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Microbial Analysis Report

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Analysis Requested: PLFA

Project: WVA #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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Microbial Analysis Report

Executive Summary

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

- Estimated viable biomass, as determined by total PLFA concentrations, was approximately 10^7 cells/gram dry weight for all samples. (Figure 1, Table 2)

- PLFA profiles showed that the microbial communities of the samples were diverse and similar to each other. All samples were primarily composed of Gram negative Proteobacteria, as shown by the proportions of monoenoic PLFA, which ranged from ~44% to ~58% of the total PLFA. Total "anaerobic" biomarkers (terminally and mid-chain branched saturated and branched monoenoic PLFA), which are attributed to the presence of Firmicutes, sulfate reducing bacteria, and anaerobic metal reducing bacteria respectively, accounted for ~19-33% of the total PLFA among these samples. (Figure 2, Table 2)

- Physiologic status ratios, indicative of starvation and microbial response to environmentally induced stress, showed that all nine samples had high levels of starvation and no notable levels of induced stress. It is important to note that starvation is a comparative measure of the growth rate of microbes, i.e., a higher starvation ratio indicates slower growth, while low starvation ratios indicate comparatively rapid growth rates. Although starvation ratios do not directly correlate to log or stationary phases of growth, they are useful for comparing changes over time and between samples. (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

Estimated viable biomass was ~10^7 cells/ gram dry weight for all samples, with only a slight difference (less than one order of magnitude) between the highest and lowest biomass levels seen.

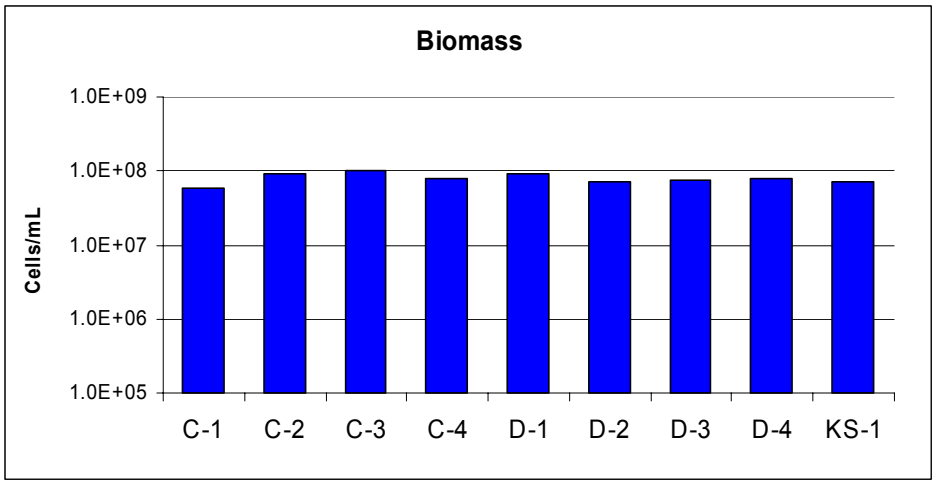

Figure 1. Biomass content is presented as a cell equivalent based on the total amount of phospholipid fatty acids (PLFA) extracted from a given sample. Total biomass is calculated based upon PLFA attributed to bacterial and eukaryotic biomass (associated with higher organisms).

The microbial communities of all the samples were similar in structure and primarily composed of Gram negative Proteobacteria, as shown by the proportions of monoenoic PLFA. Proportions of Proteobacteria in among the "D" samples ranged from ~46-50% of the total PLFA while proportions in the "C" samples showed a wider range (~44% to 58% of the total PLFA). Among the anaerobic members of the microbial community, Firmicutes (which include *Clostridia*-like fermenting bacteria) were the most abundant. Biomarkers for Firmicutes (terminally branched saturated PLFA) were present in proportions ranging from ~10-20% of the total PLFA. Firmicutes were highest in samples C-4 (~20%) and D-4 (~19%), while the lowest proportions of Firmictues were seen in samples C-2 (~11%) and C-3 (~10%). Sulfate reducing bacteria, shown by mid-chain branched saturated PLFA, were also relatively abundant, ranging from ~8-10% of the total PLFA in all samples except C-2 where these were lower (~6% of the total PLFA). Anaerobic metal reducing bacteria (branched monoenoic PLFA) represented ~3-4% of the community while eukaryotes (polyenoic PLFA) were quite low, ~1% of the community for all samples. Overall, the community structure of the "C" samples had more variation than those of the "D" samples.

