420

Pore-Size Constraints on the Activity and Survival of Subsurface Bacteria in a Late Cretaceous Shale–Sandstone Sequence, Northwestern New Mexico

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To investigate the distribution of microbial biomass and activities to gain insights into the physical controls on microbial activity and potential long-term survival in the subsurface, 24 shale and sandstone cores were collected from a site in northwestern New Mexico. Bacterial biomass in the core samples ranged from below detection to 31.9 pmol total phospholipid fatty acid (PLFA) g^{-1} of rock with no apparent relationship between lithology and PLFA abundance. No metabolic activities, as determined by anaerobic mineralization of [^{14}C]acetate and [^{14}C]glucose and $^{35}SO_4^{2-}$ reduction, were detected in core samples with pore throats <0.2 μm in diameter, smaller than the size of known bacteria. However, enrichments revealed the presence of sulfate-reducing bacteria, and $^{35}SO_4^{2-}$ reduction was detected upon extended (14 days) incubation in some small-pore-throat samples. In contrast, relatively rapid rates of metabolic activity were more common in core samples containing a significant fraction of pore throats >0.2 μm in diameter. These results suggest that subsurface bacteria require interconnected pore throats greater than 0.2 μm diameter for sustained activity but that viable bacteria can be maintained and stimulated in poorly permeable rocks, such as shales, with restrictive pore throat diameters. In addition, the detrital organic matter in the small-pore-diameter shales is not subject to direct microbial attack. Rather, bacteria in adjacent sandstones with a more open pore structure are probably sustained by endogenous nutrients that are slowly released from the shale. These results have implications for the long-term maintenance of anoxia and the impact of anaerobic biogeochemical processes on groundwater chemistry.

Keywords phospholipid fatty acid, pore size, sandstone, shale, subsurface, sulfate reduction

In 1926, Bastin et al. cultured sulfate-reducing bacteria from 17 of 19 oil-field water samples from southeastern Illinois oil fields, thus providing evidence for the role of microorganisms in the production of sulfides and carbonates in deeply buried oil fields (Bastin, 1926). The question was raised as to whether these bacteria were lineal descendants of the ones associated with the original deposits at the seafloor or whether they were transported more recently from the surface by descending groundwaters.

Bacteria have an extraordinary capacity to survive under conditions of severe nutrient limitation or even in the absence of measurable nutrients, as has been demonstrated in laboratory experiments spanning months to years (Morita, 1993). While the survival of microorganisms over geological time scales in such environments has been the subject of much interest (Kennedy et al., 1994), most of the evidence for survival of microorganisms over such time frames has been speculative or anecdotal. Compelling evidence for such long-term microbial survival is the report of culturing a *Bacillus sphaericus*-related bacterium associated with the abdominal contents of bees entrapped in Dominican amber (Cano & Borucki, 1995). It was suggested that this organism was of ancient origin and became entombed as a spore in the amber some 25–40 million years ago.

Chapelle and Lovley (1990) demonstrated that microorganisms associated with deep aquifers of the southeast coastal plain metabolize organic matter at extremely slow rates. They speculated as to whether they were remnants of microorganisms present at the time of deposition some 70 to 80 million years ago or possibly organisms transported with the groundwater that entered the subsurface between 10,000 and 50,000 years before the present. Although relatively little information is available regarding bacterial transport over rel-

atively long distances in the subsurface (hundreds to thousands of meters), significant transport of microspheres the size of bacteria has been shown to occur within weeks over a distance of 500 m under a natural hydraulic gradient in a sandy aquifer (Harvey et al., 1989).

Recently, we identified sulfate- and Fe(III)-reducing bacteria in a lacustrine sedimentary sequence in south-central Washington state (Fredrickson et al., 1995b). Geochemical and microbiological evidence suggested that these microorganisms were slowly metabolizing sedimentary organic matter, were electron-acceptor limited, and were possibly descendants of microorganisms associated with the original deposits. Bacterial transport from the surface or surrounding sediments would have been severely restricted as the permeabilities in these materials were very low, ranging from 1 mD to $<10^{-3}$ mD. Similarly, thermophilic microorganisms have been isolated from low-permeability subsurface sediments and rocks in Virginia (Boone et al., 1995) and Colorado (Colwell, Phelps, Fredrickson, unpublished data).

The purpose of the present study was to investigate the distribution of microbial biomass and metabolic activities in relation to the physical and chemical properties of the host rocks. This information could also provide insights into whether microorganisms in Late Cretaceous marine shales (90–93 million years ago) could be derived from organisms present at the time of original sedimentation. To this effect, we investigated the distribution of microbial biomass and selected microbial activities in and across shale and sandstone lithologies in relation to pore throat sizes of the rocks. The results reported herein were part of a larger study to investigate the origins of microorganisms in deep subsurface environments and to assess the interactions of the organisms with their chemical and physical environment within and across shale–sandstone lithologies. A sampling site in an undeveloped area of northwestern New Mexico was selected where a deep interstratified sequence of the Cretaceous Mancos Shale and Dakota Sandstone Formations occurs that could be readily accessed.

Materials and Methods

Drilling and Sampling Procedures

All samples were collected using either air- or mud-rotary wireline coring. Cores (10 cm diameter) were obtained from a vertically drilled corehole, designated CNV (Figure 1 and Table 1), and from an angled corehole that started at 28° from the horizontal and finished at 45° from the horizontal, designated CNA-R (Figure 2). Cores obtained from CNA-R were 6 cm in diameter. LiBr was added to drill muds or to standing water in the borehole, and perfluorocarbons were injected into the airstream during air-rotary coring to trace intrusion of solutes and gases into core samples. Fluorescent microspheres (1.0 μm, carboxylated) (Polysciences, Inc., Warrington, PA) were used to trace particle intrusion into cores using methods described elsewhere (Kieft et al., 1995). Procedures for cleaning and disinfecting drilling equipment and coring tools and general procedures for sampling are also described elsewhere (Phelps et al., 1989a; Russell et al., 1992; Fredrickson & Phelps, 1996). A control consisted of Cubero Sandstone core that was autoclaved three times.

Cores contained in clear polycarbonate (Lexan) liners were removed from core barrels immediately after they were brought to the surface. The capped cores in their liners were transferred to a glovebag filled with argon and were processed to avoid contamination of samples for analysis (Colwell et al., 1992). For microbiological and geochemical analyses, the cores were sectioned using a hydraulic core splitter (Colwell et al., 1992), pared and fragmented into cubes of approximately 2 cm, and weighed into Whirl-pak

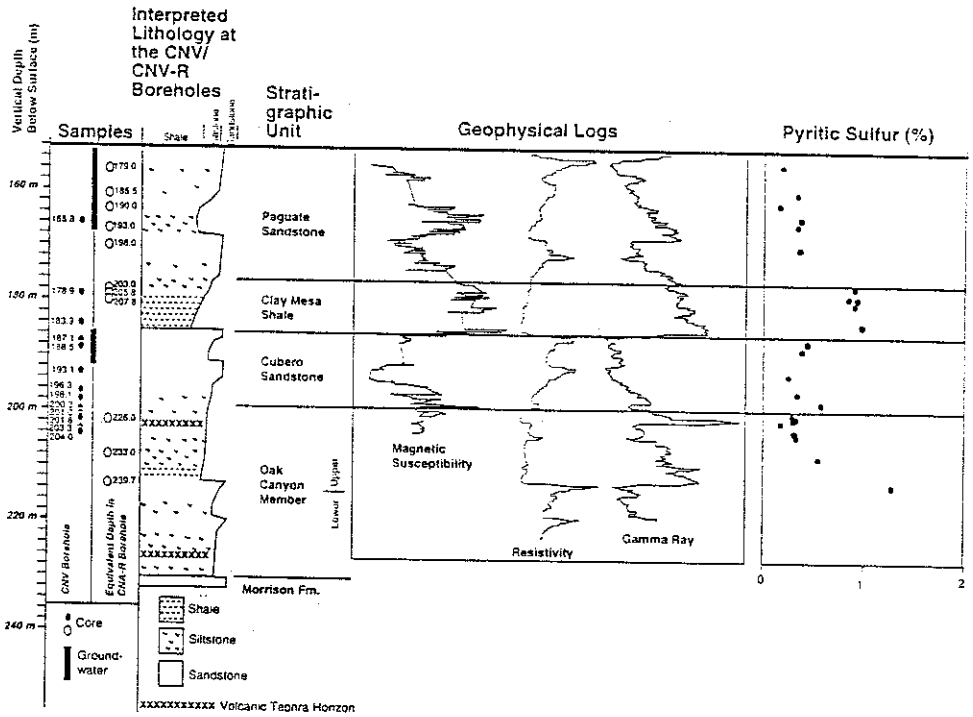

Figure 1. Interpreted lithology and stratigraphy of the interval sampled for physical, chemical, and microbiological characterization.

bags. Core fragments in Whirl-pak bags were placed in sterile, Ar-filled canning jars, sealed, and shipped on Blue Ice by overnight express to the various laboratories involved in this study. Samples for lipid analyses were frozen immediately at –20°C on site and shipped on dry ice to the University of Tennessee by overnight express. Intact cores collected for the silver foil $^{35}SO_4^{2-}$ reduction assay or for measurement of physical properties were always taken adjacent to core segments that were pared and fragmented for the microbiological and geochemical analyses. Only those sections of core that were visually homogeneous were selected. Therefore, it was assumed that the physical, microbiological, and geochemical properties within these intervals were also relatively homogeneous.

