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Microbial community structure and biogeochemistry of Miocene subsurface sediments: implications for long-term microbial survival

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Abstract

Thirty closely spaced cores were obtained from Miocene-aged fluvial, lacustrine and palaeosol subsurface sediments ranging in depth from 173 to 197 m at a site in south-central Washington to investigate the size and composition of the microbial community in relation to sediment geochemical and geophysical properties. Total phospholipid fatty acid (PLFA) analysis indicated that the greatest concentrations of microbial biomass were in low-permeability lacustrine sediments that also contained high concentrations of organic carbon. Community structure, based on lipid analyses and on *in situ* hybridization of bacterial cells with 16S RNA-directed DNA probes, also revealed the presence of metabolically active bacteria that respire sulphate and/or Fe(III) in the lacustrine sediments. Concentrations of pore water sulphate were low (4–8 mg/L) and HCl-extractable Fe was predominantly Fe(II) in the same samples where total biomass and organic carbon were highest. The low hydraulic conductivity (10^{-6} to $< 10^{-9}$ cm/s) of these sediments has likely contributed to the long term maintenance of both bacteria and organic carbon by limiting the supply of soluble electron acceptors for microbial respiration. These results suggest that the current subsurface microbial population was derived from organisms that were present during lake sedimentation ≈ 6–8 million years ago.

Keywords: 16S rRNA, Fe(III)-reducing, *in situ* hybridization, lipids, subsurface, sulphate-reducing

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Introduction

Chemoheterotrophic bacteria are now known to be common inhabitants of deep subterranean environments (Pedersen & Ekendahl 1990; Fredrickson *et al.* 1991; Haldeman & Amy 1993; Stevens *et al.* 1993), yet the origin of these bacteria remains unanswered 60 years after it was first questioned by Bastin (1926) who reported the presence of anaerobic bacteria in deep oil field waters. In aquifer sediments from the US Atlantic coastal plain, reported to be among the most oligotrophic of microbiologically active environments (Chapelle & Lovley 1990), the indigenous bacteria may be remnants of populations present at the time of deposition some 80 million years ago, they may have been transported with the groundwater over the past 10 000–50 000 years (Murphy *et al.* 1992) or they may be derived from a combination of the two.

Regardless of their origin, it is clear that micro-organisms in deep subsurface environments must be capable of surviving and functioning in an extremely resource-limited environment. However, there has been little effort to identify the source(s) of nutrients that support their *in situ* metabolism and growth. Most of the soluble nutrients carried with infiltrating water from the surface are removed by organisms residing in soil and near-surface environments before the water reaches the deep aquifers rendering much of the recharge to these aquifers oligotrophic.

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Hence, it is suspected that most of the nutrients utilized by bacteria in deep subsurface environments are obtained from adjacent rocks and sediments. An important source of electron donors for microbial respiration in deep Atlantic coastal plain aquifers is the diffusion of fermentation products from organic-rich confining layers (McMahon & Chapelle 1991) or possibly from the lignite that occurs within the sands and silts comprising some aquifers (Murphy *et al.* 1992). Hence, sedimentary organic carbon is inferred to be the principle electron donor in deep Atlantic Coastal Plain sediments.

There has been limited success (Balkwill 1989) in applying traditional microbiology techniques to characterize micro-organisms present in deep subsurface sediments. Typically, the population size of bacteria in subsurface (Kieft *et al.* 1995) or other environments, as estimated by direct microscopic counts or by total microbial lipids, is several orders of magnitude higher than the number of bacteria counted using plate count or most probable number estimations. These discrepancies make it difficult to establish the abundance and composition of subsurface microbial populations. Recently, it was demonstrated that