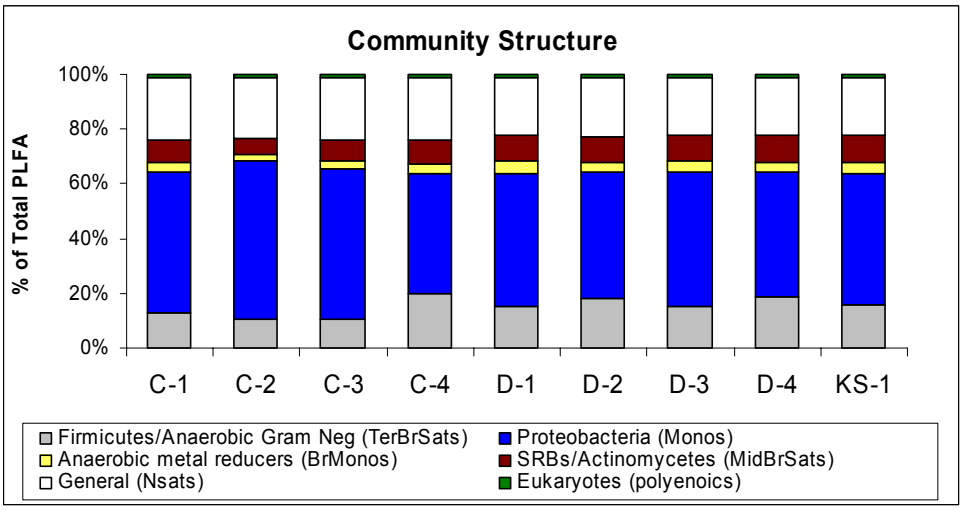

Figure 2. Relative percentages of total PLFA structural groups in the samples analyzed. Structural groups are assigned according to PLFA chemical structure, which is related to fatty acid biosynthesis. See Table 1 for detailed descriptions of structural groups.

Physiologic status ratios for starvation and microbial response to environmentally induced stress showed that none of the samples had notable levels of stress response, while all of the samples showed moderate to high starvation levels.