Table 1
Geochemical Properties of Groundwater from the Paguate and Cubero Sandstone Units

Formation	pH	DO[a] (mg L^{-1})	Fe^{2+} (mg L^{-1})	S^- (mg L^{-1})	SO_4^{2-} (mg L^{-1})	DOC[b] (mg L^{-1})
Paguate	8.46	≤0.2	bd[c]	8.0	370.4	13.9
Cubero	8.17	≤0.2	0.8	13.6	80.8	1.8

[a] DO, dissolved O_2.
[b] DOC, dissolved organic carbon.
[c] bd, below detection.

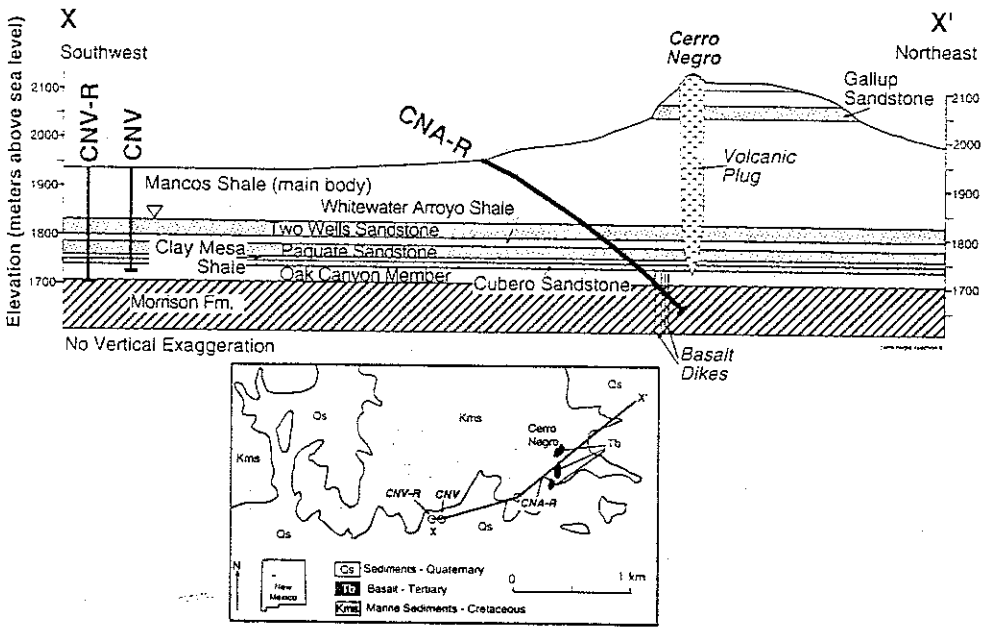

Figure 2. Geological cross section of the drill site in the vicinity of Cerro Negro.

A sealed flow cell (DS3 Data Sonde, Hydrolab, Austin, TX) was used at the surface to determine dissolved O_2 and pH of the groundwater. A bladder pump was used to obtain groundwater from chosen lithologic zones isolated by inflatable packers. Water was sampled for geochemical analyses after measurement of dissolved O_2 and pH had stabilized to constant values. Groundwater samples for sulfide were preserved using Zn acetate, and samples for Fe analyses were filtered and acidified with HCl. Fe and sulfide were analyzed by ion chromatography (McKinley et al., 1997).

Culturable Anaerobic Bacteria

Upon their arrival at Oak Ridge National Laboratory, samples were placed immediately in an anaerobic glovebag (Coy, Ann Arbor, MI), where they were pulverized using a sterile Plattner mill (Colwell et al., 1992). Counts of culturable anaerobic bacteria were performed using a single-tube dilution series and tryptone–yeast extract–glucose (TGYE) medium as previously described (Kieft et al., 1995). Sulfate-reducing bacteria were enriched in single serial-tube dilution series. The medium contained trace mineral and vitamin solutions (Stevens, 1995, number 1880), 2.0 mM bicarbonate buffer, 2.0 mM phosphate buffer, 20 mM sodium sulfate, 10 mg L^{-1} yeast extract, 10 mM each of lactate and acetate under N_2–CO_2 (80:20% v/v) headspace, and reduced with 0.3 g L^{-1} cysteine-HCl. Cultures were incubated for 1 month at 20–24°C. Positive results were indicated by turbidity and the development of a black precipitate.

PLFAME/DGFA Analyses

Lipids from approximate 75-g quantities of frozen sediments were extracted and fractionated into neutral, glyco, and polar fractions 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. Diglyceride fatty acids (DGFA) were measured as previously described (Kieft et al., 1994).

Activity Measurements

Acetate mineralization was determined by adding 0.2 ml of a solution containing 18.5 kBq of sodium [1-^{14}C]acetate (74 kBq μmol^{-1}, >98% radiopurity, NEN Research Products, Boston) and 4.8 ml of sterile artificial groundwater to 5 g of homogenized rock in sterile, 150-ml glass milk dilution bottles. The composition of the artificial groundwater was based on the geochemistry of groundwater samples from wells in the vicinity of the sampled borehole. It was prepared from deionized water by addition, in milligrams per liter, of: NaF, 3.8; KBr, 0.2; KCl, 7.2; $CaCl_2 \cdot 2H_2O$, 336.4; Na_2SO_4, 713.0; $MgCl_2 \cdot 6H_2O$, 32.7; NaCl, 200.8; $Na_2SiO_3 \cdot 9H_2O$, 82.2; and $NaHCO_3$, 899.8. All samples and solutions were manipulated in an anaerobic glovebag. Activities were measured by suspending 5-ml liquid scintillation vials containing 1 ml of 0.3 M KOH in the headspace of dilution bottles, which were sealed with butyl rubber stoppers. The bottles were incubated in the dark at 22°C for 1–3 days. The amount of $^{14}CO_2$ evolved over time was measured by liquid scintillation counting of KOH from the traps (Wallac model 1411, Pharmacia LKB, Gaithersburg, MD). Controls consisted of subsurface samples autoclaved on three consecutive days and treated in a manner identical to those described earlier. All values reported are the mean of triplicate samples. Anaerobic [U-^{14}C]glucose mineralization was measured in duplicate sterile 25-ml crimp-top tubes as previously described (Phelps et al., 1989b; Kieft et al., 1995). Briefly, tubes contained 2.0 g sediment, 1.0 ml sterile water, and 37 kBq of carrier-free [U-^{14}C]glucose (0.36 mM, Amersham Corp., Arlington Heights, IL). Isotope solutions were frozen prior to use and transferred with gas-tight syringes into anaerobic crimp-top tubes (Bellco Glass Co., Vineland, NJ). Experiments were performed in duplicate with at least three time points, of which only the longest (21 days) showed positive results. At the end of all experiments, the reaction in tubes was inhibited with 0.5 ml of 2.0 M sodium hydroxide and frozen. One hour prior to analysis, the tubes were thawed and acidified with 1 ml of 0.5 N HCl. Radioactive $^{14}CO_2$ and $^{14}CH_4$ from mineralization experiments were examined by gas chromatography–gas proportional counting (GC-GPC) using a Shimadzu gas chromatograph equipped with a thermal conductivity detector coupled to a Packard 894 GPC as described previously (Phelps et al., 1994).

