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Dissertation:

“Analysis of Bacterial Transport and Survival in the Subsurface”

by

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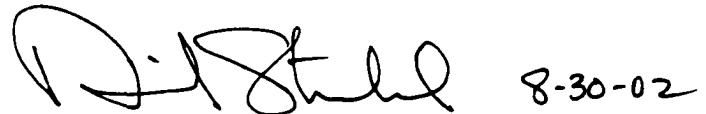
August 31, 2002

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Analysis of Bacterial Transport and Survival in the Subsurface

Advisor: William E. Holben *W. E. H.*

Many subsurface environments across the United States are contaminated from past xenobiotic discharges, much of it too deep and extensive for conventional methods of remediation. Bacteria might be the best option available for the *bioremediation* of deep subsurface contamination because of their potential to travel to distant contaminated locations, and their ability through metabolic activities to potentially detoxify or limit the further migration of contaminants. However, relatively little is known about the transport capabilities of injected microorganisms into the subsurface, nor their ability to colonize, survive and grow once they reach locations of distant contamination. A basic understanding of both processes is therefore necessary to design strategies for the use of bacteria in remediation of subsurface environments.

My dissertation research project consisted of two studies related to the ecology of bacteria in subsurface environments. The first study involved analysis of the microbial community diversity of shale and sandstone rocks located 200 meters below Cerro Negro, New Mexico. The objective was to increase our understanding of the microbial ecology of deep subsurface environments typical of many contaminated sites. The second involved studies of short-term temporal transport of bacteria, in laboratory column experiments and *in situ* injection experiments at a field site in Oyster, Virginia. The objectives were to develop methods of accurately monitoring bacterial transport and to determine the factors that control transport of bacteria in subsurface environments.

In the study of the ecology of the deep subsurface shale/sandstone interface at Cerro Negro, we found that the geochemistry of a site alone is not adequate to predict the types of organisms present. We found a predominance of organisms capable of Fe(III) reduction in an environment where sulfate reducing microbes were expected to dominate based on the geochemistry of the site. Therefore the design of remediation strategies must account for the Fe(III) reducing bacteria. In the Oyster transport study, we were able to demonstrate the *in situ* transport of adherence-deficient microbes, and their subsequent attachment and growth in aquifer sediments, demonstrating that bioremediation using injected microorganisms was feasible for subsurface contamination.

Acknowledgements and Dedication:

I would like to thank Dr. Bill Holben, my advisor, for giving me the opportunity to work on two very interesting projects. My committee members, Dr. Jim Gannon, Dr. Jim Fredrickson, Dr. Scott Samuels and Dr. Bill Woessner are also acknowledged for their constructive input and suggestions regarding my dissertation and research. Dr. Tom Kieft of New Mexico Tech is thanked for valuable advice over the last seven years or so. Karen and Lydia Schmidt are also thanked for making my last two years at the University of Montana more enjoyable and meaningful.

This dissertation is dedicated to my entire family, especially my Mother and Father, Mary Jane Kovacik and Dr. William P. Kovacik Sr. of Greensburg, Pennsylvania. Without their constant and unwavering support, this dissertation would not have been possible.

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Introduction

My dissertation research was funded by the Natural and Accelerated Bioremediation Research (NABIR) Program of the United States Department of Energy (DOE). The NABIR program is a ten-year study initiated in 1996 to determine the feasibility of using microorganisms for *in situ* bioremediation of contaminated soils, sediments and groundwater at DOE facilities (reference: <http://www.lbl.gov/NABIR/info.html>). The DOE manages over 100 sites across the U.S. that have contamination problems from past subsurface discharges. Hanford, Washington and Oak Ridge, Tennessee, are examples of especially problematic sites for the DOE. Both were created during World War II and used for development of components used in the first atomic bombs. The sites have subsurface contamination plumes that are mixtures of toxic and radioactive xenobiotics that have migrated deep into the subsurface (30). In such environments classical methods of remediation (for example, pump and treat technologies) will likely not be effective. The sites are also often located atop consolidated rock formations where preferential fracture-flow tends to complicate the transport of contaminants. The impending threats to nearby groundwater aquifers and surface waters (e.g. the Columbia River and the Tennessee River) serve to underscore the need for prompt and effective cleanup of the sites.

The DOE's interest in bacteria and bacterial transport is based on the potential of using bacteria for *in situ* bioremediation of contaminated subsurface sites. Bacteria have the ability to directly influence the transport of contaminants in the environment, either positively or negatively (9, 10, 24). Using metals as electron acceptors, microbes can change the redox potential of a contaminant, either accelerating or retarding transport (5, 26, 31). Microbes can also degrade or transform many organic compounds, either increasing or decreasing their

toxicity (1, 11, 12). Additionally, microbes can sorb contaminants to extracellular biofilms, thereby immobilizing them (4, 14, 21), or can chelate limiting nutrients, organics and metals making them more mobile (3, 18, 19). Studies describing the microbial reduction of uranium (25), and solubilization of plutonium hydroxide(32), both by iron/sulfur-reducing bacteria, demonstrate that radioactive compounds can be affected as well. Thus microbial transport and activities in the subsurface environment have important implications for DOE-managed contaminated sites.

In order to effectively use bacteria for *in situ* remediation of subsurface environments, fundamental questions regarding bacterial transport and survival need to be addressed. One question is whether injected bacteria can establish viable long-term communities in the environment. It would be problematic if xenobiotic-degrading bacteria with good transport characteristics were injected into the subsurface, but were not able to establish long-standing communities at the site of contamination. Thus, an understanding of the ecology of bacterial communities in the deep subsurface is an important consideration in remediation plans.

Research funded by the DOE's Office of Health and Environmental Research (OHER) Deep Microbiology Subsurface Science Program has addressed the questions of the origin and survival of bacteria in the deep subsurface. Two main alternate hypotheses were proposed to explain the origins of deep subsurface bacteria; 1) in environments of sedimentary origin, the bacteria present at depth arrived with the original deposition of the formations; 2) the microbes at depth were transported to the current formations from elsewhere at a later geological time. The Cerro Negro site in northwestern New Mexico was chosen by the DOE to address these hypotheses.

The lithology below Cerro Negro consists of alternating sandstone and shale sedimentary formations that were deposited during the Late Cretaceous Period nearly 100 million years ago when a shallow sea covered much of New Mexico. The shale layers consist of ocean floor sediment that was compressed and diagenized over time, forming layers with restrictive porosity that are high in organic carbon content. The sandstone layers were formed from compressed sand and have a less-restrictive porosity, providing a path for water flow and, potentially, bacterial transport. The pore throat diameter of the Clay Mesa Shale averages between 0.01 and 0.20 μm diameter (15), smaller than most known bacteria under starvation conditions which average 0.5-1.0 μm (23). It is thought that the interfaces between the shale and sandstone formations might provide a suitable environment for the long-term survival of microbes in the subsurface (9, 27, 31). One interesting hypothesis is that at least some of the bacteria present in the consolidated rock formations at depth below Cerro Negro are direct descendents of original colonizers dating to the Cretaceous Period.

Another fundamental question regarding the use of bacteria for remediation of subsurface contamination is whether bacteria injected into the subsurface can migrate to contaminated areas in sufficient numbers to be effective. Studies in laboratory columns with subsurface sediments have shown that numerous factors potentially influence the transport of bacteria. Included are the pH and ionic strength of groundwater (10, 27, 33), the presence of organics or aluminum/iron hydroxide coatings on minerals which may increase bacterial retention (13, 15, 27, 29). The size and nutritional status of the bacteria (16), the surface charge and hydrophobicity of the bacteria (7), the grain size and distribution of the subsurface media (size/filtration theory) (10, 15, 33), and bacterial motility (6, 10, 15, 19, 22, 23, 33). Studies have also demonstrated both non-reversible and reversible types of bacterial adsorption

to solid substrates related to water flow velocity (23, 33), the abundance of bacteria (3), and as a function of the residence time of bacteria in the system (17, 20).

Even less is known about the *in situ* transport behavior of bacteria in the subsurface, where many of the above factors likely come into play simultaneously. An injection of a stained, indigenous mixed collection of bacterial cells into a shallow, sandy, freshwater aquifer in Cape Cod, Massachusetts showed that DAPI-stained bacteria had similar breakthrough patterns to conservative Br⁻ tracers (bacterial C/Co~0.1), but, in general, had longer "tails" of breakthrough persisting tens of meters down-gradient (2). Although the bacteria traveled in a fairly narrow plume within the aquifer, the exact mechanisms affecting transport, and a determination of the specific types of bacteria which transported well, were not determined. There was also concern that the use of the DNA-binding dye DAPI to facilitate tracking had altered the viability and behavior of the bacteria that were being monitored. A preliminary subsurface injection into a shallow sandy aquifer at Oyster, Virginia with PL2W31 bacteria, an apparently low-adhesion bacteria indigenous to the site, showed that most (>99 %) bacteria were retained in the aquifer sediments within 0.5 meters of the injection point (8). Clearly, our ability to predict and model bacterial transport behavior based on the current state of knowledge is limited. Additional *in situ* bacterial transport experiments are necessary to help elucidate this complex and interesting phenomenon.

The Oyster, Virginia research site offers the opportunity to test specific factors which influence bacterial transport in the subsurface. The site is located on the southern tip of the DelMarVa (Delaware/Maryland/Virginia) peninsula and is owned by the Nature Conservatory of Virginia. It was chosen for a series of *in situ* injection experiments because of its physicochemical features comprised of a relatively homogeneous subsurface sandy aquifer,

consisting of unconsolidated to weakly-cemented, well-sorted, medium-to-fine-grained Late Pleistocene sands (reference: <http://www.lbl.gov/NABIR/info.html>). The site contains both aerobic and anaerobic flow fields for analysis of bacterial transport under conditions of altered iron chemistry. The physicochemical characteristics of the site have been extensively characterized by various investigators. Geochemical techniques were used to analyze groundwater and sediment core chemistry, and geophysical techniques like Ground Penetrating Radar (GPR) and Cross-Borehole Tomography (CBT) were used to derive a 3-dimensional image of the physicochemical parameters of the flow fields and to predict high and low permeability zones.

The aerobic flow field (designated NC for Narrow Channel Focus Area) is in a saturated subsurface zone with a consistent water table located between 3-6 meters below the surface, and has dissolved oxygen concentrations between 6 and 8 mg/L (28). The flow cell is comprised of relatively homogeneous and well-sorted sand with medium-sized grains composed mainly of quartz minerals (8). Clay minerals were not abundant at the site and organic carbon content was generally less than 0.5% by weight (8). Geophysical analyses of the flowfield have identified a potential zone of high conductivity based on larger pebble-sized grains. Veins of iron oxyhydroxides, which have been proposed to bind microbes and inhibit transport (24, 27) are the only potentially complicating factor identified in an otherwise homogeneous environment. The anaerobic lower flowfield (designated SOFA for South Oyster Focus Area) is more complex than the aerobic flowfield. The SOFA flow field is comprised of peat and clayey-silt organic layers in the upper regions of the aquifer, and sandy layers below, which are similar in lithology to the NC field, but with lower oxygen tensions (anoxic), presumably due to heterotrophic microbial activity in the organic layers.

Specific requirements were established by the Virginia Nature Conservatory to govern a bacterial injection at the Oyster site. The microbes used must be indigenous to the site, could not be radio-labeled, could not be genetically engineered, and could not have resistance to common clinical antibiotics (e.g. penicillin and tetracycline). Quantitative Polymerase Chain Reaction (qPCR) of genomic 16S ribosomal DNA (rDNA) was chosen as a method for bacterial enumeration because it is specific, quick and relatively inexpensive. The qPCR approach developed here was used to analyze the transport potentials of two "non-sticky" or adhesion-deficient bacterial (strains OY107 and DA001) isolated from the Oyster site.

Specific Research Goals

The specific goals of my dissertation research, as presented in my thesis dissertation proposal submitted to the Committee on December 4, 2000, were as follows:

I. Analysis of the subsurface bacterial community of Cerro Negro, New Mexico

Goal 1: Analysis of bacterial community structure and diversity of a sandstone/shale interface approximately 200 m below Cerro Negro, New Mexico, using molecular biological techniques.

Goal 2: Comparison of microbial diversity to physical, chemical, geologic and hydrological characterization of the site, to enhance our understanding of the ecology of deep subsurface microbial environments.

Goal 3: Phylogenetic analysis of DNA sequence data to assess whether the current bacterial community represents survival of the ancient marine sediment community since the time of deposition millions of years ago.

II. Bacterial transport in shallow subsurface aquifers

Goal 1: Develop gel-based and real-time fluorogenic qPCR approaches for accurately and specifically quantifying the transport of bacteria in subsurface environments.

Goal 2: Validation of qPCR versus other methods of quantification and detection in laboratory column experiments, including radioactive labeling, direct microscopic enumeration and culturable plate counts.

Goal 3: Use qPCR to monitor the transport of injected bacteria in *in situ* subsurface injection experiments into an aquifer at the field site in Oyster, Virginia.

Goal 4: Integration of microbiological results to the physical, chemical, geological and hydrological characterization data of the Oyster site to determine factors which influence the transport of bacteria.

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Preface to Research Chapters, Research Contribution:

Chapter 2 represents the results of our study of the deep subsurface shale/sandstone interface located 200 m below ground near Cerro Negro, New Mexico. The results represent a research collaboration of our laboratory (Dr. Holben) and that of Dr. James Fredrickson at Pacific Northwest National Laboratory (PNNL) in Richland, Washington. These results were submitted to the peer-reviewed journal *Applied and Environmental Microbiology* on (May 5, 2002). My contributions to that manuscript included: total community DNA isolation, PCR amplification using universal and iron/sulfur reducer-specific primers, DGGE analysis, cloning of DNA from DGGE bands for identification, generating approximately 10% of the total clones from the shotgun clone analysis and primary responsibility for writing of the manuscript on which I am lead author. Ken Takai and Melanie Mormile of PNNL performed the remainder of the shotgun cloning, the enrichment culture experiment and the RNA hybridization analysis. The chapter herein is formatted as submitted for publication.

Chapter 3 describes the development of the quantitative PCR procedures necessary for the monitoring of bacterial transport in support of column experiments and *in situ* injection experiments at the Oyster Virginia research site. This aspect of the research was performed primarily by me and represented a large portion of my total effort at the University of Montana. Similar to Chapter 2, Chapter 3 is presented as a manuscript submitted for publication with me as first author. This paper is now in the final stages of revision with Dr. Holben and will be submitted for publication to *Applied and Environmental Microbiology*.

Chapter 4 represents the culmination of four years of research involvement with the U.S. Department of Energy NABIR transport study at Oyster, Virginia. This was a large, multi-million dollar project involving many diverse research groups from around the country. At a DOE subsurface meeting in April 2002 in Virginia, it was decided that the Holben lab will take the lead role on a major publication resulting from that work based on our ability to use qPCR to relate aqueous bacterial numbers to post-injection sediment attachment rates. The paper integrates bacterial transport data from both the aqueous and solid phase (based on qPCR) with the physical and chemical characterization data from the site to determine controlling factors on bacterial transport. I will have the lead effort in writing this manuscript and will thus be lead author on this paper.

Much of the data from these experiments has only very recently become available and there major chemical and organic analysis of sediments yet to be done in other laboratories. An extensive multivariate statistical analysis is also planned at PNNL (after all data is available) to add significance to our results. Because of this, Chapter 4 of this thesis describes only my contributions to this multidisciplinary study, most notably qPCR analysis of water and sediment samples from the Oyster 2000 injection experiments. Ultimately this chapter will be expanded to become the complete manuscript described above and submitted for publication to a major journal, possibly to Science, Groundwater Research or Environmental Science and Technology.

Chapter 2: Analysis of the Microbial Community of a Shale/Sandstone

Interface 200 m below Cerro Negro, New Mexico

Molecular Analysis of Deep Subsurface Cretaceous Rock Reveals an Abundance of Fe(III)- and S⁰-Reducing Bacteria in a Sulfate-Rich Environment

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Running title: Microbial community analysis in Cretaceous rock

Keywords: Subsurface; 16S rDNA; 16S rRNA; DGGE; Community structure; Iron reducing bacteria; Sulfate- and Sulfur- reducing bacteria

Submitted to: Applied and Environmental Microbiology

For the section Geomicrobiology or General Microbial Ecology

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ABSTRACT

To analyze this subsurface microbial community, a multi-level sampler was emplaced in a borehole straddling Cretaceous-era shale and sandstone rock formations ~200 m below ground surface at Cerro Negro, New Mexico. Sterile quartzite sand in the MLS allowed *in situ* colonization in this anaerobic, sulfate-rich environment. Microbial community nucleic acids were subsequently recovered and analyzed by a suite of molecular methods and enrichment cultures for select phenotypes were established. DGGE fingerprinting was used to assess diversity and compare community structure between samples. Partial 16S rDNA gene cloning and sequence analysis was performed to survey the bacterial species present. Quantitative RNA hybridization and culture-based enrichments were used to probe community metabolic function. DGGE and rDNA gene cloning results indicated a relatively homogeneous bacterial community across the shale/sandstone interface. Based on closest-match analysis, α -Proteobacteria sequences were common at all depths, and were dominated by members of the *Geobacteraceae* family (*Pelobacter*, *Desulfuromonas*, and *Geobacter*). Other members of this group are capable of dissimilatory Fe(III) and/or S⁰ reduction, but not sulfate reduction. RNA hybridization data also suggested that Fe(III)/S⁰ reducing bacteria were predominant. Lack of significant concentrations of these electron acceptors suggests that these organisms may be growing via non-respiratory metabolism, possibly in syntrophic association with SRB or methanogens. The next most abundant bacterial group was the sulfate reducers, including *Desulfobacterium*, *Desulfocapsa* and *Desulfobulbus*. The presence of a phylogenetically and functionally diverse microbial community likely reflects the primary energy and carbon source for microbial metabolism in this subsurface environment, complex kerogen associated with the shale.

INTRODUCTION

Numerous reports within the last decade have demonstrated the presence of a diverse array of active microbial populations and communities in a variety of deep subsurface environments and suggested their importance in biogeochemical cycling and other activities (11, 15, 23, 26, 27, 31, 35, 36, 49, 52, 58, 91). These subsurface environments and their resident communities are of interest not only because of their unique nature, their lack of direct reliance on solar radiation, and the fact that they represent a large portion of the total environment available on earth, but also because subsurface environments represent perhaps the most likely location for the origin or persistence of life on other planets (12, 26). Among the many deep subsurface environments harboring microorganisms on earth, highly consolidated sediments (i.e. sedimentary rocks), some of which are comprised of highly restrictive pore spaces, are particularly intriguing because the microbes present potentially represent remnants of ancient microbial communities laid down with the original deposits (10, 16, 17, 23, 24).

In this study, the microbial community present in >90 million year old sedimentary rocks located ~200 meters below Cerro Negro, New Mexico was investigated using DNA- and RNA-based molecular analyses. The lithology at depth consists of well-defined alternating sandstone and shale sedimentary formations deposited during the late Cretaceous period when a shallow inland sea advanced and receded several times across the area (48) (Fig. 1). The shale intervals were once near-shore ocean sediment environments where anaerobic decomposition of organic material from ocean detritus occurred. The sediments were subsequently buried and diagenized over geologic time into highly consolidated rock material with low porosity (average pore

size < 0.1 μm), which severely limits water movement and microbial access within the interval (24, 93). The sandstone layers which alternate with the shale layers were formed from compressed sand and have a less-restrictive porosity (average pore size = 1 - 6 μm), providing a potential path for water and nutrient flow (24).

It has been proposed that the interfaces between shale and sandstone formations provide a suitable environment for long-term survival of microbes in such subsurface environments (16, 17, 24, 33, 35, 47). The rationale at Cerro Negro is that complex organic matter dating to the Cretaceous period is slowly being leached from the diagenized Clay Mesa Shale formation into the adjacent Cubero Sandstone formation where increased porosity and water movement provide nutrients and electron acceptors needed for microbial growth (24, 33, 35). This organic material could then be broken down by the successive action of various types of fermentative bacteria into organic acids, alcohols, H_2 and CO_2 . Based on the abundance of dissolved sulfate in the groundwater (80 - 370 mg/l), it was hypothesized that sulfate reducing bacteria (SRB) were the likely terminal degraders in the system, oxidizing the organic acids, alcohols and H_2 while reducing sulfate to H_2S or HS^- .

Prior research on intact rock cores taken from the site revealed an anaerobic environment with dissolved oxygen levels below detection and high levels of dissolved sulfate (24). Using a silver foil assay for ^{35}S -sulfate reduction on freshly fractured rock cores, discrete metabolically-active SRB communities were found *in situ* in the rock material of both the Cubero Sandstone and Clay Mesa Shale formations, although the majority of sulfate reducing activity was seen in sandstone formations located adjacent to the shales (35). Estimates of viable microbial biomass based on total phospholipid fatty

acids (PLFA) were consistent with those from other deep subsurface fluvial sand environments, with calculated bacterial densities ranging from 3.5×10^5 to 2.5×10^6 cells/g in the Cubero Sandstone and Clay Mesa Shale formations (35).

The purpose of the current study was to employ direct DNA- and RNA-based approaches to investigate the microbial community present at the shale/sandstone interface below Cerro Negro, and to determine whether SRB indeed represent the predominant terminal degraders. Although other investigators have applied activity- and culture-based approaches to study subsurface microbial communities at this site and elsewhere, it was expected that molecular approaches might provide additional insights regarding the ecology of this system based on direct analysis of community structure, diversity, and phylogeny.

Due to the relatively low biomass of bacteria in the Cerro Negro rock formations, a multilevel sampler (MLS) was placed into a vertical borehole straddling the interface between the Clay Mesa Shale and Cubero Sandstone formations 182 - 192 m below ground surface (bgs). The MLS included discrete chambers containing sterile quartzite sand to provide substantial surface area to be colonized by the microbes at depth. Total bacterial DNA and RNA were subsequently extracted from the colonized sand for molecular analysis by denaturing gradient gel electrophoresis (DGGE), cloning and sequencing of partial 16S rDNA genes, and quantitative RNA hybridization with oligonucleotide probes. Additionally, enrichment cultures were established for fermentative bacteria, SRB, and denitrifying bacteria to determine the relative abundance of the culturable component of these major functional groups.

The phylogenetic data obtained were interpreted in the framework of the existing physical and chemical data from the Cerro Negro site. There were clear examples where geochemical parameters matched the phylogenetic and functional capabilities of bacteria identified by our analyses. For example, the distribution of SRB organisms and activity correlated with sulfate and sulfide levels in the pore water. An unanticipated result of the molecular analyses was the predominance of organisms potentially capable of dissimilatory Fe(III)/S⁰ reduction in this sulfate-rich environment lacking apparent S⁰ or ferric iron. Possible explanations for this apparent paradox are considered and discussed.

MATERIALS AND METHODS

Sampling site and procedures. Groundwater samples and microbial community samples were obtained using a passive multi-level sampler (MLS) as previously described (94). The MLS was placed across the interface between the Clay Mesa Shale and Cubero Sandstone intervals (182 m - 192 m bgs) in a borehole designated as CNV-R, drilled through the Cretaceous Mancos Shale and Dakota Sandstone Formations at Cerro Negro in the southern San Juan Basin of New Mexico (24, 35). The MLS was comprised of discrete dialysis membrane-enclosed cells containing either deionized water for geochemistry samples, or a mixture of deionized water and sterile 1 mm quartzite sand (Accusand, Unimin Corp. New Canaan, CT) for microbiology samples. The sand was washed twice with 1 N HCl, rinsed extensively with deionized water, and then autoclaved twice prior to packing in the MLS cells. The MLS was emplaced and equilibrated *in situ* for 6 months prior to sampling to allow for equilibration with formation water and to provide sufficient time for microbial colonization of the sand matrix. After removal of

the MLS from the borehole, sand samples were recovered and immediately frozen on dry ice in the field, then maintained at -80°C prior to analysis. Subsamples for enrichments were collected and placed on ice prior to freezing the bulk sample.

Extraction and purification of nucleic acids. DNA was extracted from 10 g of each sand sample using the Soil DNA Kit Mega Prep (MO BIO Laboratories, Inc., Solana Beach, CA) using the manufacturer's suggested protocol. Ribonuclease A (Sigma, St. Louis, MO) was added to the preparations (0.02% w/v), which were then incubated at 37°C for 3 hours to destroy any contaminating RNA. These mixtures were subsequently extracted with an equal volume of phenol saturated with 100 mM Tris-HCl (pH 8.0), followed by sequential extraction with equal volumes of phenol/chloroform/isoamyl alcohol (24:24:1, v/v/v), and chloroform/isoamyl alcohol (24:1, v/v). DNA was precipitated from the resulting solutions using a 3X volume of ethanol in the presence of 0.3 M ammonium acetate and recovered by centrifugation using established techniques (65). To provide a negative control for potential contaminants in subsequent molecular analyses, a blank (no sand) sample was also extracted in the same manner.

Total RNA was recovered from replicate aliquots of the same samples used for DNA extraction. All plasticware, glassware and solutions used for RNA extraction and purification were treated with 0.1% DEPC to inactivate nucleases. Ten g of each sample was suspended in 7.0 ml of extraction buffer containing: 25 mM sodium acetate (pH 5.0); 5 mM EDTA; and 5% (w/v) SDS to which 5 g of sterile glass beads (0.1 mm diameter, Sigma) and 7.0 ml of phenol/chloroform/isoamyl alcohol (24:24:1) equilibrated with extraction buffer was added. The mixture was shaken on a bead-beater (BioSpec

Products, Inc., Bartlesville, OK) for 2 min, then incubated at 60 °C for 1 h, after which the bead-beating treatment was repeated. The resulting lysate was cleared by centrifugation at room temperature, then extracted with an equal volume of chloroform/isoamyl alcohol. RNA was precipitated by adding 10 M ammonium acetate, isopropyl alcohol, then incubating at -20°C, and recovered by centrifugation as previously (85). The resulting pellet was washed with 70% (v/v) ethanol, dried, and then dissolved in dH₂O. RNA was further purified using the RNeasy Midi Kit (Qiagen, Valencia, CA) according to the manufacturer's directions. Concentrations of purified RNA and DNA solutions were determined using a spectrophotometer.

Microscopic enumeration of bacteria. Microbiological samples were fixed for 12 h in 3.7% formaldehyde, then filtered through sterile 0.22 µm, irgalan-stained, 13 mm-diameter polycarbonate filters (Millipore, Bedford, MA). The filter towers and filters were rinsed twice with dH₂O, and the cells stained by treatment with dH₂O containing acridine orange (10 µg/ml) at 4 °C for 20 min. The filters were briefly rinsed with dH₂O then examined by epifluorescence microscopy to enumerate bacterial cells.

Enrichment cultures. Anaerobic enrichment cultures for organisms capable of sulfate reduction, denitrification and fermentation were set up and incubated in an anaerobic glove-bag containing a 85:20:5 mixture of N₂, CO₂, and H₂, with O₂ removed by palladium catalysts. One gram samples of MLS sand were inoculated into 15 ml of enrichment media, and four, ten-fold serial dilutions performed in the same media to estimate cell numbers. The enrichment medium contained (per l): KH₂PO₄, 54 mg; K₂HPO₄, 70 mg; CaCl₂-2H₂O, 15 mg; MgCl₂-6H₂O, 20 mg; FeSO₄-7H₂O, 5 mg; Na₂SO₄, 5 mg; MnCl₂-4H₂O, 1 mg; H₃BO₃, 0.1 mg; ZnCl₂, 0.1 mg; CoCl₂-2H₂O, 0.1

mg; NiCl₂·6H₂O, 0.1 mg; CuCl₂, 0.06 mg; Na₂MoO₄·2H₂O, 0.02 mg; NH₄Cl, 1000 mg; glucose, 500 mg; casamino acids, 500 mg; sodium lactate, 1700 mg; sodium pyruvate, 500 mg; sodium acetate, 500 mg; sodium formate, 500 mg; Na₂S·7H₂O, 100 mg; Na₂HCO₃, 240 mg. The medium was then adjusted to pH 7.5 with NaOH. For sulfate-reducers, the media was amended with 2.0 g/l Na₂SO₄. Enrichment media were prepared under strictly anaerobic conditions as described previously (80), and dispensed into 25 ml anaerobic culture tubes (Bellco Glass, Vineland, NJ). Inoculated tubes were incubated in the dark at 20 – 24°C for up to 6 months. Turbidity in a specific tube was considered as a presumptive indicator for that type of bacterial activity.

