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Combined microbial community-level analyses for quality assurance of terrestrial subsurface cores☆

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Abstract

Bacterial communities from surface soils, groundwater, drilling muds and deep subsurface cores were profiled by sole carbon source utilization and by phospholipid ester-linked fatty acid analysis. The combination of these functional and structural methods successfully distinguished communities from disparate origins. Multivariate analysis of the data showed good agreement between the results of the two methods. Subsurface communities tended to respire amino acids over carbohydrates and demonstrated preferential use of individual compounds such as acetate and Tween as sole carbon sources. PLFA profiles indicated that the groundwaters predominately contained gram negative aerobic heterotrophic populations, the drilling muds and cuttings were populated by gram negative anaerobes and the core communities were composed of anaerobic gram negative bacteria and gram positive bacteria. The utility of this approach as a component of quality assurance of core samples obtained for microbiological analysis during mud rotary coring was demonstrated. Monitoring of controlled bioprocesses, environmental remediation and detection of environmental disturbance are some of the numerous potential applications for these community-level characterization methods. Since combined analyses such as these can simultaneously provide specific information about individual community members and about community-level function, it is hoped that these methods will prove useful in answering fundamental questions in microbial ecology, such as the relationship between in situ community structure and its measurable function.

Keywords: Community-level; Drilling mud; Phospholipid fatty acid; Quality assurance; Sole carbon source; Subsurface

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1. Introduction

Interest in subsurface microbiology is expanding due to deterioration of groundwater supplies [1]. Bioremediation of aquifer contamination may be an attractive option at some sites. Indigenous microorganisms are viewed as candidates for stimulation while bioaugmentation of the subsurface microflora with specialized organisms may prove valuable. In order to effectively stimulate subsurface microorganisms to degrade a target contaminant or to estimate the interactions between added organisms and native populations, basic knowledge concerning the distribution and activity of subsurface microorganisms is required. In other subterranean habitats, the activities of microbes are known to affect the efficiency and quality of fossil fuel extraction [2] and to pose uncertainty to the stability of high-level nuclear waste stored in geological repositories [3]. Additional motivation to investigate the microbiology of the subsurface is provided by the potential of an untapped reservoir of genetic information and phenotypic expression that may be exploited for controlled bioprocessing and other biotechnological applications.

In 1986, the Department of Energy (DOE) initiated the field component of a program to systematically investigate the microbiology of deep subsurface environments with particular emphasis on DOE sites [4]. Traditional drilling and coring techniques were modified, tracers were deployed in drilling fluids and via coring tools and sample processing protocols were developed to allow recovery of microbiologically defensible samples from deep terrestrial habitats [5–7]. Ionic, liquid, gaseous and particulate tracers have been used to assess different routes of potential contaminant migration into cores; examples of successful tracers are bromide, rhodamine, perfluorocarbons, and fluorescent microspheres, respectively [5–7]. Innate chemical properties of drilling muds such as sulfate or ammonium ions have also served as conservative tracers [5,6]. Autochthonous microorganisms have been used to supplement chemical tracers. Organisms are not added (usually restricted by regulation) but the numbers and physiological types of organisms present in the drilling fluids and surface soils versus the core samples have been contrasted [5,6,8–10]. On one occasion, a specific group of organisms [non-fecal coliforms] that could be easily monitored was present in surface soils and subsequently enriched in drilling muds and therefore served as a fortuitous indicator of sample contamination [11]. In cores taken at the Savannah River Plant (SRP), the use of microbial community level analyses to compare cores with potential sources of contamination (e.g., drilling fluids, surface soils) was demonstrated by White et al. [12]. The phospholipid ester-linked fatty acid (PLFA) profile of the microbial membrane lipids extracted from drilling fluids was distinct from that extracted from sediments, suggesting little overlap between the two communities [12]. The results of this culture-independent analysis complemented isolate-based measurements and served as supporting evidence that the samples were not grossly compromised by organisms present in the drilling fluids.

In core sampling events at the SRP, Idaho National Engineering Laboratory (INEL) and the Hanford Reservation, drilling and coring was performed with the

specific purpose of obtaining samples for microbiological (with supporting physical and chemical) analyses. These dedicated boreholes allowed flexibility in the deployment of chemical and particulate tracers to ensure sample integrity. In February, 1992, investigators in DOE's Subsurface Science Program were able to receive samples from a 3100 m deep exploratory borehole drilled by Texaco, USA, Inc. in northeast Virginia. The use of fluorescent microspheres as particulate tracers was prohibited due to interference with analysis of cuttings and conservative ionic tracers such as potassium bromide and iodide were not feasible due to the quantities required to exceed background levels in the muds or formation waters in this coastal area. Alternative additions to the drilling muds such as deuterated water or specific organic compounds were eliminated due to the high cost associated with the large volume of mud circulating through the borehole or potential regulatory conflicts. Limited use of perfluorcarbon tracers (PFTs) was supplemented by intensive sampling of the drilling muds, drilling mud make-up waters and surface soils at the site in order to contrast the microbial communities in these potential sources of contamination with microbial communities found in core samples. PLFA analysis of cell membrane lipids extracted from sample matrices was used to generate profiles that reflect the microbial community structure [13]. This structural analysis was compared with a functional fingerprint of the heterotrophic communities based on community-level sole carbon source testing of sample extractants [14]. The objective of these two analyses was to provide information about the microbial communities in the different samples that could be used to determine if the core samples were grossly contaminated with microorganisms originating in drilling muds or surface locations.

2. Materials and methods

2.1. Site description

Cores were obtained from the Thorn Hill no. 1 exploration well drilled by Texaco's Frontier Exploration Department, in King George County, Virginia, USA (Fig. 1). Thorn Hill no. 1 was drilled into the Taylorsville Triassic Basin that formed during continental rifting approximately 230 million years ago. Typical basin sediments were deposited as fluvial and lacustrine sands, silts, and clays. Evidence suggests that lacustrine depositional conditions were saline, anoxic and circumneutral [15]. This material is presently characterized by black shales, sandstones and siltstones (B. Russell, personal communication). The average porosity of the formation is 2% or less (T.C. Onstott, personal communication) with a permeability on the order of 100 microdarcies [16]. Due to the low permeabilities and stratigraphic seals, it is likely that the basin has experienced limited groundwater transport suggesting an age for the groundwater in the tens of millions of years. Current porewater is circumneutral, reducing and ca. 2% (wt) NaCl (T.C. Onstott, personal communication). More detailed