To measure sulfate-reducing activity in the pared, crushed rock samples, 10 g of homogenized sample was weighed into sterile Balch tubes (Bellco Glass, Inc., Vineland, NJ) and 10 ml of sterile artificial groundwater was added (as described earlier except without Na_2SO_4), plus 0.5 ml of a filter (0.2 μm) sterilized solution of 20 mM each of sodium benzoate, ammonium formate, sodium lactate, propionic acid, isobutyric acid, and sodium acetate. One milliliter of anaerobic $^{35}SO_4^{2-}$ solution (370 kBq, carrier-free, NEN Research Products) was injected into each tube through the butyl rubber stopper. Balch tubes were incubated in the dark for 14 days at room temperature, and reactions were terminated by injecting 3.5 ml of a 20% Zn acetate solution and freezing at –20°C. Samples and controls were kept frozen until they were assayed. Controls consisted of samples to which Zn acetate was added prior to the $^{35}SO_4^{2-}$ and immediately frozen after addition of the labeled sulfate. Samples were thawed immediately before analysis and centrifuged. Aliquots of the supernatant were analyzed for $^{35}SO_4^{2-}$ by liquid scintillation counting. Sediments were washed twice with deionized water to remove residual $^{35}SO_4^{2-}$.

and the total reduced ^{35}S was determined by the single-step chromium reduction procedure (Canfield et al., 1986; Fossing & Jørgensen, 1989). All samples were analyzed in duplicate and corrected for the amount of radioactivity present in controls.

In Situ Sulfate Reduction

Silver foil (0.025 mm thick; Johnson Matthey, Ward Hill, MA) was cleaned by stepwise immersion in ethanol, acetone, and hexane. The foil was then allowed to air dry and was subsequently treated by immersion in concentrated nitric acid for 10 to 20 s. The latter step served to cover the surface of the foil with a thin layer of silver oxide. The foil was then immersed in distilled water followed by treatment with the same solvents as described earlier and subsequently air dried, autoclaved, and stored in an anaerobic glove box until use.

Intact cores were collected as described earlier and were stored under a positive-pressure nitrogen atmosphere in military surplus ammunition boxes at 4°C prior to use. The boxes were modified by drilling a single hole for placement of a rubber stopper. This allowed for flushing of the headspace and pressurization with O_2-free N_2 through a series of inlet and outlet needles passed through the stopper. The mineral solution used in these experiments was designed to simulate groundwater composition and contained (mg L^{-1}): KBr, 3; KCl, 8; Na_2HPO_4, 14; $MgCl_2 \cdot 6H_2O$, 40; $CaCl_2 \cdot 2H_2O$, 40; Na_2SO_4, 14; $NaHCO_3$, 1000; and $Na_2S \cdot 9H_2O$, 60. Trace metals were also added (Tanner, 1995). The mineral solution was prepared using the methods of Balch et al. (1979) under a nitrogen atmosphere and had a final pH of 8.1. The $Na_2{}^{35}SO_4$ solution was prepared by transferring the undiluted stock solution (ICN Biomedicals, Inc., Irvine, CA: specific activity ~43 Ci/mg S) into a serum bottle flushed with N_2. This was then autoclaved and the sterile mineral solution was added to obtain an activity of 10 μCi/100 μl.

Intact cores were removed from the ammunition boxes and fractured with a hammer and sterile chisel to expose two fresh core faces. One core face was treated by evenly distributing 100 μl of the mineral solution containing $^{35}SO_4^{2-}$ on the core face. The exposed face was then overlaid with a slightly larger piece of treated silver foil and the two rock faces were superimposed along the fracture lines. The reassembled core was clamped together, wrapped in aluminum foil, and taped to secure the core prior to clamp removal. All sample manipulations were performed inside a glovebag with H_2:N_2 (10:90) atmosphere. Cores were then placed in ammunition boxes for added protection and to prevent desiccation during incubation in the glovebag.

Following a 6-week incubation at room temperature, the cores were disassembled and the silver foil was washed overnight in distilled water. The ^{35}S-sulfide was then located and quantitated by electronic autoradiographic imaging (AMBIS 4000 radioisotopic imager; Scanalytics, Billerica, MA).

Community Level Physiological Profiles

A community-level physiological profile (CLPP) assay was used to characterize microbial communities in the rock samples on the basis of substrate utilization (Garland & Mills, 1991; Lehman et al., 1995). Microbial communities were extracted and separated from crushed 25-g samples using a flocculation procedure that yields a clarified supernatant (Lehman et al., 1995). The clarified supernatant was inoculated into Biolog-GN microplates (Biolog, Inc., Hayward, CA), which were incubated aerobically at 22°C for 1 week. Oxidation of the various carbon compounds was analyzed daily by measuring ab-

sorbance (reduction of the tetrazolium dye) at 595 nm using a model 450 microplate reader and Microplate Manager software, version 1.0 (Bio-Rad Labs, Cambridge MA).

Pore Throat Distributions and Porosity

Pore throat size distributions were determined from mercury injection capillary pressure curves. Pore throat sizes are calculated from a known relationship between mercury injection pressure and pore entry radius (Washburn, 1921; Berg, 1975):

$$R_i = [2T(\cos\theta)C]/P_c$$

where R_i is the pore radius (micrometers), P_c is the injection pressure (psia), T is the interfacial tension (dynes/cm, air:mercury), θ is the contact angle (degrees, air:mercury), and C is a conversion constant (0.145).

Median pore throat diameters are based on pore volumes. At the median pore throat diameter, 50% of the mercury injected into the sample has passed through smaller pore throats and 50% injected has passed through larger pore throats. The theoretical basis and application of mercury injection capillary pressure curves are described elsewhere (Melas & Friedman, 1992; Pittman, 1992). Total porosity is based either on the total volume of Hg injected into the sample or (for samples for which no Hg injection porosimetry is available) on Boyle's law or resaturation techniques. Total porosity estimates typically are in close agreement (<2%) for all three methods.

Results

Lithologic Characteristics and Groundwater Geochemistry

The interval selected for this study was an alternating sandstone–shale sequence consisting of four stratigraphic units: Paguate Sandstone, Clay Mesa Shale, Cubero Sandstone, and Oak Canyon Member (Figures 1 and 2). The fine-grained shales and mudstones generally contained relatively higher amounts of organic carbon (data not shown) and pyritic S (Figure 1), while the sandstones were coarse-grained and contained lower amounts of organic carbon and pyritic S. Each of these units are readily identified over a wide geographic area but, because of gradations within each unit, there is typically a wide range in the physical, chemical, and hydrologic properties of the rock samples. The trend toward progressively coarser grain size in rocks toward the top of the units is especially apparent in the Cubero Sandstone, as evidenced by a general increase in electrical resistivity and decrease in the magnetic susceptibility and natural gamma log from bottom to top (Figure 1). The correlation between the gamma log and the magnetic susceptibility measurements indicates that most of the Fe in the rock is incorporated into chlorite and is paramagnetic. The coarsening upward character displayed in the Cubero Sandstone also occurs in the Paguate Sandstone. Within the Clay Mesa Shale, which is texturally more uniform than the shale unit within the Oak Canyon Member, magnetic susceptibility and pyritic S are relatively high throughout. Of these four units, the Clay Mesa Shale was finest grained and had features most characteristic of a shale confining bed.

The analysis of groundwater samples collected from the Paguate and Cubero Sandstone units indicated that the groundwater in both of these units was anoxic and slightly alkaline, and the Cubero Sandstone was low in dissolved organic carbon (Table 1). Sulfide was present in groundwater from both formations, while sulfate was approximately threefold higher in the Paguate Sandstone than in the Cubero Sandstone.

Viable Anaerobes and Prokaryotic Biomass

The populations of culturable anaerobic bacteria were generally low or below detection with positive enrichments only in the primary dilutions (Table 2). Sulfate-reducing bacteria (SRB) were cultured from only one of the two Clay Mesa Shale samples. For samples from the other units, the occurrence of positive enrichments was equally rare except for the Pagaute Sandstone, where growth in TGYE occurred in four of seven and in SRB medium in two of seven samples.