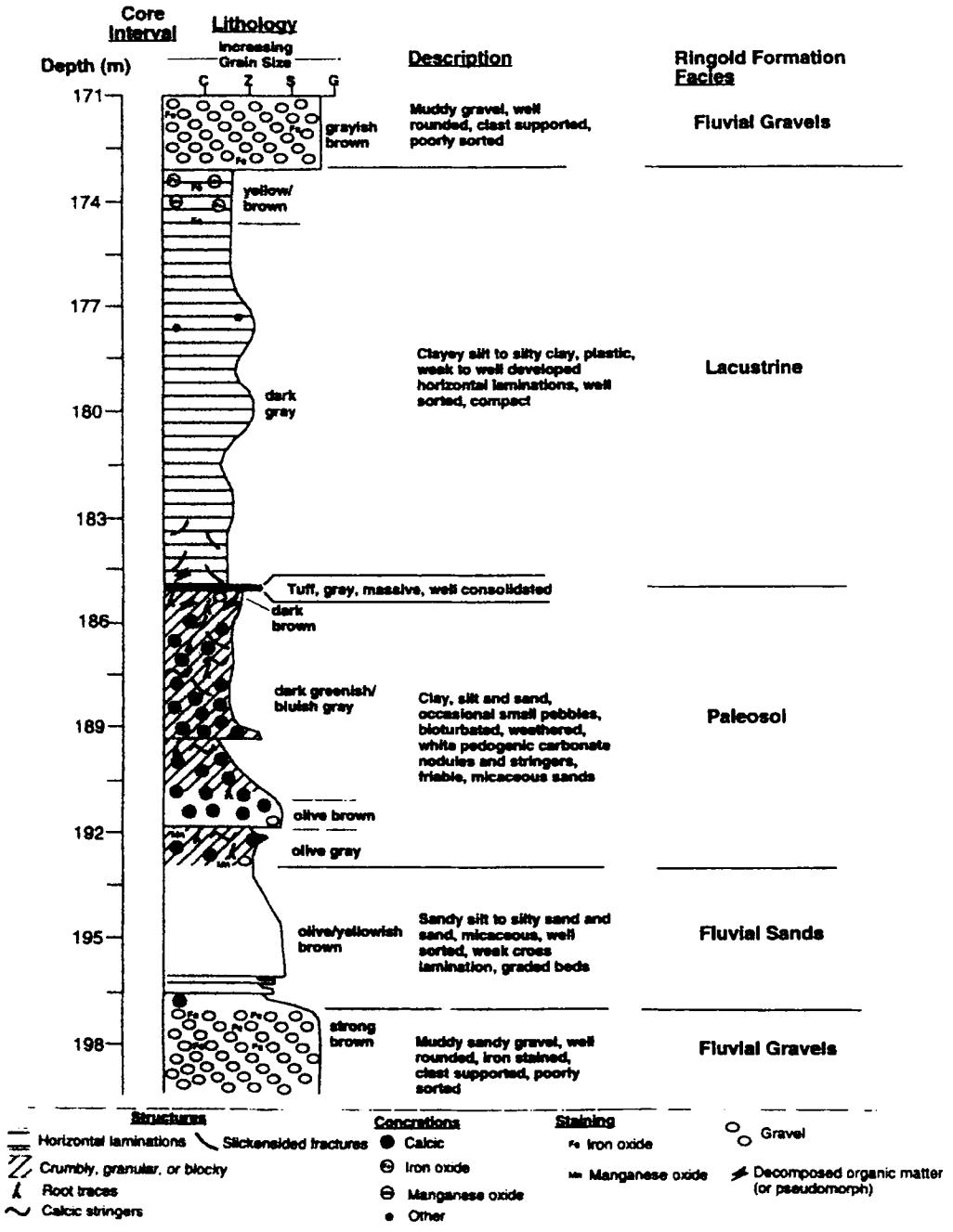

Fig. 1 Stratigraphy of subsurface lacustrine, palaeosol, and fluvial sediments from which subsurface samples were obtained, Hanford Site, south-central Washington, USA.

RNA could be extracted from low-biomass subsurface sediments as one approach for characterizing microbial communities that is independent of culturing (Ogram *et al.* 1995). In the study reported herein, two different molecular-based methods that are also independent of culturing were used to investigate the size and composition of the microbial community in deep subsurface sediments.

The relationships between the microbiology, geochemistry, and hydrology of deeply buried subsurface sediments, obtained from a site in south-central Washington, were investigated to probe the processes and properties that provide for the continued existence of micro-organisms in the deep subsurface.