The confirmatory test for SRB was conducted by extracting and measuring acid-volatile sulfide (AVS) from subsamples of the enrichment media as described previously (46). Formation of sulfide in excess of uninoculated controls was considered presumptive evidence of the presence of SRB. The presumptive test for denitrifiers employed the diphenylamine assay (89). If turbidity was observed in an enrichment tube, but confirmatory tests for SRB or nitrifiers were negative, the tube was assumed to be positive for fermentative organisms.

Denaturing gradient gel electrophoresis (DGGE). Total DNA samples were PCR-amplified using a touchdown PCR protocol (49) employing universal primers 536fc and 907r (37, 78 and Table 1). PCR reaction conditions were as follows: initial denaturation at 95°C for 5 min; followed by twenty cycles of 95°C for 45 sec, 65°C for 45 sec (decreasing 0.5°C/cycle) and 72°C for 1.5 min; followed by 10 cycles of 95°C for 45 sec, 55°C for 1.5 min and 72°C for 1.5 min. DGGE analysis was performed using a D-GENE System (Bio-Rad Laboratories, Hercules, CA) in a 14 x 14 cm format. For

these analyses, 12% acrylamide gels with a 45 - 60% gradient of urea/formamide as denaturant (100% = 42 g urea + 40 ml formamide/100 ml solution) were cast using a Hoefer SG series gradient former (Hoefer Scientific, San Francisco, CA). DGGE gels were run for 1600 Volt-hours at 70 - 100V at 60°C. Following electrophoresis, gels were stained with 5X Sybergreen I (FMC BioProducts, Rockland, ME) for 1 h at 37°C, then visualized and digitized under UV illumination using a Gel-Doc 1000 image capture system (Bio-Rad Laboratories).

Cloning and phylogenetic analysis of DGGE bands. Individual DGGE bands were recovered and analyzed using modifications of the protocol described by Sanguinetti (66). Briefly, the gel was placed on an Ultra-Lum UV transilluminator (Ultra-Lum Corp., Carson, CA) to visualize the DNA bands which were excised with a flame-sterilized razor blade and transferred to sterile 1.5 ml microcentrifuge tubes. The excised gel was thoroughly macerated with a flame-sterilized spatula, then 100 μ l of gel elution buffer (50 mM KCl; 10 mM Tris, pH 9.0; 0.1% Triton X-100) was added and the gel macerated for an additional 15 seconds. The resulting mixture was incubated overnight at 37°C, after which residual acrylamide was pelleted by centrifugation at 16,000 x g for 30 seconds. The supernatant was transferred to a sterile 1.5 ml microcentrifuge tube, purified by phenol extraction and subjected to ethanol precipitation using established protocols (65). The resulting DNA was resuspended in 20 μ l of sterile dH₂O and stored at -20 °C prior to use.

For cloning, 15 ng of DNA was ligated into the pT7Blue-3 vector and transformed into Novablue cells using the Perfectly Blunt Cloning Kit (Novagen Corp. Madison, WI) according to the manufacturer's recommendations. Plasmid DNA from

putative clones (identified by blue-white selection of colonies) was purified using QIAprep spin columns (QiaGen Corp., Valencia, CA) and screened to confirm the presence of correctly sized inserts by restriction digest analysis (65). DNA from each clone was reamplified and analyzed by DGGE to confirm clone correctness and position. Confirmed clones were then subjected to bi-directional DNA sequence analysis (MWG Biotech, High Point, NC). Potential chimeras were identified using the Chimera check function of the Ribosomal Database Project II website (RDP-II; <http://www.cme.msu.edu/RDP/>) (45) and were not considered further. Identification of the nearest known bacterial relative was performed using the Sequence Match function of the RDP II website.

Phylogenetic trees of fully aligned sequences were generated using BioNumerics ver. 2.0 software (Applied Maths, Kortrijk, Belgium). The sequences were clustered using the Neighbor Joining, Maximum Parsimony and Maximum Likelihood algorithms and Jukes and Cantor correction. The consensus tree from each algorithm was subsequently bootstrapped 1000 times.

DNA sequence analysis from MLS sand. Eubacterial rDNA was amplified by PCR using LA Taq polymerase (TaKaRa, Kyoto, Japan) and the primers Bac27f and Bac1392 (Table 1). Except for the primers, PCR reaction mixtures were essentially as described previously (86), and PCR reaction conditions were 35 cycles of: denaturation at 96°C for 25 sec, annealing at 50°C for 45 sec, and extension at 72°C for 120 sec. The resulting rDNA amplicons were cloned into vector pCR2.1 using the Original TA cloning kit (Invitrogen, Carlsbad, CA). Clones of appropriate size were identified by direct PCR analysis from picked colonies using M13 primers as described (86). The amplified

inserts were subsequently treated with exonuclease I and shrimp alkaline phosphatase (Amersham Pharmacia Biotech, Buckinghamshire, UK), then directly sequenced by the dideoxynucleotide chain-termination method using a Big Dye sequencing kit (ABI, Foster City, CA). The 907r primer (Table 1) was used to obtain single-stranded sequences for this broad phylogenetic survey. The resulting sequences were compared to other known rDNA sequences by BLAST analysis of the prokaryotic SSU rRNA database and the non-redundant nucleotide sequence database from GenBank, EMBL and DDBJ (www.ncbi.nlm.nih.gov; www.embl-heidelberg.de/; and www.ddbj.nig.ac.jp, respectively).

The affiliation of individual rDNA sequences to known phylogenetic groups was obtained using the gapped-BLAST and the SUGGEST_TREE program of the RDP II website. Individual sequences that had $\geq 99\%$ similarity by gapped-BLAST to a database sequence were assigned to the phylogenetic group of that sequence, while rDNA sequences having $< 99\%$ similarity to sequences in the RDP II database were assigned to related groups using the SUGGEST_TREE program of the RDP II website. Where rDNA sequences were included within a monophyletic cluster of database sequences by SUGGEST_TREE, the obtained rDNA clone was classified as a member of that cluster.

Northern hybridization analysis. A dilution series of RNA samples (1, 0.5 and 0.1 ng/ μ l) was denatured at 100°C for 10 min, then quickly cooled on ice. Denatured RNA samples were spotted onto Hybond-N+ nylon membranes (Amersham Pharmacia Biotech Inc., Piscataway, NJ) and UV cross-linked by exposure to 120 mJ of UV light energy with a Stratalinker 1800 (Stratagene, Torrey Pines, CA). All oligonucleotide probes (2, 4 and Table 1) were 5'-labeled with digoxigenin by the supplier (Midland

Certified Reagent Company, Midland, TX). The specificity of the probes was checked using the Probe Match function of the RDP-II and the gapped-BLAST search algorithm (3, 7) to examine whether non-targeted rDNA sequences were similar to the probe sequences. Hybridization and wash conditions were empirically optimized for each probe and defined as those giving the highest signal to the intended targets while minimizing cross-reactivity to sequences from other functional or phylogenetic groups using corresponding rDNA from known reference organisms prepared by PCR.

Northern hybridization was conducted overnight in hybridization buffer (750 mM NaCl; 75 mM sodium citrate, pH 7.0; 0.02% (w/v) SDS; 0.1% (w/v) sodium-lauroylsarcosine; and 2% (w/v) blocking reagent (Boehringer Mannheim, Indianapolis, ID)) at empirically-derived optimal hybridization temperatures (Table 1). Following hybridization, filters were washed twice for 5 min at room temperature with wash buffer I (300 mM NaCl; 30 mM sodium citrate, pH 7.0; 0.1 % SDS), and then washed twice at the optimized wash temperature (Table 1) for 30 min with wash buffer II (15 mM NaCl; 1.5 mM sodium citrate, pH 7.0; 0.1 % SDS). Hybridization signals were quantified using the DIG luminescent detection kit and a Lumi-imager F1 detection system (Boehringer Mannheim, Indianapolis, ID).

Genbank accession numbers. The twelve sequences obtained in this study that were used in the phylogenetic analysis (Fig. 4) have been deposited in GenBank under accession numbers xxxxx to xxxxx (submission in process at time of submission).

RESULTS

Geochemical characterization. MLS samples were obtained from the interface between the Clay Mesa Shale and Cubero Sandstone formations in borehole CNV-R (24, 35) following 6 months of *in situ* incubation. Prior geochemical characterization of original core samples collected during the drilling of CNV-R indicated that the fine-grained shale intervals contained higher amounts of organic carbon and sulfur (pyritic S), while the sandstone intervals were relatively coarse-grained and contained lower amounts of organic carbon and total sulfur (Fig. 1 and (24)). The average pore size in the Clay Mesa Shale interval ranged from 0.1 to 0.01 μm , while that in the Cubero Sandstone was generally $>1 \mu\text{m}$. The transition zone between the shale and sandstone was at 185 to 186 m bgs. Sulfate concentrations in MLS pore water ranged from 10 - 20 mg/l in the Clay Mesa Shale to 20 - 70 mg/l in the Cubero Sandstone (Fig. 2). The highest levels of sulfate were observed in the sandstone interval adjacent to the shale/sandstone interface. Sulfide levels were highest between 187 and 190 meters bgs, a region of the Cubero Sandstone also depleted in sulfate (Fig. 2). Soluble nitrate levels were generally near the limit of detection (0.01 - 0.05 mg/l), although two localized peaks (~ 0.4 mg/l) were detected at 184.9 m bgs (at the shale/sandstone interface) and 187.6 m bgs. Soluble iron and manganese species were below detection (data not shown).

Microbial population densities and nucleic acid extraction. Microbial population densities, determined by AODC, were relatively constant throughout the profile, at approximately 2×10^6 cells/g wet weight (Table 2). Total DNA and RNA yields ranged from 1 - 3 ng/g wet weight. Based on DNA yields from each sample, and assuming an average cellular DNA content of 2 fg (6), the estimated microbial population

densities of the MLS samples ranged from $0.8 - 1.2 \times 10^6$ cells/g wet weight, and thus were in good agreement with population densities determined by AODC.

Enrichment cultures. Based on geochemical characterization of the Cerro Negro formations, bacterial enrichment cultures for physiotypes expected to inhabit the formations were established. Enrichments for fermentative, sulfate-reducing, and denitrifying bacteria were performed to estimate the relative abundance of culturable organisms with these physiotypes colonizing the MLS samples. Organisms capable of fermentation, sulfate reduction and denitrification were found at all intervals tested (Table 3). Denitrifier and SRB populations were generally higher throughout the profile than were fermentors. Denitrifier activity was detected to the 10^{-4} dilution for all sample depths, while SRB were detected to the 10^{-3} dilution for all but the 187.24 m interval, indicating relatively high viable populations for both groups. Fermentor populations were generally lower throughout the profile, with activity detectable only to the 10^{-2} dilution in shale samples, and only to 10^{-1} in sandstone samples.

DGGE and phylogenetic analysis of cloned bands. DGGE analysis of partial 16S rDNA genes was used to determine and compare species richness across the profile. The results indicated relative homogeneity in the microbial community throughout the profile with essentially identical patterns of fourteen major bands visualized in each sample (Fig. 3).

DNA from individual DGGE bands was subjected to DNA sequence analysis and compared to known organisms in the RDPII and BLAST databases (Table 4). The results indicated phylogenetic affiliations to a suite of anaerobic bacteria, mainly within the subdivision of *Proteobacteria*. These included the sulfate reducers *Desulfobacterium*

phenolicum, *Desulfocapsa thiozymogenes* and *Desulfobulbus rhabdoformis*; the Fe(III)/S⁰ reducers *Desulforomonas acetexigens*, *Pelobacter acetylenicus*, *P. propionicus*, and *Geobacter arculus*; the syntrophic fermenter *Smithella propionica*, and the acetogen *Holophaga foetida*. The g and b subdivisions of Proteobacteria were represented by the denitrifiers *Pseudomonas stutzeri* and *Acidovorax* sp., respectively. *Clostridium perfringens*, a fermentor in the Gram positive, low G+C Division of Bacteria was the sole non-Proteobacteria representative detected. Although no sequence was 100% identical to any aligned representative on the RDP II or BLAST databases, all but two clones (bands 5 and 6) had ≥96% sequence similarity to some known organism and were identified as the same or synonymous species by both the BLAST and RDP programs (Table 4). The clone from band 9 was identified as *Azoarcus* str. BH72 by the RDP program and as *Acidovorax* sp. BSB421 in the BLAST analysis. However, both indicated species are members of the b subdivision of Proteobacteria and are involved in nitrogen metabolism. Bands 1 and 10 each produced two different sequences, indicated as different genera, which had co-migrated on the DGGE gel (Table 4). Despite multiple attempts, the unnumbered bands in Figure 3 were either heteroduplex molecules, produced no clones, or resulted in non-rDNA sequences, indicating that these were PCR artifacts.

The rDNA sequences recovered from the DGGE gel were placed into phylogenetic trees with representatives of known, related organisms and *Escherichia coli* as an outgroup to provide phylogenetic context for these deep subsurface Bacteria. Neighbor Joining, Maximum Parsimony and Maximum Likelihood phylograms were generated, bootstrapped 1000 times, and compared. All three algorithms produced very similar dendrograms with identical branching points, and, in every case, the sequences

from the isolated DGGE bands were affiliated closely with the same cohort of known sequences. The Maximum Parsimony tree is presented with bootstrap values (Fig. 4).

Phylogeny of rDNA clones from MLS samples. Because DGGE analysis provides a measure of species richness but does not reliably indicate relative abundance of individual populations, shotgun cloning of partial 16S sequences from total community rDNA was also performed. Based on the DGGE analysis, which indicated that the predominant community members were from the domain Bacteria, PCR primers targeting eubacteria were employed to produce partial rDNA clones (ca. 550 - 600 nucleotides), which were then subjected to unidirectional DNA sequence analysis. The number of clones analyzed for each MLS sample ranged between 73 and 128 (Table 5).

The rDNA phlotypes detected were relatively invariant throughout the MLS depth profile (Table 5). As with the sequences obtained from the DGGE gels, the most common rDNA clones were closely related to the d subdivision of Proteobacteria and to the low G+C, Gram-positive group. Collectively, these two groups accounted for more than one-half of the clones obtained from each sample (Table 5). Other major phlotypes included members of *Flexibacter-Cytophaga-Bacteroides* group (FCB group) and the g subdivision of Proteobacteria, represented mainly by clones related to *Syntrophus* sp. LYP (>97% similarity) and *Pseudomonas stutzeri* (>99% similarity). Several apparently novel clones were also obtained that had little apparent sequence similarity to other known rDNA sequences and no apparent phylogenetic association to any other bacterial divisions, but these were not considered further in the context of this study.

Analysis of the distribution of rDNA clones related to organisms within the d-Proteobacteria revealed certain trends corresponding to the depth of the samples. The

frequency of occurrence of the *Pelobacter-Desulfuromonas* group was higher in two of the three shale interval samples than in the transition or sandstone interval samples (Table 5). By contrast, the frequency of *Geobacter* group clones was highest in the two deepest sandstone-associated samples. The frequency of clones closely related to sulfate-respiring δ -Proteobacteria was highest in the transition sample and in 3 of 4 sandstone interval samples. Within the low G+C Gram-positive group, the dominant rDNA clones were most closely related to the sulfate reducing *Desulfotomaculum* (79), the thiosulfate-reducing fermentor *Fusibacter* (63), and the fermentor *Clostridium aldrichii* (61).

RNA hybridization analysis. In another analysis to determine whether the predominant bacterial populations detected by cloning and DGGE analysis were among the most abundant and active community members, northern hybridization analysis was performed using existing domain-specific oligonucleotide probes and other probes based on the rDNA clones detected (Table 1). With this approach, the abundance of directly-detected rRNA is presumed to be proportional to the general metabolic activity and abundance of the corresponding organisms, whereas rDNA quantification reflects only the population size.

The hybridization signal detected by the total eubacterial probe Bac338 was relatively constant throughout the profile, corresponding to a 16S rRNA signal of $\sim 6 \times 10^5$. (Fig. 5, panel A). The rRNA signal corresponding to Fe(III)/S⁰ reducers (based on the sum of the Geo989 and Pelo989 probe signals) was also relatively constant throughout the profile, comprising nearly 50% of the total Eubacterial signal. The estimated fermentor signal (based on the Fusi198 probe) ranged from $2 - 4 \times 10^4$, with the highest values seen in the Cubero Sandstone interval. The activity of the sulfate-reducing

organisms (estimated by subtracting the Geo989, Pelo989, Dstma220 and Fusi198 hybridization signals from the D+SRB385 signal) generally increased with depth, from 3×10^4 to 1×10^5 with the highest levels observed in the transition zone and sandstone intervals rather than in the shale intervals (Fig. 5, panel A). The estimated SRB and fermentative activities represented approximately 8.5 % and 5.0 % of the total community activity, respectively, as indicated by relative strength of the hybridization signals. Based on these data, the sum of the apparent activities of these three major physiological types (Fe(III)/S⁰ reducers, SRB, and fermentors) accounted for 65-78% of the overall metabolic activity of the community.

We also compiled the rDNA clone data, arranged by metabolic group, to determine whether community function could be inferred from the population densities of the corresponding groups, including denitrifiers which were not targeted in the rRNA hybridization analysis (Fig. 5, panel B). In this analysis, organisms related to those capable of Fe(III)/S⁰ respiration predominated across the entire profile. On a percentage basis, Fe(III)/S⁰ respirers were more numerous than any other group in the Clay Mesa Shale intervals. In the Cubero Sandstone intervals, sulfate reducers and Fe(III)/S⁰ reducers were present in approximately equal numbers. At the interface between the shale and sandstone formations (185.11 m bgs), SRB represented the majority of rDNA clones obtained, contrasting with the rRNA hybridization results which indicated that Fe(III)/S⁰ reducers were the most metabolically active group. Fermentative and denitrifying organisms were generally low in abundance throughout the profile, with fermentative organisms more numerous in the Clay Mesa Shale intervals. Thus, with one

exception, the distribution of metabolic types indicated in this analysis is consistent with the patterns indicated by the rRNA hybridization results shown in Figure 5, panel A.

DISCUSSION

The purpose of this study was to examine the phylogenetic and functional diversity of the bacterial community across an interface of consolidated shale and sandstone Cretaceous rock formations 200 m below Cerro Negro, New Mexico in relation to the geochemical properties of the groundwater and rock. It was hypothesized that the interfaces between organic-rich shale intervals and more permeable sandstone intervals offered the highest potential for sustained life over geologic time periods (17, 24, 33, 35, 47, 90). Based on prior chemical, physical and microbial analyses of rock cores from the site (24, 34, 35), an assemblage of sulfate reducing, fermentative, denitrifying, secondary syntrophic, and acetogenic organisms were expected to be present. Due to high sulfate concentrations in the rock pore water, sulfate reducing bacteria were expected to be the most abundant and active terminal degraders in this anaerobic ecosystem.

Geochemical analyses. Chemical analysis of the equilibrated pore water in the MLS samples indicated levels of soluble sulfate and sulfide indicative of SRB activity (Fig. 2). Sulfate levels were highest in the Cubero Sandstone interval adjacent to the boundary with the Clay Mesa Shale interval. These values decreased between 187 and 190 m in depth, corresponding to a region with elevated levels of sulfide in the pore water. Elevated SRB populations in the same region, indicated by dilution series enrichments (Table 3), and relatively higher SRB rRNA signals (Fig. 5, panel A), are also

consistent with a biogenic origin of sulfide. Nitrate levels were low but detectable in MLS pore water (0.05 to 0.40 mg/l), and enrichments for denitrifiers were positive for all depths tested (Table 3). The presence of active fermentative organisms was also supported by both the dilution series enrichment and rRNA hybridization results (Table 3 and Fig. 5, panel A), but was expected to be lower in general than the other groups tested based on lower energy yields. All of the above represent examples where the microbial abundances and activities were generally consistent with the groundwater and rock geochemical properties.

Microbial community structure. One unexpected result was the relatively homogeneous nature of the microbial community across the entire shale/sandstone interface that was indicated by our analyses. It was hypothesized that fewer and different populations of bacteria might reside in the Clay Mesa Shale interval compared to the Cubero Sandstone interval due to the restrictive porosity. It was also anticipated that a distribution of different bacterial types might exist across the shale/sandstone interface, from fermentative organisms breaking down the complex organic compounds leaching from the shale, to secondary fermentative syntrophs, acetogens and SRB utilizing the by-products of fermentation within the sandstone interval (33, 35). However, our data indicated that total bacterial numbers and the concentrations of total DNA and RNA isolated from the MLS samples were relatively constant across the shale-sandstone interface (Table 2). Further, DGGE banding patterns, which provide characteristic fingerprints representing the populations present, were also essentially identical throughout the profile (Fig. 3). The RNA hybridization results indicating the most numerous and metabolically active populations were also relatively uniform, except for

the higher SRB signals detected in the Cubero sandstone interval (Fig. 5). Even the random rDNA cloning results, indicative of the number of individuals of a given species, were similar for most samples, with the proportions of different functional and phylogenetic types being relatively constant throughout the profile (Table 5).

One possible explanation for these findings is that deep-subsurface rock environments, where *in situ* production rates are among the lowest known (17), select for a more uniform microbial community. Recent research on deep subsurface sediments in Washington State indicated a relationship between microbial diversity and sediment permeability where relatively impermeable muds and paleosols had low diversity, while more permeable sands and gravels were inhabited by a more diverse array of organisms (91). Presumably, physical and chemical isolation in the impermeable sediments limits the diversity present. Similarly, the limited porosity of the consolidated rock formations below Cerro Negro could have contributed to the homogeneity observed, resulting in a more-uniform distribution of community members and a limited number of species.

Another possible explanation is that drilling the borehole or even the multi-level sampler (MLS) itself may have contributed to the observed microbial community homogeneity. The MLS, whose use for analysis of microorganisms in low-biomass environments is first described in this study, contained sterilized quartzite sand offering a clean, extensive new surface for microbial colonization at depth. It is unlikely that there was significant vertical movement of microbes within the MLS itself since the colonization matrix was sealed into discrete compartments vertically. However, even though the MLS was designed to sample distinct zones in the vertical axis based on discrete sample chambers and inflatable sidewall baffles, the borehole walls themselves may have been uniformly colonized by the community

at depth prior to placement of the MLS. Since the borehole was capped prior to installation of the MLS, groundwater within the borehole would have been relatively stagnant prior to placement of the MLS and could have allowed movement of organisms and diffusion of solutes, including nutrients, throughout the borehole.

There might also have been a scale effect related to the MLS. The discrete cells of the MLS are positioned every 5 cm vertically, with alternating cells for chemical and microbial analysis. Thus, the microbial community was sampled every 10 cm, a fairly precise increment for biogeochemical analyses, but perhaps too large to resolve microbial community architecture and distribution, if present. Further, samples within individual MLS cells were homogenized prior to distribution, disrupting any small-scale (<5 cm) spatial heterogeneity that may have existed. Finally, recent hydrogeological data from the site indicates a possible upward flow of water in the formations, and low but measurable vertical hydraulic conductivities in the shale (93). This could also result in a "conduit" effect for water flow, and a more even distribution of organisms in the MLS, and perhaps even in the parent rock material. Any or all of these factors may have contributed to the relative homogeneity of the bacterial community over the MLS interval.

Phylogeny of the microbial community. Phylogenetic analysis indicated a community comprised of various physiological groups of anaerobic microorganisms, primarily from the δ subdivision of Proteobacteria, in this Cretaceous rock environment. This included Fe(III)- and/or S^0 -respiring chemoorganotrophs, various dissimilatory sulfate reducing bacteria, acetogens, and syntrophic fermenters. The apparent predominance of δ Proteobacteria in subsurface Cretaceous rock is first described in this study, although rDNA clones related to members of the δ Proteobacteria have been

recovered in other subsurface studies (15, 21, 55-57, 59). Denitrifying chemoorganotrophs of the b and g subdivisions of Proteobacteria were also indicated in our study, as were representatives of the low G+C Gram positive division of Bacteria. Three different algorithms were used to generate phylogenetic trees comparing the sequences obtained in these analyses to other known representatives of these groups of bacteria, and each produced comparable results. In general, the sequences obtained were closely related to other known genera and species of bacteria.

Our phylogenetic results were generally consistent with the types of organisms expected to be able to utilize the complex resources available at a shale/sandstone interface. It is possible that this community is surviving over geological time by utilizing the limited available substrates in a coordinated, syntrophic manner. Previous laboratory studies have demonstrated that shale-derived organic matter can drive sulfate reduction and acetogenesis in sandstone samples from this site (24), and bacteria displaying these physiological processes have been isolated in enrichment cultures from core samples from the site (34, 35).

Differences in the distribution of microbial types, such as the increased proportion of *Pelobacter*-like members adjacent to the Clay Mesa shale interval, may reflect the distribution of specific classes of organic compounds available for growth and energy. *Pelobacter* can grow by fermentation in the absence of either Fe(III) or S^0 as electron acceptors, but can only ferment a limited range of specific substrates including 2,3-butanediol, acetoin, ethylene glycol and acetylene (68-70, 72-74, 77). Although concentrations of the aforementioned organic compounds were not measured in this

study, it is possible that certain classes of organic compounds provided by the carbon-rich shale favored the growth and activity of *Pelobacter*-like organisms.

It has been also demonstrated *in vitro* that certain *Pelobacter* and *Syntrophus* species can grow in syntrophic association with hydrogenotrophs under some conditions (68, 69, 71, 73, 77, 92). The hydrogenotrophs, usually SRB, acetogens, or methanogens, utilize the H₂ produced so that growth of the syntrophs via fermentation is energetically favorable. A number of bacterial species previously assumed to be non-syntrophic can shift their metabolism to grow syntrophically in the absence of external terminal electron acceptors, including various SRB within the δ Proteobacteria, some *Clostridium* species and some *Geobacter* species (18, 60, 67, 70, 75).

Based on these and previous results, the microbial communities at this site are complex, interdependent, and metabolically diverse. This reflects their dependency upon the complex detrital organic matter deposited with the sediments during the Cretaceous period. We speculate that the detrital organic matter may be effectively utilized in part by syntrophic cooperation among the members of the microbial community. Although the physiology of individual members of this subsurface community was not extensively analyzed, the close phylogenetic affiliation of the rDNA clones obtained to groups of well-characterized anaerobic microorganisms indicates that the community at depth is primarily an assemblage of anaerobic microorganisms. This bacterial community contains members having a variety of metabolic strategies including primary and syntrophic fermentation, sulfate- and sulfur-respiration, acetogenesis and denitrification.

Predominance of Fe(III)/S⁰ reducing bacteria. One unanticipated finding of this study was the predominance of *Pelobacter*-, *Desulfuromonas*-, and *Geobacter*-related

bacteria in our DNA and RNA based analyses. These genera are phylogenetically related and are generally grouped as members of the *Geobacteracea* family which can couple the oxidation of organic matter to the reduction of Fe(III) and Mn(IV) (39-42, 44). Members of these same genera are also capable of utilizing elemental sulfur, but not sulfate, as an electron acceptor (39, 44).

Due to the abundance of sulfate in the pore water, and the limited availability of alternative electron acceptors, it was anticipated that SRB would represent, both numerically and in terms of activity, the predominant terminal degraders in the ecosystem. The dilution series enrichment analyses were consistent with this expectation in that viable SRB were relatively abundant. Yet, four of twelve DGGE band rDNA clones (Fig. 3), and over 41% of Eubacterial rDNA clones aligned with organisms capable of Fe(III)/S⁰ respiration, compared to three SRB DGGE band clones and 35% SRB Eubacterial clones (Fig. 5, panel B). Numerically, Fe(III)/S⁰ reducers were equal to the SRB in the Cubero Sandstone interval, and were in greater abundance than SRB in the Clay Mesa Shale (Fig. 5, panel B). The Fe(III)/S⁰ reducers group also accounted for nearly 50% of the total community abundance/activity based on RNA hybridization results (Fig. 5, panel A).

