Advantages Of Lipid Biomarkers In The Assessment Of Environmental Microbial Communities In Contaminated Aquifers And Surface Waters

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Roland Gever[1,2], Anke Bittkau[1], Margaret Gan[2], Dietmar Schlosser[1], David C. White[2]

[1] Microbiology of Subterrestrial Aquatic Systems Group, UFZ Centre for Environmental Research Leipzig-Halle, Theodor-Lieser-Str. 4, D-06120 Halle, Germany, roland@halle.ufz.de
[2] Center for Biomarker Analysis, University of Tennessee, Knoxville, TN, 37932, USA

CONDENSED ABSTRACT: Despite the fact that lipid biomarker analysis is the most satisfactory single, quantitative, and comprehensive analysis of the *in-situ* microbial community viable biomass, community composition, and physiological status it is not universally utilized as this method is labor-intensive and takes 3 days. The major drawback of the classical phospholipid fatty acid analysis (PLFA) analysis is the extraction and separation process by which lipids are recovered from environmental samples. Here we show the application of sequential accelerated solvent extraction coupled to automatable HPLC/ES-MS/MS and GC/MS analysis as a rapid (~ 3 hours) and sensitive method for the analysis of lipids from microbial cells, and of environmental sediment samples from different depths from core liners, drilled at a highly BTEX contaminated test field (Zeitz, Germany).

INTRODUCTION:

The fate of metal and organic pollutants in ground- and surface waters is often largely determined by the metabolic activities of microbial communities. Microbes may catalyze the accumulation, transformation, immobilization or release of pollutants by their activities as the key actors in remediation and natural attenuation processes. Consequentially characterization of microbial community viable biomass, composition, and nutritional status is important in defining metabolic and degradation capabilities. Spatial and temporal biodiversity and activities influence pollutant fate. Unfortunately conventional cultivation-based analyses of community biomass and community composition detect < 1-10% of the microbes identified microscopically. Therefore cultivation-independent biomarker analysis is critical to microbial ecology and remediation research. Rapid extraction and sensitive detection of the viable microbial community is helpful for an effective monitoring of contaminated sites and during remediation processes.

Biomarker analysis focuses on ubiquitous cellular components with short residence times after cell-death. Two biomarkers, DNA/RNA and lipids are components in all bacteria, fungi, algae, and protozoa. Together analyses of these two types of biomarkers are complementary and each has advantages. The DNA/RNA molecular methods require extraction of the nucleic acids, which need to be free of PCR inhibitors like tannins, and the utilization of known primer sequences to define the specificity of both rDNA or specific functional genes. Extracellular nucleic acids, especially DNA, are more persistent than polar lipids. The reagents for molecular methods are expensive and processing takes several days. Most of the environmental identifications based on sequencing and phylogenetic matching indicate heretofore-uncultured strains. Quantitative analysis is difficult and the PCR amplification is biased to specific sequences. Lipid biomarkers require extraction that both concentrates and purifies the lipids. Since all viable cells require an intact cell membrane containing phospholipids and this phospholipid membrane is metabolically labile and does not persist extracellularly, the viable biomass is quantitatively measured by the extractable phospholipid. Neutral and phospholipid fractions contain components that can change structure in specific ways depending on the micro-niche environment of the microbes. Well-defined shifts in lipids reflect the activity towards terminal electron acceptors, the bioavailable phosphate, the bioavailability of trace nutrients allowing cell division, the exposure to toxic compounds, and the stress of starvation. Phospholipids contain fatty acids (PLFA) whose composition provides quantitative insight into the community composition. By matching community composition with the degree of pollution, quantitative toxicity assessments can be utilized as a rational end-point goal for bioremediation. Specific microbial metabolic activities can be determined utilizing specifically labelled substrates. Newly developed methodology utilizing sequential high-pressure extractions that can be coupled to sophisticated tandem or ion-trap mass spectrometry with potential for automation, can greatly accelerate lipid biomarker analysis. All of these parameters may be employed in PLFA-based community fingerprints, which show advantages over molecular genetic approaches in rapidity, quantitative analyses, and cost efficiency. Molecular genetic methods have much greater specificity in detection of taxonomic and functional microbial gene families. Lipid biomarker analysis should therefore be considered as complementary to molecular genetic approaches along with activity and cultivation based methods.