Materials and methods

Site description and sample collection

The sampling site was located on the US Department of Energy's Hanford Site in south-central Washington; samples were obtained from an uncontaminated part of the aquifer that is located hydrologically up-gradient from areas affected by past site operations (Fredrickson *et al.* 1993). Thirty closely spaced cores were collected from a borehole penetrating unconsolidated sediments of the Ringold Formation. Lithofacies that were sampled included lacustrine, palaeosol, and fluvial strata from 173 to 197 m in depth (Fig. 1) (Kieft *et al.* 1995).

All subsurface samples from the Hanford Site were cored by cable-tool percussion drilling using a split-spoon core barrel containing a sterile Lexan® liner. Cable-tool drilling was the preferred sampling methodology because it does not require the use of circulating drilling fluids that can potentially contaminate samples. Procedures used to disinfect sampling tools and processing of samples were adapted from those previously described (Phelps *et al.* 1989; Colwell *et al.* 1992; Russell *et al.* 1992), have been described in detail elsewhere (Kieft *et al.* 1995). Following coring, the Lexan® liners containing the cores were immediately removed from the split-spoon core barrel and their exposed ends were capped. Cores were immediately transferred to an argon-filled glove-bag housed in an on-site laboratory trailer. Using sterile tools, the outer, potentially contaminated sediment was pared and material from the centre of the cores was retained for microbiological, chemical, and physical analyses. Intact core samples adjacent to the pared material were also retained for measurement of hydraulic properties

Lipid analyses

Samples for lipid analyses were frozen immediately on site at −20 °C and shipped overnight to the University of Tennessee on dry ice. Approximate 75-g quantities of frozen sediments were extracted and fractionated into neutral, glyco- and polar lipids using column chromatography with silicic acid (Tunlid *et al.* 1989). The polar lipids were then treated in a mild alkaline system containing methanol to transesterify the phospholipid fatty acids into methyl esters (PLFAME) (Rilfors *et al.* 1978). The PLFAME were then further separated and quantified by capillary gas chromatography/mass spectrometry.

In situ *hybridization*

One gram of sediment from each core sample was immediately placed in 1 mL of 3.7% formaldehyde in phosphate buffered saline (PBS, pH 7.4, Sigma Chemical, St. Louis, MO) and shipped overnight to Rennselaer Polytechnic Institute. Fixed samples were sonicated for two minutes and vortexed for one minute to aid in dislodging cells from particle surfaces. A 1-mL aliquot was removed immediately and washed twice with filter-sterilized PBS. Samples were resuspended in filter-sterilized 0.025% gelatin and 15 μL of sample was spotted into each of 10 wells on slides that had been baked at 94 °C for 4 h. After being spotted, samples were air-dried on slides and stored in a desiccator at room temperature until hybridized. Samples were then subjected to *in situ* hybridization according to established procedures (Braun-Howland *et al.* 1992) using a mixture of two 16S RNA-targeted DNA probes conjugated to rhodamine-x. The two probes included one targeting bacteria in the delta subdivision of the proteobacteria (Williams *et al.* 1994) and one targeting the sulphate-reducing bacteria (Amann *et al.* 1990). Cells were also stained with coumarin to estimate the total number of cells. Only cells fluorescing when viewed separately under filters for both coumarin and rhodamine were counted. Following *in situ* hybridizations, 96 fields were examined for each sample.

Geochemical and physical analyses

Core subsamples intended for geochemical analysis were placed in sterile, acid-washed canning jars which were then filled with argon and sealed before being transported to Pacific Northwest Laboratory for analysis. Total organic carbon was analysed by Huffman Laboratories, Golden, CO. Pore-water was extracted by centrifugation and the concentration of sulphate in the pore water was determined by ion chromatography (Dionex, Sunnyvale, CA). Sediments were extracted with 0.5-N HCl and Fe^{2+} and Fe^{3+} in the extracts were determined by ion chromatography.

Hydraulic conductivities as well as air permeabilities were measured on intact core segments by Core Petrophysics, Inc. (Houston, TX). Intact cores used for analyses of hydraulic properties were adjacent to core seg-

ments that were processed for microbiological and geochemical analyses.

