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Microbial Activities in Deep Subsurface Environments

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Abstract

Activities of microorganisms residing in terrestrial deep subsurface sediments were examined in forty-six sediment samples from three aseptically sampled boreholes. Radiolabeled time course experiments assessing *in situ* microbial activities were initiated within 30 minutes of core recovery. [^{14}C -1-] Acetate incorporation into lipids, [methyl- ^3H -]thymidine incorporation into DNA, [^{14}C -2-]acetate and [^{14}C -U-]glucose mineralization in addition to microbial enrichment and enumeration studies were examined in surface and subsurface sediments. Surface soils contained the greatest biomass and activities followed by the shallow aquifer zones. Water saturated subsurface sediments exhibited three to four orders of magnitude greater activity and culturable microorganisms than the dense clay zones. Regardless of depth, sediments which contained more than 20% clays exhibited the lowest activities and culturable microorganisms.

Introduction

Early twentieth century microbiological studies did not detect large microbial populations in subsurface soils (Waksman, 1916, 1932; Starc, 1942) and for decades it was generally accepted that the role of microorganisms in the terrestrial deep subsurface was insignificant (Alexander, 1977). Evidence of deep subsurface microbial populations appeared (Buswell and Larson, 1937; Zobell, 1947; Dockins et al., 1980) but little attention was focused on the presence or significance of subsurface microorganisms. A major obstacle was overcome when sampling techniques were developed which allowed aseptic recovery of sediments (Wilson et al., 1983). Recent studies incorporating these techniques have established that aquifers and subsurface sediments contain substantial microbial populations (Wilson et al., 1983; White et al., 1983; Ghiorse and Balkwill, 1983; Chapelle et al., 1987; Fliermans, et al., in press).

Although little is known about the activities of microorganisms inhabiting subsurface sediments, recent studies have shown that a variety of microbial communities are present in subsurface terrestrial environments. Heterotrophs from shallow aquifers were investigated by Ghiorse and Balkwill (1983), and sulfate-reducing anaerobes were detected in subsurface aquifers by Dockins et al., (1980). Chapelle et al., (1987) have suggested that subsurface microorganisms may impact groundwater quality through the production of carbon dioxide.

Microbiology of groundwaters and subsurface sediments have received increased attention due to severe groundwater contamination and impending loss of substantial portions of our subsurface potable water resources (Pye and Patrick, 1983; Westerick et al., 1984). Predominant

contaminants include short-chained halogenated hydrocarbons associated with defense, dry cleaning and metal finishing industries. Classical remediation technologies may not be suitable for reclaiming contaminated aquifers and vadose zones, since subsurface contaminants are difficult to quantify, unlikely to volatilize and are often recalcitrant (Schwarzenbach and Giger, 1985). Consequently, reclamation of some subsurface habitats may necessitate on-site biological remediation. Examination of the ecology, physiological potentials, *in situ* carbon and electron flow, growth and nutrient status are prerequisites to satisfactory biological remediation of subsurface environments.

The U. S. Department of Energy (DOE) and the Savannah River Laboratory have initiated a multi-phased program that examines the microbiology of terrestrial deep subsurface environments (Wobber, 1986). These institutions chose to concentrate on the deep subsurface because: 1) fundamental scientific information about microorganisms at depths greater than 30 meters was nonexistent; 2) many of the nations largest aquifers are deep systems important for potable water and future energy production needs; and 3) deep, inaccessible subsurface environments contaminated by energy and defense production activities pose serious long term restoration challenges. The program consisted of exploratory research that focused on the presence, abundance and diversity of indigenous microorganisms and the interactions that control microbial presence and activity. This phase of the program was conducted at uncontaminated sites located within the Savannah River Plant.

This report centers on the examination of subsurface microbial activities occurring within minutes of sediment recovery. Radioisotope time course experiments measured anabolism of microbial lipids and DNA,

and catabolic mineralization of acetate and glucose in surface and forty-six subsurface sediment samples. The radioisotope experiments distinguished microbial activity between lithologic formations within a given borehole or between boreholes and was effective in evaluating activities spanning five orders of magnitude.