Using the aforementioned approach we looked at two contaminated sites. Site one represents small streams and ponds and their sediments that are heavily contaminated with heavy metals such as Cu and Cd, arising from dump drainage waters at the former copper shale mining district of Mansfeld, Central Germany. These sediments

were found to contain amazingly high concentrations of Mn and showed high MPN values of Mn(II)-oxidizing bacteria. In natural environments, microbial Mn(II) oxidation is assumed to be a significant process in formation of insoluble Mn(III/IV) oxides. From biotic processes derived Mn oxides can sorptively bind heavy metals and therefore serve as sink for these pollutants. Site two represents an anoxic aquifer that is contaminated with BTEX, mainly with benzene, where the potential role of the present microbial communities for natural attenuation processes will be evaluated.

RESULTS AND DISCUSSION

Conventional extraction of lipids from sediments or lyophilized microbial cells (mixed with a incinerated sand matrix) were performed at room temperature with the one phase chloroform/methanol/buffer modified Bligh & Dyer method [1]. The extracted lipids were then fractionated into neutral, glyco- and polar lipid classes using silicic acid columns [2]. The modified Bligh and Dyer room-temperature solvent extraction has been used for direct quantification of lipids to estimate viable microbial biomass, community composition, and nutritional status *in situ* for over 15 years. Despite the fact that lipid biomarker analysis is the most satisfactory single, quantitative, and comprehensive analysis of the *in-situ* microbial community viable biomass, community composition, and physiological status it is not universally utilized as this method is labor-intensive and slow. Separation and fractionation needs 2-3 working days by a skilled lab assistant, so we tried to replace them by an automated extraction method.

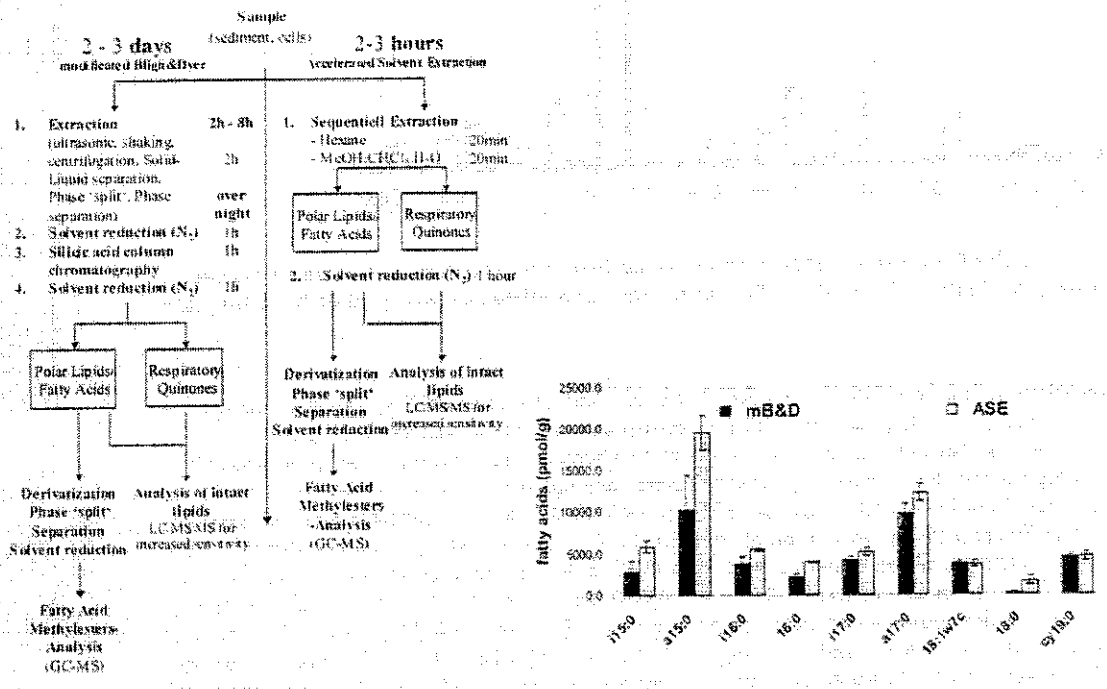

Figure 1: Comparison of lipid extraction and fractionation from sediment samples (left) performed by Bligh&Dyer method and automated Accelerated Solvent Extraction. Extraction efficiency and reproducibility of fatty acid extraction with both methods estimated with *Bacillus subtilis* cells (right)

