received 09/09/2013
Project 2130

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Identifier: 059KH **Date Rec:** 08/22/2013 **Report Date:** 09/09/2013

Client Project #: **Client Project Name:** TBAL

Purchase Order #:

Analysis Requested: CENSUS, PLFA

Reviewed By:

MICROBIAL INSIGHTS, INC.

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CENSUS

Client: **Colorado Oil and Gas Conservation Commiss**
Project: TBAL

MI Project Number: **059KH**
Date Received: 08/22/2013

Sample Information

Client Sample ID:		**752165 Goss**	**423097 WD Fed**	**750010 Bean**
Sample Date:		08/21/2013	08/21/2013	08/26/2013
Units:		cells/mL	cells/mL	cells/mL
Analyst:		RW	RW	RW
Functional Genes				
Toluene Monooxygenase	RMO	**1.00E-01 (J)**	**1.10E+01**	**1.40E+01**
Benzyl Succinate Synthase	bssA	**1.00E-01 (J)**	**9.25E+02**	**1.95E+01**
Phylogenetic Group				
Methanogen	MGN	**2.00E-01 (J)**	**2.73E+04**	**4.40E+00**
Methane Oxidizing Bacteria	MOB	**1.63E+08**	**2.98E+07**	**9.61E+06**

Legend:
NA = Not Analyzed NS = Not Sampled J = Estimated gene copies below PQL but above LQL I = Inhibited
< = Result not detected

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Comments: Please note that the total biomass for sample 423097 WD Fed fell between the reporting limit and the detection limit for the PLFA analysis.

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PLFA

Client: **Colorado Oil and Gas Conservation Commiss**
Project: TBAL

MI Project Number: **059KH**
Date Received: 08/22/2013

Sample Information

Sample Name:	**752165 Goss**	**423097 WD Fed**	**750010 Bean**
Sample Date:	08/21/2013	08/21/2013	08/26/2013
Sample Matrix:	Water	Water	Water
Analyst:	BJ	BJ	BJ

Biomass

Total Biomass (cells/mL)	**1.96E+04**	**2.45E+03**	**4.31E+04**

Community Structure (% total PLFA)

Firmicutes (TerBrSats)	**3.63**	**18.21**	**10.17**
Proteobacteria (Monos)	**75.28**	**46.42**	**70.89**
Anaerobic metal reducers (BrMonos)	**0.80**	**0.00**	**2.30**
SRB/Actinomycetes (MidBrSats)	**1.24**	**0.00**	**2.01**
General (Nsats)	**14.99**	**31.66**	**13.33**
Eukaryotes (polyenoics)	**4.05**	**3.73**	**1.28**

Physiological Status (Proteobacteria only)

Slowed Growth	**0.20**	**1.80**	**0.14**
Decreased Permeability	**0.05**	**0.00**	**0.15**