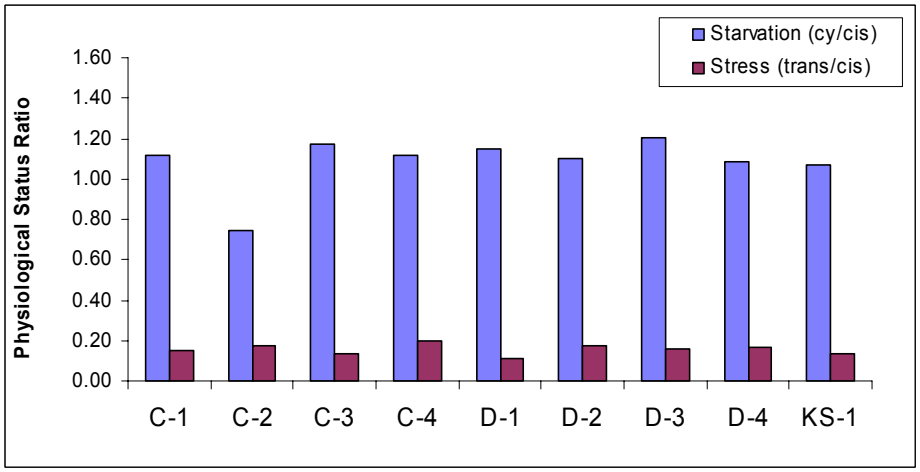

Figure 3. Microbial physiological stress markers. The starvation biomarker for the Gram-negative bacterial community is assessed by the ratios of cyclopropyl fatty acids to their metabolic precursors. An adaptation of the Gram-negative community to toxic stress is determined by the ratio of ω7t/ω7c fatty 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	7/6/04	5.75E+07	12.8	51.7	3.6	8.0	22.5	1.4	1.12	0.15
C-2	7/6/04	9.35E+07	10.8	57.6	2.5	5.7	22.6	0.9	0.74	0.18
C-3	7/6/04	9.97E+07	10.3	54.9	3.2	7.5	23.1	1.0	1.17	0.13
C-4	7/6/04	7.94E+07	19.7	44.1	3.1	8.9	23.1	1.1	1.11	0.20
D-1	7/6/04	9.20E+07	15.0	49.0	4.3	9.7	20.9	1.0	1.15	0.11
D-2	7/6/04	6.98E+07	17.9	46.4	3.5	9.3	21.7	1.3	1.10	0.17
D-3	7/6/04	7.67E+07	15.0	49.6	4.1	9.0	21.1	1.1	1.20	0.16
D-4	7/6/04	7.96E+07	18.6	45.5	3.9	9.8	21.1	1.1	1.08	0.16
KS-1	7/6/04	7.30E+07	15.5	48.5	3.8	9.7	21.4	1.1	1.07	0.13

References

1. Amann, R. I., W. Ludwig, and K.-H. Schleifer. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiological Reviews 59:143-169.
2. Cottrell, MT and David L. Kirchman. Appl Environ Microbiol. 2000 April; 66 (4): 16921697.
3. Gillis, M., V. Tran Van, R. Bardin, M. Goor, P. Hebbar, A. Willems, P. Segers, K. Kerstens, T. Heulin, and M. P. Fernadez. 1995. Polyphasic taxonomy in the genus Burkholderia leading to an amended description of the genus and proposition of Burkholderia vietnamiensis sp. nov. for N2-fixing isolates from rice in Vietnam. Int. J. Syst. Bacteriol. 45:274-289.
4. Dowling, N. J. E., F. Widdel, and D. C. White. 1986. Phospholipid ester-linked fatty acid biomarkers of acetate-oxidizing sulfate reducers and other sulfide forming bacteria. Journal of General Microbiology 132:1815-1825.
5. Edlund, A., P. D. Nichols, R. Roffey, and D. C. White. 1985. Extractable and lipopolysaccharide fatty acid and hydroxy acid profiles from Desulfovibrio species. Journal of Lipid Research 26:982-988.
6. Guckert, J. B., C. P. Antworth, P. D. Nichols, and D. C. White. 1985. Phospholipid ester-linked fatty acid profiles as reproducible assays for changes in prokaryotic community structure of estuarine sediments. FEMS Microbiol. Ecol. 31:147-158.
7. Guckert, J. B., M. A. Hood, and D. C. White. 1986. Phospholipid ester-linked fatty acid profile changes during nutrient deprivation of Vibrio cholerae: increases in the trans/cis ratio and proportions of cyclopropyl fatty acids. Appl. Environ. Microbiol. 52:794–801.
8. Hedrick, D.B., A Peacock, J.R. Stephen, S.J. Macnaughton, Julia Brüggemann, and David C. White. 2000. Measuring soil microbial community diversity using polar lipid fatty acid and denatured gradient gel electrophoresis data. J. Microbiol. Methods, 41, 235-248.
9. ITRC Internet Training on Natural Attenuation of Chlorinated Solvents in Groundwater: Principles and Practices, Apr 00.
10. Löffler, F. E., Q. Sun, et al. (2000). "16S rRNA gene-based detection of tetrachloroethene-dechlorinating Desulfuromonas and Dehalococcoides species." Appl Environ Microbiol 66(4): 1369-1374.
11. Maymo-Gatell X, Chien Y, Gossett JM, Zinder SH. 1997. Isolation of a bacterium that reductively dechlorinates tetrachloroethene to ethene. Science 276(5318):1568-71.
12. Muyzer, G., E. C. De Waal, and A. G. Uitterlinden. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Applied and Environmental Microbiology 59:695-700.

