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COMPARISION OF SELECTIVE CULTURING AND BIOCHEMICAL TECHNIQUES FOR MEASURING BIOLOGICAL ACTIVITY IN GEOTHERMAL PROCESS FLUIDS

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Introduction

For the past three years, scientists at the Idaho National Engineering and Environmental Laboratory have been conducting studies aimed at determining the presence and influence of bacteria found in geothermal plant cooling water systems. In particular, the efforts have been directed at understanding the conditions that lead to the growth and accumulation of biomass within these systems, reducing the operational and thermal efficiency. Initially, the methods selected were based upon the current practices used by the industry and included the collection of water quality parameters, the measurement of soluble carbon, and the use of selective media for the determination of the number density of various types of organisms. This data has been collected on a seasonal basis at six different facilities located at The Geysers' in Northern California. While this data is valuable in establishing biological growth trends in the facilities and providing an initial determination of upset or off-normal conditions, more detailed information about the biological activity is needed to determine what is triggering or sustaining the growth in these facilities in order to develop improved monitoring and treatment techniques.

In recent years, new biochemical approaches, based upon the analyses of phospholipid fatty acids and DNA recovered from environmental samples, have been developed and commercialized.[1-4] These techniques, in addition to allowing the determination of the quantity of biomass, also provide information on the community composition and the nutritional status of the organisms. During the past year, samples collected from the condenser effluents of four of the plants from The Geysers' were analyzed using these methods and compared with the results obtained from selective culturing techniques. The purpose of this effort was to evaluate the cost-benefit of implementing these techniques for tracking microbial activity in the plant study, in place of the selective culturing analyses that are currently the industry standard.

Analysis Techniques

Figure 1. MPN Analyses

The most commonly used method for evaluating biological activity in industrial plant systems is to determine the relative abundance, or ***most probable number (MPN)***. This method consists of making a series of dilutions of an environmental sample into selective media. The specific media are designed to test for the presence of heterotrophic bacteria, sulfate-reducing bacteria (SRB's), acid-formers, and others, such as de-nitrifying bacteria, which may be of importance in particular environments. The inoculated media are incubated for a period of time (up to 28 days), and statistical analyses are performed to determine cell populations from growth patterns in the sample vials.

The technique is widely used for a variety of reasons. First, a number of vendors supply relatively inexpensive and easy-to-use kits for making these measurements. The analyses are also straightforward, requiring simple visual observations of a color or turbidity change in the media. (For example, when SRB's are introduced into a sodium lactate solution containing an iron substrate, the sulfate is reduced to sulfide. The sulfide then reacts with the iron, producing black ferrous oxides, which are easily visible.) These techniques can be very sensitive and can be used to successfully track growth trends in facilities when

used properly. In particular, the data must be collected from the same location in the plant and incubated for the same time under the same environmental conditions to obtain consistent results. (Studies have indicated that variations in time, temperature and location can produce order of magnitude changes in readings.[5]) Even when care is taken in the measurement methodology there are some inherent disadvantages on relying on this technique to quantify microbiological growth. In particular, the selective media are, by definition, preferential to some bacterial species and may not support all types present in the stream.

Phospholipid fatty acid (PLFA) analysis is based on the extraction and separation of lipid biomarkers from cell membranes and walls of microorganisms. Upon extraction with organic solvents, the lipids are concentrated and then analyzed using gas chromatography/mass spectrometry (GC/MS). PLFA are integral components of cellular membranes. They are involved in maintaining cell fluidity and enabling the transport of nutrients into the cell and elimination of metabolic by-products. PLFA analysis provides a quantitative means to measure viable microbial biomass, community composition, and nutritional status. Since fatty acids tend to decompose quickly following cell death, the amount of PLFA found in a sample is indicative of the amount of viable biomass present. And, because different groups of microorganisms synthesize a variety of PLFA through various biochemical pathways, PLFA are also useful as taxonomic markers. Knowledge of specific lipid biosynthetic pathways can also provide insight into the nutritional status of the microbial community, as certain fatty acids provide indications of turnover and membrane permeability.

