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ASSESSING THE INFLUENCE OF PHYSICAL AND CHEMICAL PROPERTIES OF SEDIMENTS ON MICROBIAL DISTRIBUTION AND ACTIVITY IN THE TERRESTRIAL SUBSURFACE

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By
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ASSESSING THE INFLUENCE OF PHYSICAL AND CHEMICAL PROPERTIES OF SEDIMENTS ON MICROBIAL DISTRIBUTION AND ACTIVITY IN THE TERRESTRIAL SUBSURFACE

A DISSERTATION APPROVED BY THE DEPARTMENT OF BOTANY AND MICROBIOLOGY

BY

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Table of Contents

List of Tables

List of Illustrations

Preface

The life processes of microorganisms in the terrestrial subsurface are influenced by many factors, though none more strongly than the physical and chemical compositions of the soils and sediments in which the bacteria reside. Variability in the physical and chemical parameters of subsurface materials may occur at any scale, meaning that variability in microbial properties such as transport and metabolic activity may also occur at any scale. While the changes associated with different aspects of microbial life in different strata over distances of tens to hundreds of meters have been fairly well studied, heterogeneity within a single stratum or over small (cm or below) distances has received less attention in the literature. This dissertation will examine the link between variation in microbial abundance, distribution, and metabolic activity and the variability in the physical and chemical properties of the sediments surrounding the bacteria.

Chapter 1 of this dissertation is an overview of the manners in which heterogeneity in the properties of subsurface materials cause variability in bacterial transport, colonization, distribution, and metabolic activity. Many factors may affect these microbial properties, but this overview will focus on the influence of the chemical and physical properties of the materials containing the bacteria. I conclude that, along with the minor role that some physiological properties of the bacteria themselves play, the adhesiveness (as measured by the number of bacteria attached per unit area), grain size (the mean particle size), and pore size (the mean pore throat diameter) of the sediment through which the bacteria are moving determines the rate and efficiency of microbial transport (ratio of the number of bacteria

which moved completely through the porous medium to the number of bacteria available for movement) in the subsurface. Small-grained sediments (mean particle size < 0.05 mm) such as clays and silts tend to be more adhesive to bacterial cells. Additionally, these fine-grained materials have restrictive pore diameters (0.2 μm – 50 μm), making movement within them difficult for most bacteria. Varying amounts of fine-grained sediments within otherwise high permeability materials causes variations in microbial transport in the subsurface.

Heterogeneity in sediment composition and grain arrangement also controls both microbial colonization and distribution. From the literature, I found that the presence of fine-grained soils and sediments reduce the ability of bacteria to colonize a new area in the subsurface. Though these materials are more adhesive than larger-grained sediments (mean particle size > 0.05 mm), they are difficult for bacteria to colonize. The tightly packed structure of clays and silts slow microbial penetration, while the small pore sizes prevent bacteria from growing to high numbers. Materials with areas of small pores hinder the ability of microorganisms to get to nutrients, both by having slower diffusion rates and by restricting the movement of bacteria towards areas of higher nutrient concentrations. The patchiness of the areas with small pores that restrict nutrient access will determine the heterogeneity of bacterial distribution within a soil or sediment as these areas will have less cells.

From my review of the literature, I concluded that variability in the physical and chemical composition of soils and sediments causes variation in the metabolic activity of the microorganisms within the geological materials. The subsurface varies widely in parameters such as moisture content and nutrient concentration, particularly between strata. Both of these

factors are highly influential in how metabolically active microorganisms may be. Numerous studies have additionally linked the amount of fine-grained materials within a sediment to the activity level of bacteria present, with higher amount of clays and silts leading to lower metabolic activity measurements. This chapter was written in the format required for the journal *Research in Microbiology*.

In Chapter 2, I examine the microbial distribution, activity, and community composition within a shallow, homogenous, vadose zone sediment. I obtained numerous closely spaced samples along three vertical transects from a site near Oyster, VA. I measured the hydrogen uptake rates from samples that were processed both aerobically and anaerobically and the moisture content of all samples. Several collaborators also assisted with analyzing other properties of the samples. A private firm performed the geochemical analysis. The grain size analysis was done by Don Swift's laboratory at Old Dominion University. The Fe and Al extractions were done by T. C. Onstott's laboratory at Princeton University. The phospholipid fatty acid analysis was performed by D. C. White's laboratory at the University of Tennessee. The notched box plot statistical analysis was done by Chris Murray's laboratory at Pacific Northwest National Laboratory. I proposed that the lack of variability observed in the microbial metabolic activity measurements at the meter scale was due to lack of variation in the physical and chemical composition of the sediment at the site at the same scale. Further, where small areas of heterogeneity in metabolic activity where seen at the cm level, it could be explained by variations in a physical property (moisture content) and a chemical property (surface reducible Fe (III)) of the sediment. The results from this work suggest that microbial activity will be predictable in areas with little variation in the physical

and chemical properties of soils and sediments. This chapter was written in the format required by the journal *Geomicrobiology*.

For Chapter 3 I studied the distribution, abundance, and metabolic activity of sulfate reducing bacteria (SRB) in a shallow microaerophilic aquifer using an autoradiography technique. This technique allowed for visualization and quantification of SRB activity at the cm level scale. For this work I imaged over 30 core sections, performed most probable number (MPN) analysis for viable SRB, and ran a variety of experiments attempting to confirm and expand upon our results from the field samples. Don Swift's laboratory measured various physical properties of the sediments, including grain size, permeability, porosity, and hydraulic conductivity. Johnny Gilpen performed the multivariate regression analysis. Given that the sulfate-reducing activity was heterogeneously distributed throughout all the samples tested and the only measured variable that varied with SRB activity was sediment porosity, I proposed that variations in the pore diameters of the various sediments were responsible for the variability observed in SRB activity. In the sand sediments, microsites with larger pore volumes likely allowed SRB to grow to higher numbers and express higher levels of metabolic activity, resulting in areas within the sand which had much higher levels of activity than others. In the fine-grained peat and silt sediments, the same process could be occurring. In these smaller-grained sediments, small pores appear to be limiting the bacteria for nutrients. The regions within the peat and silt sediments which have larger pores, and subsequently more SRB, also likely display higher levels of metabolism due to viable cells within the large pores utilizing cellular debris as a nutrient source. This chapter was written in the format required by the journal *Microbial Ecology*.

Abstract

To maximize the possibility for success, accurate assessment of the distribution and activity of microorganisms in the subsurface is essential for implementing bioremediation strategies at contaminated sites. It is clear that large variations in microbial activity and abundance occur between strata with markedly different sedimentological features. However, the relationship between microbial activity and abundance and sedimentological properties within a single geological unit have not been quantified to the same extent as between strata. The central hypothesis driving this investigation was that the major controls of microbial activity in the subsurface are strongly correlated with a statistical description of the variation in the physical and chemical properties of the formation. To test this hypothesis, the relationships between sedimentological and microbiological parameters in the vadose zone of a barrier sediment and in a shallow, microaerophilic aquifer, both located on the eastern shore of Virginia, were examined. For the vadose zone sediment, pairs of samples were taken 10-cm apart in the vertical direction and 2 cm apart in the horizontal direction along three transects with one sample from each depth being processed aerobically and the other being processed anaerobically. Little variation was observed in the sedimentological and microbiological parameters tested. The sediment of all samples was fine to coarse sand with the grain sizes varying by less than an order of magnitude. Sediment moisture was low for all samples, but increased near the top and bottom of each sampling transect. These were regions where bioavailable Fe(III) concentrations were highest. Zero-order rates of H_2 uptake were low throughout and ranged from below detection limit to 0.064 $\mu mol\ H_2 \bullet day^{-1} \bullet g^{-1}$ of sediment with a median rate of 0.01 $\mu mol\ H_2 \bullet day^{-1} \bullet g^{-1}$ of sediment. The variation in bacterial

numbers was slightly more than an order of magnitude range over the entire sampling face. Phospholipid fatty acid analysis showed a diverse but fairly uniform microbial community from sample to sample. Statistical analysis revealed that the quartile of aerobically processed samples with the highest H_2 uptake rates had statistically higher moisture content and bioavailable iron content than did the rest of the samples. The quartile of aerobically processed samples with the lowest H_2 uptake rates had significantly more gravel, less moisture, and less bioavailable Fe(III) than did the rest of the samples. Similar trends were observed for anaerobically processed samples, but the differences were not significant. The data indicate that the spatial variation in microbial parameters is low within strata with uniform grain sizes.

In the shallow, microaerophilic aquifer, a radioimaging technique was used to visualize and quantify the activity of sulfate reducers in sediments at a centimeter-level scale. The distribution of SRB metabolic activity was heterogeneous with areas showing little activity far outnumbering areas with high activity. Variation in sulfate reducing activity was not statistically correlated with variation in depth, bacterial numbers, or the following sediment properties: sediment type (sand, peat, or silt), grain size, permeability, and hydraulic conductivity. SRB activity did vary significantly with sediment porosity (multivariate analysis, $r = 0.48$). We hypothesized that the small pore sizes associated with sediments with low porosity restricted the ability of SRB to grow to high numbers as well as their access to nutrients. To further explore the relationship between pore size and microbial metabolic activity, columns with varying pore diameters were constructed. SRB in the columns with the smallest pore diameters had the slowest rates of metabolism and SRB metabolic rates

increased as the pore diameter increased. For the aquifer studied, sediment porosities and pore sizes were the main factor controlling SRB activity.

Overall, the microbial heterogeneity, or lack thereof, at two subsurface sites could be explained by the variation in the geophysical properties of the sediments. In the case of the vadose zone site, small variations in sediment composition and microbial activity were found at the cm level. However, at larger scales, this heterogeneity became undetectable. For the more heterogeneous microaerophilic aquifer, heterogeneity in both sediment composition and SRB metabolic activity were present at scales ranging from cm to m. My work suggests that within a geophysically homogeneous sediment, the metabolic activity of the bacteria may be accurately predicted at the meter scale with relatively few samples. However, in sediments with large variations in their physical and chemical properties , the metabolic potential of the bacteria present may only be predicted to the cm level after examining numerous samples.

Chapter 1

The Influences of Subsurface Heterogeneity on Microbial Life in the Subsurface

Bacterial life in the terrestrial subsurface is controlled by a variety of factors, almost all of which are directly or indirectly related to the physical and chemical environment of the microorganisms. Subsurface soils and sediments show variability over a wide range of scales in properties such as grain size, pore size, moisture content, nutrient concentration, oxygen concentration, permeability, and hydraulic conductivity. This heterogeneity of physical and chemical properties over a wide range of scales causes subsurface bacteria to also vary widely in many parameters over the same scales. While this variability is inevitable, it must be accounted for and dealt with when bioremediating a contaminant site or tracking movement of pathogens away from sewage. Over the last decade, researchers have examined the link between the physical and chemical composition of soils and sediments and microbial heterogeneity in an attempt to better understand the variability encountered when studying microbial movement, colonization, distribution, and activity in the subsurface. This review will examine the role that the physical and chemical properties of soils and sediments play in influencing various aspects of microbial life in the subsurface.

The movement and transport of bacteria through porous media is dependent on a number of factors which vary in importance depending on the material through which the microorganism is moving. Various physiological properties of the bacteria themselves, such as the size and shape of the cell, as well as cell surface properties, influence where and how quickly the cells

move in the subsurface (12, 33). The physiologies of the bacteria seem to exert more control over the speed and distance the cells travel than do soil and sediment properties if the soil or sediment is water saturated and homogenous (6). If, however, the porous media through which the bacteria are moving is heterogeneous in nature, the physical and chemical properties of the porous material become the dominant factor determining the rate and extent of microbial movement (10). The introduction of heterogeneity in a soil or sediment, usually in the form of a small change in the physical or chemical properties of the medium, is all that is required to slow the rate of bacterial transport through a porous medium. DeFlaun et al. found that a sandy sediment containing only 9% silt and 4.3% clay (sand balance) delayed cell breakthrough up to 6 hours in 25 cm long columns, compared to columns packed with only sand. The same authors also reported that almost 5 orders of magnitude fewer cells were able to move through the heterogeneous medium compared to the entirely sand sediment (5).

The physical and chemical properties in heterogeneous soils and sediments which cause variation in microbial transport rates and efficiencies are too numerous to be fully covered in this review. Instead, the sediment physical properties adhesion, grain size, and pore size will be focused on. The presence of fine-grained sediments such as silts and clays within larger-grained materials severely retards microbial migration as the large surface areas of the silts and clays cause high amounts of bacterial adhesion (9). Additionally, as the outer surface of most bacteria are negatively charged, they tend to stick to clay because high cation concentrations on the outer surface of the clay create a net positive charge (21). Clays and silts are not the only sediments which can increase bacterial adhesion. Oxyhydroxide coatings on sand grains greatly increase their adhesive properties (30). As the presence and

concentration of these coatings are widely variable in the subsurface (30), the transport of bacteria within what appears to be a homogenous sand sediment may also be highly variable.

Along with adhesive properties, the grain size and pore size of subsurface soils and sediments influence physical properties affecting the extent and speed of bacterial movement. The pore size of sediment is related to its grain size, with small-grained sediments such as silts and clays having smaller pore diameters than larger-grained sand sediments (20). Small pores not only lead to physical exclusion of bacteria, but also tend to lower the permeability and hydraulic conductivity of soils and sediments (8) slowing microbial transport with groundwater flow. Numerous studies have linked variation in the grain size of a porous medium to variation in the ability of microbes to travel through it. Sharma and McInerney found a linear decrease in bacterial movement rates as grain size decreased (31). Entry and Farmer discovered a decrease in numbers of total coliforms and fecal coliforms in ground water when the water went from a relatively large-grained basalt aquifer to a smaller-grained sand aquifer (7). In a chemotaxis experiment, Barton and Ford observed that a ten fold decrease in grain size decreased the effective random motility coefficient almost 20 fold (1). Orders of magnitude changes in the grain sizes, and subsequently pore sizes, of soils and sediments are commonly observed in the subsurface in transitions zones between sediment types (18). However, the presence of a transition zone is not necessary to produce heterogeneities in soils and sediments. Seemingly homogenous porous media may contain numerous small areas where the physical and chemical parameters are orders of magnitude different than the surrounding material. This is the reason why the rate of bacterial movement through undisturbed aquifer soils and sediment often varies widely from the rate of movement

through the same materials after they have been homogenized or repacked (30). Harvey et al. were able to produce a range of results when comparing bacteria-sized microsphere movement through undisturbed aquifer sediment or re-packed aquifer sediment, depending on how the sediment was packed (14). This and the other studies mentioned above demonstrate the influence that physical and chemical heterogeneities found in soils and sediments exert over microbial transport and movement through these porous subsurface materials.