Mineralizable organic carbon

To estimate the fraction of total organic carbon that was susceptible to microbial metabolism, select lacustrine sediment samples were subjected to a mineralizable organic carbon assay (Burford & Bremner 1975). For this assay, 5 g of sediment was placed into 125-mL serum bottles. Five millilitres of either deionized water or a mineral nutrients solution containing 20 mM each K_2HPO_4 and KH_2PO_4 and 7.6-mM $(NH_4)_2SO_4$ was added to the bottles. The headspace was flushed with filtered (0.2-μm) air and the vials were capped with butyl rubber stoppers and crimp-sealed. Bottles were incubated at 22 °C for 84 d, at which time the concentration of CO_2 in the headspace was measured by gas chromatography. Controls containing 0.37-mM $HgCl_2$ to inhibit biological activity were included. Poisoned controls were typically 60–70% or less of those in the noninhibited samples; thus the CO_2 that accumulated in the headspace was attributed to biological activity. Each value is an average of triplicate samples. The CO_2 evolved from the Hg-poisoned samples could have been due to either dissolution of carbonate or failure of the Hg to completely inhibit microbial activity during the 84-day incubation.

Results and discussion

Total microbial biomass of individual core samples was determined by measuring the concentration of total phospholipid fatty acids (PLFA) (Tunlid & White 1992). Concentrations of total PLFA were highest in the lacustrine sediments (Fig. 2a). Using a conversion factor of 5×10^5 cells pmol^{-1} for biomass in subsurface sediments (Tunlid & White 1992), the concentration of cells in the lacustrine sediments ranged from 2×10^5 g^{-1} to 2×10^7 g^{-1}. The concentration of total PLFA declined through the upper section of the palaeosol and was at or below detection throughout the remainder of the profile.

The structures of the lipids that were directly extracted from the sediments were used as indicators of microbial community composition. Molar percentages of terminally branched saturated lipids, found in Gram-positive and Gram-negative bacteria, were elevated in the lacustrine sediments (Fig. 2b) as compared to adjacent lithologies. The presence of sulphate-reducing bacteria (SRB) can be inferred from the detection of both iso- and anteiso- (terminally) branched saturated lipids, with the iso- configuration being the more abundant one. Sulphate-reducing bacteria such as *Desulfovibrio* are known to have a greater proportion of iso- to anteiso- terminally branched saturate lipids in their membranes (Dowling *et al.* 1986; Edlund *et al.* 1986). In contrast, many Gram-positive bacteria (e.g. *Bacillus, Arthrobacter*) exhibit greater abundances of the anteiso- configuration for both 15 and 17 carbon chain lengths. In the lacustrine sediments, increases in the molar percentages of all terminally branched saturates were observed. For both the 15 (not shown) and 17 (Fig. 2c) carbon fatty acids, the iso- configuration was the more abundant one detected. While few Gram-negative bacteria, other than SRB, contain terminally branched lipids, SRB and most other Gram-negative bacteria contain monounsaturated PLFA in their membranes. Monounsaturated PLFA were detected throughout the lacustrine strata (Fig. 2b).

Evidence confirming the presence of active bacterial cells in the subsurface sediments was obtained by using 16S ribosomal RNA-targeted oligonucleotide probes spe-