PLFA are typically reported with the fatty acids designated by the total number of carbon atoms followed by the number of double bonds, with the position of the double bond indicated from the methyl end (ω) of the molecule. The configuration of the double bonds can be either *cis* (c) or *trans* (t). For example, the lipid biomarker for *Desulfobulbus* is 17:1ω6c, a PLFA with 17 total carbons with one double bond located 6 carbons from the ω end in the *cis* configuration. Branched fatty acids are designated as either *iso* (i) or *anteiso* (a) if the methyl branch is one or two carbons from the ω end, or by the position of the methyl group from the carboxylic end of the molecule. Methyl branching at undetermined positions in the molecule is indicated by the prefix "br". Cyclopropyl fatty acids are designated by the prefix "cy" followed by the total number of carbons, e.g. cy 17:0.

The community associations are determined from the distinctive patterns released by microbes, identified via the culturing and analyses of the isolates of various organisms. For complex samples, the profiles are subjected to various statistical analyses. In one technique, dendrograms from a hierarchical cluster analysis are constructed from arcsine-transformed PLFA mole percent values, with similarities based upon Euclidean distances. Two-dimensional plots based upon principal-component analysis are also used to determine similarities in profiles. These matches are taken in context with the environment from which the sample was retrieved for interpreting associations. Table 1 describes the six major structural groups employed.

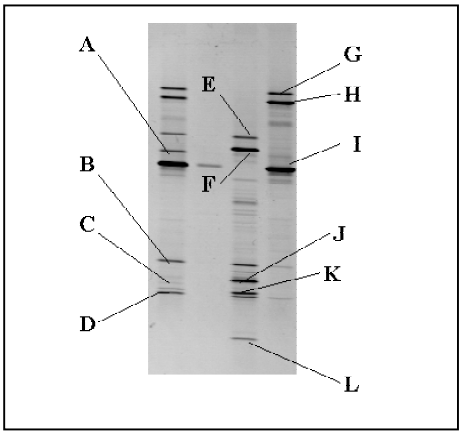
Table 1. Description of PLFA Structural Groups.

PLFA STRUCTURAL GROUP	GENERAL CLASSIFICATION
Monoenoics (Monos)	Found in Gram negative bacteria, which are fast growing, utilize many carbon sources, and adapt quickly to a variety of environments
Terminally Branched Saturated (TerBrSats)	Representative of Gram positive bacteria, but may also be found in the cell membranes of some Gram negative bacteria
Branched Monoenoic (BrMonos)	Commonly found in the cell membranes of obligate anaerobes such as sulfate or iron reducing bacteria
Mid-Chain Branched Saturated (MidBrSats)	Common in Actinomycete, sulfate reducing bacteria and certain Gram positive bacteria
Normal Saturated (NSats)	Found in both the prokaryotic and eukaryotic kingdoms
Eukaryotes (Polyenoics)	Found in organisms such as fungi, protozoa, algae, higher plants and animals

The viable biomass is determined by quantifying the organic phosphate from the polar lipid fraction of the lipid extract. The viable microbes have intact cell membranes, which contain phospholipids (and PLFA). Cellular enzymes hydrolyze and release the phosphate group within a short time after death of the cell. Therefore, by targeting these phospholipids only the cells with intact membranes, or the "viable" cells, are analyzed. In addition, certain stresses, such as starvation, can induce changes in PLFA components such as the ratio of saturated to un-saturated fatty acids, the ratio of trans to cis-monoenoic unsaturated fatty acids, and the proportion of cyclopropyl fatty acids. An increase in cyclopropyl formation has also been associated with anaerobic metabolism.

Unfortunately, PLFA analyses cannot determine the composition down to the species level. To overcome this, a complementary DNA analysis, ***denaturing gradient gel electrophoresis (DGGE***) can be used. The DGGE approach directly determines the species composition of complex microbial populations based on the amplification of 16s rDNA fragments. DNA fragments of the same length but with different base-pair sequences are separated based on their melting behavior in a polyacrylamide gel containing a linearly increasing gradient of de-naturant as illustrated in Figure 2. The banding patterns and relative intensities of the recovered bands provide a measure of change in the community. Dominant species, which constitute at least 1% of the total bacterial community, can be excised and sequenced. Sequence analysis of individual bands is used to infer the identity of the source organism based upon database searches and phylogenetic methods.