Materials and Methods

Description of experimental site. The Savannah River Plant (SRP) is a 768-km² limited access facility operated for the Department of Energy by the E. I. duPont de Nemours and Company. The SRP is located approximately 32km southeast of the geological Fall Line separating the Piedmont from the Coastal Plain. The site is located within the Upper Atlantic Coastal Plain on the Aiken Plateau adjacent to the Savannah River (Figure 1). Unconsolidated sediments extend to depths of about 300m and are underlain by crystalline metamorphic rock or consolidated mudstone. The sampled geologic formations beneath the SRP are shown in Figure 1. Sandy vadose and aquifer zones are dispersed between clay and silt formations. The two major confining clays retarding vertical water flow are in the Pee Dee and Middendorf formations.

Sample procurement. Professional Service Industries, Inc. (PSI) was contracted to drill and develop the boreholes. One week prior to the microbiological sampling, a continuously cored hole was placed 10m from the microbiology borehole site. The preliminary hole was used to ascertain the lithology at each site, to familiarize the sampling crews with the types of sediments and to determine the exact locations for sampling. Undisturbed microbiology sediment samples were collected only from strata that were consistent through a depth of 4 m so that adequate sediments were available for analyses.

The microbiology boreholes were completed in five to eight days with drill crews working 24 hours a day. Between sample depths, the borehole was drilled with a rotary bit and continuously flushed with recirculated sodium bentonite viscosifying drilling fluid (Quik-gel, NL Bariod/NL

Industries, Houston, TX) to maintain an open borehole. More detailed descriptions of sampling techniques, use of conservative drilling fluid tracers and quality assurance are published elsewhere (Phelps et al., in press).

Field studies. Undisturbed sediment cores were removed from the sampler and the core liner was immediately carried into the Mobile Microbial Ecology Laboratory (MMEL). Sediments were removed from the core liners using an extruding device (Model P-107, Soiltest, Evanston, IL). Sediments exiting the core liners, automatically entered a N₂ flushed glove bag constructed from polyethylene and slotted for gloves, core extruder, and sample removal port. All sample manipulations occurred in the glove bag which was alcohol wiped between samples and dismantled daily for thorough cleaning and disinfection. Using alcohol flamed sterilized tools, approximately half of the outermost portions of the sediment core was pared away before processing. After paring, the aseptic sample was placed into a flame sterilized pan, quartered, mixed and disbursed into sterile whirlpak bags. Bags were weighed, placed into quart canning jars, flushed with N₂, sealed and removed from the glove bag through the airlock. The entire operation was completed within 30 minutes from the time of collection. Subsamples were immediately available for pore-water chemistry measurements, initiation of on-site activity experiments, frozen for lipid analyses, prepared for express shipment to program participants, or archived.

Gases, chemicals, and isotopes. Nitrogen and N₂:CO₂ (90:10%) were greater than 99.9% pure. In the laboratory, all gases were passed through copper-filled Vycor furnaces (Sargent-Welch Scientific Co., Skokie, IL) to remove traces of oxygen. All chemicals used were of reagent grade

and were obtained from Mallinckrodt (Paris, KY) or Sigma Chemical Co. (St. Louis, MO). Glass distilled solvents and reagent were purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ). [^{14}C -1-]Acetate (56mCi/mmol) and [methyl- ^3H]thymidine (76.0mCi/mmol) were purchased from New England Nuclear Corp. (Boston, MA). [^{14}C -2-]Acetate (56 mCi/mmol) and [^{14}C -U-]glucose (2.8mCi/mmol) were obtained from Amersham Corp. (Arlington Heights, IL).

