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Microbial Analysis Report

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Analysis Requested: PLFA

Project: WVA Land Farming project #2118012

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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Executive Summary

The microbial communities of nine soil samples from the WVA Land Farming Project were characterized according to their phospholipid fatty acid content (PFLA Analysis). Results from this analysis revealed the following key observations:

- Estimated viable biomass, as determined by the total PLFA concentration, was in the range of ~10^{7-8} cells/gram dry weight for the samples. (Figure 1, Table 2)

- PLFA profiles showed that the microbial communities of these nine samples were similar in diversity and primarily composed of Gram negative Proteobacteria (as shown by the proportion of monoenoic PLFA). Total "anaerobic" biomarkers (terminally and mid-chain branched saturated and branched monoenoic PLFA) representative of Firmicutes, sulfate and metal reducers respectively, accounted for ~23-32% of the total PLFA for each of the samples. (Figure 2, Table 2)

- Physiologic status ratios for slowed growth rate (starvation) and microbial response to environmentally induced stress in the Gram negative populations showed that none of the nine samples had notable levels of stress response while all nine of the samples were experiencing high levels of starvation. (Figure 3, Table 2)

Overview of Approach

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 members of most microbial communities. There are three different types of information in PLFA profiles: biomass; community structure; and physiological status.

Biomass: PLFA analysis is the most reliable and accurate method available for the determination of viable microbial biomass. Phospholipids break down rapidly upon cell death (21, 23), so 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.

Physiological status: The membrane of a microbe adapts to the changing conditions of its 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 other microbes 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 for Starvation/Toxicity are formed by dividing the amount of the fatty acid induced by starvation and/or stress, by the amount of its 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

Biomass estimates were quite similar among the samples, with less that one order of magnitude variability between the highest and lowest biomass. Samples C-3 and C-4 had the highest biomass at ~10^8 cells/gram dry weight while sample D-1 had the lowest level of biomass.

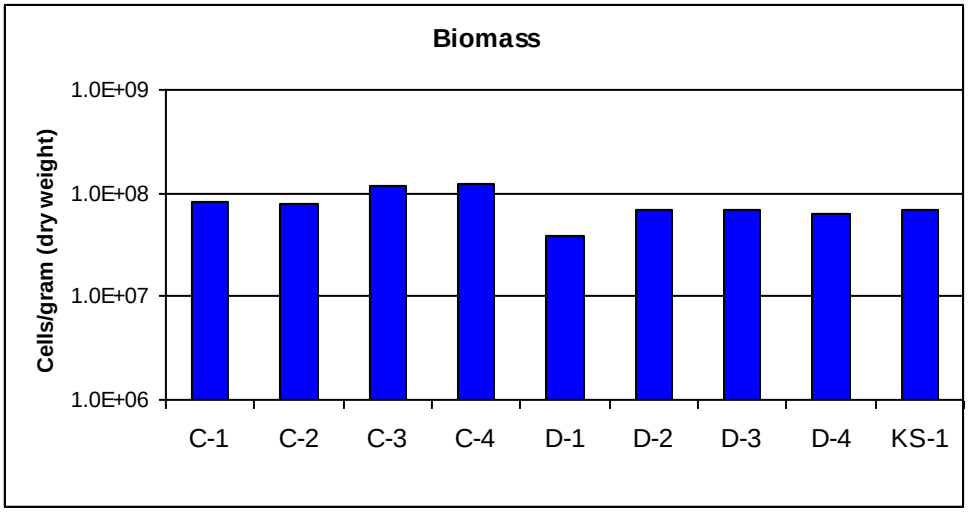

Figure 1. Biomass content is presented as a cell equivalent based on the total amount of phospholipid fatty acids (PLFA) extracted from a given sample. Total biomass is calculated based upon PLFA attributed to bacterial and eukaryotic biomass (associated with higher organisms).

The microbial communities of these nine samples were notably similar in diversity and primarily composed of Gram negative Proteobacteria (monoenoic PLFA) which were found in proportions between ~47-53% of the total PLFA for each sample. Proportions of Proteobacteria in the C-cell samples were ~48-53% of the PLFA while the D-cells samples ranged from ~47-49% of the total PLFA and in KS-1 were ~47% of the PLFA. Total "anaerobic" biomarkers were approximately one-fourth of the total PLFA in the C-cell samples (~23-29% of the total PLFA) while proportions were generally higher in the D-cell samples (~28-32% of the PLFA) and in KS-1 (~31% of the PLFA). Among the specific categories of anaerobes, Firmicutes (shown by terminally branched saturated PLFA), which include *Clostridia*-like fermenting bacteria, were the most abundant, and represented approximately 13-16% of the microbial community in the all of the samples. Sulfate reducers (mid-chain branched saturated PLFA) were also present in each sample at ~7-10% of the community. Anaerobic metal reducers were the least abundant category of anaerobes, comprising ~3-5% of the microbial community of the samples. Eukaryotes (as indicated by polyenoic PLFA) represented ≤1% of the microbial community.