Figure 2. DGGE Sequencing and Analyses Using Geysers' Samples



Band	Best Match	Similarity Index (0-1.0)
A	*Wolbachia persica*	.718
B	*Thiobacillus neapolitanus*	.917
C	*Piscirickettsia salmonis*	.700
D	*Thiobacillus neapolitanus*	.840
E	*Thiobacillus neapolitanus*	.826
F	*Thiobacillus neapolitanus*	.982
G	*Flexibacter filiformis*	.535
H	*Flexibacter filiformis*	.585
I	*Moraxella osloensis*	.720
J	*Thiobacillus sp*	.864
K	*Thiobacillus sp.*	.804
L	*Thiobacillus caldus*	.866

Experimental Results

In order to evaluate the cost-benefit of implementing the PLFA and DGGE analyses into our research, samples were collected from the effluents of four different facilities, including the Aidlin, Sulfur Springs (previously known as PG&E Unit 14), Quicksilver (previously known as PG&E Unit 16), and NCPA plants. Collections were made at the plants in September 1999, February 2000, and June 2000. During the June sampling, effluent from the Eagle Rock plant (previously known as PG&E Unit 11) was substituted for the Quicksilver sample when a maintenance shut-down at the Quicksilver facility precluded sampling of the plant. The water samples collected were immediately placed into a cooler where they were maintained at a temperature of approximately 4 °C in order to minimize

potential changes in the sample after it is taken out of the collection environment. The chilled packages were then mailed over-night to Microbial Insights, Inc. in Rockford, TN where the PLFA and DGGE analyses were performed.

The MPN data, summarized in Figure 3., were collected and analyzed using commercially-available, bacti-bottles obtained from Sherry Laboratories, Tulsa, Oklahoma. The serum bottles contained media formulations that were designed to detect the presence of various types of bacteria including heterotrophic, de-nitrifying, sulfate-reducers and acid-producers. For this part of the study, a one-millimeter aliquot was taken from each of the condenser effluent samples and injected into a vial that contained 9 milliliters of a particular growth media. The media was mixed well, and then a one-milliliter sample was withdrawn from that bottle and added to the next sterile bottle containing the same media. This procedure resulted in a 1 to 10 dilution factor, which was carried out into eight vials and replicated five times. Bacterial growth or activity, typically observed as a color or turbidity change, was noted for periods up to 28 days and used for the MPN calculations.

