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Deep Subsurface Microbial Biomass and Community Structure in Witwatersrand Basin Mines

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The extreme environments of South Africa mines were investigated to determine microbial community structure and biomass in the deep subsurface. These community parameters were determined using phospholipid fatty acid (PLFA) technique. Air, water and rock samples were collected from several levels and shafts in eight different mines. Biomass estimates ranged over nine orders of magnitude. Biofilm samples exhibited the highest biomass with quantities ranging from 10^3 to 10^7 pmol PLFA g^{-1}. Rock samples had biomass ranging from 10^3 to 10^6 pmol PLFA g^{-1}. Mine service waters and rock fracture waters had biomass estimates ranging from 10^0 to 10^6 pmol PLFA L^{-1}. Air samples biomass values ranged from 10^{-2} to 10^0 pmol PLFA L^{-1}. The biomass estimates were similar to those estimates for other deep subsurface sites. Redundancy analysis of the PLFA profiles distinguished between the sample types, where signature lipid biomarkers for aerobic and anaerobic prokaryotes, sulfate-and metal-reducing bacteria were associated with biofilms. Rock samples were enriched in 18:1ω9*c*, 18:2ω6, br17:1s and br18:1s, which are indicative of microeukaryotes and metal- reducing bacteria. Air samples were enriched with 22:0, 17:1, 18:1, and a polyunsaturated fatty acid. Service waters had monounsaturated fatty acids. Fracture waters contained i17:0 and 10Me18:0 which indicated gram-positive and other anaerobic bacteria. When the fracture and service water sample PLFA responses to changes in environmental parameters of temperature, pH, and anion concentrations were analyzed, service waters correlated with higher nitrate and sulfate concentrations and the PLFAs 18:1ω7*c* and 16:1ω7*c*. Dreifontein shaft 5 samples correlated with chloride concentrations and terminally branched saturated fatty acids and branched monounsaturated fatty acids. Kloof, Tau Tona, and Merriespruit fracture waters aligned with temperature and pH vectors and 18:0, 20:0 and 22:6ω3. The redundancy analysis provided a robust method to understand the PLFA responses to changes in environmental parameters.

Keywords extreme environment, community structure, phospholipid fatty acids, deep subsurface, fracture water

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INTRODUCTION

Extreme environments are under investigation by multidisciplinary and international scientists to increase our understanding of life processes in the Earth's deep subsurface. These studies may contribute to the search for life on other planets. Explorations of Earth's extreme environments have included nuclear waste depositories, perennially ice-covered lakes in the Antarctic, interiors of rocks, oceanic hydrothermal vents, thermal springs, and deep subsurface aquifers (Onstott et al. 1997; Sassen et al. 1998; Pederson 1999; Zierenberg et al. 2000; Rothschild and Mancinelli 2001).

South African mines provide ready access to some of the world's deepest terrestrial extreme environments. These mines allow investigators to collect water, rock and air samples for microbial and geochemical examination. Relating geochemical measurements with microbial community structure from deep mines permits investigators to determine the similarity of the communities to those in other terrestrial environments.

Phospholipids degrade rapidly upon cell death, allowing their use as a measure of living microbial communities (White et al. 1979). PLFA profiles can be used to estimate microbial biomass, determine prokaryotic and eukaryotic community composition, and determine metabolic status (Bobbie and White 1980; Federle et al. 1986; Tunlid and White 1992; Findlay and Dobbs 1993; Kieft et al. 1994; Frostagård et al. 1996). Numerous studies have shown the effects of environmental factors (e.g., contamination by hydrocarbons or metals) on PLFA-derived microbial community composition (Frostegård et al. 1996; Pfiffner et al. 1997; Bååth et al. 1998; Stephen et al. 1998; Brandt et al. 1999).

Herein, we examined PLFA profiles and estimate microbial biomass of water, rock, air, and biofilm samples collected from eight mines in South Africa. Changes in the PLFA response of the microbial communities in fracture and service water samples were related to differences in environmental parameters (temperature, pH, and anion concentration).

MATERIALS AND METHODS

Site Information and Sample Collection

The mines were located in the Witwatersrand Basin and the Bushveld Igneous Complex, South Africa (Figure 1). Herein, the mines are designated as Beatrix (BE), Driefontein (DR), Evander (EV), Kloof (KL), Merriespruit (MS), Mponeng (MP), Masimong (MM), Tau Tona (TT) and Northam (NO). If the mine designation is followed by a number, it indicates the number of the mine shaft, e.g., EV8 identifies the #8 shaft at the Evander mine. All mines were gold mines, except the Northam mine which is a platinum mine. Sample depths ranged from 720 to 3300 m below land surface. Detailed information on these mines is provided by Gihring et al. (this volume) and Onstott et al. (this volume).

A total of 162 water, biofilm, air, and rock samples were collected using aseptic technique. Service water samples, which are from the mine circulation system (i.e., water used in cooling the air supply system, lubrication of drilling rods, and suppression of dust in the stopes) or from tunnel gutters, were collected in sterile, N_2-flushed 1-L containers. One of the service water samples was collected from a cement containment dam and a second water sample was collected from a lower level borehole where upper level acidic mining water was emanating. Fracture water samples were collected using an autoclaved expanding packer and sampling manifold constructed of Delrin plastic (Dupont, DE) and equipped with quick-connect release valves attached to sterile tygon tubing with syringe tips. Geochemical field measurements were taken with a temperature, pH, and conductivity probe HI98130 (Hanna Instruments, Woonsocket, RI) and an Eh probe HI98201 (Hanna Instruments, Woonsocket, RI). Dissolved anion samples were filtered (0.22 μm Nylon Acrodisk, Gelman) into 15-mL centrifuge (Falcon) tubes. Fracture and service water (26 samples) were collected in sterile 50-mL disposable centrifuge tubes for flow cytometry measurements (Onstott et al. this volume).

Samples for lipid analysis were collected using the manifold system where the syringe tip was replaced with a quick connect valve that attached to a sterile N_2-flushed 12-L container (later filtered in laboratory) or to 0.2-μm Anodisc filters (Whatman) in sterile filter casings (filtered in mine). Air samples were collected within 3 m of the water sampling sites using the Anodisc filtering system attached to a hand-powered, bellows style, air mattress pump. Air filters were removed from the casing and stored in a muffled aluminum foil packet which was placed into a labeled Whirlpak bag prior to leaving the sampling site. Biofilm samples were collected with a 15- or 50-mL centrifuge (Falcon) tubes by scraping the film of slime and minerals formed on the mine wall where fracture water emerged. Rock samples included actively mined rock (picked up by hand wearing a sterile glove) and drill core collected from mine adits. Lipid samples were stored frozen at -40°C until analyzed. More detailed sample collection procedures are reported in Onstott et al. (2003; this volume).