Phospholipids extracted directly from the core samples were predominantly from prokaryotes (data not shown). Although the amount of prokaryotic phospholipid fatty acids (PLFA) was low, it was above the mean value for the blank plus one standard deviation (0.8 pmol g^{-1}) in all subsurface samples except for two, one at the bottom of the Paguate Sandstone and one at the bottom of the Clay Mesa Shale (Table 2). The control contained 1.8 pmol g^{-1}, a result that is consistent with the stability of phospholipids during autoclaving. In general, there was little variation in total PLFA among the samples and there was no apparent relationship to depth, lithology, or other microbiological or geochemical parameters. The concentrations and variations in the PLFA were consistent with the low populations and activities of anaerobic bacteria associated with these samples. One sample from the Paguate Sandstone had concentrations of PLFA that were approximately 10-fold greater than in any of the other samples. The reason for the high concentration of PLFA in this sample is unclear, and other microbiological assays on this sample did not indicate inordinately high levels of cells or activity.

The ratio of DGFA to PLFA provides an estimate of the proportion of nonviable to viable bacterial biomass (White et al., 1979; Kieft et al., 1994). This ratio varied from 0.1 to 13.4 for the subsurface samples, indicating that lipid associated with nonviable biomass was significant in all samples and in many cases exceeded the amount of PLFA (Table 2). As was the case for total PLFA, there was no apparent relationship between the DGFA/PLFA ratio and depth, lithology, or other measured properties. However, this ratio was 10-fold or greater for the subsurface samples than for surface soils collected at the drill site, which ranged from 0.002 to 0.017.

Activity Measurements

Anaerobic mineralization of ^{14}C-labeled acetate and of glucose and reduction of $^{35}SO_4^{2-}$ into the total reduced S pool were measured to determine if the subsurface microorganisms that were present in these samples were active or were able to respond to nutrients. Mineralization of ^{14}C-labeled acetate was detected in four of seven samples from the Paguate Sandstone but in only one of five samples from the Cubero Sandstone (Table 3). In the Oak Canyon Member, glucose and acetate mineralization were detected only in the samples from 226.0 and 233.0 m, respectively (Table 3). In general, in those samples where glucose mineralization was detected, ^{14}C-labeled acetate was also mineralized. Mineralization of [^{14}C]acetate in all sterile controls was negligible (≤0.7%).

Radiolabeled sulfate was reduced in some samples from each of the formations but was more common in samples from the Paguate Sandstone and the Oak Canyon Member; the highest activities were in the lower Oak Canyon (Table 3). There was no apparent relationship between the samples in which acetate and/or glucose were mineralized and those in which $^{35}SO_4^{2-}$ was reduced. In addition, $^{35}SO_4^{2-}$ reduction was detected in many samples for which the enrichments for SRB were negative. In the Clay Mesa Shale and Oak Canyon Member samples where $^{35}SO_4^{2-}$ reduction was detected at the exposed face of intact cores using the silver foil method, it was also detected in homogenized samples

Table 2
Enrichments for Sulfate-Reducing and Fermentative Bacteria and Biomass

Borehole distance from surface (m)	Equivalent vertical depth (m)	Formation	Br[a] (mg kg^{-1})	Growth in TGYE[b]	SRB[c]	Total PLFA[d] (pmol g^{-1})	DGFA/PLFA[e]
CNV-165.8	165.8	Paguate	bd[f]	ng[j]	+	3.3	0.2
CNV-178.9	178.9	Paguate	bd	ng	ng	31.9	0.1
CNA-R-333.7	179.0	Paguate	0.11	+[h]	nd	1.2	1.2
CNA-R-344.2	185.5	Paguate	0.13	+	nd	1.1	0.6
CNA-R-350.3	190.0	Paguate	bd	+	nd	1.2	1.7
CNA-R-355.4	193.0	Paguate	bd	+	+	1.1	1.0
CNA-R-361.1	198.0	Paguate	0.17	ng	ng	0.7	5.1
CNV-183.9	183.9	Clay Mesa	bd	ng	+	5.6	0.2
CNA-R-371.1	203.0	Clay Mesa	0.09	ng	bd	1.0	0.9
CNA-R-375.3	205.8	Clay Mesa	nd[g]	nd	nd	2.5	0.5
CNA-R-378.4	207.8	Clay Mesa	0.11	nd	nd	0.4	13.4
CNV-187.1	187.1	Cubero	0.07	ng	ng	0.9	2.1
CNV-188.5	188.5	Cubero	bd	ng	ng	1.2	0.7
CNV-193.1	193.1	Cubero	bd	ng	nd	1.2	0.3
CNV-196.3	196.3	Cubero	bd	ng	ng	1.5	0.4
CNV-198.1	198.1	Cubero	bd	ng	ng	1.0	1.1
CNV-200.3	200.3	Oak Canyon	0.08	ng	ng	3.9	0.3
CNV-201.0	201.0	Oak Canyon	bd	ng	ng	2.8	0.9
CNV-201.6	201.6	Oak Canyon	0.11	nd	nd	1.3	1.9
CNV-203.3	203.3	Oak Canyon	bd	ng	ng	1.5	1.4
CNV-204.0	204.0	Oak Canyon	bd	ng	ng	2.6	0.3
CNA-R-405.5	226.0	Oak Canyon	0.21	+	+	1.6	0.5
CNA-R-417.4	233.0	Oak Canyon	bd	nd	nd	1.6	0.6
CNA-R-425.0	239.7	Oak Canyon	bd	nd	nd	5.3	0.5
Control[i]	—	—	nd	ng	ng	1.8	1.2

[a] Concentration of Br^-, used as a conservative tracer during drilling.
[b] Anaerobic growth in trytone–glucose–yeast extract (TGYE) medium.
[c] SRB, sulfate-reducing bacteria.
[d] PLFA, phospholipid fatty acid.
[e] DGFA/PLFA, ratio of total diglyceride fatty acids to total phospholipid fatty acids.
[f] bd, Below detection. For Br, this value was 0.02 mg kg^{-1}.
[g] nd, Not done.
[h] +, Growth.
[i] Control consisted of sandstone sample that was autoclaved 3×.
[j] ng, No growth.

Table 3
Activity Measurements and Median Pore Throat Diameter

Equivalent vertical depth (m)	Formation	Acetate mineralization (dpm $^{14}CO_2$ day^{-1})	Glucose mineralization (dpm $^{14}CO_2$ day^{-1})	Sulfate reduction (nmol g^{-1} per 14 days)	Median pore throat diameter (μm)
165.8	Paguate	bd	bd	bd	0.03
178.9	Paguate	bd	bd	bd	0.03
179.0	Paguate	87,730 (3373)	nd	1.35	2.67
185.5	Paguate	87,929 (3503)	>10,000	0.23	0.07
190.0	Paguate	bd	bd	0.17	nd
193.0	Paguate	179,280 (5201)	300	bd	0.03
198.0	Paguate	185,663 (7426)	400	0.08	0.07
183.9	Clay Mesa	bd	bd	bd	0.01
203.0	Clay Mesa	bd	bd	bd	0.03
205.8	Clay Mesa	bd	nd	0.06	0.02
207.8	Clay Mesa	bd	nd	bd	nd
187.1	Cubero	bd	bd	bd	5.05
188.5	Cubero	bd	bd	1.53	3.28
193.1	Cubero	154,632 (29,424)	10,000	bd	13.36
196.3	Cubero	bd	12,000	bd	0.21
198.1	Cubero	bd	bd	bd	0.09
200.3	Oak Canyon	bd	bd	bd	0.06
201.0	Oak Canyon	bd	bd	0.23	0.05
201.6	Oak Canyon	bd	nd	nd	0.01
203.3	Oak Canyon	bd	bd	bd	0.13
204.0	Oak Canyon	bd	bd	bd	0.13
226.0	Oak Canyon	bd	300	3.79	0.05
233.0	Oak Canyon	51,709 (54,467)	nd	9.26	0.04
239.7	Oak Canyon	bd	nd	bd	0.02
Control	—	bd	nd	bd	—

Note. bd, Below detection; nd, not done.

to which $^{35}SO_4^{2-}$ was added. In the upper Clay Mesa Shale, activities were at the detection limit with the silver foil assay (data not shown) and below the detection limit using the homogenized sample assay.