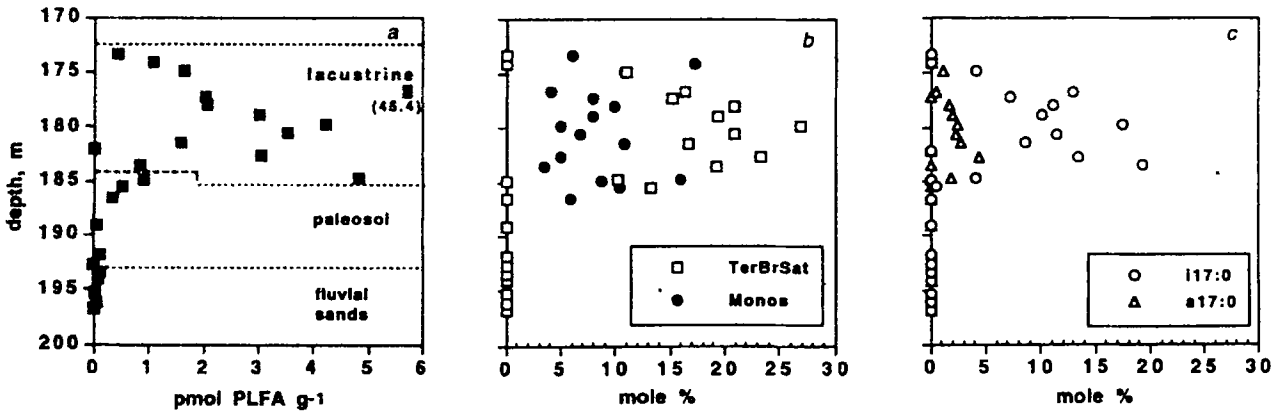

Fig. 2 (a) Total PLFA normalized to gram weight extracted in core samples, (b) composition by major structural classes including monounsaturated (Monos) and terminally branched saturated (TerBrSat) lipids, (c) and mole percentage of iso- and anteiso- 17 carbon fatty acids.

cific for SRB and for proteobacteria in the delta subdivision, of which the SRB are a subset. The dissimilatory Fe(III)-reducing bacterium (DIRB), *Geobacter metallorereducens*, is also in the delta proteobacteria (Lovley *et al.* 1993) and, if present, probably would have been detected by the delta group probe. The oligonucleotides targeting these groups of bacteria were conjugated to rhodamine and used to probe whole bacterial cells (Braun-Howland *et al.* 1992) associated with the core samples. Cells fluorescing after hybridization with the mixture of oligonucleotide probes were detected in the samples from 173.4, 176.7 (Fig. 3), 179.8, 184.7, and 184.9 m, most of which were derived from the lacustrine sediments; the 184.9-m sample came from near the interface between the lacustrine and palaeosol strata. Because the target site for the probes is rRNA, cells detected by epifluorescence microscopy contain a relatively high ribosome content and thus were metabolically active (DeLong *et al.* 1989). These results confirm the presence of SRB or closely related bacteria such as DIRB in the lacustrine sediments. In addition, SRB were cultured from samples from 181.4, 182.1, 182.7, and 183.5 m and DIRB were cultured from all 11 samples obtained from the 176.7–184.7 m interval (T. Stevens and J. Fredrickson, unpublished data).

Sediments were analysed for total organic carbon (TOC), carbonate in C, pore water sulphate, and HCl-extractable Fe. These geochemical components were selected as those likely to influence or be indicative of microbial activity. Concentrations of TOC were highest in the lacustrine sediments, except for the upper two meters of the sediments, which were almost devoid of organic carbon (Fig. 4a). In several of the core samples, TOC exceeded 1% w/w, and there was a general trend of decreasing TOC from ≈ 177 m to the thin volcanic tuff layer at 184.8 m that separates the lacustrine sediments from the palaeosol (Fig. 1). Some of the lacustrine sediment samples also had elevated concentrations of total inorganic (carbonate) carbon. Given the presence of both metabolically active bacteria and high TOC, it is likely that the source of the carbonate in these sediments was microbial respiration.

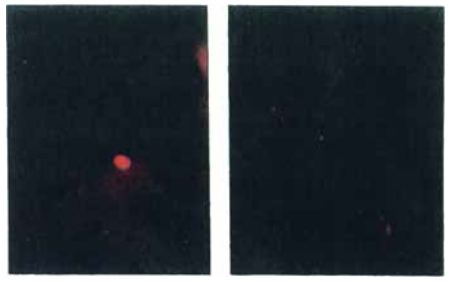

Fig. 3 Photomicrographs of bacteria from the 176.7 m lacustrine core sample hybridized with a mixture of 16S RNA-targeted oligonucleotide probes specific for SRB and for delta proteobacteria conjugated with rhodamine-x.

