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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: _____

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Microbial Analysis Report

Executive Summary

Microbial communities from 12 soil samples were characterized according to their phospholipids fatty acid content (PLFA Analysis). The samples were from 3 treatment cells (sites A, C, D) and sample K-S which was a QC sample. Results from this study revealed the following key observations:

- Estimates of viable microbial biomass, based on total PLFA content, were ~ 10^{7-8} cells/g dry weight for all samples. Sample C-2 contained the highest biomass, D-2 the lowest.
- PLFA profiles showed that all samples contained relatively diverse microbial community structures which were quite uniform among the majority of the samples. Sample D-2 had the most unique profile, consisting of the lowest proportion of monoenoics, and the highest proportion of branched monoenoic PLFA (~2-3x higher than in other samples), and also of polyenoic PLFA (~2x higher than other samples).
- Analysis of both mid-chain branched PLFA and branched monoenoic PFA indicated that anaerobic sulfate reducing bacteria were likely present in all sampling locations.
- A response to environmentally induced stress was seen in all samples, as was starvation. Among the sampling cells, cell A had the highest starvation, cell C the lowest.

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 viable biomass, was estimated using the total PLFA concentration, and was 107-8 cells per g dry weight of sample. Biomass was highest in samples A-2, A-3 and C-2 and lowest in sample D-2. Among the sampling cells, cell D contained the lowest biomass.

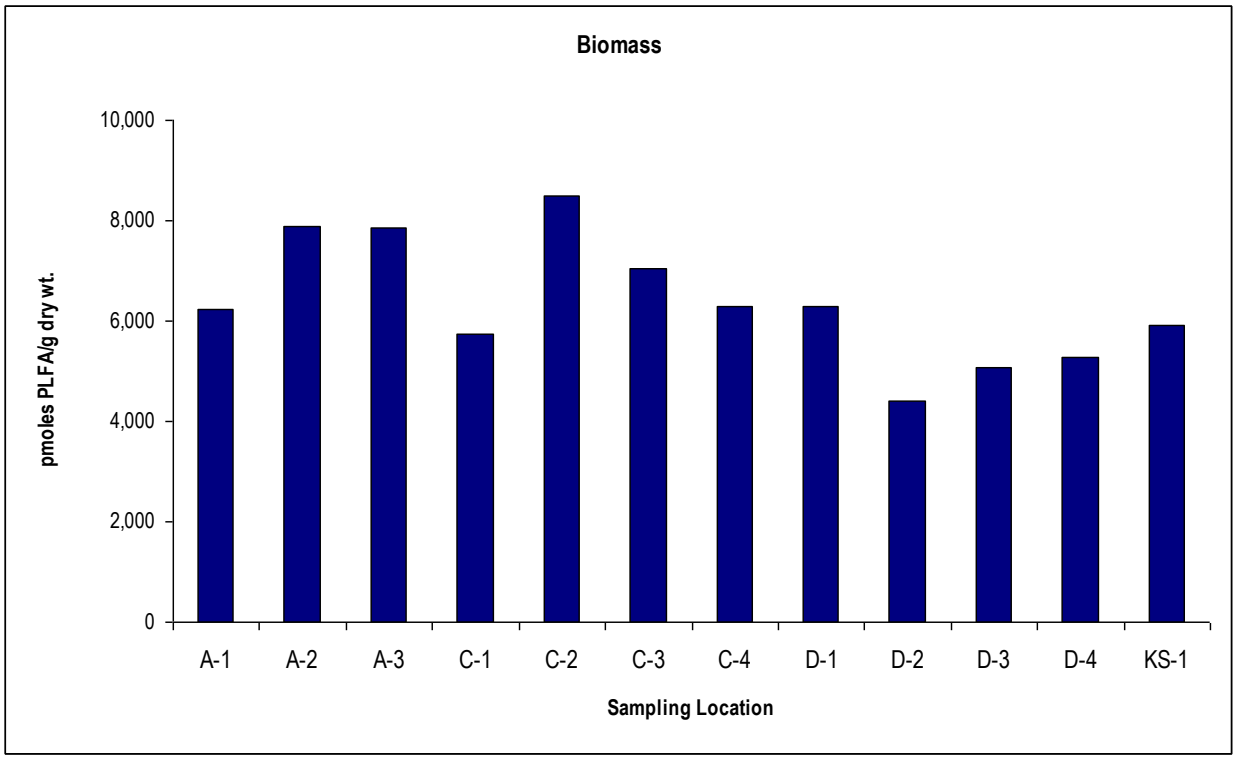

Figure 1. Biomass content is presented as the total amount of phospholipid fatty acids (PLFA) extracted from a given sample. Total biomass is calculated based upon PLFA attributed to bacterial and eukaryotic biomass (associated with higher organisms).

