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RAPID DETECTION/IDENTIFICATION OF MICROBES, BACTERIAL SPORES, MICROBIAL COMMUNITIES, AND METABOLIC ACTIVITIES IN ENVIRONMENTAL MATRICES

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1.0 Abstract

Modern molecular methods such as lipid biomarker analysis (LBA) and PCR-Denaturing Gradient Gel Electrophoresis (DGGE) of rDNA have enabled sensitive and quantitative assessment of in-situ microbial communities that is independent of isolation and culture of the microbes. Application of artificial neural network analysis (ANN) to biomarker data has demonstrated a powerful means to characterize soil, marine and deep subsurface microbial communities. This analysis has provided accurate detection of bioavailability, bioremediation effectiveness, and rational end points for remediation based on the community microbial ecology. Sequential high pressure/temperature extraction greatly shortens sample processing time and increases the recovery of lipid biomass from soils. Sequential extraction by supercritical CO_2 lyses cells and removes neutral lipids. This is followed by enhanced solvent extraction that recovers cellular polar lipids. The extracted residue is then derivatized in-situ and re-extracted with supercritical CO_2 to yield dipicolinic acid originating from bacterial spores and amide-linked hydroxy fatty acids derived from the lipopolysaccharide of Gram-negative bacteria. Once extracted, components in each fraction are separated by capillary high performance liquid chromatography (HPLC) with solvent systems compatible with electrospray ionization

and analyzed by tandem quadrupole mass spectrometry (HPLC/ESI/MS/MS). HPLC utilizing electrospray ionization (ESI) provides the potential to analyze new lipid biomarkers and provides greater insights into the viable biomass, community composition and physiological status than with previous techniques. Use of HPLC/ESI/MS/MS enables analysis of respiratory quinones, diglycerides, and sterols from the neutral lipids. Also, the structures of intact phospholipids which have been collisionally dissociated can be determined at sensitivities (for synthetic molecules) of ~ 90 attomoles/μL (the concentration of the total lipids in a single *E. coli*). Moreover HPLC/ESI/MS/MS also enables analysis/detection of the isoprenoid tetraethers of the *Archaea*, ornithine lipids of Gram-negative bacteria signaling for bioavailable phosphate, and the lysyl-esters of phosphatidyl glycerol of Gram-positive bacteria. This expanded LBA provides deeper insight into predicting in-situ metabolic activities without disturbance artifacts. In addition, the analysis is based on the responses to microniche environments—the Holy Grail of microbial ecology.

2.0. Introduction

Analysis of the cellular lipids provides a convenient quantitative way to gain insight into critical attributes of microbial communities without isolation or culture of the microbes. Lipids are cellular components recoverable by extraction in organic solvents. The extraction provides both a purification and concentration. Lipids are an essential component of the membrane of all cells and play a role as storage materials. The lipid biomarker analysis (LBA) provides quantitative insight into three important attributes of microbial communities and facilitates recovery of the DNA for increased specificity.

2.1 VIABLE BIOMASS

The determination of the total phospholipid ester-linked fatty acids (PLFA) provides a quantitative measure of the viable or potentially viable biomass. Viable microbes have an intact membrane that contains phospholipids (and PLFA). Cellular enzymes hydrolyze (release) the phosphate group from phospholipids within minutes to hours of cell death resulting in the formation of diglycerides [1]. A careful study of subsurface sediment showed the viable biomass determined by PLFA was equivalent (but with a much smaller standard deviation) to that estimated by intercellular ATP, cell wall muramic acid, and very carefully done acridine orange direct counts (AODC)[2].

2.2 COMMUNITY COMPOSITION

The analysis with lipid biomarkers provides a quantitative definition of the microbial community structure. Specific groups of microbes often contain unusual lipids [3]. For example, specific PLFA are prominent in the hydrogenase-containing *Desulfovibrio* sulfate-reducing bacteria, whereas the *Desulfobacter* types of sulfate-reducing bacteria contain distinctly different PLFA [4, 5]. Patterns of the prominent PLFA from isolated microbes after growth on standardized media are used to differentiate over 8,000 species of organisms with the Microbial Identification System (MIDI, Newark DE)[6]. Hierarchical cluster analysis of the patterns of phospholipid fatty acids shows similarities

between species of isolated methane oxidizing and sulfate-reducing bacteria that almost exactly parallel the phylogenetic relationships based on the sequence similarities of the 16S rRNA [7, 8]. Hierarchical cluster analyses of PLFA patterns of the total microbial communities quantitatively define relatedness of different microbial communities. This hierarchical cluster analysis of PLFA patterns has been done with deep subsurface sediments in which the microbial communities of permeable strata are different from surface soils, clay aqualudes, and drilling fluids used to recover the samples [9].

The analysis of other lipids such as the sterols (for the microeukaryotes--algae, protozoa) [10], glycolipids (phototrophs, gram-positive bacteria), or the hydroxy fatty acids in the lipopolysaccharide (LPS) Lipid A (gram-negative bacteria) [11] can provide an even more detailed community structure analysis.

2.3 NUTRITIONAL STATUS

The formation of poly β-hydroxyalkanoic acid (PHA in bacteria) [12], or triglyceride (in microeukaryotes) [13] relative to the PLFA provides a measure of the nutritional status. Bacteria grown with adequate carbon and terminal electron acceptors form PHA when they cannot divide because some essential component is missing (phosphate, nitrate, trace metal, etc.). Furthermore, specific patterns of PLFA can indicate physiological stress [14]. Exposure to toxic environments can lead to minicell formation and a relative increase in specific *trans* monoenoic PLFA compared to the *cis* isomers. It has been shown that for increasing concentrations of phenol toxicants, the bacteria *Pseudomonas pudita* forms increasing proportions of *trans* PLFA [15].

