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Microbial Analysis Report

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Analysis Requested: PLFA

Project: 0285929 WVA

Comments:

All samples within this data package were analyzed under U.S. EPA Good Laboratory Practice Standards: Toxic Substances Control Act (40 CFR part 790). All samples were processed according to standard operating procedures. Test results submitted in this data package meet the quality assurance requirements established by Microbial Insights, Inc.

Reported by: **Reviewed by:**

Microbial Analysis Report

Executive Summary

Microbial communities from 12 soil samples were characterized using phospholipids fatty acid content (PLFA Analysis). The samples represent 3 treatment cells (sites A, C, D). Sample KS-1 is a QC sample. Data obtained in this data set represents the baseline microbial communities present prior to treatments.

Results from this study revealed the following key observations:

- Overall viable biomass found was $\sim 10^{7\text{-}8}$ cells/ g dry wt. in all samples.
- PLFA profiles pointed to the presence of relatively diverse community structures in all samples, which were quite similar among samples.
- Microbial communities were dominated by Proteobacteria in all samples.
- The patterns seen for both mid-chain branched PLFA and branched monoenoic PLFA indicate that anaerobic sulfate reducing bacteria were likely present in all sample sites.
- The Gram-negative bacterial communities (Proteobacteria are among them) at all sites show a response to environmental stress as indicated by *trans/cis* ratios seen for particular biomarkers. The microbial communities also are starved and slow growing.
- Two differences were noted between the 18MPI pilot study and this baseline study. There was an increase in the proportion of PLFA biomarkers indicative of anaerobic sulfate-reducing bacteria, and also a decrease in the proportion of the biomarker 18:2w6, which would indicate a drop in the fungal organisms present.

Overview of Approach:

Phospholipid Fatty Acid Analysis

Examining the phospholipid fatty acids (PLFA) in environmental samples is an effective tool for monitoring microbial responses to their environment. They are essential components of the membranes of all cells (except for the Archea, a minor component of most environments), so their sum includes all important actors of most microbial communities. There are four different types of information in PLFA profiles – biomass, community structure, diversity, and physiological status.

Biomass: PLFA analysis is the most reliable and accurate method available for the determination of viable microbial biomass. Since phospholipids break down rapidly upon cell death (21, 23), the PLFA biomass does not contain 'fossil' lipids of dead cells. The sum of the PLFA, expressed as picomoles (1 picomole = 1×10^{-12} mole), is proportional to the number of cells. The proportion used in this report, 20,000 cells/pmole, is taken from cells grown in laboratory media, and varies somewhat with type of organism and environmental conditions. Starving bacterial cells have the lowest cells/pmol, and healthy eukaryotic cells have the highest.

Community Structure:. The PLFA in an environmental sample is the sum of the microbial community's PLFA, and reflects the proportions of different organisms in the sample. PLFA profiles are routinely used to classify bacteria and fungi (19) and are one of the characteristics used to describe new bacterial species (25). Broad phylogenic groups of microbes have different fatty acid profiles, making it possible to distinguish among them (4, 5, 22, 24). Table 1 describes the six major structural groups employed in this report.

Table 1. Description of PLFA structural groups.

PLFA Structural Group	General classification
Monoenoic (Monos)	Abundant in Proteobacteria (Gram negative bacteria), typically fast growing, utilize many carbon sources, and adapt quickly to a variety of environments.
Terminally Branched Saturated (TerBrSats)	Characteristic of Firmicutes (Low G+C Gram-positive bacteria), and also found in Bacteriodes, and some Gram-negative bacteria (especially anaerobes).
Branched Monoenoic (BrMonos)	Found in the cell membranes of micro-aerophiles and anaerobes, such as sulfate- or iron-reducing bacteria
Mid-Chain Branched Saturated (MidBrSats)	Common in Actinobacteria (High G+C Gram-positive bacteria), and some metal-reducing bacteria.
Normal Saturated (Nsats)	Found in all organisms.
Polyenoic	Found in eukaryotes such as fungi, protozoa, algae, higher plants, and animals.

Diversity: The diversity of a microbial community is a measure of the number of different organisms and the evenness of their distribution. Natural communities in an undisturbed environment tend to have high diversity. Contamination with toxic compounds will reduce the diversity by killing all but the resistant organisms. The addition of a large amount of a food source will initially reduce the diversity as the opportunists (usually Proteobacteria) over-grow organisms less able to reproduce rapidly. The formulas used to calculate microbial community diversity from PLFA profiles have been adapted from those applied to communities of macro-organisms (8).

Physiological status: The membrane of a microbe must adapt to the changing conditions of it's environment, and these changes are reflected in the PLFA. Toxic compounds or environmental conditions that disrupt the membrane cause some bacteria to make trans fatty acids from the usual cis fatty acids (7). Many Proteobacteria and others respond to starvation or highly toxic conditions by making cyclopropyl (7) or mid-chain branched fatty acids (20). The physiological status biomarkers for Toxic Stress and Starvation/Toxicity are formed by dividing the amount of the stress-induced fatty acid by the amount of it's biosynthetic precursor.