Accelerated Solvent Extractor (ASE 200, DIONEX) was used for a sequential extraction in a manner reported previously, where ASE recovered 3-fold and 2-fold more PLFA from *Bacillus* sp. and *Aspergillus niger* spores than did the standard one-phase extraction system [3]. We advanced the system to an automated separation of neutral and polar lipid biomarkers by sequential extraction. The samples were added into ASE extraction vessels (11ml or 33ml volume) and neutral lipids were extracted using hexane at 40°C and 1000psi with 10 min static time at a single extraction cycle. The polar lipid fraction was obtained with MeOH:$CHCl_3$:H_2O (2:1:0.8) at 50°C also in a single cycle which showed an greater than 90% estimated extraction efficiency (data not shown). Extracts were concentrated with a stream of nitrogen and derivatized by mild alkaline transesterification. The whole process can be automated in approximately 2-3 hours and is much faster than the conventional Bligh & Dyer method (Figure 1). The extraction of lyophilized cells from the gram-positive microorganism *Bacillus subtilis* showed an increased recovery of polar lipid fatty acids, especially

branched chains, with increased reproducibility for the automated ASE method (Figure 1). Comparable positive results were also obtained for the gram-negative *Escherichia coli* and the eukaryote *Saccaromyces cerevisiae* as single culture extracts or as previously mixed in a tri-culture (data not shown). The neutral lipid fractions were successful analyzed for quinones as biomarkers for respiratory chains in these organisms and the respective growth conditions by LC/IS/MS/MS. The advantage of measuring these compounds will be discussed below.

The ASE method was also tested with environmental sediment samples from different depths from core liners, drilled at a highly BTEX contaminated test field (Zeitz, Germany). Samples (30 - 40g) from 3 different wells and 4 depths (4.5m to 29 m) were extracted with ASE and fatty acids recovered from the polar lipid fraction of all depths. The residues of 12A/00 (7.4 - 7.6m depth) and 14A/00 (10.5 - 10.8m depth) were extracted additionally by the Bligh & Dyer method, which revealed extraction efficiency greater 97% for ASE (Figure 2).

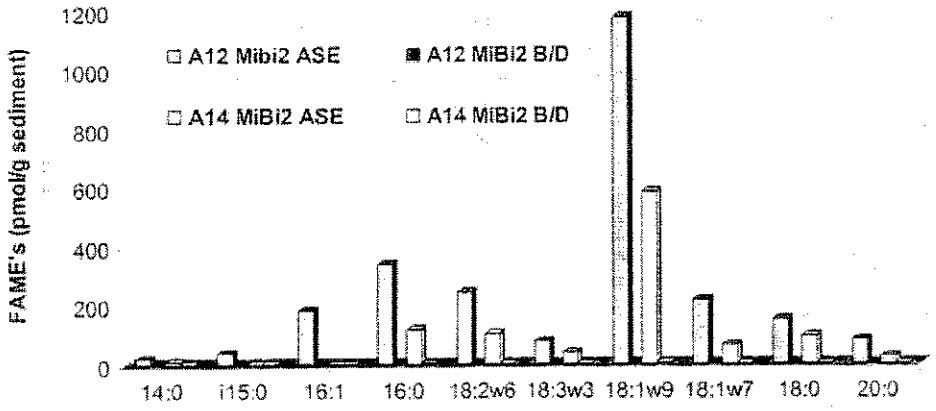

Figure 2: Sediment samples from two welliner were extracted first with ASE followed by an extraction of the residue according to Bligh & Dyer (B/D). The ASE extraction efficiency was greater 97% for both samples.