2.4 CONCOMITANT DNA EXTRACTION

This powerful quantitative assessment method developed over the past 20 years to define the viable biomass, community structure and nutritional/physiological status of environmental microbial communities based on LBA can be expanded to include analysis of DNA. The DNA probe analysis offers powerful insights because of the exquisite specificity in the detection of genes for enzyme processes and/or for16S rRNA for organism identification at the kingdom, family, genus or species levels. Extraction of DNA from soils requires lysis of the cells prior to obtaining the DNA [16]. Recent evidence indicates that the lipid extraction used for LBA also liberates the cellular DNA [17]. The combined analysis using lipid biomarkers and PCR of rDNA with subsequent separation of the amplicons by denaturing gradient gel electrophoresis (DGGE) has proved especially powerful in the analysis of microbial communities impacted by pollution [18]. One problem with DNA analysis is that it is sometimes difficult to achieve quantitative results. The concomitant DNA/lipid analysis readily provides quantitative recoveries independent of the ability to isolate or culture the microbes, and the presence of intact cellular membranes containing polar lipids provides an accurate measure of the microbial biomass.

As part of the Department of Energy Natural and Accelerated Bioremediation Research Program (NABIR), our laboratory has been able to compare the relative effectiveness of phospholipid fatty acid analysis (PLFA) *vs* the polymerase chain reaction (PCR) based techniques terminal Restriction Fragment Length Polymorphism (tRFLP)

and Denaturant Gradient Gel Electrophoresis (DGGE) for predicting the impact of chromium on the microbial community at a contaminated site. Contamination at the site ranged from 50 to 200,000 ppm [19, 20]. As a consequence of the relative lack of toxicity of Cr (III), the microbial populations were relatively diverse over this wide range. By using artificial neural network (ANN) analysis, the impact of the Cr on the microbial community down to approx 10^2-10^3 ppm Cr was detected with PLFA analysis. t-RFLP, while also able to pick up the impact of the Cr, only did so at $>10^3$ ppm Cr [21]. Preliminary analysis showed the same sensitivity of PLFA analysis in predicting effects of Cr toxicity on the microbial community in vadose-zone sediment microcosms [22].

As a membrane marker, the PLFA analysis picks up the response of not only the in situ community gene pool but also the individual physiologic response of cells to shifts in the local microniche ecosystem processes. It is this extra response that gives PLFA its advantage over the DNA based techniques, especially when analysis of the whole community response is the goal. PLFA is not as effective as DNA analysis when looking for one specific organism. It's analogous to determining the shape of a cathedral from one brick. DNA based techniques show you the shape of the individual bricks (mathematically the DNA sequence is non-compressible) but does not provide as much data the community. PLFA, on the other hand, provides an idea of how the whole community (the "cathedral") is shaped. As such, PLFA seems to provide a "holistic" answer to mapping community change, and one that takes into account any perturbation that may occur. Our proposed enhancement of lipid biomarker analysis not only greatly increases the functional insights into the community dynamics by expanding the lipids that can be readily analyzed but provides this analysis more rapidly and at greater sensitivity as well as at a lower cost.

3. Methods

One of the drawbacks of the classical PLFA analysis is the extraction process by which lipids are recovered from environmental samples. Classical room temperature/pressure extraction takes 8-12 hours to allow emulsions to settle and requires careful analytical technique by a skilled operator. Once extracted, the lipids are then separated with bulk elution on a silicic acid column into the three fractions of neutral lipid, glycolipid and polar lipid. Each fraction is then transmethylated for analysis by GC/MS [3, 23].

3.1 A RAPID POTENTIALLY AUTOMATABLE EXTRACTION SYSTEM

"Flash" sequential extraction/fractionation of neutral lipids with supercritical CO_2, followed by polar lipids with chloroform methanol, derivatization and subsequent extraction of spores and amide-linked hydroxy fatty acids represent the process. The supercritical CO_2 ($SFECO_2$) extraction of neutral lipids and the enhanced solvent extraction (ESE) of polar lipids have been reported in separate studies [24, 25]. The system achieves pressures to 10,000 psi and temperatures to 150°C on 6 different samples in parallel. This is a modification of the Applied Separations (Allentown PA) "fast PSE system" which sequentially extracts the six sample holders.

Each extraction is delivered to an ISCO System Si-10x fraction collector. The tubes containing neutral lipids after $SFECO_2$ are analyzed for respiratory ubiquinones and, after derivatization, for diglyceride and sterols. ESE of polar phospholipids is then performed on the residue of the neutral lipid extraction with one-phase aqueous chloroform/methanol/aqueous buffer and deposited in the fraction collector for analysis. Next the residue of the two sequential extractions will be derivatized in-situ and the amide-linked hydroxy fatty acids of the lipopolysaccharide (LPS-ALHFA) and the 2,6 dipicolinic acid (DPA) recovered in $SFECO_2$ for assay. The neutral lipids, polar lipids, and DPA/LPS-ALHFA fractions will be analyzed by high performance liquid chromatography/electrospray ionization/tandem mass spectrometry (HPLC/ESI/ MS/MS). Solvent will be concentrated in the nitrogen "blow-down" and the samples transferred in sequence to the HPLC autosampler. Figure 1 diagrams the system:

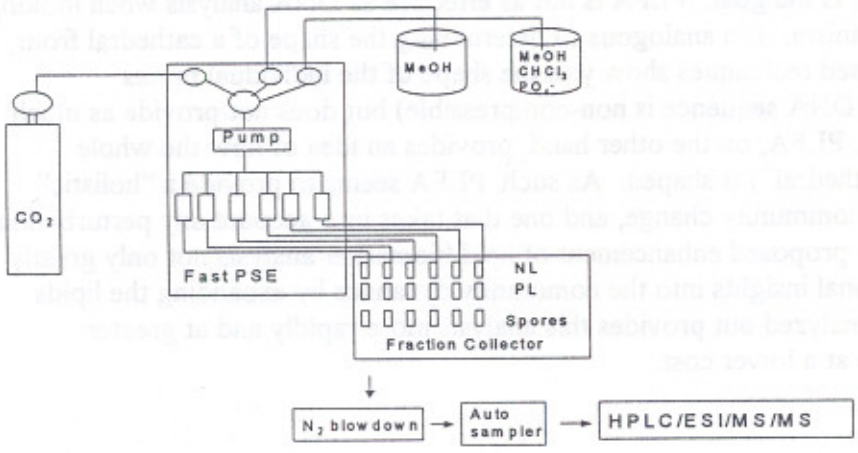

Figure 1: Schematic of the extraction apparatus

3.1.1 *$SFECO_2$ Extraction of Neutral Lipids*

Nivens and Applegate working in this laboratory [26] have shown that CO_2 when rendered supercritical at temperatures > 31.1°C and pressures greater than 72.85 atm, extracted neutral lipids (sterols, diglycerides, respiratory quinones) and pollutants/contaminants like polynuclear aromatic hydrocarbons, pesticides, and petroleum hydrocarbons.