The presence of SRB and DIRB suggested the potential for sulphate- and Fe(III)-dominated respiration within this stratum. Coinciding with the higher TOC concentrations were low pore-water sulphate concentrations in the middle section of the lacustrine strata (Fig. 4b). Pore-water sulphate concentrations were higher near the lacustrine-tuff and tuff-palaeosol boundaries and in the upper 4 m of the palaeosol than within the lacustrine sediments. Sulphate was also higher in groundwater sampled from the fluvial gravels (49 mg/L) that overlie the lacustrine strata. O_2, at a concentration of 4 mg/L, was also present in groundwater associated with the fluvial gravels. Pyrite, identified by X-ray diffraction of selected subsamples, was observed in a core sample from 177.2 m, providing additional evi-

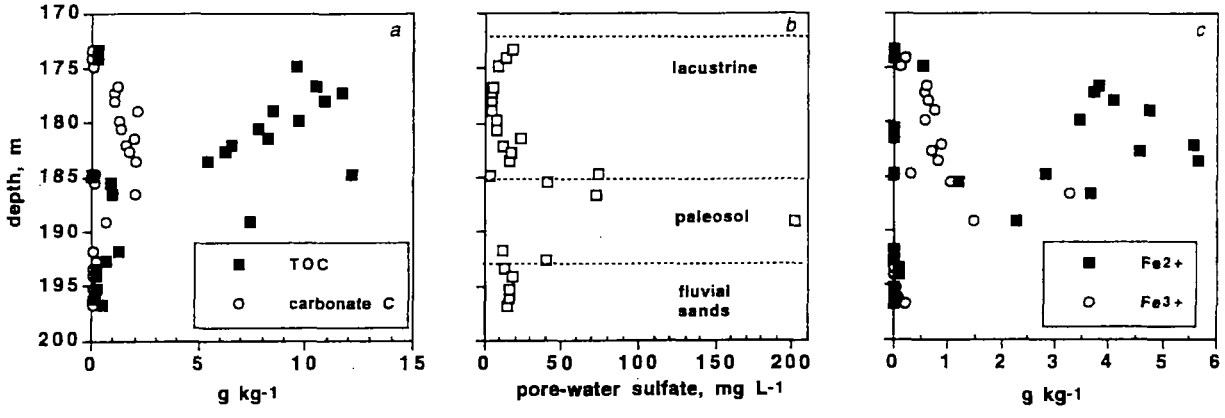

Fig. 4 (a) Total organic carbon and carbonate carbon; (b)pore-water sulphate concentration; and (c) HCl-extractable Fe of subsurface core samples.

dence for biogenic sulphide production in the lacustrine sediments at some point in time.

A number of different bacteria can couple the oxidation of organic matter or H_2 to the reduction of Fe(III) in sediments (Lovley & Phillips 1988; Myers & Nealson 1988; Caccavo *et al.* 1992). In addition, it has been shown that some SRB can reduce Fe(III) and contribute to the production of siderite ($FeCO_3$) in aquatic sediments (Coleman *et al.* 1993). Total iron extracted using 0.5-N HCl, which extracts Fe(II) from siderite, contained some Fe(III) but was predominantly Fe(II) (Fig. 4c). Fe(III), being poorly soluble at the neutral to slightly alkaline pH of the lacustrine sediments (J. McKinley, unpublished data), may limit microbial respiration even when significant quantities are present because it is less available than other more soluble terminal electron acceptors. However, the predominance of Fe(II) in the HCl-extractable fraction is consistent with microbial Fe reduction in the lacustrine sediments. In sedimentary microenvironments, the persistence of organic carbon and bacteria may be explained by physical separation of organic C, Fe(III), and bacteria. This is particularly true for low-permeability sediments that can restrict the movement of bacteria.