Analytical procedures. Sediment samples were inoculated for on-site aerobic and anaerobic activity experiments within 30 minutes of core extrusion. All isotope solutions (1-50 μCi) were frozen prior to use, thawed and transferred with gas-tight syringes (Hamilton Co., Reno, NV). Time course experiments were performed in duplicate using sterile polypropylene centrifuge tubes for aerobic incorporations and anaerobic crimp top tubes (Bellco Glass Co., Vineland, NJ) for anaerobic experiments. All incubations were at ambient temperature which was similar to the *in situ* temperature of 21° to 24°C.

Time course experiments consisted of ten incubations times. Time points of 0, 0.5 hours, 2 hours, 8 hours, 24 hours, and 96 hours, generally provided linear rates within three or more points. Experiments for acetate incorporation into lipids contained 2.0 g sediment, 5.0 μCi of [^{14}C -1-]acetate and 1.0mL sterile distilled water. Incorporations were inhibited with 3.0mL of a phosphate buffered chloroform-methanol solution. In the laboratory, acetate incorporation experiments were thawed and lipids were extracted from sediments by a modification (White et al., 1979) of the single phase chloroform-methanol method (Bligh and Dyer, 1959). The lipid extracts were evaporated to dryness, resuspended in 2.0ml chloroform and aliquots were counted by liquid scintillation counting to

determine the amount of radioactivity incorporated into microbial lipids. The earliest time points yielding measurable results were used to calculate a linear rate which was extrapolated to dpm/day.

Thymidine incorporation experiments contained 1.0g sediment, 30 μ Ci of methyl-³H-thymidine plus 2nmols of nonradioactive thymidine in 1.0mL of sterile distilled water. Incorporations were inhibited by the addition of 3.0mL 80% ethanol and samples were frozen at -20°C. Thymidine incorporation experiments were processed by thawing the reaction mixture and lysing the microorganisms with 2mL 0.3M sodium hydroxide containing 1% sodium dodecyl sulfate, 10mM thymidine and 1% humic acid. After heating at 110°C for 4 hours, the supernatants were collected and dialyzed as previously described (Moriarty and Pollard, 1982). Radioactivity incorporated into macromolecules, i.e. DNA, was determined using scintillation counting. Controls were conducted to ensure that dialysis was sufficient and to verify that radioactivity retained in the dialysis tubing (molecular weight cut off of 3500) was sensitive to DNAase and additional dialysis.

Mineralization experiments contained 2.0g sediment, 2.0 μ Ci of carrier free [¹⁴C-2-]acetate or [¹⁴C-UL-]glucose, and 1.0mL sterile water in 25mL crimp top tubes. Mineralization was inhibited with 0.5mL of 2.0M sodium hydroxide. One hour before analysis tubes were acidified with 0.5mL of 6M hydrochloric acid, and the ¹⁴CO₂ and ¹⁴CH₄ evolved during mineralization and time course experiments were examined by gas chromatography-gas proportional counting as described by Nelson and Zeikus (1974).

MPN and enumeration studies. Five tube MPN dilution series were established aerobically in screw capped tubes or anaerobically in

crimped-top tubes with medium containing 1.0g/L each of trypticase, yeast extract and glucose (TYEG) as energy sources. Single tube aerobic dilution series were also established using 0.01g/L TYEG , 0.01g/L yeast extract plus glucose, and 0.5g/L yeast extract plus 10 mmol/L methanol. All media contained trace minerals including selenium and molybdate as well as a dilute vitamin mixture and a carbonate or a phosphate buffer (Lynd et al., 1983). Experiments were incubated at ambient temperatures, which were similar to the subsurface temperatures of 21° to 24°C.

Results and Discussion

In previous studies, our laboratory examined microbial activities and biomass in subsurface sediments contaminated with trichloroethylene (Fliermans et al., 1988). Radioisotope activity measurements determined microbial activities over a range of five orders of magnitude. In this study, we examined microbial activities and culturable microorganisms in presumably uncontaminated deep subsurface sediments from three boreholes at the Savannah River Plant.