In addition to transport, the physical and chemical heterogeneities found in the subsurface affect other aspects of microbial life. The ability to colonize a new soil or sediment as well as how bacteria are distributed once a material is colonized are influenced by the structure and chemical nature of the geological medium. Though small-grained sediments like clays and silts are more adhesive to bacteria than larger-grained materials (9), they are also more difficult to colonize (16, 27). It is difficult for microorganisms to establish communities in small-grained media for several reasons. Bacteria are not easily able to penetrate the dense structure of sediments with small grain sizes. Pederson et al. (26) tested the ability of a number of bacteria to penetrate a clay sediment. They found most bacteria showed either miniscule (1-2 mm) or no measurable penetration after 8 hours. Additionally, results 15 months after inoculation indicated that numbers of cultivable bacteria dropped several orders of magnitude within a clay sediment (26). The tiny pores found in clays and silts offer less physical space for bacteria to grow, leading to lower cell numbers than are found in sediments with larger pore sizes (19). Under low nutrient conditions, bacteria may utilize debris from nearby cells as a nutrient source (13). With fewer cells present at the onset of nutrient limitation, the bacteria living in the pores of small-grained soils and sediments are less likely

to survive than are cells in larger pore spaces. Small pores further limit nutrient accessibility as they limit the ability of bacteria to move to areas of higher nutrient concentrations (11). For these reasons, the presence of even a small amount of a fine-grained soil or sediment will lead to variation in the ability of bacteria to colonize a particular area in the subsurface.

If microorganisms are able to overcome the many obstacles to colonizing a heterogeneous soil or sediment listed above, it is likely their distribution within the subsurface material will be affected. Factors causing variation in microbial distribution in the subsurface include temperature (32), moisture content (22), and nutrient availability (4). While variations in these and other factors have been shown to influence the distribution of microorganisms, other soil and sediment properties also alter bacterial distribution patterns in the subsurface. The one overriding variable in determining the extent of heterogeneity of microbial distribution in the subsurface is sediment type. The percentages of sand, silt, and clay for a particular sediment, as well as how each type is arranged, are responsible for the physical and chemical properties of that particular sediment (18). Soils and sediments with higher percentages of small-grained particles such as silts and clays tend to have lower numbers of total and culturable bacteria present than do soils and sediments with more larger-grained particles (27). In addition to being fewer in number, the bacteria within fine-grained materials also tend to have a more irregular distribution than do bacteria in larger-grained sediments (17). The reasons for this heterogeneous distribution of microorganisms, as well as their lack of abundance, in fine-grained materials is due to the smaller pore diameters associated with these sediments. Small pore diameters are inhibitory to microbial life for reasons already mentioned, including lack of physical space, restriction on movement of bacteria and

nutrients, and poor accessibility to moisture. If a subsurface material either has pockets of small-grained sediments or it contains a transition from larger-grained to smaller-grained sediments, then variability in the distribution of bacteria will be observed.

The final property of bacteria in heterogeneous soils and sediments that will be discussed is the affect soil and sediment heterogeneity has on microbial metabolic activity. Heterogeneity in subsurface materials makes it more difficult to judge how active the microorganisms present within those materials are. As heterogeneity can occur at any scale (2) the researcher must decide at what point their activity measurement is representative for the area they are studying. Parkin found that up to 85% of the denitrification activity in a 100g soil sample was associated with 0.08 to 0.4% of the soil mass (24). In a separate study, Parkin et al. concluded that 10 to 15 kg of soil would be needed to acquire a representative (mean) denitrification activity level for a particular sample, indicating large numbers of samples and a large sample volume are needed to adequately capture the microbial activity in heterogeneous sites (25). Variability in microbial metabolism can occur both in transition zones between strata and within a single stratum. Metabolic processes may vary by as much as 6 orders of magnitude between strata in the subsurface (2), though most of these studies were done at the meter scale (27, 19, 16). There is evidence that bacterial metabolic activity may also vary by several orders of magnitude within a stratum at small (cm) scales (2), but this is based on far fewer reports (2). Various physical and chemical properties of subsurface soils and sediments have been shown to correlate with the variation seen both between strata and within a stratum. Factors such as moisture content (29) and nutrient concentration (11) are both highly variable in the subsurface, leading to variability in microbial metabolic activity. Another subsurface

variable that is highly influential on microbial metabolism is the soil or sediment type that the metabolism is occurring in. Virtually every study which has examined the rate and extent of metabolic activity in fine-grained sediments such as clays or silts versus courser-grained sediments such as sands has found faster and greater metabolic activity in the sand sediments (15, 27, 28). This is despite the fact that many small-grained subsurface materials, such as clay, shale, peat, etc. tend to have higher concentrations of electron donor available in the form of organic carbon than do sands (11, 23). These small-grained materials, however, also often have very small pore diameters. These small pores limit the type and number of bacteria capable of gaining access to the organic carbon present in the clay, shale, or peat (11). The larger pore openings in larger-grained materials allows for easier access to moisture (3), faster diffusion of nutrients (8), easier movement of bacteria to nutrients and/or other bacteria (31), and offer more physical space for the bacteria to grow. These features combine to make homogenous, large-grained soils and sediments much more hospitable to microbial metabolism than either homogenous small-grained soils and sediments or heterogeneous soils and sediments with a mixture of small- and large-grained particles.

We have reviewed the manners in which heterogeneities in the physical and chemical composition of subsurface soils and sediments lead to heterogeneities in the life processes of the bacteria residing within or interacting with the soils and sediments. While a variety of different aspects of the soils and sediments influence microbial movement, colonization, distribution, and metabolic activity, the one property affecting every aspect of microbial life in the subsurface is the type of sediment in which the event is occurring. Small-grained soils and sediments tend to inhibit all of the microbial life processes listed above, while larger-

grained materials are more amenable to these processes. As homogenous regions of small-grained or large-grained soils and sediments are rare in the terrestrial subsurface, variation in microbial movement, colonization, distribution, and metabolic activity are found at every scale examined. Though the heterogeneity in microbial activities taking place in soils and sediments is unavoidable, awareness of to what extent the variability will occur and what is causing the variation will allow the researcher to better predict and model bacterial processes in the terrestrial subsurface.

From reviewing the literature on the heterogeneity of microbial processes in the subsurface, the areas most in need of more study are variability within single geological stratum and variability at small scales. Within the last 15 years, a great deal of the work done on microbial heterogeneity has been done over tens to hundreds of meters. This work has also tended to focus on differences in microbial parameters between different strata. With my work I have attempted to focus on the areas of subsurface microbial heterogeneity that are less well studied. I have examined various aspects of microbial life at the cm level scale, both within a single stratum, as well as at a location with multiple sediment types. The overall hypothesis for my work was that variation seen in microbial processes could be explained by variations in the physical and/or chemical composition of the surrounding soils and sediments.

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Chapter 2

The Factors Controlling Microbial Distribution and Activity in the Shallow Subsurface

Abstract

The relationships between sedimentological, geochemical, and microbiological parameters in the vadose zone of a barrier sediment from the eastern shore of Virginia were examined. Pairs of samples were taken 10-cm apart in the vertical direction and 2 cm apart in the horizontal direction along three transects with one sample from each depth being processed aerobically and the other being processed anaerobically. Little variation was observed in the sedimentological and microbiological parameters tested. The sediment of all samples was fine to coarse sand and the grain sizes ranged from 0.19-1.16 mm. Sediment moisture was low for all samples, but increased near the top and bottom of each sampling transect. These were regions where bioavailable Fe(III) concentrations were high. Rates of H_2 uptake ranged from below detection limit to 0.064 μmol $H_2 \bullet day^{-1} \bullet g^{-1}$ of sediment with a median rate of 0.01 μmol $H_2 \bullet day^{-1} \bullet g^{-1}$ of sediment. The variation in bacterial numbers was slightly more than an order of magnitude range over the entire sampling face. Phospholipid fatty acid analysis showed a diverse but fairly uniform microbial community from sample to sample. We found that the quartile of aerobically processed samples with the highest H_2 uptake rates had statistically higher moisture content and bioavailable iron content than did the rest of the samples. The quartile of aerobically processed samples with the lowest H_2 uptake rates had significantly more gravel, less moisture, and less bioavailable Fe(III) than did the rest of the

samples. Similar trends were observed for anaerobically processed samples, but the differences were not significant. Our data indicate that the spatial variation in microbial parameters is low within strata with uniform grain sizes.

Introduction

The ability to predict the microbial metabolic potential at a contaminated site is critical for the development of successful bioremediation strategies. However, extensive drilling and testing is both expensive and time consuming. The use of noninvasive geophysical approaches combined with a limited number of sediment cores offers a cost-effective approach to describe the geophysical properties of the aquifer. However, it is not clear how variation in the physical structure of the aquifer relates to variation in microbial activity and abundance in subsurface environments. Although early studies found little variability in total cell numbers (22), numerous later studies have shown that microorganisms are heterogeneously distributed in subsurface environments (1, 7, 11, 17, 24). In addition to abundance, diversity indices, growth rates, and metabolic activity also are highly variable in subsurface samples (9, 19, 25, 26, 37, 40, 43, 47, 48). The great majority of the studies have focused on microbial variability between geological strata in deep saturated sediments (7). Typically, microbial properties vary by 2 to 4 orders of magnitude between strata, although much larger differences have been observed (1, 9, 24, 52). This variability appears to be related to variations in physical properties, e. g., grain size, although other untested factors may also contribute. Several studies found, for example, that microbial activity is lower in clays than in sandy sediments (38, 45). Similarly, poorly permeable shales had little or no sulfate reducing activity whereas

the more permeable adjacent sandstones exhibited high levels of sulfate reducing activity (30). It is clear that large variations in microbial activity and abundance will be observed between strata with markedly different sedimentological features. However, the relationship between microbial activity and abundance and sedimentological properties within a single geological unit have not been quantified. If the sedimentological features between different strata are the cause of the variability seen in microbial activity and abundance between strata, then much less variability in microbial activity and abundance should occur within a single geological stratum.

Several studies have recently been completed examining the microbiology of unsaturated or vadose zone sediments. The numbers of culturable bacteria as well as microbial phospholipid fatty acids (PLFA) decreased with depth in a variety of vadose zone environments (5), including deep vadose zone sediments from a semiarid high desert (10). Many studies implicate moisture as the most important factor in controlling microbial activity and numbers in the vadose zone (39, 26, 50). The addition of moisture to deep vadose zone samples from Nevada, Idaho, and Washington stimulated microbial activity (26). However, not all the samples were stimulated by the addition of water, suggesting that other nutrients may also have been limiting. After fractionating vadose zone sediment into different grain sizes, Nacro et al. (34) found that most of the sediment's organic carbon and organic nitrogen were located in the smaller grain size fractions while the larger grain size fractions contained the most metabolic activity.

The variation in microbial activity, abundance and community structure was examined on a centimeter-level scale in a vadose zone sediment near Oyster, VA. The central hypothesis driving this investigation was that the major controls of microbial activity in the subsurface are strongly correlated with a statistical description of the variation in the physical and chemical properties of the formation. Our prediction was that within strata, the variation in the physical parameters would be low, as would the variability in microbial parameters. Hydrogen was chosen as an electron donor to assay microbial activity for several reasons; 1) it is a widely utilized substrate by both aerobic and anaerobic bacteria (6, 27), 2) it may be added without altering the sediment's physical properties, and 3) it is possible to monitor its microbial uptake without sample destruction. We found that the sediment at the test site had relatively uniform geological features over scales of tens of meters. The measured microbial properties within this sediment were also relatively uniform. Local variations in microbial activity did occur, which could be explained by local changes in sediment chemistry and/or sediment moisture content.

Materials and Methods

Sampling Site Description and Sample Processing

The sampling site is located near the small fishing village of Oyster, VA (Figure 1) on the eastern coast of the United States on the southern Delmarva peninsula about 19 km north of the Chesapeake Bay Bridge Tunnel. The sampling site is located in a barrier complex that is underlain by the Late Pleistocene Wachapreague Formation, composed of repetitive deposits

of prograding shoreface sand. The sediment consisted of unconsolidated to weakly cemented, well-sorted, medium- to fine-grained sand and pebbly sand (21). On the edge of an agricultural field, an excavator was used to dig a pit approximately 2 m deep. A 1.5 m high sampling face was established and smoothed utilizing hand tools, with the top of the sampling face located below the root zone, approximately 0.5 m below ground surface (bgs). Samples were taken along three vertical transects located about 5.5 m apart in the horizontal direction at 3.3 m, 8.7 m, and 14.3 m from the left (south) side of the sampling face (subsequently referred to as transects A, B, and C, respectively) (Figure 2). Because of the sloping surface topography, vertical sample position was designated relative to the floor of the trench. All samples were taken aseptically within 3 hours after the excavation was completed, using an ethanol-rinsed trowel to remove the sediment on the surface of the sampling face after which a sterile polycarbonate tube (10 cm in length, 4.5 cm inner diameter) was driven into the sampling face. After the sediment was removed, both ends of the polycarbonate tube were sealed with sterile rubber stoppers. Samples were taken 2 cm from each other in the horizontal direction and 10 cm from each other in the vertical direction. At each sampling depth, one sample was processed aerobically while the other sample was processed anaerobically. Aerobically processed samples were immediately placed into a cooler at 4^{o} C while the anaerobically processed samples were placed in a nitrogen-filled portable anaerobic chamber. The rubber stoppers were removed from the ends of the anaerobically processed samples and the samples were left in the chamber for several minutes before the stoppers were placed back on the ends of the tube. After removal from the anaerobic chamber, the anaerobically processed samples were placed into a cooler at 4^{o} C. Samples were stored at 4^{o} C for no longer than 48 hrs before being shipped to the University of Oklahoma. Samples

were processed within 48 hrs of arrival in Oklahoma. Additionally, samples were taken as described above from throughout the sampled face for phospholipid fatty acid (PLFA) analysis. The PLFA samples were placed on dry ice immediately after removal from the sampling face and shipped to the University of Tennessee the same day where they were stored at -80^{o} C until processed.