The predominance of organisms related to Fe(III)/S⁰ reducers is intriguing and not readily explained by the measured geochemical properties of the site. In other studies of subsurface environments, community structure usually reflected the geochemistry of the site and availability of suitable electron acceptors utilized in order of reduction potential: O₂ > Mn(IV) > nitrate > Fe(III) > sulfate > CO₂ (16, 17, 19, 53, 54, 60, 88, 90). Although iron is a common component of the Clay Mesa Shale formation, it is present

predominantly as the secondary mineral pyrite (Fig. 1). Pyrite dissolution is generally only possible under highly acidic, oxic conditions and is thought to not be possible under strictly anoxic conditions (5, 9, 29, 51, 76, 84, 90). Even if dissolution were possible, the iron would be in the $+2$ oxidation state and not available as an electron acceptor for microbial respiration unless iron oxidizing bacteria were present (90) and these were not indicated in our results.

Nitrate was present in limited amounts in this system, but only a few Fe(III)/S⁰ reducers have been shown to utilize nitrate as an electron acceptor (41). Concentrations of Mn(IV) were not determined in our study but would be less likely to be present than Fe(III) because the presence of Mn(IV) would be even less thermodynamically favorable than Fe(III). The relative insolubility of S⁰, and the fact that it is generated mainly from biotic and abiotic oxidation of reduced S species, suggests that that S⁰ was probably not a major electron acceptor for microbial metabolism in the reducing environment of the shale/sandstone interface either. The lack of soluble Fe(III), Mn(IV) or elemental S⁰ essentially eliminates them as significant electron acceptors supporting the growth and metabolism of *Pelobacter*, *Desulfuromonas* and *Geobacter* in this environment.

It is possible, however, that Fe(III)/S⁰ reducers are proliferating at the shale/sandstone formation through some novel and as yet undescribed type of metabolism. Recently, it has been demonstrated *in vitro* that certain denitrifiers isolated from sediments can couple nitrate reduction with the oxidation of ferrous iron (8, 81, 82), although the identities of the organisms involved were not determined. A similar type of metabolism might function at Cerro Negro to utilize by-products of pyrite dissolution (sulfate and ferrous iron) if it were occurring in micro-aerobic environments at depth.

However, energetically, it seems unlikely that this metabolic strategy could support the relatively large population of Fe(III)/S⁰ reducers detected.

Alternatively, the specific d Proteobacteria in this deep subsurface environment may have additional metabolic capabilities beyond Fe(III)/S⁰ reduction that are not represented by other known members of this group, potentially being capable of reducing sulfate or other electron acceptors. Recently an organism was isolated from a 3000 m deep gold mine in Africa that was able to utilize O₂, nitrate, Fe(III), Mn(IV), Co(III)-EDTA, Cr(VI), U(VI) and S⁰ as electron acceptors (32), demonstrating the wide range of potential metabolic capabilities inherent in microorganisms from subsurface environments. Another possibility is that the rDNA sequences recovered, although most closely affiliated with known Fe(III)/S⁰ reducers, may instead represent a novel group of SRB that are related phylogenetically, but not functionally, to known Fe(III)/S⁰ reducers.

We believe, however, that the most likely explanation for these results is that the organisms related to Fe(III)/S⁰ reducers are growing via a fermentative metabolism in syntrophic association with respiratory organisms, utilizing the complex organic material available and secreting H₂ or simple organic acids which are then utilized by SRB and/or methanogenic bacteria (68-70, 72-74, 77, 92). Viable sulfate-reducing bacteria were present in relatively high numbers throughout the MLS interval and, although not included as part of this study, organisms closely related to archaeal methanogens were also present throughout the MLS interval (Takai et al. submitted for publication). Some were phylogenetically related to *Methanosarcinaceae* members that are capable of autotrophic growth with H₂. The presence of active methanogens also suggests that the community members related to Fe(III)/S⁰ were not growing with Fe, Mn or S as electron

acceptors because they would have likely outcompeted the methanogens in this environment if these electron acceptors were non-limiting. Our results indicate that microbial metabolism in the deep-subsurface environment at Cerro Negro is likely limited by the availability of readily usable electron donors, relying on the interactions of many types of organisms to utilize the complex organic material dating to the Cretaceous Period.

Origins and survival of indigenous bacteria. An original objective of the research at Cerro Negro was to determine the origin and potential time of arrival of the bacteria at depth. One intriguing scenario to explain the abundance of Fe(III)/S⁰ reducers at Cerro Negro is that these organisms could be remnants of original community inhabiting the near-shore ocean sediments during the Cretaceous Period. During that time, a large ocean covered much of the southwestern United States, and the sediments derived from this near-shore ocean sediment environment would eventually become the Clay Mesa Shale rock formation that was examined in this study (48). Based on the restrictive porosity of the formations, especially the Clay Mesa Shale, it was postulated that at least some of these microbes might represent or be descended from the original microbes associated with the sediments at the time of deposition. It was hypothesized that some of those bacteria could have subsequently become entrapped during sediment deposition and diagenesis (24).

Consistent with that hypothesis, it has previously been shown that Fe(III) and sometimes Mn(IV) reduction are important for organic matter oxidation in near-shore ocean sediments. These processes account for 30-90% of organic carbon oxidation under conditions where the sediment is physically mixed, such that any reduced Fe or Mn

species would be re-oxidized (1, 14, 28, 43, 50, 88). Indeed, prior research from modern marine sediment environments indicates an abundance of δ Proteobacteria (20), *Cytophaga-Flavobacterium-Bacteroides* (CFB)-related organisms (38, 62, 87), as well as organisms capable of denitrification (13, 25) and fermentation (22, 30, 83). Thus, the metabolic profile of the Cerro Negro microbial community at depth indicated by our analyses is consistent with an origin based on near-shore ocean sediments. This is particularly striking considering that the surface landscape is currently an arid terrestrial environment.

Although our results are consistent with microbial phylotypes and metabolic activities found in near-shore ocean sediment environments, no firm conclusions concerning the origin and arrival times of the current microbial community can be made based solely on these analyses. Many of the nearest relatives to our 16S rDNA clones were isolated from a variety of anaerobic sediment environments including marine, estuarine, and freshwater sediments, as well as contaminated subsurface environments and digester sludge. Identification and alignment of the sequences obtained in this study with other known organisms revealed no obvious correlation or bias toward marine sediment organisms. This is perhaps not surprising since a survey of the literature related to the phylogeny of Fe(III)/S^0 reducers, SRB, and other organisms reveals no compelling evidence of discrete phylogenetic groupings for organisms of marine versus terrestrial/freshwater origin. Often the only detectable difference between marine and terrestrial/freshwater bacterial populations for a variety of bacterial groups is the requirement for NaCl and facultatively psychrophilic growth observed in marine organisms (64). It is possible, maybe even likely, that the current microbial community

residing at the shale/sandstone interface below Cerro Negro is a mixture of surviving bacterial populations that colonized the sediments at the time of deposition, and others that arrived via migration at some later geological time.

Summary. Collectively, our results and those of prior studies at this site (24, 35) indicate the presence of an active, phylogenetically and physiologically diverse microbial community in the deep subsurface Cretaceous rock environment at this site. This diversity probably reflects the complex nature of the kerogen associated with the shale. The geochemical parameters measured were generally consistent with the phylogenetic and functional capabilities of bacteria identified by our analyses. For example, the relationship between sulfate and sulfide levels in pore water and the presence of SRB organisms and activity. Two unanticipated findings were the homogenous nature of the microbial community traversing the shale sandstone interface, and the apparent predominance of Fe(III)/S⁰ reducers in an environment expected to be dominated by SRB. Further analyses are required to provide additional insights into the biogeochemical processes occurring in this Cretaceous rock environment and the factors responsible for the apparent predominance of *Pelobacter-Desulfuromonas-Geobacter*-type organisms. Applying methods for the cultivation and study of syntrophs to samples from this site might also provide additional insights into the *in situ* microbial processes and interactions, and may represent a strategy for culturing previously uncultivated and unidentified microorganisms from this and other subsurface sites.

ACKNOWLEDGEMENTS

This research was supported by the Subsurface Science Program, Office of Energy Research, U.S. Department of Energy Grant No. DE-FG03-96ER62154 and by the Natural and Accelerated Bioremediation Research Program-Acceleration Element Grant No. DE-FG02-97ER62472. Pacific Northwest National Laboratory is operated for the DOE by Battelle Memorial Institute under Contract DE-AC06-76RLO 1830. We also acknowledge Tom Kieft of the New Mexico Institute of Mining and Technology for valuable insights and discussions on these topics.

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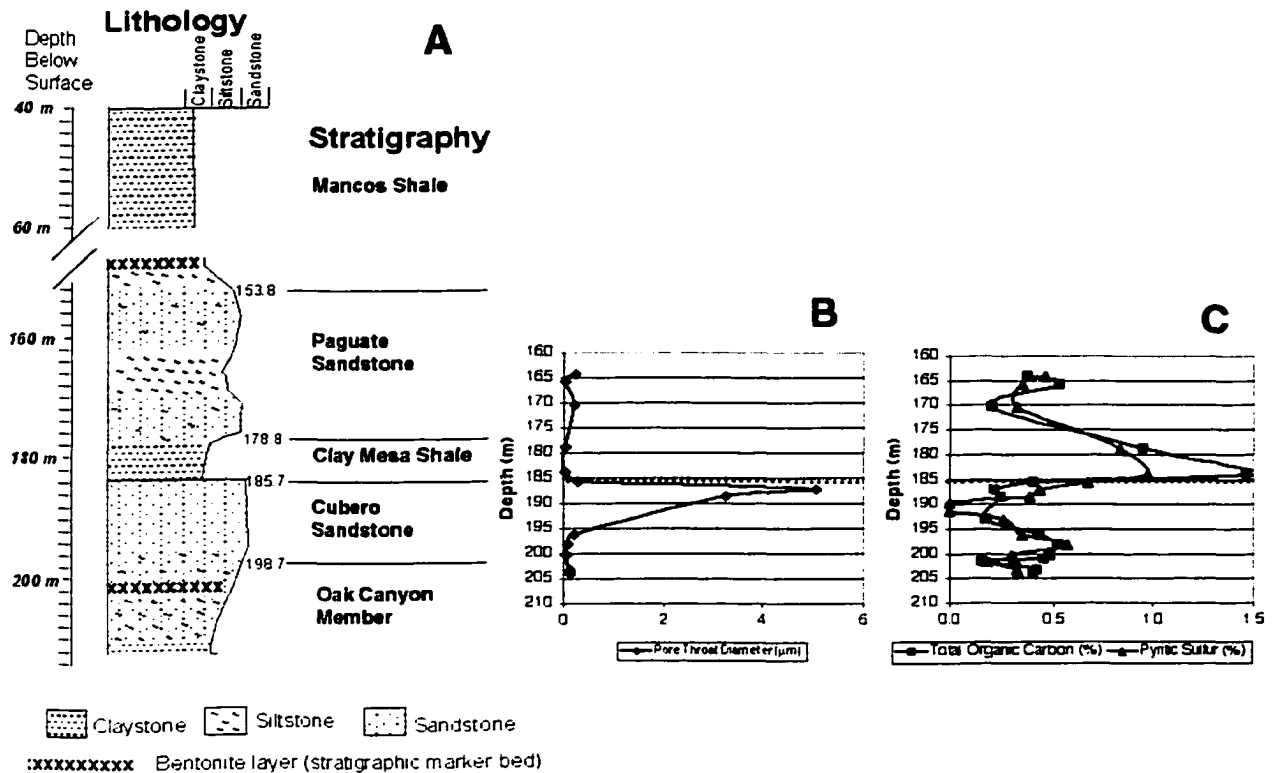


Fig. 1. Study site characteristics. A MLS was placed across the interface between Clay Mesa Shale and Cubero Sandstone formations at the depth range of 182 to 190 m. The transition zone between the shale and sandstone is indicated by dashed line. (A) Interpreted lithology and stratigraphy of the CNV-R borehole. (B) Pore throat diameter (μm) of rock cores (◆). (C) Concentrations of total organic carbon (■) and pyritic sulfur (□) of rock cores.

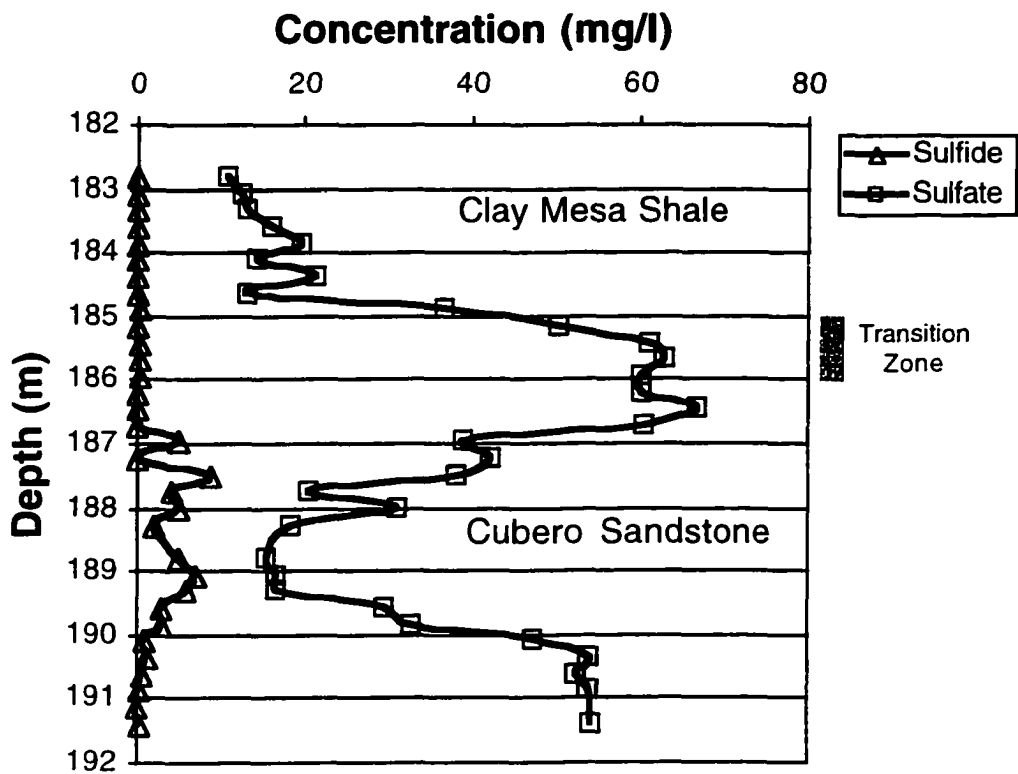


Fig. 2. Sulfate (□) and sulfide (Δ) concentrations (μg/l) in MLS pore water versus depth below ground surface. The transition zone between the shale and sandstone is indicated by shaded rectangle.

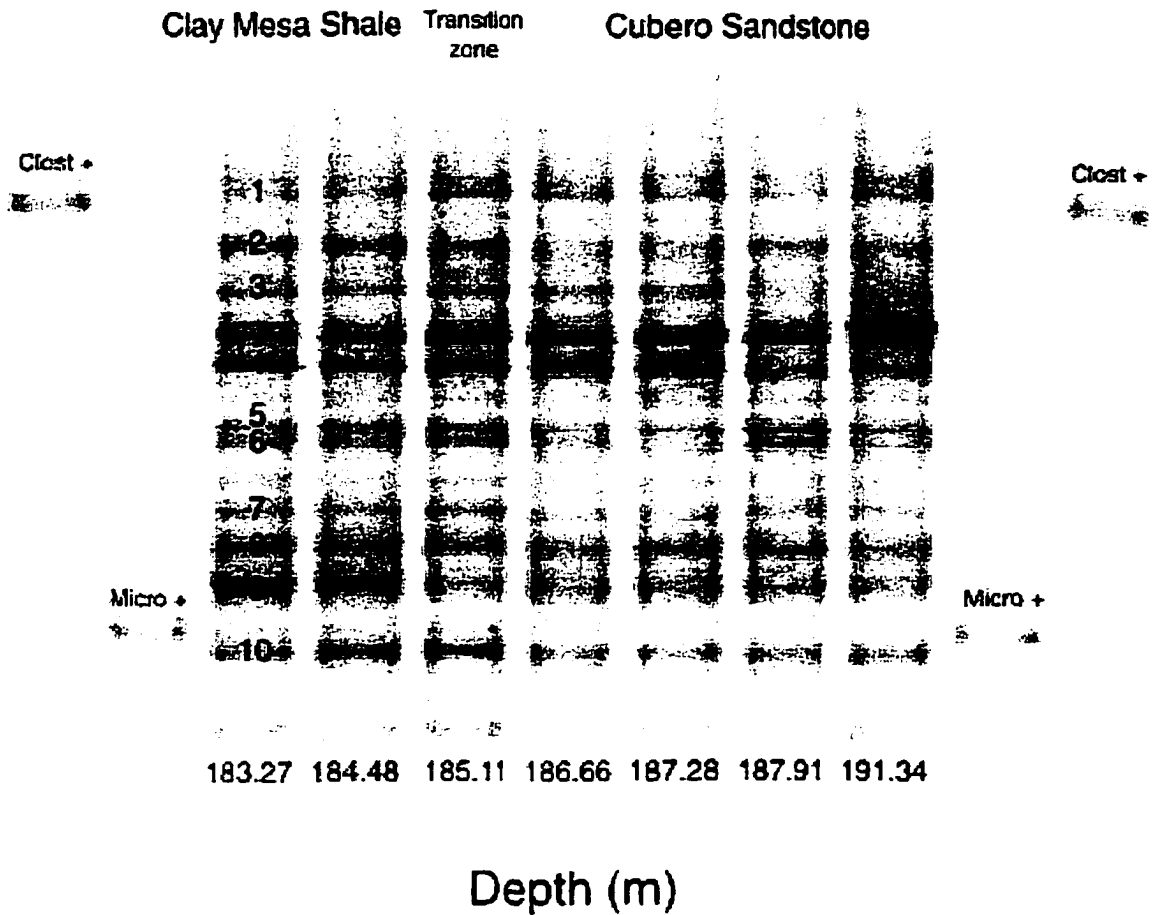


Fig. 3. DGGE profile of PCR-amplified 16S rDNA from MLS samples across the shale/sandstone interface using universal primers. Numbered bands correspond to those bands yielding valid 16S rDNA sequence information; bands 1 and 10 each produced two valid sequences. Unnumbered bands were either heteroduplex rDNA molecules or did not produce valid 16S rDNA sequences. PCR amplified rDNA from ATCC strains *Clostridium perfringens* (Clost +) and *Micrococcus lysodeikticus* (Micro +) were used as markers for low (28%) and high (72%) G+C content DNA, respectively.

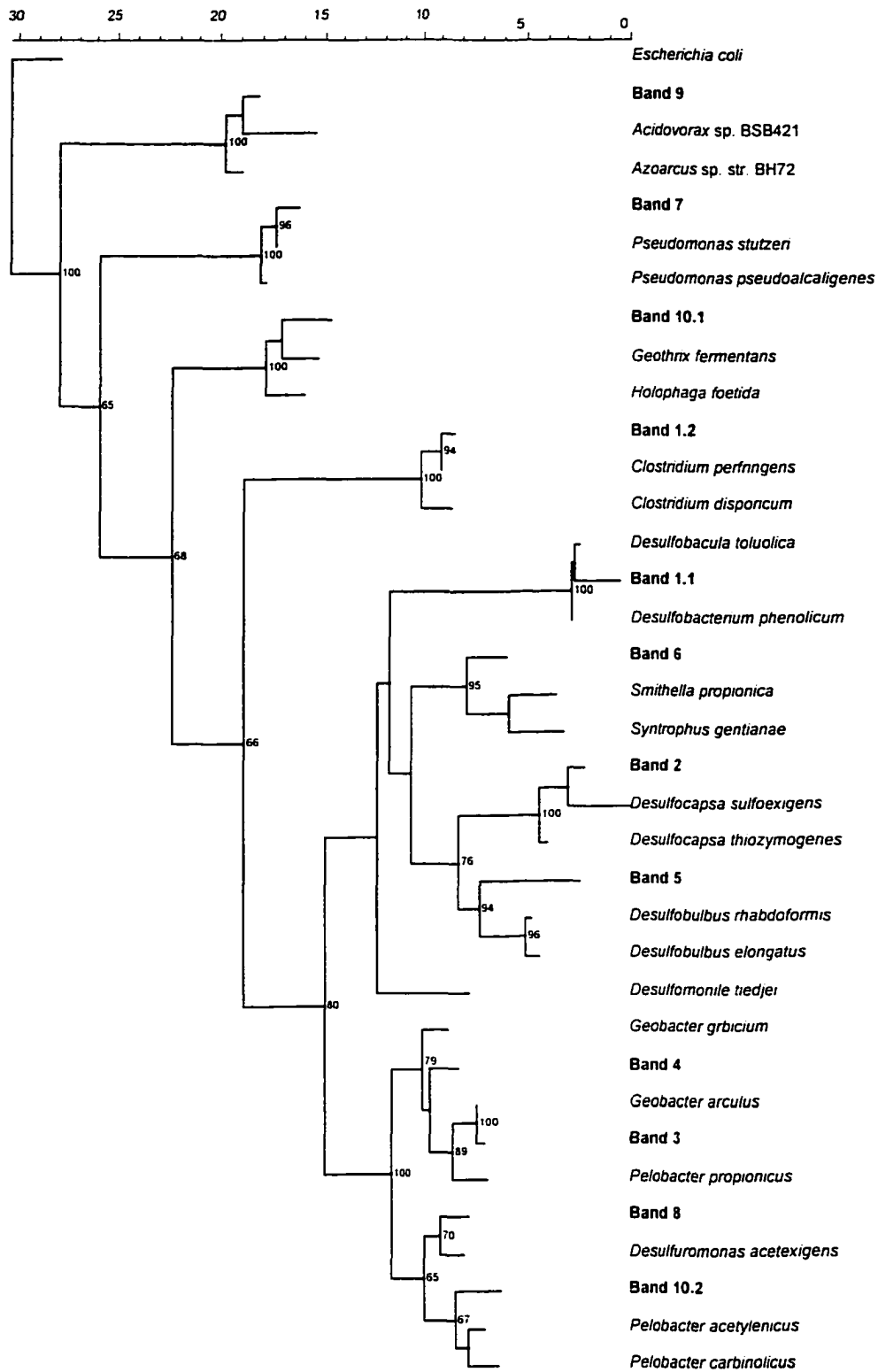


Fig 4. Representative phylogenetic tree generated from DGGE band sequences and related known sequences. Maximum Parsimony phylogram rooted with *E. coli*. Sequences from this study are in bold type. The phylogram was bootstrapped 1000 times with branch-points supported $\geq 65\%$ of the time indicated.

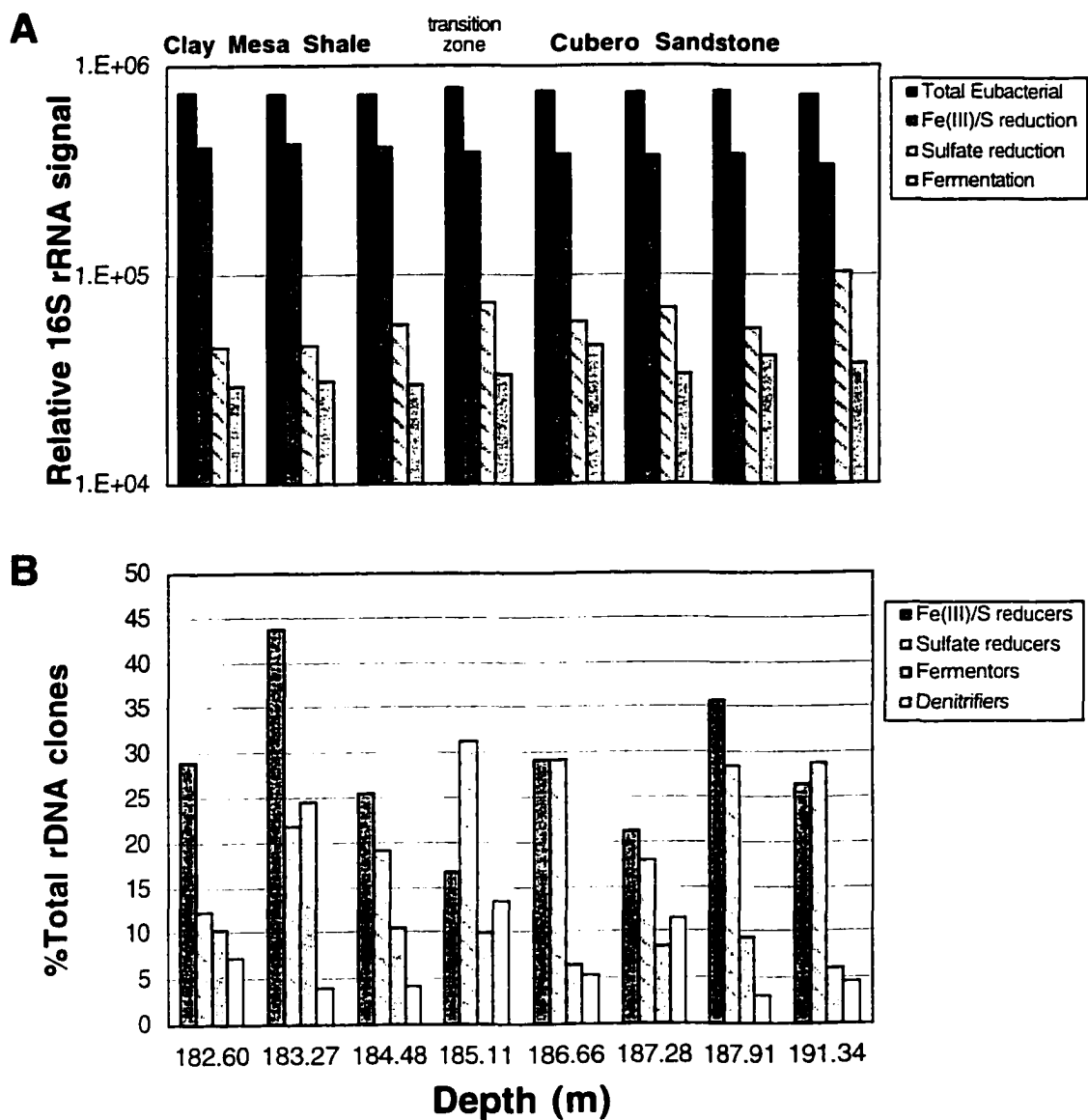


Figure 5. (A) General metabolic activity associated with microbial physiologies, as inferred from Northern rRNA hybridization with a set of relevant probes identified from partial sequencing of library clones, and from the literature (see Table 1). (B) Distribution of rDNA clones by functional group; function inferred by rDNA clones to nearest relative on RDP and BLAST databases (complete clone data shown in Table 5).

Table 1. PCR primer and oligonucleotide probe sequences.

Name	Sequence (5' to 3')	Target	Temp (°C) ¹	Reference
DGGE PCR primers				
536fz ₃	CGCCGGCCGGCCCGCCGGCCGGCCGGCCGGC- CGCCGGCCCGCAGC(AC)GCCGGGTAAT(AT)C	most Eubacteria, Eukaryotes, Archaeobacteria	65-65 (-0.5' /cycle)4	(37)
907r	CCCGTCAATTCTTGAGTTT	most Eubacteria, Eukaryotes, Archaeobacteria	65-65 (-0.5' /cycle)4	(37)
Cloning primers				
27f (PCR)	AGAGTTGATC(CA)TGGCTCAG	most Eubacteria	55	(37)
1392r (PCR)	ACGGGCGGTGTGT(AG)C	most Eubacteria, Eukaryotes, Archaeobacteria	55	(37)
907r (sequencing)	CCCGTCAATTCTTGAGTTT	most Eubacteria, Eukaryotes, Archaeobacteria	55	(37)
Oligonucleotide probes				
Bac336	GCTGCCTCCCGTAGGAGT	Eubacteria	50	(2)
D-SR18385	CGCGTCGCTGCGTCAGG	most d-Proteobacteria, some low %G+C gram positives	55	(4)
Geo869	GGTTCCG(CT)(AG)GATGTCAA	Geobacter	53	this study
Pelo969	GT(AG)CGAT(CT)GGGATGTCAA	Palaeobacter, Desulfuromonas, Desulfurobium	50	this study
Dsrma220	ACGCGGACCCCACTCC(AT)T(AT)AG	Desulfotomaculum	57	this study
Fusi198	CCGATAAATCTTGCCA	Fusibacter	50	this study

¹Temperature listed is the optimum annealing temperature for PCR primers, and optimum hybridization/wash temperature for oligonucleotide probes, empirically derived.