3.1.2 *ESE Extraction of Polar Lipid*

The second phase in the rapid recovery will be to use the Applied Separations enhanced solvent extraction of the residue after $SFECO_2$ cell lysis and neutral lipid extraction. The yield will be compared to classical Bligh and Dyer one-phase chloroform/methanol/buffer solvent extraction at room temperature and pressure as published previously [3, 23]. The lipid patterns and concentrations are well known, having been determined using the classical methods. Using the Dionex ASE-200 Accelerated Extraction system with 2 cycles, 80°C and 1200 PSI produced recovery of 3 fold more PLFA from *Bacillus* sp. spores and 2-fold more PLFA from *Aspergillus niger* spores than did the standard one-

phase extraction system [24]. Preliminary studies [23] showed that increases in pressure to 8000 psi at 80°C in a hand operated ISCO SFX apparatus further increased the recovery of lipids from *Bacillus* spores. The lipids were detectable after a minute exposure to high pressure/temperature solvent.

3.2 RECOVERY OF DPA AND LPS-ALHFA AFTER *IN- SITU* DERIVATIZATION

The chelator, 2, 6-dipicolinic acid (DPA), is specific to all known bacterial spores and is primarily responsible for their remarkable resistance to heat and dryness. A combined derivatization/extraction procedure that quantitatively forms the dimethyl ester of spore 2,6-DPA *in situ* and allows extraction has been developed [27]. ESI/MS results in the formation of the protonated molecular ion corresponding to DPA. Up-front collisionally induced dissociation (CID) produces two major ions, a protonated molecular ion $(M+H)^+$ at m/z 196 plus a sodiated molecular ion $(M+Na)^+$ at m/z 218. The limits of detection are 100 fg/μL or 2.7×10^3 *B. subtilis* spores, s/n = 2.6. The assay is linear over three orders of magnitude $R^2 = 0.9997$, y = 0.5525, and takes less than one hour to perform.

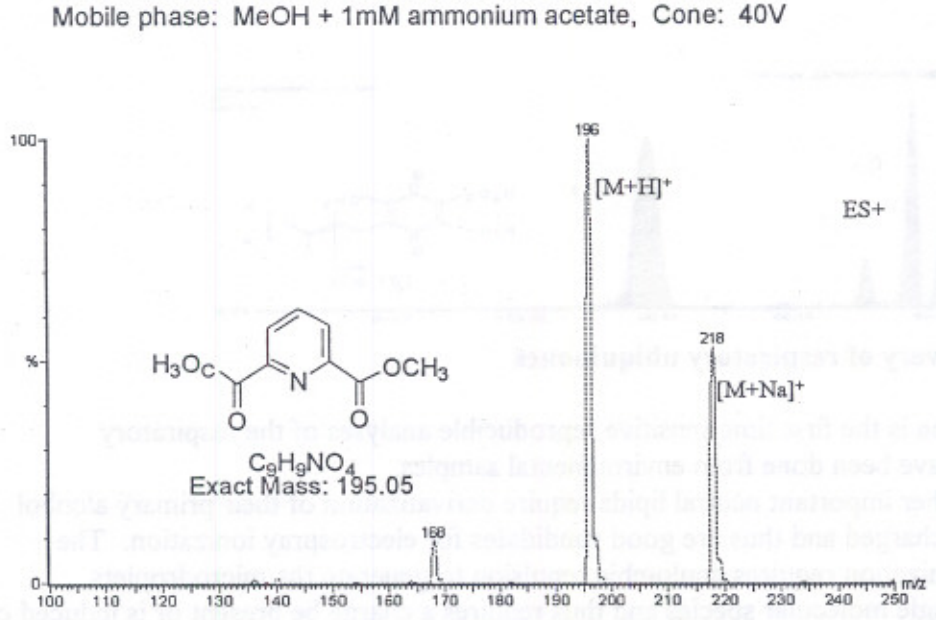

Figure 2 illustrates the HPLC/ESI/MS spectrum of DPA.

3.3. HPLC/ESI/MS OF NEUTRAL LIPID

One of the primary considerations in the bioremediation or the immobilization of U and Pu contaminants is the ability to biologically remove high potential electron donors such as oxygen and nitrate thereby decreasing the redox potential. This results in a reduction of the nuclides to insoluble compounds following interactions with metal or sulfate reducing bacteria or by geochemical processes. Estimating the terminal electron acceptor in the

subsurface microbial microniche has proved especially difficult as the recovery of unaltered samples or the placement of microsensors from the subsurface has generally been unsatisfactory. Gram-negative bacteria form respiratory ubiquinones (UQ) when the terminal electron acceptor is oxygen [28]. Under anaerobic growth conditions Gram-negative bacteria may form respiratory naphthoquinones or no quinones. Gram-positive bacteria form respiratory naphthoquinones when grown aerobically. Respiratory ubiquinones are found in concentrations about 200 times less than the PLFA or about 0.5 μmole/g dry weight [29]. Knowing the isoprenoid side chain length has taxonomic implications. Eukaryote mitochondria contain UQ-10 (80 carbon side chain). Gram-negative bacteria contain isoprenologues from UQ-4 to UQ-14 [30]. Figure 3 illustrates an HPLC method for the separation UQ isoprenologues with methanol 1% ammonium acetate. The limit of Detection (LOD) is 200 pmoles/μL of UQ-6 (LOQ 500 pmoles/μL using a positive ion of m/z = 197. The LOD with this single quadrupole MS system represents about 10^4 bacteria the size of *E. coli* growing aerobically. Figure 3 illustrates the analysis of respiratory ubiquinones:

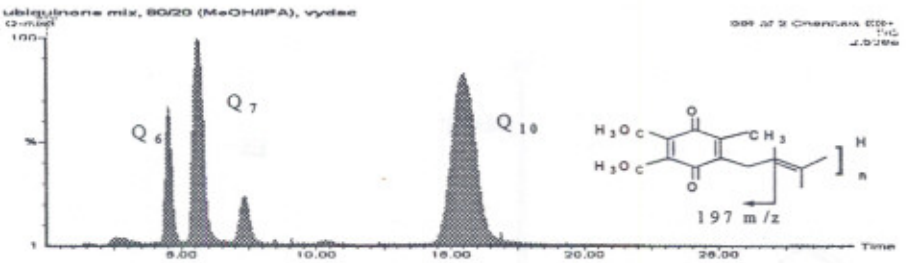

Figure 3.Recovery of respiratory ubiquinones

This application is the first time sensitive reproducible analyses of the respiratory ubiquinones have been done from environmental samples.