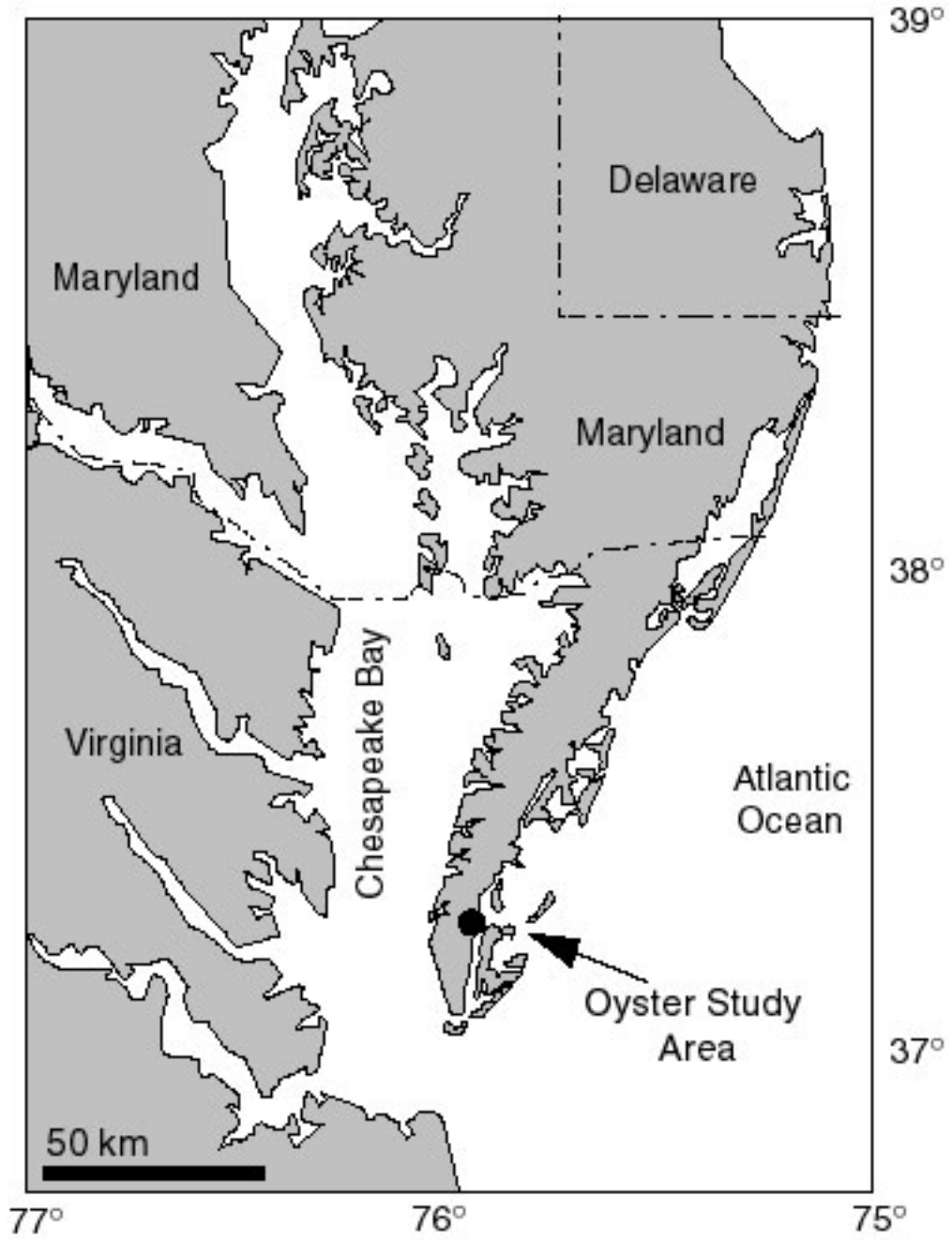


Figure 1. Location of Oyster, VA Study Site

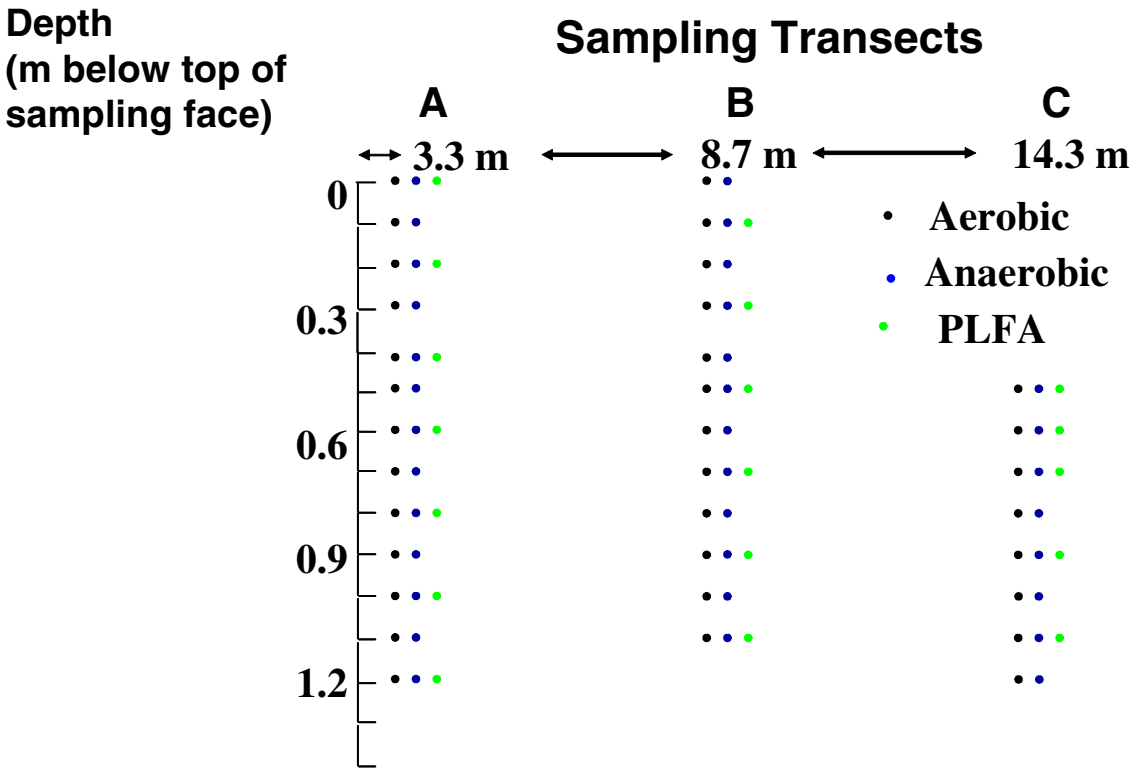


Figure 2. Diagram of the sampling transects. Locations where the aerobically processed, anaerobically processed, and PLFA samples were taken are indicated

Hydrogen Uptake Activity

Standard anaerobic and aseptic techniques were used throughout this study (2). All samples were processed in an anaerobic chamber (Coy Laboratory Products Grass Lake, MI) filled with a 100% N_2 atmosphere. In the chamber, 3 g of sediment from each sample was added to a sterile 30 ml serum bottle. The serum bottle was sealed with a sterile butyl rubber stopper before being removed from the chamber. Each serum bottle was then aseptically evacuated with vacuum and repressurized with an O_2-free, 100% N_2 gas phase 5 times using sterile gassing probes. For the aerobically collected samples, O_2 was added into the headspace of the serum bottle by transferring air from a sterile bottle using a sterile syringe and needle. The final O_2 concentration was 13% (14.42 kPa). Selected samples from both the aerobically and anaerobically processed samples were used as sterile controls by autoclaving them at 121^0C for 20 minutes. At the start of the experiment, H_2 was transferred from a sterile bottle filled with 100% H_2 (110.66 kPa) using a sterile, 1-ml syringe into the headspace of each serum bottle to give a final concentration of 0.026% (2.877×10^{-2} kPa) H_2 in the gas phase (12). The disappearance of H_2 over time was measured by using a gas chromatograph (GC) equipped with a 100-µl sample loop and a mercury vapor reduction detector (12). The H_2 concentration of each sample was checked every 24 hours until the rapid disappearance of H_2 was detected, after which H_2 concentrations were checked every 8-12 hours until the H_2 levels became undetectable. The H_2 concentration of samples showing no detectable H_2 uptake activity was checked every 24 hours for 30 days. At each sampling time, 0.3 ml of gas was aseptically removed from the headspace of each sample and injected into the sample loop of the GC. The linear portion of the change in H_2 concentration with time was then used to

calculate the zero order rate of H_2 uptake. A minimum of 3 data points were used to calculate each rate, while 5 or more data points were used to calculate most rates. Linear regression was performed on all samples, and samples with correlation coefficients below 0.9 were not used in subsequent analysis. To ensure that mass transfer did not limit the rate of H_2 uptake, the amount of sediment was varied in the serum bottle while the concentration of H_2 was held constant. The amounts of sediment tested were 0.5g, 1 g, 3g, 5g, 10g, and 30 g. The zero order rate of hydrogen uptake varied linearly with the amount of sediment added up to the 10g sample, at which point it leveled off (data not shown). This suggests the system was biomass and not mass transfer limited at the sediment concentrations were our assays were run.

Moisture and Nutrient Additions

To determine the effect of moisture and electron acceptors on H_2 uptake, a bulk sample of sediment was collected by digging below the root zone and aseptically filling sterile mason jars with sediment. Bottles for H_2 uptake measurements were prepared as described above with each bottle receiving 3 g of sediment. Sterile water was added to duplicate bottles to give a final moisture content of 10%, 20%, or 100% (of saturation), while another set of duplicate bottles was left at their original moisture content (approximately 5%). Another set of duplicate bottles was heated at 105^{o}C for 48 hr to serve as an abiotic control. Different electron acceptors were added to other sets of bottles. Nitrate, sulfate, and Fe(III) were added in concentrations of 0 mM, 1 mM, 5mM, and 10 mM with NaCl added in the appropriate amount to ensure that the ionic strength was equal under all conditions tested. The amount of moisture in each bottle was also held constant.

Phospholipid Fatty Acid Analysis

Each sample taken for PLFA analysis was homogenized then split into triplicate subsamples. Lipids were extracted using the modified Bligh/Dyer extraction (49), with fractionation of polar, neutral, and glyco-lipids and methylation of the polar fraction performed as described by Guckert et al. (23). Fatty acid methyl esters (FAMES) were separated and quantified by gas chromatography (GC) with peak identification by mass spectrometry (MS). PLFA analysis was used to estimate biomass and community structure over the sampling face.

Bacterial Enumeration

Estimations of bacterial numbers were performed via two methods. Biomass content was estimated from each sample's PLFA using a conversion factor described by Balkwill et al. (4) to convert the total amount of PLFA in each sample to cell abundance per gram dry weight of the sediment. Three tube most probable number (MPN) analyses were also performed on selected samples to enumerate aerobic heterotrophic bacteria, anaerobic heterotrophic bacteria, and sulfate-reducing bacteria (SRB). One gram of sediment from the selected samples was placed into 9 ml of sterile 0.1% sodium pyrophosphate at pH 7 (3). The liquid-sediment solution was subjected to two 30-second mixing cycles on a test tube mixer. One milliliter was removed from the resulting suspension and used to inoculate the first set of tubes. After mixing, one milliliter was aseptically withdrawn with a sterile syringe and needle and inoculated into the next tube in the series. This procedure was repeated until all of the tubes were inoculated. Degassed syringes and needles were used for the anaerobic

heterotrophic and sulfate-reducing cultures (2). The medium used for both the aerobic and anaerobic heterotrophic bacteria was a 1:10 dilution of the peptone, trypticase, yeast extract, glucose (PTYG) medium described by Balkwill and Ghiorse (3). Standard anaerobic techniques were used to prepare the anaerobic heterotrophic medium (2). The SRB medium was API-RST medium and was prepared as described by Tanner (44). For each medium, an organism known to grow in the medium was added to additional MPN tubes that had been inoculated with sediment to insure that the medium was capable of supporting bacterial growth and inhibitory compounds were not present. The positive control for the aerobic heterotrophic medium was *Bacillus subtilis*, for the anaerobic heterotrophic medium was *Clostridium difficile*, and for the SRB medium was *Desulfovibrio vulgaris*. All of the positive controls grew to 10^8 to 10^9 cells/ml. Uninoculated media served as the negative controls, and none of the negative controls showed any signs of growth after 6 months of incubation.

All tubes were incubated at room temperature without shaking and were checked periodically for growth. For the aerobic and anaerobic heterotrophic cultures, tubes that had an increase in absorbance of 0.1 units at 600 nm above the uninoculated medium and contained viable cells as determined by plating on agar medium of the same composition were scored as positive. For sulfate reducers, tubes with blackening indicative of iron sulfide production were scored as positive.

Geochemical Analysis

The total C, N and S were determined on selected samples by dry combustion using an EA 1110 Elemental Analyzer (CE Elantech, Inc., Lakewood, NJ). Aliquots of the sediment

samples were placed in Sn capsules and combusted at 1800°C with O_2. The oxidized gases are then reduced, separated chromatographically and detected with a thermal conductivity detector. The volumes of CO_2, N_2 and SO_2 released were then converted into weight percent of C, N and S by calibration with standards (35).

To measure organic C, a separate aliquot of each sample was acidified with 1 M HCl in an Ag capsule to convert any carbonates to CO_2. The sediment and capsule were then dried at 80 to 100°C overnight, and then dry combusted at 1000°C and analyzed. The volume of CO_2 released was then converted into weight percent of organic C by calibration with standards. The difference between the total C and the organic C represents the weight percent of inorganic C.

The bioavailable (surface reducible) Fe(III) was estimated by a modified version of the hydroxylamine-HCl procedure (32). Aliquots of the sediments, 0.1 to 0.5 g, were immersed in 5 ml of 0.5 M HCl, shaken for 30 seconds and then heated at 50°C for 30 minutes in the dark. The mixture was allowed to cool to room temperature, centrifuged at 3000 rpm for 8 minutes, and the supernatant decanted into a beaker. The Fe in the extract was measured by ferrozine (31) and by ICP-AES. A split of the same sample was acidified with 5 ml of 0.25 M HCl and 0.25 M hydroxylamine-HCl. The mixture was shaken for 30 seconds, and then equilibrated at 50°C for 30 minutes in the dark, followed by centrifugation and removal of the supernate. The concentration of Fe in the supernate was determined by ferrozine and ICP-AES. The difference between the Fe concentration of the 0.25 M HCl+0.25 M hydroxylamine extract and that of the 0.5 M HCl extract was converted into mg kg^{-1} sediment of Fe. According to Lovley and Phillips (32) this Fe concentration is equivalent to the

amount of amorphous Fe(III)oxide or bioavailable Fe(III). Of the 78 samples analyzed, only 2 samples yielded higher Fe concentrations in the 0.5 M HCl extraction than in the hydroxylamine-HCl extraction. These two discrepancies are attributed to the release of Fe from clay in the samples. The Al content associated with the 0.5 M HCl extraction was also quantified on the same extracts by ICP-AES.