The anion samples were measured by ion chromatography (DX-320, Dionex, Sunnyvale, CA) using EG40 and LC25 columns and an AS40 autosampler. Cell abundance and sizes were determined by flow cytometry. A formaldehyde-fixed sample was concentrated 10-fold by centrifugation, stained with Syto-13 DNA stain (Molecular Probe, CA) and cells were enumerated using a flow cytometer (FACScan, Becton-Dickinson). The cell density and size were calibrated to 1-μm fluorescent microspheres at known concentrations (Molecular Probes, Eugene, OR). The uncertainty for enumeration was $\pm 10\%$. Physical and geochemical measurements procedures are reported in Onstott et al. (this volume).

Phospholipid Fatty Acid Analysis

Lipids were extracted using a modified Bligh and Dyer extraction (Bligh and Dyer 1959; White et al. 1979) and subsequently fractionated with the polar-lipids then subjected to a mild alkaline methanolysis. The resulting PLFA methyl esters were separated, quantified, and identified by gas chromatography-mass spectrometry (White and Ringelberg 1998). The identity of PLFA was verified using GC/MS in comparison with standards (Matreya Inc. Pleasant Gap, PA, USA). Fatty acid nomenclature is as described by Ringelberg et al. (1989).

Biomass estimates were calculated as pmol PLFA g^{-1} for rock and biofilm samples and as pmol PLFA L^{-1} for water and air samples (data not shown). Biomass ranges were displayed as box and whisker plots in Figure 2A and 2B. For comparison with flow cytometry counts, the concentrations of total PLFA

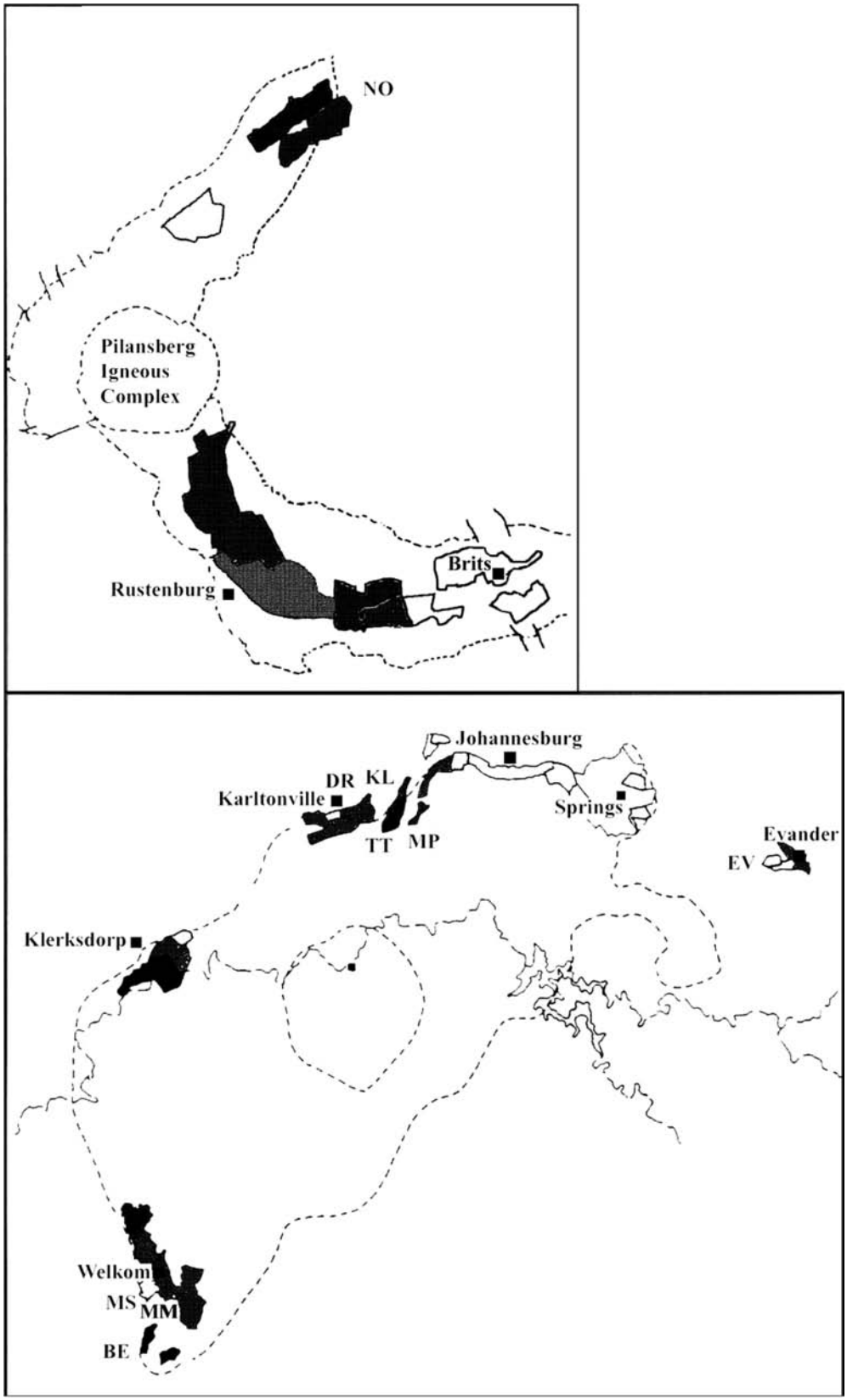

FIG. 1. Location of South African gold and platinum mines are indicated on the map with the abbreviation as NO = Northam platinum mine, and gold mines as BE = Beatrix, DR = Dreifontein, EV = Evander, KL = Kloof, MM = Masimong, MP = Mponeng, MS = Merriespruit, and TT = Tau Tona.