Two other important neutral lipids require derivatization of their primary alcohol moieties to be charged and thus are good candidates for electrospray ionization. The electrospray ionization requires coulombic repulsion to generate the microdroplets containing a single molecular species and thus requires a charge be present or is induced on the analyte molecule. Primary alcohols have been particularly difficult to successfully electrospray. Two alcohols, sterols and diglycerides are especially valuable indicators of the microbial community composition and physiological status. Sterols are valuable in assessing microeukaryotes. In work done in this laboratory analysis of sterols allowed the diets of filter feeding marine invertebrates to be assessed [10]. With cell death the bacterial phospholipids rapidly form diglycerides by the action of endogenous and exogenous phospholipases [1]. The ratio of Diglyceride/PLFA is a valuable indicator of conditions promoting cell lysis in the subsurface [31]. Recently a derivatizing agent was developed for primary alcohols based on the formation of ferrocenoyl carbamates from ferrocenoyl azide that have subfemtomolar sensitivities in ESI/MS [32]. Figure 4 illustrates the mass spectrum of the ferrocenyl derivative of cholesterol after HPLC/ESI/MS:

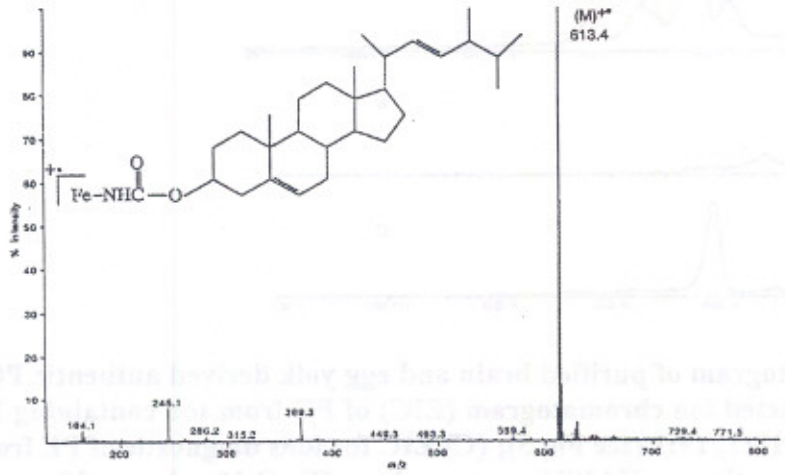

Figure 4. The mass spectrum of the ferrocenyl derivative of cholesterol after HPLC/ESI/MS.

With cell death the bacterial phospholipids rapidly form diglycerides by the action of endogenous and exogenous phospholipases [1]. The primary alcohol of the 1,2 diglycerides can be derivatized with another charge promoting moiety. This derivatization of uncharged alcohols based on the formation of ESI active N-methylpyridyl alcohol salt. The N-methylpyridyl salt is formed from the diglyceride by direct reaction with 2-fluoro-1-methylpyridinium p-toluenesulfonate (24). The N-methylpyridyl ether salt will give an $(M + 92)^+$ (where M is the mass of the diglyceride or sterol) (24).

3.4 HPLC SEPARATION OF POLAR LIPIDS

There is a rich literature on the HPLC separation of lipids [34]. Unfortunately the columns and elution gradients used on normal-phase HPLC columns that give excellent separation of polar lipid classes are incompatible with the conditions for ESI. For optimal ESI the analytes must either be charged in solution, or readily ionized electrolytically in the ES capillary and the presence of non- volatile ions in the eluent and certain solvents greatly compromises the robustness of the ESI source. To provide a negative charge on the intact phospholipids for ready ionization the pH should be above 9.0. However, at this pH the silicic acid based column material used in the normal phase separation of polar lipids dissolves. Use of a post column addition of ammonium acetate (which is volatile) achieves the pH but generates an emulsion that compromises the ESI source. The use of polar solvents with chloroform greatly depressed ionization in the ESI inlet. Therefore, reverse phase HPLC with a resistant C-18 column material provided base line separation of intact polar lipids in a methanol solvent with 0.002% piperidine as base with a post column addition of 0.2% piperidine in methanol (Figure 5). This has proved a significant advance in providing rapid (> 8 min) separation of intact polar lipids with ESI compatible solvents isocratically with no re-equilibration [35].

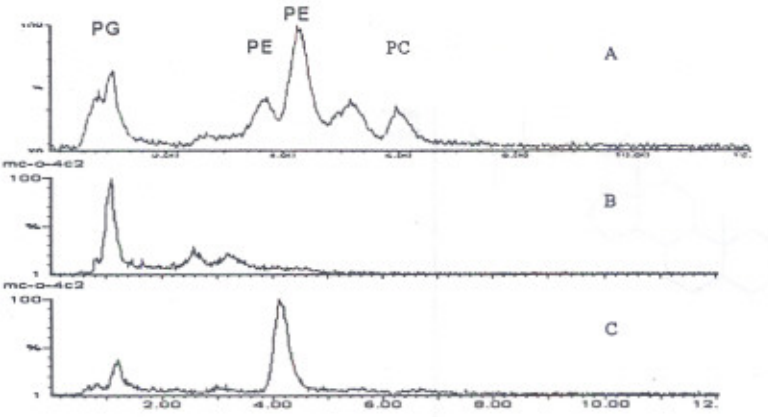

Figure 5: (A) Chromatogram of purified brain and egg yolk derived authentic PG, PE, and PC; (B) Extracted ion chromatogram (EIC) of PG from soil containing 15:0*, 16:0, 16:1, 17:0, 17:1, 18:1, 19:1 (see Fig 5); (C) EIC for ions diagnostic of PE from the soil used in B. ***Separation on HAISIL reverse phase HL C-18 column, 30 mm x 1mm x 3 μ, 95/5 methanol + 0.002% piperidine/water, 50 μL/min, post-column modifier 0.02% piperidine in methanol at 10 μL/min.

