatings. Migration greater than observed here would be expected in vadose zone systems where flow occurs in fractures.
- Addition of soluble nutrients to vadose zones with patchy and low density microbial populations appears to be an effective technology for driving microbial colonization in the field. Aqueous-based nutrient delivery systems are potentially acceptable technologies at DOE sites; thus, results of these laboratory experiments have potential application to vadose zone bioremediation.

## Objective 2. Characterize microbiological properties of a carbon tetrachloride-contaminated deep vadose zone site at the DOE Hanford Site.

Characterization was performed at two field sites: the DOE Hanford Site in Washington state and a hydrologically similar site at the Amargosa Desert Research Site (ADRS) in Nevada. The latter effort was an interaction with the U.S. Geological Survey Toxics Program, studying the microbiology in a borehole near a shallow low-level radioactive and mixed waste burial site.

### I. Hanford Site 216-Z-9 Trench.

Site description. The 216-Z-9 Trench is located in the 200 West area of the Hanford Site. It is an engineered waste disposal facility designed to discharge liquid wastes to the soil column and has an active floor area of 9.1 x 18.3 m. From July 1955 to June 1962 the Z-9 Trench received the contaminant stream from the Recuplex facility and the Plutonium Reclamation Facility (234-5 Z Plant) that succeeded it. The Z-9 Trench received a total of 4.09 million liters of discharged liquids (Johnson 1993) from the Recuplex process, a solvent extraction system that recovered plutonium from many types of scrap or wastes such as casting skulls, slag and crucible, button line supernates, and fabrication oil (a mixture of lard oil and 75% carbon tetrachloride). The process used nitric and hydrofluoric acids, and carbon tetrachloride as an organic diluent for the plutonium extractants tributyl phosphate dibutyl butyl phosphonate. Most of the organic contaminants at the Z-9 Trench were disposed as radiologically contaminated spent solvent mixtures, consisting of either 85:15 volume percent CT to tributyl phosphate or

50:50 volume percent CT to dibutyl butyl phosphonate. The organic solvent stream was usually recycled, but periodically the solvents were disposed of and discharged to the ground. Contaminant inventories disposed to the Z-9 Trench included approximately 83,000 L (21,912 gal) to 300,000 L (79,200 gal) of CT; 27,900 L (7,366 gal) of tributyl phosphate; 46,500 L (12,276 gal) of dibutyl butyl phosphonate; 9,300 L (2,455 gal) of lard oil; and 106 kg of Plutonium and 2.5 kg of Americium (Johnson 1993). Large quantities of primarily non-organic wastes – metal nitrate salts, plutonium-contaminated wastewater, acidic solutions, and dilute solutions of the target nonaqueous liquids and other radionuclides – were also discharged to the Z-9 Trench.

CT and nitrate migrated to groundwater and the residual solvent in the soil column beneath the Z-9 Trench is suspected to be continuing source of groundwater contamination. A small portion of this contaminant (~ 2%, according to Rohay et al. [1994]), is present in the groundwater, but even this small percentage has resulted in a CT groundwater plume that covers 10 $km^2$, with maximum concentrations of 4000-7000 μg/L. The zone of highest CT concentration in the groundwater is still located under the 216-Z-9 Trench even though it has been inactive since 1962, indicating that CT in the vadose zone is likely a continuing source for groundwater contamination.

Results from vadose zone gas surveys in the vicinity of the 216-Z-9 Trench in fiscal year 1992 identified the presence of carbon tetrachloride, chloroform, trichloroethylene, and tetrachloroethylene. In response to the need for control of residual contamination sources in the vadose zone, a soil vapor extraction (SVE) system was installed in the vicinity of the Z-9 Trench and began operation in 1993. During the operating period from 1993 through September 1999, the carbon tetrachloride concentration in soil vapor at the Z-9 Trench SVE system declined from 30,000 parts per million by volume (ppmv) to approximately 25 ppmv. An estimated 53,000 kg of carbon tetrachloride were removed from the Z-9 Trench SVE well field during this time period (Rohay 2000).

Concentrations of contaminants rebound with time after SVE halts. Thus, the Z-9 Trench area remained a high priority for Hanford site cleanup and was the focus of a DOE/EPA Innovative Treatment Remediation Demonstration (ITRD) Program effort to evaluate suitable technologies to speed up the remediation of the CT contamination in both the vadose zone and the groundwater. At the time the proposal for this EMSP project was written (spring 1999) it was expected that drilling activities would occur in early 2000 (M. Hightower, ITRD Technical Coordinator, personal communication, March, 1999). Drilling was delayed for over a year until May and June of 2001, when two 100-foot-deep existing boreholes adjacent to the Z-9 trench site were extended to a depth of 195 to 200 ft depth. The boreholes were 299-W15-95 (6 ft north of the trench) and 299-W15-84 (30 feet west of the trench). These holes were deepened to enable a potential partition interwell tracer test and to install screened zones in the region containing the highest CT for subsequent soil vapor extraction.

Vadose zone sediments at the field site are about 200-210 ft thick at the 216-Z-9 trench. Three geologic strata exist in the region of the vadose zone that was cored in 2001. The upper strata is the bottom of the Hanford Formation, composed of sand and silty sand derived from catastrophic late Pleistocene floods. The middle strata is the low permeability Plio-Pleistocene unit, composed of fine-grained paleosols (ancient soils) and containing a zone which is indurated with calcium carbonate deposits. The lower strata is Ringold Unit E riverbank deposits that are composed of sands and gravel.

Sample acquisition. The primary sampling targets for microbiology were in the Plio-Pleistocene unit and immediately above and below the unit, because this was the zone expected to have the highest levels of CT, moisture, and microbial activity. Secondary sampling targets for microbiology in the Ringold formation were at approximately 20 ft. Because of uncertainties regarding amount of core recovery, drilling was performed as if all cores were microbiological cores. Specialized procedures were taken to minimize the potential for microbial contaminants to be introduced into the sediments during coring and handling in the field. These included steam-sterilized core barrels, autoclaved 4-inch-diameter and 6-inch-long stainless steel liners (4 per barrel), and autoclaved end caps and drive-shoe assemblies. Sterilized components were unwrapped and assembled immediately prior to going downhole by a geologist familiar with aseptic microbiological sampling methods, and a sterile bag was taped to the open end of the core for the downhole trip.

Microbiological analysis had to be conducted in a specialized radiological laboratory because (a) analysis of the potential presence of plutonium and other transuranics in the cores by other parties was not completed until many months after core retrieval, and (b) rapid (within one week) radiochemical analysis for these elements would have been borne by this project and was cost-prohibitive. Cores were opened and handled in a hood using aseptic procedures and sterile gloves and implements. Sediments from the top 1 to 2 inches were aseptically removed and discarded. Sediment from the outer 1 inch of core was avoided, and only material (minus solids >2 mm) from the center portion of each core was homogenized.

This EMSP project paid for 3 weeks of labor at the borehole (core barrel preparation and recovery of microbiology samples). In addition, substantial costs were incurred because the sediment was a F-001 listed waste and all sediment, and all containers and solutions that the sediment came into contact with in the laboratory, had to be disposed of as mixed organic-radioactive waste.

A total of 24 samples were analyzed for microbiological properties and moisture content.

Methods

*Estimate of heterotrophic population.* Past studies in the deep vadose and saturated zones at the Hanford Site have shown that heterotrophic plate counts and liquid enrichments that rely on visible growth are poor indicators of the number of viable microbes. Phospholipid fatty acid analysis is the preferred method to determine viable biomass but was problematic due to the F-001 listing. As denitrifiers typically represent well over 10% of the total population, and would be expected to a larger fraction of the total population in nitrate-contaminated sediments, a good proxy to estimate viable heterotrophs was determined to be a sensitive colorimetric denitrification assay. The assay was setup in a most-probable-number (MPN) format consisting of 3 tubes at each of 3 serial 10-fold dilutions, and allowed to incubate at room temperature for 6 weeks. The assay was initiated with 10 grams of sediment. The medium was 10% Difco R2A plus 0.05% $KNO_3$. Disappearance of nitrate and/or appearance of nitrite were scored as positive and the population determined using a MPN table.

*Heterotrophic activity.* Potential heterotrophic microbial activity was determined by measuring the time-course evolution of $^{14}C\text{-}CO_2$ after addition of spiked substrates. mineralization of assayed by adding a mixture solution prepared from was used as carbon substrates. One ml of a solution containing [1,2-$^{14}$C] sodium acetate (specific activity 57.0 mCi/mmole, Amersham Pharmacia Biotech, England) and D-UL-$^{14}$C glucose (specific activity 248 mCi/mmole, Sigma, St. Louis, MO) was evenly distributed over 10 g sediment inside a 160 ml milk dilution bottle to preserve unsaturated conditions during the incubation. The substrate mixture was amended with non-labeled compounds to give a final concentration of 3 µmole acetate/ml (0.25 µCi/ml) and 1 µmole glucose/ml (0.25 µCi/ml). Evolved $^{14}CO_2$ was trapped in 1 ml of 0.3 M KOH hung inside the bottle. Mineralization rates were calculated by regression of the linear portion of the $^{14}CO_2$ evolution curves. Assays were performed in triplicate and included sterile controls containing sediments autoclaved on three consecutive days.

*Water content.* Gravimetric water content was measured by immediately transferring sediment from the center of the core to sealed tins and weighing before and after oven drying for 2 days at 60 degrees Centigrade.

Results

The PPU was at 108-121 feet in hole 1 and above 110 to 130.5 feet in hole 2. The Hanford formation was present in hole 1 above 108 feet but was encountered in the top of hole 2 (where the borehole extension began at 100 feet). The PPU is thicker in hle 2 because some of the unit was removed by erosion during Pleistocene floods at hole 1.

Contaminant levels were measured by Bechtel Hanford Incorporated (Rohay et al., 2002). Detection of CT and chloroform in sediment samples was rare, and maximum values were 9 µg/kg and 5 µg/kg respectively. All positives were immediately above the PPU in hole 1 (102 and 107 ft) and in the middle of the PPU in hole 2 (119 and 121 ft). These levels were three orders of magnitude lower than in 1992 before SVE occurred. CT was detected in 12 of 13 sediment gas samples was highest at the bottom of the PPU in hole 1 (174 and 95 ppmv at 122 and 114 ft respectively) and in the middle of the PPU in hole 2 (519 and 539 ppmv at 109 and 120 ft respectively) (Figure 19). The next highest CT concentrations were 21 and 38 ppmv at 184 and 181 ft in holes 1 and 2 respectively. These concentrations were two orders of magnitude lower than in the initial SVE operations in 1993. Chloroform was detected in 10 of 13 sediment gas samples at 1-5 ppmv. Other volatile and semivolatile polyaromatic hydrocarbons and chlorinated compounds (89 compounds) and tributyl phosphate (detection level of about 400 µg/kg) were not detected in any of the sediment samples. Analysis for "oil and grease" by gravimetric determination of hexane-extractable materials was used to potentially indicate presence of lard oil and breakdown products of tributyl phosphate and dibutyl butyl phosphonate, but results were inconclusive because they did not match the pattern of other contamination and naturally occurring organic and (potentially) inorganic material may be extracted with the method. Target radionuclides (americium-241, plutonium-238, plutonium-239/240, cobalt-60, antimony-125, and strontium-90) were not present in the core samples.

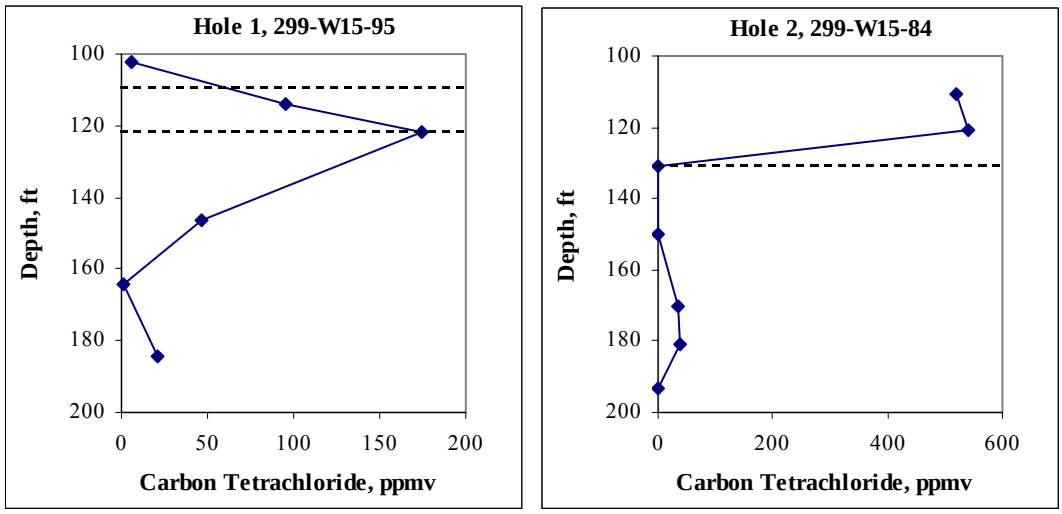



Figure 19. CT concentrations in downhole soil gas samples taken during drilling.