Data for microbial activities and MPN's from the sediments of borehole P-24 are shown in Table 1. Significant anabolic activity and culturable microorganisms were detected 260 m below the surface. Activity measurements based on radiolabel incorporations were calculated from linear rates of isotope accumulation from duplicate tubes at five or more time points. Acetate incorporation activities varied more than three orders of magnitude between confining layers and aquifers. Three depths (34, 146, and 239m) exhibited no acetate incorporation during time course experiments.

Thymidine measures were expressed as - to ++++ based upon the magnitude of activity above background which averaged 900 dpm/day. Thymidine analyses showed large deviations between replicates but were useful as a qualitative measure of microbial activity. Thymidine incorporation experiments generally agreed with acetate activities in that zones which did not incorporate acetate incorporated little thymidine. Similarly, those zones which incorporated the greatest amount of acetate were also high for thymidine activity. Sandy sediment materials at a depth of 92m gave the maximum subsurface acetate and thymidine incorporation rates as well as the largest MPN densities, while confining clay sediments at 164m exhibited the lowest activities and MPN. In all boreholes examined, surface soil samples exhibited the greatest MPN and radiotracer incorporation activities followed by the shallow Congaree formation sediments. Lowest activities and MPN densities were always associated with confining clay zones. Depth did not appear to determine the subsurface biomass or radiotracer incorporation rates, since zones deep beneath the surface exhibited large and active microbial communities.

Mineralization of acetate and glucose were performed under both aerobic and anaerobic conditions as time course experiments at selected depths (Table 2). Anaerobic activity was never significantly greater than the aerobic activity and radioactive methane was never detected. These findings agreed with chemical analyses which suggested that these subsurface environments were aerobic to microaerophilic and that methanogenesis was not significant (Fliermans et al., submitted). Similar to the radiolabeled incorporation studies, high rates of mineralization were observed at 59, 112, 182 and 261m (Table 2). Confining clay layers and some unsaturated zones (71, 146 and 205m) exhibited the lowest

mineralization rates. Considerable mineralization of glucose and acetate were observed in the deepest formations. Scarcity of pore waters for pool size determinations impeded efforts to compare acetate turnover rates between subsurface formations.

Three formations, the Ellenton silt, a shallow unsaturated zone with >20% silt and clay, the Pee Dee variegated clays and the Middendorf middle clays with >50% clays, consistently exhibited less activity and less culturable biomass. As shown in Table 3, only one of nine samples from these formations displayed acetate incorporation rates greater than 1000 dpm/day. Previous studies that examined the Ellenton silt and Pee Dee clay corroborated these findings (Phelps et al., in press). The results from P-29 at 112m are likely the result of process contamination since it required three persons three hours to extrude and process this dense and compacted clay. Thymidine incorporation values were more variable yet low for the confining clay zones.

Culturable microorganisms were three to five orders of magnitude less in the confining layers than in shallow aquifers or surface soils. Other investigators reported substantially lower culturable numbers in confining formations than those reported here (Fliermans, et al., in press). Several factors may account for the differences. First, the MPN values reported here are from enrichments performed in the field after priority on-site functions were completed or in some cases, the MPN's were initiated four weeks later at our home laboratory. Such delays likely caused growth of some populations and death of others, consequently we view these MPN results as enrichment values indicative of the samples and refer to the investigator's whose tasks were prioritized towards enumerations for greater detail (Fliermans et al., submitted). Secondly, there were large

differences within subsamples. Within any subsample patchiness of moist and dry sands, silts or clays can occur. Finally, our enrichments began with one gram inocula and subsequent dilutions whereas some investigator's started enumerations at 10^{-2} dilution. Nonetheless, it was obvious that samples with the lowest culturable microorganisms and activities were from sediments with the highest clay content.