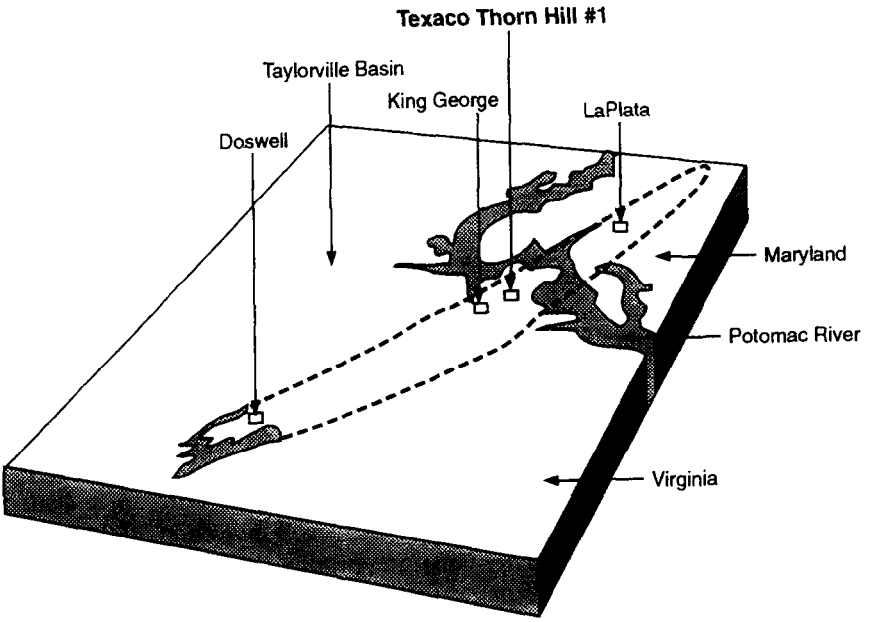

Fig. 1. Location of Taylorsville Basin and Texaco Thorn Hill no. 1 exploratory well (courtesy Golder and Associates).

information on the geology [17,18] and hydrology [16] of the sampling location is available.

2.2. Sample acquisition

The well was drilled to a total depth of 3113 m using conventional mud rotary drilling. Samples of the drilling fluids and makeup waters were collected frequently as the borehole was deepened and whenever a change in lithology was noted in the cuttings. Drilling muds were sampled at the inflow to the first shaker and make-up waters were withdrawn from a well installed in the local aquifer at ca. 130 m. Surface soil samples were collected from a depth of 7 cm at the start of field activities at an undisturbed location proximal to the drill site. Sidewall cores were collected with both rotary sidewall (7.6 × 1.9 cm) and percussion coring tools (5.1 × 1.9 cm) in April 1992 when the borehole attained total depth. The cores were taken from the Lower Falling Creek Member of the Doswell formation between 2621 and 2804 m below ground surface. Black shale and sandstone were the dominant lithologies at the sampling interval. Core samples were pared and crushed under argon atmosphere to reduce sample contamination from drilling fluids and surface materials while limiting access of oxygen to the samples [5]. Processing of rotary sidewall cores resulted in three types of samples: outer parings (CS), inner parings (RP) and inner core sample (RS). Outer parings and inner core for percussion core samples were designated PP and PC,

respectively. In the glove bag, small amounts (1–15 g) of processed core samples were sealed in sterile bags and the bags deposited in argon-flushed mason jars. Blank cores prepared by repeated overnight combustion of Berea sandstone at 550°C to eliminate organic material were processed in an identical manner to authentic samples. After processing, samples were either placed on ice or frozen and placed on ice (PLFA) and sent to investigators by overnight mail. Upon receipt at the laboratory, samples were immediately refrigerated (4°C) or frozen (−20°C) until analysis.

2.3. Community-level physiological profile (CLPP)

Microbial communities from the samples were submitted to sole-carbon source utilization testing [14] to produce a community-level physiological profile (CLPP) for each sample. This method utilizes the Biolog GN microplate (Biolog, Inc., Hayward, CA), a microtiter plate composed of 95 wells each containing a different sole carbon source (and nutrients) and a 96^{th} well with no carbon source (control). Oxidation of the carbon source is indicated by reduction of 2,3,5-triphenyl tetrazolium chloride (TTC) to colored, insoluble formazan [19]. A relatively clear, carbon-free inoculum is required for accurate results. In the case of aquifer waters, whole samples were inoculated directly into the microplates. For solid samples, two to twenty-five grams of material were blended with 100 ml of 0.1% sodium pyrophosphate (two 30 s blending intervals with 30 s rest in between) [20]. The use of this extractant [20,21] and this blending regime [20] has been shown to result in high yields of viable cells from soils and sediments. Following blending, sample slurries were transferred to 250-ml Erlenmeyer flasks and placed on a controlled temperature shaker (22°C) at 150 rpm for 24 h to maximize desorption of bacteria from particles. Previous studies have shown enhancement of biomass in subsurface sediments by prolonged hydration prior to assays [22,23]. Since this method of physiological profiling requires some critical amount of activity to obtain a signal, this enrichment was performed but without added carbon or nutrients that may cause marked shifts in the community [14]. Following the 24 h contact period with the extractant, suspensions were decanted to 100-ml graduated cylinders and flocculated with 0.5 g of an 8:5 mixture of $CaCl_2.2H_2O$ and $MgCO_3$ salts [24] for 2 h. This procedure has been used successfully by other researchers for CLPP on soils [14,25] and fatty-acid methyl ester (FAME) analysis of the flocculent has indicated that little of the bacterial community was lost from the extractant in agricultural soils [25]. Drilling muds resisted settling and were subsequently centrifuged at 1000*g* for 10 min to produce a cell-containing supernatant that was inoculated into the microplates. It is recognized that viable cell recovery from solids will vary with soil/sediment type. It was assumed that a representative sample of the dominant members of the bacterial community was obtained by these methods.

Duplicate microplates for each sample were incubated aerobically in the dark under humidification at 22°C. Color development in the plates was monitored at

24 h intervals up to 1 week by measurement of absorbance at 590 nm using a microplate reader and Microplate Manager Software (Biorad, Inc., Hercules, CA). The raw data was then exported to spreadsheet software for further manipulation. The raw absorbance data for each reading was converted to net absorbance data by subtracting the control well reading. The average well color development (AWCD) for each plate reading was calculated by averaging all 95 responses. For each sample, the AWCD for both replicates was graphed versus time revealing a classic sigmoidal growth curve. In order to account for variations between samples in lag time prior to color development and rate of color development, the plate reading closest to the peak of linear color development was chosen for each sample; these 95 values were normalized by dividing by the AWCD for this plate reading. The normalized data for all 95 carbon sources for each sample replicate was analyzed by principal components analysis (PCA) and cluster analysis. Identical analyses were performed on groupings of the 95 sole carbon sources into 11 variables reflecting the different carbon source types (by averaging responses): carbohydrates, amino acids, carboxylic acids, polymers, amines, amides, phosphorylated compounds, brominated compounds, aromatics, esters, and alcohols. Approximately 75% of the 95 carbon sources fall into one of the first three groups (carbohydrates, amino acids, or carboxylic acids). PCA was performed on the covariance matrix followed by varimax rotation of the derived components. Exclusive, hierarchical clustering was performed on the correlation matrix using the complete linkage method with the Pearson correlations as the basis for dissimilarities. All statistical analyses for CLPP data were performed with the Systat software package (Systat, Inc., Evanston, IL).