In addition, several freshly exposed faces of intact cores that were incubated with $^{35}SO_4^{2-}$ using the silver foil assay confirmed that sulfate reduction, in the interior of the cores, occurred in samples from 205.8 m in the Clay Mesa Shale unit and 233.0 m in the Oak Canyon Member (Figure 3). The locations of $^{35}SO_4^{2-}$ reduction in the Clay Mesa Shale sample were confined to the interior of the core and hence SRB were probably not contaminants introduced during drilling and sampling. Interestingly, SRB were not cultured from either of these samples (Table 2).

The community-level physiological profile (CLPP) results indicated that the only samples where a positive response could be detected by this method, as measured by the ability to oxidize individual carbon compounds, were in the Paguate Sandstone. Three of

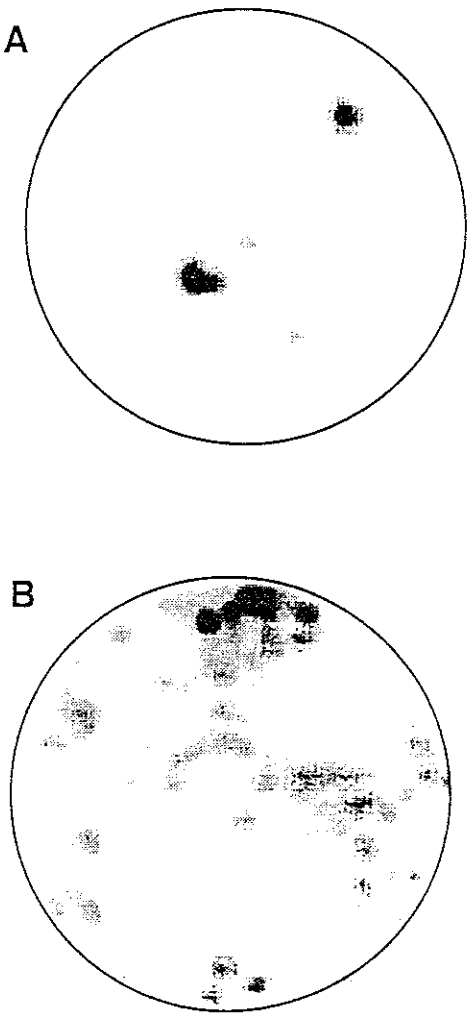

Figure 3. Radioisotopic image of silver foil contacted with exposed faces of cores from (A) the Clay Mesa Shale (205.8 m) and (B) the Oak Canyon Member (226.0 m) showing regions of sulfate-reducing activity. The diameter of these cores was 6 cm.

the 7 Paguate Sandstone samples showed a response to 1 or more of the 95 carbon substrates in the BIOLOG plate, whereas none of the samples from the other units gave even a single positive response. In contrast, the CLPP of surface soil and groundwater samples at the site yielded positive responses, on average, to 90 and 73 of the carbon compounds, respectively. Organic compounds commonly used by the microorganisms in the Paguate Sandstone samples included Tween compounds, methyl pyruvate, acetic acid, propionic acid, and several amino acids. In contrast, the most commonly metabolized compounds in the soil and groundwater samples collected from the site were carbohydrates (data not shown). One possible reason for the inability to detect activity in these samples using the Biolog plates is the fact that this method generally detects only the activity of aerobic metabolism. The geochemical analyses indicate that the environments sampled in this study were predominantly anaerobic (Table 1).

Pore Throat Size Distributions

The coarsening upward gradation in particle size that occurs within some of the stratigraphic units is reflected in the pore throat distributions (Figure 4) and the mean pore size diameters (Table 3). For example, the range of mean pore throat diameters in the Cubero Sandstone unit decreased from 3–13 μm at a depth of 187.1–193.1 m toward the top of the unit to 0.21–0.06 μm at a depth of 196.3–200.3 m toward the base. One of the reasons for the small pore throats in some of the sandstones with relatively coarse grain sizes, such as the Paguate Sandstone, is infilling of pores with secondary quartz or calcite (T. C. Onstott, unpublished data). The pore throats were very small in the fine-grained Clay Mesa Shale and the Oak Canyon Member sample from a depth of 201.6 m where the mean pore throat diameter was less than 0.03 μm (Table 3), and all the pore throats were less than 0.2 μm in diameter (Figure 4). The largest pore throats observed (ranging up to 12 μm in diameter) were in the Cubero Sandstone samples.

It is interesting to note that even though the pore throats in the Clay Mesa Shale and the Oak Canyon Member sample from a depth of 201.6 m were extremely small, samples from this formation contained as much PLFA as samples from the other units. Also, SRB were present in the Clay Mesa sample from a depth of 183.9 m, as determined by enrichment culture. With the exception of several of the sandstone samples, the pore throats in these samples were considerably smaller than the size of most bacteria, even those subjected to starvation.

Discussion

The origin of the microorganisms in the shale–sandstone sequence is an intriguing question that requires consideration of the geological and microbiological histories of these formations. The sediments that now form the sandstone–shale units of this study were deposited about 100 million years (my) ago during the Cretaceous period in a shallow marine environment that was part of a large inland sea covering much of the western United States. Anaerobic microbial processes including fermentation, Fe/Mn reduction, sulfate reduction, and methanogenesis would have been active in the marine sediments during the time of their deposition, as they are in modern near-shore marine sediments. During burial and diagenesis, sediments would have become compacted, organic carbon and electron acceptors consumed, and the populations of bacteria and their activities would have been expected to decline. Parkes et al. (1990) showed that although the population of bacteria declined with depth in marine sediments, populations of $>10^8$ total bacteria

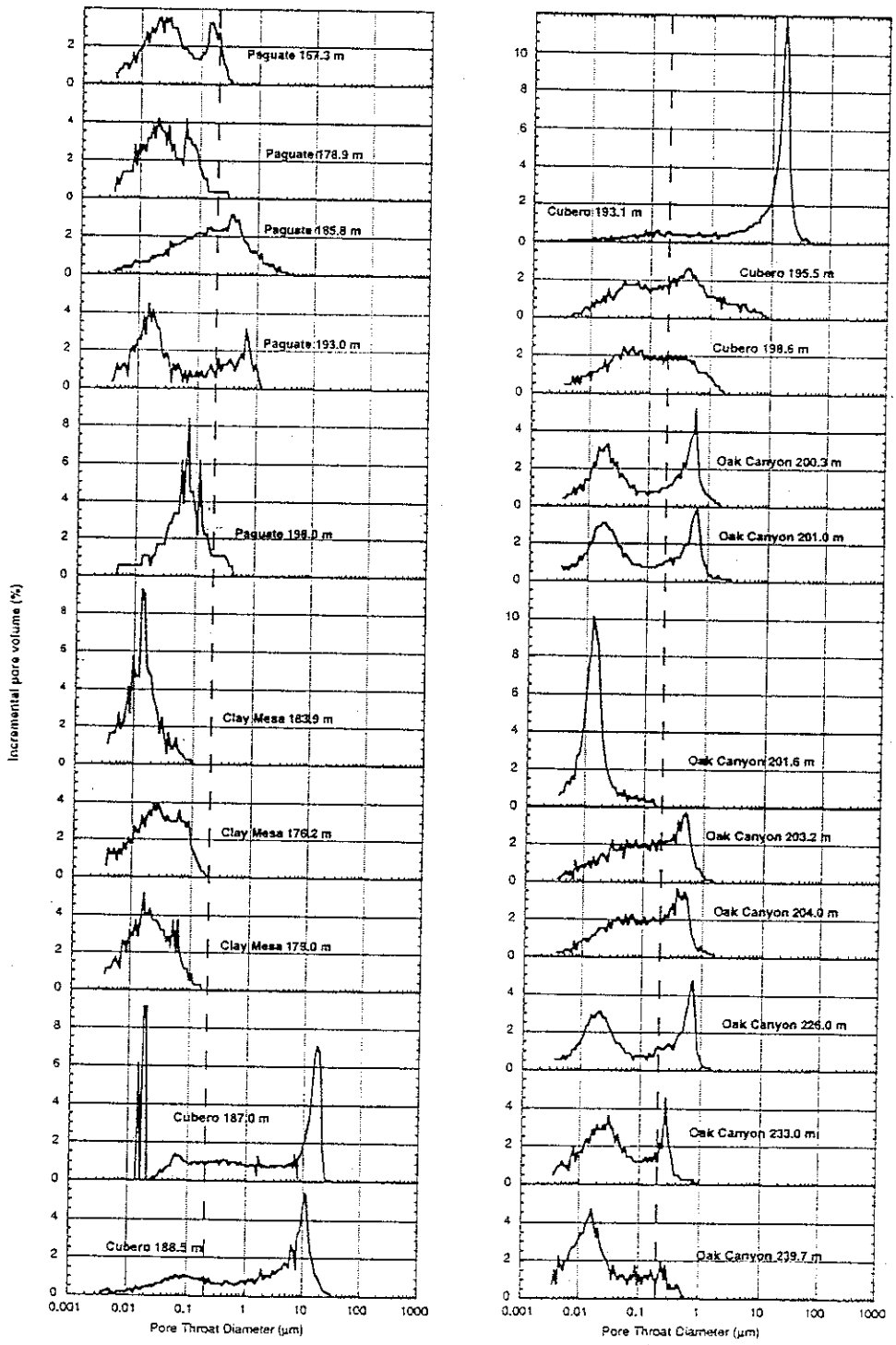

Figure 4. Distribution of pore throat sizes as determined by Hg porosimetry in intact cores adjacent to samples collected and used for microbiological analyses.