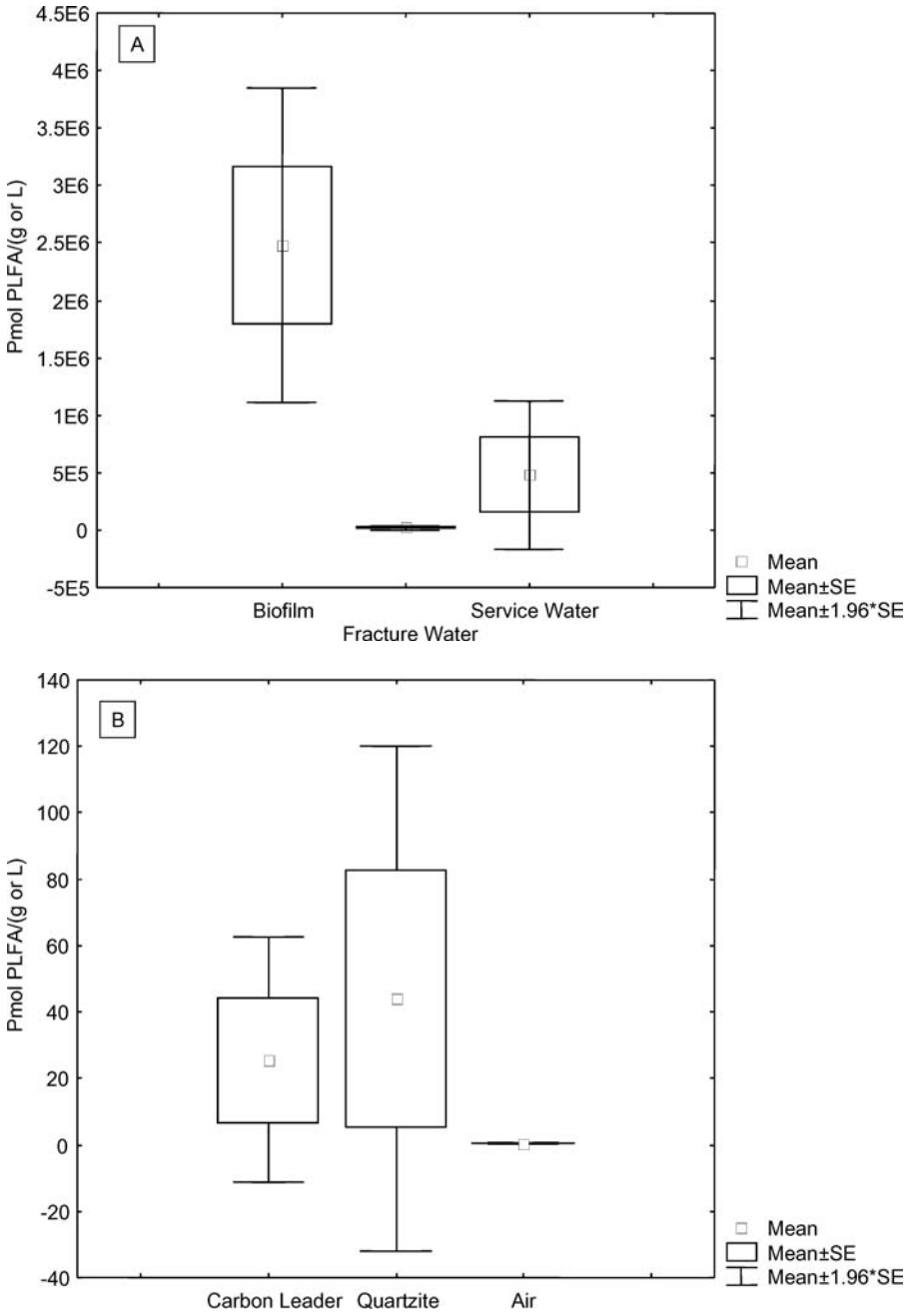

FIG. 2. Box and Wisker plots of PLFA biomass distribution for biofilm, fracture water and service water samples are shown in plot A, with carbon leader, quartzite and air samples shown in plot B. The units were pmol PLFA L^{-1} for air and water and pmol PLFA g^{-1} for rock and biofilm.

were converted to abundances (Table 1) using the conversion factor of 2.5×10^5 cells $pmol^{-1}$ PLFA (Balkwill et al. 1988).

Redundancy Analyses

Samples with PLFA profiles containing 90 mol% or greater straight chain saturated fatty acids or fewer than 5 identified PLFA were considered below detection limits and were removed from the data set. The redundancy analysis (RDA) was first performed on the data set containing 110 PLFA profiles using CANOCO for Windows, version 4.02 (ter Braak 1998). The second RDA was preformed on a data set containing PLFA profiles and geochemical parameters (Table 2) for 57 fracture and service water samples. The Mponeng and Northam mine samples lacked sufficient geochemical parameters to be included in this RDA. A mean imputation was used for missing values of pH and temperature. Eh measurements were not included in the RDA because only seven samples had recorded values.

TABLE 1
Geochemical parameters for fracture and service water samples[a]