PLFA were analyzed by extraction of the total lipid (21) and then separation of the polar lipids by column chromatography (6). The polar lipid fatty acids were derivatized to fatty acid methyl esters, which were quantified using gas chromatography (15). Fatty acid structures were verified by chromatography/mass spectrometry and equivalent chain length analysis.

Results and Discussion

Phospholipid Fatty Acid Analysis

Overall biomass estimates, determined by PLFA concentrations were ~$10^{7\text{-}8}$ cells per g dry wt of soil sample. Biomass seen was highest for samples A-1 and A-3 and D-4. Other samples generally had a slightly lower biomass.

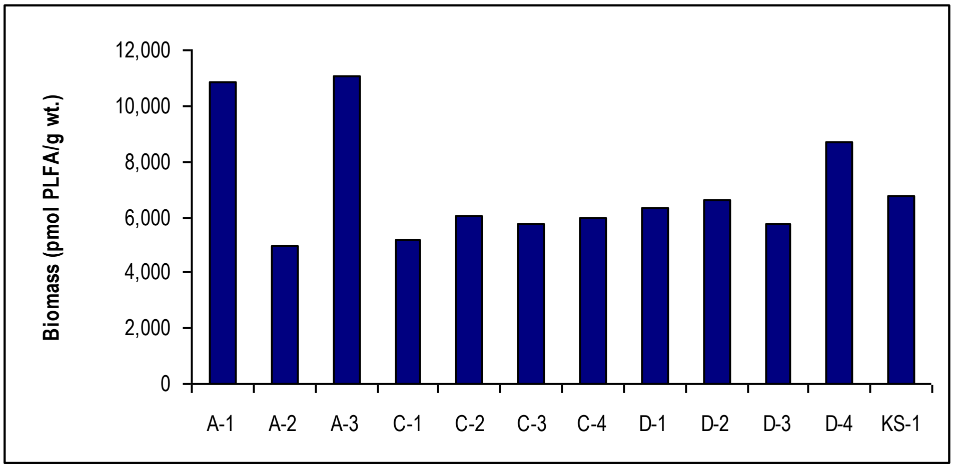

Figure 1. Biomass content is presented as the total amount of phospholipid fatty acids (PLFA) extracted from a given sample. Total biomass is calculated based upon PLFA attributed to bacterial and eukaryotic biomass (associated with higher organisms).

The PLFA profiles seen indicated the presence of diverse microbial communities which were similar among all samples (Figure 2). Profiles were primarily composed of monoenoic PLFA (46-55%), which are common in Proteobacteria. Gram-negative bacteria are of particular interest in contaminated sites due to their ability to utilize a variety of carbon sources and to adapt quickly to changes in environmental conditions.

Terminally branched saturated PLFA was present in amounts ranging from 12-17% of the total PLFA. These biomarkers are most commonly attributed to *Firmicutes* (*Clostridia*-like Gram-positive bacteria). These biomarkers can also indicate the presence of anaerobic sulfate-reducing bacteria such as *Desulfovibrio*.

Mid-chain branched PLFA were present in amounts that ranged from ~7% (sample A-3) to ~13% (sample A-2). These biomarkers are common in aerobic *Actinomycetes*, but can also represent the presence of anaerobic Gram-negative *Desulfobacter* -type sulfate reducers. The high ratio of 10Me16:0 to 10Me18:0 found in all samples indicates that these biomarkers support the presence of sulfate-reducing Gram-negative bacteria.

The proportion of branched monoenoic PLFA ranged from 2-4% of the total PLFA. These biomarkers (particularly i17:1w7c), are indicative of anaerobic *Desulfovibrio*-type metal-reducing bacteria.

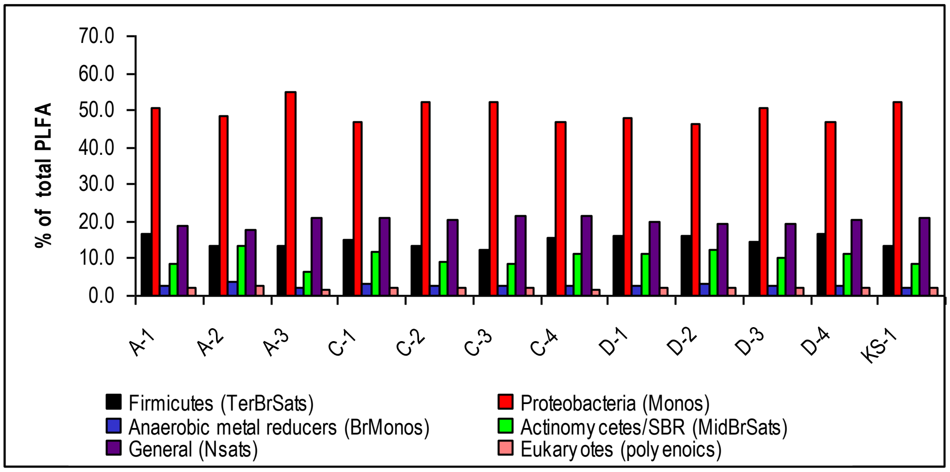

Figure 2. Relative percentages of total PLFA structural groups in the samples analyzed. Structural groups are assigned according to PLFA chemical structure, which is related to fatty acid biosynthesis. See Table 1 for detailed descriptions of structural groups.