Moisture content within the upper portion of the fine-textured Plio-Pleistoecene unit (PPU) was 15-21% and higher than in the bottom of the PPU (2-4%) and underlying Ringold (2-7%) (Figure 20, top panels). The highest moisture content was observed just above the carbonate cemented zone of the PPU which begins at 121 and 113 ft in holes 1 and 2 respectively. The moisture data shows that water from disposed liquids reached the PPU.

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Populations of heterotrophic denitrifiers ranged from >log3.3 in 5 samples to <log0.3 in 7 samples (Figure 20, center panels). High levels of denitrifiers correlated well with the zone of high moisture. The high denitrifier value at 142 ft in hole 2 is likely due to contamination as this sample was of the poorest quality; mixing of sediment from the outside of the core into the center of the core used for microbiological analysis was observed during opening of the core.

Potential heterotrophic microbial activity was generally highest in the upper part of the PPU (Figure 20, bottom panels). This pattern was very evident in hole 1 and present but less evident in hole 2. The pattern of potential heterotrophic microbial activity correlated well with the patterns seen in moisture content and denitrifier population estimates. After a lag time, mineralization showed a linear pattern over the first week. The lag times were from 1-3 days. The lag times and amount of mineralization are consistent with earlier results (Fredrickson et al 1993; Kieft et al 1993) from boreholes slightly further from the Z-9 trench than the boreholes studied here. The results from these earlier mineralization studies and other studies of anthropogenic moisture-impacted (Brockman et al. 1992) and native moisture recharge (Brockman et al. 1997) deep vadose zone sediments at Hanford indicate that the viable microbial populations in the high moisture zone within the PPU are on the order of $10^6$ cells/gram and $10^4$ cells/gram in the Ringold sediments.

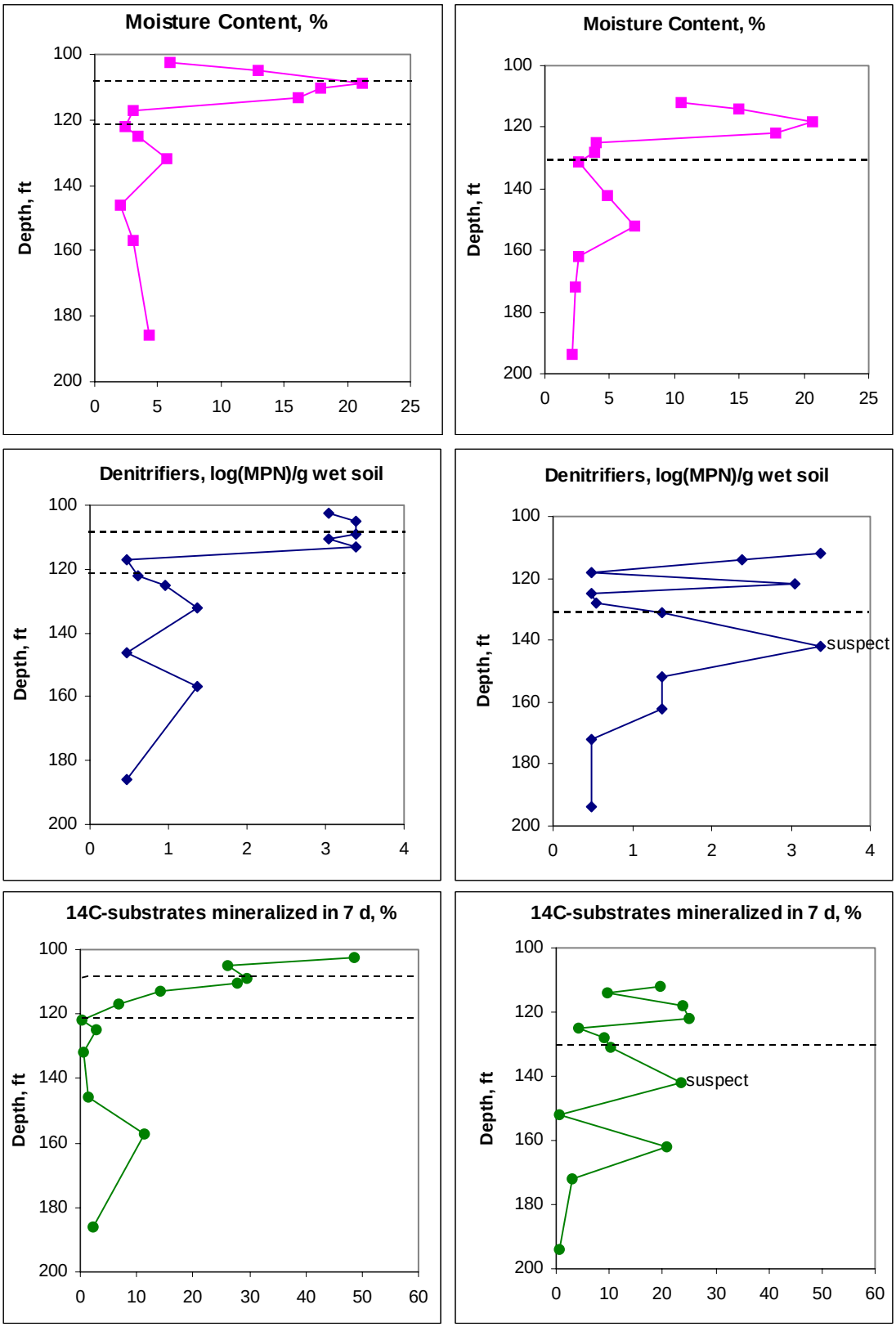


Figure 20. Moisture, denitrifying microbial population, and microbial activity profiles in hole 1 (299-W15-95, left panels) and hole 2 (299-W15-84, right panels).

Conclusions.

- The microbiological analysis reported here represents 6 times more samples than the sum from the previous investigations and provides a detailed picture of microbial populations and activities and their relationship to the distribution of water content and CT.
- The highest concentrations of CT are associated with the top and middle portions of the PPU. The upper portion of the PPU has the highest moisture content and highest microbial populations. Thus the highest microbial populations and the highest moisture and CT and roughly co-located.
- The ability of microbial populations in these same sediment samples to utilize gaseous hydrocarbons and potentially transform CT under unsaturated conditions was also tested; see objective 3 below.

## II. Amargosa Desert Research Site

Site description. The Amargosa Desert Research Site (ADRS) is located 17 km south of Beatty, Nevada and 20 km east of Death Valley National Park, and is part of the Mohave Desert province. The ADRS is established at a shallow low-level radioactive and mixed waste burial site. Waste materials were put in the bottom of a trench and covered with previously excavated soil. Annual precipitation is 10.8 cm per year but no recharge occurs below several meters in undisturbed desert at this location (Stonestrom et al. 2003). Vadose zone moisture content ranges from 5-15% in the 360 ft vadose zone at the site (Prudic et al. 1997). The U. S. Geological Survey has been researching the site, and a large amount of hydrologic and chemical data exists for the site. Tritium and $^{14}C$-$CO_2$ has been observed in sediments several hundred yards from the trench site (Prudic et al. 1977; D. Stonestrom, personal communication). Because no microbiology data existed for the site the USGS asked for our participation in determining if the $^{14}C$-$CO_2$ several hundred yards from the trench site was the result of vapor phase transport of $^{14}C$-contaminants and microbial degradation at the location of the borehole, or whether $^{14}C$-$CO_2$ was being produced in or below the trench and being transported to the boreholes located several hundred yards from the trench.

Sample acquisition. Borehole UZB-3 was drilled in December 1999. No procedures were used to prevent introduction of microbial contaminants. A segment (6 inches long by 4 inches wide) of each core was shipped to PNNL in early August 2000. Cores were opened inside a sterile laminar flow hood. Sterile implements were used to remove approximately 1-2 cm of material from all exposed areas of the core. A second set of sterile implements was used to remove another 1-2 cm of material to expose undisturbed (hopefully uncontaminated) sample from the inner portion of the core. A third set of sterile implements was used to remove 100 g of sediment for microbiological analysis and the sediment was homogenized.

Methods.

*Heterotrophic plate counts.* Homogenized sediment (10 g) was added to 95 ml 0.1% Na-pyrophosphate and processed in a Waring blendor for 1 minute. Serial dilutions of log1, 3, and 5 plated in duplicate to 10% R2A medium. In addition, aliquots of sediment

that were briefly air dried were crushed and disaggregated using a sterile implement and duplicate 0.1 and 1.0 gram aliquots of sediment were sprinkled directly onto plates of the same medium. Blank control plates spread with and without sterile diluent were also incubated to ensure sterility. Plates were incubated at room temperature for 3 weeks.

*Water content.* Gravimetric water content was measured by immediately transferring sediment from the center of the core to sealed tins and weighing before and after oven drying for 2 days at 60 degrees Centigrade.

Results

Gravimetric water content in samples 1-10 ranged from 5-12%, with a value of 18% for sample 13. Sample 1-C (7 feet) contained many cuttings and was probably contaminated with material from higher in the profile. Plate count populations in samples between 18 and 188 feet ranged from 20 to 905 CFU/gram sediment (Table 1). Samples 2 feet above and 6 feet below the water table had the highest populations.

Table 1. Heterotrophic plate counts in the ADRS UZB-3 borehole samples.

| Sample | Depth (feet) | Log colony forming units/g sediment |
|---|---|---|
| 1-C | 7 | 5.24 |
| 2-B | 18 | 1.74 |
| 3-B | 38 | 2.47 |
| 4-B | 58 | 1.30 |
| 5-B | 78 | 2.82 |
| 6-B | 118 | 2.73 |
| 7-B | 158 | 2.57 |
| 8-B | 188 | 2.96 |
| 9-B | 238 | 3.49 |
| 10-B | 288 | 4.29 |
| 13-B | 359 | 5.50 |
| 14-B | 367 | 6.02 |

Both the ADRS and native recharge studies at the Hanford Site have similar annual precipitation and average annual recharge. The heterotrophic plate counts for samples shallower than 200-250 feet are similar to one order of magnitude higher than non-paleosol (non ancient soil horizons) native recharge samples from the Hanford deep vadose zone. Due to the nature of the coring, it is impossible to know the fraction of cultured microbes that represent indigenous versus introduced microbes. It is probably that some or many of the microbes represent contamination introduced during drilling and coring. Far-field migration of tritium and/or $^{14}C$-$CO_2$ at the ADRS occurs in coarse-grained high permeability zones shallower than 65 feet (D. Stonestrom, personal communication). The data shows these zones contain the lowest water contents and are associated with the lowest number of culturable microbes. If $^{14}C$-contaminants were being transported to the borehole through high permeability horizontal strata and being

degraded in the borehole sediments, cultured microbial populations would be expected to be much higher than was found.

Although additional analyses were planned on the samples, analyses were not conducted on the sediments because of the uncertainty regarding the levels of microbial contamination in the samples.

Conclusion

- Based on the similarity in hydrologic and microbiological properties at the ADRS and native recharge study sites at the Hanford Site, it is unlikely that $^{14}C$-contaminants were being degraded in the borehole sediments. The much more likely explanation is that the presence of $^{14}C$-$CO_2$ several hundred yards from the trench site was the result of vapor phase transport of $^{14}C$-organic contaminants that were degraded in or below the trench.