Legend:
NA = Not Analyzed NS = Not Sampled

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PLFA

Client: **Colorado Oil and Gas Conservation Commiss**
Project: TBAL

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Date Received: 08/22/2013

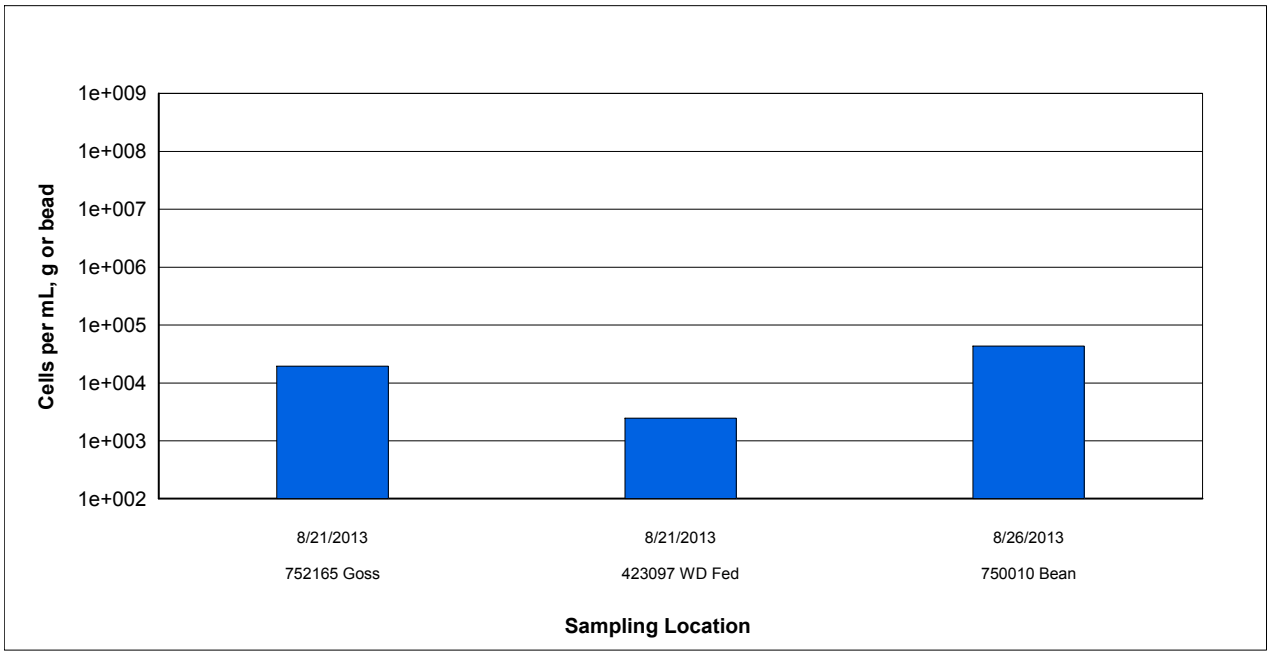

Figure 1. Biomass content is presented as a cell equivalent based on the total amount of phospholipid fatty acids (PLFA) extracted from a given sample. Total biomass is calculated based upon PLFA attributed to bacterial and eukaryotic biomass