Sample #	Name	pH	Eh (mv)	T°C	Cl[b]	SO_4^{2-}	NO_3^-
1	BE116 FW 121801	N.A.	N.A.	N.A.	3.40 × −02	2.16 × −05	3.02 × −07
4	BE225 FW1 121598	9.5	N.A.	35.0	3.66 × −02	7.12 × −06	<1.61 × −07
6	E4 IPC DW2 111698	7.0	398	25.0	4.37 × −04	1.69 × −03	<1.61× −07
18	DR546 BH1 103001	7.9	26	32.0	6.76 × −01	1.11 × −03	<1.61 × −07
19	DR546 BH1 120798	5.5	N.A.	37.0	6.34 × −01	4.81 × −04	<1.61 × −07
20	DR546 BH1 020199	7.4	N.A.	37.2	6.37 × −01	9.64 × −04	<1.61 × −07
34	W638 FW3 111598	9.0	N.A.	45.0	1.19 × −02	9.49 × −04	2.31 × −05
35	DR638 BH2a 111598	10	N.A.	45.0	1.72 × −02	1.17 × −03	7.05 × −06
38	DR938 H3 110701	9.0	−90	60.5	3.16 × −02	1.35 × −04	1.06 × −06
39	DR938 HI 082001	9.7	N.A.	42.6	3.00 × −03	1.38 × −04	<1.61 × −07
40	DR938 H2 083001	9.4	N.A.	44.0	2.17 × −02	4.82 × −05	7.45 × −07
41	DR938 CH 071102	9.2	−40	42.0	3.00 × −03	1.35 × −04	7.26 × −08
43	DR938 H3 125M 102401	8.2	N.A.	46.0	2.63 × −02	3.27 × −05	2.79 × −06
44	DR938 H3 125M 102501	8.2	N.A.	46.0	2.58 × −02	3.62 × −05	1.44 × −07
45	DR938 H3 250M 102401	7.8	N.A.	54.0	2.64 × −02	3.49 × −05	1.48 × −06
46	DR938 H3 390M 102401	7.7	N.A.	54.0	2.61 × −02	2.63 × −05	7.39 × −07
47	DR938 H3 550M 102501	7.4	N.A.	54.0	2.62 × −02	1.58 × −05	3.64 × −07
48	DR938 H3 648M 102501	7.3	N.A.	48.0	2.67 × −02	4.02 × −05	<1.61 × −07
49	DR938 CH 110701	7.5	N.A.	43.0	3.13 × −03	1.75 × −04	5.89 × −08
57	EV219 H3 081601	7.6	N.A.	25.0	9.04 × −03	2.57 × −04	2.37 × −06
58	EV221 H1 111601	6.9	N.A.	N.A.	1.56 × −02	3.30 × −04	3.10 × −07
59	EV221 H2 111601	2.8	678	32.0	2.98 × −02	7.88 × −03	3.07 × −04
60	EV221 H3 111601	8.7	−62	36.0	2.66 × −02	9.14 × −04	<1.61 × −07
61	EV221 H3 121702	8.9	N.A.	35.5	2.95 × −02	7.85 × −04	<1.61 × −07
62	EV221 H4 121702	9.3	28	33.0	2.86 × −02	6.74 × −04	<1.61 × −07
64	EV522 FW030801HWD	7.2	190	36.7	3.68 × −02	1.32 × −03	1.12 × −06
65	EV522 HI 041801CTS	7.0	N.A.	32.0	6.50 × −02	7.38 × −06	1.19 × −06
69	EV818 H5 102502	7.3	−112	40.5	1.71 × −01	2.62 × −03	8.39 × −06
70	EV821 FW 101601	8.3	N.A.	45.0	9.77 × −02	7.60 × −05	6.34 × −07
71	EV818 FW 062101	7.8	−52	45.0	1.47 × −01	5.46 × −04	4.43 × −07
72	EV818 H5 102702	8.8	−48	42.0	1.73 × −01	2.97 × −03	<1.61 × −07
75	EV820 FW 121401	8.5	N.A.	37.5	4.32 × −02	3.45 × −05	9.68 × −08
79	EV914 FW022801 ED	10.0	N.A.	33.0	1.00 × −02	2.11 × −05	<1.61 × −07
80	KL441HWDS H1 120198	8.4	N.A.	60.0	5.79 × −02	1.48 × −06	<8.06 × −06
81	KL441 BH1 022801	5.5	N.A.	N.A.	2.14 × −01	1.02 × −03	9.14 × −07
82	KL441 HWDS H2 100201	9.0	−12	55.0	3.99 × −02	6.57 × −05	1.01 × −06
83	KL443 HWDN FW 050801	8.2	−100	59.0	2.90 × −01	7.29 × −04	8.29 × −07
86	KL739 FW 062901	8.4	−41	61.1	2.31 × −01	1.08 × −04	9.71 × −07
87	MM51870 FW 030402	8.0	N.A.	42.0	4.40 × −02	1.91 × −05	1.33 × −05
104	MS149 BH1 200603	7.8	−98	36.3	4.04 × −02	6.38 × −05	9.68 × −08
105	MS149 BH2 200603	7.8	−39	37.6	4.07 × −02	5.46 × −05	2.26 × −07
110	TT104 FW 080703	7.0	15	48.0	1.73 × −03	8.10 × −05	6.90 × −07
23	DR546 SW 103001	N.A.	N.A.	N.A.	7.97 × −03	9.11 × −03	1.64 × −03
25	Dr546 sump	11.6	337	34.2	5.01 × −02	9.47 × −04	1.24 × −03
26	DR548 SW1 090198	7.9	N.A.	17.6	6.25 × −03	4. 16 × −04	7.58 × −04
28	DR550 30 SW-1 101502	7.0	349	24.8	1.05 × −02	1.52 × −02	1.30 × −03

(*Continued on next page*)

TABLE 1
Geochemical parameters for fracture and service water samples[a] *(Continued)*

Sample #	Name	pH	Eh (mv)	T°C	Cl[b]	SO_4^{2-}	NO_3^-
30	DR550 30 SW-3 101502	2.6	665	35.0	2.80×-02	1.94×-02	1.41×-03
37	W638 SW 120899	7.5	617	20.0	9.18×-04	1.16×-03	6.42×-05
	WDF1 acid service water						
56	SW2 121098	3.0	N.A.	30.0	2.89×-03	8.01×-03	7.10×-04
51	DR938 SW 110201	N.A.	N.A.	30.0	2.01×-03	2.08×-04	$<1.61 \times -07$
52	DR938 SW 091202	N.A.	N.A.	30.0	1.38×-03	1.74×-03	5.47×-06
63	EV221 SW 103002	N.A.	N.A.	N.A.	2.66×-02	3.89×-03	3.17×-04
76	EV818 H5 SW2 102502	8.0	N.A.	37.8	4.00×-02	3.08×-03	5.64×-04
77	EV818 SW1 102502	6.4	N.A.	33.0	4.70×-02	3.08×-03	7.50×-04
78	EV821 SW 101601	N.A.	N.A.	N.A.	4.13×-02	2.38×-03	7.28×-04
84	KL441 SW 120198	6.8	N.A.	19.0	4.20×-03	6.61×-03	9.06×-04
85	KL441 HWDN SW 022801	N.A.	N.A.	N.A.	5.56×-03	6.97×-03	2.95×-04

[a]Only fracture and service waters with values for at least three geochemical parameters were selected.
[b]Cl, SO_4^{2-}, and NO_3^- are listed as M concentrations.