**Objective 3. Evaluate the potential for gas phase feeding of carbon, nitrogen, and phosphorus to deep vadose zone microbial communities.**

Viable microbial populations are low, typically $10^4$ cells per gram, and with a patchy distribution in deep vadose zones in arid climates. Thus, microbial populations and degradation rates must be stimulated orders of magnitude for bioremediation to be effective. The advantage of gas phase delivery of nutrients over the traditional liquid phase injection or infiltration is that gases can travel through the unsaturated zone more rapidly and are less likely to clog injection wells and drive contaminants to underlying aquifers. While the practice of introducing limiting nutrients other than oxygen via the vapor phase has relatively little precedence, the generation of gas flow through the vadose zone to enhance remediation is not in itself new. A widely used practice is the method of soil-vapor extraction, in which air is pumped though the unsaturated zone to volatilize contaminants which can then be collected above ground. Bioventing, alternatively, is a bioremediation technique in which air is pumped into the subsurface to increase the availability of the terminal electron acceptor oxygen, to stimulate *in situ* microbial degradation.

The introduction of a more complete suite of nutrients has been shown to stimulate the growth of native microbial degraders to a greater extent than would bioventing or soil-vapor extraction methods (Palumbo et al, 1995). The injection of methane, triethyl phosphate (TEP, which exists as a gas when a gaseous mixture is heated prior to injection) and nitrous oxide in addition to air was repeatedly found to be quite successful in encouraging groundwater bioremediation (Brockman, Payne et al. 1995; Palumbo, Scarborough et al. 1995; Brigmon, Altman et al. 1998). Degradation in the vadose zone has also been enhanced by gaseous nutrient delivery (Brigmon 2001). However, other researchers (Bogan, Lahner et al. 2001) found only limited success in using organic phosphates (TEP and tributylphosphate(TBP)) and $N_20$ to stimulate polyaromatic hydrocarbon degradation in inoculated soil slurries in the laboratory. They suggested that the success of the nitrogen and phosphorous additions may depend on microbiology, soil chemistry, or soil structure. Thus, it is apparent that the dynamics of and constraints on gaseous-phase biostimulation are minimally understood.

The objective is composed of multiple laboratory activities. These are:

- Efficacy of gaseous carbon, nitrogen, and phosphorus additions to Hanford deep vadose zone sediments.
- Ability of gaseous carbon additions to sponsor carbon tetrachloride degradation in unsaturated incubations using Hanford deep vadose zone sediments.
- Studies to support investigation of gas transport, distribution, and utilization in one-dimensional columns and a two-dimensional chamber.

## I. Efficacy of gaseous carbon, nitrogen, and phosphorus additions to Hanford deep vadose zone sediments.

Methods

Sediments were from the 299-W15-95 and 299-W15-84 boreholes described in Objective 2 above. An aqueous solution (5 ml) of triethyl phosphate (TEP) (Aldrich, Milwaukee, WI) and tributyl phosphate (TBP) (Sigma, St. Louis, MO) was evenly distributed over 50 g of homogenized sediment in serum bottles (160 ml size) to give 7 μmoles each of TEP and TBP. Each bottles was crimp-sealed with aluminum caps and thick rubber stoppers. Gaseous nitrogen and carbon was provided by injecting 1 ml $N_2O$ and 5 mls of a gas mixture containing equal amounts of methane, ethane, propylene, propane, and butane, providing 41 μmoles of each of the substrates. Both gas cylinders were certified grade from Air Liquide, LaPorte, TX. The hydrocarbon mixture supplied was an NIST traceable standard by Air Liquide (nominally 20% per component ±1% relative). A set of positive controls received traditional N and P nutrients (20 μmoles $NH_4NO_3$ and 7 μmoles $K_2HPO_4$) plus the gaseous carbon mixture. A set of negative controls received the gaseous C mixture but no N or P sources. Experiments were set up in duplicate for each of the 3 treatments from each of 12 sediment depths from both holes, for a total of 144 bottles. In addition, 6 sediments representing the 3 geologic strata were autoclaved and setup in duplicate as sterile controls. Bottles were incubated in the dark at ambient temperature for 6 months and analyzed for loss of the 5 carbon substrates and production of carbon dioxide by gas chromatography (GC).

At sampling, the septa was pierced, the pressure within each bottle automatically measured, a portion of the headspace gas was directed to a gas-sampling valve, and a prescribed volume of gas was injected into the GC for separation of the components and quantitation by thermal conductivity detection. Analysis of the gas samples was performed in a similar manner as that described in ASTM Method D1945-96: Analysis of Natural Gas. The ASTM method is used for analysis of hydrocarbons C1 through C7 and carbon dioxide using a Porapak column. The gas chromatograph was an MTI Model 200 that included a heated inlet, integral sample pump, and three-way sample valve for piercing the septa, measuring the pressure within each bottle via a pressure transducer, and injecting a prescribed volume of gas into a gas chromatograph equipped with a thermal conductivity detector. A data system which controlled the GC operation and storage of data was used. The GC was initially calibrated by performing direct analysis of standard gases or by performing serial dilutions using gas tight syringes. The gas standards used were UHP carbon dioxide and the hydrocarbon mixture described above. A three-point calibration was obtained over the expected concentration range (200 to

20000 ppmv for the hydrocarbons, 370 to 100300 ppmv for carbon dioxide) with an $r^2 \geq 0.99$ for each of the analytes. Prior to analysis, a continuing calibration verification standard (CCV) was analyzed and at an interval of approximately every 12 samples, a blank analysis was performed and another CCV. At the end of each day, a closing CCV was also analyzed. Duplicate sample analysis was also performed approximately every 12 samples. A pressure measurement of the sample bottle was obtained both before and after sampling to verify that an adequate sample had been obtained. Positives were determined based on concentrations greater than one standard deviation less than the concentration measured in sterile controls.

Results

The results are shown for each bottle in Table 2. Methane was not used in any of the bottles. Nineteen of the 24 sediments (79%) showed degradation of at least one of the hydrocarbons in at least one of the three nutrient conditions tested. Three or 4 hydrocarbons were degraded in 30% of the bottles, representing 71% of the sediments. Even though sediments were homogenized, results in duplicate bottles were generally different highlighting the low population and patchy distribution of microbes in these samples. Eighteen of the 24 sediments (75%) showed degradation of at least one of the hydrocarbons in the inorganic salt N/P addition, while only 58% of the sediment showed degradation of at least one of the hydrocarbons in the gaseous N/P addition and in the no exogenous N/P treatment. Thus, gaseous N and P did not stimulate removal of gaseous C substrates. The inorganic salt N/P addition increased the frequency of positives by 36%, from 58% to 79%; however, the increase was not dramatic suggesting the microbial community was not particularly limited by N or P in their in situ habitat.

Table 2. Microbial utilization of gaseous hydrocarbons in Hanford deep vadose zone sediments. Yellow, orange, and blue indicates Hanford, Plio-Pleistocene, and Ringold sediments, respectively.

| **Hole 1** | $NH_4NO_3/K_2HPO4$ | | | | | $N_2O/TEP/TBP$ | | | | | no nutrient addition | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| depth,ft | M | E | Ppl | P | B | M | E | Ppl | P | B | M | E | Ppl | P | B |
| **102.5** | - | - | + | - | - | - | - | + | - | - | - | - | + | - | - |
| **102.5** | - | - | + | - | + | - | - | + | - | + | - | - | + | - | - |
| **105** | - | - | + | - | - | - | - | + | - | + | - | - | - | - | - |
| **105** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **109** | - | + | + | + | + | - | - | - | - | - | - | - | - | - | - |
| **109** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | + |
| **110.5** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **110.5** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **113** | - | + | + | + | + | - | - | - | - | - | - | - | - | - | - |
| **113** | - | + | + | + | + | - | - | - | - | - | - | - | - | - | - |
| **117** | - | - | - | - | - | - | + | + | + | + | - | + | + | + | + |
| **117** | - | + | + | + | + | - | - | - | - | - | - | - | - | - | + |
| **122** | - | + | + | + | + | - | - | - | - | - | - | - | - | - | - |
| **122** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **125** | - | - | + | + | + | - | + | + | + | + | - | + | + | + | + |
| **125** | - | - | - | - | - | - | - | - | - | - | - | + | + | + | + |
| **132** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **132** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **146** | - | + | + | + | + | - | - | + | + | + | - | - | - | - | + |
| **146** | - | + | + | + | + | - | - | + | + | + | - | - | - | - | - |
| **157** | - | - | - | - | - | - | - | + | + | + | - | - | + | + | + |
| **157** | - | + | + | + | + | - | - | + | + | + | - | - | + | - | + |
| **186** | - | + | + | + | + | - | - | - | - | - | - | + | + | + | + |
| **186** | - | + | + | + | + | - | - | - | - | + | - | - | + | - | + |

| **Hole 2** | $NH_4NO_3/K_2HPO4$ | | | | | $N_2O/TEP/TBP$ | | | | | no nutrient addition | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| depth,ft | M | E | Ppl | P | B | M | E | Ppl | P | B | M | E | Ppl | P | B |
| **112** | - | + | + | + | + | - | + | + | + | + | - | + | + | + | + |
| **112** | - | + | + | + | + | - | + | + | + | + | - | + | + | + | + |
| **114** | - | + | + | + | + | - | - | + | + | + | - | - | + | - | - |
| **114** | - | - | - | - | - | - | - | + | - | - | - | - | + | - | + |
| **118** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **118** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **122** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **122** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **125** | - | + | + | + | + | - | - | - | - | - | - | + | + | + | + |
| **125** | - | - | - | - | + | - | - | - | - | - | - | - | - | - | - |
| **128** | - | - | - | - | - | - | + | + | + | + | - | - | - | - | - |
| **128** | - | + | + | + | + | - | - | - | - | - | - | - | + | - | + |
| **131** | - | + | + | + | + | - | - | + | - | + | - | - | - | - | - |
| **131** | - | + | + | + | + | - | - | + | + | + | - | - | - | - | - |
| **142** | - | - | - | - | - | - | - | + | + | + | - | - | - | - | - |
| **142** | - | + | + | + | + | - | - | + | + | + | - | - | - | - | - |
| **152** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **152** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **162** | - | - | - | - | - | - | - | - | - | + | - | + | + | + | + |
| **162** | - | - | - | - | - | - | - | - | - | - | - | - | + | - | + |
| **172** | - | + | + | + | + | - | - | - | - | - | - | - | - | - | + |
| **172** | - | - | + | + | + | - | - | - | - | + | - | - | - | - | + |
| **194** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **194** | - | - | - | - | + | - | - | - | - | - | - | - | + | + | + |

M=methane; E=ethane; Ppl=propylene; P=propane; B=butane

There was no pattern evident in the 5 sediments that lacked the ability to utilize the gaseous hydrocarbons. The 110.5 ft sample in borehole 1 (299-W15-95) had the second highest field moisture and high mineralization and denitrifier levels, and the 118 and 122 ft samples in borehole 2 (299-W15-84) had the highest field moisture, highest mineralization (the 122 ft sample also had high denitrifier numbers), and highest downhole CT levels (ca. 500 ppm). The other two samples (132 and 152 ft depths in holes 1 and 2, respectively) that lacked the ability to utilize gaseous hydrocarbons had quite low mineralization potential and denitrifier levels. Conversely, many of the samples with low mineralization potential and/or denitrifier levels and low field moisture degraded several gaseous hydrocarbons. Thus, there did not appear to be a pattern explaining the ability of the microbial community to utilize the gaseous hydrocarbons.

Table 3 summarizes the Table 2 results by N/P treatment and by gaseous hydrocarbon. Butane was the most commonly used substrate (44% of bottles), followed by propylene (42%), propane (31%), and ethane (22%) (Table 3). There was essentially no difference between sediments from boreholes 1 and 2 in the frequency of positive results overall or positive results by nutrient treatment (inorganic salt N/P addition, gaseous N/P addition, no exogenous N/P). The inorganic salt N/P treatment performed the best for all 4 of the gaseous hydrocarbons that were degraded, and had about twice as many positives overall compared to the other two treatments. The gaseous N/P treatment did not stimulate removal of any of the individual gaseous hydrocarbons as compared to the no exogenous N/P treatment.