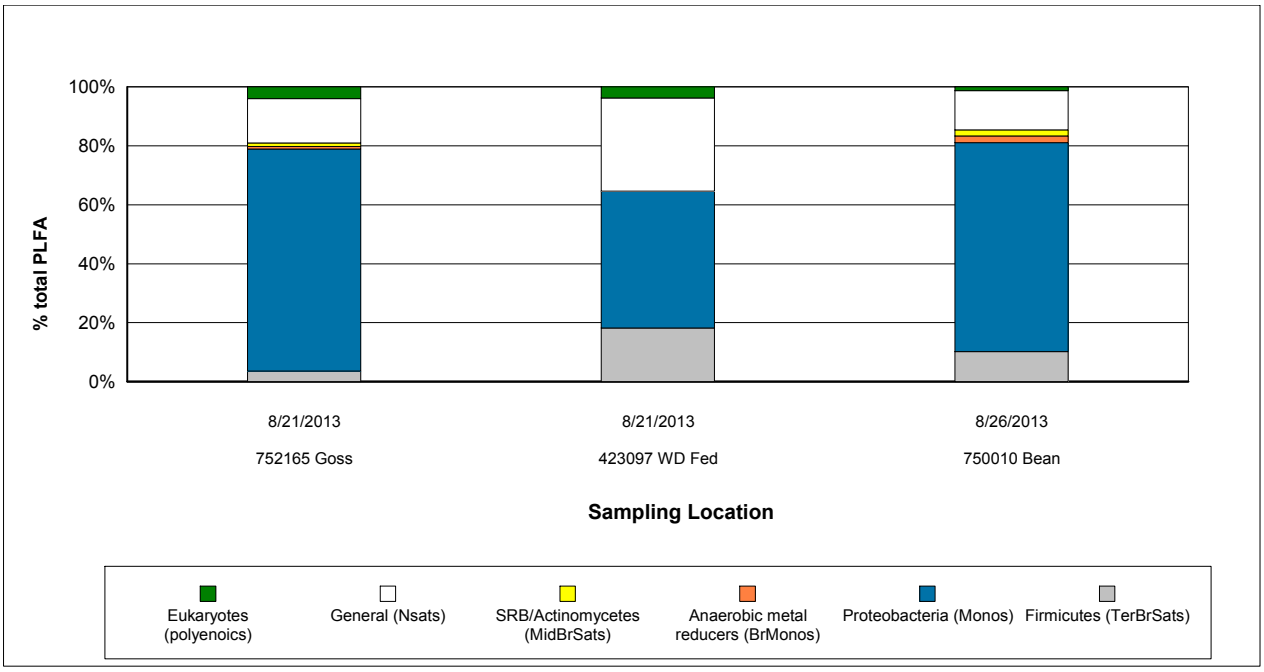

Figure 2. Relative percentages of total PLFA structural groups in the samples analyzed. Structural groups are assigned according to PLFA chemical structure, which is related to fatty acid biosynthesis.

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Phospholipid Fatty Acid Analysis

Interpretation Guidelines

Phospholipids fatty acids (PLFA) are a main component of the membrane (essentially the "skin") of microbes and provide a powerful tool for assessing microbial responses to changes in their environment. This type of analysis provides direct information for assessing and monitoring sites where bioremediation processes, including natural attenuation, are of interest. Analysis of the types and amount of PLFA provides a broad based understanding of the entire microbial community with information obtained in three key areas viable biomass, community structure and metabolic activity.

What is the detection limit for PLFA?

Our limit of detection for PLFA analysis is ~150 picomoles of total PLFA and our limit of quantification is ~500 picomoles of total PLFA. Samples which contain PLFA amounts at or below 150 pmol cannot be used to determine biomass, likewise samples with PLFA content below ~500 pmol are generally considered to contain too few fatty acids to discuss community composition.

How should I interpret the PLFA results?

Interpreting the results obtained from PLFA analysis can be somewhat difficult, so this document was designed to provide a technical guideline. For convenience, this guideline has been divided into the three key areas.

Viable Biomass

PLFA analysis is one of the most reliable and accurate methods available for the determination of viable microbial biomass. Phospholipids break down rapidly upon cell death (21, 23), so biomass calculations based on PLFA content do not contain 'fossil' lipids of dead cells.

How is biomass measured?

Viable biomass is determined from the total amount of PLFA detected in a given sample. Since, phospholipids are an essential part of intact cell membranes they provide an accurate measure of viable cells.

How is biomass calculated?

Biomass levels are reported as cells per gram, mL or bead, and are calculated using a conversion factor of 20,000 cells/pmole of PLFA. This conversation factor is based upon cells grown in laboratory media, and varies somewhat with the type of organism and environmental conditions.

What does the concentration of biomass mean?

The overall abundance of microbes within a given sample is often used as an indicator of the potential for bioremediation to occur, but understanding the levels of biomass within each sample can be cumbersome. The following are benchmarks that can be used to understand whether the biomass levels are low, moderate or high.

Low	Moderate	High
10^3 to 10^4 cells	10^5 to 10^6 cells	10^7 to 10^8 cells

How do I know if a change in biomass is significant?

One of the primary functions of using PLFA analysis at contaminated sites is to evaluate how a community responds following a given treatment, but how does one know if the changes observed between two events are significant? As a general rule, biomass levels which increase or decrease by at least an order of magnitude are considered to be significant. However, changes in biomass levels of less than an order of magnitude may still show a trend. It is important to remember that many factors can affect microbial growth, so factors other than the treatment could be influencing the changes observed between sampling events. Some of the factors to consider are: temperature, moisture, pH, etc. The following illustration depicts three types of changes that occurred over time and the conclusions that could be drawn.