2.4. Ester-linked phospholipid fatty acid analysis of microbial communities

Following overnight delivery of frozen samples, the lipids were extracted in a single-phase, chloroform-methanol system (Bligh and Dyer technique [26]) modified to include a phosphate buffer [27]. Samples were extracted at room temperature using the microtechnique (28). The total lipid extract was fractionated on silicic acid columns into neutral, glyco- and polar lipids [29]. The polar lipid fraction, recovered in methanol, was transesterified by mild alkaline methanolysis [27], and the resultant methyl esters separated, quantified, and identified by capillary gas chromatography mass spectrometry (GC/MS). A Restek RT_x-1 (nonpolar methyl silicone) capillary column (60 m × 0.25 mm ID × 0.1 mm film thickness) was used to separate the fatty acid esters on a Hewlett-Packard 5890 Series II GC equipped with a HP5971 mass selective detector. The MS was operated in the positive ion electron impact mode with a single ion monitoring program which scanned for the base peaks of saturated, monounsaturated and polyunsaturated fatty acid methyl esters at an electron energy of 70 eV. The compounds were introduced using a 1 μl splitless injection (100 ml min^{-1} after 0.5 min) with a column flow rate of 1 ml min^{-1} using helium as the carrier gas. The column temperature was programmed with an initial temperature of 100°C and increased 10°C min^{-1} to 150°C, held 1 min and

increased to 282°C at 3°C min^{-1} and maintained at this temperature for 5 min. The injector temperature was maintained at 270°C and detector temperature at 290°C. Phospholipid ester-linked fatty acid methyl esters were identified based on the relative percentages of the ions scanned and by comparison of retention times to those obtained from standards. Fatty acids were designated by the total number of carbon atoms: number of double bonds with the position of the double bond closest to the methyl end (w) of the molecule. Configuration of the double bonds is indicated as *cis* (c) or *trans* (t). For example, 16:1w7c is a PLFA with 16 total carbons with one double bond 7 carbons from the methyl end in the cis configuration. Branched fatty acids are designated as *iso* (i) or *anteiso* (a) if the methyl branch is one or two carbons respectively from the w end (ex-i15:0) or by the position of the methyl group from the carboxylic end of the molecule (ex-10me16:0). Methyl branching at undetermined positions in the molecule is indicated as br. Cyclopropyl (cy) fatty acids are designated by the total number of carbons (ex-cy17:0). The PLFA profiles, as molar percentages were treated as multivariate data and analyzed using hierarchical cluster analysis and PCA using the PC-based software package, Ein*Sight (Infometrix; Seattle, WA). Capillary grade GC/GC/MS solvents were obtained from Burdick and Jackson (McGraw Park, IL). Reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI), Sigma Chemical Co. (St. Louis, Mo.) and Pierce Chemical Co. (Rockford, IL).

3. Results

3.1. CLPP

A total of 23 samples were analyzed: 1 surface soil, 5 drilling mud make-up waters from the 130 m deep well, 6 drilling muds circulated to depths ranging from 1725 m to 2798 m, 2 drilling muds taken from core sampling tools upon core retrieval, 7 cores or core parings, and 2 control cores. Aquifer (make-up water) communities showed rapid oxidation (within 24 h) of a wide variety of sugars, amino acids, and carboxylic acids and eventually respired about 90% of the carbon sources offered. Surface soil communities oxidized nearly all 95 carbon sources fairly rapidly (within 48 h). Drilling mud communities oxidized substrates at a slower rate but were eventually able to utilize about three-quarters of the 96 substrates within 144 h of inoculation. Extractants from five of the seven core or core parings did not oxidize any of the carbon sources. These five samples were all obtained by rotary sidewall coring. Communities from two samples obtained by percussion sidewall coring were able to oxidize from 30 to 50% of the 95 carbon sources after a 2 wk incubation period. Both control cores were negative. Top scoring carbon sources averaged and arranged by sample media type are listed in Table 1.

PCA of the normalized data from all positive samples distinguished between communities from the groundwaters, drilling muds and cores based on their

Table 1
Top fifteen sole carbon sources respired by communities the four sample types

Surface soils	*Ground waters*
L-Asparagine	L-Proline
L-Proline	D-Glucose
L-Fucose	*m*-Inositol
Turanose	D-Fructose
D-Trehalose	D-Sorbitol
D-Sorbitol	L-Glutamic acid
L-Glutamic acid	Sucrose
D-Fructose	D-Galactose
cis-Aconitic acid	L-Aspartic acid
D-Glucosaminic acid	L-Pryroglutamic acid
D-Lactose	Tween 40
L-Rhamnose	L-Asparagine
D-Galactose	L-Leucine
Gentiobiose	D-Mannitol
Maltose	Xylitol
Drilling muds	*Percussion core*
L-Asparagine	L-Asparagine
L-Proline	L-Glutamic acid
L-Aspartic acid	L-Alanine
L-Glutamic acid	L-Aspartic acid
Hydroxy-L-proline	D-Alanine
cis-Aconitic acid	L-Proline
L-Pyroglutamic acid	Citric acid
Itaconic acid	*cis*-Aconitic acid
Quinic acid	Aminobutyric acid
Tween 40	Tween 80
Aminobutyric acid	α-keto-glutaric acid
D-Saccharic acid	L-Histidine
Tween 80	Tween 40
β-Hydroxybutyric acid	Bromosuccinic acid
L-Alanine	D-Saccharic acid

Carbon sources arranged in descending order with most-utilized at head of list.

response to the 95 sole carbon tests (Fig. 2). Although the surface soils grouped with the groundwaters, these two communities can be easily resolved by analysis of a dataset that includes only these two sample media (data not shown). In this diagram (Fig. 2), 29.9% of the total variance in the dataset was accounted for by factor 1 and 17.7% by factor 2, meaning that a total of 47.6% of the variance in the data was retained while the dimensionality of the dataset was reduced from 95 to 2. Carbon sources exhibiting a strong negative correlation with principal component 1 indicating preferential use by core sample microorganisms were (correlation coefficients in parentheses): acetate (−0.74), Tween 40 (−0.82) and 80 (−0.79), citric acid (−0.85), propionic acid (−0.84), bromosuccinate (−0.74), and several amino acids: D-alanine (−0.72), L-alanine (−0.72), L-proline (−0.81), L-aspartic acid (−0.94), L-glutamic acid (−0.95) and L-asparagine (−0.95).