Table 3. Count of bottles showing degradation of gaseous hydrocarbons in Hanford deep vadose zone sediments by N/P treatment and individual gaseous hydrocarbon.

| | methane | | ethane | | propylene | | propane | | butane | |
|---|---|---|---|---|---|---|---|---|---|---|
| Treatments | hole 1 | hole 2 | hole 1 | hole 2 | hole 1 | hole 2 | hole 1 | hole 2 | hole 1 | hole 2 |
| $NH_4NO_3/K_2HPO_4$ | 0 | 0 | 10 | 9 | 14 | 10 | 11 | 10 | 12 | 12 |
| $N_2O$/TEP/TBP | 0 | 0 | 2 | 3 | 9 | 9 | 6 | 7 | 9 | 10 |
| no nutrient | 0 | 0 | 4 | 4 | 9 | 9 | 5 | 5 | 10 | 10 |
| # of positive bottles---> | 0 | | 32 | | 60 | | 44 | | 63 | |
| % positive bottles---> | 0 | | 22 | | 42 | | 31 | | 44 | |

Calculations show that very large amounts of biomass were produced. For example, at 50% utilization of a single gaseous hydrocarbon and assuming 50% of the carbon is lost to carbon dioxide, 3 x $10^9$ cells would be produced, equivalent to 6x$10^7$ cells per gram sediment. It is possible that cells in the gaseous N/P and no exogenous N/P treatments simply converted the carbon to storage products and increased in size, rather than growing by cell division. The evidence indicates this was unlikely. Based on past investigations at Hanford, many of these sediments contained only $10^4$ viable cells per gram; no more than 10% (i.e., $10^3$ per gram) are likely to be capable of gaseous hydrocarbon utilization. Starved cells can increase their volume up to 10-50 fold. Thus, if cells were not dividing, the equivalent of $10^4$ to $10^5$ (normal-sized) cells would be present per gram. In contrast, as shown above, removal of 50% of a single hydrocarbon – a common result in the positive bottles – yields $10^7$ (normal-sized) cells per gram. This 100-fold difference indicates most of hydrocarbon utilization is coupled to cell division.

This conclusion is consistent with the observations that the inorganic salt N/P treatment only increased the frequency of positives by 36% (from 58% to 79%) and the average % of removal was not higher than the other treatments (data not shown), yet would support the synthesis of $10^8$ cells per gram. The conclusion that very large numbers of cells were produced in the absence of added N or P is important because (a) gaseous N and P did not stimulate hydrocarbon utilization, and (b) rates of contaminant degradation in the field will be much higher if microbial growth is due to cell division versus cell enlargement.

Conclusions

- Subsurface microorganisms in these low biomass sediments utilize a range of gaseous hydrocarbons. ~80% of the sediments degraded at least one of the hydrocarbons. ~20 to 45% of the sediment samples showed the ability to biodegrade a specific hydrocarbon. Greater than 3 hydrocarbons were degraded in ~70% of the sediments.
- Gaseous N and gaseous P did not stimulate removal of gaseous C sources compared to no addition of N and P, however, the microbial community appeared to have adequate levels of sediment-associated N and P to convert large amounts of hydrocarbon utilization to cell mass.
- Use of gaseous hydrocarbons to increase microbial populations in situ has great promise in Hanford deep vadose zone sediments. The ability to successfully deliver the gases to the in situ subsurface microsites where microbes exist should be tested in intact Hanford deep vadose zone cores.

## II. Ability of gaseous carbon additions to sponsor carbon tetrachloride degradation in unsaturated incubations with Hanford deep vadose zone sediments.

Carbon tetrachloride is degraded by various microbes via anaerobic respiration. Anaerobic metabolisms can exist in bulk phase aerobic environments such as soils and vadose zones. This is possible because facultatively aerobic microbes can switch to anaerobic metabolisms (fermentation or use other terminal electron acceptors when oxygen is not available) as anaerobic microsites form, such as within active biofilms, in pores with blocked pore throats, or other locations where diffusion is limited and aerobic microbial activity is high. Some obligate anaerobes can survive aerobic conditions for long periods of time if they are inactive, and resuscitate when anaerobic conditions reform. If CT is anerobically degraded to chloroform, methane and butane-oxidizing bacteria and potentially other gaseous hydrocarbons can degrade the chloroform using oxygen. The objective of the following experiment was to determine if microbial communities in select sediments from the above experiment would, when re-supplied with gaseous hydrocarbons, degrade carbon tetrachloride under unsaturated (i.e., bulk-phase aerobic) conditions. It was assumed that carbon and energy substrates needed by anaerobes would be generated from decay of the biomass produced during feeding with the gaseous hydrocarbons.

Methods

All bottles that showed removal of at least one gas hydrocarbon (36 from Hole 1 and 35 from Hole 2) were selected for subsequent CT degradation studies. In addition, one

bottle from hole 1 (157 feet, replicate 1, inorganic nutrient treatment) and 3 bottles from hole 2 (114 feet, replicate 2; 122 feet, replicate 1; 142 feet replicate 1; all in the inorganic nutrient treatment) that did not show any removal of gaseous hydrocarbons were included to determine if sediment initially negative for removal could become positive with a double enrichment. Bottles were opened and equilibrated with air for one day, resealed, spiked with the 5-component hydrocarbon mix as described above, and finally spiked with 0.26 µmoles CT delivered in 10.0 µl methanol to give a final headspace concentration of approximately 30 ppm. Autoclaved sediments also received the hydrocarbon mix and served as sterile controls.

Gaseous hydrocarbons, CT, and carbon dioxide were analyzed the same day and after 1, 4, and 10 months using an Agilent 6890 Gas Chromatograph/5973 Mass Spectrometer (GC/MS) system. The MS was tuned each day of operation using perfluorotetrabutylamine and air to insure proper mass assignments. Calibration standards and samples were analyzed by manually injecting 10 µL of gas into a programmable temperature vaporization inlet. A three point initial calibration was performed to establish linearity of the instrument. A continuing calibration standard was prepared each day of operation. Injections of the continuing calibration standard were performed prior to and at the end of sample analysis each day and at a frequency of at least every ten sample injections. Sample quantitation was performed using the extracted ion instrument response obtained from the continuing calibrations. Chromatograms were reviewed for non-target analytes to detect CT degradation compounds.

For gaseous hydrocarbons, positives were determined based on concentrations greater than one standard deviation less than the concentration measured in sterile controls. For CT, positives were determined based on concentrations greater than two standard deviations less than the concentration measured in sterile controls.

Results

The results are shown for each bottle in Table 4 and summarized in Table 5. Only 4 of the 75 bottles showed CT degradation, with three of these occurring in the inorganic salt N/P treatment. Only 50-60% of the CT was degraded in these 4 bottles after 10 months. One of the bottles was positive after 4 months incubation but failed to further degrade CT in the ensuing 6 months. In all 4 bottles, three hydrocarbons were near or below detection after 10 months incubation. However other bottles degraded multiple hydrocarbons equally or more rapidly, and did not degrade CT. Thus it is not clear what controlled the low frequency at which CT degradation was observed. Because methane was rarely used, it was used as an internal standard to ensure CT and other gases has not leaked from the bottles over the extended incubation. Mass spectra were scanned for chloroform or other CT degradation products but were not detected in any of the bottles. This is somewhat surprising as metabolites of CT would be expected. However it is possible that CT degradation products were degraded using oxygen as the terminal electron acceptor. Methanotrophs and butane-oxidizers are known to degrade chloroform; butane was degraded to below (or very near to) detection in the 4 bottles and may explain the observation.

Table 4. Microbial degradation of gaseous hydrocarbons and CT in Hanford deep vadose zone sediments by N/P treatment and compound. Hole 1, top panel; hole 2, bottom panel. Only cells with a plus or minus were included in the assay. Yellow, orange, and blue indicates Hanford, Plio-Pleistocene, and Ringold sediments, respectively. Results are after 10 months of incubation.

| **Hole 1** | $NH_4NO_3/K_2HPO4$ | | | | | | $N_2O/TEP/TBP$ | | | | | | no nutrient addition | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| depth,ft | M | E | Ppl | P | B | CT | M | E | Ppl | P | B | CT | M | E | Ppl | P | B | CT |
| **102.5** | - | + | + | + | + | - | - | - | + | - | - | - | - | - | - | - | - | - |
| **102.5** | - | + | + | + | + | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **105** | - | + | + | + | + | - | - | - | - | - | - | - | | | | | | |
| **105** | | | | | | | | | | | | | | | | | | |
| **109** | + | + | + | + | + | - | | | | | | | | | | | | |
| **109** | | | | | | | | | | | | | - | - | - | - | - | - |
| **110.5** | | | | | | | | | | | | | | | | | | |
| **110.5** | | | | | | | | | | | | | | | | | | |
| **113** | - | + | + | + | + | - | | | | | | | | | | | | |
| **113** | - | + | + | + | + | - | | | | | | | | | | | | |
| **117** | | | | | | | - | - | - | - | - | - | - | - | - | - | - | - |
| **117** | + | + | + | + | + | + | | | | | | | - | - | - | - | - | - |
| **122** | + | + | + | + | + | - | | | | | | | | | | | | |
| **122** | | | | | | | | | | | | | | | | | | |
| **125** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **125** | | | | | | | | | | | | | - | - | - | - | - | - |
| **132** | | | | | | | | | | | | | | | | | | |
| **132** | | | | | | | | | | | | | | | | | | |
| **146** | - | + | + | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **146** | - | + | + | + | + | - | - | - | - | - | - | - | | | | | | |
| **157** | - | - | - | - | - | - | - | - | - | - | - | - | + | + | + | - | + | - |
| **157** | - | - | + | + | + | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **186** | - | + | + | + | + | - | | | | | | | - | - | - | - | - | - |
| **186** | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |

| **Hole 2** | $NH_4NO_3/K_2HPO4$ | | | | | | $N_2O/TEP/TBP$ | | | | | | no nutrient addition | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| depth,ft | M | E | Ppl | P | B | CT | M | E | Ppl | P | B | CT | M | E | Ppl | P | B | CT |
| **112** | - | + | + | + | + | - | - | + | + | + | + | - | - | - | + | + | + | - |
| **112** | - | + | + | + | + | - | - | + | + | + | + | - | - | - | - | - | - | - |
| **114** | - | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **114** | - | - | + | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| **118** | | | | | | | | | | | | | | | | | | |
| **118** | | | | | | | | | | | | | | | | | | |
| **122** | - | + | + | + | + | - | | | | | | | | | | | | |
| **122** | | | | | | | | | | | | | | | | | | |
| **125** | - | + | + | + | + | + | | | | | | | - | - | - | - | - | - |
| **125** | - | + | + | + | + | - | + | + | + | + | + | - | | | | | | |
| **128** | | | | | | | - | + | + | + | + | - | | | | | | |
| **128** | + | + | + | + | + | - | | | | | | | - | + | + | + | + | - |
| **131** | - | - | - | - | - | - | - | - | + | - | - | - | | | | | | |
| **131** | - | + | + | + | + | + | - | + | + | + | + | + | | | | | | |
| **142** | - | - | + | + | - | - | - | - | - | - | - | - | | | | | | |
| **142** | + | + | + | + | + | - | - | - | - | - | - | - | | | | | | |
| **152** | | | | | | | | | | | | | | | | | | |
| **152** | | | | | | | | | | | | | | | | | | |
| **162** | | | | | | | - | - | - | - | - | - | - | - | - | - | - | - |
| **162** | | | | | | | | | | | | | - | - | - | - | - | - |
| **172** | - | - | - | - | - | - | | | | | | | - | - | - | - | - | - |
| **172** | - | + | + | - | + | - | - | - | - | - | + | - | - | - | - | - | - | - |
| **194** | | | | | | | | | | | | | | | | | | |
| **194** | - | + | + | - | + | - | | | | | | | + | + | + | + | + | - |

Table 5. Count of bottles showing degradation of gaseous hydrocarbons and CT in Hanford deep vadose zone sediments by N/P treatment and compound. Hole 1, top panel; hole 2, bottom panel. Results are after 10 months of incubation.

