# REVIEW OF PHA AND SIGNATURE LIPID BIOMARKER ANALYSIS FOR QUANTITATIVE ASSESSMENT OF *IN SITU* ENVIRONMENTAL MICROBIAL ECOLOGY

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**Abstract:** Signature lipid biomarker analysis (SLB) provides quantitative analysis of the viable microbial biomass with indications of the community composition and nutritional/physiological status. SLB is based on extraction of the membrane lipids, fractionation into lipid classes, derivatization, and analysis by gas chromatography/mass spectrometry (GC/MS). The analysis is not dependent on the recovery and subsequent growth of the microbes but samples the lipids of the extant *in situ* community. Poly β-hydroxyalkonate (PHA) accumulates under conditions of unbalanced growth with sufficient terminal electron acceptor and carbon source but without all the components necessary for cell division. Changes in the ratio of PHA to phospholipid ester-linked fatty acids (PLFA) are detected in disturbance of anaerobic estaurine sediments and in grazing of epiphytic detrital microbial growth. Unbalanced growth occurs in the rhizosphere but not the rhizoplane and in subsurface sediments. Shifts in the PHA/PLFA ratio characterise effective bioremediation of pollutants and can be useful in the quantitative assessment of toxicity in streams and soils.

**Key Words:** signature lipid biomarkers (SLB), phospholipid fatty acids (PLFA), non-culturable viable biomass, *in situ* microbial biomass, nutritional/physiological status.

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## Introduction

The analysis of microbial communities *in situ* presents a significant challenge. Classical methods based on culture or microscopy are inadequate (White, 1995). Unfortunately often less than 1% of the microbes that can be detected in stained microscopic preparations can be cultured. Staining microbes in environmental samples like soils can be difficult as many are attached to soil granules and may be hidden. Agents that release attached microbes are often selective and do not release them quantitatively. The morphology of the microbes does not often reflect the function or activity so very little insight into the community structure or nutritional status is possible. Measurements of metabolic processes are complicated by the facts that most microbes in the soil are inactive, but poised for activity when nutrients appear. Adding labeled substrates to determine rates of metabolic activity induces major disturbance artifacts giving much higher rates than actually exist in the environment. This is possibly best exemplified in studies of the deep subsurface microbiota where oxygen and inorganic carbon are found in groundwater with a ground water age of greater than $1.1 \times 10^5$ years. Measurements of metabolic activity based on isotope incorporation experiments by the microbes in subsurface sediments were $10^3$ to $10^6$ times greater than the geochemical evidence would predict. The metabolic activities by the subsurface microbiota indicate growth rates in centuries (Phelps et al. 1994) and subsurface microbes often accumulate PHA.

## Methods

A solution to the quantitative detection of microbes in the environment is in signature lipid biomarker analysis (SLB). Every living cell is surrounded by a lipid membrane. These lipids are quantitatively extracted from the microbiota *in situ* with a one-phase chloroform- methanol-aqueous buffer solvent system (White et al. 1979), the lipids fractionated into neutral lipids, glycolipids, and phospholipids with silicic acid chromatography (Gehron et al. 1982), and derivatized and analyzed using gas chromatography/mass spectrometry (GC/MS) (Guckert et al. 1985, Tunlid and White 1991). Several unique classes of lipids, including steroids, diglycerides, triglycerides, respiratory quinones, poly-β-hydroxyalkanoate (PHA), phospholipid lipid fatty acids (PLFA), lipo-amino acids, plasmalogens, acyl ethers, sphingolipids, and lipopolysaccharide hydroxy fatty acids can be used as signature lipid biomarkers to characterize microorganisms or communities of microorganisms. Recently the lipid extraction has been shown to yield DNA suitable for gene probing and enzymatic amplification (Kermeyer et al. 1996).

PHA is extracted with the other lipids from environmental samples and biofilms with the one phase solvent extraction, recovered from the lipids in the acetone fraction from a silicic acid column, subjected to acid ethanolysis and analyzed by GC/MS (Nickels et al.1979, Findlay and White 1983). A rapid and convenient method for determining the ratios of the rates of synthesis of PHA and PLFA from $^{14}C$ labeled acetate utilized the one/phase extraction, fractionation on a disposable silicic acid column, recovery of the triglycerides by elution with chloroform, recovery of the PHA by elution with acetone, and recovery of the PLFA in methanol (Findlay and White 1987). The PHA fraction is fixed to cellulose disks with heat and then washed with ethanol and ether prior to radioactive counting with a scintillation spectrometer.