cm^{-3} were present in marine sediments from depths as great as 80 m beneath the seafloor. Viable sulfate-reducing and methanogenic (H_2/CO_2) bacteria were found in these deep marine sediments, and although the rates of sulfate reduction and methanogenesis declined with depth, they remained above detection limits. Perhaps even more intriguing is the report of viable bacterial cells at depths as great as 518 m beneath the seafloor (Parkes et al., 1994). Sulfate-reducing bacteria, tentatively identified as *Desulfovibrio* sp., and sulfate-reducing activities measured using $^{35}SO_4^{2-}$ were also detected in these same deep marine sediments. The oldest deep marine sediment containing bacteria in the study by Parkes et al. (1990) was approximately 10 my.

The shale-sandstone sequence that was the subject of this study is Cretaceous (90–100 my). Viable SRB that were capable of transforming $^{35}SO_4^{2-}$ into reduced sulfur species were detected in these formations, although the in situ rate of sulfate reduction is unknown. Phelps et al. (1994) estimated that the rate of sulfate reduction was less than 1 nmol kg^{-1} $years^{-1}$ in Atlantic Coastal Plain subsurface sediments, and it is likely that the rates of sulfate reduction in the strata included in this study were equally low or lower.

In contrast to the deep marine sediments studied by the Parkes group, the shale and sandstone samples of this study have undergone considerable diagenesis including cementation of particles by quartz overgrowths and the formation of secondary minerals such as chlorite, calcite, pyrite, and gypsum (data not shown). These processes, in addition to compaction, would have contributed to reductions in permeability and pore throat size over time. Stable isotope analyses of these rocks (T. C. Onstott, unpublished data) indicated that this sequence was in contact with meteoric (i.e., fresh) water as early as the Late Cretaceous period and that ^{13}C-depleted calcite and ^{34}S-depleted pyrite were the result of past microbial activity. Bacterial transport could have occurred during early diagenesis when pore throats may not have been as restrictive as they are presently. The thermal history of this sequence indicates that it was never exposed to temperatures greater than 65°C (data not shown).

Although the recent transport of bacteria from the surface cannot be completely ruled out, it is unlikely, especially in those lithologies where the mean pore throat diameter was smaller than the diameter of starved or ultramicrobacteria (0.2–0.4 μm) (Novistsky & Morita, 1976; Cusack et al., 1992). It has been suggested that the pore throat size must be double the diameter of cells in order for bacteria to effectively pass through (Updegraff, 1982). Jenneman et al. (1985) observed that bacterial penetration into sandstone with permeabilities <100 mD was superficial and occurred slowly, if at all. They also suggested that pore throat size was a better indicator of the potential for bacterial penetration of rock than was permeability.

Microorganisms in this shale–sandstone sequence probably reflect a combination of those organisms transported from the surface and those associated with the original deposits. Microorganisms in the Paguate and Cubero Sandstone units have a greater likelihood of having been transported because the pore throats in some of the regions of these units are large enough for the passage of bacterial cells. In addition, the age of the groundwater in the sandstone units is young at <3500 years (P. Pegram, personal communication) relative to the age of the rock. In contrast, recent bacterial transport in the Clay Mesa Shale is unlikely given the restrictive pore size (e.g., <0.2 μm), yet viable bacteria and PLFA were detected in these samples that were consistent with the types of organisms (e.g., sulfate-reducing bacteria) that would have been present during deposition and early diagenesis. One possible explanation is that the PLFA in these low-permeability sediments are associated with nonviable biomass. The turnover rate of PLFA (i.e., phosphate group removal) in low-biomass subsurface sediments and rocks is unknown. Pyrite

was common throughout all of the units but was highest in the Clay Mesa Shale, where pyritic S comprised as much as 1% of the rock by weight (Figure 1). Pyrite typically forms in shales during the early stages of diagenesis as the result of bacterial sulfate reduction concomitant with the consumption of sedimentary organic C. The isotopic fractionation of S during sulfate reduction can range from 2‰ to 50‰ (Kemp & Thode, 1968); hence pyrite produced as the result of bacterial sulfate reduction typically will have low $\delta^{34}S$ values. The $\delta^{34}S$ of framboidal pyrite ranged from –42‰ to –3‰, a value that is consistent with biological reduction of Late Cretaceous marine sulfate. Hence, sulfate reduction occurred early in the history of these rocks during deposition of sediments and early diagenesis. In spite of transport-restricting pore throat sizes, SRB can still be recovered from these rocks.

Microbial activities in these samples, as measured using isotopically labeled acetate, glucose, or sulfate, were generally low or below detection except for a few samples. Of these assays, only the incubations with [^{14}C]acetate and [^{14}C]glucose were short term (2 days) and thus represented those samples in which microorganisms would be likely to have been metabolically active at the time of sampling. The sulfate reduction assay was incubated over a 14-day period and thus could represent cells that were inactive at the time of sampling but were subsequently stimulated over the incubation period in addition to metabolically active organisms. It has been observed that the number of culturable bacteria as well as metabolic activities can increase dramatically after sampling subsurface sediment or rock (Haldeman et al., 1994, 1995; Fredrickson et al., 1995a). Metabolic activities, as measured using isotopically labeled compounds, can greatly overestimate in situ process rates relative to geochemical methods for the same process (Chapelle & Lovley, 1990; Phelps et al., 1994). The situation is no doubt exacerbated by the high degree of spatial heterogeneity associated with metabolic activities as evidenced by the pockets of sulfate reduction detected in our studies. To be conservative, the activities measured in this study should be considered as potential activities and confirmatory evidence for the presence of viable bacteria rather than as actual measures of in situ rates.

Metabolic activities were greatest in the Paguate and Cubero Sandstone units, where the pore throat diameters were typically larger than those found in the fine-grained rocks. Nine of the 15 samples collected for Hg porosimetry (Figure 4, Paguate samples from depths of 167.3, 185.8, 193.0, and 198.0 m; Cubero samples from depths of 187.0, 188.5, 193.1, and 195.5 m; and Oak Canyon samples from depths of 200.3, 201.0, 203.2, 204.0, 226.0, 233.0, and 239.7 m) with pore throats greater than 0.2 μm in diameter were immediately adjacent to samples where microbial activity, as measured by [^{14}C]glucose or acetate mineralization or $^{35}SO_4^{2-}$ reduction, was detected. In contrast, there was no activity detected in any of the microbiology samples adjacent to the 6 samples (Paguate at depths of 167.3 and 178.9 m; Clay Mesa at depths of 183.9, 203.0, and 206.2 m; Oak Canyon at a depth of 201.6 m) that had pore throats less than 0.2 μm in diameter, except for a low level of $^{35}SO_4^{2-}$ reducing activity in the Clay Mesa Shale sample from 205.8 m. As pore throat size and interconnectivity vary directly with permeability (Swanson, 1981; Pittman, 1992), it follows that the lithologies with the smallest pore throats, and thus low permeabilities, also had the lowest metabolic activities. The flux of nutrients and metabolic products into and out of poorly permeable rock such as the Clay Mesa Shale would be essentially diffusion controlled except for possible movement along relatively rare fractures.