Physiological biomarkers that provide an indication of starvation and response to environmental stress showed that the bacterial communities in all samples were showing signs of starvation, i.e. they are not actively growing, but in a relatively slow-growing or stationary phase. Starvation was highest for sample A-2. The ratio of *trans/cis* biomarkers indicated that for all samples there was a response of the bacterial community to environmental stress. The response to stress appears to be relatively uniform among sites.

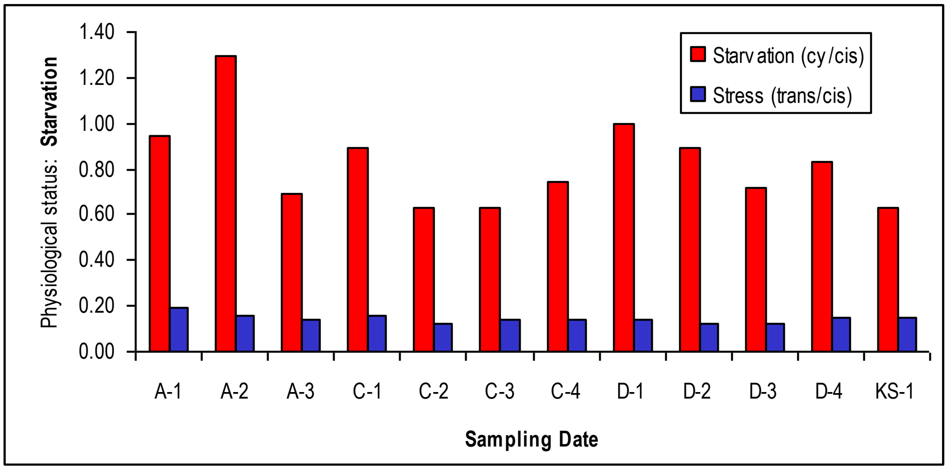

Figure 3. Microbial physiological stress markers. The starvation biomarker for the Gram-negative bacterial community is assessed by the ratios of cyclopropyl fatty acids to their metabolic precursors. An adaptation of the Gram-negative community to toxic stress is determined by the ratio of ω7t/ω7c fatty acids. Gram-negative bacteria generate *trans* fatty acids to minimize the permeability of their cellular membranes as an adaptation to a less favorable environment. Ratios (16:1ω7t/16:1ω7c and 18:1ω7t/18:1ω7c) greater than 0.1 have been shown to indicate an adaptation to a toxic or stressful environment, resulting in decreased membrane permeability.

Table 2 Values below are: viable microbial biomass expressed as picomoles of PLFA per mL of sample and as cells per mL of sample, fatty acid structural groups as percent of total PLFA, and physiological status biomarkers as mole ratio. "-" indicates data not available.

Samples		**Biomass**		**Community Structure (% of total PLFA)**						**Physiological Status**	
Sample Name	Sample Date	pmol//g dry wt.	cells/g dry wt.	Anaerobic Gram Neg./ Firmicutes (TerBrSats)	Proteobacteria (Monos)	Anaerobic metal reducers (BrMonos)	Actinomycetes/ SRB (MidBrSats)	General (Nsats)	Eukaryotes (polyenoics)	Starved *cy/cis*	Membrane Stress, *trans/cis*
A-1	6/11/03	10,886	2.18E+08	16.5	50.6	2.7	8.8	19.1	2.4	0.95	0.19
A-2	6/11/03	4,953	9.91E+07	13.4	48.6	4.0	13.4	17.7	2.9	1.29	0.16
A-3	6/11/03	11,076	2.22E+08	13.4	55.0	2.1	6.6	21.2	1.8	0.70	0.14
C-1	6/11/03	5,184	1.04E+08	15.2	46.9	3.1	11.7	20.9	2.2	0.89	0.16
C-2	6/11/03	6,014	1.20E+08	13.5	52.4	2.5	9.3	20.4	1.9	0.63	0.13
C-3	6/11/03	5,714	1.14E+08	12.4	52.4	2.4	8.7	21.8	2.3	0.63	0.14
C-4	6/11/03	5,935	1.19E+08	15.5	46.8	2.7	11.3	21.8	1.9	0.74	0.14
D-1	6/11/03	6,338	1.27E+08	16.2	47.9	2.9	11.2	19.8	2.0	1.00	0.14
D-2	6/11/03	6,580	1.32E+08	16.3	46.1	3.4	12.2	19.6	2.4	0.90	0.12
D-3	6/11/03	5,738	1.15E+08	14.8	50.7	2.8	10.2	19.6	2.0	0.71	0.12
D-4	6/11/03	8,687	1.74E+08	16.6	46.6	2.9	11.3	20.5	2.1	0.83	0.15
KS-1	6/11/03	6,765	1.35E+08	13.7	52.2	2.4	8.5	21.2	2.0	0.63	0.15

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