## Results

Phospholipids are one of the most important SLB classes, and are essential membrane components of living cells. Unlike most other biomarkers, phospholipids are typically degraded within hours following cell death (White et al. 1979). This rapid degradation of the phospholipids establishes the PLFA as ideal biomarkers for viable cells, thus, the quantification of total PLFA is an accurate measurement of living biomass and the estimates agree well with other chemical and microscopic estimates of subsurface microbiota (Balkwill et al. 1988). Because different groups of microorganisms synthesize a variety of PLFA through various biochemical pathways, the PLFA are effective taxonomic markers and can be utilized to provide insight into the community composition. PLFA analysis can provide insight into the phylogenetic relationships between organisms similar to phylogenetic analysis based on the sequence homology of 16S ribosomal RNA (Guckert et al. 1991; Kohring et al. 1994). Knowledge of specific lipid biosynthetic pathways can provide insight into the nutritional status of the microbial community as certain fatty acids, such as *trans* and cyclopropyl fatty acids, provide indications of environmental stress. Other components indicate unbalanced growth where carbon sources and terminal electron acceptors abound but a critical nutrient prevents cell division but not growth, or bioavailable phosphate is insufficient (White 1995, White et al. 1996 ). The redox level of the microbiota can be determined *in situ* by shifts in the composition of lipids in specific indicator microbes. The signature lipid biomarker techniques have been successfully applied to subsurface materials (White and Ringelberg 1996, 1997).

## PHA and Unbalanced growth

Microbial biofilms on estaurine detritus can be induced to accumulate PHA under conditions suggesting unbalanced growth such as limitation of a critical factor(s) in the presence of adequate carbon sources and terminal electron acceptors (Nickels et al. 1979). Incubating the detritus in the laboratory under conditions of low pH or acute anoxia depresses the synthesis and catabolism of PHA without change in lipid phosphate. Balanced growth induced with nutrient supplementation results in increased PLFA, decreased PHA synthesis and increased PHA catabolism resulting in a low PHA/PLFA ratio. Unbalanced growth induced by high salinity or to greater extent by the chelators found in tannin rich upland runoff results in rapid PHA accumulation, slowing of PHA catabolism and little change in PLFA. Unbalanced growth results in a high PHA/PLFA ratio. It was determined later that adding ethylenediaminetetraacetic acid (EDTA) to a growing culture prompted a rapid increase in the PHA/PLFA ratio.

## Disturbance in Estaurine Sediments

Where aerobic seawater overlies anaerobic sediments it is possible to readily demonstrate a disturbance artifact in attempts to measure metabolic activities. The process of injection of a labeled precursor into the sediment disturbs the sediment. The disturbance of injection allows oxygen to interact with the facultatively aerobic bacteria which explosively oxidize the reduced carbon in the sediments (Moriarty et al. 1985). Ratios of the rates of incorporation of $^{14}$C-acetate into PHA and PLFA proved to be an excellent way to monitor disturbance. Gently placing a trail of $^{14}$C acetate in a sediment with the greatest care produced a PHA/PLFA ratio of $3.1 \pm 0.2$ after a 10 minute incubation (Findlay and White

1984). Performing the same experiment on sediment that had just been raked with a garden rake produced a PHA/PLFA ratio of 1.7 ± 0.2 whereas sand slurried in an Erlenmeyer flask as often done in field determination of rates produced a PHA/PLFA ratio of 0.6 ± 0.2. The disturbance doubled the rates of PLFA synthesis and decreased the rate of PHA biosynthesis. This reaction to disturbance has a complex response in sediments recovered from the estuary and placed in a microcosm. In the formation of a seawater microcosm and its subsequent incubation resulted in shifts in the PHA/PLFA ratio (Findlay et al. 1990a). The disturbance caused in the making of the microcosm slightly decreased the total PLFA and decreased the PHA by half. Incubating the microcosm without disturbance showed a continued decrease in PLFA until the 4$^{th}$ hour to levels of about a third of microcosm starting level. By the 8$^{th}$ hour the PLFA level had reached slightly below the starting level and remained at that level for 120 hours. The PHA level decreased rapidly until it was undetectable by the 8$^{th}$ hour. PHA level rose to a maximum in 12 hours which was equivalent to that found in undisturbed sediment prior to microcosm formation. This was followed by a steady decrease to a level a tenth that of the highest level. Growth rates measured by acetate and thymidine incorporation showed an increase for 2 hours followed by a slow decrease. The ratio of PHA to PLFA synthesis in this period showed an initial low rate followed by a steady increase suggesting unbalanced growth. Natural sediments disturbed by feeding stingrays and enteropneust worms were complex (Findlay et al. 1990b). The ray pits showed effects of mechanical disturbance with an increase in PLFA synthesis and little change in the PHA/PLFA ratio. Ingestion and subsequent defecation of sediment by the enteropneust worms profoundly affected the biomass and growth rate of the community in the fecal castings. There was a profound decrease in biomass but a high rate of balanced growth that continued for 6 hours. At that time the microbial biomass reached a level half that of the ambient sediment. The PHA/PLFA ratio remained half that of the undisturbed sediment.