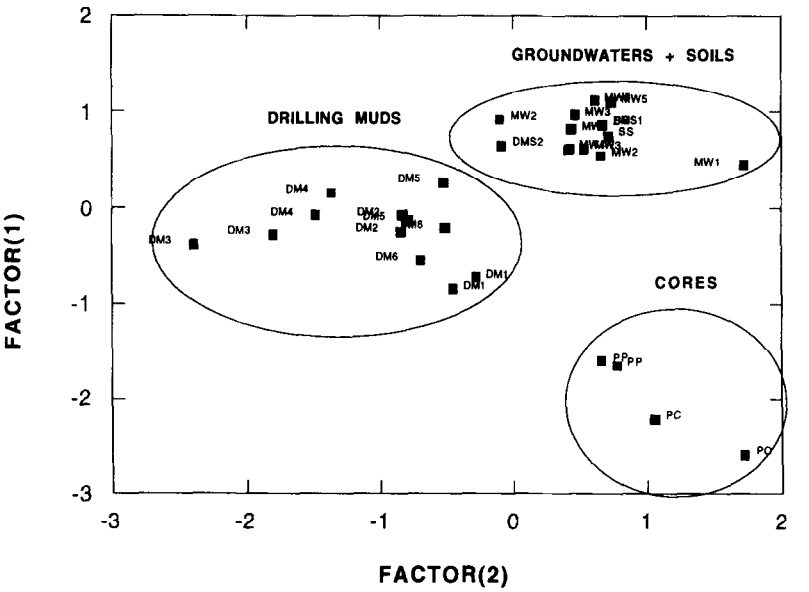

Fig. 2. Plot of Factor 1 versus Factor 2 scores from principal components analysis of CLPP data (respiration of 95 sole carbon sources) distinguished between samples based on origin. SS = surface soil; PC = percussion core; PP = percussion core parings; MW = drilling mud make-up water; DM = circulating drilling mud; DMS = drilling mud taken from core sampling tools. DM, DMS, and MW designations are followed by integer indicating sample number; identical sample numbers signify duplicate analyses.

Cluster analysis (doesn't assume statistical distributions) on this same dataset showed a similar result as the PCA (figure not shown). Plotting of the normalized data (prior to PCA) by sample type per carbon source showed that communities from the drilling mud and core samples had a strong affinity for acetate and Tweens 40 and 80 compared to the surface soil and groundwater communities (data not shown).

When the 95 carbon sources were grouped into 11 carbon source groups and the PCA repeated, the samples were again separated by origin (data not shown). This result reinforced the PCA on all 95 substrates and provides some structure to explain the differences between samples. Factors 1 and 2 accounted for 47.3% and 15.8% of the total variance in the dataset, respectively. Two carbon source groups were more strongly correlated with principal component 1 than the other groups: carbohydrates (0.85) and amino acids (−0.87). Plotting the normalized response to these substrate groups showed that sample types could be separated solely on the basis of their relative use of amino acids and carbohydrates (Fig. 3). Communities from the cores showed a near complete inability to oxidize any of the carbohydrates but respired a number of amino acids to great extent.

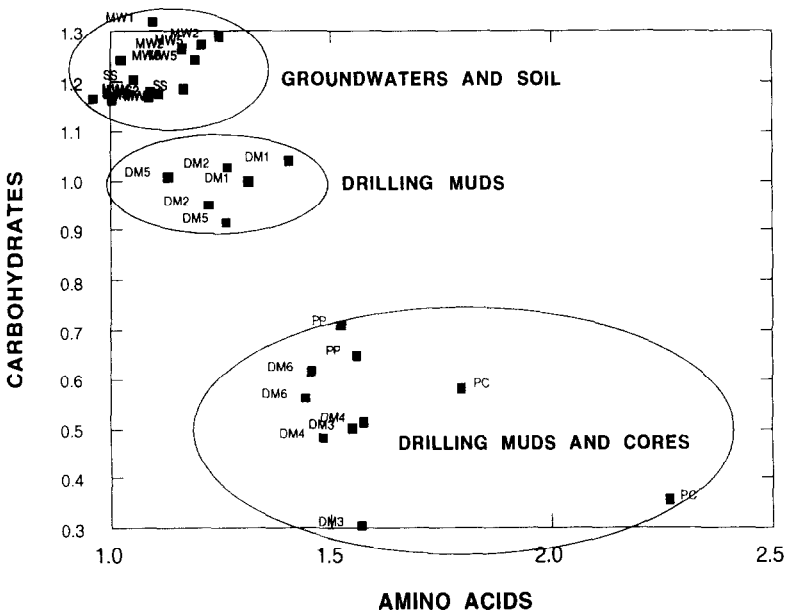

Fig. 3. Sample media type plotted based on their averaged relative use (absorbance units) of 28 carbohydrates and 20 amino acids as sole carbon sources. SS = surface soil; PC = percussion core; PP = percussion core parings; MW = drilling mud make-up water; DM = circulating drilling mud; DMS = drilling mud taken from core sampling tools. DM, DMS, and MW designations are followed by integer indicating sample number; identical sample numbers signify duplicate analyses.

3.2. PLFA analysis

A total of 31 samples were analyzed for PLFA, including one surface soil collected from an area adjacent to the drilling site. Drilling muds contained the greatest concentration of PLFA per gram of all the different sample types analyzed, an average of 124 pmol PLFA gram^{-1}. Cuttings and make-up waters contained an order of magnitude less, an average of 33 and 12 pmol PLFA gram^{-1}, respectively. Outer and inner parings averaged 7 and 13 pmol PLFA gram^{-1}, respectively. Concentrations of PLFA at the center of the rotary sidewall core, 16 pmol PLFA gram^{-1}, collected from the 2798 meter depth were greater than those observed in this core's inner and outer parings (10 and 1 pmol PLFA gram^{-1} respectively) as well as being an order of magnitude greater than the average levels observed in the combusted sandstone blanks, an average of 2.5 pmol PLFA gram^{-1}.