TABLE 2
Cell counts as determined by PLFA and flow cytometry analyses

Sample #	Fracture Water Samples	Cytometry cells/mL	PLFA[a] cells/mL
1	Be 116 FW 121801 CXN	3.9×10^4	4.0×10^4
6	Dr4 IPC	5.4×10^5	1.1×10^4
18	DR546 BH1 103001–110801	4.4×10^4	4.0×10^4
19	DR546 BH1 120798	6.1×10^5	6.3×10^4
42	DR938 H3 0M 102401	3.6×10^3	2.0×10^4
43	DR938 H3 125M 102401	3.0×10^3	6.6×10^3
44	DR938 H3 125M 102501	1.2×10^4	2.1×10^4
45	DR938 H3 250M 102401	1.7×10^4	1.3×10^4
46	DR938 H3 390M 102401	1.2×10^4	8.1×10^3
47	DR938 H3 550M 102501	3.9×10^4	5.8×10^3
48	DR938 H3 648M 102501	3.3×10^4	5.6×10^3
50	DR938 H1 110201	3.6×10^3	2.1×10^4
57	EV219 H3 080601	$<5.0 \times 10^3$	4.5×10^2
60	EV221 H3 111601	1.1×10^4	1.9×10^5
64	EV522 FW 030801HWD	$<5.0 \times 10^3$	4.2×10^3
65	EV522 FW H1 041801CTS	$<5.0 \times 10^3$	2.6×10^4
69	EV818 H5 102502	$<1.0 \times 10^3$	2.2×10^3
71	EV818 FW 062101	5.4×10^4	1.3×10^3
72	EV818 H5 102702	$<1.0 \times 10^3$	4.3×10^2
73	EV818 H6A 102702	$<1.0 \times 10^3$	4.5×10^2
79	EV914 FW 022801 ED	2.4×10^6	1.9×10^5
82	KL441 HWDS H2 100201	$<5.0 \times 10^3$	5.0×10^2
83	KL443 HWDN FW 050801	$<5.0 \times 10^3$	5.6×10^3
86	KL739 FW 062901	4.1×10^3	9.3×10^2
87	MM51870 FW 030402	9.0×10^4	6.2×10^4
97	MSI51 FW 022202 XCT8EAST	9.5×10^4	3.1×10^3

[a]The conversion factor, 2.5×10^5 cells $pmol^{-1}$ PLFA, was used to convert biomass concentrations to cells (Balkwill et al. 1988).

RDA is a linear canonical community ordination method that is similar to canonical correspondence analysis (CCA), except that RDA is a linear model, whereas CCA is unimodal (the PLFA data in this study did not conform to a unimodal distribution). The RDA method has been previously used with PLFA profiles and geochemical parameters by McKinley et al. (2005). In RDA, the ordination axes are constrained to be linear combinations of the environmental variables allowing the relationships between the environmental variables and the PLFA response variables to be directly compared. The samples are represented by points in the ordination space (a distance diagram), and the PLFA response variables and environmental variables are represented by arrows projecting from the origin (ter Braak and Prentice 1988). The arrows in the resulting ordination diagrams point in the direction of maximum variation in the PLFA, and the arrow length is proportional to the rate of change. PLFA arrow heads near the edge of the plot are most important in explaining sample differences, whereas PLFA arrow heads near the center of the plot are of less importance. PLFA arrows pointing in the same general direction as environmental gradient arrows can be interpreted as correlating well with that variable, and the longer the arrows, the greater the confidence in that correlation (ter Braak 1994, 1998). The sample-type point (Figure 3) and the mine-type point (Figure 4) in the ordination diagram were located at the centroid of the sample points where it occurs.

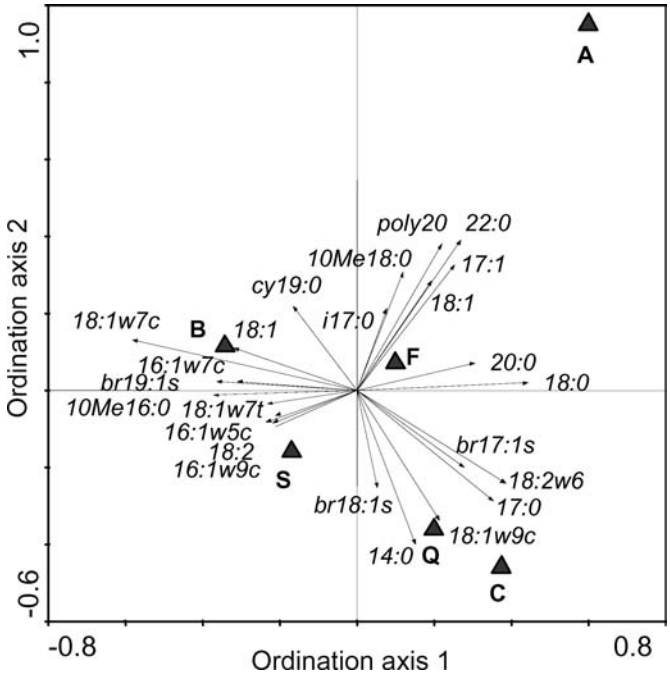

FIG. 3. Redundancy analysis of the PLFA data set for 110 samples used 61 PLFA as species. For ease of viewing, the 15 PLFAs with the largest vector magnitudes are shown. The arrows point in the direction of maximum variation in the species' abundance, and the arrow length is proportional to the rate of change. For example, 18:1ω7*c* had higher abundances in biofilm samples and lower abundances in rock samples. The sample type point (triangle symbols) were designated as fracture water (F), service water (S), biofilm (B), carbon leader (C), quartzite (Q), and air (A).