Water bearing formations contained the most culturable microorganisms and the greatest activities as shown in Table 4. Surface soils, which recharged the shallowest aquifers contained microbial populations of $10^7/\text{gdw}$ whereas sediments from subsurface aquifers contained culturable bacteria at 10^5 to $10^7/\text{gdw}$. Surface soils at each site exhibited radiolabel incorporation rates greater than 10^6 dpm/day for both acetate and thymidine. Sediments from the Congaree aquifer displayed the highest activities of the subsurface formations, although they were an order of magnitude less than surface soils. These data agreed with previous studies of Congaree sediments (Fliermans et al., submitted; Phelps et al., 1987) in that activities were an order of magnitude less than surface soils yet greater than the deeper aquifers used for agricultural and potable waters. Fully developed wells in the Congaree produced 400-1,000L/min whereas wells developed in the Black Creek aquifer produced 40,000L/min (Fliermans et al., submitted). Culturable microorganisms and significant microbial activities were assessed in 200m deep sediments from the Middendorf formation. All Middendorf samples examined in this study were aerobic to microaerophilic (Hazen et al., unpublished results). It was not clear whether microbial activities observed in the Middendorf aquifer were sufficient to remove oxygen, but it appeared that these waters had not been recharged with respect to oxygen for considerable

periods of time (Bledsoe, personal communication). The detection of isotope incorporation and mineralization activities within minutes of sediment recovery suggested that microorganisms residing in the deep subsurface were poised for microbial metabolism. Sampling and processing may have resulted in an increased rate of isotope utilization over time (White et al., 1985), since the rate of acetate incorporation into lipids increased more than three fold within 24hr in four of eight Middendorf aquifer samples (data not shown). Thus, the observed increase in activity by deep subsurface microorganisms may have been an overestimate of the absolute activity. It is notable that water bearing sediments from greater than 200 m exhibited more culturable microorganisms and greater activities than silt and clay layers much closer to the surface (Tables 3 and 4).

Initial evidence suggested that moisture content and sediment texture were major determinants of subsurface microbial populations. Independent of depth, sediments were separated into groups based on sediment texture and qualitative measures of moisture availability. Sediment textures were of two major types of particle distributions, either greater than 20% clays or greater than 70% sands. Moisture content of sandy samples was a qualitative determination. Samples were considered greater than 70% saturated with water if they were recorded as being saturated at the time of recovery or if the initial sediment moisture content was 70% of the saturated moisture content used for pore water extraction procedures. Eleven samples contained greater than 20% clays, fourteen were unsaturated sands, while 20 were judged as being more than 70% saturated. The data in Figure 2 show the relationship between sediment texture and moisture content with respect to microbial activities

and MPN data. Regardless of depth, sediments containing greater than 20% clay averaged less than 100 dpm/day [^{14}C]-acetate incorporation into lipids and MPN values of $10^3/\text{gdw}$. Saturated and unsaturated sandy sediments averaged approximately 10^5 bacteria per gram but saturated sands averaged ten times greater acetate incorporation into lipids. There were six samples which showed no incorporation of ^{14}C -acetate into lipids, four of which were clays and none of which were saturated sands. There were twelve samples which incorporated acetate at rates greater than 10^5 dpm/day; ten of which were saturated sands while none were clays. Culturable biomass followed similar trends, in that clays consistently exhibited lower biomass than sands regardless of depth. These results suggested that although many sandy sediments contained similar microbial populations, those saturated with water exhibited greater activities. In contrast, sediments containing greater than 20% of fine clay particles revealed less culturable biomass and activity.

Although previous subsurface studies have reported the presence of microorganisms, it was only recently suggested that microorganisms may alter groundwater chemistry (Chapelle et al., 1987). Harvey et al. (1987) demonstrated that microorganisms could be transported through subsurface materials, thus providing a source for inocula. This and collaborating works have demonstrated an abundant microflora deep beneath the surface. This research has shown that subsurface microorganisms are metabolically active. They possess the ability to mineralize acetate and glucose as energy sources, and to assimilate acetate into lipid fatty acids or thymidine into DNA within minutes or hours of incubation initiation.