The viable biomass at different depths, estimated as polar lipid fatty acids (PLFA), showed changes in a range of 300 to 7000 pmol fatty acids g^{-1} sediment. PLFA decreased with depth in well 9B/00 (Figure 3).
The biomass and PLFA (microbial community) is determined by chemical and (hydro)geological parameters of the subsurface. Geological profiles revealed lignite-clay layers ranging from 13.4 to 14.9 and 17.7 to 21.7m depth in well 14A/00 and from 11.3 to 12.1 and 18.5 to 25m in well 9B/00. Benzene concentrations of sediments at the depths shown in Figure 3 (from top to bottom) were 30, 14, 0.08 and 4.2mg kg^{-1} sediment (9B/00) and < 0.05mg kg^{-1} sediment (14A/00). Groundwater derived from these wells had an approximately neutral pH and were nearly anoxic (redox potential around –200mV in 14A/00 and between –230 to –350mV in 9B/00). Benzene concentrations in this groundwater were 33 and 4µg l^{-1} in 9B/00 and 14A/00, respectively. The total organic hydrocarbon was 1.7 (9B/00) and < 0.1mg l^{-1} (14A/00), indicating that well 14A/00 was less contaminated with a lower content of carbon. The microbial biomass in well 14A/00 was an order of magnitude lower compared to well 9B/00 with high benzene concentrations in groundwater and sediments. Well 14A/00 showed the elevated proportion of normal saturated fatty acids (N-Sats) at the -18m layer in well 14A/00 were dominated by long-chain fatty acids (20:0 to 28:0). Saturated fatty acids with more than 19:0 carbons are indicative of plant biomass and not of microbial origin and were here extracted from compact brown coal deposit at this subsurface layer (data not shown). The PLFA of microbial origin were less than 600pmol g^{-1}. The changes in the PLFA composition (pie plots in Figure 3) are an excellent mirror for the microbial community composition in the changing sediments and properties of the subsurface.
The occurrence of polyunsaturated fatty acids in the samples from -10 to -15m core layers indicate eukaryotic microorganisms (protozoa, fungi).

The groundwater pumped from several depths in this area contains up to 0.1mg l^{-1} oxygen whereas the redox potential (see above) showed a more anoxic subsurface environment. The oxygen levels in the sediments itself are difficult to estimate. In monitoring accelerated attenuation during bioremediation processes it could be very helpful to know if there are aerobic or anaerobic environments or are micro-niches established. Ubiquinones (UQ) are a biomarker for the actual *in-situ* redox-state as Gram-negative bacteria form respiratory ubiquinones (UQ) when the terminal electron acceptor is oxygen [4]. Under anaerobic growth conditions Gram-negative bacteria may form respiratory menaquinones (MK, contains naphtoquinone nuclei) or no quinones. Gram-positive bacteria form respiratory menaquinones when grown aerobically. Knowing the isoprenoid side

chain length has important taxonomic implications. Eukaryote mitochondria contain UQ-10 (80 carbon side chain). Gram-negative bacteria contain isoprenologues from UQ-4 to UQ-14 [4]. Respiratory ubiquinones are found in concentrations of about 200 times less than the PLFA or about 0.5μmol g^{-1} cell dry weight [5]. UQ isoprenologues can be detected utilizing HPLC/ES/MS with 95.5/0.5 v/v methanol containing 1 mM ammonium acetate/H_2O on C-18 LC columns in isocratic mode. This does not suppress electrospray ionization in mass spectral detection and has Limit-of-Detection (LOD) of 75 fmoles μL^{-1} of UQ-7 and Limit-of-Quantification (LOQ) of 225 fmoles μL^{-1} using a positive ion of m/z = 197. The LOD with this single quadrupole MS system represents about 150 bacteria the size of *E. coli* growing aerobically [5]. The menaquinones (MK) are not ionizeable with an electrospray ion source (ES) and can not be measured with this method. A simultaneous measurement of UQ and MK with detection of positive ions of m/z = 197 and m/z=187, respectively with atmospheric pressure chemical ionization tandem mass spectroscopy (LC/APCI/MS/MS) was utilized. A linear detection range of about 3 orders of magnitude and detection limits lower than 1ppm for both compound classes was achieved. The quantification of the UQ and MK profiles and contents in the core samples, which were extracted in the first phase of ASE extraction as neutral lipids, are still in progress. In samples with low biomass the extractable quinones could be near or below the current detection limit. Application atmospheric pressure photionization ion source prototype from SCIEX (Canada), that expands the detection limits for quinones to approximately 1ppb, is in progress.