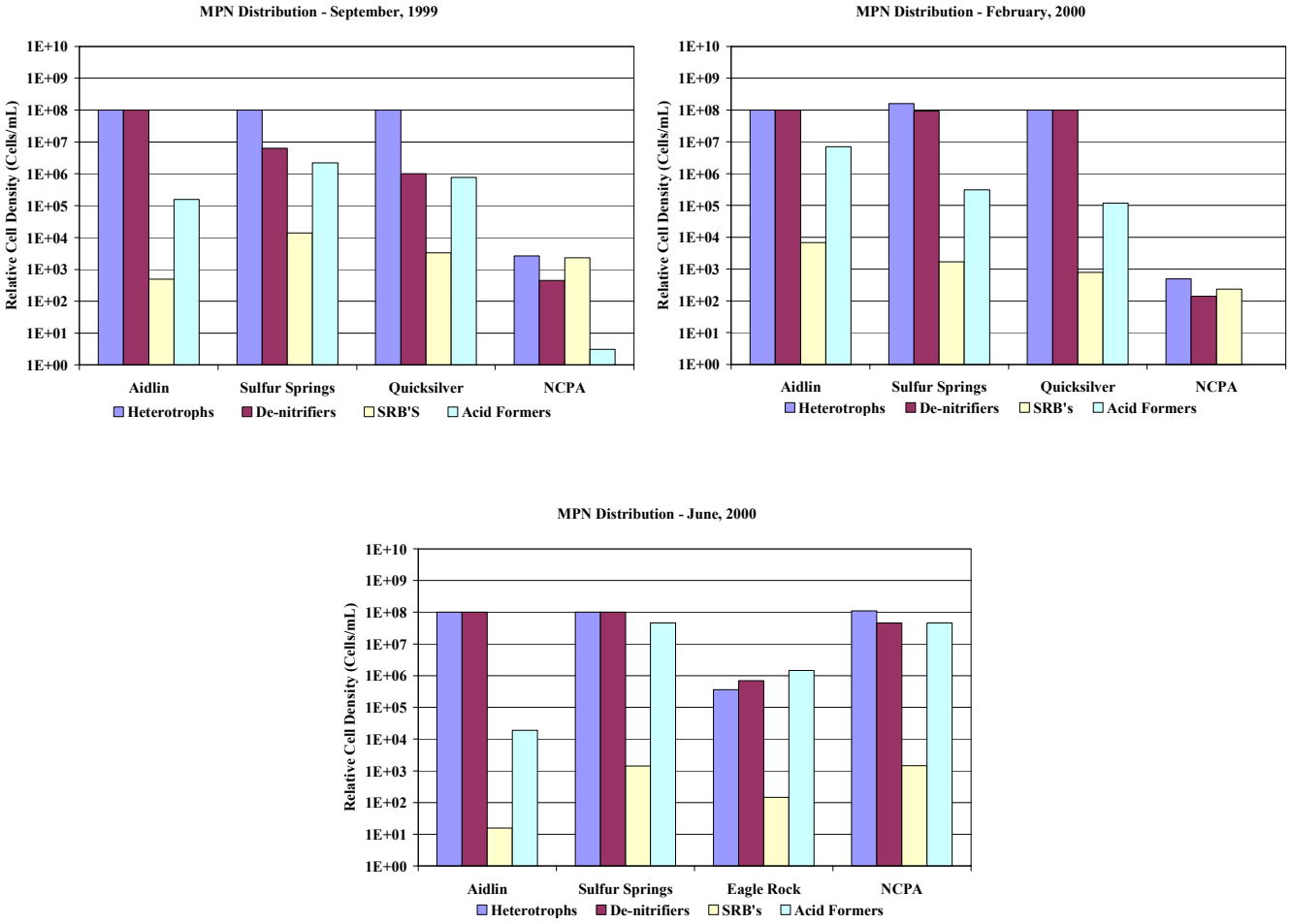


Figure 3. Seasonal MPN Data

The community structures detected using the PLFA technique (defined in Table 1) are summarized below.