²The forward DGGE primer incorporates a 5', 40-bp, G-C rich clamp (underlined) as described by Sheffield et al. (78)

³The 536f primer sequence used here is the reverse-complement of the 518r primer described by Lane (37)

⁴DGGE utilizes a touchdown protocol (49), with annealing temperature ramped from 65-65' (-0.5' /cycle) for 20 cycles, followed by 10 cycles at 55'

Table 2. Sample intervals, bacterial numbers, and nucleic acid yields from MLS samples

Depth (m)¹	Total counts	DNA (ng/g)²	RNA (ng/g)² (x 10⁶/g)²
183.27	1.70	1.80	2.50
183.85	2.00	1.80	2.30
184.48	1.50	1.50	2.80
185.11	1.80	1.90	2.20
185.74	1.90	2.20	2.40
186.66	2.00	2.10	2.20
187.28	1.80	1.40	2.00
187.91	2.10	2.10	2.50
188.54	2.20	1.90	2.30
189.17	1.60	2.30	2.40
189.80	2.50	2.00	2.10
190.71	2.20	1.90	2.50
191.34	1.90	2.20	2.40

1Depths are in meters below ground surface

2All yields are per gram wet weight

Table 3. Bacterial enrichments from MLS samples. 1

183.27 m, Clay Mesa Shale		1.E+00	1.E-04	1.E-02	1.E-03	1.E-01
Fermentors	Turbidity	+++	-	+/-	-	++
Fermentors	Gas production	-	-	-	-	-
Denitrifiers	Turbidity	++	+	++	++	++
Denitrifiers	NO2	-	-	-	-	-
Denitrifiers	NO3	-	-	-	-	-
SO4 reducers	blk ppt	+++	-	+++	+++	+++
184.48 m, Clay Mesa Shale		1.E+00	1.E-04	1.E-02	1.E-03	1.E-01
Fermentors	Turbidity	++	-	+/-	-	+
Fermentors	Gas production	+	-	-	-	-
Denitrifiers	Turbidity	++	+	++	++	++
Denitrifiers	NO2	-	-	-	-	-
Denitrifiers	NO3	-	-	-	-	-
SO4 reducers	Black precipitate	+++	-	+++	-	+++
185.11 m, Transition zone		1.E+00	1.E-04	1.E-02	1.E-03	1.E-01
Fermentors	Turbidity	++	-	-	-	+
Fermentors	Gas production	-	-	-	-	-
Denitrifiers	Turbidity	++	+	++	++	++
Denitrifiers	NO2	-	-	-	-	-
Denitrifiers	NO3	-	-	-	-	-
SO4 reducers	Black precipitate	+++	-	+++	-	+++
186.66, m Cubero Sandstone		1.E+00	1.E-04	1.E-02	1.E-03	1.E-01
Fermentors	Turbidity	++	-	-	-	+
Fermentors	Gas production	-	-	-	-	-
Denitrifiers	Turbidity	++	+	++	++	++
Denitrifiers	NO2	-	-	-	-	-
Denitrifiers	NO3	-	-	-	-	-
SO4 reducers	Black precipitate	+++	-	+++	+/-	+++
187.28, m Cubero Sandstone		1.E+00	1.E-04	1.E-02	1.E-03	1.E-01
Fermentors	Turbidity	++	-	-	-	+
Fermentors	Gas production	+	-	-	-	-
Denitrifiers	Turbidity	++	+	++	++	++
Denitrifiers	NO2	-	-	-	+/-	-
Denitrifiers	NO3	-	-	-	-	-
SO4 reducers	Black precipitate	+	-	-	-	+/-
187.91 m, Cubero Sandstone		1.E+00	1.E-04	1.E-02	1.E-03	1.E-01
Fermentors	Turbidity	++	-	-	-	+
Fermentors	Gas production	-	-	-	-	-
Denitrifiers	Turbidity	++	+	++	++	++
Denitrifiers	NO2	-	-	-	-	-
Denitrifiers	NO3	-	-	-	-	-
SO4 reducers	Black precipitate	+++	-	+++	+++	+++
191.34 m, Cubero Sandstone		1.E+00	1.E-04	1.E-02	1.E-03	1.E-01
Fermentors	Turbidity	++	-	-	-	+
Fermentors	Gas production	-	-	-	-	-
Denitrifiers	Turbidity	++	+	++	++	++
Denitrifiers	NO2	ND2	-	-	-	ND
Denitrifiers	NO3	ND	-	-	-	ND
SO4 reducers	Black precipitate	+++	-	+++	+++	+++

1Each sample was serially diluted in tenfold increments out to 1.E-04

2ND = not done

Table 4. Organism identification from DGGE band rDNA.

Band	Nearest Relative by RDP	Sub score	Nearest Relative by BLAST ID	Similarity (%)	Phylogenetic group	Metabolic type
1.1	<i>Desulfobacterium phenolicum</i>	0.884	<i>Desulfobacterium phenolicum</i>	88	Proteobacteria, d subdivision	sulfate reduction
1.2	<i>Clostridium perfringens</i>	0.982	<i>Clostridium perfringens</i> 16S rRNA	89	Gram positive, low G+C division	fermentation
2	<i>Desulfocapsa thiozymogenes</i> str. Bra2	0.874	<i>Desulfocapsa thiozymogenes</i>	97	Proteobacteria, d subdivision	sulfate reduction
3	<i>Geobacter arcuatus</i> str. Dtr1	0.993	<i>Geobacter bremensis</i>	99	Proteobacteria, d subdivision	Fe(III) SO reduction
4	<i>Pelobacter propionicus</i> str. OrBd1	0.835	<i>Geobacter gibbicus</i>	97	Proteobacteria, d subdivision	Fe(III) SO reduction
5	<i>Desulfobulbus rhabdofornis</i> 16S	0.658	<i>Desulfobulbus rhabdofornis</i>	91	Proteobacteria, d subdivision	sulfate reduction
6	<i>Smithella propionica</i> str. LYP	0.744	<i>Syntrophus</i> sp. LYP 16S rRNA	84	Proteobacteria, d subdivision	fermentation
7	<i>Pseudomonas stutzeri</i> str. StanW 224	0.853	<i>Pseudomonas</i> sp. JPL-1	89	Proteobacteria, d subdivision	denitrification
8	<i>Desulfuramonas acetatidgens</i>	0.825	<i>Desulfuramonas acetatidgens</i>	97	Proteobacteria, d subdivision	Fe(III) reduction
9	<i>Azoarcus</i> str. BH72	0.858	<i>Azotomonas</i> sp. BSB421	86	Proteobacteria, d subdivision	denitrification
10.1	<i>Holophaga foetida</i> str. TMBS4	0.855	<i>Holophaga foetida</i> str. TMBS4-T	86	Proteobacteria, d subdivision	acetogenesis
10.2	<i>Pelobacter acetylenicus</i> str. WoAcyl	0.825	<i>Pelobacter acetylenicus</i> str. WoAcyl	86	Proteobacteria, d subdivision	Fe(III) reduction

Table 5. Distribution of representative bacterial rDNA clones in MLS samples.

Phylogenetic group / Dominant species †	Metabolic type ‡	Number of clones by depth							
		Clay Mesa Shale		(transition zone)		Cubero Sandstone		Cubero Sandstone	
		182.6 m	183.27 m	184.48 m	185.11 m	186.66 m	187.28 m	187.91 m	191.34 m
Proteobacteria, d subdivision	total =	34	31	29	32	44	22	44	57
Pelobacter sp. (<i>P. acetylenicus</i> , <i>P. venetianus</i>)	Fe(III) reduction, fermentation (limited)	23	5	14	4	3	8	11	12
Desulfuromonas acetabuligena	Fe(III) reduction	5	15	3	4	11	3	6	2
Geobacter arculus str. Dfr1	Fe(III)/So reduction	0	11	7	5	13	6	17	20
Desulfobacterium sp.	sulfate reduction	6	0	5	19	18	8	16	25
Gram-type positive, low G+C group	total =	33	18	20	14	15	19	23	28
Desulfotomaculum sp.	sulfate reduction	3	3	4	3	3	3	5	6
Clostridium aldrichii	fermentation	0	8	6	5	0	5	0	3
Fusibacter paucivorans	fermentation, thiosulfate reducing	3	5	2	3	5	2	7	5
others		27	2	8	3	7	14	11	15
Flexibacter-Cytophaga-Bacterioides group	total =	4	17	11	7	11	10	14	10
uncultivated rDNA clone WCHB1-29	(unknown)	4	13	11	7	10	10	14	10
others		0	4	0	0	1	0	0	0
Proteobacteria, g subdivision	total =	7	3	2	12	6	10	2	4
Pseudomonas stutzeri	denitrification	7	3	2	12	5	10	2	4
Others (non-aligned)		18	14	32	24	17	33	12	28
Total clones per depth =		96	73	94	89	92	94	95	128

† The affiliation of each rDNA clone to known phylogenetic groups was carried out using the gapped-BLAST program and the SUGGEST_TREE program of RDPII.

‡ Metabolic type inferred from nearest relative by gapped-BLAST or RDP

Chapter 3: Development and Application of qPCR Approaches for Monitoring Bacterial Transport in the Subsurface

**Monitoring Subsurface Bacterial Transport Through Direct Quantitative PCR (qPCR):
Development and Comparison of Gel-Based Competitive qPCR and Fluorogenic Real-
Time qPCR Methods.**

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Running title: qPCR Monitoring of Bacterial Transport

**Keywords: Subsurface bacterial transport, Quantitative PCR, qPCR, Competitive qPCR,
Real-time qPCR.**

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ABSTRACT

To facilitate monitoring of bacterial transport during *in situ* subsurface bacterial injection experiments, two quantitative PCR (qPCR) methods for enumerating specific bacteria in aqueous environmental samples were developed and compared, a gel-based competitive qPCR method, and a fluorogenic, real-time qPCR method. These protocols allow direct determination of bacterial numbers in aqueous samples without requiring any prior sample processing or DNA purification. The competitive qPCR approach is based on PCR amplification using primer sets specific for a unique region of the 16S rDNA gene of the organism of interest. Target bacterial DNA and a constant amount of competitor DNA are simultaneously amplified in a single PCR reaction mixture, permitting quantification of bacterial numbers based on ratios of target and competitor products. The fluorogenic method employs primers and fluorogenic probes targeting unique 16S rDNA sequences, thereby increasing the specificity and range of detection. Signal detection is accomplished in real-time, permitting a wider dynamic range of detection and more precise quantification without sample dilution or concentration. Validation of these methods was accomplished in comparison to other established methods in pre-packed columns and intact sediment cores using *Comamonas* sp. DA001 and *Acidovorax* sp. OY107, low-adhesion bacterial strains indigenous to the test aquifer. Preliminary results of *in situ* bacterial injection experiments into the aquifer are also presented. Our results indicate that the qPCR strategies described herein allow rapid, specific and accurate quantification of transported bacteria, and represent useful tools for rapid, long-term and far-field analysis of bacterial movement and survival in the environment.

INTRODUCTION

Knowledge regarding the transport behavior of bacteria in saturated and unsaturated porous media has broad implications in diverse areas ranging from agriculture to groundwater quality, risk assessment and bioremediation (14). For example, bioremediation using introduced bacteria represents a potential method for dealing with deep subsurface contamination since injected bacteria can potentially travel by water flow to distant contaminated sites. However, relatively little is known about the transport capabilities of bacteria injected into the subsurface. Much of our knowledge base comes from laboratory studies using intact cores or repacked columns of subsurface material (10, 11, 20, 32, 33). Fewer studies address the *in situ* transport properties of bacteria (1, 13),

Important to any bacterial transport study is the availability of accurate and specific methods of bacterial enumeration in environmental samples. Bacterial transport experiments performed to date have, for the most part, employed selective plating approaches based on natural or modified bacterial traits (32), nucleic acid-stained bacterial cells (1, 2, 13), DNA-based detection of engineered DNA sequences specific to the organism of interest (34) or radiolabeling of cells (12). These approaches have proved invaluable for monitoring bacterial transport, but may not be applicable in all experimental situations. For example, it is generally not feasible to employ radioactively-labeled or genetically-modified organisms for *in situ* studies, and other methods of “tagging” organisms may alter the physiological or transport properties of the bacteria (13, 28).

To overcome these limitations, a new suite of bacterial tracking tools that are more suitable for *in situ* experiments is required. Vital dyes that appear not to affect bacterial viability or transport have recently been successfully used for laboratory (9) and field (8) transport experiments. Ferrographic tracking of organisms in the subsurface,

based on magnetic recovery of ferric iron-linked antibodies, has been developed and deployed (17). Detection of ^{13}C -enriched bacterial cells by isotope ratio mass spectrometry has also recently been successfully employed to monitor bacterial transport in an East Coast aquifer (14).

Here, we describe the development of direct qPCR techniques to monitor *in situ* bacterial transport in subsurface aquifers. This approach was developed in support of studies where indigenous organisms were injected into a shallow aquifer at the DOE bacterial transport study site on the Eastern Shore of Virginia, and transport behavior subsequently monitored. Requirements for these injection experiments were the use of non-engineered, indigenous bacterial strains not having resistance to clinically important antibiotics. The use of radioactive or other potentially harmful chemical tags was also not allowed. The qPCR approaches described herein satisfy those requirements because detection is based on PCR amplification of a naturally-occurring sequence in the genomic DNA of the test organism. The specificity of detection is based on the binding of PCR primers and in some cases fluorogenic probes to unique regions of the 16S rDNA gene in the target organism. These approaches provide for rapid, sensitive and specific detection of bacteria of interest in groundwater samples.

The gel-based competitive qPCR (hereafter competitive qPCR) method relies on simultaneous amplification of target DNA with a known amount of internal competitor template in the reaction mixture, allowing precise quantification of cell numbers through determination of the ratio of target (unknown) to competitor (known) PCR product. . The added competitor DNA has identical primer-binding regions as the target sequence of the organism of interest, but has a 100 base pair insert in the middle of the target sequence, resulting in the production of two PCR products of different size in a single reaction. This allows simultaneous separation and quantification of competitor and target PCR products in an agarose gel. The competitive qPCR concept was first developed to monitor HIV long-terminal repeats (35) and has been previously used in past microbial

ecology research for the quantification of 16S rDNA genes (16, 21-24), 16S rRNA copy numbers (6, 7, 29), and for the detection of specific functional markers (18, 24, 25).

The fluorogenic real-time qPCR (hereafter fluorogenic qPCR) method is based on the real-time detection of fluorescence increase from an oligonucleotide probe molecule when degraded by the 5' exonuclease activity of the polymerase enzyme used in PCR (15, 21). The oligonucleotide probe molecule contains a fluorochrome moiety covalently attached to one end of the molecule (5' or 3' end), and a quencher moiety attached to the other end. The quencher prevents emission of light by the fluorochrome when the probe is intact (e.g. bound to sample DNA or free in solution). During each cycle of PCR, double-stranded DNA is denatured at 95°C, followed by specific binding of the PCR primers and oligonucleotide probe to complementary regions of the denatured DNA strands. The polymerase enzyme then traverses the template from the 3' end of each primer, degrading bound probe with its 5' exonuclease activity producing a light emission increase in proportion to the exponential increase in DNA copies during PCR amplification. A threshold cycle (T_C) is then determined corresponding to the point at which fluorescence begins to increase in a linear fashion. Samples with higher target cell numbers will have a lower T_C , while those with lower target cell numbers will have a higher T_C . By inclusion of a dilution series of known target cell numbers, a regression formula is generated to enumerate the number of target cells in each sample. The fluorogenic qPCR concept has been previously used in past microbial ecology research for the quantification of 16S rDNA and rRNA (3, 4, 31). We report an approach for direct qPCR that eliminates sample processing and DNA purification. This direct approach was validated by comparison to data obtained from direct microscopic enumeration, plate count enumeration, and radioactively labeled bacteria in experiments with repacked columns and intact cores of sediment from the DOE Oyster bacterial transport site. Representative data from *in situ* injection experiments at the site are also presented in comparison to other detection methods used in the field. To our knowledge,

this work represents the first direct application of competitive and fluorogenic qPCR methods to unpurified environmental samples, and the first use of qPCR to study subsurface bacterial transport.

MATERIALS AND METHODS

Bacterial culture conditions. The bacterial strains *Comamonas* sp. DA001 and *Acidovorax* sp. OY107, were isolated from groundwater as indigenous members of the microbial community at the DOE bacterial transport site as previously described (5). The identification of these aerobic heterotrophic strains was based on partial 16S rDNA sequence information. To initiate each experiment, the cells were streaked from a permanent glycerol stock culture (15% glycerol, -70°C) onto R2A agar (Difco, Detroit, MI) and incubated at 25°C for 48 h. For radioactive labeling, colonies growing on R2A agar were scraped off with a sterile loop and thoroughly resuspended to an OD₅₅₀ of 5.0 in M9 medium (26). This cell suspension was diluted 1:100 in 250 ml of M9 supplemented with 250 µl of ¹⁴C-acetate (1.0 mCi/ml, 2.0 mCi/mmol, New England Nuclear, Boston, MA) and incubated overnight at 25°C on a rotary shaking platform at 250 rpm. Unlabeled acetate was then added to a final concentration of 0.1% (w/v) followed by an additional 48 h incubation with shaking at 25°C. Following incubation, the cells were harvested by centrifugation at 16,000 x g at 10°C for 10 min, then resuspended in the same volume of unsupplemented M9 to remove unincorporated glucose. This wash step was repeated and the cells resuspended in one-fourth volume of un-supplemented M9, then starved at room temperature in the dark for 48-72 h prior to use in experiments. Following starvation, the cells were once again washed as described above. The degree of ¹⁴C enrichment in the cultures was determined by analysis of an aliquot of the starved bacterial suspension as described below.

Study site description. The DOE bacterial transport site is located at the tip of the Delmarva Peninsula on the southern end of the Eastern Shore of Virginia. The site has been fully characterized and is described in detail elsewhere (5). The sediments in the aquifer are comprised of unconsolidated to weakly cemented sand that is well-sorted and ranges from fine- to medium-grained and pebbly sand. The sediments were deposited by wind-, wave-, and tidally-driven currents (5).

Intact and repacked cores. Native-matrix intact and repacked core experiments were performed to validate the qPCR approaches for specific detection of bacteria in aquifer samples. Sediment material, intact cores and groundwater used in these experiments were taken from the DOE bacterial transport site. Intact cores (7.5 x 70 cm) were recovered from an exposed outcrop (the “borrow pit”) and represent lithologies similar to those in the flow field used for the *in situ* experiment (27). As described previously (14), each exposed end of the cores was trimmed to provide a final length of 50 cm and sealed with a ported PVC end cap milled and screened to provide uniform access to the entire core diameter for influent and effluent water. Prior to initiating experiments, cores were perfused extensively with 5-10 pore volumes of site groundwater (SGW) in an up-flow configuration.

Repacked cores were constructed of polycarbonate cylinders (3.5 x 45 cm) which were packed to uniform density with bulk sediment (thoroughly homogenized) from the borrow pit. The repacked cores were capped with rubber stoppers containing inlet and outlet ports and sealed with silicon caulking. Repacked cores were sterilized by autoclaving three times for 1 h with 24 h intervals between each round of autoclaving. Prior to initiating experiments, the repacked cores were perfused in an up-flow configuration with 5-10 pore volumes of Oyster artificial groundwater (OAGW), the composition of which is based on the groundwater chemistry of the site and has been described previously (14).

For all core experiments, radioactively labeled cells were introduced at a density of approximately 10^9 cells/ml (exact concentrations were precisely determined for each experiment) in 0.5 PV of SGW or OAGW at a flow rate of 5.0 ml/min in the up-flow configuration. Samples (0.1 pore volumes each) were collected from the core outlet during this process using a Bio-Rad Model 2128 fraction collector (Bio-Rad Laboratories, Hercules, CA). Individual samples were processed for enumeration of bacterial cells by direct microscopic enumeration, plate count enumeration, radioactive isotopic analysis, or qPCR as indicated.

Direct microscopic enumeration. Direct microscopic enumeration of cells in the cultures and core eluent samples was based on fluorescence microscopy of DAPI stained cells, essentially as described by Schallenberg et al., (30).

Plate count enumeration. Enumeration of strains DA001 and OY107 based on colony forming units (CFU) was accomplished by spread plating appropriate dilutions of samples onto R2A agar plates followed by incubation at 25°C for 48 h.

Quantification of cell numbers based on ^{14}C label. Radioactively labeled cells in OAGW or SGW were enumerated by filtering 5 ml of water sample (unknown or regression samples) onto a 0.2 μm Poretics filter (Osmonics, Livermore, CA). The filter was subsequently added to 10 ml of EcoLite scintillation fluid (ICN Biomedicals, Inc., Costa Mesa, CA), mixed vigorously, and then counted in a Beckman LS 6500 scintillation counter (Beckman Instruments, Fullerton, CA) for 10 min. To determine the numbers of radioactively labeled cells in unknown samples, the relationship between ^{14}C cpm (counts per minute) values and cell numbers was established as described previously (14). Briefly, a subsample of the starved cells for injection was analyzed by direct microscopic enumeration to determine the true number of cells, and then subjected to serial dilution in triplicate to provide a series of samples containing 10^9 , 10^8 , 10^7 , 10^6 , 10^5 , 10^4 , 10^3 , 10^2 , 10^1 and 0 cells/ml. These dilutions were then analyzed by liquid scintillation counting and the data used to generate a regression plot of log cell numbers

vs. $\log^{14}\text{C}$ CPM. The regression formula from this plot was subsequently used to determine the number of cells/ml in unknown samples based on measured ^{14}C cpm.

Development of the competitive qPCR system. Strains DA001 and OY107 were streaked for single colony isolation onto R2A agar and incubated at 25°C for 48 h. A small, sterile inoculating loop was touched to a single colony and the intact cells transferred to a 600 μl microfuge tube containing PCR reaction mix. The primers in this reaction were 16S rDNA "universal" primers 536f (5'-CAGC(CA)GCCGCGGTAAT(AT)C-3', *E. coli* positions 519-536) and 1392r (5'-ACGGGCGGTGTGT(AG)C-3', *E. coli* positions 1406-1392). The PCR reaction mix contained: 20pM of each primer; 200 μM dNTPs; 1x PCR buffer containing 1.5mM MgCl_2 (Boehringer-Mannheim, Mannheim, Germany); and 5 units of Taq polymerase (Boehringer-Mannheim) in a total reaction volume of 50 μl . A "touchdown" PCR protocol was used to increase specificity of amplification (5), with an initial denaturation at 95°C for 15 minutes, followed by 20 cycles of: 1 min denaturation at 95°C; 1 min annealing at 68°-58°C (starting at 68°C and decreasing by 0.5°C/ cycle); 3 min extension at 72°C; and then 10 additional cycles of 1 min denaturation at 95°C; 1 min annealing at 58°C; 3 min extension at 72°C; and a final extension for 10 min at 72°C.

The PCR products were purified using the QiaQuick PCR purification kit (Qiagen, Valencia, CA), ligated into the pT7Blue-3 plasmid vector and transformed into NovaBlue competent cells (Perfectly Blunt Cloning Kit, Novagen, Madison, WI) using the manufacturer's specifications. Blue/white screening was used to identify potential clones. Plasmid mini-preps of putative clones were performed using the QiaPrep Spin Mini-prep kit (Qiagen) and proper-sized inserts confirmed by *EcoRI* (Gibco Life Science Products, Grand Island, NY) restriction digestion resolved on 1.5% agarose gels (SeaKem GTG agarose, FMC Bioproducts, Rockland, ME).

DNA inserts were sequenced from both directions and a consensus sequence generated using the AssemblyLign program (Eastman Kodak, New Haven, CT).

Potential primer pairs for specific detection of strains DA001 and OY107 were generated using the MacVector program (Eastman Kodak), which selects primer pairs based on GC content, compatible melting temperatures, and secondary structure considerations. The candidate qPCR target sequence was then submitted to the RDPII database (Ribosomal Database Project II, Center for Microbial Ecology, Michigan State University (www.msu.edu/RDP/html)), checked for potential chimeras using the "Chimera Check" program, and aligned to the nearest relatives in the RDPII database using the "Sequence Match" program. The sequences of the five most closely related organisms represented in the database were downloaded from the RDP site and manually aligned with the corresponding DA001 sequence using AssemblyLign. Based on this approach, primer sets specific for strains DA001 and OY107 were obtained comprised of the specific primers DA001f and OY107f (positions 471-494 based on *E. coli* positions) and the universally-conserved reverse primer 1392r (19). As a final confirmation of primer specificity, the primer sequences were analyzed by the "Probe Match" analysis of RDPII, which compares potential primer sequences to all known 16S sequences in its database. Each primer was determined to be unique to strains DA001 and OY107, respectively.

For development of the competitor sequence for the qPCR procedure, the DNA Strider 1.0 (internet shareware) program was used to determine potential restriction enzyme sites in the target sequence. A 100 bp segment of DNA was subsequently inserted into the qPCR target sequence using the restriction enzyme Aat II. For this purpose, the 100 bp fragment of the Gibco Low Mass Ladder (Gibco Life Science Products, Grand Island, NY) was excised from a low-melt 1.5% NuSieve GTG agarose gel (FMC Bioproducts, Rockland, ME), purified by phenol/chloroform extraction and ethanol precipitation using standard protocols, and ligated into the target DNA at the *Aat II* restriction site. The correctness of this construct was subsequently confirmed by DNA sequence analysis.

Competitive qPCR analysis. A standard curve of known numbers of DA001 cells was generated for regression analysis based on a serial dilution of target cells with a fixed amount of competitor DNA. A dilution series of known amounts of competitor DNA in either SGW or OAGW (as appropriate) was first amplified alone to determine the linear concentration range of amplification based on subsequent detection of product on 1.5% SeaKem GTG agarose gels (FMC Bioproducts, Rockland, ME) with EtBr staining (125 $\mu\text{g}/\text{ml}$). The amount of competitor DNA template corresponding to the midpoint of the linear range (0.6 ng of competitor plasmid DNA or approximately 1×10^5 copies per PCR reaction) was then chosen as the fixed amount of competitor for future regression sample and unknown sample analyses.

For the regression analysis, a subsample of the starved cells for injection was analyzed by direct microscopic enumeration to determine the true number of cells and then subjected to serial dilution in triplicate to produce a series of samples containing 10^9 , 10^8 , 10^7 , 10^6 , 10^5 , 10^4 , 10^3 , 10^2 , 10^1 and 0 cells/ml. These dilutions were then used in qPCR reactions and the data used to generate a linear regression plot of cell numbers vs. target:competitor ratio as described below. For all qPCR reactions 12.5 μl of water sample was added to 12.5 μl of PCR reaction buffer containing 20 pM each of primer sets DA001f and 1392r or OY107f and 1392r; 200 μM dNTPs; 1x PCR buffer containing 1.5mM MgCl_2 (Boehringer-Mannheim, Mannheim, Germany) and 5.0 units of Taq polymerase (Boehringer-Mannheim) in a total reaction volume of 50 μl . Touchdown PCR was then performed as described above. Following PCR, 8 μl of PCR product and 2 μl of sample loading buffer were mixed and loaded on a 1.5% TAE agarose gel and subjected to electrophoresis. A digital image of the resulting ethidium bromide-stained DNA bands was captured using a Gel Doc 1000 digital system and Molecular Analyst software (Bio-Rad Laboratories, Hercules, CA) and then exported as a TIFF file into RFLP Scan Plus 3.0 software (Scanalytics Inc., Billerica, MA) for quantification by densitometry. A plot was made of the log of known cell numbers in the dilution series to

the log of target:competitor product ratio. The regression formula from that plot was subsequently used to determine the numbers of cells in unknown samples based on their target:competitor ratio. New dilutions and regression analyses were performed for each different experiment.