Sediment Grain Size and Moisture Content Analyses

The moisture content of each sample was determined gravimetrically by drying 5 g of triplicate samples of wet sediment for 48 hr at 105^{o}C (28). The grain sizes of the samples were determined by the Sedimentary Dynamics Laboratory at Old Dominion University. Samples were sieved using standard methods of sieve analysis with Endecott sieves ranging from 4 mm to 0.15 mm and spaced at an interval of 0.84 mm (18). Sediments that were finer than 0.15 mm were subsequently analyzed using an Elzone 280PC Particle Size Analyzer (Micromeritics, Norcross, GA). Additionally, percent gravel and percent silt were determined for all samples.

Statistical Analysis

Standard statistical methods were used to analyze the H_2 uptake rate data and to compare it to the geological and geochemical properties of the samples. Histograms of H_2 uptake rates and grain size were prepared to examine the shape and the degree of spread of their frequency distributions. Bivariate scatterplots and Pearson product moment correlation coefficients (51) were used to compare the H_2 uptake rates to the geological and geochemical properties of the

samples. The H_2 uptake rate samples were divided into 4 classes using the quartiles of the data (i.e., the 25^{th}, 50^{th}, and 75^{th} percentiles). Notched box plots (33) were then used to graphically compare 95% confidence intervals for the medians of the geological and geochemical properties of the samples for the 4 classes of H_2 uptake data. Analysis of variance (ANOVA) was used to determine if there was a significant difference in H_2 uptake rates in samples with different amounts of total carbon, nitrogen, sulfur, and organic carbon.

Results

Relationship between Initial Rates of H_2 Uptake and Sediment Properties with Depth

The change in H_2 uptake rate, moisture content, surface reducible (bioavailable) Fe(III), grain size, and biomass for all samples with depth is shown in Figure 3a-c. In general, H_2 uptake rates varied less than 10 fold from sample to sample, regardless of either depth, transect location, or whether the sample was processed aerobically or anaerobically. Moisture content was highest at the top and bottom of all three transects, as was the amount of bioavailable Fe(III). Grain size varied less than an order of magnitude from sample to sample, both with depth and horizontally across the sampling face. The PLFA biomass measurements exhibited

a range of slightly more than an order of magnitude for all samples tested, with 16 of 18 samples falling within the 10^6 bacteria/g sediment range. No significant correlation ($\alpha = 0.05$) was found by linear regression between PLFA biomass concentrations and H_2 uptake rates.

The areas which exhibited higher H_2 uptake rates were located at the top and bottom of each transect, where moisture contents and bioavailable Fe(III) concentrations were highest.

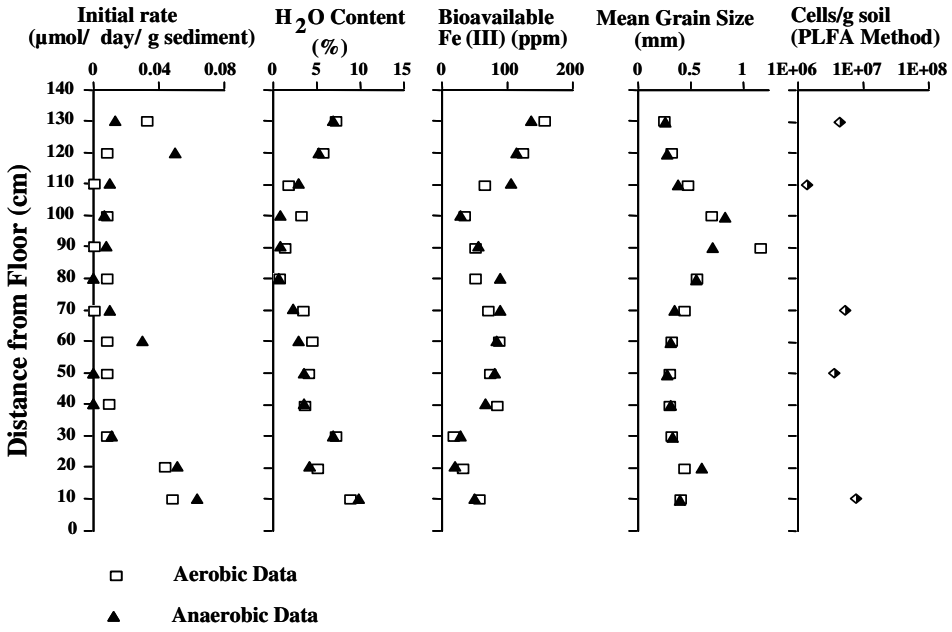


Figure 3a. Initial rates of hydrogen uptake, moisture contents, bioavailable iron concentrations, mean grain sizes, and estimates of total bacterial cell numbers for all samples from transect A. Note: Distance from floor is the distance from the bottom of the sampling face to the point where the samples were taken

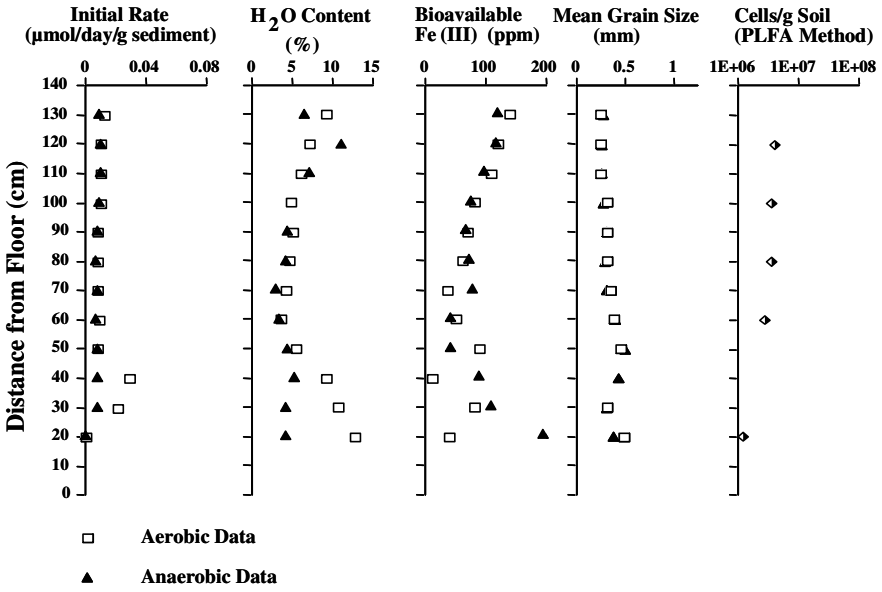


Figure 3b. Initial rates of hydrogen uptake, moisture contents, bioavailable iron concentrations, mean grain sizes, and estimates of total bacterial cell numbers for all samples from transect B. Note: Distance from floor is the distance from the bottom of the sampling face to the point where the samples were taken

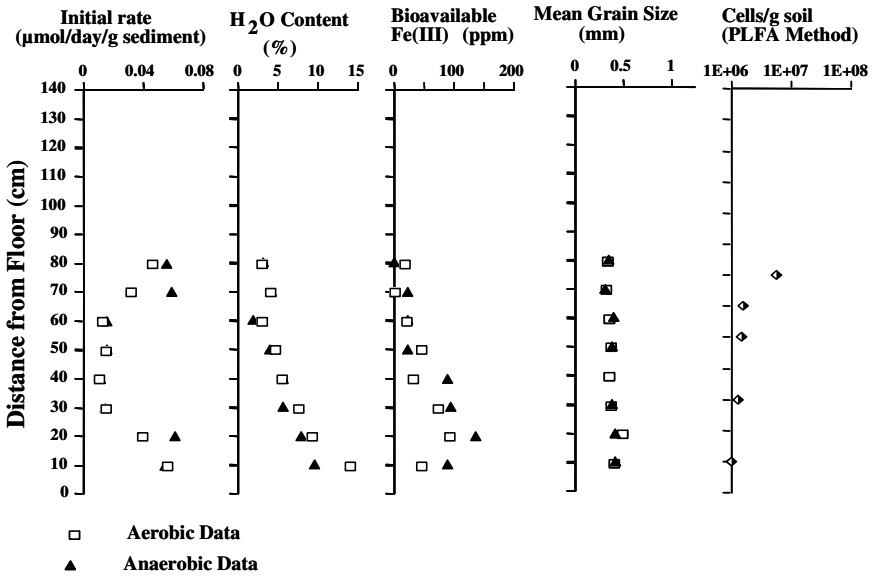


Figure 3c. Initial rates of hydrogen uptake, moisture contents, bioavailable iron concentrations, mean grain sizes, and estimates of total bacterial cell numbers for all samples from transect C. Note: Distance from floor is the distance from the bottom of the sampling face to the point where the samples were taken

More than 80% of the samples tested had low H_2 uptake rates, varying from 0.005 to 0.05 µmol H_2 consumed/ day /g sediment, with a median H_2 uptake rate of 0.01 µmol H_2 consumed/day /g sediment (Figure 4). No autoclaved control showed any loss of H_2 after 60 days of incubation. The values exhibited by the live samples were orders of magnitude lower than H_2 oxidation rates measured for different soils (42) or sediments (41). The coefficient of variation for all H_2 uptake rate measurements was 101.7%. The mean grain sizes tended to be uniform both throughout each sampling transect and between transects. The range of mean grain sizes for all samples spanned about an order of magnitude, with all samples falling into the fine to coarse sand classification (Figure 4).

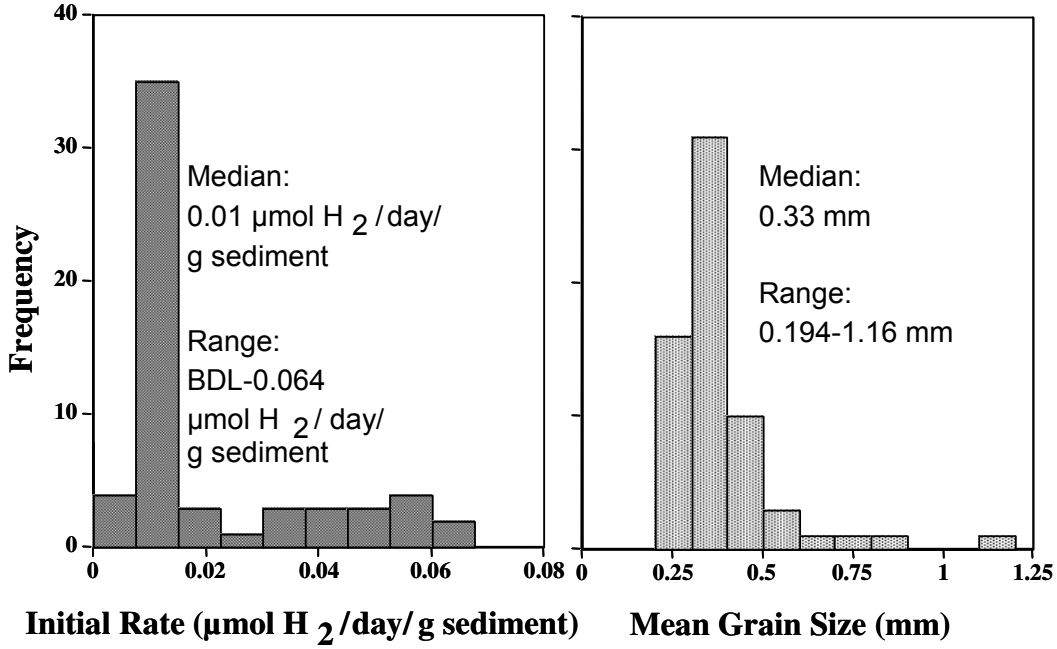


Figure 4. Distribution of hydrogen uptake rates and mean grain sizes for all aerobic and anaerobic samples.
BDL - Below Detection Limit (0.001 µmol H_2 /day/g sediment).

To assess whether significant relationships between H_2 uptake rates of the aerobically and anaerobically processed samples and the geological and geochemical properties of the sediment existed, the samples were separated into four classes, based on quartiles of the H_2 uptake rates. Notched box plots were then used to graphically compare 95% confidence intervals for the medians of the geological and geochemical properties of the samples for the 4 classes of H_2 uptake data. Notched box plots provide graphical displays of the central tendency and variability of groups of data (33) and can be used to perform the graphical equivalent of a t-test or analysis of variance. Notched box plots showed that, for the case of the aerobically processed samples, the class with the lowest rate of H_2 uptake had significantly lower moisture content, lower bioavailable iron, and significantly higher gravel content compared to the other classes (Figure 5). Additionally, the class with the highest H_2 uptake rate had significantly higher moisture content and bioavailable iron than the other classes. For anaerobically processed samples, the class with the highest H_2 uptake rate had less gravel and greater moisture content than the other classes, but the differences were not significant (data not shown).