There are several difficulties in drawing direct relationships between the presence and activity of microorganisms and physical properties of the rocks such as pore throat size and permeability. First, due to the nature of the analyses it is difficult, if not impossi-

ble, to measure microbiological and physical properties on the same sample. Although only those core sections that were homogeneous in appearance (e.g., color, texture) were selected for this study, considerable heterogeneity in both the physical and microbiological properties of the core could exist but not be visually apparent. This was especially evident in the sandstone units where there was a wide range in the pore throat size distributions. The results of this study also indicate that the distribution of microorganisms and microbial activities was patchy in these formations. The distribution of sulfate-reducing activity across freshly exposed faces of intact cores from the Clay Mesa Shale and Oak Canyon Member as shown in Figure 3 also attests to this fact. Heterogeneous distributions of microbial activity in these samples are not surprising, given the overall low biomass concentrations. Previous studies have shown that microbiological properties such as methanogenesis (Adrian et al., 1994) and denitrification (Smith et al., 1991) can vary substantially over relatively short (e.g., centimeters to meters) distances in the subsurface.

In spite of very low permeability, the Clay Mesa Shale could potentially serve as a source of electron donors (i.e., organic carbon) and/or electron acceptors (i.e., sulfate) for microorganisms in the sandstone units. McMahon and Chapelle (1991) reported that a principal source of electron donors for microbial respiration in deep Atlantic coastal plain aquifer sands was the diffusion of fermentation products from organic-rich confining layers. McMahon et al. (1992) also suggested that sulfate diffusion from confining beds provided the electron acceptor for microbial respiration of organic acids in the Black Creek sands of southeast coastal plain subsurface sediments. They suggested that sulfate within Gulf Coast shales may maintain microbial sulfate reduction at depth in sandstones. It is possible that there are analogous processes occurring in the low-permeability organic-rich shales and adjacent sandstones reported in this study. Although very few of the samples gave positive response to the CLPP assay, it is interesting to note that the organic compounds most commonly used by samples from the Paguate Sandstone were organic acids, some of which are common fermentation products. Additional investigations are underway to determine if there are geochemical and microbial interactions between the shale and sandstone units.

In contrast to the sandstone units, neither [^{14}C]acetate nor [^{14}C]glucose was mineralized during short-term incubation of samples from the Clay Mesa Shale. However, small amounts of $^{35}SO_4^{2-}$ reduction were detected after periods of incubation of 14 days or longer. These results along with the enrichment of SRB from the Clay Mesa shale suggest that although such microorganisms are present they are likely dormant, possibly existing as spores of bacteria such as *Desulfotomaculum*. It is suspected that sporulation contributed to the long-term survival of *Bacillus* in Dominican amber (Cano & Borucki, 1995).

The microbial biomass in samples throughout the cored interval, as determined by total bacterial PLFA, was similar to levels measured in deep subsurface fluvial sand and lacustrine sediments in Washington State (Fredrickson et al., 1995). Using a conversion factor of 5×10^5 cells pmol^{-1} for bacterial biomass in subsurface sediments (Tunlid & White, 1992), the estimated population density of bacteria in these samples ranged from 3.5×10^5 to 1.8×10^7 cells g^{-1}. In addition to viable biomass as determined by PLFA, there were significant quantities of nonviable biomass as measured by DGFA. Kieft et al. (1994) used DGFA/PLFA ratios as a relative measure of nonviable and viable cells in a sand microcosm experiment to examine the effects of desiccation and starvation on the maintenance of viable cells in porous medium. The DGFA/PLFA ratios for *Pseudomonas aureofaciens* introduced into the microcosms remained relatively constant at 0.12 or below during a 16-day incubation period, while the same ratio increased to 0.2 in the

dessicated microcosms containing *Arthrobacter protophormiae*. It is assumed that a ratio of 1 would indicate equivalent levels of viable and nonviable biomass. Therefore, in many of the samples from both the shale and sandstone units in this study, the amount of nonviable biomass approached or exceeded the viable biomass. Once bacteria lose viability in sediment where microorganisms are active, their phospholipids are rapidly turned over (White et al., 1979). It is unlikely that the PLFA in these subsurface samples represented preserved phospholipids of nonviable bacteria, although the rate of phospholipid turnover in subsurface sediments is unknown. The recovery of low numbers of viable anaerobic bacteria suggests that PLFA measured in these subsurface samples are not preserved phospholipids of nonviable cells.

In summary, the results from this study indicate that a major factor controlling the sustained activities of bacteria in a deep shale–sandstone sedimentary sequence in northwestern New Mexico is the size distribution of pores. Metabolic activities and viable bacteria were observed in sandstone samples containing a significant volume of >0.2 μm diameter pores but were generally below detection in shale samples with little or no >0.2 μm diameter pores. A notable exception was the detection of $^{35}SO_4^{2-}$-reducing activity in some shale samples upon extended incubation. An important implication of these results is that the detrital organic matter in the small-pore-diameter shales is not subject to direct microbial attack. However, bacteria in adjacent sandstone with a more open pore structure are likely sustained for geological time scales by endogenous nutrients that are slowly released from the shale. An interesting result of this process is that anaerobic conditions are continuously maintained in the subsurface, and metals such as uranium that are present naturally or may be introduced as contaminants remain immobile. Further work is needed to unravel the detailed biogeochemical processes, probably occurring at shale–sandstone interfaces, responsible for long-term sustenance of these microbial populations.

References

Adrian, N. R., Robinson, J. A., and Suflita, J. M. 1994. Spatial variability in biodegradation rates as evidenced by methane production from an aquifer. *Appl. Environ. Microbiol.* 60:3632–3639.

Balch, W. E., Fox, G. E., Magrum, L. J., Woese, C. R., and Wolfe, R. S. 1979. Methanogens: Reevaluation of a unique biological group. *Microbiol. Rev.* 43:260–296.

Bastin, E. 1926. The presence of sulphate reducing bacteria in oil field waters. *Science* 63:21–24.

Berg, R. R. 1975. Capillary pressure in stratigraphic traps. *AAPG Bull.* 59:939–956.

Boone, D. R., Liu, Y., Zhao, Z.-J., Balkwill, D. L., Drake, G. R., Stevens, T. O., and Aldrich, H. C. 1995. *Bacillus infernus* sp. nov., an Fe(III)- and Mn(IV)-reducing anaerobe from the deep terrestrial subsurface. *Int. J. Syst. Bacteriol.* 45:441–448.

Canfield, D. E., Raiswell, R., Westrich, J. T., Reaves, C. M., and Berner, R. A. 1986. The use of chromium reduction in the analysis of reduced inorganic sulfur in sediments and shales. *Chem. Geol.* 54:149–155.

Cano, R. J., and Borucki, M. K. 1995. Revival and identification of bacterial spores in 25- to 40-million-year-old Dominican amber. *Science* 268:1060–1064.

Chapelle, F. H., and Lovley, D. R. 1990. Rates of microbial metabolism in deep coastal plain aquifers. *Appl. Environ. Microbiol.* 56:1865–1874.

Colwell, F. S., Stormberg, G. J., Phelps, T. J., Birnbaum, S. A., Mckinley, J., Rawson, S. A., Veverka, C., Goodwin, S., Long, P. E., Russell, B. F., Garland, T., Thompson, D., Skinner, P., and Grover, S. 1992. Innovative techniques for collection of saturated and unsaturated subsurface basalts and sediments for microbiological characterization. *J. Microbiol. Methods* 15:279–292.

Cusack, F., Singh, S., McCarthy, C., Grieco, J., de Rocco, M., Nguyen, D., Lappin-Scott, H., and Costerton, J. W. 1992. Enhanced oil recovery: three-dimensional sandpack simulation of ultramicrobacteria resuscitation in reservoir formation. *J. Gen. Microbiol.* 138:647–655.

Fossing, H., and Jørgensen, B. B. 1989. Measurement of bacterial sulfate reduction in sediments: Evaluation of a single-step chromium reduction method. *Biogeochemistry* 8:205–222.

Fredrickson, J. K., and Phelps, T. J. 1996. Subsurface drilling and sampling. In *Manual of environmental microbiology*, eds. G. Knudsen, L. Stetzenbach, M. McInerney, and M. Walter, pp. 526–540. Washington, DC: American Society for Microbiology.