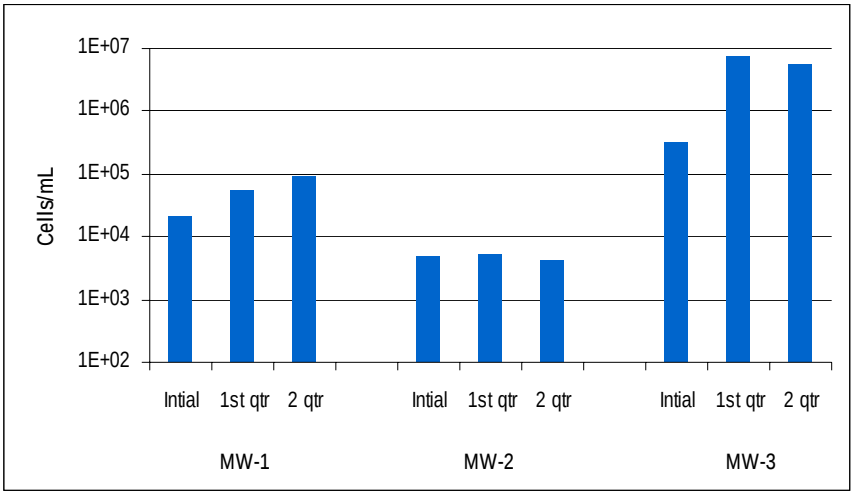

Figure 1. Biomass content is presented as a cell equivalent based on the total amount of phospholipid fatty acids (PLFA) extracted from a given sample. Total biomass is calculated based upon PLFA attributed to bacterial and eukaryotic biomass (associated with higher organisms).

Conclusions from graph above:

- MW-1 showed a trend of biomass levels increasing steadily over time, although cell concentrations were ~10^4 cells/mL at each sampling event.
- MW-2 showed no notable trends or significant changes in biomass concentrations.
- MW-3 showed a significant increase in biomass levels between the initial and 1st quarter sampling events (from ~10^5 to ~10^6 cells/mL).

Community Structure:

The PLFA in a sample can be separated into particular types, and the resulting PLFA "profile" reflects the proportions of the categories of organisms present in the sample. Because groups of bacteria differ in their metabolic capabilities, determining which bacterial groups are present and their relative distributions within the community can provide information on what metabolic processes are occurring at that location. This in turn can also provide information on the subsurface conditions (i.e oxidation/reduction status, etc.). Table 1 describes the six major structural groups used and their potential relevance to site specific projects.

Table 1. Description of PLFA structural groups.

PLFA Structural Group	General classification	Potential Relevance to Bioremediation Studies
Monoenoic (Monos)	Abundant in Proteobacteria (Gram negative bacteria), typically fast growing, utilize many carbon sources, and adapt quickly to a variety of environments.	Proteobacteria is one of the largest groups of bacteria and represents a wide variety of both aerobes and anaerobes. The majority of Hydrocarbon utilizing bacteria fall within the Proteobacteria
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).	Firmicutes are indicative of presence of anaerobic fermenting bacteria (mainly *Clostridia/Bacteriodes*-like), which produce the H_2 necessary for reductive dechlorination
Branched Monoenoic (BrMonos)	Found in the cell membranes of micro-aerophiles and anaerobes, such as sulfate- or iron-reducing bacteria	In contaminated environments high proportions are often associated with anaerobic sulfate and iron reducing bacteria
Mid-Chain Branched Saturated (MidBrSats)	Common in sulfate reducing bacteria and also Actinobacteria (High G+C Gram-positive bacteria).	In contaminated environments high proportions are often associated with anaerobic sulfate and iron reducing bacteria
Normal Saturated (Nsats)	Found in all organisms.	High proportions often indicate less diverse populations.
Polyenoic	Found in eukaryotes such as fungi, protozoa, algae, higher plants, and animals.	Eukaryotic scavengers will often rise up and prey on contaminant utilizing bacteria

Following are answers to some of the common questions about community composition and some detailed descriptions of some typical shifts which can be observed between sampling events.

How is the community structure data presented?

Community structure data is presented as percentage (%) of the total amount of PLFA. In order to relate the complex mixture of PLFA to the organisms present, the ratio of a specific PLFA group is determined (detailed in Table 1 above), and this corresponds to the proportion of the related bacterial classification within the overall community structure. Because normal saturated PLFA are found in both prokaryotes (bacteria) and eukaryotes (fungi, protozoa, diatoms etc), their distribution provides little insight into the types of microbes that are present at a sampling location. However, high proportions of normal saturates are often associated with less diverse microbial populations.

