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Quantitative Comparison of the *in situ* Microbial Communities in Different Biomes

D. C. White*[1, 2,3], D. B. Ringelberg[1], & R. J. Palmer[1]

[1]*Center for Environmental Biotechnology, University of Tennessee, 10515 Research Drive, Suite 300, Knoxville, TN 37932-2575, USA*
Environmental Sciences Division, Oak Ridge National Laboratory, Oak Ridge, TN 3783
[3] *Department of Microbiology, University of Tennessee, Knoxville, TN 37932-2575*

INTRODUCTION

A system to quantitatively and comprehensively define microbial communities in different biomes requires the application of non-traditional methodology. Classical microbiological methods, that are successful with infectious disease, have severe limitations for the analysis of environmental samples. Pure-culture isolation, biochemical testing, and/or enumeration by direct microscopic counting or most probable number (MPN) are not well suited for the estimation of total biomass or the assessment of community composition within environmental samples. These classical methods provide little insight into the *in situ* phenotypic activity of the extant microbiota since several of these techniques are dependent on microbial growth and, thus, select against many environmental microorganisms which are non-culturable under a wide range of conditions. It has been repeatedly documented in the literature that viable counts or direct counts of bacteria attached to sediment grains are difficult to quantitate and may grossly underestimate the extent of the existing community [1-5]. The traditional tests provide little indication of the *in situ* nutritional status or for evidence of toxicity within the microbial community.

A more recent development, known as the MIDI Microbial Identification System (Microbial ID, Inc., Newark, DE), measures free and ester-linked fatty acids from isolated microorganisms. Microbial ID, Inc. has commercialized the MIDI system worldwide and sells a comprehensive database. Bacterial isolates are identified by comparing their fatty acid profiles to the MIDI database which contains over 8000 entries. The utilization of this system for identification of clinical isolates has been remarkably successful; however, the application of the MIDI system to the analysis of environmental samples has significant drawbacks. The MIDI system was developed to identify clinical microorganisms and requires their isolation and culture on trypticase soy agar at 27°C. Since many isolates are unable to grow at these restrictive growth conditions, the system does not lend itself to identification of some environmental organisms.

A more applicable methodology for environmental microbial analysis is based on the liquid extraction and separation of microbial lipids from environmental samples, followed by quantitative analysis using gas chromatography/mass spectrometry (GC/MS) [1-5]. Several

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unique classes of lipids, including steroids, diglycerides (DG), triglycerides (TG), respiratory quinones (RQ), poly β-hydroxyalkanoate (PHA), phospholipid lipid fatty acids (PLFA), lipo-amino acids, plasmalogens, acyl ethers, sphingolipids, and lipopolysaccharide hydroxy fatty acids (LPS-OHFA) can be used as signature lipid biomarkers (SLB) to characterize microorganisms or communities of microorganisms.

Phospholipids, one of the most important SLB classes, are essential membrane components of living cells. Unlike most other biomarkers, phospholipids are typically degraded within hours following cell death. 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. Because different groups of microorganisms synthesize a variety of PLFA through various biochemical pathways, the PLFA are effective taxonomic markers. PLFA analysis can provide insight into the phylogenetic relationships between organisms similar to phylogenetic analysis based on the sequence homology of 16S ribosomal RNA [6,7]. 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 cyclopropyls, provide an indications of environmental stress. PLFA and other biomarkers have been successfully extracted from environmental matrices such as soils and sediments providing a means for direct *in situ* measurements.

The SLB methodology is not widely utilized for microbial characterizations since lipid extraction, fractionation and derivatization procedures are time consuming and labor intensive. Current SLB extraction procedures also require extensive attention to detail in the purification of solvents, reagents and glassware. The interpretation of the SLB analysis requires an extensive understanding of a widely dispersed data-base. All of these factors have prevented the widespread usage of this quantitative definition of soil and sediment microbiota in long-term ecological studies. SLB analytical techniques can be difficult for graduate students or investigators interested in ecological interactions involving soil or sediment microbiology to take up as a task, secondary to their primary ecological interest. Commercial ventures (such as Microbial Insights, Inc., Knoxville, TN) have made the SLB analysis available to such users though its highly trained staff and the singular dedication to this labor intensive process.

METHODS

Lipids are extracted with liquid organic solvents and then separated into fractions using column chromatography. Fractions containing SLB may be further separated by thin layer or liquid chromatography and individual SLB determined using analytical instrumentation. The analysis was developed for quantitatively assessing microbial communities (bacteria, fungi, protozoa, and metazoa) in slimes, muds, soils, filter retentates, bioreactors, and sediments [1-5,8]. Specifically, the signature lipid biomarker methodology provides a quantitative means to measure: 1) viable microbial biomass, 2) microbial community composition, and 3) community nutritional status.