Fredrickson, J. K., Li, S. W., Brockman, F. J., Haldeman, D. L., Amy, P. S., and Balkwill, D. L. 1995a. Time-dependent changes in viable numbers and activities of aerobic heterotrophic bacteria in subsurface samples. *J. Microbiol. Methods* 21:253–265.

Fredrickson, J. K., McKinley, J. P., Nierzwicki-Bauer, S. A., White, D. C., Ringelberg, D. B., Rawson, S. A., Li, S.-W., Brockman, F. J., and Bjornstad, B. N. 1995b. Microbial community structure and biogeochemistry of Miocene subsurface sediment: Implications for long-term microbial survival. *Mol. Ecol.* 4:619–626.

Garland, J. A., and Mills, A. L. 1991. Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilization. *Appl. Environ. Microbiol.* 57:2351–2359.

Haldeman, D. L., Amy, P. S., White, D. C., and Ringelberg, D. B. 1994. Changes in bacteria recoverable from subsurface volcanic rock samples during storage at 4°C. *Appl. Environ. Microbiol.* 60:2697–2703.

Haldeman, D. L., Penny, S. A., Ringelberg, D., White, D. C., Garen, R. E., and Ghiorse, W. C. 1995. Microbial growth and resuscitation alter community structure after perturbation. *FEMS Microbiol. Ecol.* 17:27–37.

Harvey, R. W., George, L. H., Smith, R. L., and LaBlanc, D. R. 1989. Transport of microspheres and indigenous bacteria through a sandy aquifer: Results of natural- and forced-gradient tracer experiments. *Environ. Sci. Technol.* 23:51–56.

Jenneman, G. E., McInerney, M. J., and Knapp, R. M. 1985. Microbial penetration through nutrient-saturated Berea Sandstone. *Appl. Environ. Microbiol.* 50:383–391.

Kemp, A. L. W., and Thode, H. G. 1968. The mechanism of bacterial reduction of sulphate and of sulphite from isotope fractionation studies. *Geochim. Cosmochim. Acta* 32:71–91.

Kennedy, M. J., Reader, S. L., and Swierczynnski, L. M. 1994. Preservation records of micro-organisms: Evidence of the tenacity of life. *Microbiology* 140:2513–2529.

Kieft, T. L., Ringelberg, D. B., and White, D. C. 1994. Changes in ester-linked phospholipid fatty acid profiles of subsurface bacteria during starvation and desiccation in a porous medium. *Appl. Environ. Microbiol.* 60:3292–3299.

Kieft, T. L., Fredrickson, J. K., McKinley, J. P., Bjornstad, B. N., Rawson, S. A., Phelps, T. J., Brockman, F. J., and Pfiffner, S. M. 1995. Microbiological comparisons within and across contiguous lacustrine, paleosol, and fluvial subsurface sediments. *Appl. Environ. Microbiol.* 61:749–757.

Lehman, R. M., Colwell, F. S., Ringelberg, D., and White, D. C. 1995. Combined microbial community-level analyses for quality assurance of terrestrial subsurface cores. *J. Microbiol. Methods* 22:263–281.

McKinley, J. P., Stevens, T. O., Fredrickson, J. K., Zachara, J. M., Colwell, F. S., Wagnon, K. B., Rawson, S. A., and Bjornstad, B. N. 1997. The biogeochemistry of anaerobic lacustrine and paleosol sediments within an aerobic unconfined aquifer. *Geomicrobiol. J.*, 14:23–39.

McMahon, P. B., and Chapelle, F. H. 1991. Microbial production of organic acids in aquitard sediments and its role in aquifer geochemistry. *Nature* 349:233–235.

McMahon, P. B., Chapelle, F. H., Falls, F. W., and Bradley, P. M. 1992. Role of microbial processes in linking sandstone diagenesis with organic-rich clays. *J. Sediment. Petrol.* 62:1–10.

Melas, F. F., and Friedman, G. M. 1992. Petrophysical characteristics of the Jurassic Smackover Formation, Jay Field, Conecuh Embayment, Alabama and Florida. *Am. Assoc. Petrol. Geol. Bulletin.* 76:81–100.

Morita, R. Y. 1993. Bioavailability of energy and the starvation state. In *Starvation in bacteria*, ed. S. Kjellberg, pp. 1–23. New York: Plenum Press.

Novistsky, J. A., and Morita, R. Y. 1976. Morphological characterization of small cells resulting from nutrient starvation of a psychrotrophic marine vibrio. *Appl. Environ. Microbiol.* 32:617–622.

Parkes, R. J., Cragg, B. A., Fry, J. C., Herbert, R. A., and Wimpenny, J. W. T. 1990. Bacterial biomass and activity in deep sediment layers from the Peru margin. *Phil. Trans. R. Soc. Lond. A* 331:139–153.

Parkes, R. J., Cragg, B. A., Bale, S. J., Getliff, J. M., Goodman, K., Rochelle, P. A., Fry, J. C., Weightman, A. J., and Harvey, S. M. 1994. Deep bacterial biosphere in Pacific Ocean sediments. *Nature* 371:410–413.

Phelps, T. J., Fliermans, C. B., Garland, T. R., Pfiffner, S. M., and White, D. C. 1989a. Recovery of deep subsurface sediments for microbiological studies. *J. Microbiol. Methods* 9:267–280.

Phelps, T. J., Raione, E. G., White, D. C., and Fliermans, C. B. 1989b. Microbial activities in deep subsurface environments. *Geomicrobiol. J.* 7:79–91.

Phelps, T. J., Murphy, E. M., Pfiffner, S. M., and White, D. C. 1994. Comparison between geochemical and biological estimates of subsurface microbial activities. *Microb. Ecol.* 28:335–349.

Pittman, E. D. 1992. Relationship of porosity and permeability to various parameters derived from mercury injection-capillary pressure curves for sandstone. *Am. Assoc. Petrol. Geol. Bull.* 76: 191–198.

Rilfors, L., Weislander, A., and Stahl, S. 1978. Lipid and protein composition of the membrane of *Bacillus megaterium* variants in the temperature range 5 to 70°C. *J. Bacteriol.* 135:1043–1052.

Russell, B. F., Phelps, T. J., Griffin, W. T., and Sargent, K. A. 1992. Procedures for sampling deep subsurface microbial communities in unconsolidated sediments. *Ground Water Monitor Rev.* 12:96–104.

Smith, R. L., Harvey, R. W., and Leblanc, D. R. 1991. Importance of closely-spaced vertical sampling in delineating chemical and microbiological gradients in groundwater studies. *J. Contam. Hydrol.* 7:285–300.

Stevens, T. O. 1995. Optimization of media for enumeration and isolation of aerobic heterotrophic bacteria from the deep terrestrial subsurface. *J. Microbiol. Methods* 21:293–303.

Swanson, B. F. 1981. A simple correlation between permeabilities and mercury capillary pressures. *J. Petrol. Technol.* December:2488–2504.

Tanner, R. S. 1995. Monitoring sulfide and sulfate reducing bacteria. *Fifth Int. Conf. Microbial Enhanced Oil Recovery and Related Biotechnology for Solving Environmental Problems* (pp. 353–362). Springfield, VA: National Technical Information Service.

Tunlid, A., and White, D. C. 1992. Biochemical analysis of biomass, community structure, nutritional status, and metabolic activity of microbial communities in soil. In *Soil biochemistry,* pp. 229–262. New York: Marcel Dekker.

Tunlid, A., Ringelberg, D., Phelps, T. J., Low, C., and White, D. C. 1989. Measurement of phospholipid fatty acids at picomolar concentrations in biofilms and deep subsurface sediments using gas chromatography and chemical ionization mass spectrometry. *J. Microbiol. Methods* 10:139–153.

Updegraff, D. M. 1982. Plugging and penetration of reservoir rock by microorganisms. *Int. Conf. Microbial Enhancement of Oil Recovery,* Bartlesville, OK. Washington, DC: U.S. Department of Energy.

Washburn, F. W. 1921. Note on a method of determining pore sizes in porous material. *Proc. Natl. Acad. Sci. USA* 7:115–116.

White, D. C., Davis, W. M., Nickels, J. S., King, J. D., and Bobbie, R. J. 1979. Determination of the sedimentary microbial biomass by extractable lipid phosphate. *Oecologia* 40:51–62.