| Treatments | methane | ethane | propylene | propane | butane | carbon tetrachloride |
|---|---|---|---|---|---|---|
| $NH_4NO_3/K_2HPO3$, n=16 | 3 | 11 | 12 | 11 | 12 | 1 |
| $N_2O$/TEP/TBP, n=9 | 0 | 0 | 1 | 0 | 0 | 0 |
| no nutrient, n=12 | 1 | 1 | 1 | 0 | 1 | 0 |
| # of positive bottles ---> | 4 | 12 | 14 | 11 | 13 | 1 |
| % of positive bottles ---> | 11% | 32% | 38% | 30% | 35% | 3% |

| Treatments | methane | ethane | propylene | propane | butane | carbon tetrachloride |
|---|---|---|---|---|---|---|
| $NH_4NO_3/K_2HPO3$, n=16 | 2 | 10 | 13 | 9 | 10 | 2 |
| $N_2O$/TEP/TBP, n=11 | 1 | 5 | 6 | 5 | 6 | 1 |
| no nutrient, n=11 | 1 | 2 | 3 | 3 | 3 | 0 |
| # of positive bottles ---> | 4 | 17 | 22 | 17 | 19 | 3 |
| % of positive bottles ---> | 11% | 45% | 58% | 45% | 50% | 8% |

Table 6. Degree of degradation of individual hydrocarbons in Hanford deep vadose zone sediments. Results are after 10 months of incubation.

| | Percent of bottles (n=75) with indicated amount of degradation, by compound: | | | | |
|---|---|---|---|---|---|
| % of compound degraded | methane | ethane | propylene | propane | butane |
| >90% | 1% | 20% | 20% | 15% | 25% |
| 76-90% | 1% | 4% | 4% | 7% | 5% |
| 51-75% | 5% | 7% | 11% | 11% | 11% |
| positive but <50% | 3% | 8% | 7% | 5% | 1% |

Degradation of hydrocarbons increased over time. By 10 months, most of the positives for ethane, propylene, propane, and butane were showing >90% degradation (Table 6). Three or 4 hydrocarbons were degraded in 41% of the bottles after 10 months. Nearly all positives for gaseous hydrocarbon degradation in hole 1 occurred in the inorganic salt N/P treatment (Tables 4 and 5). In hole 2, the inorganic salt N/P treatment had a greater fraction of positives than the gaseous N/P treatment, which is turn had a greater fraction of positives than the no exogenous N/P treatment. Thus, in contrast to the initial experiment with hydrocarbons (Tables 2 and 3) in this experiment the gaseous N/P treatment did appear to stimulate removal of ethane, propylene, propane, and butane as compared to the no exogenous N/P treatment in sediments from one of the holes. The inorganic salt N/P treatment outperformed the other treatments to a greater extent in this experiment than the initial experiment, and there were greater differences between the three treatments in this experiment than in the initial experiment. This suggests that the cumulative biomass production in the double incubation resulted in N and P limitation due to inadequate levels of naturally occurring sediment-associated N and P.

In the 4 bottles that were included to determine if sediment initially negative for hydrocarbon degradation would show degradation following a double enrichment (see

methods for their identity), 3 of the 4 bottles showed degradation; either one, two, or 4 hydrocarbons were degraded in the 3 bottles. Similarly, methane was degraded in a few of the bottles, in contrast to the initial experiment (Tables 2 and 3) where no methane degradation was detected. Bottle by bottle comparisons of results in the initial experiment and this experiment do not indicate an overall high correlation. While results in many individual bottles were highly correlated (plus and plus for a given substrate, or negative and negative for a given substrate), many results in other individual bottles reflected the acquisition of degradation of a hydrocarbon or loss of ability to degrade a hydrocarbon. These changes are likely due to community dynamics such as competition between organisms, or slow resuscitation and growth of organisms that were not initially active or able to grow. This is particularly true given that 5 different growth substrates were present in the same bottle.

Conclusions

- CT degradation occurred in only 5% of the bottles and only 50-60% was degraded after a 10 month period. Degradation products were not detected, but may have been degraded by butane-oxidizing organisms. The stochiometry occurring in the incubations needs to be compared to other reports to further evaluate these results.
- It is likely CT degradation would occur more readily in the subsurface vadose zone where porosity and oxygen diffusion rates are lower and anoxic sites would be more readily formed as a result of aerobic degradation of gaseous hydrocarbons. Similar studies to those conducted here, but conducted in closed columns, are warranted to further investigate the potential for degradation of CT and other DOE organic contaminants in the vadose zone using gaseous hydrocarbon-promoted bioremediation.

## III. Studies to support investigation of gas transport, distribution, and utilization in one-dimensional columns and a two-dimensional chamber.

### A. Substrate screening experiments

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A series of screening experiments were conducted to select suitable carbon, nitrogen and phosphorus substrates for two model organisms, *Pseudomonas fluorescens* HK44 and *Pseudomonas stuzeri* KC. *Pseudomonas butanovora* was used as a control organism.

Methods

*Carbon Source*:

*Screen 1*: Cultures of the above three organisms were grown overnight on phosphate buffered modified mineral salts (MMS) amended with lactate (0.5 g/L). Cells were harvested by centrifugation, washed, and resuspended in 0.85% NaCl to an optical density at 600 nm ($OD_{600}$) of about 0.5. Twenty µL of cell suspension were added to 70 mL serum vials containing 20 mL basal medium without carbon. Vials were sealed with butyl rubber stoppers and aluminum crimp seals, with a needle inserted through the stopper as a vent to maintain atmospheric pressure within the vial. Five mL of gas (20 C, 1 atm) were injected into the headspace, upon which the vial was inverted and incubated at 25°C with shaking at 150 rpm for 90 hours. Injected gas was methane, ethylene,

propane, propylene, or butane. Each of these five treatments was replicated 3 times with each model organism. This replication procedure was used throughout the following screens.

*Screen 2*: Cultures were grown as described above. Cells were suspended in 20 mL of 50mM MMS in a sealed anaerobic vial. Carbon sources were added to Konte cups suspended within the vials, and were of one of the following: hexane, heptane, acetate (as acetic acid), propionate, methylamine, diethylamine, and naphthalene.

*Screen 3*: HK44 cultures were grown in a sodium-acetate solution for 24 hours, and then centrifuged, washed and resuspended to an $OD_{600}$ of about 0.25. 70 mL vials with 20 mL of 50mM phosphate-buffered MMS were inoculated with 100 µL of cells. 100 µL aliquots of aqueous dilutions of acetic acid were then added to Konte cups suspended in the vials' headspaces, and allowed to volatilize. *P. stuzeri* KC cultures were grown and treated as described for HK44, except that no tetracycline was added, and the inoculum was 200 µL of a 48 hour culture ($OD_{600}$ = 0.11).

*Nitrogen and Phosphorus Sources:*

*Screen 1*: Cultures were prepared as in the carbon Screen 1, except that HK44 and KC were grown on MOPS media with lactate. Serum vials contained 20 mL of 0.5 g/L lactate in 50 mM MOPS. While $N_2O$ was injected as above (0.1 mL of gas, 20 C, 1 atm), triethyl phosphate (TEP) was added as a liquid. After sealing and injections, vials were inverted and incubated at 25 C with shaking at 150 rpm. Treatments were of the following: $NH_4NO_3$, $K_2HPO_4$; $NH_4NO_3$, TEP; $NH_4NO_3$, no P; $N_2O$, $K_2HPO_4$; no N, $K_2HPO_4$

*Screen 2*: Cultures were prepared as for the carbon experiments, with 20 mL of 0.5 g/L lactate in 50 mM MOPS in the serum vials. Nitrogen sources examined were methylamine, diethylamine, nitrous oxide and ammonia. Tributyl phosphate and triethyl phosphate were examined as phosphorus sources, this time with an unbuffered mineral salts media to reduce negative impacts on *P. butanovora*.

*Screen 3*: Cultures of HK44 and KC were prepared as in the carbon Screen 3, except that they were resuspended in N-free MMS, and 100 µL of inoculum was used for both strains. Nitrogen was added in the following forms: aqueous $NH_4NO_3$, $NH_3$ gas (5mL injected in the headspace) and $NH_4OH$ (200 µL added to a Konte cup in the headspace). No phosphorus sources were tested.

Results

Results are summarized in Table 7. Acetic acid and ammonia were the most successful carbon and nitrogen sources, respectively, in these trials, and thus were selected as gaseous substrates for *P. fluorescens* HK44. TEP and TBP were the only sources of phosphorus thought to be potential gaseous substrates due to the difficulties encountered in getting phosphorus compounds into the vapor phase, and both failed to induce microbial growth.

**Table 7.** Microbial utilization of carbon, nitrogen and phosphorus substrates. *P. butanovora* was the control organism, and either +N+P, $NH_4$ or$K_2HPO_4$ was used as the control substrate.

| | **No Cells** | ***P. fluoresc.* HK44** | ***P. stutzeri* KC** | ***P. butanovora*** |
|---|---|---|---|---|
| **Carbon** | | | | |
| methane | - | - | - | - |
| ethylene | - | - | - | - |
| propane | - | - | - | + |
| propylene | - | - | - | - |
| butane | - | - | - | + |
| hexane | - | - | - | - |
| heptane | - | - | - | - |
| acetate | - | - | - | - |
| propionate | - | - | - | - |
| naphthalene | - | + | - | - |
| MAM | - | - | - | - |
| DAM | - | - | - | - |
| **Nitrogen & Phosphorus** | | | | |
| +N+P | - | + | + | - |
| +N-P | - | - | - | - |
| -N+P | - | - | - | - |
| +$N_2O$+P | - | - | - | - |
| MAM | - | - | - | - |
| DAM | - | - | - | - |
| $N_2O$ | - | - | - | - |
| $NH_4$ | - | + | ? | + |
| no N | - | - | - | - |
| + N + TEP | - | - | ? | - |
| TBP | - | - | - | - |
| TEP | - | - | - | - |
| $K_2HPO_4$ | - | + | ? | + |
| no P | - | - | - | - |

(-) indicates no growth, (+) growth, and (?) unclear results.

B. Feeding experiments in chamber and microcosm

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In these experiments, the substrates selected above were introduced into sand-packed systems to induce the growth of *P. fluorescens* HK44, which was chosen over *P. stuzeri* KC due to its bioluminesence abilities amenable to the light transmission chamber system. Original experiments were conducted in the chamber, which was described above, while later experiments were run in small columns.

Methods

*Chamber Experiments*

The light transmission chamber was packed as described in Yarwood et al (2002) with 3772 grams of sterile sand, yielding a porosity of 0.333 (utilizing inner volume of dimensions 1 cm thick x 48.3 cm wide x 44.0 cm high**)**. The sand used was a 40/50 mesh size Accusand silica sand. Inoculum containing approximately 5.3 x $10^8$ CFU/mL HK44

was injected through a port in the glass 12.5 cm below the surface of the sand pack. Two feed solutions, 500 mL of 3.5 mM ammonium hydroxide and 500 mL of 3.5 mM acetic acid, were prepared in 1 liter bottles, thus allowing 500 mL of headspace for volatilization of the compounds. These were then pumped through the left-hand port at flow rates of 634.7mL/hour (ammonium hydroxide) and 664.5 mL/hour (acetic acid).

In order to visualize the effect of the gaseous substrates on microbial growth using HK44's *lux* response to naphthalene, air was pumped through a cartridge of naphthalene crystals into the chamber for various periods of time (Table 8). This method was chosen over liquid pulses of salicylate, as used by Yarwood (Rockhold, Yarwood et al. 2002), so as not to alter gas flow dynamics. Using a fixed liquid-cooled, 16 bit, grey scale, 512 x 512 pixel CCD camera (Princeton Instruments, Trenton, New Jersey), images were taken at appropriate intervals. For water content images, an f-stop of 5.6 was used with a 500 nm red wide bandpass filter. Microbial light emission images were taken at 300 second intervals over each period, using an f-stop of 5.6, a 300 second exposure, and no filter. Due to minimal microbial response thought to be a result of oxygen limitation, supplemental oxygen was pumped into the chamber for the fourth and fifth imaging periods.