13. Ribosomal Database Project (http://rdp.cme.msu.edu. National Center for Biotechnology Information. (http://www.ncbi.nlm.nih.gov/)
14. Overman, J., "Family Chlorobiaceae," in M. Dworkin et al., eds., The Prokaryotes: An Evolving Electronic Resource for the Microbiological Community, 3rd edition, release 3.7, November 2, 2001, Springer-Verlag, New York, www.prokaryotes.com.
15. Ringelberg, D. B., G. T. Townsend, K. A. DeWeerd, J. M. Sulita, and D. C. White. 1994. Detection of the anaerobic dechlorinating microorganism Desulfomonile tiedjei in environmental matrices by its signature lipopolysaccharide branch-long-chain hydroxy fatty acids. FEMS Microbiol. Ecol. 14:9-18.
16. Schlötelburg, C. 2001. Mikrobielle Diversität und Dynamik einer 1,2-Dichlorpropan dechlorierenden Mischkultur (Microbial Diversity and Dynamics in a 1,2-Dichloropropane Dechlorinating Mixed Culture). Dissertation, Humbolt University, Berlin, Germany. In German: http://edoc.hu-berlin.de/dissertationen/schloetelburg-cord-2001-12-07/PDF/Schloetelburg.pdf
17. Sharp, R., D. Cossar, and R. Williams. 1995. Physiology and metabolism of Thermus. Biotechnol. Handb. 9:67-91.
18. Stephen, J. R., Y.-J. Chang, Y. D. Gan, A. Peacock, S. Pfiffner, M. Barcelona, D. C. White, and S. J. Macnaughton. 1999. Microbial characterization of a JP-4 fuel-contaminated site using a combined lipid biomarker/polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) based approach. Environmental Microbiology 1:231-241.
19. Tighe, S.W., de Lajudie, P., Dipietro, K., Lindström, K., Nick, G. & Jarvis, B.D.W. (2000). Analysis of cellular fatty acids and phenotypic relationships of Agrobacterium, Bradyrhizobium, Mesorhizobium, Rhizobium and Sinorhizobium species using the Sherlock Microbial Identification System. Int J Syst Evol Microbiol 50, 787-801.
20. Tsitko, I.V. Gennadi M. Zaitsev, Anatoli G. Lobanok, and Mirja S. Salkinoja-Salonen. 1999. Applied and Environmental Microbiology 65(2) 853-855.
21. White, D. C., W. M. Davis, J. S. Nickels, J. D. King, and R. J. Bobbie. 1979. Determination of the sedimentary microbial biomass by extractable lipid phosphate. Oecologia 40:51-62.
22. White, D. C., H. C. Pinkart, and D. B. Ringelberg. 1997. Biomass measurements: Biochemical approaches, p. 91-101. In C. J. Hurst, G. R. Knudsen, M. J. McInerney, L. D. Stetzenbach, and M. V. Walter (ed.), Manual of Environmental Microbiology. ASM Press, Washington.
23. White, D. C., and D. B. Ringelberg. 1995. Utility of signature lipid biomarker analysis in determining in situ viable biomass, community structure, and nutritional / physiological status of the deep subsurface microbiota. In P. S. Amy and D. L. Halderman (ed.), The microbiology of the terrestrial subsurface. CRC Press, Boca Raton.
24. White, D. C., J. O. Stair, and D. B. Ringelberg. 1996. Quantitative comparisons of in situ microbial biodiversity by signature biomarker analysis. Journal of Industrial Microbiology 17:185-196.
25. Vandamme P, Pot B, Gillis M, de Vos P, Kersters K, Swings J. Polyphasic taxonomy, a consensus approach to bacterial systematics. Microbiol Rev 1996 Jun;60(2):407-38.