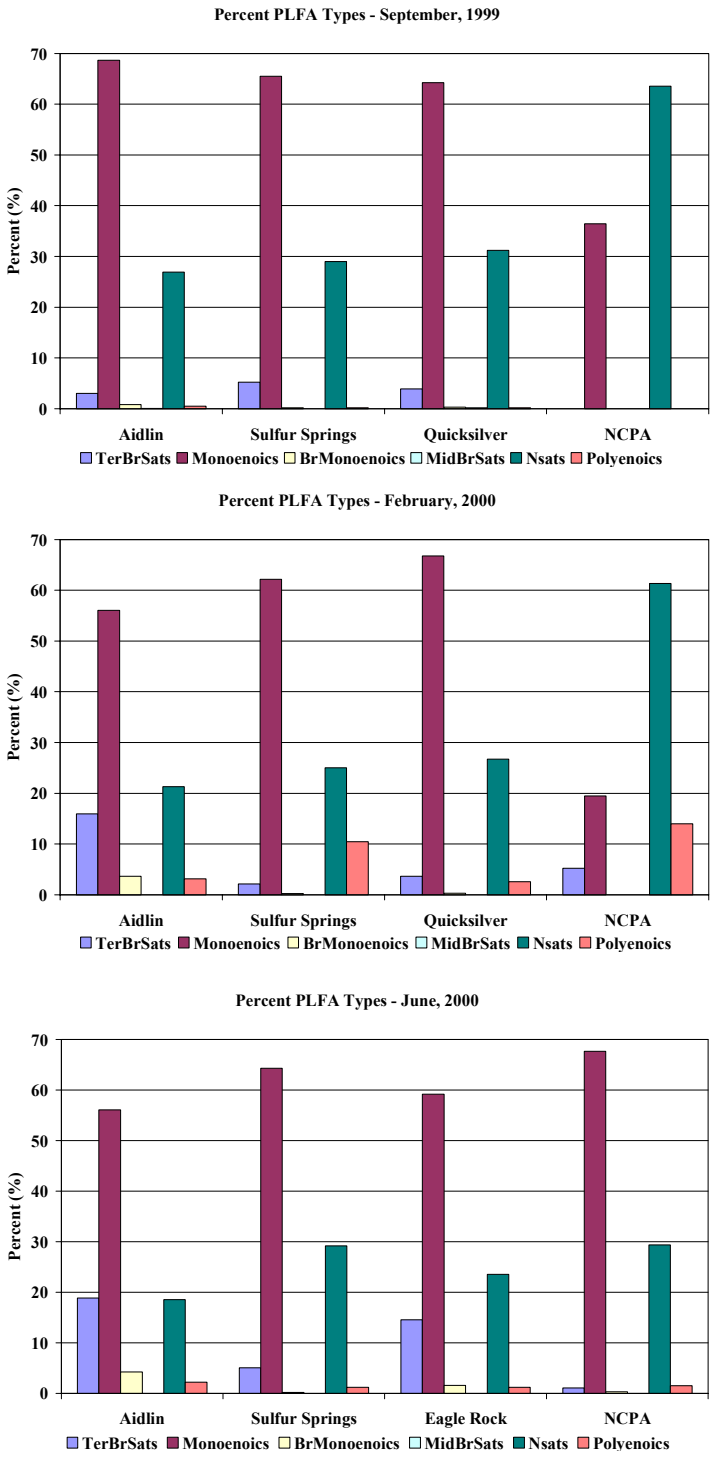


Figure 4. Community Structure Based Upon PLFA Analyses

A comparison of the PLFA profiles indicate moderately diverse microbial communities at all of the plants sampled with the exception of NCPA, which was composed primarily of normal saturated (NSat) PLFA. Normal saturated PLFA are found throughout both the prokaryotic and eukaryotic kingdoms. High levels of NSats are typically

associated with less diverse populations. The Aidlin, Quicksilver and Sulfur Springs communities were dominated by Gram negative bacteria, indicated by the high percentage of monoenoic PLFA found in these samples. Terminally-branched saturated PLFA (TerBrSats) were also detected in all of these samples. Terminally-branched PLFA are representative of Gram positive bacteria, but may also be found in the cell membranes of many anaerobic Gram negative species, such as sulfate reducing bacteria. Generally, Gram positive bacteria are slower growing than Gram negative bacteria, more resilient and are capable of degrading more complex compounds. Anaerobic metal or iron reducing bacteria (BrMonos and MidBrSats) were also detected with the highest proportion observable in the samples from Aidlin.

As previously discussed, the lipid composition of microorganisms is a product of metabolic activity and thus reflects the phenotypic response of the organisms to their environment. The physiological status of Gram-negative communities can be assessed from ratios of different monoenoic biomarkers. Specifically, 16:1ω7c and 18:1ω7c are converted to cyclopropyl fatty acids (cy17:0 & cy19:0) as microbes move from a logarithmic, or rapid growth phase, to a stationary, or slowing, phase of growth. This change is expressed in the two ratios cy17:0/16:1ω7c and cy19:0/18:1ω7c, which may vary from organism to organism or environment to environment but usually will fall within the range of 0.1 (log phase) to 5.0 (stationary phase) when summed. This ratio is inversely proportional to the turnover rate, i.e. a lower ratio infers a higher turnover rate. An increase in cyclopropyl formation also has been associated with anaerobic metabolism. Gram-negative bacteria also generate *trans* fatty acids to minimize the permeability of their cellular membranes as adaptation to a more hostile environment. Ratios (16:1ω7t/16:1ω7c and 18:1ω7t/18:1ω7c) greater than 0.1 (when summed) have been shown to indicate an adaptation to a toxic or stressful environment resulting in decreased cellular membrane fluidity, which decreases membrane permeability. Figure 5 shows the growth phase for the Gram negative bacteria in the samples.