**Table 8.** Timing of naphthalene pulses to induce microbial *lux* response.

| Date | Time since inoculation (hours) | Length of naphthalene pulse | Oxygen flow? | Naphthalene pump setting |
|---|---|---|---|---|
| 9/20 | 22 | 3.5 | No | 1 |
| 9/23 | 89.5 | 10 | No | 1 |
| 9/26 | 160 | 17.25 | No | 1 |
| 9/30-10/1 | 267 | 14 | Yes | 1 |
| 10/1-10/2 | 286 | 18.5 | Yes | 2 |
| 10/2-10/4 | 309 | 43.5 | No | 3 ** |
| 10/7-10/8 | 430 | 19 | No | 3 ** |

** indicates switched flow direction across chamber (R to L).

*Microcosm experiments*

Our use of naphthalene vapor to induce the microbial *lux* reaction in the above gas-phase feeding chamber experiments was limited in its success, potentially due to transport issues discussed below. To simplify the system and pinpoint problems, we next used miniature columns made from modified 30 mL plastic syringes. Using these microcosms, we ran experiments looking solely at the addition of acetic acid vapor, with all other nutrient sources present in the inoculum. The microcosm's construction allowed us to easily remove and plate samples to determine microbial growth as a result of gaseous feeding. The conical injection ends were removed from the syringes which were then packed with 40/50 Accusand. Both ends of each syringe were fitted with rubber stoppers attached to nylon mesh which prevented sand loss and drying. All components of these systems were autoclaved, and experiments proceeded as follows:

*Experiment 1:* Twelve microcosms were packed volumetrically with 25 mL 40/50 Accusand. Eleven were inoculated drop-wise with 4 mL of an HK44 culture in an MMS-no carbon solution, with the control system receiving only MMS. The microcosms were

connected to a 3.5 M acetic acid solution (1 L solution in a 2 L bottle with a sparging apparatus) via a Masterflex L/S® multi-channel 12 cartridge pumphead (model # 7519-15) fitted with Masterflex PharMed tubing (Saint Goban) (06485-16). Acetic acid vapor flowed through the systems at an approximate rate of 600 mL/hour for 6 days.

Two microcosms were dismantled every day, their pH measured, contents added to buffer solution, and the supernatant used to plate serial dilutions according to standard methods. The inoculum was also plated as a control. Plate counts were made after two days of incubation at 25° C.

*Experiment 2:* Microcosms were packed with sand as above, but only the MMS buffer solution was added. Two sample acetic acid solutions were prepared (Solution 1 = 1.49 mM, pH = 4.5, Solution 2 = $3.5 \times 10^{-3}$mM, pH = 6.6) and pumped through the abiotic microcosms for three days at a flow rate of 600 mL/hour, at the end of which pH values were measured.

*Experiment 3:* Microcosms were packed with 34 g of sand, inoculated, and gaseous acetic acid was introduced as above. Both the inlet and the outlet ends of the column were sampled volumetrically to examine potential effects of retardation. Sample pH was measured, buffer was added to the sample, and the supernatant was vortexed and plated. Plate counts were made after two days.

## Results

### *Chamber experiments*

Images taken during naphthalene pulses revealed *lux*-induced glow at the injection port at the beginning of the pulse, as expected. However, instead of the light response gradually increasing in area from this central point, a thin "leading edge" of glow gradually migrated away from the injection port (Figure 21). This was originally understood to be a result of oxygen limitation, as observed by (Rockhold, Yarwood et al. 2002). In subsequent experiments, oxygen was pulsed simultaneously with naphthalene, however similar results were observed. It is probable that the naphthalene vapor either solubilized too readily or was trapped by microbial communities (Zhang, Bouwer et al. 1998) (Gamst, Moldrup et al. 2003). Further experiments addressing this situation may be in order.

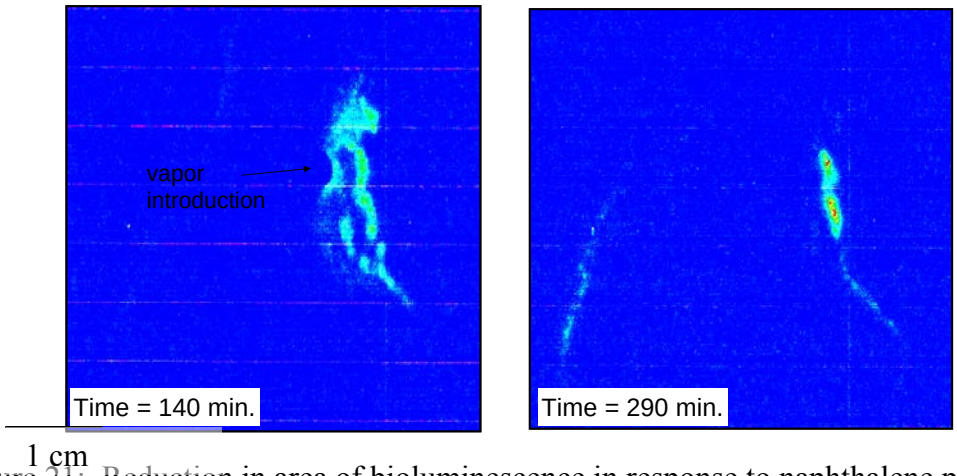


Figure 21: Reduction in area of bioluminescence in response to naphthalene plume over time.

*Microcosm experiments*

Although microbial growth was observed in one sample on the 5th day (Figure 22), acidification restrained growth in most cases, and left no viable organisms in all cases where the pH was below 6.4. These results led us to seek a more appropriate starting acetic acid concentration. Using acetic acid concentrations that produced pHs above 6.4 in abiotic trials, we next conducted a biotic experiment which appeared to have been plagued by contamination, as unexpected growth was observed. However, an acetic acid concentration-dependent growth pattern was noted, as was a general trend of greater density of colonies localized near the incoming gas. This last result supports a trend of maximum utilization of the substrate at the injection point which could become problematic over larger distances.

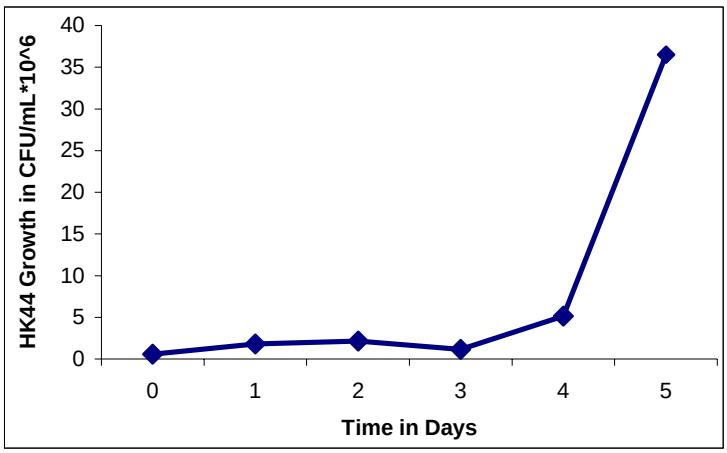


Figure 22: Growth of HK44 on vapor-phase acetic acid over time

Using acetic acid concentrations that produced pHs above 6.4 in abiotic trials, we next conducted a biotic experiment which appeared to have been plagued by contamination, as unexpected growth was observed. However, an acetic acid concentration-dependent growth pattern was noted, as was a general trend of greater density of colonies localized near the incoming gas. This last result supports a trend of maximum utilization of the substrate at the injection point which could become problematic over larger distances.

C. Gas transport visualization

In order to approximately predict gaseous nutrient distribution in the chamber system, and to test whether the system would serve to further understanding of the distribution of gases in unsaturated media, we traced the movement of carbon dioxide using the pH indicator methyl red.

Methods

The chamber was packed and prepared as described above, either with a homogenous sand pack, or with an inclusion of coarse sand in the midst of finer sand. The system was then saturated with a 0.005% methyl red solution and upon ponding a lower siphon was opened to flush out the chamber. The volume and pH of liquid flushed was recorded.

Sparging stones were installed and the upper manifold replaced with an aluminum cover. $CO_2$ gas was then pumped through the system for a period of time between 35 and 90 minutes. During this interval, one or two series of images was made using the camera equipment described above with a 500 nm wideband filter ; to make 120 images we used an f-stop of 5.6, an exposure time of 1s, and 15 seconds delay between each frame; to make 144 images the exposure time was changed to 0.5 seconds . $CO_2$ flow rates varied between 39 ± 2 mL/h to 281 ± 6 mL/h. In some experiments, two separate carbon dioxide pulses were separated by an oxygen pulse in an attempt to flush out residual carbon dioxide. Twelve experiments were run at hydrostatic conditions, while the other five had liquid flow at the same rate as gas flow, 160 mL/h. Relative humidity and temperature measurements of the room were made throughout.

The images captured by the camera were expressed in WinView (Princeton Instruments, Princeton, NJ) in terms of pixel intensity. In order to create images displaying $CO_2$ concentrations, it was necessary to create a standard curve for methyl red relating percent transmittance to pH. Factoring in water content and Beer's Law, it was then possible to transform pixel intensity values into $CO_2$ concentration values.

*Standard Curve*

Methyl red stock solution (0.005%) was prepared as for the $CO_2$ tracing experiments, with NaOH added drop-wise as needed to facilitate dissolution, resulting in a solution with a pH value above 8.0. HCl was used drop-wise to create a series of 16 5-10 mL samples spanning the desired range of pH 7.05 to pH 3.82. All pH readings were taken uniformly with a Orion Research pH meter (model SA250).

The Bausch & Lomb Spectronic 21 was calibrated to 0% using a specially machined occluder of black composite material. The 100% calibration was based on transmittance through distilled water. Three mL of each pH-adjusted solution was pipetted into the same cylindrical cuvette and read for percent transmittance at 520nm. Between readings the cuvette was rinsed with solution from the next sample. This full procedure of pH adjustments and spectrophotometer readings was repeated three times, each starting with the same stock solution. The result was 3 sets of 16 values, for a total of 48 data points.

pH and % transmittance values were averaged over the three sets of data. The variation in the independent pH variable was minimal, as pH adjustment approximated uniformity. The variation in the dependent variable, % transmittance, produced standard error values between 0.0002 and 1.58. The maximum error occurred around pH 5.2 where methyl red was undergoing the most abrupt color change. pH values were converted to an exponential scale (10^pH) in order to accurately represent their relationship to % transmittance.

Upon graphing % transmittance vs. 10^pH it was noted that the resulting curve resembled the plot of the van Genuchten curve for water retention (Figure 23),

$$\theta_h=\theta_r + (\theta_s - \theta_r)/ [1+ (\alpha h)^{n}]^{1-(1/n)}$$

For ease, the equation was re-symbolized as

$$t = r + (s-r)/[1 + (ap)^{n}]^{1-(1/n)}$$

where t = % transmittance, p = pH, and a, s, r and n are fitting parameters.
Using Microsoft Excel's Solver, and constraining parameters as 0<= s<= 1, 0<= r<= 1, a>0, n>1, a solution was found for the following parameter values: s = 1, r = 0.472, a

= 2.27 x $10^{-6}$, n = 1.972. The equation was then algebraically rearranged to solve for pH so as to be applicable to $CO_2$ tracking data (in the form of %transmittance).

$$p = \{[((s-r)/(t-r))^{\wedge 1/m} - 1]^{1/n}\}/a$$

The fit of this equation to collected standard curve data is shown in Figure 23.

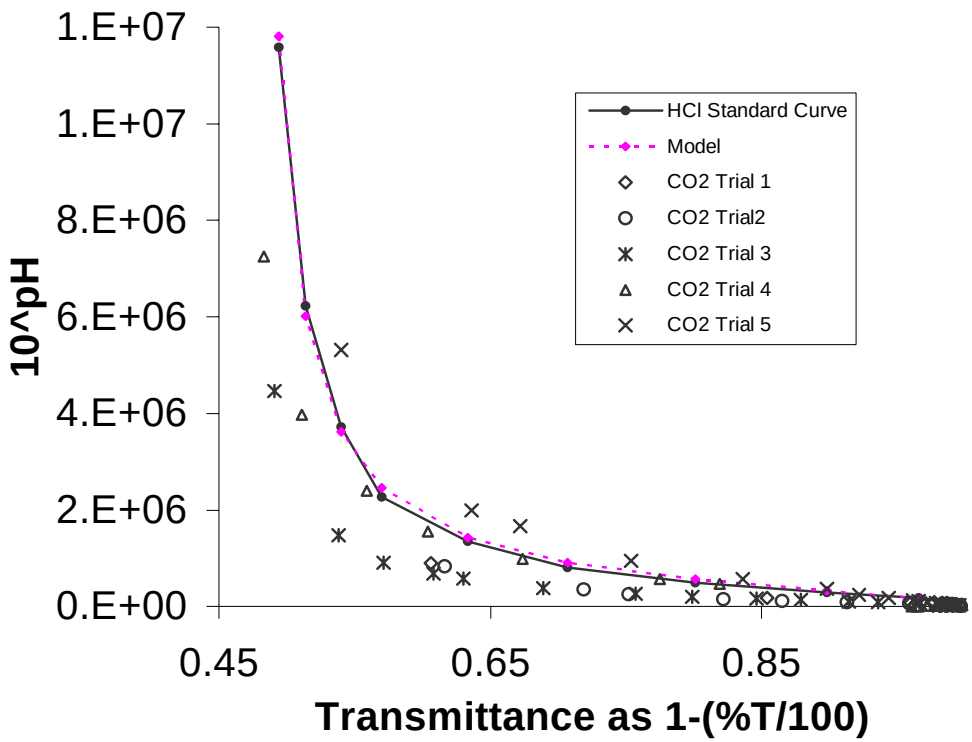


Figure 23. Standard curve for gas tracing experiment relates pH of 0.005% methyl red solution to its %transmittance at 520nm. Solid line represents averaged values from HCl trials, scatter shows data cloud of trials using sparged $CO_2$, and the dashed line represents the fitted model equation.