RESULTS

General Physical/Chemical Patterns

Fracture water temperature ranged from 19 to >60°C, whereas the service water temperature ranged from 17 to 33°C (Table 2). Fracture water pH ranged from 5.5 to 10, whereas service water tended to be either circumneutral (6.4 to 7.9) or highly acidic (2.6 to 3.0). Differences in anion concentrations between fracture waters within a mine and between mines were observed to range over 3 to 5 orders of magnitude (Table 2). DR546 BH1 from 2001 showed the highest Cl^- concentration at 6.76×10^{-1} M, whereas E4 IPC DW2 (DR shaft 4) had the lowest Cl^- concentration (4.37×10^{-4} M). The lowest sulfate concentrations (10^{-5} to 10^{-6} M) were found in fracture water from BE, EV and KL mines. Higher sulfate and nitrate concentrations (10^{-2} and 10^{-3} molar, respectively) were observed in DR550 service waters. The sulfate concentration of DR548 SW1 service water was low when the shaft was first opened in 1998, but over the subsequent 4 years of continuous mining activity, the sulfate and nitrate concentrations increased by a factor of 50 and 2, respectively. The service water sulfate concentration for DR938 SW, which has never been opened to mining, was quite low at 2.08×10^{-4} M (Table 2). The service water from EV221, which has been an operating mine for many years, exhibited higher concentrations of sulfate and nitrate than the fracture water (EV219 H1, H2, H3 and H4 samples). The service water for EV818, which represents an exploration tunnel where no mining was occurring, had sulfate concentrations comparable to that of the EV818 fracture water, but much higher nitrate levels (10^{-4} M). EV821 service water came from an older part of EV8 and both the sulfate and nitrate concentrations (10^{-3} and 10^{-4} M, respectively) were two- to three-fold higher than the EV821 fracture water. In the Kloof mine, which had been active for several years, both the sulfate and nitrate concentrations of KL441 and KL441 HWDN service waters were higher than the fracture waters. In general, service water has elevated nitrate concentrations compared to those of fracture water, but only service water from areas of the mine, which have been in operation for more than a year or two exhibit sulfate concentrations that are elevated with respect to the fracture water.

Biomass Estimates

Estimates of microbial biomass for the 110 samples are displayed in Figures 2A and 2B. Biofilm samples had the largest biomass ranging from 1×10^3 to 1×10^7 pmol PLFA g^{-1}. Most service water samples had higher biomass values (highest value was 6×10^6 pmol PLFA L^{-1}) than fracture water, yet both types of samples had biomass ranging over 5 orders of magnitude (10^0 to 10^5 pmol PLFA L^{-1}). Air samples had the lowest biomass at 1×10^{-2} to 1×10^0 pmol PLFA L^{-1}. Carbon leader and quartzite

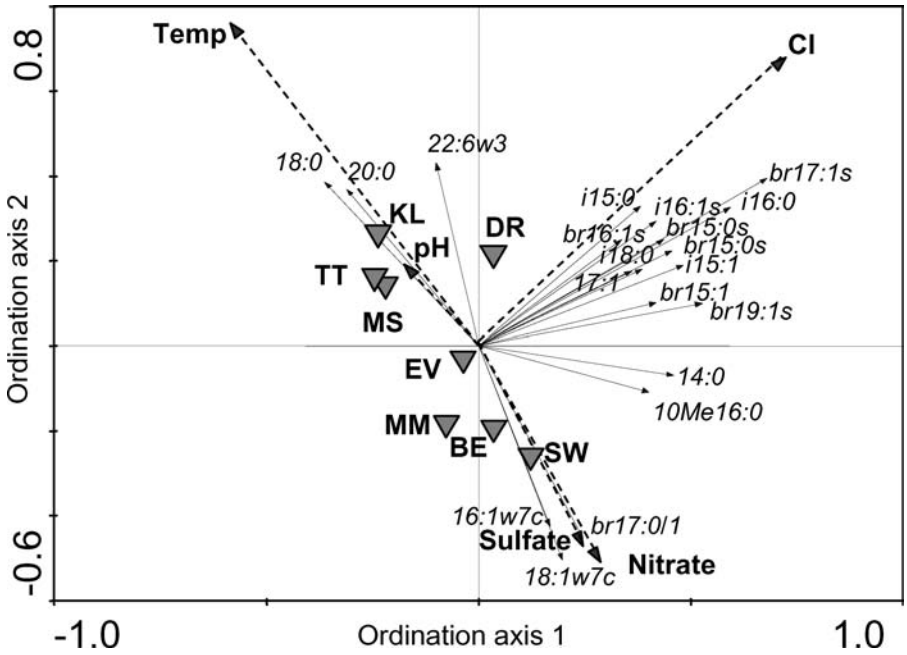

FIG. 4. Redundancy analysis of the PLFA subset of 57 fracture water and service water samples used 61 PLFA as species and 4 environmental parameters. The triangles representing the fracture waters from the various mines were identified as BE = Beatrix, DR = Dreifontein, EV = Evander, KL = Klook, MM = Masimong, MS = Merrispruit, and TT = TauTona. The Mponeng and Northam mine samples lacked sufficient geochemical data to be included in this RDA. Service waters from any of the mines were represented by SW and a triangle. Environmental parameters and PLFA were plotted as vectors. For ease of viewing, the 20 PLFAs with the largest vector magnitudes are shown. The arrows point in the direction of maximum variation in the environmental and species' abundance, with the arrow lengths being proportional to the rate of change of these parameters. For example, the service water point was closely related to increased concentrations of sulfate and nitrate and increased PLFA abundances of br 17:0/1, 16:1ω7*c* and 18:lω7*c*.

rock samples had PLFA biomass estimates of 3×10^{-1} to 2×10^{2} pmol PLFA g^{-1}.

Flow cytometry cell counts and PLFA abundances (1) were analyzed by linear regression analysis. One data point was determined to have excess leverage and it was removed from the analysis. The re-run of the analysis dropped the R^2 value from 0.41 to 0.004 and the slope from 0.066 to 0.0154. Based on these results, there is no relationship between flow cytometry and PLFA abundance.

Redundancy Analyses

Figure 3 shows PLFA gradient arrows and the sample-type points for the RDA ordination of the 110 PLFA profiles. The total variation in PLFA that was explained by sample type was 9.9%. The first canonical axis of the model was significant ($F = 4.008$ and $p = 0.012$). Biofilm samples were enriched in 18:1ω7c, 18:1, 16:1ω7*c* and br19:1s, and to a lesser extent 10Me16:0 and cy19:0. Carbon leader and quartzite rock samples were enriched in 18:1ω9*c*, 14:0, 17:0, 18:2ω6, br18:1s, br17:1s and depleted in cy19:0. Air samples were enriched with 22:0, 17:1, 18:1 and a 20-carbon polyunsaturated fatty acid, whereas fracture waters were intermediately enrich and service waters were depleted. Likewise, the service water samples were enriched with 16:1ω9c, 18:2, 16:1ω5*c*, and 18:1ω7*t* whereas fracture and air samples were depleted. The fracture water point located nearest to the origin and could be intermediately enriched by most of the PLFAs.