3.4.1 *Bioavailable Phosphate*

Other phospholipids provide significant insight into the microbial communities nutritional/physiological states. Bioavailable phosphate has been a particularly difficult parameter to estimate [36] and a wide variety of extractants have been utilized to quantify soil phosphorous status. These methods vary in chemical aggressiveness. Those methods utilizing water, acid or alkali do not accurately reflect the bioavailable P as reflected in plant productivity. Estimates of mineralization have utilized a sequence of bicarbonate extracts over time to gain a truer insight into the extent of mineralization and immobilization processes [37, 38]. An alternative to chemical extractants is the application of isotopic techniques. A long series of experiments are reviewed in Frossare et al, [39], who came to the conclusion that great caution should be taken before extrapolating the quantity of isotopically exchangeable phosphate to isotopic exchange times longer than a minute. Suffice it to say the estimation of bioavailable P in soils and sediments is highly problematic. The HPLC/ESI/MS can provide a biological sensor for phosphate bioavailability. Sedimentary universally distributed Gram-negative soil organisms like *Pseudomonas* form acyl-ornithine lipids when grown with limited bioavailable phosphate [40]. The acyl-ornithine lipids replace phospholipids as the predominating polar lipids in the cell membranes.

3.4.2 *Amino Acid PG Esters*

Another phospholipid class lipid is the lysyl ester of PG that is formed by *Micrococci* when grown at acid pH [41]. Both the acyl ornithine lipids and lysyl-PG should be readily analyzed by HPLC/ESI/MS and the separation conditions are currently being developed in our laboratory.

3.4.3 *Archaea*

Another important group of organisms are the *Archaea*. The *Archaea* contain isoprenoid ether lipids that have been difficult to measure by GC/MS. For example the principal lipid of from the membrane of *T. acidophilum* is a Tetraether lipid comprised of two diphantyl (saturated methyl branched C-40 isoprenoid) chains (many with cyclopentane rings) covalently linked at their terminal carbon atoms with a sugar molecule at one end and a phosphoglycerol ester at the opposite end with masses approaching m/z 1800. We have demonstrated that isoprenoid tetraethers can be injected using a mobile phase of 95/5 (methanol +0.002% piperidine/water) at a flow rate of 50 µL/min. Post column injection of 0.2% piperidine in methanol added through a zero dead volume tee at a rate of 10 µL/min provided the alkaline pH. In the positive ion mode two distinct ions were observed. The $[M+H]^+$ at m/z 1641 was approximately 20% abundant, while the most intense ion in the spectrum $[M-2H+Na+K]^+$ at m/z 1700 corresponded to an adduct ion containing both sodium and potassium at a cone voltage of 60V.

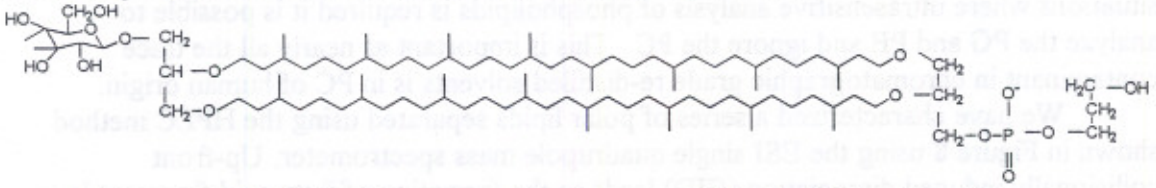

Figure 6. ESI/MS of the tetraether isoprenoid lipid of *T. acidophilum*

The sodium and potassium adducts agree with the findings of Qiu *et al* [42]. Qiu *et al* [42] investigated lipids from *Archaea* using tandem MS/MS and showed many high mass fragments, diagnostic of their components of interest which will be invaluable when the proposed system can be applied to MS/MS analysis.

3.4.4 *Extraction Recovery, Acid Methanolysis/Derivatization of Poly β-Hydroxyalkonate (PHA)*

The detection of the bacterial polyester storage product is a measure of bacterial unbalanced growth which occurs when bacteria have an adequate carbon and terminal acceptor supply but lack some essential trace nutrient such as phosphate, nitrogen or trace elements [12]. The most sensitive and specific assay for PHA, developed in this laboratory, unfortunately requires hydrolysis of the polyester with derivatization to a volatile ethyl ester for GC/MS [45]. Unless great care is taken, the esterification often leads to losses. In addition PHA in its most common form is remarkably insoluble and maximal recovery at room pressure requires boiling chloroform. PHA precipitates on glass when the solvent cools and the transfer to the GC/MS is often not quantitative. Preliminary experience has shown that much more PHA is recovered by the "flash" high temperature/pressure extraction method proposed herein. We propose in-situ transesterification to form the isopropanol ester of the β-hydroxyacid monomer subunits of the PHA in the $SFECO_2$ chamber prior to "flash high temperature/high pressure extraction. We have demonstrated effectiveness of in-situ $SFECO_2$ chamber transesterification derivatization under the high pressure/high temperature of refractory substances such as the 2,6 diaminopimelic acid of bacterial spores to form the dimethyl

ester is possible in $SFECO_2$ [27]. We pioneered in-situ derivatization to form methyl esters of phospholipids with trimethylphenylammonium hydroxide under conditions of $SFECO_2$ [25]. We propose to transesterify the PHA with isopropanol, recover the β-hydroxy alkanyl isopropanol ester and then derivatize the primary alcohol with the ester directly with 2-fluoro-1-methylpyridinium p-toluenesulfonate to form the ESI active N-methylpyridyl ester salt. The N-methylpyridyl ether salt will give an $(M+92)^+$ (where M is the mass of the β-hydroxy acid monomers the PHA hydroxy alkanyl isopropyl ester) derivative [33]. Measuring PHA is particularly important as we have shown bacteria in the rhizosphere attached to fine roots show no evidence of unbalanced growth (PHA/PLFA < 0.02), whereas bacteria from the surrounding soil show evidence of unbalanced growth (PHA/PLFA >0.1), [44].