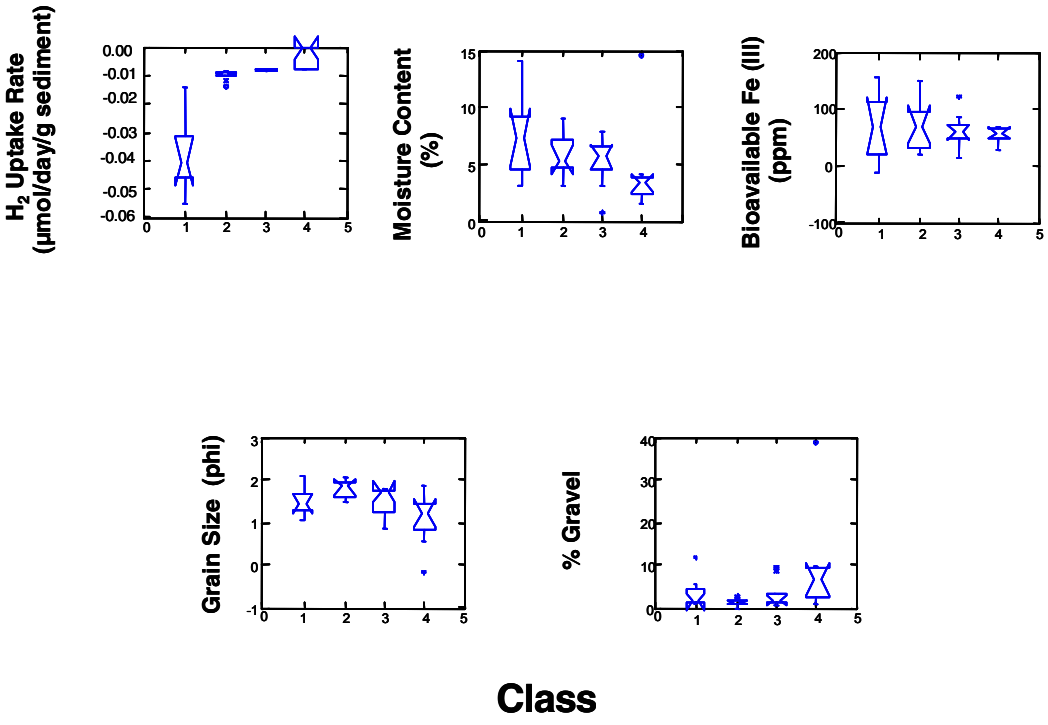


Figure 5. Notched box plots of metabolic and geochemical data for the aerobically processed samples. The medians of the distributions are shown as the center of the notches, and the lower and upper quartiles are the hinges (upper and lower ends) of the boxes. Asterisks and circles represent outlier data points at 1.5 and 3 times the inter-quartile range, respectively. The notches themselves represent an approximate 95% confidence interval around the median, so statistically significant differences exist between groups for which the notches do not overlap. Note: For this figure, a greater negative number for the hydrogen uptake rate represents a faster hydrogen uptake rate.

Enumeration of Bacteria

The MPN of aerobic heterotrophs ranged from 10^4 to 10^7 cells/g sediment and that of the anaerobic heterotrophs ranged from 10^3 to 10^6 cells/g sediment (data not shown). Most of the MPN counts were in the 10^5 to the 10^6 range for both analyses. Estimates of total bacterial cell numbers from the PLFA analysis were in the 10^5 to 10^6 cells/g sediment range (Figure 3a-c). Regression analysis revealed no significant correlation between cell numbers obtained from either the MPN or the PLFA methods and any other variable tested (r values ranged from -0.25 to 0.23). Most samples tested had no detectable SRB while a few samples had counts in the 10 to 10^2 cells/g sediment range.

Effect of the Addition of Moisture and Electron Donors and Acceptors on Metabolic Activity

The data in Figure 3 indicated that metabolic activity may be related to moisture content. To test this hypothesis, an experiment was performed to determine the effect of varying levels of sediment moisture on H_2 uptake rate. The addition of moisture stimulated the level of microbial metabolic activity within the sediment. Bacteria within sediment which either had no additional moisture added or was heated at 105^o C until all moisture was removed displayed no detectable metabolic activity (<0.001 µmol H_2/ day /g sediment). However, samples which had moisture added (the 10%, 20%, and 100% moisture samples) all had detectable metabolic activity (0.004 to 0.005 µmol H_2/ day /g sediment). The metabolic activity detected in the samples with added moisture was low (half of the median hydrogen uptake rate of the field samples), however, suggesting that other nutrients might be limiting.

The addition of 1 to 10 mM sodium nitrate increased the rate of H_2 uptake 4 to 5 fold, relative to controls with equal ionic strength of sodium chloride. The addition of sodium sulfate or Fe(III) did not affect H_2 uptake rates relative to sodium chloride controls.

Site Geochemistry

The amount of total organic carbon, total nitrogen, and total carbon present in select samples is shown in Table 1. The low levels of nitrogen and organic carbon are consistent with the beach sand type of sediment typified by low amounts of clay, silt, and organic matter (14). An analysis of variance (paired ANOVA) revealed no significant relationship between the levels of the above nutrients and sampling depth, transect location, H_2 uptake rates, or cell numbers.

Table 1. Geochemical Analysis of Selected Samples

Sample	*Transect*	*Total Organic C (%)*	*Total N (%)*	*Total C (%)*
8	14.3 m	0.059	0.007	0.066
16	14.3	0.084	0.014	0.123
18	14.3	0.053	0.01	0.083
28	8.7	0.08	0.016	0.113
35	8.7	0.078	0.091	0.073
45	8.7	0.035	0.026	0.045
47	8.7	0.06	0.008	0.091
48	8.7	0.056	0.003	0.005
56	3.3	0.054	0.011	0.078
78	3.3	0.036	0.004	0.046
79	3.3	0.051	0.007	0.066

The acid-extractable Fe(III) concentrations ranged from 10 to 155 ppm whereas, the acid-extractable Al concentrations ranged from 87 to 1037 ppm. These values are a mere fraction of the total Fe and Al concentrations of for these sediments, which are 4300 and 17,400 ppm, respectively. The majority of the Fe and Al resides in smectitic grain coatings identified by extensive SEM analyses of intact cores recovered from the same excavation with a minor portion of the Fe locally residing in goethitic pseudomorphs of pyrite framboids (15). Extraction experiments revealed that the Fe and Al associated with these smectite phases could be solubilized by 0.5 M HCl (data not shown) as was found to be the case for Fe by Tuccillo et al. (46). The Fe(III) released by the hydroxylamine-HCl treatment is likely to be comprised of the Fe found in Si and Al-bearing amorphous grain coatings and in Halloysite-like grain coatings by TEM analyses (15, 36).

Phospholipid Fatty Acid Analysis

The microbial community structure of the site was relatively diverse, but uniform between samples (Figure 6). All samples tested contained high relative proportions of terminally branched, saturated PLFA. These PLFA are indicative of the presence of Gram-positive and anaerobic Gram-negative bacteria. The markers indicative of both aerobic and anaerobic Gram-negative bacteria (monoenoic fatty acids) were relatively low. Specific biomarkers for both sulfate-reducing bacteria and for actinomycetes (mid-chain branched saturated PLFA) were found in all samples tested. Likewise, PLFA markers for eukaryotic organisms, specifically fungi, algae, plant and animal matter, were detected throughout the sampling face.

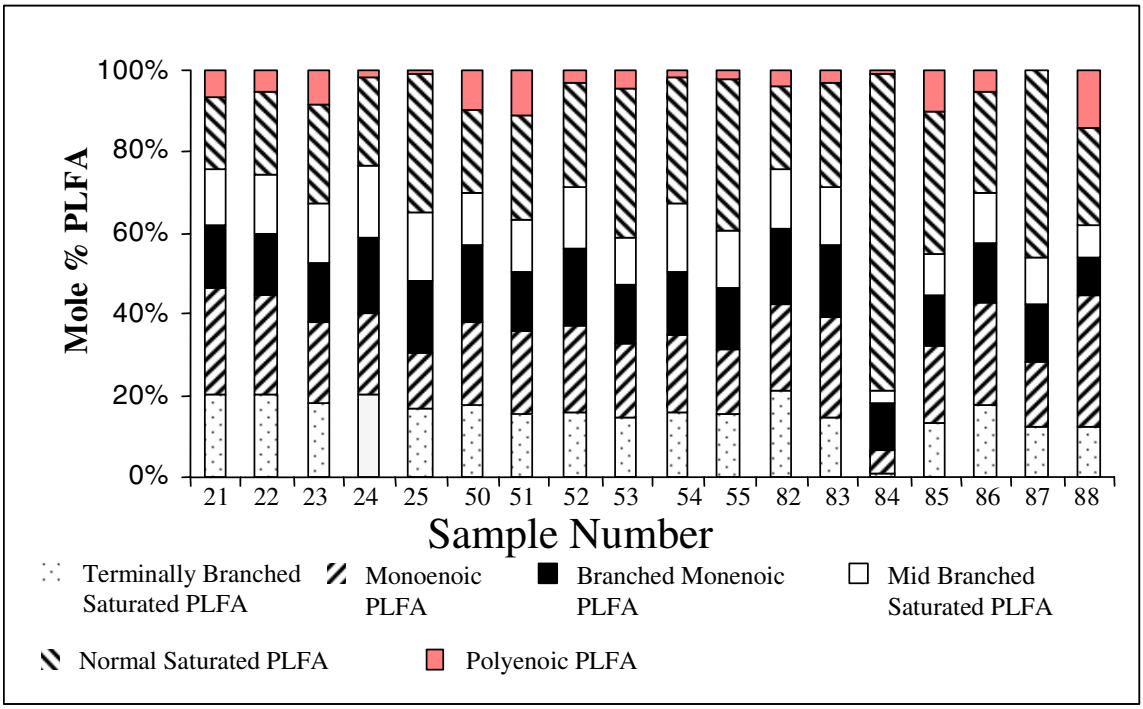
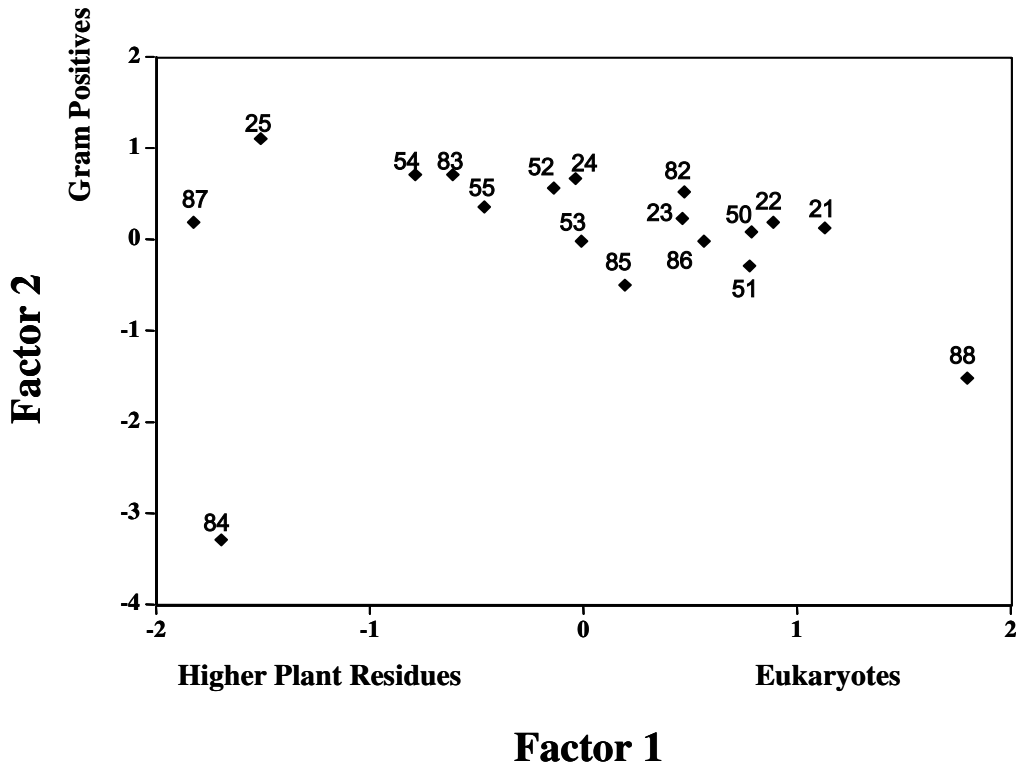


Figure 6. Community structure as determined by PLFA analysis

*Samples 21-25: Transect C, Samples 50-55: Transect B, Samples 82-88: Transect A

Principal components analysis of the PLFA data showed that the majority of samples clustered together with only two samples as outliers (Figure 7). One of these samples contained both the highest relative proportion of high molecular weight normal saturated PLFA as well as high amounts of dicarboxylic acids and high molecular weight hydroxy fatty acids. Taken together, these PLFA are indicative of relatively large amounts of higher plant matter in this sample. The other outlying sample, conversely, contained higher relative portions of the biomarkers for Gram-negative bacteria and fungi than did any other sample analyzed. The percent of the total variance explained by factor 1 is 52.4% and the percent explained by factor 2 is 12.6%.



*Samples 21-25: Transect C, Samples 50-55 Transect B, Samples 82-88 Transect A

Figure 7. Principal Component Analysis of PLFA. Influential biomass-types were inferred from the presence of specific PLFA and are shown in the appropriate positions.

Discussion

Several studies have attributed the high degree of spatial variability in microbial activity and abundance in subsurface sediments to differences in grain size or pore size between strata (19, 24, 25, 30). If the major controls of microbial activity in the subsurface are strongly correlated with the statistical description of the physical and chemical variation of the formation, then one would predict that sites with little variation in grain size should have little variation in microbial parameters. Our data are consistent with this hypothesis. Not only were microbial activity and abundance uniform throughout the site, but the microbial community structure as inferred from PLFA analysis was also uniform, except for two samples (Figure 7). Other workers have shown similar relationships between grain size and microbial activity. Albrechtsen and Winding (1) found large variations (orders of magnitude differences) in metabolic activity between different grain size fractions from an unconsolidated, sandy aquifer. Metabolic activity was within an order of magnitude for samples taken from the same grain-size fraction, however, indicating less variability in regions with similar grain sizes.

Although some nutrients at the Oyster site varied by up to two orders of magnitude from sample to sample (Table 1), the concentration of all of the measured nutrients was low regardless of the sampling location. Organic carbon was ten-fold and total nitrogen was 10^3 to10^6 fold lower at the Oyster site than in a typical, Midwestern, agricultural, vadose-zone soil (29). Konopka and Turco (29) found that total phosphorous, total nitrogen, exchangeable K^+, and organic C decreased with depth in the vadose zone, whereas an analysis of variance

revealed no relationship between sampling depth and the concentration of any nutrient tested at the Oyster site. The low moisture content (94 % of samples under 10% moisture) at the Oyster site would compound the effects of low nutrient content (no sample above 0.09% total organic carbon or total nitrogen) to limit microbial activity. Low moisture in unsaturated sediments would slow cell growth due to increased energy expenditure to maintain turgor pressure, slow diffusion of nutrients to cells, and would inhibit bacterial movement towards nutrients (50). Not surprisingly, researchers found that vadose-zone samples with higher moisture have higher metabolic activity than those with low moisture (50). The majority of the samples at the Oyster site had very low moisture content, with many samples containing less than 5% moisture (Figure 3a-c). These samples were located in the middle of the sampling transects, where microbial activity was also the lowest. In a subsequent sampling trip, we did not detect metabolic activity, in the form of hydrogen uptake, in samples taken from the vadose zone with moisture contents less than or equal to the original moisture content, even after extended incubation. However, vadose zone sediment samples to which moisture was added all had measurable metabolic activity. Similarly, others have found that the addition of moisture markedly increases metabolic activity (8, 26) and bacterial abundance (8) in vadose-zone soils and sediments. Given that nutrient diffusion is probably limiting in the vadose zone (26), the combination of low levels of nutrients and moisture may explain, in part, the low levels of metabolic activity observed at the Oyster site. While some nutrients may be higher at one location compared to another, the low concentration of all nutrients along with limiting moisture would result in generally low and uniform rates of microbial activity.