Relative proportions of PLFA detected in each sample are given in Table 2. The surface soil contained the most diverse PLFA profile indicating a diverse microbial community to be present. The PLFA detected included: terminally branched saturated PLFA indicating either a gram positive or obligate anaerobic gram negative bacterial input, monoenoic PLFA synthesized by either the aerobic (w9) or anaerobic desaturase pathways (w7) and found in gram negative

Table 2
PLFA profiles (expressed as mol%) recovered from different subsurface and drilling operation samples

	SS	DM	MW	CU	CS	RP	RS	CB
14:0					7	3	2	4
15:0		1			3	1	3	3
16:0	14	23	25	22	37	34	40	41
17:0		4		3	4	2	2	3
18:0	3	14	5	16	25	33	23	12
20:0		2		2	3	3	5	5
21:0					1	1		
22:0						1	3	2
24:0						2	3	2
Total	17	44	30	42	80	81	81	72
i14:0								1
i15:0	10	7		4				
a15:0	6	1					1	1
i16:0	3	3		1			2	
i17:0	2	6		5				
a17:0/17:1w8c	2	2		1			2	
i18:0/18:3w3							2	1
Total	23	20		11			7	3
16:1w7c	8	4	22	4				
16:1w7t			2					
16:1w5c	5							
cy17:0	2	4	1	3				
18:1w9c	8	4	10	8	9	8	6	11
18:1w7c	12	8	25	13		1	2	2
18:1w7t					1			
cy19:0	6	5		6				
i17:1	4							
Total	45	24	61	34	10	9	7	13
10me16:0	6	1						
10me17:0		1		1				
br18:0	2							
10me18:0		2		3				
Total	9	4		4				
18:2w6	2	2	2	2	5	5	1	8
Trace Compounds	4	5	7	7	6	5	3	4
Total mol%	100	100	100	100	100	100	100	100

SS, surface soil; DM, drilling mud; MW, make-up water; CU, cutting; CS, outer paring; RP, inner paring; RS, rotary core sidewall; CB, combusted blank.
Each value represents a mean for a given sample type, *n* varies.

heterotrophs, mid-chain branched saturates and terminally branched monoenoics were also detected indicating a likely input from actinomycetes into these aerobic surface soils ([30]; Ringelberg and White, unpublished data]. The microbiota of

the make-up waters showed PLFA patterns typical of gram negative heterotrophic bacteria like the pseudomonads as indicated by the high proportions of 16:1w7c (>24%) and 18:1w7c (>14%). The muds also contained a variety of PLFA but in proportions different from those observed in the surface soils. Percentages of terminally branched saturated PLFA were decreased with respect to the surface soil and contained greater percentages of the *iso* vs the *anteiso* configuration. Within the prokaryotes, terminally branched saturated PLFA in the *iso* configuration have been shown to be greater in gram negative obligate anaerobes such as the sulfate reducing bacteria (SRB) [31,32]. In contrast, *anteiso* PLFA have been observed at greater concentrations in gram positive species such as *Arthrobacter* [33]. A number of thermophilic bacteria have also been shown to contain *iso* and *anteiso* PLFA with the *iso* configuration being the more abundant branching [P.D. Nichols, personal communication].

The cuttings contained similar PLFA profiles to those observed in the drilling muds. The similarity can be attributed to the fact that the cuttings were recovered from drilling muds by sieving and had become saturated. The rotary sidewall parings and core contained simple PLFA profiles when compared to those observed in the drilling muds and cuttings. Terminally branched saturates were detected showing the *anteiso* configuration to be more abundant than the *iso*. *Anteiso* fatty acids are known to comprise up to 65% of the total fatty acids in gram positive bacteria such as *Arthrobacter globiformis*, 61% in *Micrococcus luteus* and 50% in *Bacillus subtilis* (aerobe database version 3.7, 1993 Microbial ID, Inc. Newark, DE). Monounsaturates were detected including both oleic (18:1w9c) and vaccenic acids (18:1w7c). Both monoenoics have been observed in methanotrophic species at mole percentages of >30% [P.D. Nichols, personal communication]. Both acids have also been observed in other species of gram negative bacteria. Oleic acid is also prominent in a number of microeukaryotic organisms.

A principal components analysis (PCA) of the PLFA mole percent data (Fig. 4) indicated three major groups to occur among the samples analyzed. Rotary sidewall parings, core and a combusted blank were plotted together and away from the drilling muds and cuttings which formed another group distinct from that of the make-up waters. The PCA indicated that the surface PLFA profile was not closely related to those of the drilling muds and cuttings. Drilling mud DM2027 and one combusted blank CB3353 were identified as having PLFA profiles dissimilar to either the drilling muds or the rotary sidewall samples. The first principal component captured 76% of the variance in the data set. Palmitic and stearic acids (16:0 and 18:0) were assigned the two greatest positive coefficients (0.78 and 0.44, respectively) followed by 18:1w7c (vaccenic acid; 0.27). The two normal saturates, 16:0 and 18:0, are common constituents in the cell membranes of most bacterial genera, limiting their diagnostic utility. As mentioned previously, 18:1w7c is synthesized via an anaerobic desaturase pathway which is utilized by a number of gram negative obligate anaerobes. These three acids carried the greatest weight in defining the rotary sidewall parings and core as well as the one combusted blank. The second principal component captured an additional 11% of

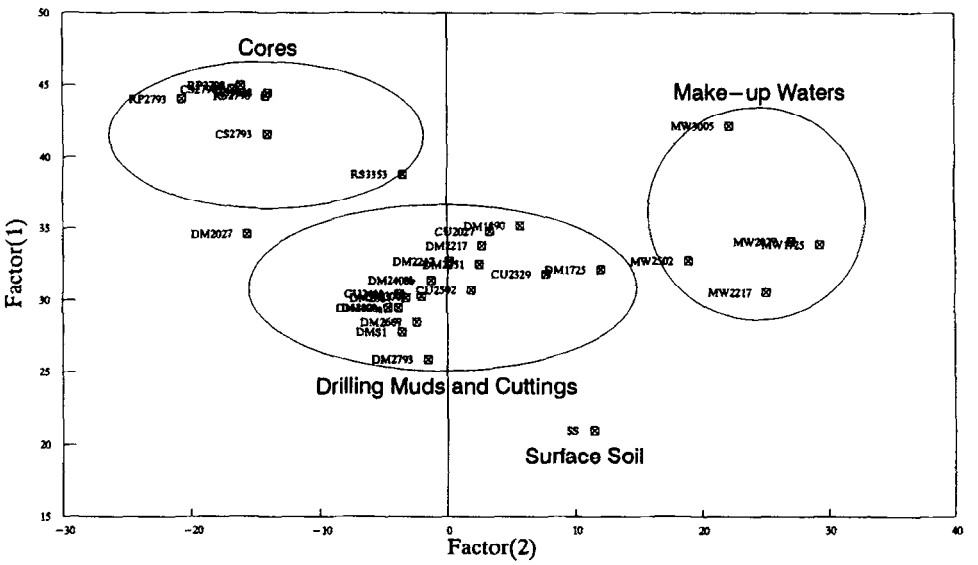

Fig. 4. Plot of Factor 1 versus Factor 2 scores from principal components analysis of PLFA mole percent patterns of the different sample types. SS = surface soil; DM = drilling mud followed by depth in meters to which the mud was circulated; DMS = drilling mud taken from core sampling tools; MW = drilling mud make-up water followed by depth in meters of the borehole at the time of sampling; RP = rotary sidewall core followed by depth in meters that was core; RS = rotary core inner parings followed by depth in meters of core; CS = rotary core outer parings followed by depth in meters of core; CU = drill bit cuttings followed by depth too which the mud was circulated. Samples with identical depths are duplicate tests of samples.

the variability and assigned the greatest positive coefficients to 18:1w7c and 16:1w7c (0.66 and 0.54) and the greatest negative coefficients to 18:0 and 16:0 (−0.468 and × minus;0.09). The make-up water PLFA profiles were dominated by the presence of the two positive variables 16:1w7c and 18:1w7c. Both are found in a number of gram negative aerobic heterotrophic bacteria. The rotary parings and core contained higher percentages of the negative variables, palmitic and stearic acids. A hierarchical cluster analysis (HCA, incremental method with no preprocessing of the data) of the PLFA mole percent data indicated similar relationships between samples as that observed in the PCA (data not shown).