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Table 1

Microbial Activities and MPN's from Deep Subsurface Sediments of
Borehole P-24

Depth (m)	^{14}C -Acetate into Lipids (dpm/day) ¹	^3H -Thymidine into DNA (dpm/day) ²	MPN (log/gdw)
0.0	3.00×10^7	++++	7
34	0.0	-	7
45	2.45×10^4	-	4
58	3.00×10^3	-	6
92	6.26×10^5	+++	7
119	1.20×10^3	+++	4
140	9.30×10^3	++	2
146	0.0	-	2
182	5.00×10^3	++	3
202	5.18×10^4	+++	3
205	3.50×10^2	+++	2
239	0.0	+	3
247	4.02×10^4	++	4
257	4.30×10^2	++	4
261	1.02×10^5	+++	5
264	2.54×10^2	nd	4

nd. not determined.

1 Data recorded as dpm/day calculated from linear rates of isotope accumulation during time course experiments.

2 linear rates less than $10^3/\text{day}$ = -; $<10^4$ = +; $<10^5$ = ++; $<10^6$ = +++; $>10^6$ = ++++.

Table 2

Aerobic and Anaerobic Mineralization of ^{14}C -Acetate and ^{14}C -glucose to $^{14}\text{CO}_2$ in Subsurface Sediments from Borehole P-24.

Depth (m)	dpm/day $\times 10^3$			
	Aerobic		Anaerobic	
	Glucose	Acetate	Glucose	Acetate
45	291 $\pm$ 165	678 $\pm$ 60	nd	nd
58	66 $\pm$ 36	70 $\pm$ 41	67 $\pm$ 29	51 $\pm$ 4
59 ¹	720 $\pm$ 72	165 $\pm$ 54	nd	nd
71 ¹	17 $\pm$ 12	9 $\pm$ 8	38 $\pm$ 29	11 $\pm$ 4
112 ¹	792	720	nd	nd
146	92 $\pm$ 25	18 $\pm$ 6	nd	nd
182	720 $\pm$ 10	436 $\pm$ 99	nd	nd
205	49 $\pm$ 33	12 $\pm$ 8	35 $\pm$ 2	7 $\pm$ 3
247	120 $\pm$ 62	660 $\pm$ 220	61 $\pm$ 43	16 $\pm$ 3
257	203 $\pm$ 72	52 $\pm$ 47	17 $\pm$ 2	12 $\pm$ 3
261	864 $\pm$ 192	1065 $\pm$ 288	132 $\pm$ 51	1036 $\pm$ 4

nd. not determined.

¹Data are from borehole P-28 and correspond to sediment lithologies at depths of 92, 119, and 146m, respectively, in P-24.

Table 3

Microbial Activities and MPN's in Subsurface Confining Layers

Description	Borehole	Depth (m)	¹⁴ C-Lipids (dpm x 10 ³ /day)	³ H-DNA (log/gdw)	MPN (log/gdw)
Ellenton silt	24	119	1	207	4
Ellenton silt	28	72	1	180	3
Ellenton silt	29	70	0	67	3
Pee Dee clay	24	146	0	0	2
Pee Dee clay	28	115	0	88	3
Pee Dee clay	29	112	34	55	2
Middendorf	24	239	0	7	3
middle clays	28	184	1	0	4
	29	183	0	18	2

Table 4

Microbial Activities and MPN's in Water Bearing Formations

Description	Borehole	Depth (m)	¹⁴ C-Lipids (dpm x 10 ³ /day)	³ H-DNA	MPN (log/gdw)
Surface	24	0	3000	4665	7
Surface	28	0	1128	2920	7
Surface	29	0	2602	3375	6
Congaree	24	92	626	281	7
Congaree	28	59	167	23	6
Congaree	29	39	606	625	6
Black Creek	24	202	52	73	5
Black Creek	28	164	192	140	3
Black Creek	29	152	199	0	5
Middendorf	24	261	102	33	5
Middendorf	28	217	104	248	5
Middendorf	29	201	12	24	4

Figure 1. Location of Deep Core Sampling Sites on the Savannah River Plant.

Figure 2. Influence of particle size and moisture content on microbial activity and biomass.

FIGURE 1. Location of Deep Core Sampling Sites on the Savannah River Plant

FIGURE 2.