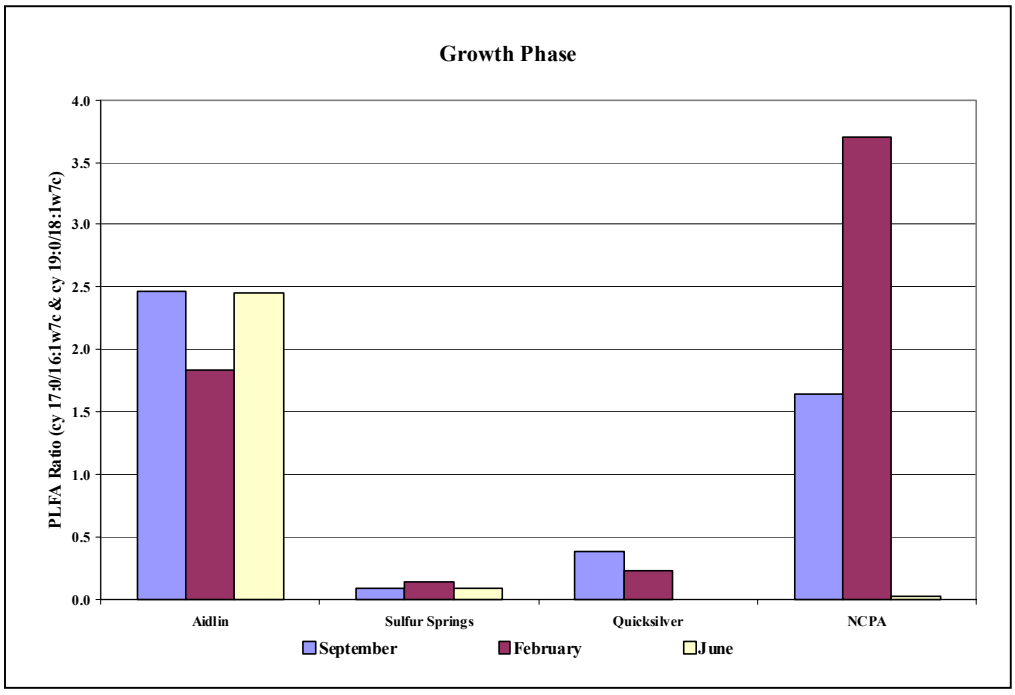


Figure 5. Growth Phase of Gram Negative Bacteria

This data indicates that the Aidlin communities were in the stationary phase of growth during the sampling periods, as were the communities at NCPA during September 1999 and February 2000. The Sulfur Springs and Quicksilver plants were experiencing a more rapid turnover and faster growth during the sampling. NCPA's growth was also

recorded in the log phase during the June 2000 period. A transition from stationary to log phase may indicate a change in environmental conditions in the plant that is triggering a change in the growth kinetics. The biomass measurements varied from 550 picomoles of PLFA per milliliter of filtered sample from the Aidlin plant in February 2000 to trace amounts 0.04 picomoles of PLFA per milliliter of filter sample detected in the NCPA samples in September 1999.

The results of the DGGE analyses are presented in Table 2, which displays the dominant species based on DNA profiling, found at the various plants during the sampling periods. These results complement the PLFA results, indicating more diverse populations at the Aidlin, Quicksilver, and Sulfur Springs plants. These species can be generally characterized as moderately thermophilic, sulfate and iron metabolizers as might be expected of bacteria found in the plant environments.