4. Discussion

The results of PCA analyses of the community-level data using structural (PLFA) and functional (CLPP) methods were remarkably similar (Figs. 2 and 4). Communities from samples of surface soils, aquifer waters, drilling muds and cores taken at one site were distinguished with either approach. The equivalency of the minimal PLFA response noted in the rotary sidewall cores and the control cores (Fig. 4) testifies to the care taken in processing and handling of the samples.

PLFA concentrations in the inner paring and the central rotary core material (RP and RS2798) were determined to be 10 and 16 pmol PLFA gram^{-1} respectively, an order of magnitude higher than that observed in the outer paring (CS2798, 1 pmol PLFA gram^{-1}) and an order of magnitude less than that observed in the drilling fluid (124 pmol PLFA gram^{-1}). Each sample type contained a PLFA profile indicative of a different microbial community: make-up waters contained a predominately aerobic gram negative heterotrophic population, drilling muds and cuttings a predominately anaerobic gram negative community and rotary sidewall samples a community composed of anaerobic gram negative bacteria and also of gram positive bacteria. The sample PLFA concentrations and the observed differences in the PLFA profiles between sample types (Fig. 4) provide some reassurance that the thermophilic anaerobic microorganisms cultured from some rotary cores [34] are representative of the deep formations that were cored. The CLPP results suggest that the cores obtained by rotary sidewall coring were not contaminated (no response seen) but allowed for the occurrence of incremental contamination of percussion sidewall cores by the muds. The absence of CLPP response by the rotary cores implies that these cores were not heavily colonized with organisms that respond under mesophilic aerobic conditions, e.g., surface conditions. The small amounts of core received and processed (2 to 10 g) may have influenced the ability to detect activity in these samples. The lack of response does not rule out the presence of viable organisms that may not respond under the incubation conditions, that do not use an electron transport system (ETS) (e.g., exclusive use of substrate-level phosphorylation) or that possess ETS activities not compatible with detection by TTC.

Although the CLPP responses from the percussion cores were different from the muds (Fig. 2), this outcome could have been achieved by the introduction of some fraction of the mud community to the percussion cores. Visual inspection of the percussion cores indicated less physical integrity compared to the rotary sidewall cores and therefore an increased possibility of contamination. The predominance of thermophilic anaerobic organisms cultured from the high-integrity rotary sidewall cores [34], corroborate the suggestion that mesophilic aerobic organisms may have been introduced to the percussion cores from the drilling fluids. On the other hand, it is possible that the community in the percussion core is authentic and that any similarity with the drilling mud communities is due to entrainment of subsurface [formation] organisms into the muds during drilling. While factor 1 of the PCA of CLPP results (Fig. 2) distinguished between make-up waters, drilling muds and cores, this factor was somewhat related ($r^2 = 0.67$) to AWCD. So despite normalization attempts to account for sample biomass differences, factor 1 may partially reflect differences in the magnitudes of relative use of individual carbon sources that depend on the overall number of strong responses per sample, not necessarily the pattern. However, biomass effects in this dataset were not detected by the relation of inoculum cell density (by acridine orange direct count) to AWCD ($r^2 = 0.047$, data not shown). Factor 2 (Fig. 2) was not related to AWCD ($r^2 = 0.049$) and it was this factor that distinguished between cores and the likeliest source of

contamination, the drilling muds. In many cases, the top carbon sources utilized by communities from percussion cores were different than those corresponding to communities from muds, soils and groundwaters (Table 1). The carbon sources utilized by the percussion core microorganisms were very similar to the limited number of sole carbon sources oxidized by communities from unsaturated sediments at the INEL and Hanford sites (Colwell, unpublished data). The lack of accompanying perfluorocarbon tracer measurements [35] and PLFA data on percussion cores makes it difficult to determine if the organisms from percussion cores that responded under aerobic mesophilic conditions represent autochthonous populations. This ambiguity underscores the need for multiple tracers to assure the quality of microbiological data on deep terrestrial subsurface cores.

When the original 95 carbon sources were grouped into 11 carbon source groups and PCA performed, the amino acid and carbohydrate groups were found to correlate strongly (with opposite sign) with factor 1. A scatterplot of the relative responses of communities to these two groups of carbon sources showed that communities from surface soils and the aquifer 'preferred' carbohydrates over amino acids compared to the communities in the percussion cores (Fig. 3). If populations in the percussion cores were not representative of the formation that was cored, as some evidence suggests, the organisms that colonized these cores could have originated from some shallower subsurface strata and were introduced to the cores from the circulating drilling fluids. The unique CLPP of the percussion core communities supports the conclusion that these organisms were not primary constitituents of the surface soils or make-up waters microflora. The mud populations were split in their preferences, possibly indicating an intermediate composition of these communities between microorganisms characteristic of the surface and those from some depth. The type of PLFA (monounsaturates and teminally-branched saturates) detected in the muds results from two very different biosynthetic pathways and can be indicative of very different microbial populations. An increase in the mole percentages of terminally and mid-chain branched saturated PLFA in the muds with increasing borehole depth was also seen (data not shown). The shifting dominance of these populations may be reflected by the bimodal CLPP result. The appearance of two groups of mud communities may also be related to the schedule of additions to the mud reservoir; however, the information required to establish a relationship is not available. The core communities were able to respire only a limited number of the 28 carbohydrates tested as sole carbon sources. In another CLPP analysis of subsurface cores, a similar lack of oxidation of carbohydrates was seen in unsaturated sediments from the INEL and Hanford (Colwell, unpublished data). Physiological testing of SRP subsurface isolates by Fredrickson et al. [36] showed that a relatively low percentage of these isolates metabolized sugars.