How can community structure data be used to manage my site?

It is important to understand that microbial communities are often a mixture of different types of bacteria (e.g. aerobes, sulfate reducers, methanogens, etc) with the abundance of each group behaving like a seesaw, i.e. as the population of one group increases, another is likely decreasing, mostly due to competition for available resources. The PLFA profile of a sample provides a "fingerprint" of the microbial community, showing relative proportions of the specific bacterial types at the time of sampling. This is a great tool for detecting shifts within the community over time and also to evaluate similarities/differences between sampling locations. It is important to note that PLFA analysis of community structure is analyzing the microbes directly, not just secondary breakdown products. So this provides evidence of how the entire microbial community is responding to the treatment.

How do I recognize community shifts and what they mean?

Shifts in the community structure are indications of changing conditions and their effect on the microbial community, and, by extension on the metabolic processes occurring at the sampling location. Some of the more commonly seen shifts within the community are illustrated and discussed below:

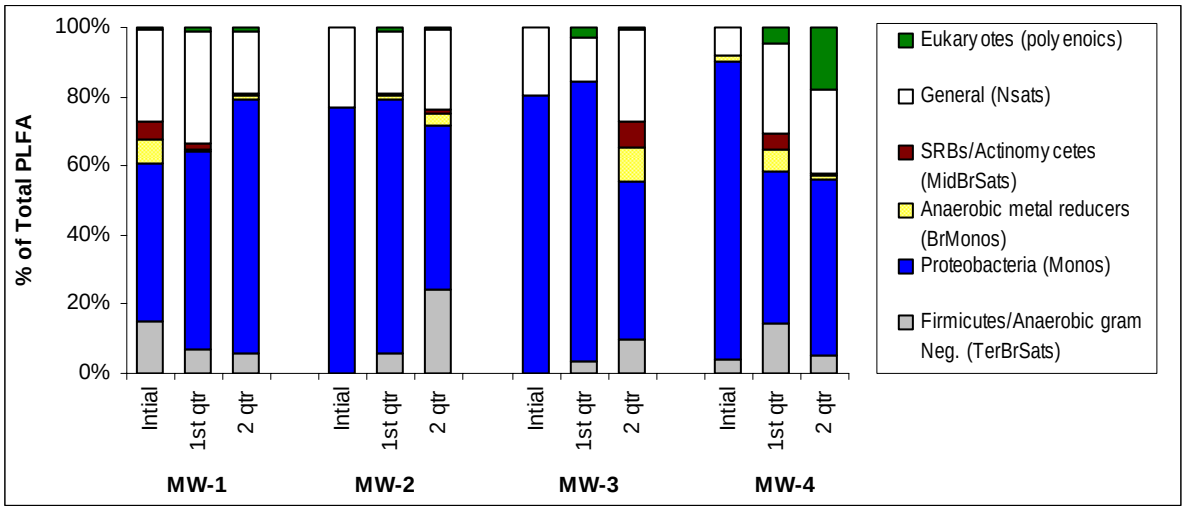

Figure 2. Relative percentages of total PLFA structural groups in the samples analyzed. Structural groups are assigned according to PLFA chemical structure, which is related to fatty acid biosynthesis. See Table 1 for detailed descriptions of structural groups.

- **Increased Proteobacteria**

Proportions of Proteobacteria are of interest because it is one of the largest groups of bacteria and represents a wide variety of both aerobe and anaerobes. The majority of hydrocarbons (including benzene and naphthalene) are metabolized by some member of Proteobacteria, mainly due to their ability to grow opportunistically, quickly taking advantage of available food (i.e. hydrocarbons), and adapting quickly to changes in the environment. The detection of increased proportions of Proteobacteria coupled with increased biomass suggests that the Proteobacteria are consuming something. In situations where it is important to determine the extent to which the Proteobacteria are utilizing anaerobic or aerobic pathways, it is possible to measure relative proportions of specific biomarkers that are associated with anaerobic or aerobic pathways thus separating the Proteobacteria into different groups, based on pathways used. Sample MW-1 from Figure 2 depicts a shift in community structure where the proportion of Proteobacteria has increased over time.