Fluorogenic probe qPCR. For this approach, the consensus sequence of DA001 and OY107 was used for developing the probe sequences for fluorogenic qPCR. For analysis of samples, a subsample of the starved cells for injection was analyzed by direct microscopic enumeration to determine the true number of cells and then subjected to serial dilution in triplicate to produce a series of samples containing 10^9 , 10^8 , 10^7 , 10^6 , 10^5 , 10^4 , 10^3 , 10^2 , 10^1 and 0 cells/ml. These dilutions were then used in qPCR reactions and the data used to generate a linear regression plot of cell numbers vs. T_c (threshold cycle), and the regression formula used for determination of cell numbers. For all qPCR reactions, 5.0 µl of water sample was added to 12.5 µl of Platinum Quantitative PCR SuperMix-UDP (2X solution containing 60 U/ul Platinum Taq DNA polymerase, 40mM Tris-HCL (pH 8.4), 100mM KCl, 6 mM MgCl₂, 400uM dGTP, dATP and dCTP, and 800 uM dUTP, 40 U/ul UDG and stabilizers), with 0.2uM forward and reverse primers, and 0.2uM 5' FAM-labeled fluorogenic probe with 3' QSY7 quencher (MWG Biotech, High Point, NC), in a total volume of 25 ul. The real-time detection of PCR product accumulation was accomplished using an iCycler PCR thermocycler / fluorometer (Bio-Rad Labs, Hercules, CA). A two-step PCR protocol was utilized, 45 cycles of (950 for 45 sec., 720 for 1min. 30 sec.), with FAM-specific fluorescent detection during the annealing cycles.

RESULTS AND DISCUSSION

Long-term monitoring of injected bacteria into the deep subsurface is possible with qPCR because of specific detection of genomic 16S rDNA of the target organism. The gel-based competitive and fluorogenic probe qPCR methods are both accurate and specific PCR-based methods for the enumeration of bacteria in environmental samples. Although both methods rely on PCR amplification of 16S rDNA genes, the gel-based method requires many more steps and man-hours, both in the development of the procedure, as well as in sample analysis (Fig.1).

Sample analysis using the gel-based qPCR method requires PCR amplification of sample DNA, followed by separation of PCR products on a TAE agarose gel containing EtBr, scanning of the gel image into a densitometry program, determination of absorbance of genomic and competitor band intensities, and manual entry of absorbance data into a spreadsheet program for determination of cell numbers. Although labor- and time-intensive, gel-based competitive qPCR is a cost-effective and accurate method for bacterial enumeration by PCR, and can be accomplished using equipment found commonly in any molecular biology laboratory.

The main advantage of the fluorogenic probe approach is the ability to process many samples simultaneously and very quickly. Samples are loaded into the wells of a 96 well microtiter plate containing PCR master mix (all PCR components except primers, probe, and sample template) and PCR is performed with a combination PCR thermocycler / fluorometer. Results are obtained under real-time conditions, with calculated cell numbers directly downloaded to a spreadsheet program. Sample analysis which took days to complete using the gel-based method requires only a few hours for 96 samples using the fluorogenic probe method. This approach also has the advantage of a much broader linear range of detection, virtually eliminating the need for sample dilution (see Standardization and Controls).

Development of Methods

Development of both the gel-based and fluorogenic qPCR methods begins with insertion of the gene of interest into a plasmid vector for ease of manipulation and sequence analysis. In the 16S rDNA genome, there are a number of potential regions to choose from, and it is best to consider as many regions as possible for potential primers and probe locations. For this reason, it is advantageous to initially clone as big of fragment of the 16S genome into the vector system as possible. We used the universal primers 536f and 1392r to clone approximately 60% of the 16S genome into our working vector.

Development of the gel-based qPCR involves construction of a plasmid competitor that competes with sample template DNA in a PCR reaction tube. The construction of the competitor sequence requires either an internal insertion or deletion in the target sequence to facilitate discrimination of the two fragments (target and competitor) by agarose gel electrophoresis. Upon examination of the DNA sequence of DA001, no single restriction enzyme or pair of enzymes could be identified to remove the desired 50-100 bp of DNA by deletion, an artificial 100 bp DNA fragment (gel-purified from the Gibco Low Mass DNA Ladder) was cloned into the single *Aat* II site of the target sequence. The resulting construct sequence was not similar to any organism found in the RDP database when analyzed by the Sequence Match or Sequence Align programs. Thus, the inserted piece of DNA was not expected to significantly affect its PCR amplification qualities since the primers employed do not bind in that region, and no secondary structures were predicted that might inhibit or otherwise affect the polymerase chain reaction. The success of PCR amplification in yielding the proper-sized product in predicted amounts was further confirmation that the insertion caused no functional problem for the qPCR procedure. The construction of the competitor sequence and an

example agarose gel showing the separation of target and competitor fragments are depicted in Fig. 2.

Unlike the gel-based method, the majority of developmental time for the fluorogenic probe method involves sequence analysis for determination of suitable primer and probe locations. For use of the fluorogenic probe method on 16S rDNA genes, this can be problematic for a number of reasons. First, the PCR amplicon must be relatively small in size (≤ 150 bp) for real-time detection of fluorogenic probes (PE Applied Biosystems bulletin # 777904). This limits the choice of suitable primer and probe locations because variable regions of the rDNA genome (specific to a given organism) are often hundreds of base pairs apart. Because of potential secondary formation in the sequence of bases in the 16S rDNA (which might cause the oligonucleotide probe to fold onto itself) there is a need to run the PCR analysis at relatively high annealing temperatures as well. For our analysis, we used a 2-step PCR protocol of 45 cycles of 30 sec. at 95°C for denaturation and annealing/extension for 1 min. at 72°C . And because of the high temperature conditions needed for annealing, the sequence chosen for primers and probe had to have a relatively high T_m as well, which in turn requires fairly long oligonucleotides to be designed (personal communication, Research and Development, Bio-Rad Laboratories, Hercules, CA). For the fluorogenic method, the resultant primer and probe sequences were approximately 40 bp long with an average G-C content near 60%. One advantage of the relatively high T_m 's required is being able to run the annealing/extension step at the T_m maximum for Taq polymerase.

PCR primers and probe selection

The specificity of detection for both the gel-based and fluorogenic qPCR methods is based upon the regions of DNA selected for primer and probe location. In general, selection of suitable primers and probes for qPCR involves the following steps:

1. Choosing potential PCR primer and probe location

2. Adjustment of the length and position of primers/probe so that T_m matches within 2°
- C.
3. Analysis of sequences for potential secondary structure and self-complimentary regions
4. Check of specificity by comparison of primer and probe sequence to known organisms

The approximate location on the 16S genome sequence where the primers / probe are to be located was first chosen: more variable regions for specific detection of an organism, or more-conserved regions for group-specific detection. A computer program was then used (MacVector, Kodak, Rochester, N.Y.) to identify potential primer pairs based on desired amplicon and primer length, %G-C content, ionic content of PCR buffers, as well as compatibility as primers. Both the size and base composition of the DNA chosen (G-C content) determine the T_m ; increasing the size and/or the G-C content of the primer/probe increases its T_m as well.

Gel-based competitive qPCR. For our experiments, the goal was to develop a specific detection system for the quantification of the transport of bacteria injected into subsurface aquifers at a research site located in Oyster, Virginia. The gel-based competitive method was developed for an injection of the bacterial strain DA001 into an aerobic subsurface aquifer (Narrow Channel or NC) in Nov. 1999. For strain DA001, only one highly specific region was identified by MacVector that was both specific for the organism and usable as a functional primer. We therefore decided to pair this specific primer (designated DA001-F) with a universally-conserved reverse primer, 1392r. Compatibility of the specific forward primer and universal reverse primer for PCR reactions was determined by T_m analysis (MacVector), and searching for regions of self-complimentarity by analysis of folding (Zuker RNAfold). The primers should have T_m 's within 2°C of one another, and the ∂G of folding should be positive. Based on analysis of the DA001f primer sequence using the Sequence Match and Probe Match components

of the RDP II website, and functional specificity tests in column studies in the presence of the indigenous community described below, one specific primer was sufficient to allow sensitive and quantitative detection of strain DA001.

For the injection of DA001 into the NC flowfield, it was important to distinguish DA001 from other background organisms in the NC flowfield. The specificity of the gel-based qPCR approach for detecting the target organism was first assessed by comparison of the DNA target sequence of the DA001 cells to other known sequences in the RDP database. Using the "Sequence Match" analysis tool of the RDP, the DA001 sequence of interest was aligned to its nearest relatives on the database. The nearest known relative was *Comamonas testosteroni* with an Sab score of 0.897. The true basis for specificity in these qPCR reactions stems from differences between the sequence of the selected forward PCR primer and the corresponding regions of the related organisms, where there are three or more base differences between the DA001 forward primer and all other organisms (Table 1).

The validity and utility of the gel-based qPCR approach was tested in replicate columns of re-packed, sterilized sediment obtained from the Oyster site using artificial groundwater for the water phase (Fig. 3). Bacterial breakthrough (elution profile) results for DA001 were very similar for qPCR, direct microscopic enumeration, culturable counts (except at very low cell densities) and liquid scintillation counting. Thus, the comparatively rapid and inexpensive gel-based qPCR approach reliably and reproducibly detected bacterial breakthrough producing results that compared very favorably to three other mechanistically independent bacterial tracking approaches. These results demonstrate that this approach can be employed to accurately, reliably and accurately monitor bacterial transport in the subsurface.

Fluorogenic probe qPCR. Because of the large number of samples generated during a typical subsurface injection, fluorogenic qPCR was developed to replace the gel-based competitive qPCR in support of a bacterial injection into the anaerobic flowfield

(designated SOFA) at the Oyster site in the summer of 2000. The goal for this injection was to distinguish and quantify two bacterial types co-injected into the flowfield for a comparative analysis of transport behavior. Therefore, the most important factor in choosing of primers and probes for each strain was to identify primer and probe sequences that were different between the test organisms. The choice of primer and probe sequences was complicated by the need to keep the PCR product size < 150 bp to reduce PCR errors (PE Applied Biosystems bulletin# 777904). Fig. 4 compares the partial 16S and DNA sequences of DA001 and OY107, showing the location of forward and reverse primers, and the regions chosen for the fluorogenic qPCR probes. As can be seen, there are many potential areas for primer and probe location to distinguish DA001 and OY107 (DNA degeneracies are indicated by black boxes in Fig. 4) As with development of the gel-based qPCR, potential primer pairs were determined using MacVector software, but adjustments to the sequences had to be made to account for relatively high T_m needed for the fluorogenic qPCR method (personal communication, Research and Development, Bio-Rad Laboratories, Hercules, CA). Through a combination of shifting the probe/primer sequence toward the 5' or 3' direction (changing the G-C content), and changing the the sequence length (usually lengthening), a combination of primers and probe regions were chosen that have similar high T_m 's. Compatibility of the specific forward primer and universal reverse primer for PCR reactions was determined by T_m analysis (MacVector), and searching for regions of self-complimentaryness by analysis of folding (Zukar RNAfold). Similar to development of the gel-based qPCR (based on the DNA sequence, size constraints, and other factors) it was decided to pair specific forward primers with a reverse primer that was identical for both organisms. The internal flurogenic probe binding region has four base differences between the two strains, which, coupled with the specificity of the forward primer region (three base differences), confers specificity to the fluorogenic qPCR method allowing specific detection and enumeration of both strains, when both are present in the same sample.

As a test for specificity, an experiment was conducted in which the concentration of either DA001 or OY107 was kept constant in a PCR reaction, while increasing the amount of the other organism in the reaction was varied over orders of magnitude. If the primers and probes chosen are totally specific, no non-specific increase in the apparent number of non-target cells should be seen when the target strain is present in excess. The OY107 detection system was specific to a ratio of one OY107 cell to 10,000 DA001 cells while the DA001 selection system was specific up to a ratio of one DA001 cell to 1,000 OY107 cells (Fig. 5). Both systems were considered acceptable since cell ratios in this range are unlikely in our injection experiments.

Controls and standardization

Our qPCR study of the subsurface transport bacteria at the Oyster research site was benefited by the conditions of the site aquifer. In all of the studies to date involving qPCR detection of bacteria from environmental samples, a separate DNA purification step was utilized prior to PCR amplification to remove contaminants such as humic acids and organic compounds that could inhibit DNA polymerase. The use of a separate DNA purification step requires calculation of "cell lysis efficiency" and "DNA recovery efficiencies" in order to estimate true rDNA copy numbers in environmental samples. Most studies to date have not directly addressed this concern, and report copy numbers normalized to the amount of DNA recovered, or per weight or volume of sample.

Since the groundwater of the Oyster aquifer is relatively free of organic compounds and contaminants (< 0.5% DOC (5)) extensive processing of samples and purification of DNA was not required. In fact, we were able to reliably perform qPCR directly on water samples from the aquifer without any type of DNA purification. Serial dilutions of known numbers of cells in Oyster aquifer site water and in distilled water gave essentially identical results (data not shown), indicating that no inhibition of the PCR reaction was occurring due to organics or other contaminants in the aquifer water.

Interestingly, inhibition of PCR reactions was observed only in a few samples where concentration of cells from water samples via centrifugation was attempted in an effort to increase PCR detection levels.

To quantify the number of bacteria in a sample by gel-based competitive qPCR, a regression analysis of data from a serial dilution series of known numbers of target cells amplified with a standardized amount of competitor DNA was performed. Consistently, through a number of such regression analyses, there was approximately three orders of magnitude of linear range of detection (Fig. 6A). This linear response typically ranged from approximately 5×10^2 to 5×10^5 cells per PCR reaction. This corresponds to a lower limit of detection of approximately 2×10^4 cells/ml, with a linear response to 2×10^7 cells/ml for unconcentrated water samples. Typical linear regressions for DA001 and OY-107 (a second bacterial strain used in the SOFA 2000 injection) by the fluorogenic probe method are shown in Fig. 6B. Noticeable immediately is the much broader linear range of detection with the fluorogenic qPCR for both cell types, typically 10^2 to 10^8 cells/ml. Unlike gel-based end-point analysis, where one has to predict a range of proper dilution to be in the linear range of detection, the fluorogenic method usually requires no sample dilution or other processing.

Also important to our injection experiments are the concentrations of indigenous DA001 and OY107 in the Oyster flowfields. Since qPCR is based on detection of 16S rDNA genes, the method detects both injected bacteria and indigenous cells in the flowfield. Concentration of indigenous DA001 in the NC flowfield immediately prior to the November 1999 injection averaged $\log 4.18$ cells/ml (Table 2). Prior to the co-injection of DA001 and OY107 into the anaerobic SOFA flowfield in July 2000, DA001 cells averaged $\log 3.30$ cells/ml and OY107 cells (isolated from the SOFA flowfield) averaged $\log 4.19$ cells/ml in the SOFA field (Table 2). The concentration of bacteria employed for *in situ* injection experiments is typically 10^8 to 10^9 cells/ml. Thus, for a typical *in situ* injection experiment, this approach should reliably allow for a linear range

of detection of bacterial breakthrough in unconcentrated samples that spans four orders of magnitude from 10^4 - 10^8 cells/ml.

Finally, the ability to store and later retrieve samples for analysis is of great importance in microbial ecology for studies such as *in situ* injection experiments where the use of several multi-level samplers that can generate thousands of individual samples for analysis. To assess the ability to archive groundwater samples for later analysis by qPCR, two experiments were performed. In the first experiment, column fractions from a laboratory column injection experiment were analyzed at times $t = 0, 2, 3$ and 4 weeks after storage at 4°C . Sample numbers were consistent through 3 weeks of storage at 4°C and only after 4 weeks were sample numbers significantly different from original detected values. In the second experiment, DA001 cells used for the NC '99 injection were diluted in site ground water to known cellular concentrations (by microscopic count), and stored at 4°C and -20°C for up to one year, and then analyzed by qPCR (Table 3). Cell numbers from archived samples stored at 4°C were stable for approximately 1 month, while those stored at -20° were nearly identical in magnitude to input cell numbers, indicating that long-term storage at -20°C was possible with archived samples from the Oyster site.

Field validation of qPCR methods:

The truest test of the qPCR approach is bacterial enumeration in *in situ* field injections at the Oyster site. The gel-based competitive qPCR technology was used to monitor the transport of DA001 bacteria in an *in situ* injection into the Narrow Channel aerobic flowfield at the Oyster field site, in November of 1999. DA001 cells were injected at a concentration of 10^8 /ml concurrently with a bromide tracer (100 ppm) during a 12 hour injection pulse. Results of DA001 breakthrough at MLS located 1.5 meters, 3.5 meters and 5.5 meters from the injection port are shown in Fig. 8A. Due to the lengthy amount of time needed for sample processing and analysis by the gel-based

competitive qPCR method, only samples from a single port located 6.5 meters below ground were processed from the injection. The fluorogenic probe PCR method was field-tested during a co-injection of DA001 and OY107 cells into the Oyster anaerobic SOFA flowfield in July/August of 2000. The shorter processing times afforded by this approach allowed rapid analysis of hundreds of samples in just a few weeks compared to the gel-based competitive qPCR method. Fig. 8B shows the breakthrough results for DA001 and OY107 cells at all twelve sampling ports (4-6 meter depth) from a single MLS located 1.5 meters from the injection port, with other qPCR data from in-line MLS at 3.5 and 5.5 meters excluded for figure clarity. The fluorogenic probe qPCR method thus allowed for complete analysis of in-line MLS water samples from the SOFA injection of 2000. An interesting and important extension of this technology comes from its application to solid phase (sediment) samples obtained by coring the flowfield following the injection experiment. Multivariate analysis of sediment and water qPCR results is being used to determine the factors which influence the transport and attachment of injected bacteria into shallow subsurface aquifers (publication in preparation).

The gel-based competitive and fluorogenic probe qPCR methods are both able to accurately and specifically detect bacteria that are transported in shallow subsurface aquifers. The gel-based method is more laborious, time consuming and has a more limited detection range than the fluorogenic method, but has the advantage of being a low cost qPCR alternative. Both methods offer an accurate portrayal of bacterial breakthrough in comparison to established and novel methods of bacterial enumeration. The methods were used directly on site water samples containing transported bacteria, without the need for DNA purification prior to PCR amplification. The methods reported should benefit other environmental studies with the goal of enumerating bacteria.

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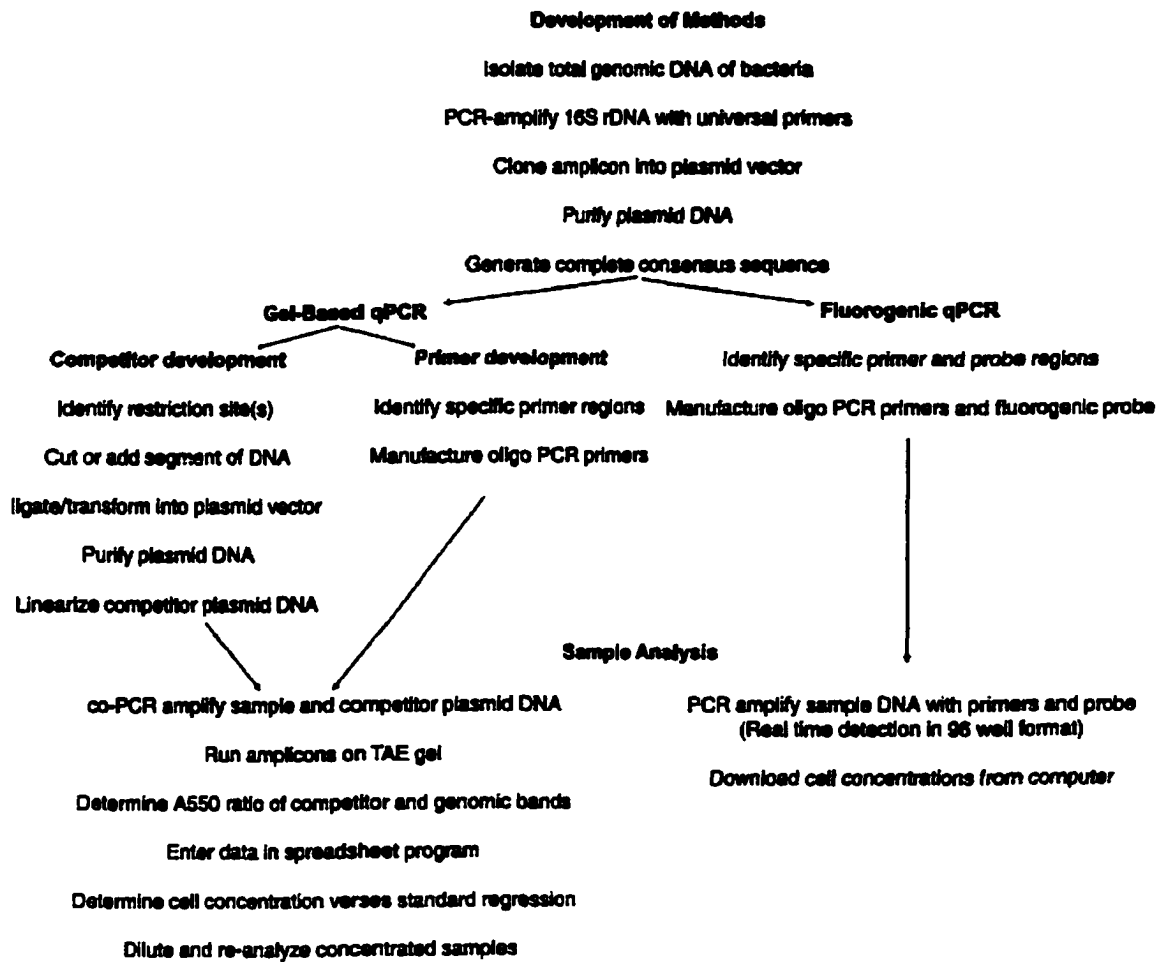


Fig. 1. Flowchart for development of gel-based competitive and fluorogenic probe qPCR methods.

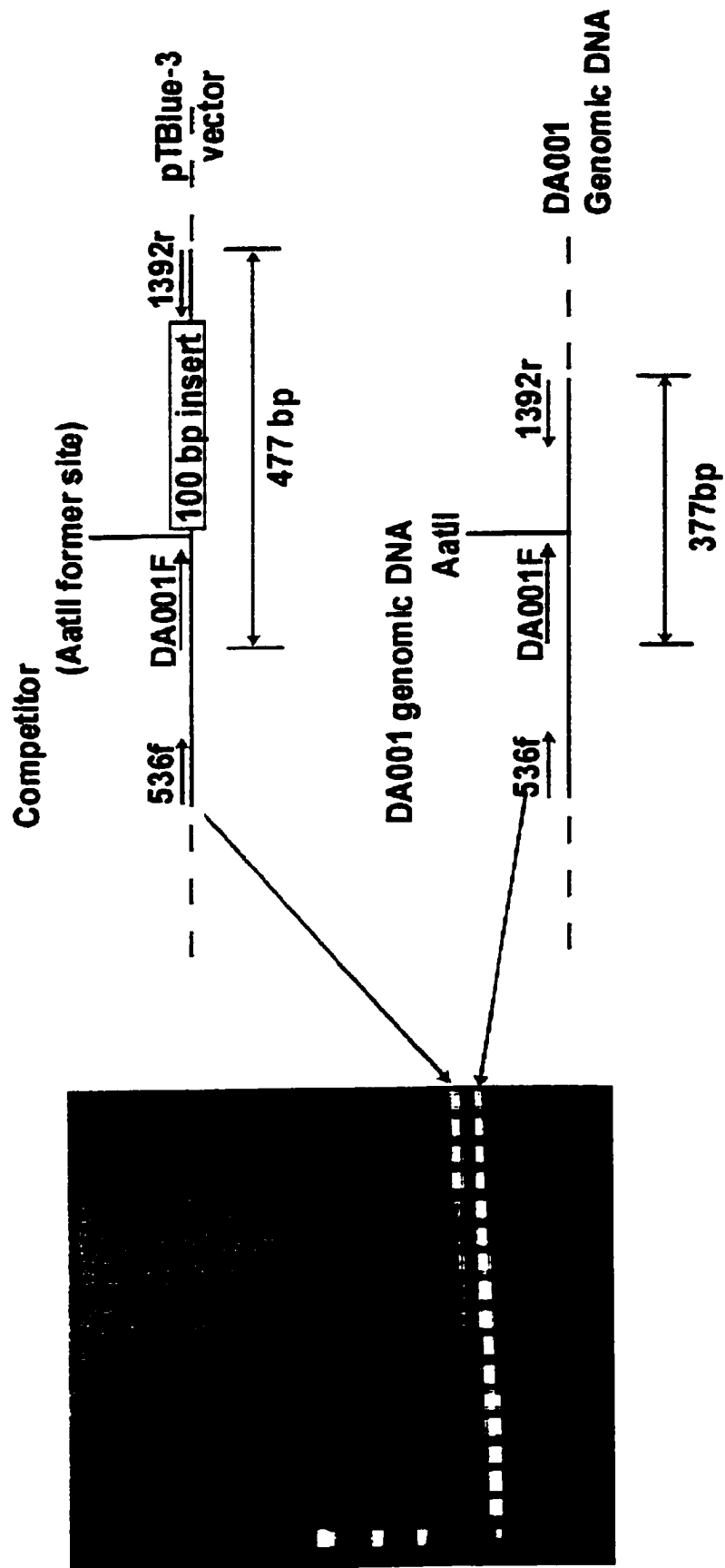


Fig. 2. Comparison of DA001 competitor construct and DA001 genomic DNA, with 1.5% TAE gel image showing location of competitor and DA001 genomic DNA bands.

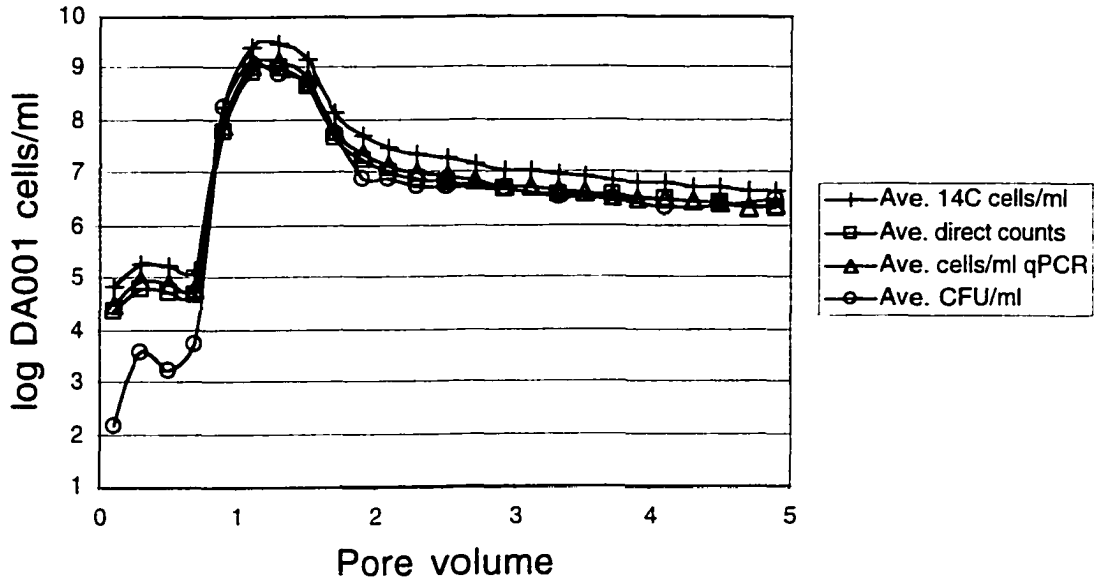


Fig. 3. DA001 bacterial breakthrough on sterile, re-packed column of Oyster aquifer sand, by gel-based competitive qPCR (Δ), direct microscopic counts (\square), culturable colony CFU on R2A plates (\circ), and ^{14}C labeling of cellular components(+).