3.4.5 *ESI/MS Analysis of PolarLipids*

The HPLC/ESI/MS greatly facilitates ultrasensitive analysis of intact polar lipids. In situations where ultrasensitive analysis of phospholipids is required it is possible to analyze the PG and PE and ignore the PC. This is important as nearly all the trace contaminant in chromatographic grade re-distilled solvents is in PC of human origin.

We have characterized a series of polar lipids separated using the HPLC method shown in Figure 8 using the ESI single quadrupole mass spectrometer. Up-front collisionally induced dissociation (CID) leads to the formation of fatty acid fragment ions in the negative on mode, *e.g.* the m/z 255 ion corresponding to palmitic acid (16:0), the m/z 253 ion corresponding to palmitoleic acid (16:1), and the m/z 267 ion corresponding to cyclopropane 17 (cy17:0). PG also gives rise to ions corresponding to phosphoylglycerol at m/z 171 and glycerol phosphoryl glycerol (less water and hydrogen) at m/z 227. PE gives the corresponding ions at m/z 140 and 197. The phosphoryl fragments are 10 times less abundant than the fatty acid negative ions when using up-front CID. With the single quadrupole HPLC/ESI/MS system we have achieved a limit of detection (LOD) of 8 femtomoles/μL and a limit of quantitation (LOQ) 10 fold higher with a standard deviation of ~10% with *B. subtilis*. This corresponds to detection of roughly 10^2 bacteria the size of *E. coli* and is in contrast to GC/MS with LOD of at best 2000 femtomoles/μL.

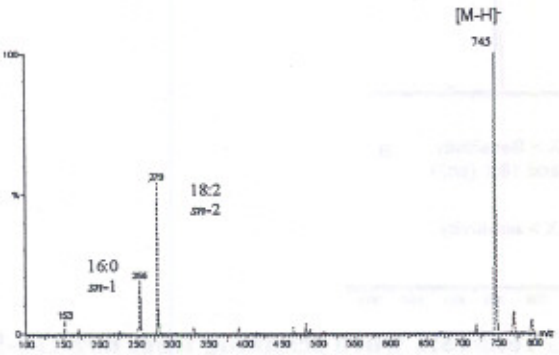

Figure 8: The mass spectrum of synthetic PG with negative ion fragments for 16:0 at m/z 255 for *sn*-1, 18:2 at m/z 279 for *sn*-2, and m/z 153 for glycerol-P.

The ions we detect with HPLC/ESI/MS of phospholipids precisely match those reported by Fang and Barcelona [44].

3.4.6 *ESI/MS/MS*

Tandem mass spectrometry will greatly increase the sensitivity, and specificity of this environmental analysis system without any increase in the analysis time. The effectiveness of detection can be greatly increased [45-48]. Neutral loss scans are particularly useful for the rapid screening of targeted lipid ions as demonstrated by Cole and Enke [48] who showed that the PE and PG could be readily detected using a neutral loss of m/z =141 and 154, respectively. In the negative ion mode, it proved possible to detect the position of each ester linked fatty acid *i.e.* the fatty acid component at the *sn*1 position had 20% of the abundance of the fatty acid at the *sn*2 position of the glycerol (as in Figure 8). Tandem mass spectrometry, ESI/MS/MS, provides great advantages in the structural analysis of phospholipids. Product ion spectra and multiple reaction monitoring (MRM) were used to investigate PG containing a 16:0 fatty acid at the *sn*-1 position and an 18:0 fatty acid at the *sn*-2 position. Figure 9A shows the ESI spectrum for 1 ppm PG when scanning from m/z 110-900; figure 9B shows the product ion spectra for m/z 747 with collision-induced dissociation (CID) in the second quadrupole in the presence of 3.7 x 10-3 mbar Ar yielding the product ions that were analyzed by scanning over m/z 110-900 in the third quadrupole. MS/MS decreases chemical noise in the product ion spectra thereby increasing the signal-to-noise (s/n) ratio and the resulting sensitivity. Comparing the s/n ratio of the up-front CID of the *sn*1 and *sn*2 fatty acids in the upper panel of Figure 9 (A) to the product ion spectra from CAD in the second quadrupole, the product ion spectrum for PG is more sensitive by a factor of 50 (Figure 9 (B) lower panel). By scanning over a narrower range, the sensitivity can be increased. When acquiring product ion spectra, the LOD and LOQ were experimentally determined to be 446 attomol/μL and 1.3 fmol/μL, respectively. If the third quadrupole is scanned for m/z 281 product of m/z 745, a roughly 5-fold additional gain in sensitivity was achieved. This is multiple reaction monitoring (MRM), m/z 747 → m/z 281 and represents the most sensitive application of this HPLC/ESI/MS/MS.

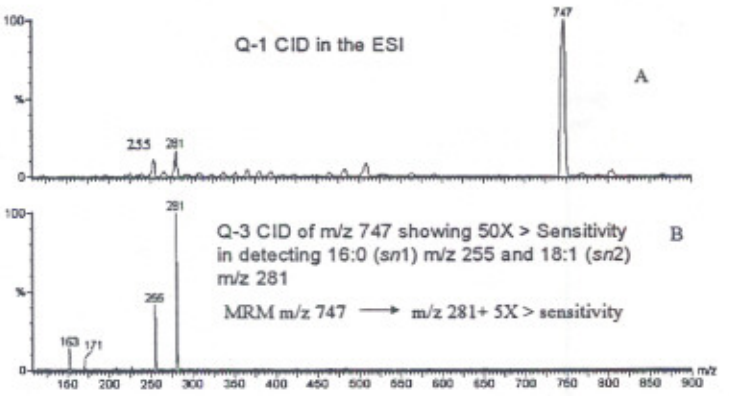

Figure 9: (A) ESI spectra for 1 ppm PG 16:0/18:1 when scanning from m/z 110-900. (B) ESI product ion spectra for m/z 747 of 1 ppm PG 16:0/18:1 when scanning from m/z 110-900.