The RDA ordination of the PLFA and geochemical data set containing 57 fracture and service water samples are shown in Figures 4 and 5. The total variation in PLFA that was explained by environment factors was 17.1%. The first canonical axis of the model was significant ($F = 3.770$ and $p = 0.002$). Samples taken from Kloof and Tau Tona mines were enriched in the fatty acids (18:0, 20:0 and 22:6ω3) and were located near temperature and pH vectors and depleted in fatty acids (16:1ω7*c*, 18:1ω7*c*. and br17:0/1), which were positioned near the sulfate and nitrate vectors. Service waters and Beatrix fracture water samples exhibited the opposite relationship where 18:0, 20:0 and 22:6ω3 were depleted, while 16:1ω7*c*, 18:1ω7*c* and br17:0/1 were enriched. These samples were positioned near the sulfate and nitrate vectors. Dreifontein fracture water samples were enriched in terminally branched and mid-chain branched saturated fatty acids and plotted between the temperature, pH, and chloride vectors. Evander fracture water samples were not as enriched in the previously PLFAs and as such they were located in-between these sample groups and near the center of the plot.

Figure 5 shows RDA ordination with environmental gradient vectors and sample points. Chloride was an important geochemical parameter for distinguishing the DR5 fracture water samples

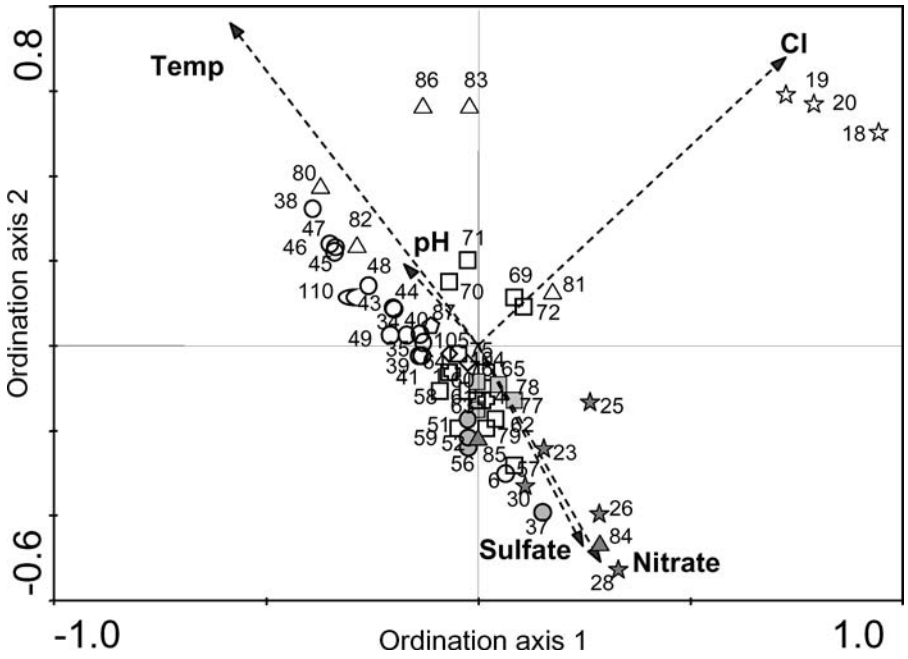

FIG. 5. Redundancy analysis on the PLFA subset of 57 fracture water and service water samples used 61 PLFA as species and 4 environmental parameters. Arrows representing environmental variable abundance were shown with fracture water samples (open symbols) and service water samples (closed symbols). Mines were identified with a cross for Beatrix, star for Dreifontein shaft 5, circle for Dreifontein shafts 4, 6 and 9, square for Evander, triangle for Kloof, pentagon for Mponeng, diamond for Merriespruit, and crescent for Tau Tona. The sample number may be used to identify the exact water sample by cross-referencing with Table 2. Sample 19 was DR546 BH1 120798 and had a higher concentration of chloride compared with other water samples.

from those of the other mines. Likewise, higher water temperature was important for separating KL441 HWDS, KL443, KL7 and TT104 from other fracture water. Fracture water samples from EV8 appear to associate with both temperature and Cl vectors. Sulfate and nitrate correlated highly with service water. Fracture water from BE, DR (shafts 4, 6 and 9) and EV (shafts 2 and 5) did not exhibit any strong correlation with these environmental parameters.

DISCUSSION

Biomass

The 9 orders of magnitude range for biomass exhibited in the mine samples indicated large variability between sample type and considerable variation among biofilm, fracture and service water samples. However a similar variation, especially a steady decline in microbial biomass with depth, has been shown by other terrestrial deep subsurface drilling projects and at the ASPO Hard Rock Laboratory (Kieft et al. 1995; Kotelnikova and Pedersen 1997; Onstott et al. 1998, 1999). When cell abundance-based PLFA and flow cytometry were analyzed by linear regression, no relationship was found. One speculation as to why these techniques were not correlated is that Archaea, which have been shown to be present in the South African fracture water (Takai et al. 2001a; Gihring et al. this issue), were not accounted for by the PLFA technique. A second explanation is that the flow cytometry data may have overestimated cell counts because up to four morphological types were used to estimate total cells. Some of these morphologies may have represented particulate matter other than bacterial cells. Another consideration is that the amount of lipid membrane per cell may be altered under the temperature and pressure constraints in these deep environs, as has been shown for cell size variation and lipid content in surface and subsurface microorganisms (Balkwill et al. 1988).

Microbial Community Structure and Environmental Factors

The RDA analysis allowed us to examine the patterns in the PLFA data in terms of both the sample types and the measured environmental parameters (Figures 3–5). In Figure 3, the PLFA ordinations clearly indicated that specific PLFA were important for distinguishing sample types. These PLFAs were assigned to functional groups of microorganisms using previous documented approaches (Findlay and Dobbs 1993; White et al. 1996).

The PLFAs associated with biofilm were 18:1ω7c, 16:1ω7c, 18:1, cy19:0, and 10Me16:0. The first three monounsaturated fatty acids are indicative of aerobic or anaerobic prokaryotes and, in combination with cy19:0, may be used as a marker for gram-negative bacteria. Cy19:0 and 10Me16:0 are associated with sulfate- and metal-reducing bacteria and other anaerobic prokaryotes. The PLFAs important to distinguishing carbon leader and quartzite rock samples included 18:2ω6, 17:0, 18:1ω9c, br17:1s, and br18:1s. The polyunsaturated and

monounsaturated fatty acids represented microeukaryotic populations; whereas the branch chained monounsaturated fatty acid indicated metal reducing bacteria.