When PCA of the samples based on the original 95 carbon sources was completed (Fig. 2), Tween 40 (−0.82) and 80 (−0.79) and acetate (−0.74) were found to correlate well with the principal component axes indicating their importance in separating the samples. Relative respiration of these compounds showed that mud and core communities tended to utilize Tween compounds and

acetate to a much greater extent than surface soil or aquifer communities. Tween compounds have been shown to be oxidized by subsurface microorganisms in investigations with isolates from SRP sediments [36], by communities from INEL and Hanford unsaturated sediments (Colwell, unpublished data), from Hanford saturated sediments (Colwell and Lehman, unpublished data), from basalt aquifers (Colwell and Lehman, unpublished data) [37] and from groundwater and sediments from a shallow sand aquifer [38]. Fredrickson et al. [36] observed strong use of acetate as a sole carbon source by a collection of subsurface isolates from boreholes at SRP. Subtle differences in the community-level oxidation of single carbon sources, e.g., acetate or Tween, may be a sensitive tool for discriminating between microbial communities.

Other compounds that were used preferentially by percussion core communities were citric acid, propionic acid, bromosuccinate and several amino acids. Additional compounds were utilized by core communities but were not helpful in distinguishing among communities from the different media sampled. Differentiating communities with PCA based on their normalized or relative responses to carbon sources or groups differs from basing distinctions on extent of use. The normalization procedure used by Garland and Mills [14] was used in an effort to reduce the influence of biomass effects on the response and to allow the patterns of responses to be evaluated. It is important that manipulation of the raw data be done to fit the question that is being asked, the dataset being analyzed and the type of analysis that is being employed. In our case, communities were distinguished based on their patterns of relative use of compounds. Compounds that show higher relative use by one community versus another are not necessarily used to a greater overall extent. The result of normalization is that the data is expressed as a ratio and high ratios may mean increased use of that compound(s) or decreased use of other compounds(s). In the case of the core samples, it was the near complete absence of response to carbohydrates that caused the relative use of amino acids to increase in value.

The use of drilling muds during rotary drilling and coring introduces considerable uncertainty into the ability to obtain uncompromised core samples. The microbial communities in the drilling muds originate from local surface soils, make-up water, dry mud ingredients (potash, bentonite, barite, and other additives), drilling steel and the formation. Recirculating drilling muds continually entrain organisms from the uncased borehole and cuttings while additions to the mixture to optimize its functions are made at the surface. The repeated transit of the muds from the surface conditions (e.g., temperature, pressure) to subsurface conditions presents an enrichment regime allowing only a certain fraction of the overall community to proliferate to significant levels. Balkwill et al. [39] reasoned that core samples obtained from a mud rotary-drilled borehole at SRP were uncompromised because: (i) isolates cultured from surface and subsurface materials were physiologically dissimilar; (ii) 84% of subsurface isolates characterized by physiological testing were unique to one strata; and, (iii) the proportion of physiologically distinct biotypes did not decrease with depth. Zachara et al. [9] compared isolates cultured from drilling muds and core samples

at SRP by DNA homology and physiological testing and concluded that there was little similarity between these groups of cultures.

The evidence presented by Balkwill et al. [39] and Zachara et al. [9] supports the integrity of SRP core samples, although dependence on isolate-based approaches under mud-rotary drilling conditions could be risky. Particular isolates, either added or indigenous, may not survive, be extractable or be recoverable (culturable) from muds or core samples under a given set of conditions. The response of pure cultures to physiological testing may be variable. Further, it is likely that there will be some similarity in the types of isolates recovered from uncompromised core samples and drilling muds due to autochthonous organisms contributed to the muds from borehole cuttings and slough. Researchers cannot rely on the fortuitous presence of easily trackable and culturable microorganisms like coliforms [10,11]. The use of CFUs or biomass as indicators of sample quality yields very limited information [5,6] and differences in these quantities are probably inadequate for the basis of estimates of contamination. In order to acquire subsurface core samples, investigators may look increasingly to opportunities provided by commercial drilling operations. In these instances, mud rotary drilling (versus air rotary or cable tool) is most common and restrictions may exist for the application of inert chemical and particulate tracers. Parallel examination of drilling mud microorganisms should be included in the battery of QA/QC approaches that could be implemented at a particular site where cores for microbiological analyses are being collected. The use of community-level characterization techniques can increase the ability to detect contamination over isolate-based methods and provide insight into the structure and function of microbial communities in diverse environments.

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References

[1] Keswick, B.H. (1984) Sources of groundwater pollution. In: Groundwater Pollution Microbiology, (G. Bitton and C.P. Gerba, eds.), pp. 39–64, Wiley-Interscience, New York.

[2] Jack, T.R. (1993) M.O.R.E. To M.E.O.R.: An overview of microbially-enhanced oil recovery. In: Microbial Enhancement of Oil Recovery – Recent Advances, (E. Premuzic and A. Woodhead, eds.), pp. 7–16, Elsevier, Amsterdam.

[3] West, J.M., McKinley, I.G. and Chapman, N.A. (1982) Microbes in deep geological systems and their possible influence on radioactive waste disposal. Radioactive Waste Management and the Nuclear Fuel Cycle 3, 1–15.

[4] Wobber, F.J. (1986) Microbiology of Subsurface Environments. DOE/ER-0293. U.S. Department of Energy, Washington, D.C.

[5] Phelps, T.J., Fliermans, C.B., Pfiffner, S.M. and White, D.C. (1989) Recovery of deep subsurface sediments for microbiological studies. J. Microbiol. Methods 9, 267–279.

[6] Russell, B.F., Phelps, T.J., Griffin, W.T. and Sargent, K.A. (1992) Procedures for sampling deep subsurface microbial communities in unconsolidated sediments. GWMR (Winter), 96–104.

[7] 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.

[8] Balkwill, D.L., Fredrickson, J.K. and Thomas, J.M. (1989) Vertical and horizontal variations in the physiological diversity of aerobic chemoheterotrophic bacterial microflora in deep southeast coastal plain subsurface sediments. Appl. Environ. Microbiol. 55, 1058–1065.

[9] Zachara, J.M, Fredrickson, J.K. and Balkwill, D.L. (1993) Assessment of solute and microbiological contamination in deep cores from the South Carolina coastal plain. 1993 International Symposium on Subsurface Microbiology, Bath, UK, September, 1993.

[10] Jones, R.E., Beeman, R.E. and Suflita, J.M. (1989) Anaerobic metabolic processes in the deep terrestrial subsurface. Geomicrobiol. J. 7, 117–130.

[11] Beeman, R.E. and Suflita, J.M. (1989) Evaluation of deep subsurface sampling procedures using serendipitous microbial contaminants as tracer organisms. Geomicrobiol. J. 7, 223–233.

[12] White, D.C., Ringelberg, D.B., Guckert, J.B. and Phelps, T.J. (1991) Biochemical markers for in situ microbial community structure. In: Proceedings of the First International Symposium on the Microbiology of the Deep Subsurface. (C.B. Fliermans and T.C. Hazen, eds.), pp. 4/41–4/51, Westinghouse Savannah River Company, Aiken, South Carolina.