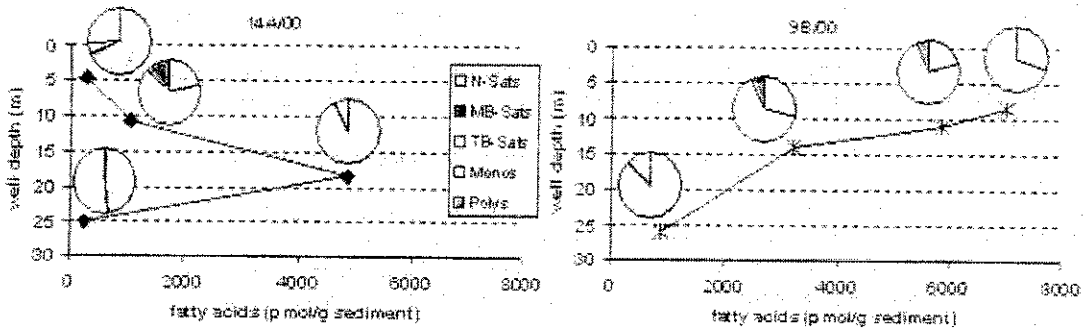

Figure 3: Sediment samples of two wellliners showed different behavior in viable biomass (expressed as pmol fatty acid g^{-1} sediment; scatter plot) and fatty acid composition (% of total FA's; pie plots) with increasing well depth. The fatty acids at –18m in well 14A/00 are dominated by long-chain monounsaturated fatty acids, which were derived from unconsolidated plant material out of an brown coal layer. The indicated fatty acid groups are: N-sats – normal saturated, MB-Sats – mid-chain branched, TB-Sats – terminal-branched, Monos – monounsaturated, Polys – polyunsaturated fatty acids.

Polar lipids can be extracted at low temperatures with the ASE method. The fatty acid chains and polar head-groups of glycerophospholipids (main polar lipids in eubacterial membranes) remain intact. These 'intact lipids' can be readily detected with LC/ES/MS/MS [6] using a neutral loss of m/z =141 and 154 for phosphatidylethanolamine (PE) and phosphatidylglycerol (PG), respectively. Tandem mass spectrometry, ES/MS/MS, provides great advantages in the structural analysis of phospholipids. 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 1 position had 20% of the abundance of the fatty acid at the 2 position of the glycerol (which offers an higher degree of specificity). Lytle et al. demonstrated limits of detection of 90 amoles/μL using a single molecular species of phosphatidylglycerol, [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 for collisionally induced dissociation by monitoring m/z = 284 negative ions [6]. That is essentially equivalent to the total phospholipids in a single *E. coli* cell providing the high sensitivity measurements necessary for aquifers with low biomass (see Figure 3).

The ASE residues can be subjected to strong acid hydrolysis followed by a third extraction recovering the ester-linked hydroxy-fatty acids of the lipopolysaccharides (LPS-HFA) of Gram-negative bacteria. Water biofilm organisms, such as *Pseudomonas*, have 3-OH 10:0 and 3-OH 12:0 [*10 and 12 carbon n- saturated fatty acid with OH 3 carbons from carboxyl] as LPS HFA in contrast to enteric pathogens, such as *Salmonella* and *E. coli*, which contain 3-OH 14:0 [7]. The rapid extraction, separation and detection procedure can be applied for analysis of biofilms generated on coupons to deliver important information to a decision tree in water resource [8]. The advantage of rapid extraction and measuring the viable microbial biomass in sediments from aquifers or filtered surface waters can be increased by recovering DNA remaining in the initial extraction residue [9] so that

specific bacteria can be identified off-line. Further extractable target compounds are diglyceride and poly-hydroxyalkanoate as biomarkers for non-viable biomass and nutritional status of the microbial community.

CONCLUSIONS

The application of sequential accelerated solvent extraction coupled to automatable HPLC/ES/MS/MS analysis yields both DNA (for rDNA and specific genes) and lipid biomarkers for quantitative and specific analyses of extant microbial communities in a system that is potentially automatable and more sensitive and comprehensive. The system is useable for aquifer sediments and microbial biomass from biofilms on coupons or filters.

ACKNOWLEDGEMENTS

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