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. The viable microbes have an intact membrane which contains phospholipids (and PLFA). Cellular enzymes hydrolyze and release the phosphate group within minutes to hours following cell death [9]. The lipid remaining is diglyceride (DG). The resulting diglyceride contains the same signature fatty acids as the original phospholipid, at least for some period of time. Consequently, a comparison of the ratio of phospholipid fatty acid profiles to diglyceride fatty acid profiles provides a measure of the viable to non-viable microbial abundance and composition. A study of subsurface sediment showed that viable biomass as 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 conducted acridine orange direct counts (AODC) [10].

2). **Community Composition** The presence of certain groups of microorganisms can be inferred by the detection of unique lipids that originate from specific biosynthetic pathways [1-5]. Consequently, the analysis of SLB classes provides a quantitative definition of the microbial community. For example, specific PLFA are prominent in *Desulfovibrio* sulfate-reducing bacteria, whereas the *Desulfobacter* type of sulfate-reducing bacteria contain distinctly different PLFA [11,12]. The analysis of other lipids such as the sterols (for the microeukaryotes--nematodes, algae, protozoa) [13], glycolipids (phototrophs, gram-positive bacteria), or the hydroxy fatty acids from the lipid A component of lipopolysaccharide of gram-negative bacteria [14,15], sphinganines from sphingolipids [16], fatty dimethyl acetals derived from vinyl ether containing plasmalogens [4], and alkyl ether polar lipids derived from the *Archae* [17] can provide a more detailed community composition analysis (Figure 1).

3). **Nutritional Status** Bacterial poly β-hydroxyalkanoic acid (PHA) [18-20] and microeucaryotic triglyceride [21] are endogenous storage lipids. The relative amounts of these compounds, as compared to the PLFA, provides a measure of the nutritional status. Many bacteria form PHA under conditions of unbalanced growth when a carbon source and terminal electron acceptor(s) is present but cell division is limited by the lack of some essential nutrient [18, 20]. Specific patterns of PLFA can indicate physiological stress [22]. Exposure to toxic environments can lead to minicell formation and a relative increase in specific PLFA. Increased conversion from *cis* to *trans* PLFA occurs in *Pseudomonas* species with exposure to higher concentrations of phenol in the absence of bacterial growth [23]. Prolonged exposure to conditions inducing stationary growth phase induce the formation of cyclopropane PLFA [4, 22, 27]. Respiratory quinone composition can be utilized to indicate the degree of microbial aerobic activity [24]. Environments with high potential terminal electron acceptors (oxygen, nitrate) induce formation of benzoquinones in bacteria in contrast to microbes respiring on organic substrates which form naphthoquinones. Some specific but useful insights come from analysis of organisms like the *Pseudomonas* species which form acyl-ornithine lipids when growing with limited bioavailable phosphate [25] while some gram-positive bacteria form increased levels of acylamino acid phosphatidylglycerols when grown at sub-optimal acid pH levels [26].

RESULTS

Quantitative comparisons of microbial viable biomass and community composition in biomes: Marine neotropical, tropical and Antarctic sandy surface marine sediments have very similar viable biomass [28] (20 to 70 nmoles PLFA/gm dry wt. corresponding to between 10^8-10^9 bacteria the size of *E. coli*/gm.). Surface sediments from the Antarctic which were permanently covered by 10 m of snow-covered ice and which were far from any nutrient inputs and deep sea (~4000 m deep) sediments contained an order of magnitude less viable microbial biomass (Figure 2). The different sandy marine sediments also contained distinct microbial community compositions (Figure 3). In Figure 3, the short terminally branched saturated PLFA are used as indicators of aerobic gram-positive and anaerobic gram-negative sulfate-reducing bacteria, the polyenoic PLFA for microeukaryotes, and the monoenoic as characteristic of gram-negative heterotrophic bacteria. The detailed analysis of these PLFA patterns, as presented in the original publications [29-32], indicated that specific differences in community compositions existed. Metabolic activities (measured with injected substrates *in situ* with as small a disturbance artifact as possible) showed the neo-tropical sediments to be at least 300-fold more active in terms of DNA synthesis from ^{3}H-thymidine incorporation rates than those in the Antarctic [31].