Another microeukaryotic PLFA biomarker is 18:2, which was associated with service water samples. These water samples also contained prokaryotic PLFAs 16:1ω9*c*, 16:1ω5*c* and 18:1ω7*t*. The *trans* monounsaturated fatty acid has been produced when microbial communities are starved or exposed to toxic compounds (Guckert et al. 1986; Kieft et al. 1994; Frostagård et al. 1996). The appearance of this stress indicator in service water is not surprising since service water is treated with hypochlorite. Air samples had PLFA associated with aerobic prokaryotes and eukaryotes. The PLFA associated with the fracture waters are less well defined but could include i17:0 and 10Me18:0, which indicate gram-positive prokaryotes and other anaerobic bacteria. These PLFA data are supported by the phylogenetic 16S rDNA analysis performed on service water, air, and carbon leader samples, which showed that clones from service water belonged to the α-, β- and γ-*Proteobacteria* division and that the carbon leader clones were more closely related to those from service water than air samples (Onstott et al. 2003).

Temperature, pH, and concentrations of anions were included in a second RDA with the PLFA profiles for a subset of the fracture and service water samples. In this RDA, different mine samples were related to specific fatty acids and correlated with environmental parameters. The service water sample point (Figure 4) and the water samples (filled symbols on Figure 5) were highly correlated with sulfate and nitrate concentrations and with 18:1ω7*c* and 16:1ω7*c*. These monounsaturated fatty acids have been associated with aerobic heterotrophic bacteria, a finding which is consistent with the service water clones that belong to the α-, β- and γ-) *Proteobacteria* (Onstott et al. 2003).

Both MM and BE facture water samples were located near the service water and have a weaker correlation with sulfate and nitrate concentrations and the PLFAs (18:1ω7*c* and 16:1ω7*c*). These PLFA results were substantiated by the 16S rDNA clone libraries from the Beatrix mine fracture water, in which *Proteobacteria* dominated, with sequences resembling those found in the service water (Lin et al. 2005).

In Figure 5, fracture water samples EV8 (square symbols numbered 69 through 72) are located between the temperature and chloride vectors, while DR5 (star symbols numbered 18 through 20) had chloride concentrations of 0.6 M and were highly correlated with the chloride vector. The chloride vector was strongly associated with the terminally branched saturated fatty acids and the branched monounsaturated fatty acids (Figure 4). These PLFA strongly indicated gram-positive prokaryotes and other anaerobic bacteria such as sulfate- and metal-reducing bacteria. This finding was supported by phylogenetic analysis of DNA extract from fracture water that showed the presence of sulfate reducing bacteria, including *Desulfotomaculum*-like organisms (Baker et al. 2003; Moser et al. 2003). PLFA profiles from these DR5 fracture waters samples were similar to the PLFA profile for *Desulfotomaculum putei* which was isolated from the Taylorsville Triassic Basin in Virginia (Liu et al. 1997).

The temperature and pH vectors were strongly correlated with the Kloof, TauTona, and Merrispruit mine fracture waters. The temperatures of Kloof fracture waters were 55–61 °C and the pH values were 8.2–9.0. These water samples aligned with two straight-chain saturated fatty acids (18:0 and 20:0) and a microeukarote lipid biomarker, 22:3ω6. This observation corresponded with the high amounts of normal saturates detected in these mines and may denote low biomass or a more homogeneous community.

Interestingly, eukaryotic PLFAs including 18:2ω6, 18:2, 18:3ω3, 20:1's, 20:5ω3, 22:4ω6, and 22:6ω3 have been detected in the South African samples (data not shown). 18:2ω6 generally indicates fungi or cyanobacteria (Weete 1974; Stahl and Klug 1996); however, cyanobacteria were absent from the 16S rDNA clone libraries (Lin et al. this volume). Bacteria have been shown to produce PLFA of 20 or more carbons in length, with one or more double bonds. Takai et al. (2001b) demonstrated that a thermophilic heterotroph isolated from deep subsurface geothermal water produced 20:1ω9 as 50% of its total PLFA profile. Furthermore, piezophiles have been shown to contain polyunsaturates, 20:ω3 and 22:6ω3 (DeLong and Yayanos 1985; Fang et al. 2000b). Bartlett (2002) suggested that polyunsaturates maintain membrane stability and fluidity. In the South African mines, we were unable to assess the partial pressures or the combination of temperature and pressure experienced by microorganisms in the fracture waters. However, we speculate that the deep subsurface communities may alter PLFA compositions to maintain membrane integrity under these extreme environmental conditions. 18S rRNA gene-based analysis was recently used to document the predominance of microeukaryotes in anaerobic sulfide and sulfur-rich springs (Luo et al. 2005). Future studies using this genetic analysis (PCR amplification of 18S rRNA genes) or microscopic observation will be necessary to confirm the presence of eukaryotes.

CONCLUSIONS

The mines provide access to the deep subsurface and life in extreme environments. The PLFA approach provided a sensitive and meaningful measure of microbial biomass and of PLFA responses to changes in environmental factors. Biofilm samples had the most lipid biomass, followed by (in decreasing concentration of total PLFA) service water samples, fracture water samples, rock samples, and air samples. PLFA profiles revealed a diverse microbial community structure for most samples. The RDA analysis provided a robust method to understand the PLFA responses to changes in environmental parameters. Community structures varied among mines and were related to chemistry and physical parameters. PLFA signature biomarkers in conjunction with molecular analyses confirmed the presence of mesophilic heterotrophic bacteria, thermophiles, sulfate reducers, and metal reducers. Mesophilic heterotrophic bacteria

were associated with mine samples that had lower temperatures and higher concentrations of nitrate and sulfate. Metal reducers and sulfate reducers were more prominent in mine water samples with higher chloride concentrations. Samples at higher temperature had lower PLFA biomass and the PLFA profiles were not indicative of a specific group of bacteria. Biofilm samples contained a broad spectrum of bacteria from aerobic heterotrophic bacteria to anaerobic sulfate-reducing bacteria. Continued investigations into the deep terrestrial subsurface will provide a better understanding of the microbial communities and their biogeochemical processes.

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