**Effects of Grazing the Estaurine Detrital Microbiota**

Estuarine gammaridean amphipods grazing at natural population density on the detrital microbial community showed an increased microbial biomass and higher rates of oxygen consumption, PHA synthesis, lipid synthesis and $^{14}CO_2$ release from pre-labeled detrital microbiota than the ungrazed detrital microbial community (Morrison and White 1980). Loss of $^{14}C$ from pre-labeled detrital microbiota showed an initial half life of 0.8 days over days 3 to 8 compared to 1.8 days for the ungrazed detritus. Rates of loss of $^{14}C$-from pre-labeled detrital microbiota polar lipids showed initial half lives of 1.2 days with grazing and 2.0 days for the ungrazed detrital microbiota. Scanning electron microscopy showed the grazed community was dominated by bacteria rather than a bacterial/fungal community seen in the ungrazed system. Grazing increases the microbial biomass, and the metabolic activities of the community with a shift to a more vigorous community typical of the initial stages of colonization. This vigorous grazed detrital microbial community has a low PHA/PLFA ratio.

**Rhizosphere and Rhizoplane**

Seeds of the rape plant, *Brassica napus*, were sterilized and planted in sterile sand. In some experiments the sand was inoculated with a consortium of bacteria recovered from the rootlets of *Brassica* with the seeds (Tunlid et al. 1985). The vessels were then incubated in the light and the seedlings recovered. The PHA/PLFA of the rhizoplane bacteria was < 0.00001. The bacteria not associated with the roots had a PHA/PLFA ratio of 6.6 ± 0.5. The rhizosphere was modeled with an "artificial" root system in which a polyvinylidene membrane with 0.22 μm pores was used as the surrogate rootlet (Odham et al. 1986). A dilute glucose/sucrose and amino acid solution that mimicked *Brassica* root secretion was allowed to flow into 100 ml sterile buffer at rate of 0.5 ml/h and the buffer was inoculated with a gram-negative bacteria isolated from the rhizosphere of *Brassica*. A biofilm formed on the membrane which contained organisms in which the PHA/PLFA ratio decreased from 1.8 in the inoculum to 0.6 in the biofilm and 0.00002 in the bacteria recovered from the buffer. This model clearly did not parallel the response in the rhizosphere in defining the PHA/PLFA ratio of the microbiota.

**Subsurface Microbiota**

Subsurface microbiota can be recovered from subsurface sediments with excellent assurance that the microbiota represent the extant microbiota and not contaminants introduced during the drilling and sample recovery program (Lehman et al. 1995, Colwell et al.1992, Phelps et al. 1989). Samples recovered from the Bucatunna clay (near Pensicola, FL) aquitard 410 m below the surface showed a PHA/PLFA ratio of 3.6 ± 0.3 (White et al. 1983). A shallower clay aquifer from Fort Polk, LA contained microbiota with a PHA/PLFA ratio of 0.13 ± 0.2 with almost twice the viable biomass. Clay surface soils contain microbiota with PHA/PLFA ratios of 0.8 ± 3.0 (Ringelberg et al. 1988). In general the lower the permeability the higher the PHA/PLFA ratio. Transmission electron micrographs of subsurface microbes often show extensive intracellular PHA granules (Ghiorse and Balkwill 1983).

**Unbalanced Growth in Phytoremediation**

The presence of a xenobiotic has also been shown to affect both microbial community composition and physiological status (Smith et al. 1986). The presence of about 30 ppm trichloroethylene (TCE) in topsoils where both a legume *Lespedeza intermidis*, (bush clover) and *Pimus taeda* (Loblolly pine) were growing, induced a shift in the rhizosphere soil communities of both plants (Anderson et al. 1995). In addition to a significant increase in the biomass of the legume rhizosphere microbiota, a significant increase in the proportion of terminally branched saturated PLFA (Gram-positive bacteria), was apparent. Although no significant increase in the biomass of the pine rhizosphere microbiota was detected, a significant increase in the percentage of monounsaturated PLFA, (suggestive for Gram-negative heterotrophic bacteria), was measured. The two plants and their associated below ground rhizosphere soil microbiota showed different responses to the presence of the contaminant. Both plants did, however, show a similar physiological response to the TCE presence with an increase in the ratio of PHA/PLFA from < 0.0002 to 7.2 ± 2.4 for the legume rhizosphere and from <0.0002 to 5.9 ± 4.0 for the pine. This

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