The hydraulic conductivities of intact core segments, and therefore their permeabilities, were generally low, ranging from 10^{-6} to $< 10^{-9}$ cm/s (Fig. 5). The zones of lowest hydraulic conductivity, in fact below that which could be measured ($< 10^{-9}$ cm/s), were encountered near the top of the lacustrine sediment and at the bottom of the palaeosol. It is between these regions of low hydraulic conductivity that the highest concentrations of microbial biomass and organic carbon were found. The region of relatively high hydraulic conductivity at 179–180 m in the lacustrine is also the zone where the sulphate concentration was the lowest. In those lacustrine core samples with high concentrations of TOC and PLFA, bounded at the top and at the bottom of the palaeosol by zones of very low hydraulic conductivity, the transport of electron acceptors such as sulphate or O_2 from the more permeable to the less permeable zones is likely to be dominated by diffusion. Thus, microbial metabolism in the lacustrine sediments containing the highest concentrations of organic carbon would be controlled by the diffusion of electron acceptors from adjacent strata. Kieft *et al.* (1995) found relatively high rates of microbial respiration in these same lacustrine sediments when incubated aerobically, suggesting that O_2 was limiting *in situ* metabolism. In addition to limiting the transport of solutes, the low permeability of these sediments can also limit bacterial movement. Jenneman *et al.* 1985) in studies with vegetative bacterial cells found that the penetration of cells into nutrient-saturated sandstone cores declined rapidly below 100 mD. Assuming that 1 D is equivalent to 10^{-3} cm/s (Freeze & Cherry 1979), the permeabilities of the cores in this study ranged from 1 mD to $< 10^{-3}$ mD. These low permeabilities suggest bacterial movement would be severely limited in the sediments studied.

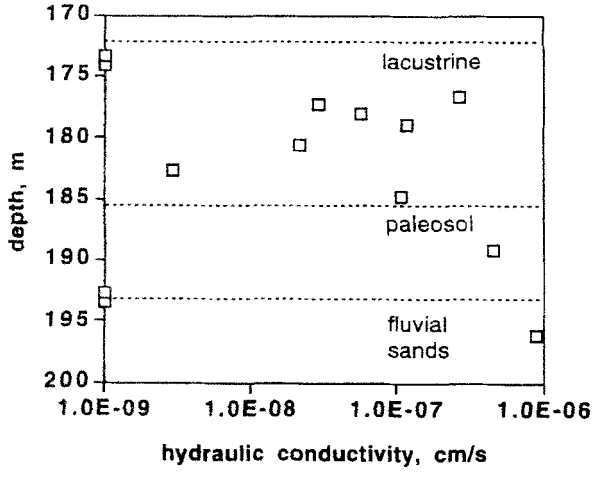

Fig. 5 Hydraulic conductivities of intact core segments from select core intervals.

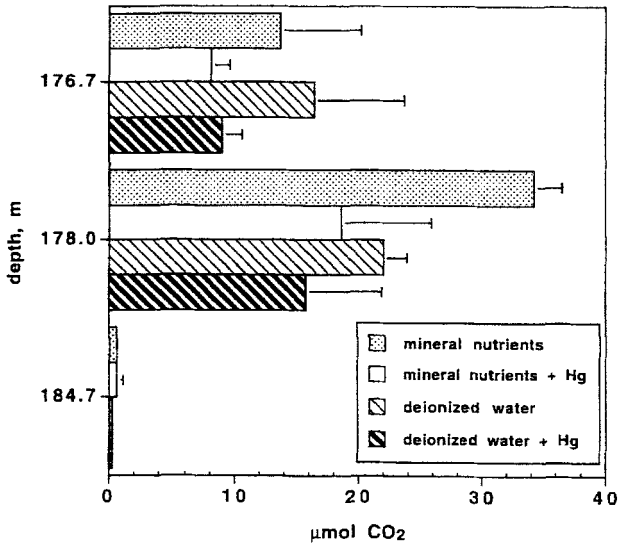

Fig. 6 Total CO_2 respired from selected lacustrine sediments. The TOC concentrations were 10.5, 10.9, and 12.2 g/kg C for samples from 176.7, 178.0 and 184.7 m, respectively.