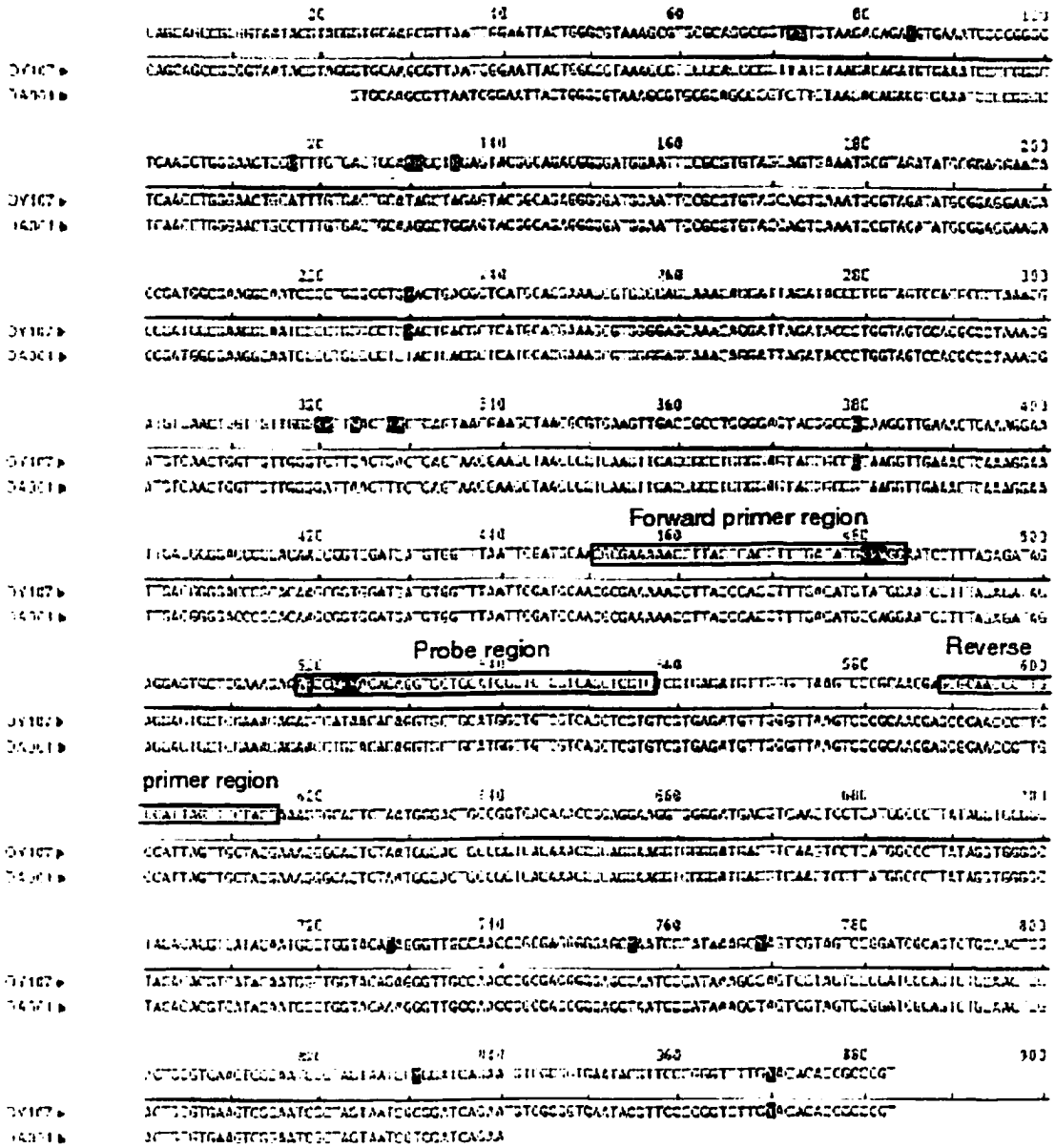


Figure 4. Comparison of DA001 and OY107 16S rDNA sequences showing locations of forward and reverse primers and flourogenic probe.

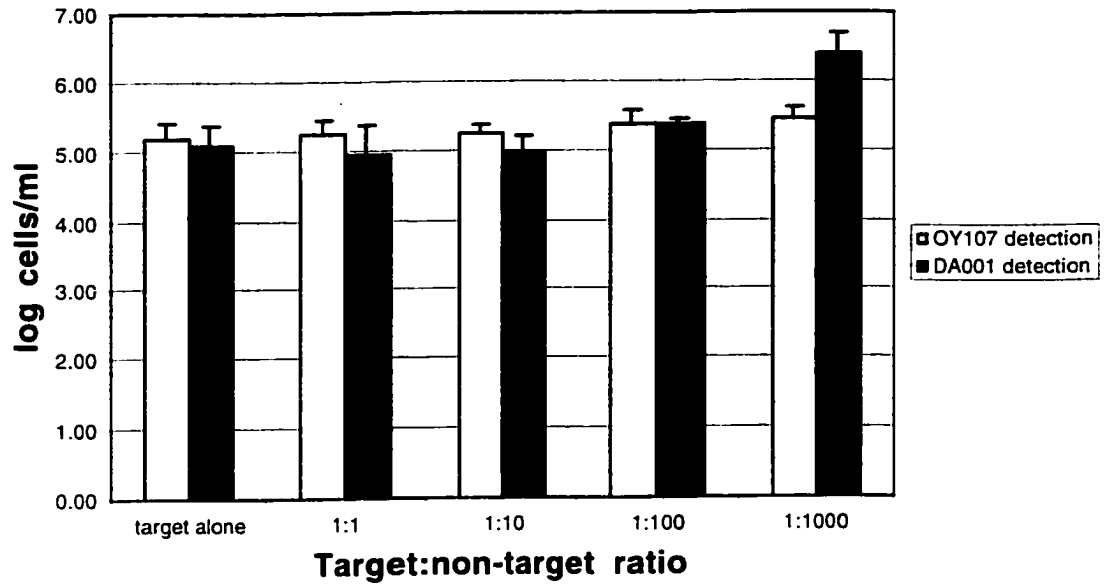


Fig. 5. Test of specificity of detection for DA001 and OY107 cells using fluorogenic probe qPCR method.

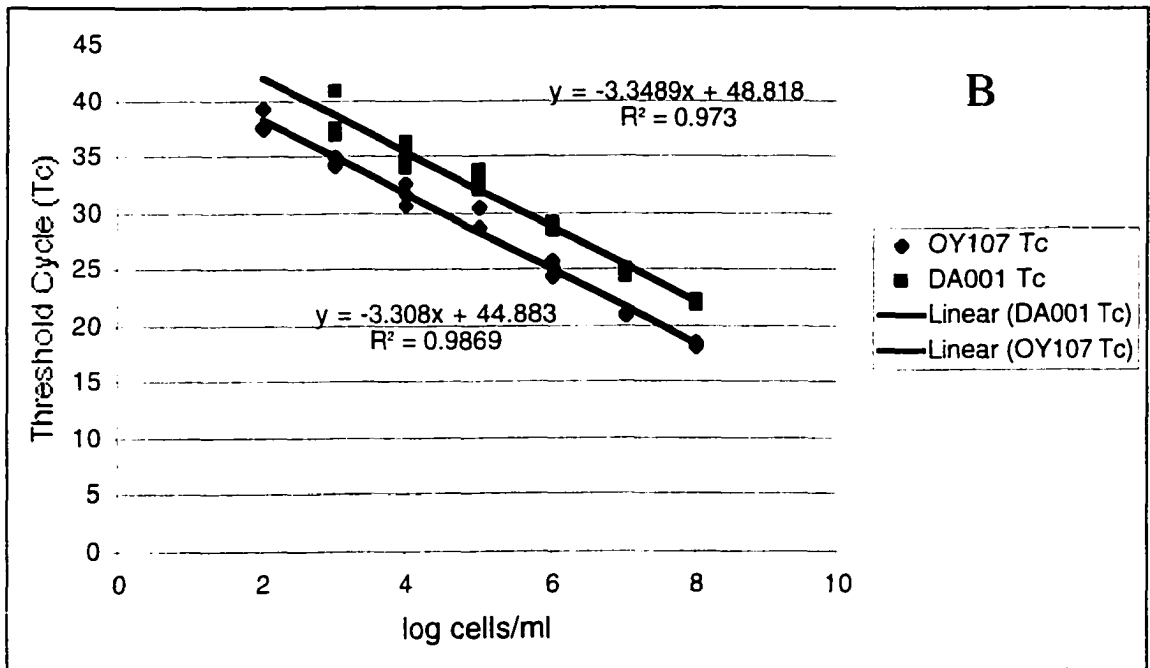
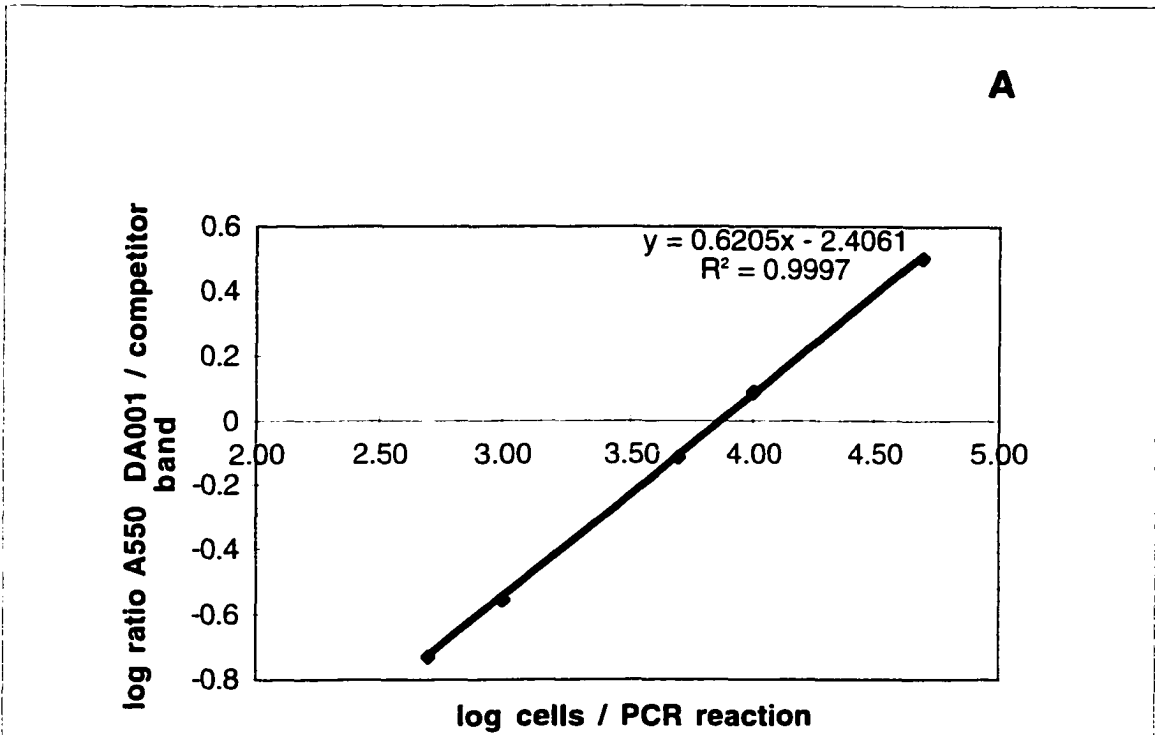


Fig. 6. Linear range of detection, gel-based competitive qPCR method (A), and fluorogenic probe qPCR method (B).

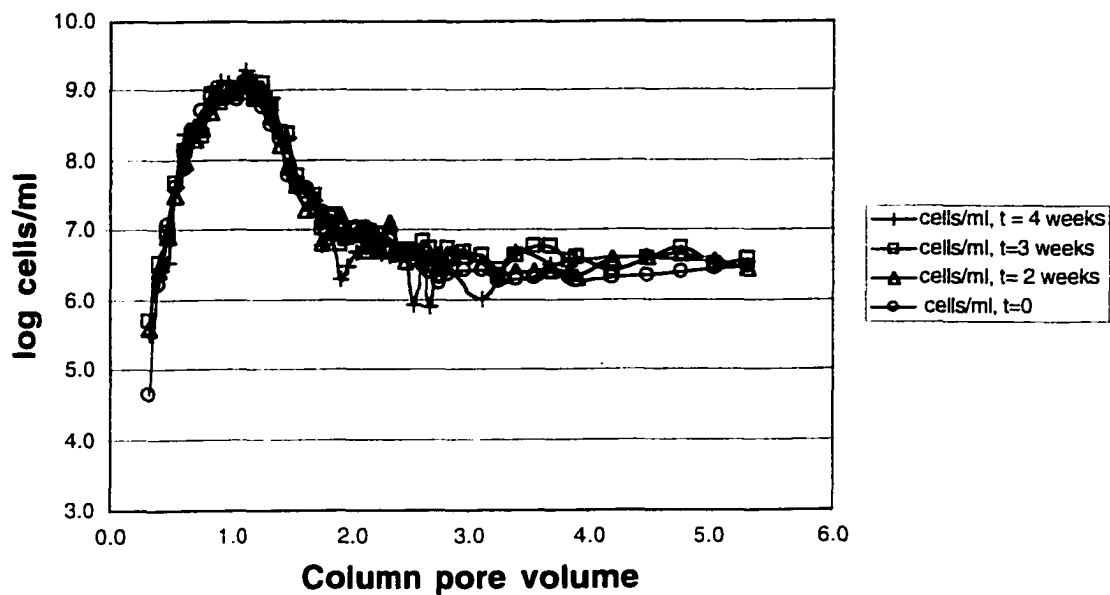


Fig. 7. Short-term stability of archived samples for qPCR detection at 4°C, time zero (o), storage for 2 weeks (Δ), 3 weeks () and 4 weeks (+).

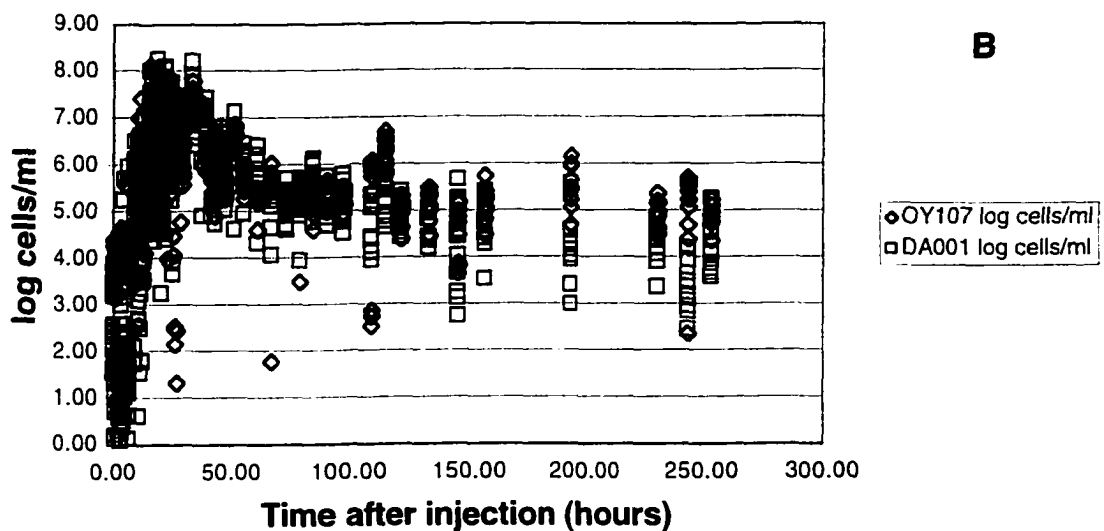
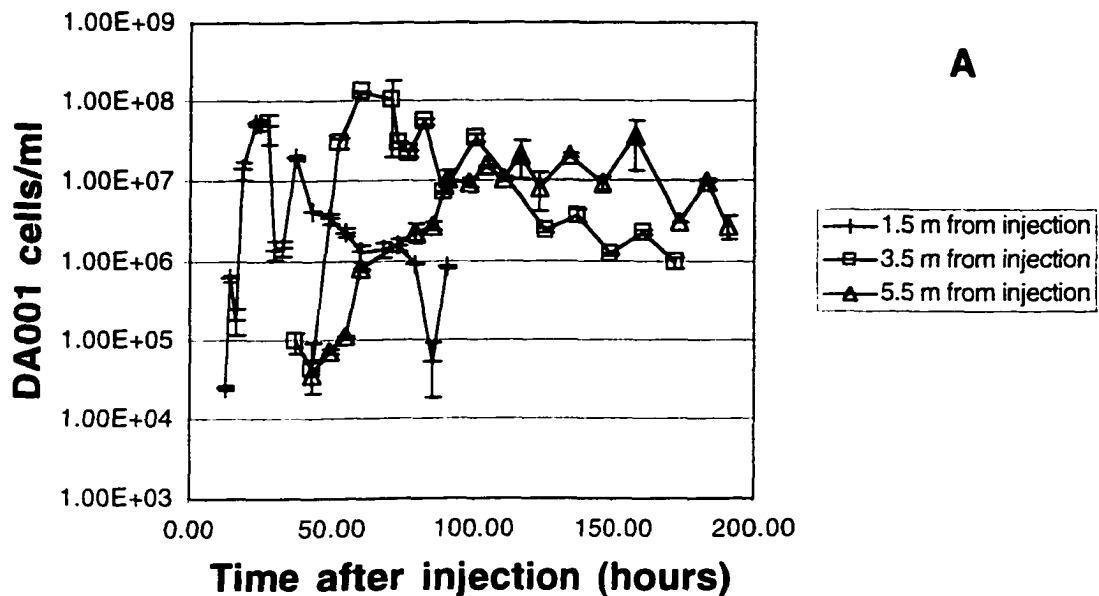


Fig. 8. Field validation of qPCR methods. (A) gel-based, competitive qPCR results of *in situ* injection of DA001 into the Oyster aerobic flow field, October/November 1999, comparing DA001 breakthrough at multi-level samplers located 1.5 meters (+), 3.5 meters (□) and 5.5 meters (△) from the injection port (at 6.5 meters below ground surface). (B) fluorogenic probe qPCR results of *in situ* injection of DA001 and OY107 into the Oyster anaerobic flow field (designated SOFA) in July/August of 2000, showing bacterial breakthrough at a single MLS located 1.5 meters from the injection port (all sample depths from 4 to 8 meters below ground surface).

Table 1: Comparison of DA001 16S sequence to nearest relatives, gel-based qPCR

organism	sequence (DA001 specific primer region*)
DA001	G A G A T <u>A G A G G</u> A G T G C T C G A A A G A G
C. testosteroni	G A G A T <u>G G T T T</u> G G T G C T C G A A A G A G
B. denitrificans	G A G A T T <u>G G G A A T</u> G C T C G T A A G A G
Leptothrix	G A G A T <u>G T G G G A</u> G T G C T C G A A A G A G
V. paradoxus	G A G A T <u>G G C T T A</u> G T G C T C G A A A G A G
I. dechloritans	G A G A T T T <u>G G G A G</u> T G C T C G A A A G A G
L. discophora	G A G A T T T <u>G G G A</u> G T G C T C G A A A G A G

* underlined letters indicate region of specificity

Table 2 : Pre-injection background numbers, DA001 and OY107

Well #	Port #	log ave. DA001/ml	SD	log ave. OY107/ml	SD
pre-Narrow Channel 1989 Injection					
2	10	4.22	0.03	(not determined for NC'99)	
6	10	4.10	0.04		
9	8	4.07	0.08		
9	10	4.89	0.00		
10	12	4.25	0.04		
11	10	4.22	0.04		
16	7	3.96	0.00		
18	10	4.63	0.05		
19	12	3.89	0.18		
21	12	4.24	0.07		
21	7	3.87	0.10		
23	7	4.08	0.24		
	Average=	4.18	0.07		
pre-SOFA 2000 Injection					
10	1	1.68	0.81	3.59	0.24
10	2	2.18	0.41	3.79	0.27
10	3	2.41	0.00	3.35	0.13
10	4	2.34	0.54	3.49	0.35
10	5	2.42	0.01	3.87	0.24
10	6	2.46	0.58	4.29	0.23
10	7	2.60	0.28	4.05	0.18
10	8	2.53	0.17	3.94	0.24
10	9	1.55	0.40	3.68	0.09
10	10	1.59	0.21	3.41	0.46
10	11	2.02	0.70	4.42	0.11
10	12	3.23	0.11	3.40	0.54
14	1	3.27	0.27	4.26	0.23
14	2	2.41	0.61	4.12	0.08
14	3	2.43	0.02	3.96	0.05
14	4	2.49	0.11	4.43	0.15
14	5	2.41	0.73	3.56	0.12
14	6	2.78	0.26	4.03	0.23
14	7	2.45	0.05	4.09	0.03
14	8	3.02	0.38	4.04	0.07
14	9	1.82	0.55	3.88	0.30
14	10	2.62	0.24	4.27	0.20
14	11	2.66	0.04	4.50	0.08
14	12	3.63	0.73	3.42	0.20
T2	1	3.28	0.50	3.97	0.44
T2	2	2.71	0.40	4.23	0.14
T2	3	3.55	0.08	4.21	0.30
T2	4	2.65	0.48	3.74	0.33
T2	5	3.25	0.40	4.45	0.43
T2	8	2.86	0.19	4.29	0.10
T2	7	3.34	0.24	4.28	0.08
T2	8	3.02	0.24	3.95	0.57
T2	9	2.93	0.59	4.16	0.33
T2	10	3.65	0.18	4.16	0.03
T2	11	3.41	0.68	4.41	0.23
T2	12	5.00	0.28	4.38	0.31
	Average=	3.30	0.35	4.19	0.28

Table 3: Long-term storage of archived samples

Time point	input (cell/ml)	-20°C		4°C	
		qPCR cell/ml*	C.I. (p=0.05)	qPCR cell/ml*	C.I. (p=0.05)
t = 1 week	1.00E+09	1.17E+09	2.81E+08	1.14E+09	1.41E+08
	1.00E+07	1.19E+07	2.25E+08	1.32E+07	2.70E+08
	1.00E+05	9.43E+04	4.02E+04	4.68E+04	2.97E+04
t = 6 weeks	1.00E+09	9.60E+08	1.54E+08	1.38E+09	4.33E+08
	1.00E+07	1.07E+07	2.27E+08	3.33E+06	1.10E+08
	1.00E+05	7.46E+04	4.27E+04	6.30E+04	5.66E+04
t = 1 year	1.00E+09	1.04E+09	1.59E+08	3.01E+08	5.14E+07
	1.00E+07	1.04E+07	1.45E+08	2.91E+05	1.22E+05
	1.00E+05	9.54E+04	8.31E+03	1.03E+04	8.76E+03

*Mean and confidence interval based on triplicate 10ml samples, analyzed with triplicate PCR reactions each

Chapter 4: Monitoring of a co-injection of adherent-deficient bacteria into a shallow subsurface aquifer at Oyster Virginia by quantitative PCR (qPCR).

Introduction

Many subsurface aquifers across the United States are contaminated from past xenobiotic discharges, much of it too deep and extensive for conventional methods of remediation. Bacteria might be the best option available for *bioremediation* of deep subsurface contamination because their potential through metabolic activity to directly influence the transport of contaminants, and their potential to travel by groundwater flow to distant contaminated locations.

Most of our knowledge to date regarding the transport behavior of bacteria comes from laboratory column experiments under controlled conditions (see Chapter 1 Introduction). Little is known about the *in situ* transport behavior of bacteria in the subsurface, where many environmental variables such as water chemistry, sedimentology, and hydrology likely come into play simultaneously to determine the final fate of injected bacteria. An injection of a stained, indigenous collection of bacterial cells into a shallow, sandy, freshwater aquifer in Cape Cod, Massachusetts showed that DAPI-stained bacteria had similar breakthrough patterns to conservative Br^- tracers (bacterial $C/\text{Co} \sim 0.1$), but, in general, had longer "tails" of breakthrough persisting tens of meters down-gradient (1). Although the bacteria traveled in a fairly narrow plume within the aquifer, the exact mechanisms affecting transport, and a determination of the specific types of bacteria which transported well, were not determined. There was also concern that the use of the DNA-binding dye DAPI to facilitate tracking had altered the viability and behavior of the bacteria that were being monitored. A preliminary subsurface injection into an aerobic shallow sandy aquifer at Oyster, Virginia with PL2W31 bacteria, a candidate low-adhesion bacteria indigenous to the site, showed that most (>99 %) bacteria were retained in the aquifer sediments within 0.5 meters of the injection point (4).

Clearly, our ability to predict and model bacterial transport behavior based on the current state of knowledge is limited. Additional *in situ* bacterial transport experiments are necessary to shed additional light on this complex interesting phenomenon.

The Oyster, Virginia research site offered the opportunity to test specific factors which influence bacterial transport in the subsurface. The site is located on the southern tip of the DelMarVa (Delaware/Maryland/Virginia) peninsula and is owned by the Nature Conservatory of Virginia. It was chosen for a series of *in situ* injection experiments because of its physicochemical features comprised of a relatively homogeneous subsurface sandy aquifer, consisting of unconsolidated to weakly-cemented, well-sorted, medium-to-fine-grained Late Pleistocene sands (reference: <http://www.lbl.gov/NABIR/info.html>). The site contains both aerobic and anaerobic flow fields for analysis of bacterial transport under conditions of altered iron and metal chemistry. The anaerobic lower flowfield (designated SOFA for South Oyster Focus Area) was the focus of our current study. The SOFA flow field is comprised of peat and clayey-silt organic layers in the upper regions of the aquifer, with sandy layers below comprised of relatively homogeneous and well-sorted sand with medium-sized grains composed mainly of quartz minerals (4). Veins of iron and Mn oxyhydroxides and organics, which have been proposed to bind microbes and inhibit transport (10, 11) are also interspersed throughout the field.

Three research hypotheses were proposed by the Natural and Accelerated Bioremediation (NABIR) Bacterial Transport Research Group of the U. S. Department of Energy to examine bacterial transport behavior in the subsurface:

- 1) Physical heterogeneity in the porous medium controls bacterial transport. Bacterial transport is controlled primarily by physical heterogeneity within in the aquifer, with grain size and pore throat diameters being the most important factors.
- 2) Chemical heterogeneity in the porous medium controls bacterial transport. Surface charges of the aquifer matrix, and overall charge on the bacteria, are the primary factors which control transport, with negatively charged bacteria traveling faster through negatively charged quartz material and adhering preferentially to positively charged Fe, Al and Mn oxyhydroxides.
- 3) Microbial iron (Fe(III)) reduction will indirectly enhance iron-reducing bacteria (IRB) transport. It was proposed (2) that iron-reducing bacteria are hydrophobic and reversibly adhere to hydrophobic Fe, Mn and Al oxyhydroxide minerals. Enhancing of Fe(III) reduction rates may increase the desorption rate (and thus increase transport) by reducing the bioaccessible mineral surface area, encouraging IRB, and presumably other bacteria to desorb. Also, it was postulated that IRB activity could lead to localized increases in pH, promoting desorption of metals, radionuclides and other charged elemental compounds by reducing the positive surface charge on the mineral surfaces of the sediments.

Specific requirements were established by the Virginia Nature Conservatory to govern a bacterial injection at the Oyster site. The microbes used had to be indigenous to the site, could not be radio-labeled, could not be genetically engineered and could not have resistance to common clinical antibiotics (e.g. penicillin and tetracycline). In this study, quantitative PCR (qPCR) of genomic 16S ribosomal DNA (rDNA) was used to monitor the co-injection of adhesion-deficient bacterial strains OY107 and DA001, indigenous to the Oyster site, into the SOFA flowfield. Aqueous phase breakthrough data was analyzed from in-line Multi-level-samplers (MLS) implanted between 4-8 m below mean sea level in the flowfield. Post-

injection cores were extracted, and the levels of bacteria in the sediment determined by qPCR. Geochemical techniques were used to analyze groundwater and sediment core chemistry, and the geophysical techniques Ground Penetrating Radar (GPR) and Cross-Borehole Tomography (CBT) were used to derive a 3-dimensional image of the physicochemical parameters of the flow fields, and to predict high and low permeability zones (3, 6-9, 14). Statistical multivariate analysis by Partial Least Square (PLS) Regression and other modeling techniques were used to correlate the chemico-physical conditions of the Oyster SOFA flowfield to bacterial numbers detected in sediment samples (5, 12, 13).