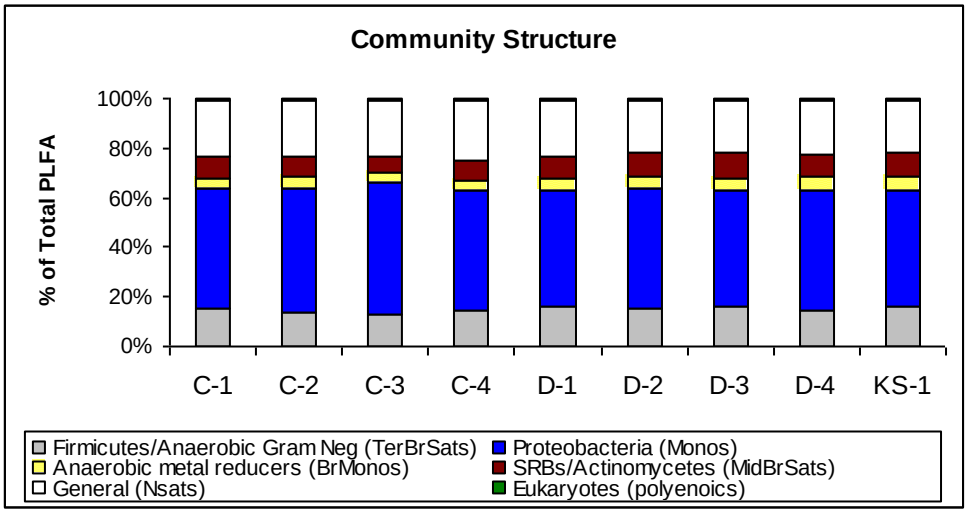

Figure 2. Relative percentages of total PLFA structural groups in the samples analyzed. Structural groups are assigned according to PLFA chemical structure, which is related to fatty acid biosynthesis. See Table 1 for detailed descriptions of structural groups.

Physiologic status ratios indicated that none of the samples showed any notable levels of stress response while the Gram negative microbial communities of all samples were experiencing high levels of starvation. It is important to note that starvation is a comparative measure of the growth rate of microbes, i.e., a high starvation ratio may indicate slowed growth. Although these ratios do not correspond to the log or stationary phases of growth can be useful for comparisons over time and among samples.

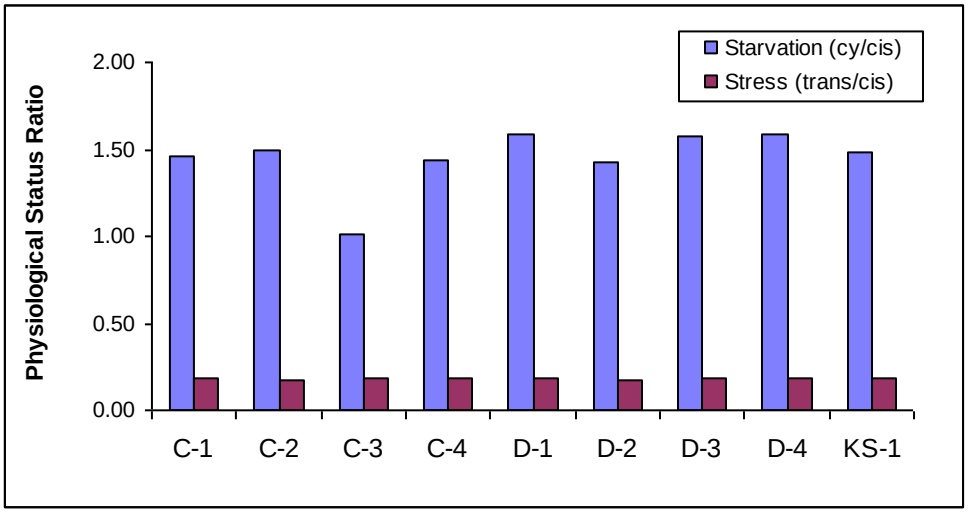

Figure 3. Microbial physiological stress markers. The starvation biomarker for the Gram-negative bacterial community is assessed by the ratios of cyclopropyl fatty acids to their metabolic precursors. An adaptation of the Gram-negative community to toxic stress is determined by the ratio of ω7t/ω7c fatty 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 0.2 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 (based on total PLFA content) is expressed as cells per g of sample; fatty acid structural groups as percent of total PLFA; and physiological status biomarkers as mole ratio.

Sample		Biomass	Community Structure (% of total PLFA)						Physiological Status	
Sample Name	Sample Date	Cells/ g	Firmicutes Anaerobic Gram Neg./ (TerBrSats)	Proteobacteria (Monos)	Anaerobic metal reducers (BrMonos)	SRBs/ Actinomycetes (MidBrSats)	General (Nsats)	Eukaryotes (polyenoics)	Starved *cy/cis*	Membrane Stress, *trans/cis*
C-1	9/22/04	8.15E+07	15.7	47.7	4.4	9.1	22.5	0.8	1.46	0.19
C-2	9/22/04	7.99E+07	13.4	50.4	4.5	8.0	22.9	0.8	1.49	0.18
C-3	9/22/04	1.19E+08	13.1	53.4	3.4	6.9	22.5	0.8	1.02	0.18
C-4	9/22/04	1.21E+08	14.5	48.7	3.9	8.2	23.6	1.1	1.44	0.19
D-1	9/22/04	3.90E+07	16.2	46.7	4.6	8.8	23.2	0.5	1.59	0.19
D-2	9/22/04	6.78E+07	15.7	47.9	5.2	9.4	21.0	0.8	1.43	0.17
D-3	9/22/04	6.97E+07	16.5	46.5	4.8	10.3	21.3	0.8	1.57	0.19
D-4	9/22/04	6.22E+07	14.2	49.1	5.2	9.0	21.8	0.8	1.58	0.18
KS-1	9/22/04	6.96E+07	16.0	47.2	5.3	9.8	21.1	0.7	1.48	0.18

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