- **Increased Firmicutes/Anaerobic Gram negative bacteria**

Increased proportions of Firmicutes/Anaerobic Gram negative bacteria generally indicate that conditions are becoming more reductive (i.e. more anaerobic). Proportions of Firmicutes are of particular interest in sites contaminated with chlorinated hydrocarbons because Firmicutes include anaerobic fermenting bacteria (mainly *Clostridia/Bacteriodes*-like), which produce the H_2 necessary for reductive dechlorination.

Enhanced bioremediation of chlorinated solvents often employs the injection of fermentable substrates which, when utilized by fermenting bacteria, results in the release of H_2. Engineered shifts in the microbial community can be shown by observing increased proportions Firmicutes following an injection of fermentable substrate. Through long-term monitoring of the community structure it is possible to know when re-injection may be necessary or desirable. Sample MW-2 from Figure 2 depicts a shift in community structure where the proportion of Firmicutes has increased over time.

- **Increased anaerobic metal reducing bacteria (BrMonos) and SRB/Actinomycetes (MidBrSats)**

An increase in the proportions of metal and sulfate reducing bacterial groups, especially when combined with shifts in the other bacterial groups, can provide information helpful to monitoring bioremediation. Generally, an increase in metal and sulfate reducers points to more reduced (anaerobic) conditions at the sampled location. This is especially true if there is an increase in Firmicutes at the same time. Large increases in either metal and sulfate reducers, particularly if accompanied by a decrease in Firmicutes, may suggest that conditions are becoming increasingly reduced. In this situation the metal and sulfate reducers may be out-competing dechlorinators for available H_2, thereby limiting the potential for reductive dechlorination at that location. Sample MW-3 from Figure 2 depicts a shift in community structure where the proportion of metal reducing bacteria has increased over time.

- **Increased Eukaryotes**

Eukaryotes include organisms such as fungi, protozoa, and diatoms. At a contaminated location, an increase in eukaryotes, particularly if seen with a decrease in the contaminant utilizing bacteria, suggests that eukaryotic scavengers are preying upon what had been an abundance of bacteria which were consuming the contaminant. Sample MW-4 from Figure 2 depicts a shift in community structure where the proportion of eukaryotes has increased over time.

Physiological status of Proteobacteria

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 may disrupt the membrane and some bacteria respond by making *trans* fatty acids instead of the usual *cis* fatty acids (7) in order to strengthen the cell membrane, making it less permeable. Many Proteobacteria respond to lack of available substrate or to highly toxic conditions by making cyclopropyl (7) or mid-chain branched fatty acids (20) which point to less energy expenditure and a slowed growth rate. The physiological status ratios for Decreased Permeability (trans/cis ratio) and for Slowed Growth (cy/cis ratio) are based on dividing the amount of the fatty acid induced by environmental conditions by the amount of its biosynthetic precursor.

What does slowed growth or decreased permeability mean?

Ratios for slowed growth and for decreased permeability of the cell membrane provide information on the “health” of the Gram negative community, that is, how this population is responding to the conditions present in the environment. It should be noted that one must be cautious when interpreting these measures from only one sampling event. The most effective way to use the physiological status indicators is in long term monitoring and comparing how these ratios increase/decrease over time.

A marked increase in either of these ratios suggests a change in environment which is less favorable to the Gram negative Proteobacteria population. The ratio for slowed growth is a relative measure, and does not directly correspond to log or stationary phases of growth, but is useful as a comparison of growth rates among sampling locations and also over time. An increase in this ratio (i.e. slower growth rate) suggests a change in conditions which is not as supportive of rapid, “healthy” growth of the Gram negative population, often due to reduced available substrate (food). A larger ratio for decreased permeability suggests that the environment has become more toxic to the Gram negative population, requiring energy expenditure to produce *trans* fatty acids in order to make the membrane more rigid.

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