PLFA profiles were used to determine the microbial community structures of each sample. These community structures were quite similar among the samples, and indicated the presence of diverse microbial communities. Monoenoic PLFA are indicative of the presence of Gram-negative *Proteobacteria*, and were present in proportions of ranging from ~36%-~51% of the total PLFA concentration. The second most prevalent PLFA structural group was normal saturated PLFA, which is present in all living organisms, and therefore does not yield useful information on microbial community structure.

Anaerobic biomarkers were present in all samples. Terminally branched PLFA, is indicative of Firmicutes (*Clostridia*-like bacteria) and also of some Gram-negative anaerobes, and was seen in proportions that ranged

from ~11-20% of the total PLFA. The proportion of these PLFA was highest in samples from cell A. Branched monoenoic PLFA is indicative of the presence of anaerobic metal reducers and was seen in proportions of ~2.6-4.6% of the total PLFA for the majority of the samples. Branched monoenoics were 2-3x higher in sample D-2 (~10.5% of the total PLFA). This higher proportion included a 2x higher concentration of the biomarker i17:1w7c, which is indicative of anaerobic *Desulfovibrio*-type sulfate reducers.

Mid-chain branched saturated PLFA are indicative of the presence of Actinomycetes and also of some anaerobic sulfate reducers such *Desulfobacter*. Analysis of the ratio of 10Me16:0 to 10Me18:0 indicated that all samples likely contained sulfate reducing bacteria.

Eukaryotic biomarkers (polyenoics) made up ~1.7-5.0% of the total PLFA. This PLFA structural group occurred in proportions ~2% of the total PLFA for the majority of the samples, and was highest (~5%) in sample D-2.

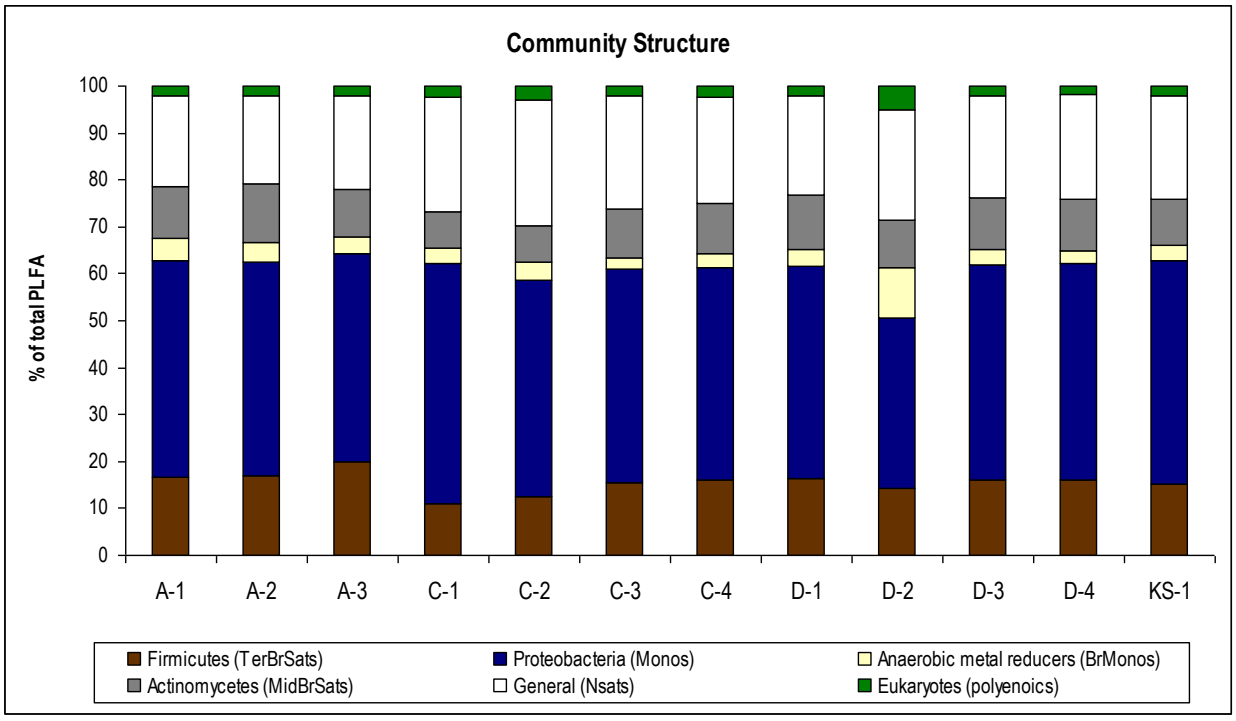

Figure 2. Relative percentages of total PLFA structural groups in the samples analyzed. Structural groups are assigned according to PLFA chemical structure, which is related to fatty acid biosynthesis. See Table 1 for detailed descriptions of structural groups.