Materials and Methods

Fluorogenic probe qPCR. Similar to the gel-based qPCR approach, the first step was to PCR amplify genomic DNA using universal primers 536f and 1392r. The amplicon was ligated into the pT7Blue-3 plasmid vector, and transformed into NovaBlue competent cells. As before, plasmid preparations were made of potential clones using QIAprep columns, and the DNA sequenced from both directions. The consensus sequence obtained was submitted to the RDP database and aligned to the nearest organism on the database for OY107, *Acidovorax temperans* (Sab = 0.943) was the closest known relative. The consensus sequence of DA001 determined for the gel-based qPCR approach was used for developing the primers and probe sequence for fluorogenic qPCR.

For analysis of samples, a subsample of the starved cells for injection was analyzed by direct microscopic enumeration to determine the true number of cells and then subjected to serial dilution in triplicate to produce a series of samples containing

10^9 , 10^8 , 10^7 , 10^6 , 10^5 , 10^4 , 10^3 , 10^2 , 10^1 and 0 cells/ml. These dilutions were then used in qPCR reactions and the data used to generate a linear regression plot of cell numbers vs. Tc (threshold cycle), and the regression formula used for determination of cell numbers. For all qPCR reactions, 5.0 μ l of water sample was added to 12.5 μ l of Platinum Quantitative PCR SuperMix-UDP (2X solution containing 60 U/ μ l Platinum Taq DNA polymerase, 40mM Tris-HCL (pH 8.4), 100mM KCl, 6 mM MgCl₂, 400uM dGTP, dATP and dCTP, and 800 uM dUTP, 40 U/ μ l UDG and stabilizers), with 0.2uM forward and reverse primers, and 0.2uM 5' FAM-labeled fluorogenic probe with 3' QSY7 quencher (MWG Biotech, High Point, NC), in a total volume of 25 μ l. The real-time detection of PCR product accumulation was accomplished using an iCycler PCR thermocycler / fluorometer (Bio-Rad Labs, Hercules, CA). A two-step PCR protocol was utilized, 45 cycles of (95° for 45 sec., 72° for 1min. 30 sec.), with FAM-specific fluorescent detection during the annealing cycles. Total genomic DNA was isolated from 1 gram sediment samples using the Soil DNA Purification Kit (MoBio Inc.), according to manufactures' instructions, except final dilution volume was 50 μ l of dH₂O.

Results and Discussion

Validation experiment: DNA extraction from sediment

The fluorogenic qPCR method used in these analyses was validated for use with aqueous water samples from the Oyster site as described in a separate publication (Kovacik and Holben 2002, Chapter 3). Controls for linear range of detection (approx. seven orders of magnitude for both OY and DA001 detection systems), water chemical interference of PCR (none detected), storage conditions (>1 year at -20° C) and specificity of detection (specific to 1 in 100-1000 cells), demonstrated that the qPCR method could accurately and specifically quantify DA001 and OY107 cells in aqueous samples from the Oyster site. To further validate the qPCR method for use with sediment samples, a separate control experiment was conducted in which three different sediment samples (organic, sandy, and Fe-rich) from a pre-injection coring of MLS 14 in May 1999, were spiked with known concentrations of DA001 and OY107 cells (based on microscopic cell counts) (Fig. 1). The samples were frozen for 1 week, thawed, the total DNA isolated from the sediments using a commercially available kit (MoBio Soil DNA Kit), and then subjected to fluorogenic qPCR analysis for cell quantification. For all sediment types, the detected cell concentrations by qPCR decreased with decreasing number of input cells, positive indication of specific detection of DA001 and OY107 cells in the sediment samples (Fig.1). The DNA recovery efficiencies (based on qPCR enumeration) were nearly 100% from the organic rich and sandy sediment types, while the Fe-rich sediment was only approximately 10% of input concentrations. Regression analysis was then used to relate detected qPCR values to actual cell numbers, and a

correction factor determined to normalize detected qPCR concentrations detected from sediments.

Background bacterial levels: water and sediment

The background levels of indigenous DA001 and OY107 cells in the water column and sediment were also important considerations for this experiment because qPCR detects both indigenous and injected bacteria. Table 1 shows the background levels of indigenous DA001 and OY107 detected by qPCR prior to the SOFA 2000 injection. In general, the levels of OY107 detected in both water and sediment samples were higher than detected for strain DA001, with aqueous bacterial levels (per ml) larger by an order of magnitude than sediment levels (per gram). Sediment levels for DA001 were near or below detection in most pre-injection sediments.

Aqueous qPCR results

To analyze the transport behavior of the adhesion deficient strains OY107 and DA001 in the Oyster sofa flowfield, breakthrough patterns for the bacteria were compared to the breakthrough of the conservative tracer, bromide, injected concurrent with the bacteria during a pulse injection in the first 12 hours of the experiment. First, a series of “snapshots” of bromide and bacterial breakthroughs at 25, 50, 100 and 150 hours post-injection were plotted to visualize the general distribution and extent of breakthrough by depth over time (Fig. 2). At 25 hours post injection, the bromide arrived in a concise and focused plume with center of mass at MLS 10 (1.5 m from injection), with highest concentrations observed between ports 4 and 7, the approximate screened interval of the B2 injection port. The breakthrough patterns for strains DA001 and

OY107, on the other hand, were spread more uniformly throughout the depth profile, with detectable bacterial concentrations reaching nearly to MLS T2 (4.5 m from injection). At 50 hours post injection, the bromide plume is centered at MLS 14 (2.5 m from injection), with a preferred flow path indicated toward lower portions of the flowfield. The concentration of bromide detected has also decreased to approximately 1/3 of the injected levels of 100 ppm. Breakthrough patterns for strains DA001 and OY107 are similarly centered near MLS 14, but are present in greater numbers, and are more uniformly distributed throughout all sample depths than bromide. At 100 hours post-injection, the bromide plume is nearly non-detectable in the flowfield, while the center of the bacterial plumes appears to be just reaching MLS T2 located 4.5 m from injection. Again, higher than background levels of bacteria were detected throughout all sample depths tested. At 150 hours post-injection, the bromide plume has re-appeared in barely detectable levels near MLS T2, while both bacterial strains persist in greater than background levels throughout the flowfield.

For a more precise indicator of bacterial transport behavior within the SOFA flowfield, average bacterial and bromide breakthroughs were plotted on a C/Co basis verses time, as detected at MLS 10, 14, T2 and S24 (1.5, 2.5, 4.5 and 7.0 m from injection). Differences in transport behavior of bacteria verses bromide were detected related to the distance from the injection port, as well as to the depth interval in the flowfield. When breakthrough was averaged over all 12 port depths, the centers of mass of the plumes can be seen moving through the flowfield in almost linear fashion (Fig. 3). In general, peak breakthroughs for the bacteria and bromide occurred at the same time, at approximately 25 hours post injection at SOS 10 located 1.5 m from injection, at

approximately 45 hours at 2.5 m, and approximately 225 hours at 4.5 m from injection. It was difficult to determine the approximate peak breakthrough at SOS 24 located 7.0 m from injection, as the peak likely occurred sometime after 300 hours post injection. The bacterial profiles were almost entirely bracketed by the bromide peak indicating similar transport characteristics for both, although the bacterial peaks had many more peaks and troughs than the smoother bromide profile. The patterns of breakthrough for the two strains OY107 and DA001 were more similar to each other than to the bromide, but in general the DA001 acted more conservatively (less up and down) than the OY107 strain.

Because of differences noted in the lithology and geology of the Oyster SOFA flowfield by depth, with upper regions dominated by an organic rich peat layer, and lower regions by a mixed assemblage of sand and gravel sized particles, with interspersed organic, and Fe and Al oxyhydroxide coatings, we decided to analyze average bacterial breakthrough verses bromide in four quadrants by depth (ports 1-3, 4-6, 7-9 and 10-12, corresponding to depths of 2.81-3.41, 3.71-4.31, 4.61-5.21 and 5.51-6.11 m below sea level). The results indicated different transport behavior at different depths within the flowfield. At SOS 10 (1.5 m), the profiles for both bacteria in Quads 2 and 3 more resembled the bromide, indicating nearly-conservative breakthrough behavior through the middle regions of the flowfield (Fig. 4). In contrast, bacterial breakthrough patterns in the uppermost and lowermost quadrants at SOS 10 were very different than bromide, with large regions of deviation especially in "tail" regions of the breakthroughs, indicating possible bacterial attachment or differential flow paths. At SOS 14 (2.5 m from injection) the pattern had changed somewhat (Fig.5). The bacterial breakthrough patterns in the lower two quadrants now resembled the bromide profile more closely,

while in the upper two quadrants there were more indicators of binding and differential flow of the bacteria. The preferential flow toward the bottom of the flowfield was reflected in peak C/C_0 levels as well, with significantly higher levels detected in the lower two quadrants. At SOS T2 (4.5 m from injection) again the pattern changed (Fig. 6). Bacterial breakthrough patterns at all three lower quadrants were more conservative in profile and in peak C/C_0 , than the uppermost quadrant. The breakthrough pattern for the OY107 strain had more peaks and troughs than the DA001 strain, indicating greater levels of interactions with the environment, and more conservative transport behavior by the DA001 strain. At SOS 24 (7.0 m from injection) discernable bacterial breakthrough versus bromide was only possible in Quad 3, where the bacterial and bromide profiles were very similar (Fig. 7). At the other three quadrants, the levels of bromide have reached undetectable levels (<0.5 ppm) so that comparisons of bacterial breakthrough to bromide were not possible. Due to the wider and lower linear range of detection with the qPCR procedure, it is likely that the patterns observed reflect actual bacterial breakthrough.

To quantify the extent of bacterial transport as it relates to distance from injection and depth in the field, the Relative Breakthrough (RB) levels of bacteria were calculated for each MLS and the four quadrants (Table 2). The RB results supported and quantified trends observed in the breakthrough plots, such as less transport, and by inference, greater levels of attachment for both organisms in the upper quadrant I of the flowfield. Also noticeable at many locations was the greater breakthrough of DA001 in comparison to OY107, especially at SOS 10 nearest the injection port, indicating more conservative transport by DA001 and greater attachment levels of OY107 in that region of the

flowfield. At the three MLS further from injection (except for Quad II at SOS T2), transport behavior in terms of RB was very similar between the two organisms.

Sediment qPCR Results

In addition to estimating attached bacterial levels remaining in the flowfield by inference from aqueous breakthrough data, we also wanted to determine the actual bacterial numbers remaining in the sediments two weeks after the start of the injection (when the cores were extracted). In theory, these values would represent a more accurate indicator of final attachment levels, with less contribution from transient attachment/detachment events during the peak breakthrough hours. The bacterial concentrations were then directly compared to chemical and sedimentology data derived for each sediment sample, including measures of hydraulic conductivity, grain size and surface chemistry.

As a first analysis, scatter plots were generated comparing bacterial concentrations and certain sediment characteristics or analyses as a function of depth and location in the flowfield. In the SOS2 core located 0.5 m from injection, based on polynomial trendline patterns (Fig. 8), there appears to be a relationship between OY107 and DA001 and measures of conductivity and permeability (+), as well as for total organic carbon in the sediments (+). This would be consistent with seeing less bacterial breakthrough at MLS 10, and more attachment in those regions. OY107 has greater levels of attachment than DA001 in general, especially nearer upper levels of the sediments. In SOS 17 core samples, the patterns had changed (Fig. 9). OY107 and DA001 patterns were nearly identical 3.5 m from injection, and resembled patterns for Fe

and Mn, and possibly % silt (an indicator of organics). (Total organic carbon on SOS 17 sediments and SOS 21 sediments has not been done yet). In SOS 21 cores (5.5m from injection), the OY107 and DA001 patterns are still similar, but possible relationships to variable factors were not readily apparent (Fig. 10). It is possible, and likely, that many factors are involved simultaneously in determining which sediments the bacteria ultimately bind to when being transported through the flowfield. A complete multivariate statistical analysis is planned at PNNL to determine the complex interactions involved in transport and bacterial attachment. In a very preliminary analysis of SOS 2 sediments only using Partial Least Squares (PLS) analysis (Fig. 11), a very strong correlation existed between measured chemical variables (organics, metals, streaming potential).

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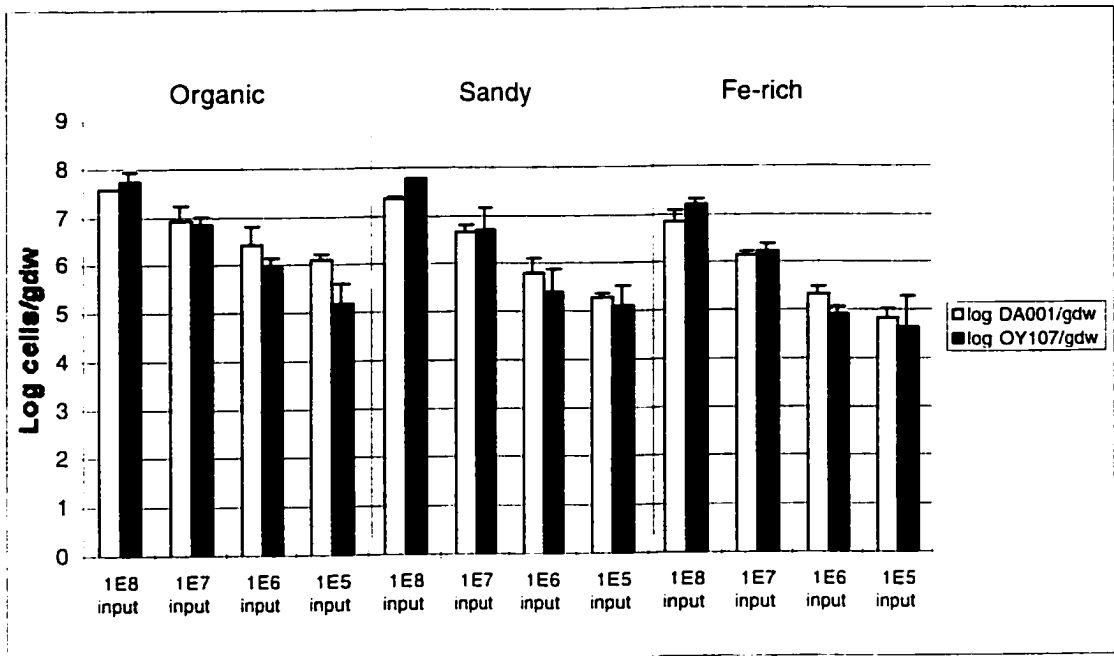


Figure 1. qPCR validation for sediments. Three sediments types (Organic rich, Sandy loam, Fe-rich) spiked with varying input levels of bacteria and analyzed by qPCR.

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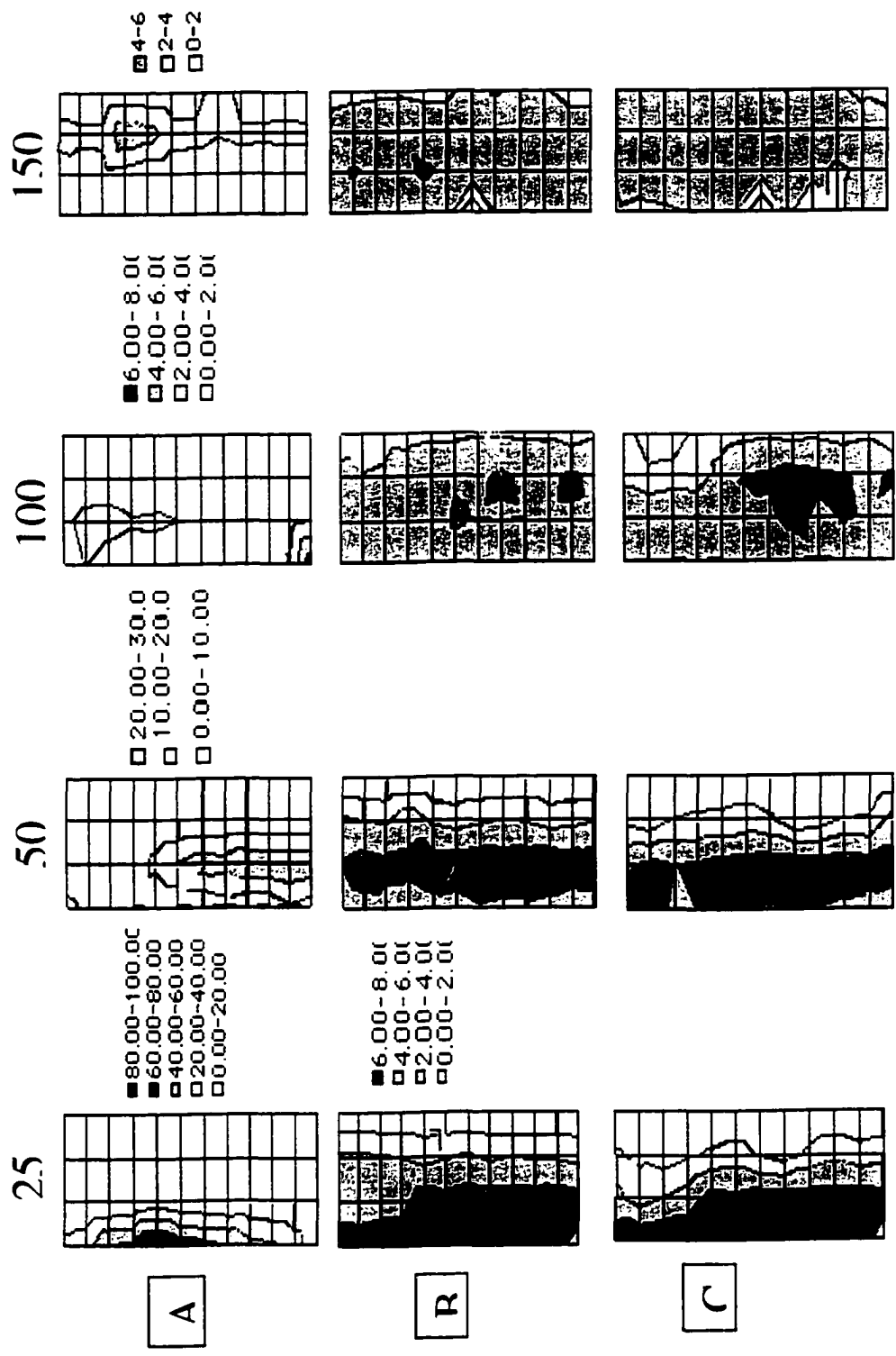


Figure 2. Bromide (A) and Oy107(B), and DA001 (C) breakthrough at 25, 50, 100, and 150 hours post-injection.

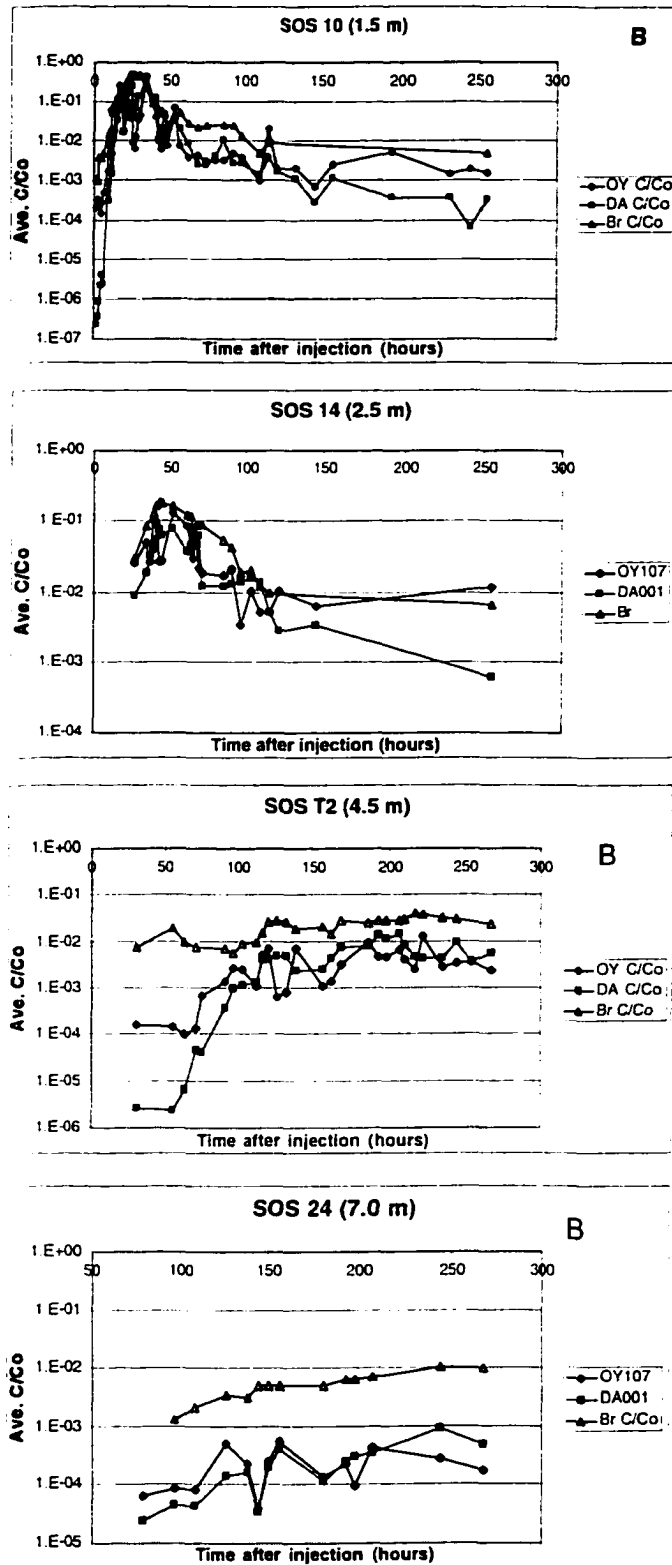


Figure 3. Average bacterial breakthrough vs Bromide (all ports) at 1.5, 2.5, 4.5, and 7 m from injection.

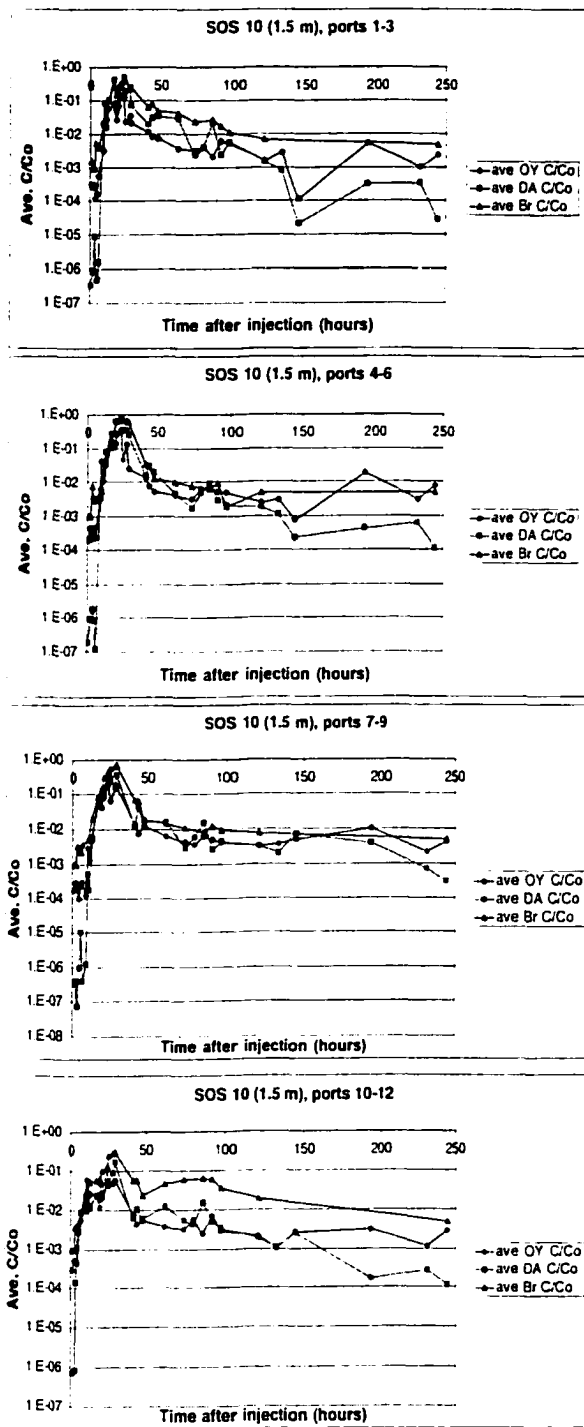


Figure 4. Average bacterial breakthrough by quadrants 1.5m from injection.

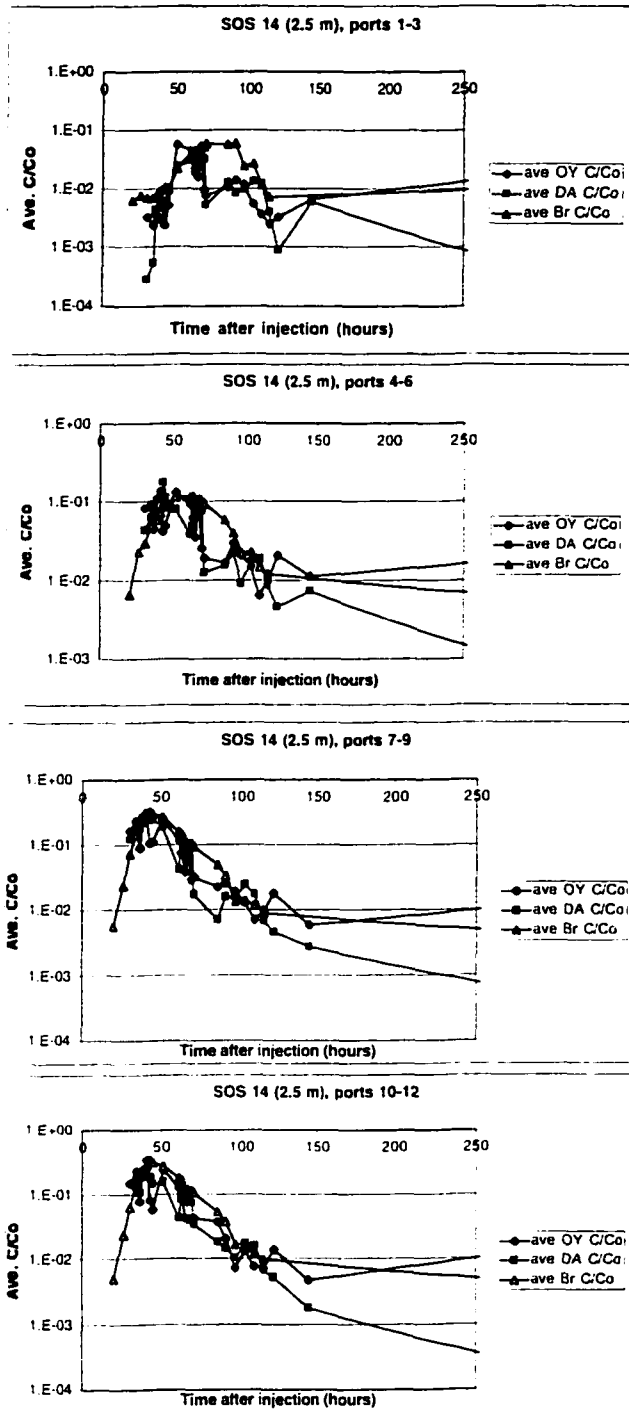


Figure 5. Average bacterial breakthrough by quadrants 2.5m from injection.