[13] Tunlid, A. and White, D.C. (1991) Biochemical analysis of biomass, community structure, nutritional status, and metabolic activity of the microbial communities in soil. In: Soil Biochemistry 7, (J.-M. Bollag, G. Stotzky, eds.), pp. 229–262.

[14] Garland, J.L. 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.

[15] Ressetar, R. and Taylor, G.K. (1988) Late Triassic depositional history of the Richmond and Taylorsville Basins, eastern Virginia. In: Triassic-Jurassic Rifting, Continental Breakup and the Origin of the Atlantic Ocean and Passive Margins, (W. Manspeizer, ed.), Elsevier, Amsterdam, pp. 423–443.

[16] Bain, G.L. and Brown, C.E. (1981) Evaluation of the Durham Triassic basin of North Carolina and technique used to characterize its waste-storage potential. U.S. Geological Survey Open-file Report 80–1295, 132 pp.

[17] Robbins, E.I. and Weems, R.E. (1988) Preliminary analysis of unusual palynomorphs from the Taylorsville and Deep Run basins in the eastern piedmont of Virginia. In: Studies of the early Mesozoic basins of the eastern United States, A.K. Froelich and G.R. Robinson, (eds.). U.S. Geological Survey Bulletin 1776, pp. 40–57.

[18] Milici, R.C., Bayer, K.C., Pappano, P.A., Costanin, J.K., Coruh, C. and Nolde, J.E. (1991) Preliminary geologic section across the buried part of the Taylorsville Basin, Essex and Caroline counties, Virginia. Virginia Division of Mineral Resources Open-File Report 91–1, 31pp.

[19] Bochner, B.R. and Savageau, M. (1977) Generalized indicator plate for genetic, metabolic and taxonomic studies with microorganisms. Appl. Environ. Microbiol. 33, 434–444.

[20] Balkwill, D.L. and Ghiorse, W.C. (1985) Characterization of subsurface bacteria associated with two shallow aquifers in Oklahoma. Appl. Environ. Microbiol. 50, 580–588.

[21] Victorio, L., Allen, D.G. and Liss, S.N. (1993) Phenotypic fingerprinting of biologically-based wastewater treatment systems. Abstract, 93rd American Society for Microbiology General Meeting, Atlanta, GA, May, 1993.
[22] Brockman, F.J., Kieft, T.L., Fredrickson, J.K., Bjornstad, B.N., Li, S.W., Spangenburg, W. and Long, P.E. (1992) Microbiology of vadose zone paleosols in south-central Washington state. Microb. Ecol. 23, 279–301.
[23] Brockman, F.J., Fredrickson, J.K., Li, S.W., Spadoni, C.M., Pfiffner, S.M., Johannesen, J.M. and Phelps, T.J. (1993) Stimulation of the microbial community by nutrient amendment and storage of saturated sediments with low biomass and numbers of culturable microorganisms. 1993 International Symposium on Subsurface Microbiology, Bath, UK, September, 1993.
[24] Demezas, D.H. and Bottomley, P.J. (1986) Autecology in rhizosphere and nodulating behavior of indigenous *Rhizobium trifolii*. Appl. Environ. Microbiol. 52, 1014–1019.
[25] Garchow, H.L., Collins, H.P. and Klug, M.J. (1993) Characterization of whole soil microbial communities using Biolog GN microplates. 3rd Annual Center for Microbial Ecology Forum, Michigan State University, East Lansing, MI., September, 1993.
[26] Bligh, E.G. and Dyer, W.M. (1959) A rapid method of lipid extraction and purification. Can. J. Biochem. Physiol. 35, 911–917.
[27] White, D.C., Davis, W.M., Nickels, J.S., King, J.D. and Bobbie, R.J. (1979) Determination of sedimentary microbial biomass by extractable lipid phosphate. Oecologia 40, 51–62.
[28] 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.
[29] Guckert, J.B., Antworth, C.P., Nichols, P.D. and White, D.C. (1985) Phospholipid, ester-linked fatty acid profiles as reproducible assays for changes in prokaryotic community structure of estuarine sediments. FEMS Microb. Ecol. 31, 147–158.
[30] Kroppenstdedt, R.M. (1985) Fatty acids and menaquinone analysis of actinomycetes and related organisms. In: Chemical Methods in Bacterial Systematics, (M. Goodfellow and D.E. Minnikin, eds), pp. 173–199, Academic Press, London.
[31] Dowling, N.J.E., Widdel, F. and White, D.C. (1986) Phospholipid ester-linked fatty acid biomarkers of acetate-oxidizing sulfate reducers and other sulfide forming bacteria. J. Gen. Microbiol. 132, 1815–1825.
[32] Edlund, A., Nichols, P.D., Roffey, R. and White, D.C. (1985) Extractable and lipopolysaccharide fatty acid and hydroxy acid profiles from *Desulfovibrio* species. J. Lipid Res. 26, 982–988.
[33] Kaneda, T. (1991) Iso- and *anteiso*-fatty acids in bacteria: Biosynthesis, function, and taxonomic significance. In: Microbiological Reviews, pp. 288–302, American Society for Microbiology.
[34] Stevens, T.O. and Boone, D.R. (1993) Thermophilic anaerobic bacteria in 2800-m-deep samples from the terrestrial subsurface. 1993 International Symposium on Subsurface Microbiology, Bath, UK, September, 1993.
[35] McKinley, J.P., Colwell, F.S., Long, P.E., Phelps, T.J. and Veverka, C. (1993) The application of perfluorocarbon tracers to subsurface microbial sampling. 1993 International Symposium on Subsurface Microbiology, Bath, UK, September, 1993.
[36] Fredrickson, J.K., Balkwill, D.L., Zachara, J.M., Li, S.W., Brockman, F.J. and Simmons, M.A. (1991) Physiological diversity and distributions of heterotrophic bacteria in deep Cretaceous sediments of the Atlantic coastal plain. Appl. Environ. Microbiol. 57, 402–411.
[37] Zheng, M. and Kellogg, S.T. (1992) Microbial nutritional analysis in a basalt aquifer. Abstract of American Society of Microbiology 92nd General Meeting, p. 247; New Orleans, LA, May, 1992.
[38] Kolbel-Boelke, J., Anders, E. and Nehrkorn, A. (1988) Microbial communities in the saturated groundwater environment II: Diversity of bacterial communities in a Pleistocene sand aquifer and their in vitro activities. Microb. Ecol. 16, 31–48.
[39] Balkwill, D.L. (1989) Numbers, diversity, and morphological characteristics of aerobic, chemoheterotrophic bacteria in deep subsurface sediments from a site in South Carolina. Geomicrobiol. J. 7, 33–52.