*Relating pixel intensity to carbon dioxide concentration*

The relationship between pH and carbon dioxide concentration was derived from the ionization and equilibrium equations describing how $CO_2$ hydrates to form $H_2CO_3$ (carbonic acid), which in turn quickly ionizes to the three species $HCO_3$- (biocarbonate ion), H+ (hydrated protons) and $CO_3^{--}$ (carbonate ion). We assume that at pH <=7.5, where we are working, the concentrations of $[OH^-]$ and $[CO3^{--}]$ are negligible. Using substitution and the definition of pH yields the equation

$$\frac{[\log^{-1}(-pH)]^2}{K_{a1}} = [CO_2] \qquad \text{where } K_{a1} = 10^{-6.381} \text{ (reported value at 20° C, Butler, 1982).}$$

Finally, percent transmittance was correlated to relative pixel intensity (α, accounts for water content as described below) using Beer's Law, $I = I_o \exp(-\alpha x)$. Path length, x, was set at 1.14 cm, or the thickness of the cuvette with which percent transmittance measurements were made. The equation was rearranged as follows:

$$\alpha = \frac{1}{1.14} * \ln\left(\frac{1}{T}\right) \qquad \text{where T = \% transmittance}$$

These three equations were combined into a single equation which would calculate carbon dioxide concentration given pixel intensity at a point.

$$[CO_2] = 10^{6.381} * \log^{-1}\left\{-\log\left[\left(\left\{\frac{0.528}{0.528 - \frac{1}{100e^{1.14\alpha}}}\right\}^{2.029} - 1\right)^{0.507} * 2.27 * 10^6\right]\right\}^2$$

*Image processing*

Collected images of pixel intensity were converted to carbon dioxide concentration maps using the software Transform 3.4 (Fortner Software LLC, Sterling, VA). The first step depended on the assumption under Beer's Law that two major factors influenced pixel intensity: pH-induced methyl red color (absorbance, α) and volumetric water content (path length, x). Each individual frame was processed using a modified Beer's Law macro to isolate the former effect, creating an "alpha distribution" image. These images were then reprocessed with a macro involving the general equation which solves for carbon dioxide concentration given an alpha value. Sample images showing $CO_2$ plume migration over time are shown in Figure 24.

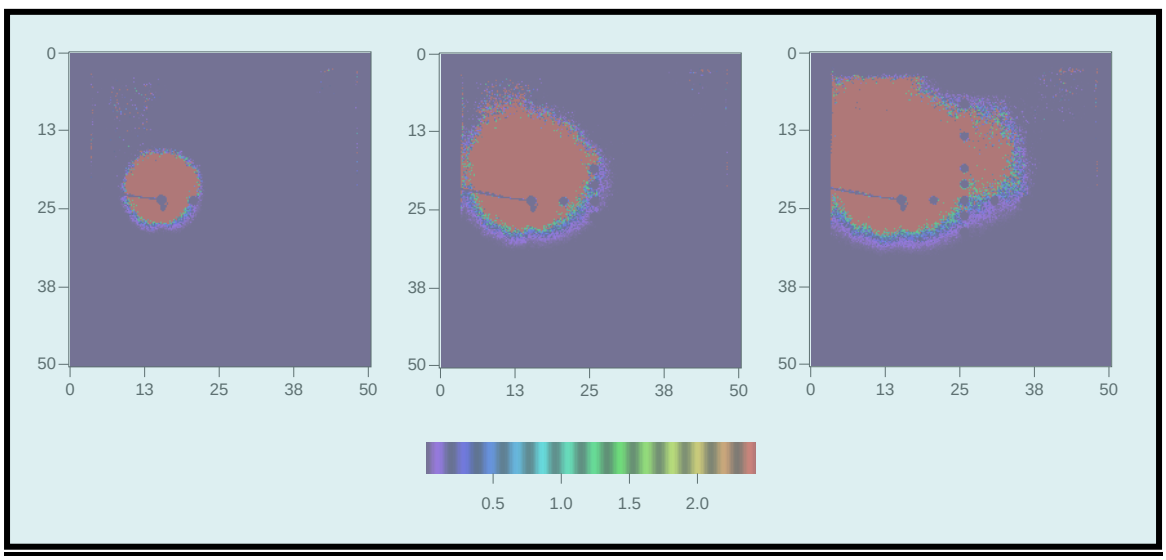


Figure 24. Migration of carbon dioxide introduced at the left-hand sparging stone at a constant rate of 110 mL/h. Image times are (L to R) 4.5 minutes, 10.5 minutes, and 20 minutes.

In conclusion, in this research task a colorimetric readout method was developed for real-time monitoring of $CO_2$ movement (as a proxy for tracking movement of gaseous microbial nutrients) through unsaturated 2-d chambers. This work is being extended to track movement of gaseous microbial nutrients in the 2-d chambers, and to investigate relationships between hydraulic processes, gaseous nutrient delivery to microorganisms, and microbiological processes.

**Relevance, Impact, and Technology Transfer**

**a.** Traditional biostimulation methods involving the use of liquid-phase nutrients to stimulate indigenous microorganisms are more effective in the saturated zone than the unsaturated zone. However, the Department of Environmental Management must deal with numerous sites in which the contamination is concentrated in the vadose zone. Thus, development of highly effective techniques for vadose zone bioremediation, such as gaseous-nutrient stimulation, would benefit the Department's efforts in site clean-up. The microbial research herein focused on research to understand (i) the degree to which microbes migrate and colonize unoccupied pore networks and regions of the vadose zone, (ii) the coupling of hydrologic processes and microbial growth during aqueous delivery of nutrients and developing models of these processes for future use in field studies, (iii) the ability of microbial communities from contaminated deep vadose zones at the DOE Hanford Site to utilize gaseous sources of carbon, nitrogen, and phosphorus and degrade carbon tetrachloride under bulk-phase aerobic conditions, and (iv) gas movement in the unsaturated zone to assist future development of vapor-phase (gas) feeding methodology for vadose zone bioremediation.

**b.** The focus of this project tended to be basic rather than aimed at developing applied technologies in the near-term. This was in part due to the fact that field samples from the Hanford Site deep vadose zone were not made available until 21 months after the project started, in contrast to our original plan to use these samples in the first 6 months of the project. This necessitated major changes in the research objectives of the project. Never the less, portions of this project involved improvement of numerical models to simulate and better understand interacting vadose zone processes, numerical values describing microbial movement and colonization in the vadose zone, and the ability of deep vadose zone communities to utilize gaseous nutrients for vadose zone bioremediation. While the results of this work aid identification of future research and development for vadose zone bioremediation, they will not reduce immediate costs, schedules, and risks relative to DOE compliance requirements.

**c.** While field studies have shown that vapor-phase stimulated bioremediation has potential, its success relies on the appropriate match between substrates and contaminants and specific contaminant-degraders, the selection of moderately volatile compounds which can travel sufficient distances in the vadose zone, and conducive subsurface hydrologic and physical properties. We believe that laboratory studies such as those conducted in this project are a crucial first step before attempting a field-scale remediation using volatile nutrients, and that this research is an important step to bridge the gap between fundamental research and applied technology development.

**d.** As stated in b, this particular project focused on basic research into the development of new methodologies, rather than more applied research which would have an immediate impact. However, we believe that numerous individuals and institutions will utilize outputs of this project including: use of our improved numerical models to simulate and better understand interacting vadose zone processes, numerical values describing microbial movement and colonization in the vadose zone, and the knowledge that (even though microbial populations are low in these environments) gaseous hydrocarbons can be effectively used to grow biomass in Hanford sediments and likely in sediments from other arid environments. These results will be used in the near-term by applied scientists for advancing the modeling and study of vadoze zone processes.

**e.** The 2-dimensional chamber experiments conducted at Oregon State University represent an important capability and expertise for the study of interacting vadoze zone processes. Further studies in such chambers will be critical for building a stronger predictive modeling and interpretive capability, which is necessary for a high probability of success in implementating vadose zone gaseous nutrient feeding strategies in the field.
**f.** Collaborating scientists have gained an increased understanding of gas transport dynamics in the vadose zone, microbial behavior in unsaturated systems, and methods of utilizing light-transmission chambers to understand vadose zone dynamics.
**g.** This research has advanced our understanding of (i) the degree to which microbes migrate and colonize unoccupied pore networks and regions of the vadose zone, (ii) the coupling of hydrologic processes and microbial growth during aqueous delivery of nutrients and developing models of these processes for future use in field studies, (iii) the ability of microbial communities from contaminated deep vadose zones at the DOE Hanford Site to utilize gaseous sources of carbon, nitrogen, and phosphorus and degrade carbon tetrachloride under bulk-phase aerobic conditions, and (iv) gas movement in the unsaturated zone to assist future development of vapor-phase (gas) feeding methodology for vadose zone bioremediation.
**h.** Use of gaseous hydrocarbons to increase microbial populations in situ has great promise in Hanford deep vadose zone sediments. The ability to successfully deliver the gases to the in situ subsurface microsites where microbes exist should be tested in intact Hanford deep vadose zone cores. These studies with intact cores should also further investigate the potential for degradation of carbon tetrachloride and/or other DOE organic contaminants. Additional 2-d chamber experiments to better understand gas transport and hydrological-microbial-gas transport dynamics are also needed before considering field tests.
**i.** Not to date.

**Project Productivity**

The project was successful in producing publications at a normal rate. Three book chapters and three papers were published, and a fourth paper is in press. At least 6 other papers are in preparation. (see below). Because it was a rather high-risk research effort which pursued undeveloped technologies, a number of tactics were unsuccessful, leading to explorations in new directions and revisions of the work plan. Certain efforts which were not originally planned, such as visual imaging of gas flow through the sand chamber, were more successful than initial directives. The project also proceeded more slowly than anticipated, due to the complex nature of the problem, and a high rate of turnover within the personnel. Regardless, the project yielded a greater understanding of unsaturated zone dynamics, as described elsewhere.

**Personnel Supported**

Oregon State University: John Selker (faculty), Mark Rockhold (graduate student), Rockie Yarwood (graduate student), Mike Niemet (technician), Laila Parker (graduate student), Erika Kraft (graduate student), Chris Vick (technician), Nick McGinnis (student worker)

PNNL: Fred Brockman (PI), Shu-mei Li and Andy Plymale (scientists, microbiology), Eric Hoppe (scientist, analytical chemistry), Mark Rockhold (scientist, hydrology), Tina Spadoni (technical specialist); Nisha Kapadia, Ginny Williams, and Thomas Perry (post-Bachelors students).