PLFA biomarkers which point to starvation and to a microbial response to environmental stress showed that the microbial communities of all samples were undergoing both starvation and stress. Starvation was highest in samples A-2, A-3 and D-2. Among the study cells, starvation was highest in cell A, and lowest in cell C. Response to environmentally induced stress was moderate, and was generally uniform among the samples.

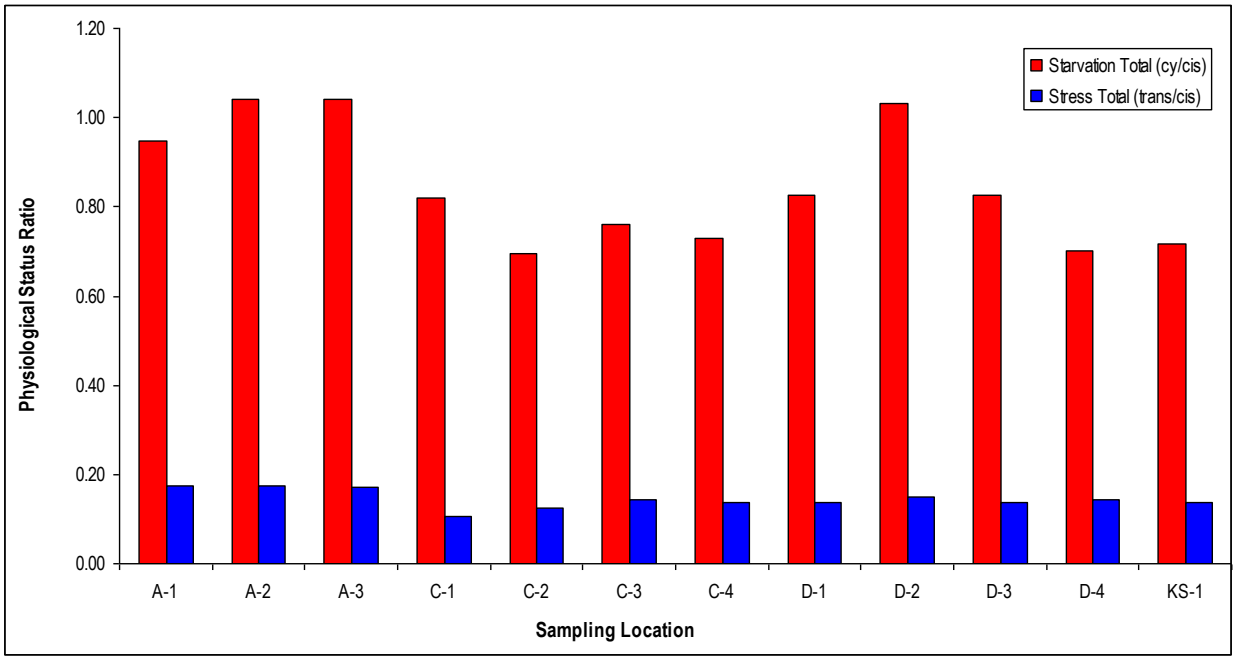

Figure 3. Microbial physiological stress markers. The starvation biomarker for the Gram-negative bacterial community is assessed by the ratios of cyclopropyl fatty acids to their metabolic precursors. An adaptation of the Gram-negative community to toxic stress is determined by the ratio of ω7t/ω7c fatty 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 g dry weight of sample and as cells per g dry weight 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 weight	cells/g dry weight	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	7/29/03	6,219	1.24E+08	16.8	46.1	4.6	11.2	19.3	2.1	0.95	0.17
A-2	7/29/03	7,876	1.58E+08	17.0	45.5	4.3	12.4	18.9	1.9	1.04	0.17
A-3	7/29/03	7,862	1.57E+08	20.0	44.5	3.5	9.9	20.0	2.1	1.04	0.17
C-1	7/29/03	5,732	1.15E+08	11.1	51.2	3.2	7.8	24.4	2.3	0.82	0.11
C-2	7/29/03	8,493	1.70E+08	12.6	46.2	3.8	7.5	26.9	2.9	0.70	0.13
C-3	7/29/03	7,054	1.41E+08	15.6	45.4	2.6	10.3	24.1	2.1	0.76	0.14
C-4	7/29/03	6,304	1.26E+08	16.0	45.5	3.0	10.5	22.8	2.3	0.73	0.14
D-1	7/29/03	6,295	1.26E+08	16.3	45.3	3.7	11.4	21.4	2.0	0.82	0.14
D-2	7/29/03	4,400	8.80E+07	14.4	36.3	10.5	10.3	23.5	5.0	1.03	0.15
D-3	7/29/03	5,082	1.02E+08	16.1	45.7	3.5	11.0	21.7	2.0	0.83	0.14
D-4	7/29/03	5,286	1.06E+08	16.2	46.0	2.9	10.8	22.5	1.7	0.70	0.14
KS-1	7/29/03	5,907	1.18E+08	15.2	47.5	3.5	9.8	22.1	2.0	0.72	0.14

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