The few samples that did exhibit somewhat higher H_2 uptake rates than the rest of the samples were from areas where moisture and bioavailable Fe(III) were high (Figure 3 and 5). The iron hydroxides at this site coat the sand grains (16). The iron hydroxides are positively charged and very fine-grained, on the order of 10-30 µm. Since bacterial cells are negatively charged, bacteria would attach to and accumulate on the iron-coated sand grains (20). This may explain the large number of cells found in fine-grained sediments (1, 13) as well as the increased H_2 uptake rates observed in samples with high Fe content at the Oyster site.

We have shown multiple lines of evidence supporting the hypothesis that sediments with little sedimentological variation harbor microbial communities with little spatial variation in activity and abundance. Grain size is often one of the key factors controlling microbial abundance and activity since grain size is a major determinant of the permeability and porosity of the porous matrix. Porosity and permeability are parameters which are measures of the amount of water that a porous material can hold and the ease with which water can move through the material. These parameters would control the flux of nutrients and microbial cells. In regions where the grain size is uniform, nutrient content and flux would be fairly uniform, and, as a consequence, the microbiology of such environments should be relatively uniform. This is consistent with our observations at the Oyster site, which include uniform grain size, uniformly low moisture and nutrient levels, and uniformly low microbial activity. The few samples that did show higher levels of microbial activity were from areas that had local variations in grain size and geochemistry. Our data suggest that spatial variability will be low within strata with uniform sedimentological properties. Extensive

drilling and sampling may not be necessary within strata with uniform geophysical properties to obtain an adequate assessment of the spatial variability of microbial properties.

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Chapter 3

Spatial Variability of Sulfate Reduction in a Shallow Aquifer

Abstract

The distribution and metabolic activity of sulfate-reducing bacteria (SRB) in a shallow, microaerophilic aquifer were studied. A radioimaging technique was used to visualize and quantify the activity of sulfate reducers in sediments at a centimeter-level scale. The distribution of SRB metabolic activity was heterogeneous with areas showing little activity far outnumbering areas with high activity. Variation in sulfate reducing activity was not statistically correlated with variation in depth, bacterial numbers, or the following sediment properties: sediment type (sand, peat, or silt), grain size, permeability, and hydraulic conductivity. SRB activity did vary significantly with sediment porosity (multivariate analysis, $r = 0.48$). We hypothesized that the small pore sizes associated with sediments with low porosity restricted the ability of SRB to grow to high numbers as well as their access to nutrients. To further explore the relationship between pore size and microbial metabolic activity, columns with varying pore diameters were constructed. SRB in the columns with the smallest pore diameters had the slowest rates of metabolism and SRB metabolic rates increased as the pore diameter increased. For the aquifer studied, sediment porosities and pore sizes were the main factor controlling SRB activity.

Introduction

Sulfate reducing bacteria are widely found in a variety of anaerobic and transition-zone environments (2, 4, 6, 25). In these environments, the distributions, numbers, and metabolic activities of the SRB vary widely. Some of the factors contributing to this heterogeneity include availability and location of nutrient sources (5, 9, 19), availability of water (14), redox conditions (10, 22), and relative ease of transport of bacteria through the surrounding medium (21). Many of these factors can be grouped into a larger, more general class, the physical and chemical composition of the sediment in which the bacteria resides. The physical and chemical structure of the subsurface medium in which SRB live contribute greatly to the distribution, number, and metabolic activity of these bacteria. Numerous studies have found significant shifts in the composition and metabolic activity of bacterial populations when the physical properties of the surrounding sediments change. Chapelle and Lovley found higher rates of metabolism in a sandy sediment than in a finer grained clay sediment (3). Likewise, Fredrickson et al. (8) reported that after a shift in aquifer sediment type from a relatively porous sandstone to a less porous material, there was a decrease in the ability of the bacterial populations to utilize different substrates as well as an overall decrease in bacterial numbers.

Other researchers have focused their work on the areas were changes in physical properties of sediments occur. Work done on deep subsurface formations in New Mexico discovered differing levels of SRB activity at the transition zones between

sediment types. The researchers hypothesized that the high levels of metabolism found in sandstones located just beneath shale layers was due to nutrients leaching from the shale into the more porous and more easily colonized sandstone strata (17). Similar results were obtained by Ulrich et al. who found narrow zones of high sulfate reducing activity in sands that were positioned next to either clays or lignite-rich sediments (31). Their explanation for these results was that organic matter was being fermented within the clay and lignite-rich layers and the resulting small organic molecules were driving sulfate reduction in the sand strata.

While these and other studies have contributed greatly to our understanding of the variability present in the terrestrial subsurface, they have focused on large (tens of meters) to very large (hundreds of meters) scales. In their review, Brockman and Murray point out that there are very few studies examining microbial variability at the centimeter-level scale due to the incorrect assumption that such small scale variability has little effect on field-scale problems (1). In this work, we examined the influence sediment grain size, permeability, porosity, hydraulic conductivity, and type have on abundance and activity of SRB at a centimeter-level scale. The objective was to understand the factors that influence the distribution of SRB activity at the study site. The main hypothesis was that this variability in SRB metabolic activity can be explained by the changes in the geophysical properties of the surrounding aquifer sediments.

Materials and Methods

Site Description and Sediment Core Acquisition

Sediment cores were taken from a shallow, microaerophilic aquifer located at the Department of Energy/Natural and Accelerated Bioremediation Research Program South Oyster Focus Area on the southern Delmarva Peninsula near the town of Oyster, VA. The groundwater at the site is suboxic, with O_2 concentrations typically in the 0.5 to 1 mg/L range. The pH of the groundwater is acidic, ranging from 5.6 to 5.9 while sulfate is present in concentrations of 0.4-0.7 mM. The sedimentology of the site has been described previously (13). Briefly, the sediment from the surface to a depth of about 3 meters consists of well-sorted, medium- to fine-grained sand and pebbly sand. A fine-grained, poorly porous, organic-rich peat layer is located underneath this top sandy sediment. From about 4 to 6.5 meters below ground surface is another higher permeability sand stratum which is confined below by a fine grained silt layer. Sediment cores were obtained using a rotosonic drilling method which minimized disturbance to the sediment structure. Three cores, SO-ODU2, SO-ODU3, and SO-ODU4, were taken at locations approximately 10 m from each other. Ten meters of sediment were taken for each core, starting at the ground surface. We utilized portions of cores SO-ODU2 and SO-ODU3 and the entirety of SO-ODU4 for our study.

Core Processing

The processing of the sediment cores began immediately after they were removed from the drilling holes. Each core was marked for orientation and taken to a nearby work area. The core liner was cut in half lengthwise using a circular saw and a specially constructed jig, the cuts were sealed with tape, and then the core was cut into 26-cm sections. The core sections were brought inside of a portable anaerobic chamber with a 100% nitrogen gas phase and an oxygen removing catalyst. Once inside of the chamber, the tape was removed and a sterile blade was used to divide the core sections into two halves lengthwise. One half of each core section was covered with plastic wrap to prevent drying and then placed into an ammunition box. Once sealed, the ammunition box provided a gas-tight means for maintaining an anaerobic environment during transport back to Oklahoma. The other half of the core sections was removed from the anaerobic chamber and used for geophysical measurements at Old Dominion University.

Geophysical Measurements

Sediment samples were removed every 10 cm from the Old Dominion core halves for a variety of geophysical assays. Any discreet interval of approximately 10 cm or more that consisted of fine grained organic matter was labeled peat. The percentages of sand, clay, silt, and gravel were determined by the individual size distributions of the sediment particles. These grain sizes were determined by a standard dry mechanical sieve procedure to a minimum sieve size of 50 µm. Sediments with grain sizes between 50 and 1 µm were analyzed using an Elzone particle size analyzer, as

described previously (20). Sediment porosity and hydraulic conductivity were calculated using standard methods (7). Sediment permeability was determined using the falling head method (7).

SRB Activity Measurements and Bacterial Enumeration

A radioimaging technique was used to visualize and quantify the metabolic activity of sulfate reducing bacteria at a small scale (32). The technique is a modification of the silver foil assay used by other researchers to visualize SRB activity (17). Within an anaerobic chamber, a one cm thick sediment slice was cut from the center of a core section using a sterile knife. All samples were taken from the center of the core sections to minimize the impact of any contaminants associated with the core liner. The sediment slice was placed onto a rigid plastic sheet for support. At this time, a small amount (0.1 g) of sediment was aseptically removed from various locations on the sediment slice to use in a most probable number (MPN) enumeration analysis. The medium used for enumeration was (API)-RST (28) and a standard 3 tube MPN protocol was employed (15). Sterile filter paper (Fisher P8) was placed over the entire top surface of the sediment slice. The filter paper was used to insure that radioactive sulfate was evenly distributed over the entire top surface of the core slice. Radioactive sulfate (100 µCi $S^{35}O_4$ in 5 ml H_2O) was slowly added dropwise onto the filter paper. The sediment slice was then placed into an ammunition box and taken out of the anaerobic chamber. The atmosphere within the ammunition box was changed to 80% N_2/20% CO_2. The sediment slices were allowed to incubate with the radioactive

sulfate for 48 hrs inside of the ammunition box before being taken back into the anaerobic chamber. After applying new filter paper, 10mM molybdate was dripped onto the sediment slice surface to halt SRB metabolism and to wash away any unreacted $S^{35}O_4$. During the incubation period, metabolically active SRB will reduce the $S^{35}O_4$ to H_2S^{35}. The newly formed sulfide will react with ferrous iron present in the sediment at the spot it was created. The sediment slice was subsequently placed into an Instant Imager autoradiographer (Packard Instrument Company, Meridan, CT) and imaged for 2 hours. The resulting image showed the locations and relative intensity of SRB metabolic activity, with red representing the highest levels of activity followed by yellow, then green, then blue. The image was analyzed using software supplied by the autoradiographer manufacturer. A matrix was placed over the images with each grid within the matrix measuring 8 x 8 mm. The software recorded the number of counts in each grid per time, providing a measure of the amount of radioactive sulfide present (in counts per minute), and subsequently the relative level of SRB activity at that particular location. A control was run to insure unreacted sulfate was adequately removed by the washing procedure. Sand sediment from the site was sterilized and put through the radioimaging procedure.. The sodium molybdate washing procedure removed 98% of the added radioactivity from the sterilized sediment, with the remaining 2% of the radioactivity being evenly distributed over the surface of the sediment. To insure ferrous iron was present throughout the tested sediments, actively metabolizing SRB (*Desulfovibrio desulfuricans* G20, 10^9 cells) were added to portions of each sediment type (sand, peat, and silt) that had been sterilized. A uniform distribution of radioactivity was

observed after the washing procedure in the areas where the bacteria were added on all three sediment types, with the coefficients of variation for these areas being 20.6% for the sand sediment, 15.2% for the peat sediment, and 12.9% for the silt sediment while the coefficient of variation for the entirety of core SO-ODU 4 was 155.3%. The areas where the SRB were not added had little radioactivity present. These results indicate that the heterogeneous distribution of radioactivity we observed in our live sediments were the result of SRB metabolic activity, not of an uneven distribution of ferrous iron in the sediments. In additional controls, sterile sediments showed sulfate reducing activity that was not statistically different from background levels. Also, selected areas in live sediment core segments with high levels of activity were analyzed using a sulfide extraction method (30). The results indicated that the radioactivity was in the form of sulfide minerals and not unreacted sulfate.

Nutrient and Cell Additions

To further investigate if SRB activity was limited by cell numbers or nutrients, experiments were done on homogenized sediment segments. Live sand or peat sediments were removed from core SO-ODU 4 and homogenized to disperse any microsites containing higher cell numbers or nutrient concentrations. In one group of experiments, the homogenized sediments were used to construct three small sediment slices. Either live or heat-killed SRB (*Desulfovibrio desulfuricans* G20) were added to portions of two of the sediment slices while no additions were made to the third slice. All sediment slices were then radioimaged. In the other group of experiments, the live

homogenized sediments were again used to construct three small sediment slices. To one of the slices lactate (1 mM) was added, the second sediment slice received sulfate (1 mM), while sterile water was added to the final slice to help differentiate the stimulatory effects of the added nutrients compared to sediment moisture.

Effects of Varying Pore Sizes on SRB Metabolism

To study the influence of grain size, and corresponding pore size, on SRB activity, a model porous system was constructed by using solid glass beads of varying diameters (Biospec Products, Inc., Bartlesville, OK). Glass chambers were constructed by fusing together the top ends of two serum tubes (26). All glass chambers used were 10 cm long and triplicate chambers were used for each bead size in addition to a heat-killed control. The chambers were completely filled with glass beads and SRB media inside an anaerobic chamber, sealed with serum stoppers, removed from the anaerobic chamber, and autoclaved. The sulfate-reducing bacterium, *Desulfovibrio desulfuricans* G20, was injected into one end of the chamber and allowed to incubate at 37^{o} C under static conditions. Over the next 96 hours, liquid samples were removed from the end of the core chamber opposite of where the bacterium was injected. These liquid samples were analyzed for the concentrations of acetate and lactate by HPLC. The glass beads used had diameters of 0.1, 0.5, 1, and 3.5 mm which corresponds to pore sizes of 8.5, 42.5, 85, and 300 μm respectively (16, 27). The pore volume of the chambers for all bead sizes was 5.0 ml with a porosity of 57.5%.