Table 2. Dominant Species Distributions Identified From DGGE Analyses

Aidlin	**Sulfur Springs**	**Quicksilver**	**NCPA**
September 1999			
	Flexibacter filiformis	*Flexibacter filiformis*	
	Moraxella osloensis	*Moraxella osloensis*	*Moraxella osloensis*
		Piscrickettsia salmonis	
Thiobacillus caldus			
Thiobacillus neapolitanus	*Thiobacillus neapolitanus*	*Thiobacillus neapolitanus*	
		Wobachia persica	
Thiobacillus sp.			
February 2000			
Thermicanus aegypticus			
Thiobacillus caldus		*Thiobacillus caldus*	
Thiobacillus neapolitanus	*Thiobacillus neapolitanus*		
	Methylococcus sp.	*Methylococcus sp.*	
Ralstonia sp.		*Ralstonia sp.*	
Thiobacillus sp.			
June 2000		**Eagle Rock**	
	Methylococcus capsulatus	*Methylococcus capsulatus*	*Methylococcus capsulatus*
	Morexella osloensis		*Moraxella osloensis*
Thiobacillus neapolitanus			
		Thiomonas thermosulfata	
Bacillus sp.		*Bacillus sp.*	
			Chromobacterium sp.
Rhizobium sp.			
Thiobacillus sp.		*Thiobacillus sp.*	

Results and Discussion

The PLFA and DGGE analyses were seen to support the growth trends determined by the selective culturing, or MPN methods. Generally, both sets of data found the bacterial populations to be similar at the Aidlin, Sulfur Springs and Quicksilver plants, with NCPA showing considerably lower numbers, particularly during the September and February sampling periods. A plot in which biomass measured via the PLFA technique is compared to biomass measured via the MPN method is presented in Figure 6. For the comparison, the quantity of PLFA measured in the various samples is converted to cell numbers. The cell equivalent value has been derived from standard experiments with typical bacteria isolated from soil and water. This value is based on 2.0×10^{12} cells per gram dry weight of cells and 10^8 picomoles of phospholipid/gram dry weight of cells. This number is approximate and may vary with the environmental conditions from which microorganisms were recovered by a factor of up to 5. (This relates to the fact that organisms under nutritionally stressed conditions may be smaller in volume; and therefore, represent less biomass and contain less PLFA.)

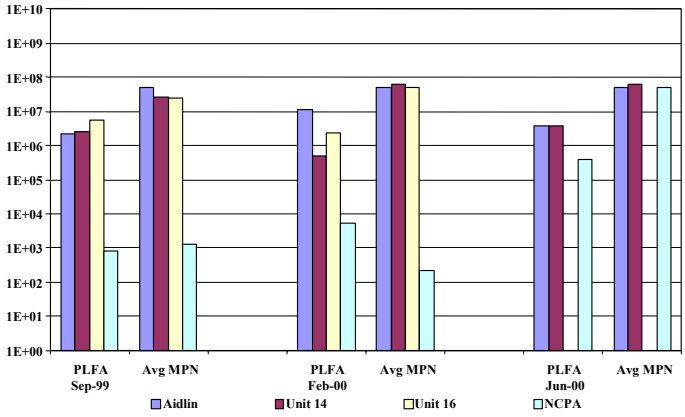


Figure 6. PLFA Comparison to Average MPN Values

Referring to the plot, the PLFA and MPN numbers are seen to track with the highest numbers recorded at the Aidlin, Quicksilver, and Sulfur Springs plants and considerably lower numbers recorded at NCPA during the September 1999 and February 2000 sampling periods. The number values do exhibit a 1 to1 ½ order of magnitude difference between the two techniques. This variance can be explained by differences in the handling and processing of data used by the techniques. As noted above, the conversion of PLFA to number density is approximate, due to uncertainties in the biovolumes used for the conversion. In addition, the MPN method relies on the culturing of organisms, which is a highly time-dependent process.

In conclusion, these studies indicate that microbial growth trends at the Geysers' facilities can be observed by the both the selective culturing and the PLFA analyses. However, the PLFA and DGGE techniques can provide much more information about microbial activity at the plants including the dominant species forming the microbiological community at the plant and their growth status. This information, while more expensive to obtain ($1000 per sample versus $100 per sample), is very valuable to the research program in achieving the long-term goals of determining what conditions trigger increased growth at the plants and in developing monitoring and treatment programs targeted at these activities. In particular, a knowledge of the species present in the environment as a function of plant conditions could lead to the development of improved control agents, targeting key species and/or nutrient conditions. And while these types of analyses are presently not affordable for a typical plant operation, current efforts directed toward automating these techniques could eventually reduce costs, making them competitive with other methods.

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