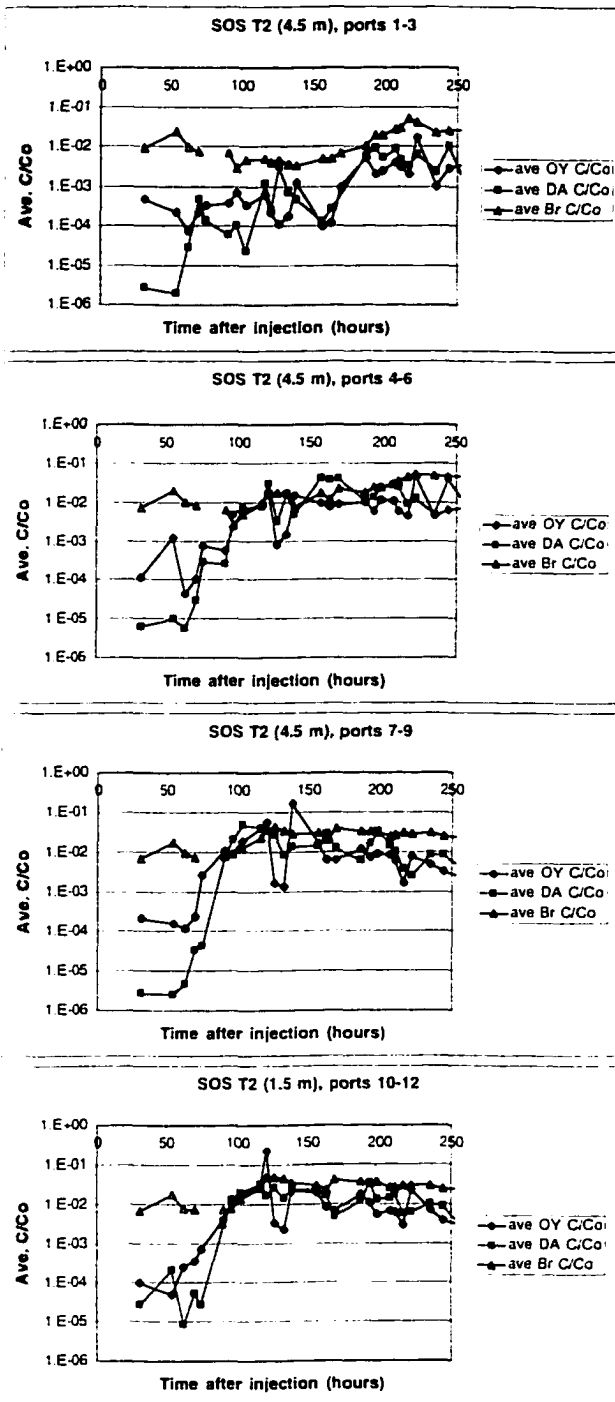


Figure 6. Average bacterial breakthrough by quadrants 4.5m from injection.

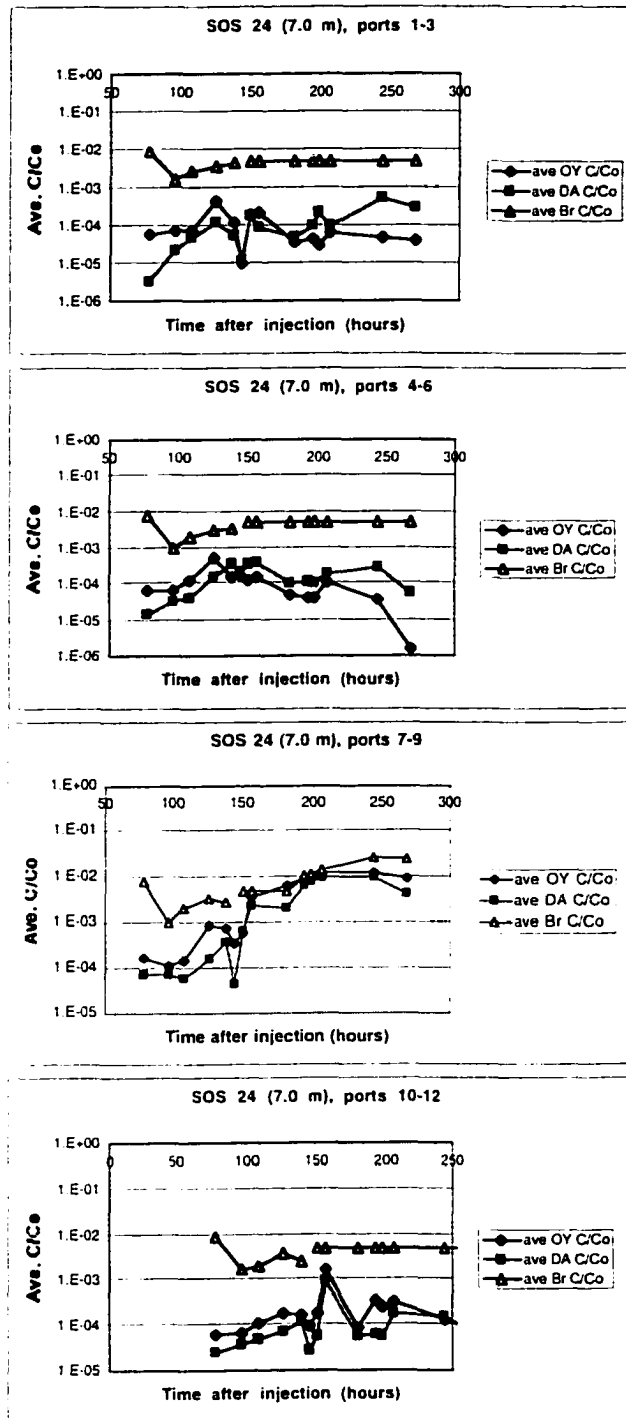


Figure 7. Average bacterial breakthrough by quadrants 7m from injection.

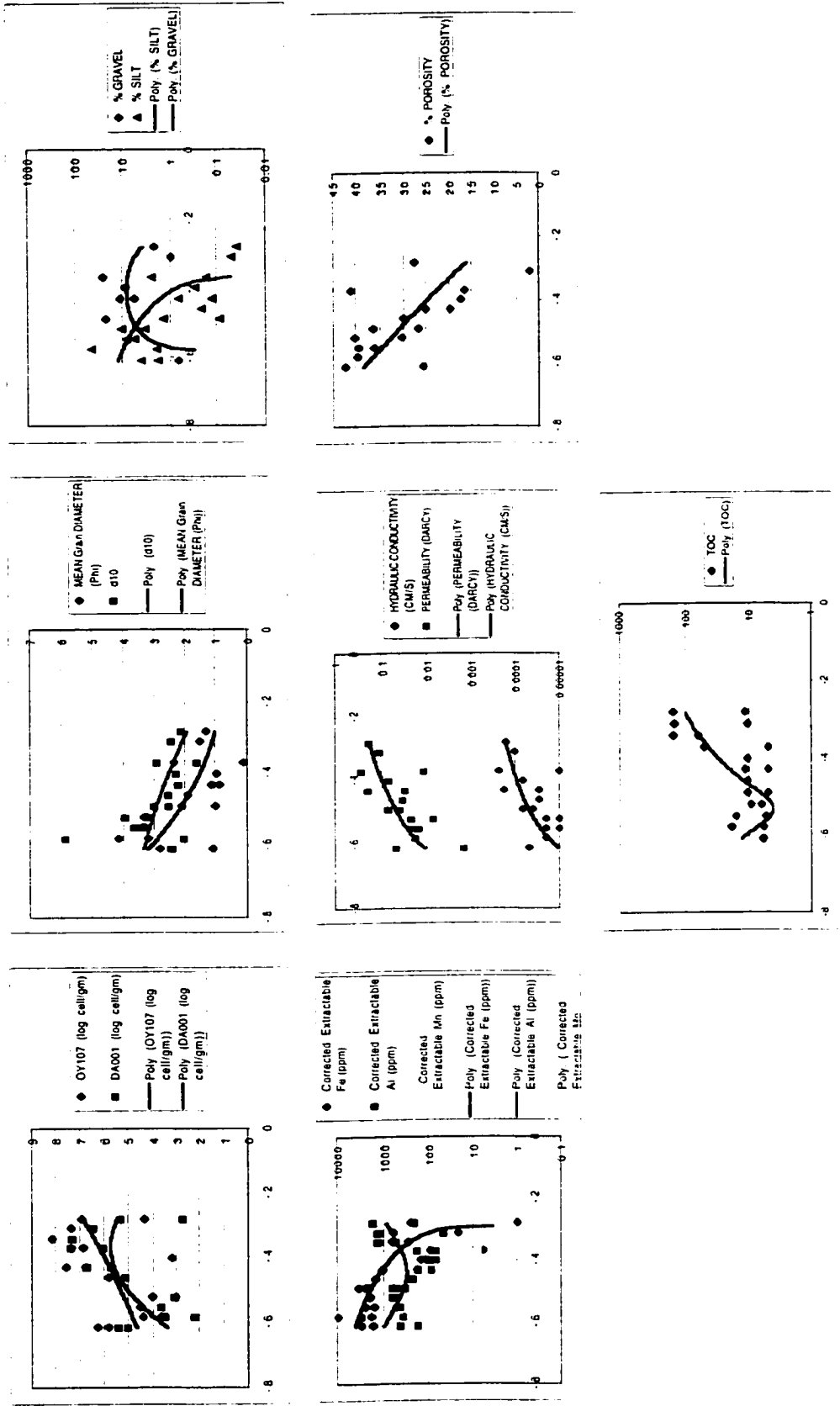


Figure 8. SOS 2 sediment core (0.5m from injection). Relationships to sediment physical and chemical parameters.

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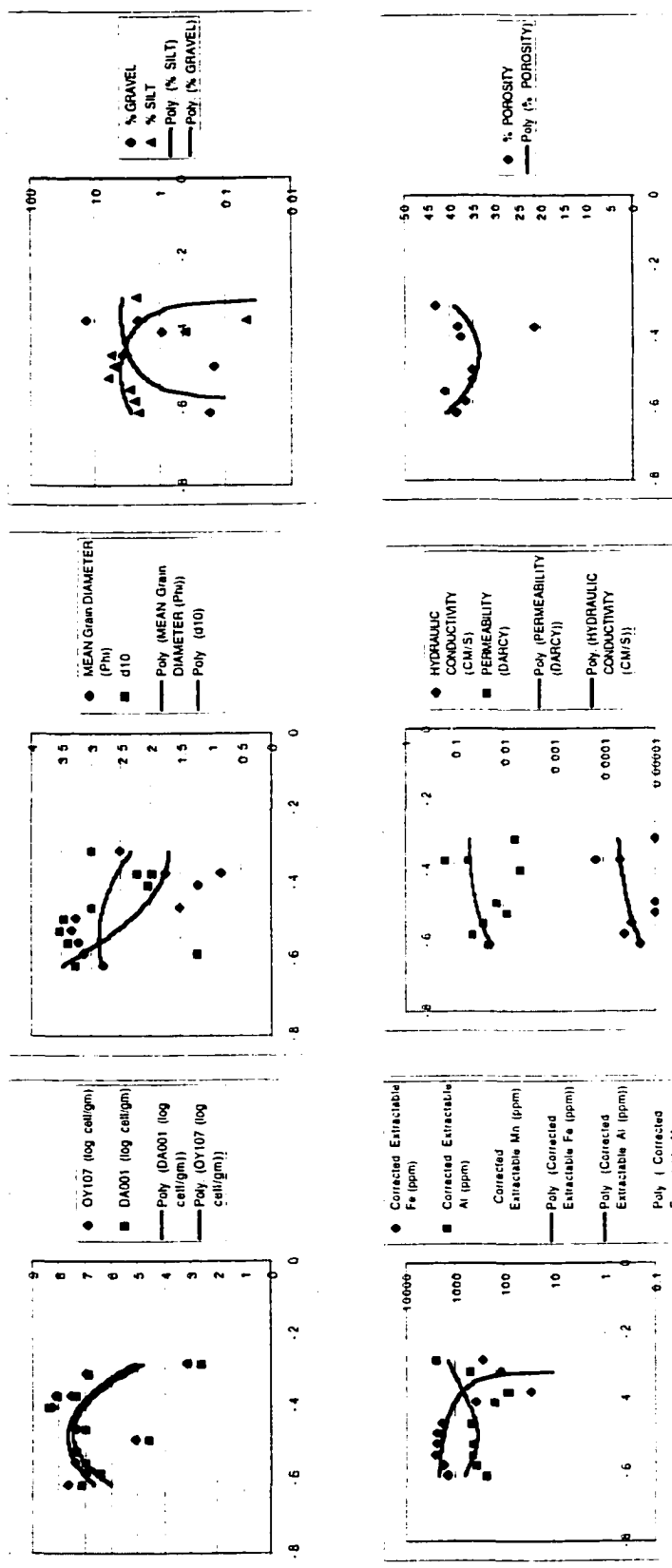


Figure 9. SOS 17 sediment core (3.5m from injection). Relationships to sediment physical and chemical parameters.

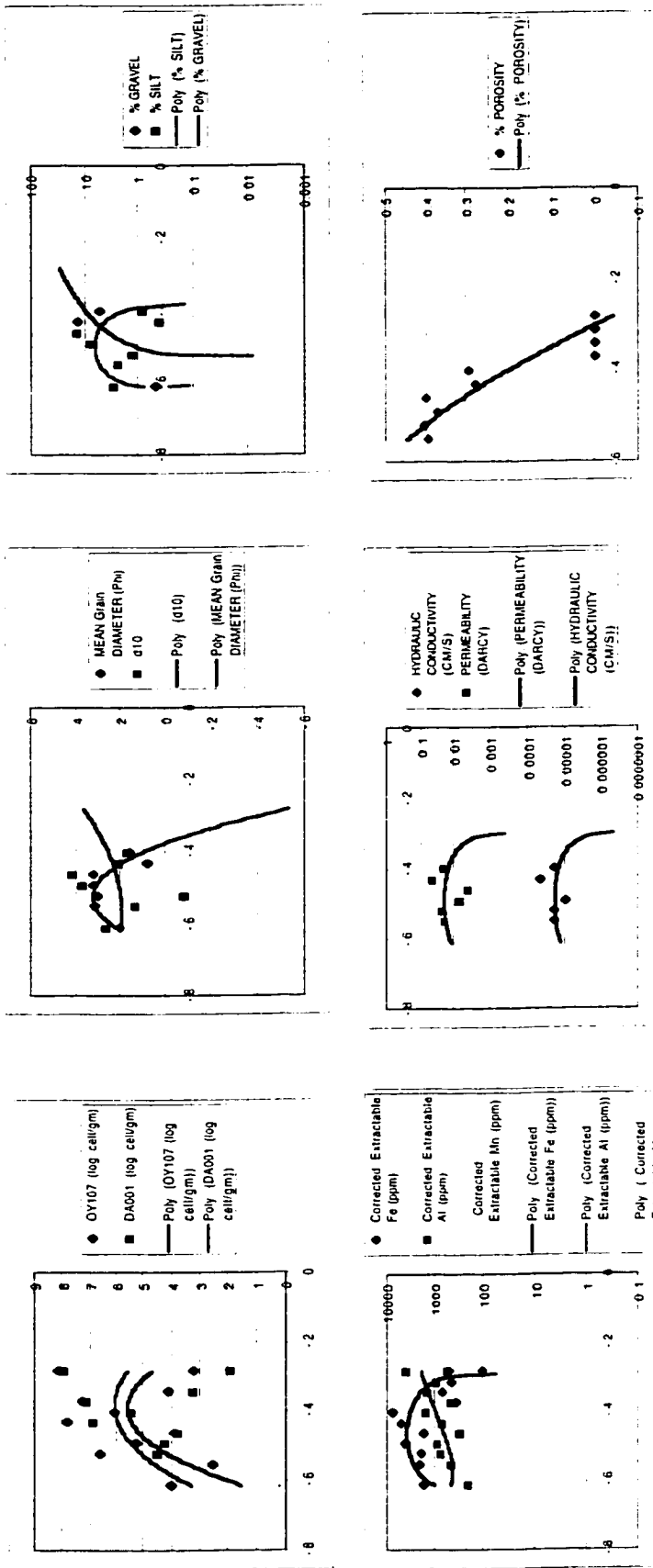


Figure 10. SOS 21 sediment core (5.5m from injection). Relationships to sediment physical and chemical parameters.

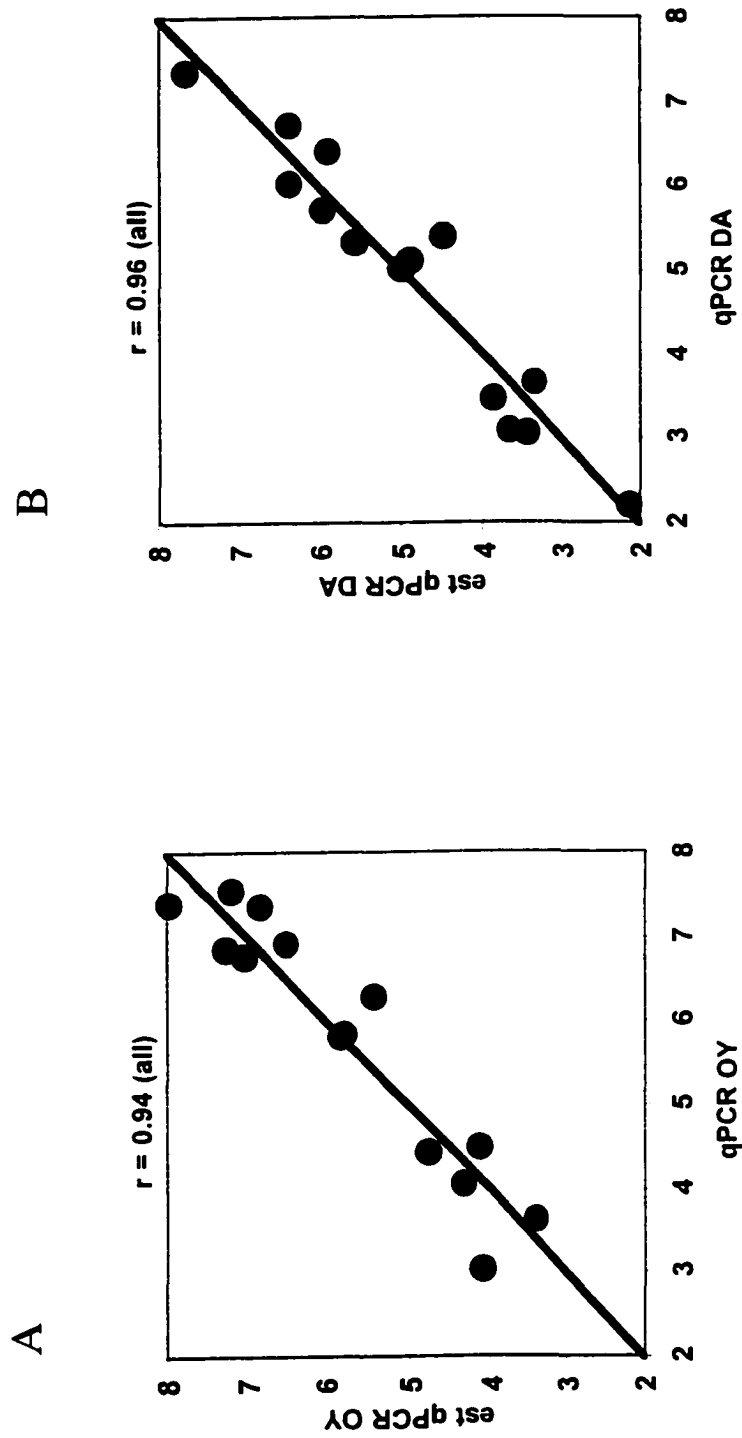


Figure 11. PLS modeling, qPCR raw data (X-axes) of SOS2 sediment vs. PLS predicted values (Y-axes). A. Measured OY107 vs predicted OY107. B. Measured DA001 vs predicted DA001.

Table 1. Pre-injection background number, water and sediment samples.

pre-injection water samples, July 31, 2000 *SOS14 sediment samples, May '99 coring*

Well #	Port #	log ave. DA001/ml	SD	log ave. OY107/ml	SD	Depth (m)	log DA001/g	log OY107/g
10	1	1.88	0.81	3.59	0.24	4.5	(can't locate data)	2.00
10	2	2.18	0.41	3.79	0.27	4.6		2.60
10	3	2.41	0.00	3.35	0.13	4.7		2.00
10	4	2.34	0.54	3.49	0.35			4.88
10	5	2.42	0.01	3.87	0.24	4.8		2.00
10	6	2.46	0.58	4.29	0.23	4.9		2.00
10	7	2.80	0.28	4.05	0.18	5.0		5.40
10	8	2.53	0.17	3.94	0.24	5.2		2.84
10	9	1.55	0.40	3.68	0.09	5.3		2.68
10	10	1.59	0.21	3.41	0.46	5.4		5.65
10	11	2.02	0.70	4.42	0.11	5.5		3.41
10	12	3.23	0.11	3.40	0.54	5.6		3.78
14	1	3.27	0.27	4.26	0.23	5.7		2.00
14	2	2.41	0.61	4.12	0.08	5.8		2.60
14	3	2.43	0.02	3.96	0.05	6.2		2.00
14	4	2.49	0.11	4.43	0.15	6.3		3.87
14	5	2.41	0.73	3.56	0.12	6.4		3.22
14	6	2.78	0.28	4.03	0.23	6.5		2.25
14	7	2.45	0.05	4.09	0.03	6.6		3.23
14	8	3.02	0.38	4.04	0.07	6.7		3.32
14	9	1.82	0.55	3.88	0.30	6.8		2.66
14	10	2.62	0.24	4.27	0.20	6.9		2.00
14	11	2.66	0.04	4.50	0.08	7.0		2.53
14	12	3.63	0.73	3.42	0.20	7.1		3.08
T2	1	3.28	0.50	3.97	0.44	7.2		3.61
T2	2	2.71	0.40	4.23	0.14	7.3		2.44
T2	3	3.55	0.06	4.21	0.30	7.4		2.57
T2	4	2.65	0.48	3.74	0.33	7.5		3.25
T2	5	3.25	0.40	4.45	0.43	7.6		2.00
T2	6	2.88	0.19	4.29	0.10	7.7		3.48
T2	7	3.34	0.24	4.28	0.08	7.8		2.00
T2	8	3.02	0.24	3.95	0.57	7.9		2.83
T2	9	2.93	0.58	4.16	0.33			
T2	10	3.65	0.18	4.16	0.03			
T2	11	3.41	0.68	4.41	0.23			
T2	12	5.00	0.28	4.38	0.31			
Average=		3.30	0.35	4.19	0.28	Average		2.97

Samples below detection assigned default value = log 2.0

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Table 2. Relative Breakthrough (RB) / Relative Attachment (RA) of OY107 and DA001

MLS (m from injection)>	SOS 10 (1.5 m)	SOS 14 (2.5 m)	SOS T2 (4.5 m)	SOS 24 (7.0 m)
Depth interval (m bmsl)	OY107 % RB	DA001 % RB	OY107 % RB	DA001 % RB
Quadrant I (2.81 to 3.41 m)	29.6	85.0	14.6	19.1
Quadrant II (3.71 to 4.31 m)	32.8	93.2	38.0	70.2
Quadrant III (4.61 to 5.21 m)	28.7	55.4	69.3	64.4
Quadrant IV (5.51 to 6.11 m)	26.4	39.2	67.9	53.1
Depth interval (m bmsl)	OY107 % RA	DA001 % RA	OY107 % RA	DA001 % RA
Quadrant I (2.81 to 3.41 m)	70.4	15.0	85.4	80.9
Quadrant II (3.71 to 4.31 m)	67.2	6.8	62.0	29.8
Quadrant III (4.61 to 5.21 m)	71.3	44.6	30.7	35.6
Quadrant IV (5.51 to 6.11 m)	73.6	60.8	32.1	46.9
			OY107 % RB	DA001 % RB
			ND	ND
			ND	ND
			52.6	36.7
			ND	ND
			OY107 % RA	DA001 % RA
			ND	ND
			47.4	63.3
			ND	ND

Chapter 5: Summary and Implications for Bioremediation:

The goals of my thesis research were focused toward an understanding of what factors control the fate and transport of bacteria in the subsurface. A major portion of my research effort was focused toward development of the new techniques necessary for the analysis and quantification of bacteria in environmental samples. In the past, culture-based analyses or specific labeling of the cells were used to monitor the transport and fate of injected microbes, but in general these methods were inadequate to specifically identify and quantify injected bacteria. In addition, one of the requirements for the *in situ* injection into the Oyster Virginia subsurface aquifer was the use of non-engineered, indigenous organisms from the site, precluding specific labeling of cells. The quantitative PCR methods developed here, specific to the 16S rDNA genes of bacteria, proved of great benefit for effectively and efficiently monitoring levels of added bacteria. This rapid and inexpensive approach facilitated transport experiments involving thousands of samples per experiment in support of modeling efforts and afforded fine-point resolution of bacterial transport behavior. We were also able to utilize the qPCR technology to monitor levels of bacteria on solid phase sediments, representing the first time this has been accomplished during an *in situ* bacterial injection. This represents a substantial increase in bacterial monitoring capabilities over what was considered state-of-the-art just a few years ago.

The long-term goals of my research related to the effective utilization of bacteria for the bioremediation of deep subsurface contamination. In order for microbes to be effective for remediation, four processes must occur: 1) The injected bacteria must travel to the distant contaminated site, 2) The bacteria must then attach to substrates at the site of contamination to remain resident in the plume or source of the contaminant, 3) The

bacteria must compete and survive with indigenous organisms at the site of contamination and 4) The bacteria must actively remediate the contaminated environment. The results of my thesis research suggest that at least three of the requirements for subsurface remediation by bacteria have been demonstrated (requirement #4 was not addressed by my studies).

An original goal of our study of a sandstone/shale interface 200 m below Cerro Negro, New Mexico (Chapter 2) was to potentially determine the arrival time of the bacteria present. Due to a very restrictive porosity in the rocks, with pores smaller than any characterized bacteria, it is possible that some of the bacteria existing at depth might be descendants of the original colonizers laid down in sediments during the Cretaceous Period nearly 100 million years ago. Although the use of an MLS with a “bacterial colonizing substrate” may have eliminated fine-point resolution of the distribution of individual types of microbes at the shale-sandstone interface, the bacterial phylotypes detected were phenotypically consistent with those found in near-shore ocean environments. This suggests that at least some of the bacteria present have been surviving over geological time periods, potentially allowing for the long-term remediation of deep subsurface environments with only a finite number of injections necessary.

The results of our Oyster Virginia bacterial injection experiments (Chapters 3 and 4) demonstrated that injected adhesion-deficient bacteria can migrate in the environment (satisfying requirement #1), although the scale of our study was only approximately 10 meters before the injected bacterial strains DA001 and OY107 reached indigenous background levels of approximately log 3-5 cells per ml. It is likely that injected bacteria

travel much greater distances in the subsurface in the long term, although such questions were beyond the scope of these studies.

Our newly-developed ability to quantify bacteria on sediments (i.e. the solid phase) allowed demonstration of requirement #2 for the injected bacteria- that they need to attach to sediments at the site of contamination, in order to be effective. Our results were potentially very exciting because relationships seemed to exist between bacterial numbers and concentrations of organics and metals on sediments. This would likely be the scenario encountered at contaminated sites, especially DOE managed ones with mixed organic and radioactive metal plumes of contamination. Complete multivariate analysis is necessary however before this conclusion can be substantiated, due to the many variables involved. This major advance in bacterial tracking technology is also exciting in the context of basic research on bacterial transport because, for the first time, it should be possible to perform true mass balance experiments in the field where all bacteria in the aqueous and solid phases are accounted for through direct measurement rather than through mathematical subtraction of the numbers detected from the total added.

Requirement #3-- that the injected bacteria survive and grow, could not be addressed directly because the injection period and coring of sediments occurred only over a three-week period. Elevated bacterial numbers on sediments with higher organic and metal concentrations (potential electron donors and acceptors) suggests that preferential adhesion and possibly growth of injected bacteria is occurring in the field. Further, our spiked sediment experiment, in which the sediments were incubated at 15° C rather than frozen, showed that increasing numbers of bacteria were detected above input

levels on most sediments after only one week. The most likely explanation for this observation is that growth of organisms on the sediments had occurred during the course of the experiment. Thus, it seems likely that, at least in this case, the injected bacteria are surviving and growing in the environment. This has important implications for bioremediation applications through bioaugmentation, especially where long-term residence and activity of the added organisms is a factor.

The results of my thesis research therefore demonstrated that remediation of deep subsurface contamination is possible utilizing microorganisms. Utilizing novel DNA molecular methods based on the 16S rDNA genome, we were able to specifically monitor injected bacteria in water phase samples, as well as demonstrate the attachment, and potential growth, of bacteria on solid phase aquifer sediments. These results significantly increase our understanding of subsurface microbiology, as well as demonstrate the potential of using microorganisms for the remediation of deep subsurface contamination.