We have demonstrated limits of detection of 90 amoles/μL using a single molecular species of phosphatidyl glycerol, [sn-1 palmitic acid m/z = 255, sn-2 oleic acid 18:1 m/z = 284 parent ion m/z = 747] as the parent ion selected CID by monitoring m/z = 284 negative ions. That is essentially equivalent to the total phospholipid in a single *E. coli* cell!

In the positive ion mode, intact molecular ions are seen [48]. The introduction of ethyl vinyl ether into the CID chamber at a pressure of 3.5-4.0 mTorr resulted in the addition of ethyne at 26 au to the polar head groups of the molecular ions of PG. Each phospholipid class resulted in a unique neutral gain upon reaction with ethyl vinyl ether due to differences in the polar head groups [48]. With MS/MS it is possible to form the fatty acid negative ions to help determine the position of unsaturation, branching and other structures [49]. Such details of the phospholipid fatty acid structure including position and chirality of methyl branching, unsaturation, hydroxylation, cyclopropyl ring formation all have great significance for identification and physiological status [3, 15].

4. Expanded Lipid Biomarker Analysis

Figure 10 represents a diagram of the possibilities for lipid analysis. We have included the standard PLFA analysis as it continues to be very useful.

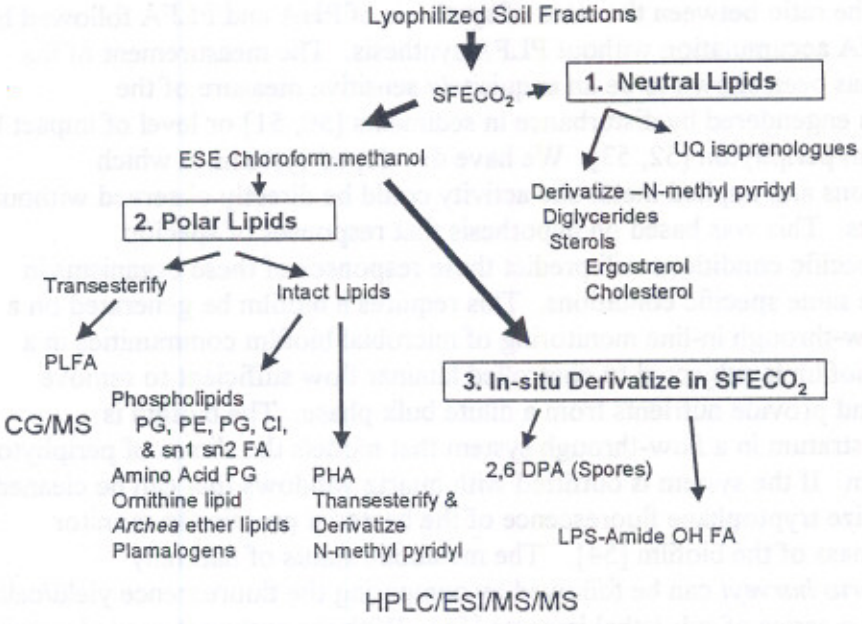

Figure 10: Schematic for the expanded lipid biomarker analysis.

The sequential "flash" extraction begins with 1. The $SFECO_2$ extraction of the neutral lipid. The respiratory benzoquinones are analyzed directly and the diglycerides, and sterols derivatized with charged derivatives. 2. ESE to recover the polar lipids. A portion of the intact lipids would be separated by capillary HPLC for ESI/MS/MS analysis. A second portion of the polar lipid would be transesterified and the methyl esters of the PLFA analyzed by GC/MS. A third portion of the polar lipids would be transesterified under high pressure and temperature and the hydroxyl derivatized for analysis of the PHA monomers by HPLC/ESI/MS/MS. 3. The extracted residue is derivatized in-situ in $SFECO_2$ and the 2,6 dipicolinic acid recovered for analysis of bacterial spores, and the amide-linked 2- and 3- hydroxyl fatty acids from the lipopolysaccaharides analyzed by HPCL/ESI/MS/MS. The presence of tannins in soils could complicate interpretation of hydroxy fatty acid analysis after the derivatization.

6. Interpretation

The expanded LBA allows insight into not only the community composition and viable biomass but into the physiological status of the microbial community. From the analysis of the community physiological status the microniche environment can be ascertained and metabolic activities predicted. The most difficult measurements in microbial ecology are estimations of in-situ metabolic activity. The problem is the determination of metabolic activity of a community poised for rapid acquisition of any resources presented by disturbance. We have shown that LBA provided a quantitative means to measure the effects of disturbance in sediments where an aerobic surface overlies anaerobic sediment rich in reduced substrates. Disturbance introduces aerated water to the anaerobic sediment and a burst of activity ensues with rapid growth until a critical nutrient again limits cell division. However, carbon accumulation continues

without cell division and new cell membrane synthesis. This was readily detectable as an initial decrease in the ratio between the rates of synthesis of PHA and PLFA followed by a rebound with PHA accumulation without PLFA synthesis. The measurement of the PHA/PLFA ratio has been shown to be an exquisitely sensitive measure of the unbalanced growth engendered by disturbance in sediments [50, 51] or level of impact by pollutants on stream periphyton [52, 53]. We have developed systems in which microniche conditions and implied metabolic activity could be directly observed without disturbance artifacts. This was based on hypothesis that responses of specific monocultures to specific conditions will predict these responses of these organisms in communities to the same specific conditions. This requires a biofilm be generated on a substratum in a flow-through in-line monitoring of microbial biofilm communities in a device where the biofilm is subjected to controlled laminar flow sufficient to remove pelagic microbes and provide nutrients from a dilute bulk phase. The biofilm is generated on a substratum in a flow-through system that models the slimes of periphyton on rocks in a stream. If the system is outfitted with quartz windows that can be cleaned, it is possible to utilize tryptophane fluorescence of the bacterial proteins to monitor formation and biomass of the biofilm [54]. The metabolic status of naturally bioluminescent *Vibrio harveyi* can be followed by comparing the fluorescence yield/cell of monocultures to a series of sub-lethal impacts [55]. With the system it proved possible to clearly correlate the changes in metabolism to the antifouling and fouling-release properties of coatings [55].