Results

Quantification of SRB Metabolism

A radioimage of a core section is shown in Figure 1. This core section was taken from a depth of 4.08 to 4.34 m below ground surface (BGS) and is composed entirely of sand and is thus representative of a majority of the sediment sample taken at this site. Both the location and the intensity of sulfate-reducing activity varied widely over millimeter distances within this particular core section and in virtually all other core sections analyzed (data not shown). SRB activity was relatively low at both the top and bottom of this particular core section, with an area of 10-fold higher activity located in the central portion of the core section. MPN of SRB ranged from 1.5×10^3 per g of sediment to 7.5×10^6 per g sediment in the core section, with the highest numbers located near the region with the highest SRB activity.

Figure 1.

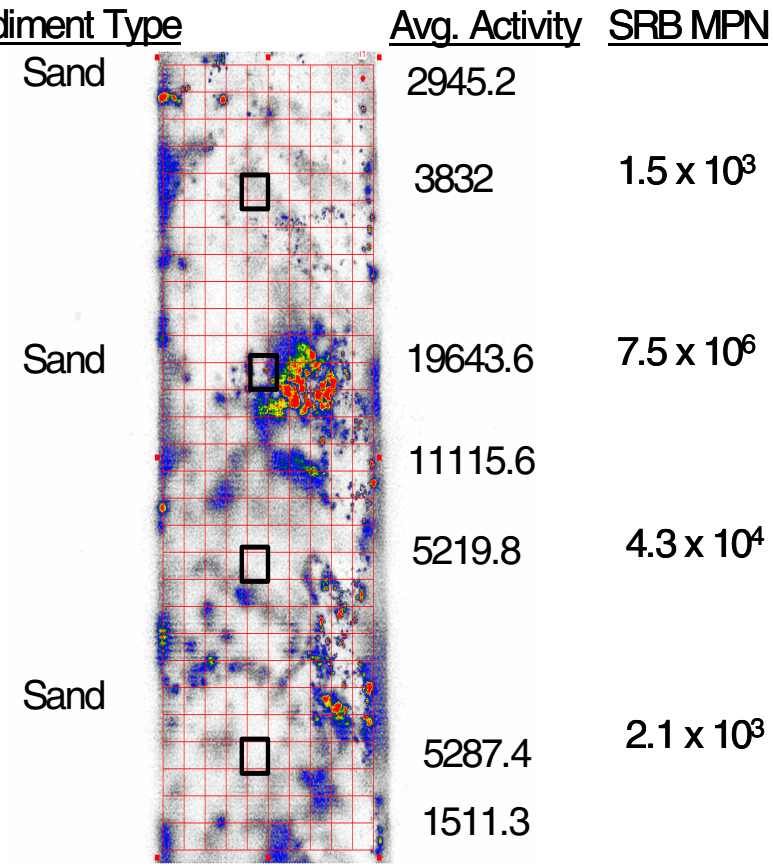

Figure 1. Radioimage of the core section of SO-ODU 4 from 4.08 to 4.34 m below ground surface. The areas in red represent the highest activity, followed by yellow, green, and blue respectively. Average activity measurements are the mean of the measurements for each grid at a particular depth (i.e. across the width of a core section) and are in counts per minute. The black boxes represent the area were sediment was removed for MPN analysis.

Figure 2 shows a region of the core that contains a transition zone from a relatively porous, large-grained, sand sediment to a smaller-grained, peat sediment. In both sediment types, the intensity of SRB metabolic activity is heterogeneously distributed. The metabolic activity varied by two orders of magnitude within this core section, with most areas on the lower portion of the core section having little to no activity while a few regions had high levels of activity. The variation in metabolic activity seemed to be independent of sediment type, as both the sand and peat sediments contain areas of negligible activity as well as a few regions of high activity. It was somewhat surprising to detect high levels of metabolism in peat as many studies have reported little to no bacterial metabolism in fine grained sediments due to the restrictive pore throat diameter (9, 12, 23). No statistically significant change in SRB metabolism was observed near the transition zone between the sand and peat sediments.

Figure 2.

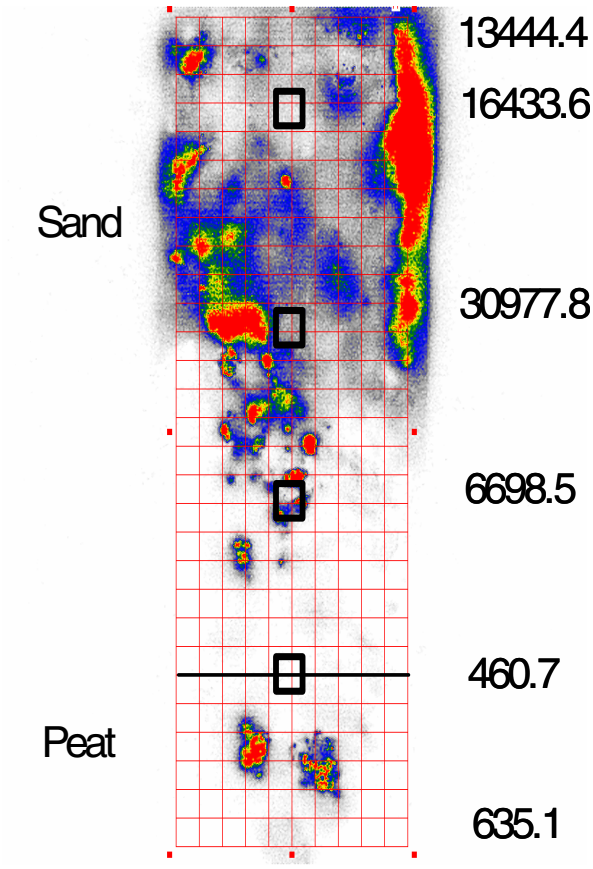

Figure 2. Radioimage of the core section of SO-ODU 3 from 5.25 to 5.48 m below ground surface. The areas in red represent the highest activity, followed by yellow, green, and blue respectively. Average activity measurements are the mean of the measurements for each grid at a particular depth (i.e. across the width of a core section) and are in counts per minute. The black boxes represent the area were sediment was removed for MPN analysis. BDL = Below Detection Limit

Relationship of SRB Activity to Bacterial Abundance and Sediment Properties

The core sections in Figures 1 and 2 are two of more than thirty that were analyzed. In order to better visualize the distribution of sulfate-reducing activity at the site, we plotted the distribution of SRB activity from each grid for the entire length of core SO-ODU4, a distance of more than 7.5 m (Figure 3). This histogram revealed a non-normal distribution of sulfate reducing metabolic activity where the vast majority of all grids showed little to no metabolic activity. The first interval, which represents these grids, actually goes up to a frequency of almost 6000 but had to be cropped so that the "hot spots" (areas with much higher metabolic activity) on the far right would be visible. Using the frequency distribution of activity we were able to further quantify the heterogeneity seen at the site. Half of the more than the 8000 grids examined had low metabolic activity levels of 3500 cpm or less. Approximately 17% of the grids had almost no detectable metabolic activity, in the range of 100 – 1000 cpm. The areas with high metabolic activities of greater that 50, 000 cpm consisted of just over 1% of all the grids tested. The main question is why these few small regions have up to three orders of magnitude higher activity than other regions of the same core.

Figure 3.

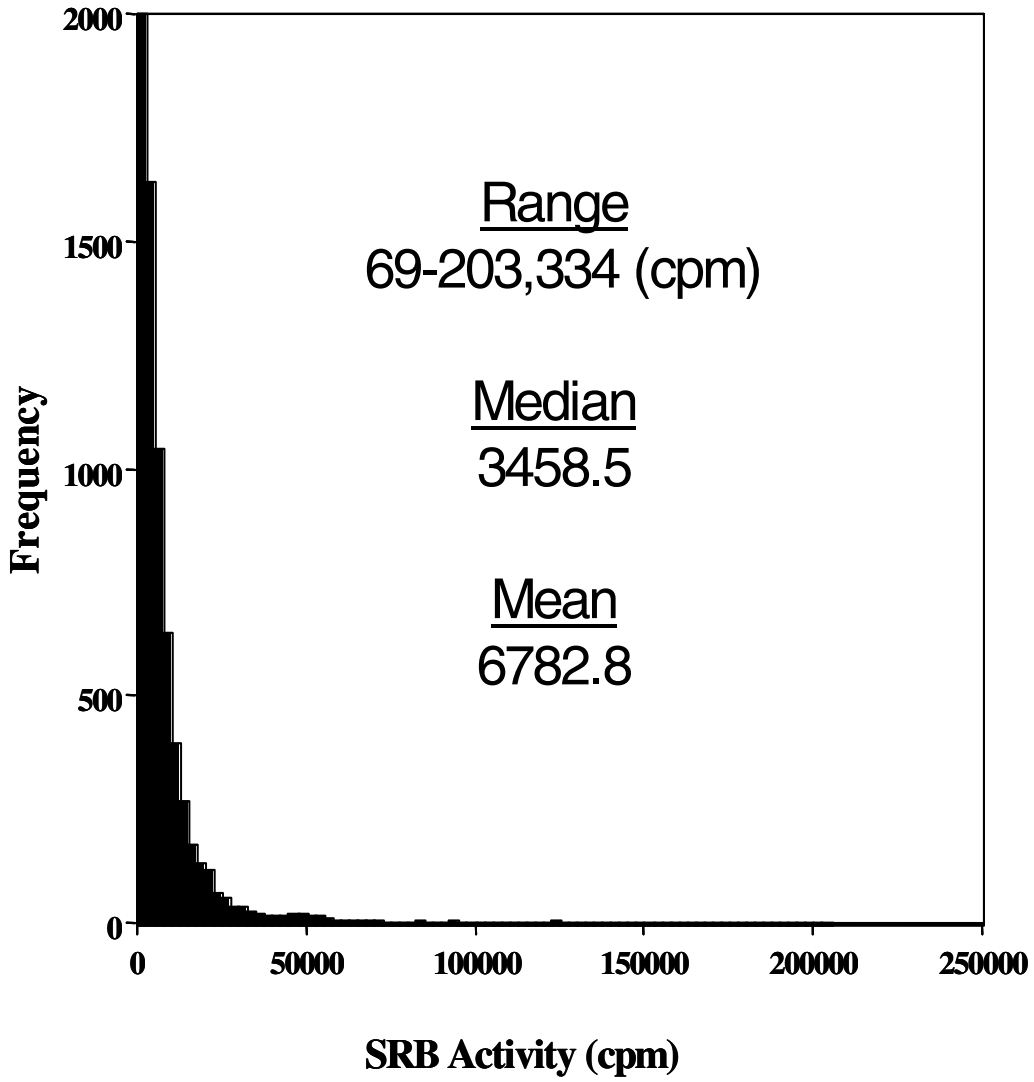

Figure 3. Distribution of metabolic activity in core SO-ODU 4. The range, mean, and median of metabolic activity are in counts per minute (cpm).

One possibility is that a particular sediment type strongly favors a heterogeneous distribution of activity. To test this possibility, we plotted the distribution of SRB metabolic activity measurements for each type of sediment present, sand, peat, and silt (Figure 4). Sand was the most common sediment type at this site and showed the widest range of SRB activity. The peat and silt sediments at this site were similar in many physical properties and the mean and median levels of SRB activity in the two sediments were almost identical. However, Kolmogorov-Smirnov tests revealed that distributions of activity were not the same between any of the sediment types (sand vs. silt – D = 0.600 and P = 0.000, peat vs. silt – D = 0.218 and P = 0.000, sand vs. peat – D = 0.530 and P = 0.000). Overall, the histograms show a skewed distribution for the three sediment types, with all three displaying a heterogeneous distribution of SRB activity. Within sand, peat, and silt, the vast majority of grids had little to no metabolic activity, while only a few had higher levels of activity often by several orders of magnitude.

Figure 4.

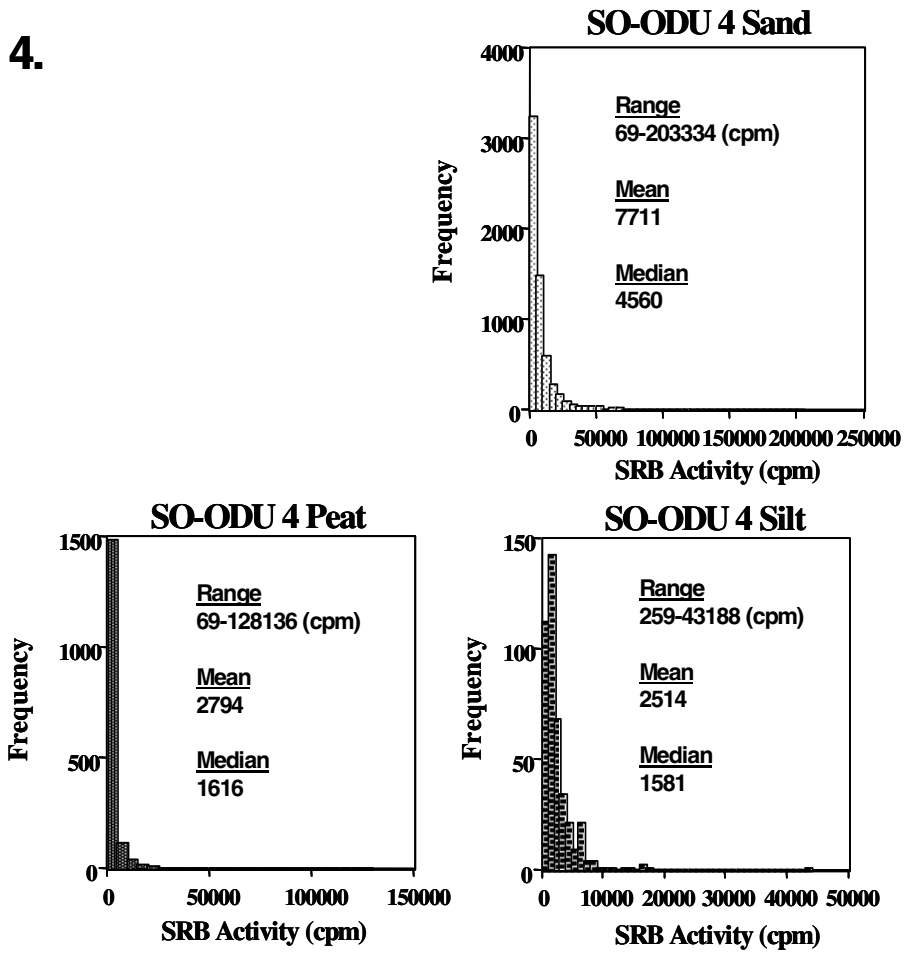

Figure 4. Distribution of metabolic activity by sediment types. The range, mean, and median of metabolic activity for each sediment type are in counts per minute (cpm).