**Publications**


1. Ainsworth CC, FJ Brockman, and PM Jardine. 2000. Biogeochemical considerations and complexities, pp. 829-923. *In* Vadose Zone Science and Technology Solutions, Eds. BB Looney and RW Falta. Battelle Press, Columbus OH.
2. Kieft TL and FJ Brockman. 2001. Vadose zone microbiology, pp. 141-169. *In* Subsurface Microbial Ecology and Biogeochemistry, Eds. JK Fredrickson and M Fletcher.
3. Brockman FJ, SN Bradley and TL Kieft. 2002. Vadose zone microbiology. *In* Encyclopedia of Environmental Microbiology. John Wiley and Sons, New York.
4. Niemet, M. R., M. L. Rockhold, et al. (2002). "Relationships between gas-liquid interfacial surface area, liquid saturation, and light transmission in variably saturated porous media." Water Resources Research 38(8).
5. Rockhold, M. L., R. R. Yarwood, et al. (2002). "Considerations for modeling bacterial induced changes in hydraulic properties of variably saturated porous media." Advances in Water Resources 25: 477-495.
6. Yarwood, R. R., M. L. Rockhold, et al. (2002). "Noninvasive Quantitative Measurement of Bacterial Growth in Porous Media under Unsaturated-Flow Conditions." Applied and Environmental Microbiology 68(7): 3597-3605.
7. Rockhold, M. L., R. R. Yarwood, et al. (2003). "Modeling interactions between microbial dynamics and transport processes in soils." Vadose Zone Journal (submitted).
8. Rockhold, M.L., R.R. Yarwood, and J.S. Selker. Interactions between microbial dynamics and transport processes in variably saturated porous media, 1. Model development. **In progress**.
9. Rockhold, M.L., R.R. Yarwood, M.R. Niemet, P.J. Bottomley, and J.S. Selker. Interactions between microbial dynamics and transport processes in variably saturated porous media, 2. Observed and simulated results. **In progress**.
10. Brockman FJ, N Kapadia, G Williams, JS Selker, and ML Rockhold. Microbial colonization in unsaturated porous media. **In progress.**
11. Brockman FJ, SW Li, AE Plymale, and E Hoppe. Gaseous hydrocarbon utilization by vadose zone microbial communities under unsaturated conditions. **In progress.**
12. Yarwood RR., ML Rockhold, MR Niemet, FJ Brockman, PJ Bottomley, and JS Selker. Impact of microbial growth on water flow and solute transport in unsaturated porous media. **In progress.**
13. Parker L, RR Yarwood, FJ Brockman, and J.S. Selker. Methodology for real-time visualization of gas transport in unsaturated porous media. **In progress.**

**Interactions**

**a.** Assistance was provided to the U.S. Geological Survey Toxics Program, which was studying far-field migration of $^{14}C\text{-}CO_2$ near a shallow low-level radioactive and mixed waste burial site at the Amargosa Desert Research Site in Nevada. The site had extensive physical characterization but no microbial characterization or USGS microbiologists to help with microbial characterization. Microbial characterization was performed in a dozen borehole samples by this project to address the question of whether the $^{14}C\text{-}CO_2$ was being produced by microbes. The characterization led to the conclusion that degradation was occurring in or below the trench, and not near the far-field borehole. This was an important conclusion that helped direct future USGS research at the site.

**b.** A list of 26 presentations from this project is given below.

Seminars: 10/01 Washington State University (John Selker); 10/01 University of Idaho (John Selker); 11/02 Keynote, Annual Chilean Society of Soil Scientists Meeting, Talca, Chile (John Selker).

**c.** Answered queries (John Selker) from researchers including Karsten Preuss at Lawrence Berkeley National Labs regarding gas transport modeling, and Pengfei Zhang at the University of West Florida regarding imaging methods.

**d.** This research involved collaborations between researchers at Oregon State University and Pacific Northwest National Lab, as well as interdepartmental collaborations at Oregon State University. Dr. Mark Rockhold, a PNNL employee prior to this project, conducted part of his Ph.D. research in Dr. Selker's lab during this project, and also worked on this project after his return to PNNL.

*Presentations*

1. Murray CJ, GP Streile, FJ Brockman, T Scheiebe, and A Chilakapati. Potential effects of the spatial distribution of bacteria on bioremediation in subsurface sediments. International Symposium on Subsurface Microbiology, Vail CO, Aug. 22-27 1999.
2. Rockhold, M.L., R.R. Yarwood, M.R. Niemet, P.J. Bottomley, and J.S. Selker. 1999. Interactions between microbial dynamics, water flow, and transport of biologically reactive solutes in unsaturated porous media: 2. Numerical modeling. Presented at the annual meeting of the Soil Science Society of America, Oct. 31 - Nov. 4, 1999, Salt Lake City, Utah.
3. Rockhold, M.L., R.R. Yarwood, M.R. Niemet, P.J. Bottomley, F. J. Brockman, and J.S. Selker. 1999. Interactions Between Microbial Dynamics and Transport Processes in Variably Saturated Porous Media, 1. Numerical modeling.
4. Brockman FJ, CJ Murray, and J Selker. Understanding engineered vadose zone bioremediation. EMSP PI meeting, Richland WA, Nov. 16-18 1999.
5. Rockhold, M. L., R. R. Yarwood, M. R. Niemet, P. J. Bottomley, and J. S. Selker, Interactions between microbial dynamics, water flow, and transport of biologically reactive solutes in porous media: II. numerical modeling, presented at Soil Science Society of America Annual Meeting, 1999.
6. Yarwood, R. R., M. L. Rockhold, M. R. Niemet, J. S. Selker, and P. J. Bottomley, Interactions between microbial dynamics, water flow, and transport of biologically reactive solutes in porous media: I. experimental system and results, presented at Soil Science Society of America Annual Meeting, 1999.

7. Rockhold, M. R., Interactions between microbial dynamics, water flow and solute transport in soils, presented at the Oregon State University James A. Vomocil Water Quality Conference 2000.
8. Niemet, M. R., Oregon State University's Vadose Visualization Laboratory: A Brief Overview, presented at the Oregon State University James A. Vomocil Water Quality Conference 2000.
9. Brockman FJ, CJ Murray, M Rockhold, G Streile, J Selker, R Yarwood, and P Bottomley. Integrated field, laboratory, and modeling studies to determine the effects of linked microbial and physical spatial heterogeneity on engineered vadose zone bioremediation. EMSP National Workshop, Atlanta, GA, April 24-28 2000. (Talk and poster presentation).
10. Brockman FJ. Potential role of microorganisms in enhanced gas-phase transport of radionuclides. *Invited*, USGS Arid Desert Research Site annual meeting, Menlo Park CA, Aug 24-25 2000.
11. FJ Brockman, J Selker, M Rockhold, R Yarwood, M Niemet, S Bradley, and T Spadoni. Integrated field, laboratory, and modeling studies to determine the effects of linked microbial and physical spatial heterogeneity on engineered vadose zone bioremediation. National EMSP PI meeting, Nov 28-30 2000 (Richland, WA).
12. Niemet, M. R., A new method for quantification of liquid saturation in 2D translucent porous media systems using light transmission, presented at AGU Fall Meeting, San Francisco CA, 2000.
13. SN Bradley, TS Spadoni, and FJ Brockman. Colonization behavior of *Pseudomonas stutzeri* KC in static unsaturated porous media columns. American Society for Microbiology annual meeting, May 20-24 2001 (Orlando, FL).
14. FJ Brockman. Biological considerations for successful vadose zone remediation. Eighth Biannual Unsaturated Zone Interest Group Meeting, July 30-Aug 2 2001 (Idaho Falls, ID).
15. FJ Brockman, M Rockhold, R Yarwood, M Niemet, T Spadoni, S Bradley, and J Selker. Microbial dynamics in unsaturated porous media under static and flow conditions. Abstracts of papers of the American Chemical Society 121-NUCL, Part 2, Aug 2001
16. DA Stonestrom, RL Michel, WC Evans, TR Smith, DE Prudic, RG Striegl, Jr. , H Haas, FJ Brockman, and BJ Andraski. Carbon isotopes in unsaturated-zone gases and groundwater near a radioactive-waste disposal area, Amargosa Desert Research Site, Nye County, Nevada. Eighth Biannual Unsaturated Zone Interest Group Meeting, July 30-Aug 2 2001 (Idaho Falls, ID).
17. Yarwood, R. R., M. L. Rockhold, M. R. Niemet, J. S. Selker, P. J. Bottomley and F. J. Brockman. Interactions Between Microbial Dynamics and Transport Processes in Variably Saturated Porous Media: 1. Experimental Systems and Results. American Geophysical Union annual meeting, Dec 10-14 2001 (San Francisco CA).
18. Rockhold, M. L., RR Yarwood, MR Niemet, PJ Bottomley, JS Selker, and FJ Brockman. Interactions between microbial dynamics and transport processes in variably saturated porous media. 2. Model development and evaluation. American Geophysical Union annual meeting, Dec 10-14 2001 (San Francisco CA).
19. DA Stonestrom, RL Michel, WC Evans, TR Smith, DE Prudic, RG Striegl, Jr., H Haas, FJ Brockman, and BJ Andraski. Carbon isotopes in unsaturated-zone gases and

ground water near a radioactive-waste disposal area, Amargosa Desert Research Site, Nye County, Nevada. American Geophysical Union annual meeting, Dec 10-14 2001 (San Francisco CA).

20. Brockman, FJ. Invited speaker. Hydrologic and chemical controls on vadose zone microbiology, American Geophysical Union annual meeting, Dec 10-14 2001 (San Francisco CA).
21. Brockman, FJ, TC Perry, N Kapadia, ML Rockhold, and JS Selker. Movement of motile bacteria in unsaturated porous media. American Geophysical Union annual meeting, Dec 10-14 2001 (San Francisco CA).
22. Brockman FJ. Invited. The unsaturated zone in arid climates: an extreme environment for microorganisms. Arizona branch of American Society for Microbiology, April 6 2002 (Flagstaff AZ).
23. Brockman FJ. Invited. Microbiology of the vadose zone: Extremophiles to bioremediation. Southeastern branch of the American Society for Microbiology. November 7 2002 (Gainesville, FL).
24. Brockman FJ. Invited. Microbiology of the Vadose Zone: Extremophiles to Bioremediation. April 26, 2003. Alaska Branch ASM meeting. Anchorage AK.
25. Brockman FJ, N Kapadia, G Williams, and M Rockhold. Microcosm experiments and modeling of microbial movement under unsaturated conditions. ASM, May 18-22, 2003, Washington DC
26. Li SW, AE Plymale, and FJ Brockman. Potential for microbial stimulation in deep vadose zone sediments by gas-phase injection. ASM, May 18-22, 2003, Washington DC.

**Transitions**

None

**Patents**

None

**Future Work**

We intend to complete the map of $CO_2$ transport in the chamber based on the available images, the standard curve, and disassociation equations for CO2 in water. Additionally, the modeling software HYDRUS-2D will be used to simulate carbon dioxide movement in the chamber, taking into account textural inclusions. This should complete the gas-tracing task.

Laila Parker and Erika Kraft intend to focus on related issues for their PhD theses. Topics may include analysis of the 'leading edge' effect to explore whether naphthalene retardation is a function of microbial density, how microbial growth affects fluid dynamics, and how species diversity in the microbial community may alter or enhance the methods here described.

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Brigmon, R. L., D. J. Altman, et al. (1998). "Evaluation of Methanotrophic Bacteria during Injection of Gaseous Nutrients for *in situ* Trichloroethylene Bioremediation in a Sanitory Landfill." DOE/WSRC-MS-98-00854 U.S. Department of Energy, Springfield, VA.

Brockman, F. J., W. Payne, et al. (1995). "Effect of gaseous nitrogen and phosphorus injection on *in situ* bioremediation of a trichloroethylene-contaminated site." Journal of Hazardous Materials **41**: 287-298.

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Rockhold, M. L., R. R. Yarwood, et al. (2002). "Considerations for modeling bacterial induced changes in hydraulic properties of variably saturated porous media." Advances in Water Resources **25**: 477-495.

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## Feedback

The project was significantly hampered by schedule problems. When the proposal was written, we had reson to believe field samples from the 216-Z-9 site would be available in the first 6 months of the project. Most of the original research tasks were sequenced based on having these samples at the beginning of the project. Drilling and

samples were not available until 21 months after the project started. This necessitated major changes in the research objectives of the project.

The research described here was more basic than applied and supported the greater understanding of interacting hydrologic and microbial processes in the deep vadose zone, and the possibility of gas-phase stimulation of bioremediation in the vadose zone. The EMSP funding was critical to allow such high-risk research, and the team was allowed, within the logical constraints of contracting and reporting requirements, to follow a natural and productive line of research.

## Appendices

None