With LBA not only can metabolic activities be predicted but also the longer-term responses by the total community used in estimates of bioavailability and as ecologically defensible end-points in remediation [56]. The rapid responses of periphyton to pollution abatement in surface run-off waters [52, 53] seem to be matched by responses to hydrocarbon degradation and putative return of communities to the uncontaminated status of the aquifer up-gradient of the pollution source [57].

7. Predictions

Based on expanded LBA and judicious use of the PCR of rDNA with DGGE and sequence analysis for identification of specific organisms in communities where dominance of < 50 "species units" is sufficient to make it practical, the following predictions of consequences of microniche environments/and thus metabolic activities can be made:

1. The microniche redox potential and terminal electron acceptor will be correlated to the respiratory ubiquinone/PLFA ratio. Aerobic micronitches will be found with high respiratory ubiquinone/PLFA ratios, the absence of plasmalogens, and phosphatidyl glycerol (PG) with ratios of i15:0* to a15:0 <0.1 characteristic of the aerobic Gram positive *cocci* and the presence of Actinomycete biomarkers (high 10 Me 17:0 and 10 Me 18:0. Straight-chain sphinganines from *Sphingomonas* are found in aerobic microsites.
2. Anaerobic micronitches will be correlated to the plasmalogen/PLFA ratio (plasmalogens are characteristic *Clostridia* spp.) and the isoprenoid ether lipids of the methanogenic *Archaea*. In the presence of sulfate the anaerobic microniches will have a ratio of i15:0 to a15:0 > 2.0 and of 17:1ω7c in the phosphatidyl ethanolamine (PE)

(characteristic of Gram-negative *Desulfovibrio*) and high relative proportions of 10Me16:0 (characteristic of sulfate/metal reducing bacteria).
3. Microniches with low pH will have elevated concentrations of lysyl esters of PG characteristic of Gram-positive *Micrococci* type bacteria; micronitches with low bioavailable phosphate will show high ratios of acyl ornithine polar lipids characteristic of Gram-negative bacteria such as *Pseudomonas*.
4. Microniches with suboptimal growth conditions (low water activity, missing nutrients or trace components) will show high (> 1) cyclopropane to monoenoic fatty acid ratios in the phosphatidylglycerol (PG) and phosphatidylethanolamine (PE). They will show greater ratios of cardiolipin (CL) to PG ratios.
5. Microniches will show lower viable biomass but increased activity under conditions inducing high levels of microeukaryote predation as indicated by higher proportions of phospholipid polyenoic fatty acids in phosphatidylcholine (PC) and cardiolipin (CL). Viable biomass (total PLFA) will decrease, but there will be an increase in the mineralization/unit viable biomass.
6. Reductive process will increase with increases in the microniches with non-growth-limiting-carbon sources and terminal electron acceptors but with limiting nitrogen or trace growth factors as reflected in PHA/PLFA > 0.8. Conditions favoring cometabolic fortuitous biodegradation of refractory compounds are associated with very high PHA/PLFA ratios (> 10) [58].
7. In microniches with suboptimal growth conditions (low water activity, missing nutrients or trace components), high (> 1) cyclopropane to monoenoic fatty acid ratios in the PG, PE and PLFA, as well as greater ratios of cardiolipin (CL) to PG ratios will be detected.
8. Conditions of maximal fine root proliferation will mirror the bacterial viable biomass (PLFA) and evidence of balanced growth (PHA/PLFA <0.06), whereas conditions of fine root dieback will be associated with a much slower loss of viable bacterial biomass and higher fungal biomass expressed as increases in ergosterol and 18:2ω6 in PC and UQ-10 vs bacteria which contain no sterols, little PC, 16:1ω7c, br 15:0 and other bacterial PLFA in PG and PE, with the range of UQ-4-13.
9. Microniches with surfactants high bacteriophage multiplicities or other cell-lysis conditions can be detected as increased diglyceride/PLFA. Desiccation and suboptimal growth will be detected as cyclopropane/monoenoic precursors >0.1 in the PLFA or PG/PE.
10. Microniches exposed to toxic solvents will results in ratios of *trans* to *cis* PLFA (16:1ω7t/16:1ω7c) > 0.05 . As the bacteria make membranes less permeable, they produce membranes with higher relative levels of *trans* PLFA most prominently in PE. Gram-negative bacteria reversibly make *trans* fatty acids as a result of changes in the environment, usually as a result of stress (*i.e.* toxicity or starvation)
11. Microniches with high activity in anaerobic iron reduction and contain α and ω diacids, dioics-chemical species as well as PLFA in PE and PG typical of sulfate-reducing bacteria.
12. Microniches exposed to oxidative biocides or reactive agents show epoxide (oxirane) PLFA in PE in conditions *e.g.* in mine service water and wastewater treatment facilities. They also occur in the fungal rust parasite *Pneumocystis carni*. The presence of oxirane PLFA result in Gram-negative bacteria that are rendered not culturable [59].

13. Microniches exposed to high temperatures show ω-Cyclohexane PLFA and short chain < 14 carbon PLFA. Average PLFA chain length and average degree unsaturation (ACL & ADU) has been shown to increase with soil temperature, but may also be related to substrate availability
14. Metabolic stress can be reflected in Iso10me16:0/i17:1ω7c in Gram-positive bacteria in PG. This indicator is similar to the cyclopropyl to monoenoic PLFA ratio in Gram-negative bacteria.

* Fatty acids are defined as the number of carbon atoms in the longest chain: the number of double bonds with the position of the first double bond counting from the hydrocarbon end (omega (ω) end) of the carbon chain. Double bond conformation is indicated by the suffix *c* for *cis* or *t* for *trans*. The prefix indicating methyl branching is Me. The prefix number indicates by the position of the mid chain methyl branching or br if not known. Terminal methyl branching is *i* for *iso* and *a* for *anteiso* for 2 carbons from the hydrocarbon terminal. Cyclopropane rings are indicated by the prefix *cy*.

8. Acknowledgements

This work was supported by National Science Foundation grant DEB 9814813, EAR 9714215, and EAR 9978161 together with the Department of Energy, Office of Energy Research, grant number DE-FC02-96ER62278 (White) (part of the Assessment Component of the Natural and Accelerated Bioremediation Research Program, NABIR).

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