Multiple regression analysis was performed to determine whether the variation in SRB activity could be explained by the variation in any of the measured properties in this shallow aquifer. The correlation coefficients for different sedimentological parameters and bacterial abundance against metabolic activity were determined (Table 1). The results indicate that variability in SRB metabolic activity could not be explained by variations in depth, bacterial number, grain size, permeability, or hydraulic conductivity at this site. For this aquifer, SRB metabolic activity did vary significantly with porosity (Table 1), which is the proportion of the non-solid volume to the total volume of the material.

Table 1.

Parameter	Value	Metabolic Activity	Mean	Median	Range
Metabolic Activity	r p #	1.0 N/A 87	6782.8 (cpm)	3458.5 (cpm)	69-203,334 (cpm)
Depth	r p #	0.03 0.76 87	N/A	N/A	N/A
MPN	r p #	0.16 0.15 87	2.2×10^6 cells/g sediment	1.5×10^3 cells/g sediment	BDL – 9.3×10^7 cells/g sediment
Grain Size	r p #	-0.03 0.81 87	0.27 (mm)	0.31 (mm)	0.05 – 0.65 (mm)
Porosity	r p #	0.48 <0.0001 87	28.6 %	35.1 %	BDL – 47.8 %
Permeability	r p #	-0.13 0.50 30	0.05 (Darcys)	0.02 (Darcys)	BDL – 0.17 (Darcys)
Hydraulic Conductivity	r p #	0.02 0.85 87	5.0×10^{-5} (cm/s)	2.2×10^{-5} (cm/s)	BDL – 1.8×10^{-4} (cm/s)

Table 1. Correlations between metabolic activity and other variables tested with the means, medians, and ranges of the variables listed. CPM = Counts per Minute, N/A = Not applicable, BDL = Below Detection Limit

Effects of Nutrients and Bacterial Cells

The results from the addition of live or killed sulfate-reducing bacteria and nutrients to homogenized sand and peat sediments are shown in Table 2. Student's t-tests were run to assess statistical significance. For the sand sediment, the addition of heatkilled or live SRB had no significant affect on the sulfate-reducing activity when compared to the sediment slice where no additions were made. Likewise, neither lactate nor sulfate significantly increased activity compared to a water addition. Additions to the peat sediment, however, did cause significant changes in metabolic activity. Both heat-killed and live SRB added to peat resulted in significantly higher levels of sulfate-reducing activity than was seen in the sediment slice with no additions. Lactate also stimulated SRB activity compared to a peat sediment slice with water as the only addition. Sulfate, however, did not produce a significant change in the level of SRB activity.

Table 2.

Sediment	Condition	Median Activity (cpm)
Sand	+ Heat Killed Cells	6422
	+ Live Cells	7135
	No Additions	7091
	+ Lactate	8988
	+ Sulfate	8245
	+ Water	7969
Peat	+ Heat Killed Cells	15119
	+ Live Cells	13139
	No Additions	6315
	+ Lactate	11988
	+ Sulfate	7014
	+ Water	6733

Table 2. Effect on SRB metabolic activity of the addition of bacterial cells or nutrients to homogenized sediment.

The distributions of activity for the live homogenized sand and peat sediment slices were more normally distributed than the distributions from a typical core section (SO-ODU 4 1.98-2.24 m BGS) or core SO-ODU 4 as a whole as measured by the Kolmogorov-Smirnov test. Histograms of the distributions are shown in Figure 5. The majority of grids for both sediment types were towards the middle of the activity range. Neither homogenized sediment showed large numbers of grids with very high or very low levels of SRB activity, with activity measurements varying less than 10 fold. This is in contrast to both the example core section given in Figure 5 and the entirety of core SO-ODU 4, which both displayed highly skewed activity distributions. Both distributions had the majority of their grids in areas representing low amounts of SRB activity. Both distributions also had a few areas with much higher levels of activity. These outliers caused wide ranges of metabolic activity, over 20 fold in the case of the example core segment and almost 3000 fold in the case of core SO-ODU 4 as a whole.

Figure 5.

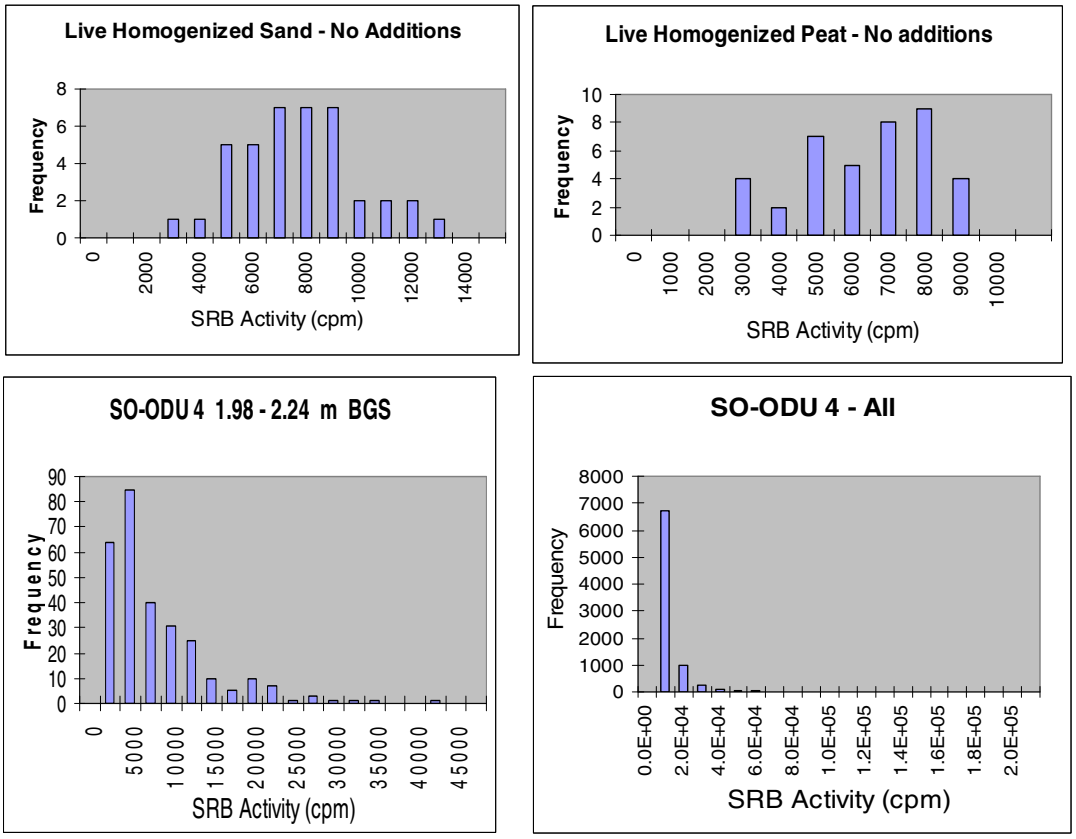

Figure 5. Frequency distributions of SRB activity for live, homogenized sand, live homogenized peat, a representative section of core SO-ODU 4, and the entirety of core SO-ODU 4. All activity measurements are in counts per minute (cpm). Depth is meters below ground surface (m BGS).

Influence of Pore Size on SRB Metabolism

Since SRB activity varied with porosity, we hypothesized that the small pore sizes associated with sediments with low porosity restricted the space needed by SRB to grow and their access to nutrients. To further investigate the role of pore size on SRB metabolism, the rates of lactate metabolism and acetate production by a sulfate-reducer in a porous medium with varying pore sizes were measured (Table 3). By testing a single species of a sulfate-reducing bacterium in a controlled laboratory setting, many of the uncontrolled variables found in the field were eliminated and the role of pore diameter on metabolic activity could be more directly studied. The same inoculum size was used for each condition (4.7×10^9 cells). The pore sizes tested ranged from a relatively small pore diameter of 8.5 µm to a much larger opening of 300 µm. This gave a range of pore sizes that can be found in silt and sand sediments (18). The rates for both measures of SRB activity more than doubled over the range of pore sizes tested.

Table 3.

Bead Diameter (mm)	Pore Size (µm)	Lactate (mM/hr)	Acetate (mM/hr)
0.1	8.5	-0.215	0.1
0.5	42.5	-0.307	0.146
1	85	-0.373	0.184
3.5	300	-0.459	0.241

Table 3. Effect of varying pore throat diameter on rates of lactate degradation and acetate accumulation. Negative rates represent disappearance of a substrate while positive rates represent accumulation of a substrate.

Discussion

The radioimaging process we employed allowed us to visualize and quantify microbial metabolic activity at scales much smaller than typically studied in the subsurface. The results of our experiments show that SRB activity is heterogeneously distributed over distances ranging from millimeters to meters in the shallow subsurface at this site. Within undisturbed sand sediment, the addition of nutrients to sand sediments did not stimulate metabolic activity. This was the case regardless of whether the nutrient addition was in the form of cellular debris, an electron donor, or an electron acceptor (Table 2). The lack of nutrient limitation argues against variation in either the hydraulic conductivity or permeability of the sediment causing the variation we observed in SRB activity. This was consistent with multivariate analysis that showed no correlation between variation in metabolic activity and variation in sediment permeability or hydraulic conductivity (Table 1). Both of these sediment parameters are closely correlated with movement of nutrients in porous materials, with high permeability and hydraulic conductivity sediments having faster rates of nutrient diffusion (7). Our results argue that SRB within sand sediments were not nutrient limited, indicating that other factors were causing the observed heterogeneity.

A likely cause of the heterogeneous distribution of SRB activity in sand sediments was microsites of large pores within the sediment. Such differences in pore sizes are fairly common in sediments as pore diameters change over small distances within sediment types due to how the soil grains are shaped and arranged (29). Areas with larger pore

volumes would have allowed SRB to congregate and grow to higher numbers than in the more numerous areas that had smaller pore sizes. These few areas of higher pore volumes would have higher cell numbers and metabolic activity than regions with smaller pore sizes. This explanation accounts for the heterogeneous distribution observed for the SRB activity measurements in sand seen in Figures 1 and 2 and plotted in Figure 4. Further evidence for the hypothesis that variation in pore size caused the variation in SRB activity in sand sediment was seen in the frequency distribution of metabolic activity of homogenized sand shown in Figure 5. When the sand sediment was undisturbed and the large pore volume microsites were left intact, a heterogeneous distribution of SRB activity was seen in the sand sediment (Figure 4). However, when the sand was homogenized and the microsites were destroyed, a near normal distribution of activity was seen (Figure 5). If this hypothesis is true, it is unclear why SRB activity did not vary with culturable sulfate-reducers. Perhaps the sensitivity of our MPN methodology was not great enough to detect small changes in cell numbers or changes in cell numbers over small distances. Previous work has shown that less than an order of magnitude change in SRB numbers may cause differences of several fold in measured metabolic activity using the autoradiography method (32). Another possibility is that the MPN medium did not allow for cultivation of all the SRB present.

Variation in pore sizes was likely responsible for the variation seen in the SRB activity within the fine grained peat and silt sediments. These types of sediments are known to have smaller pore sizes than sand sediments (9). The small pores restricted microbial

activity in the manner described for the sand sediment, as seen by comparing the near normal activity distribution seen in the homogenized peat (Figure 5) with the heterogeneous activity distribution displayed for undisturbed peat and silt sediments (Figure 4). Additionally, the small pores within the peat and silt sediments at this site limited microbial access to nutrients. The permeability of the peat and silt sediments at this site was below our detection limit (data not shown) so it is not possible to determine if a correlation between activity and permeability exists. However, it is likely that the influx of nutrients is very slow within these sediments. When nutrients were added to homogenized peat sediment, whether in the form of live cells, killed cells, or lactate, metabolic activity was significantly stimulated (Table 2). This suggests that SRB residing in fine grained sediments at this site were limited for electron donors. The lack of stimulation by the addition of sulfate indicates they were not electron acceptor limited (Table 2). When pore sizes within a porous medium were varied, SRB living within chambers with the smallest pore diameter displayed metabolic rates less than half of those seen for the cells within the chambers with the largest diameters (Table 3). Taken together, these results argue that low activity levels seen in a majority of the grids for peat and silt sediments were caused not only by a lack of physical space for the bacteria to grow, but also by electron donor limitations caused by the small pore size of the fine grained sediments.

The relatively few areas of much higher activity found within peat and silt sediments at this site were likely the result of microsites within these sediments which had larger pore volumes than did most regions of these sediments. These microsites benefit the

bacteria not only by increasing the volume of water available for holding nutrients, but also by providing the SRB with a means of surviving long term nutrient limitation. SRB residing within small pores not only have less nutrient rich water available to them, but also have less physical room in which to grow. The sulfate-reducers in large pore volume microsites, however, have a larger volume of dissolved nutrients available as well as more room to divide after consuming the nutrients. This accumulation of bacterial cells allows continued metabolism by some SRB during nutrient limitation as they consume cellular debris of dead cells. This survival strategy works better in the microsites with larger pore volumes as more cells are initially present. SRB within fine grained sediments at this site seem adapted to the metabolism of cellular debris as their activity was significantly increased by the addition of dead bacteria (Table 2). Harris et al. (11) also showed that heat-killed cells could serve as an electron donor for sulfate reduction. Adding live bacteria also significantly increased SRB activity within peat, consistent with other studies that have shown that fine grained sediments are difficult to colonize and generally inhospitable to microbial growth and survival (8, 21, 24).

We have shown that SRB metabolic activity in the shallow subsurface at this site was heterogeneously distributed. The heterogeneity was not correlated with sediment type, depth, culturable bacterial numbers, sediment grain size, sediment permeability, or sediment hydraulic conductivity. The variation that we observed in the distribution of sulfate-reducing metabolism was correlated with variation in sediment porosity. We believe that the pore spaces within the sediments controlled where measurable

metabolic activity was located. In undisturbed sand sediments, most areas would have pore diameters too small to hold enough cells for high levels of metabolic activity. However, microsites with larger pores would allow for the growth of greater numbers of bacteria, which, in turn, would have higher levels of metabolic activity. The same phenomenon was seen when examining the fine grained peat and silt sediments from the site. Additionally, small pores within these sediment types seemed to limit the availability of nutrients to the SRB. Microsites with larger pore volumes allow for higher cell numbers, some of whose components can be subsequently utilized by viable SRB as nutrients to sustain metabolism.

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