To assess whether the organic carbon in the lacustrine sediments was bioavailable, experiments were conducted to determine the amount of organic carbon that could be respired aerobically using sediment from three samples. In core samples from 176.7 and 178.0 m, as much as 0.74% of the total organic carbon was respired to CO_2 (Fig. 6). In

comparison, the amount of mineralizable organic carbon in surface soils can range from 0.4% to 1.3% (Burford & Bremner 1975). The addition of mineral nutrients stimulated respiration in the sample from 178.0 m but not in the sample from 176.7 m (Fig. 6). Despite similarities in TOC concentration, little organic carbon was respired in the 184.7-m sample from near the tuff layer. The reason for these differences is not clear but it may be that the organic C in the deeper sample was more refractile to microbial metabolism.

Low concentrations of pore-water sulphate and low sediment hydraulic conductivities in combination with the presence of respirable organic carbon suggests that microbial metabolism in the lacustrine sediments is electron acceptor limited. Although it is not possible to calculate the flux of sulphate through these sediments with the data available, the consumption of organic matter in these sediments is likely controlled by the diffusion of sulphate and/or O_2 from adjacent, more permeable sediments and by the availability of Fe(III). Ultimately, it is the flux of electron acceptor that maintains slow *in situ* rates of microbial respiration. Extremely slow rates of *in situ* microbial metabolism have been noted for deep sediments of the Atlantic Coastal Plain. Phelps *et al.* (1994) estimated that the rate of CO_2 production from microbial oxidation of organic matter in Atlantic Coastal Plain deep subsurface sediments ranged from 1 to 107 nmol/kg/year. Assuming that the rates of oxidation in the lacustrine sediments of this study were in this same range, it would take from between 8 and 800 million years to effect oxidation of 10 g/kg total organic carbon. Phelps *et al.* also estimated the rate of sulphate reduction to be less than 1 nmol/kg/year in Atlantic Coastal Plain subsurface sediments. Assuming a 1 nmol/kg/year sulphate reduction rate for the lacustrine sediments, it would take ≈ 300 million years to consume 10 g/kg total organic carbon coupled to sulphate respiration.

Although, not sampled at a scale that would allow resolution, it is probable that zones of O_2 and sulphate/Fe(III) respiration are spatially separated in these sediments. O_2 diffusing from groundwater in the overlying sediments is probably consumed in the region between 174 and 175 m. The low organic carbon and the yellow-brown colour of the upper portion of the lacustrine strata indicates that this zone is oxidized. The zone of sulphate consumption lies immediately below 175 m and probably at around 184 m at the bottom of the lacustrine strata as well. Fe(III) reduction is limited by the low solubility of Fe(III) and probably occurs unevenly throughout the zone where organic carbon is present.

The low permeability of the lacustrine sediments, by limiting transport of bacteria and electron acceptors, has provided conditions for the long term maintenance of both organic carbon and a bacterial population containing SRB or closely related bacteria. Phelps *et al.* (1994) estimated that the average doubling time of microbial biomass in Atlantic Coastal Plain subsurface sediments is on the order of centuries and Murphy *et al.* (1992) estimated the groundwater age in some deep Atlantic Coastal Plain aquifers to be 10 000 years. This suggests that microbial populations in these deep subsurface environments have been isolated for at least 10 000 years. The results presented in this study suggest that the micro-organisms in the lacustrine core samples were derived from organisms associated with the lake sediments as they were deposited 6–8 million years ago. The low permeability physical conditions in the lacustrine sediments would be conducive to the preservation of organic matter but not to the transport of micro-organisms. Thus, in many deep subsurface environments comprised of low permeability sediments, the micro-organisms may be remnants of populations associated with the original deposits. Some of these organisms have likely survived for extensive periods by metabolizing and growing at an extremely slow rates.

Acknowledgements

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This paper is a result of a multidisciplinary, multi-institutional collaboration between scientists in the Deep Subsurface Microbiology Subprogram of the US Department of Energy's Subsurface Science Program. The results presented herein are a component of a larger effort to understand the complex interactions between the subsurface microbiota and their surrounding subsurface environment at DOE's Hanford Site in Washington State. The approaches used in this field research program are currently being used to investigate the origins of micro-organisms in the deep subsurface at two sites in the Upper Colorado River Basin in New Mexico and Colorado.