Surface soils tend to have a wide variation in viable biomass and community composition (Figure 4). Although the figure illustrates no obvious correlations between viable microbial biomass and community composition, those soils with the lowest biomass also tended to have the lowest proportions of polyenoic PLFA (representing the microeukaryote community). More detailed analyses of PLFA patterns show significant differences between surface and subsurface sediments and, more specifically, between soils with a high clay content and those with a high permeability coefficient [33]. A comparison of differing biomes along a vertical global transect as well as similar biomes subjected to different disturbances show great variations in viable biomass and community composition (Figure 5). Indoor air biocontaminants collected on a glass fiber from 10^3 m^3 of indoor air show inputs of human dandruff and other eukaryotic particulates which are high in normal saturated PLFA. A comparison of forest leaf litter from Oak Ridge, TN with the fine root rhizosphere of a longneedle pine grown in sand under 400 kg/ha/yr nitrogen and 720 ppm CO_2 and 40 kg/ha/yr nitrogen and ambient CO_2 [34] showed a progressive decrease in the terminally branched saturated PLFA which are characteristic of the gram-positive *Micrococci/Arthrobacter* genera. Conversely, there was a progressive increase in polyenoic PLFA which are characteristic of the microeukaryotes, in particular, the fungi. Only the litter showed a substantial percentage of the mid-chain branched saturated PLFA which are characteristic of the Actinomycetes. Surface soils near Decatur, GA supporting annual and perennial weeds showed a similar community composition to soils which supported a dimorphic community of sedges although decreased proportions of gram-positive-bacteria (terminally branched saturated PLFA) and increased proportions of microeukaryotes (polyenoic PLFA) were quantified in the "richer" dimorphic sedge supporting soils. A marine tidal salt marsh in England [35] showed the highest proportion of the branched monoenoic PLFA characteristic of sulfate-reducing bacteria. The bivalve clam, *Potamocorbula amurensis,* changed the PLFA composition of its tissue depending on whether a supply of food from a phytoplankton bloom was available or

not [36]. Periphyton from stones in an uncontaminated freshwater stream contained a PLFA pattern distinctly different from periphyton recovered from a contaminated freshwater stream. The differences were manifest in the individual components of the polyenoic (microalgae) classification [37]. Marine deep sea sediment recovered in the Pacific ocean (208°E at the Equator) contained a substantially different PLFA profile from that detected in sediment recovered from the Endeavor Ridge hydrothermal vent 47°57'N, 129° 06' W, also in the Pacific ocean. A deep subsurface Miocene lacustrine sediments recovered from 186 m below the surface in Richmond, WA contained a microbial community different from that of the surface soils located in Decatur, GA. Note that both the indoor air and the deep subsurface lacustrine sediment showed the largest proportion of normal saturated PLFA which are often characteristic of a community of low diversity.

Detection of disturbance with the SLB analysis: Problems in comparing microbial communities in different biomes can be further complicated by dynamic responses of the microbiota to disturbances. Microbial communities can show remarkable changes in community ecology as a result of disturbance. Since the SLB technique involves the separation and assay of distinctive lipid biomarkers, rates of formation from precursors or turnover during growth can be determined [38]. In a series of studies [39-41] it was shown that the addition of a labeled precursor to sediments in slurries or by injection with various degrees of disturbance resulted in progressively greater apparent metabolic rates.

Natural disturbances by benthic invertebrates (i.e. sting ray feeding) or wave action can be detected both as increased rates of incorporation of labeled precursors and/or shifts in PHA/PLFA ratios provided the gentlest methods of labeled precursor applications are utilized [41]. Utilizing these methods, sedimentary microbiota have been shown to be remarkably responsive to disturbances. Microbes in sediments exist like coiled springs awaiting metabolic opportunities resulting in activities that are much greater than the actual basal rates. Activity measurements made in subsurface sediment slurries yielded metabolic rates 5-orders of magnitude greater than those which could have possibly been maintain in the subsurface as indicated by the oxygen content in the ground waters at the depth sampled and due to minimal recharge rates [42]. Since estimates of carbon dioxide and methane production by soil and benthic microbes are important in calculations of greenhouse effects, these disturbance artifacts can introduced serious errors.

Toxicity Assessments: The SLB analysis can be used to provide a quantitative measure of toxicity. At Oak Ridge National Laboratory a system based on multi-trophic levels and multi-species in periphyton (the slime covering rocks in streams) was developed to monitor disturbances involving pollution abatement in streams [37]. Unglazed tiles or rocks were incubated in an unpolluted stream for a month and then transferred to three sites in a local creek containing different levels of toxicity as estimated by responses to *Ceriodaphnia* and *Pimephales pomelas* larval assays. The ratio of rates of PLFA synthesis (membrane) to PHA (storage lipid) synthesis showed an increase in the ratio as the system became more highly impacted. A multivariate analysis of the PLFA profiles indicated there were three dominate profile patterns containing

signature PLFA of: i) diatoms which were associated with the least impacted location, ii) of green algae which were associated with the most impacted location, and of iv) a diverse PLFA profile which was associated with the intermediately impacted location. The experiment was repeated three times at different seasons with identical results. Similar to identical results were observed in two other separate studies [43, 44].

Soil and Sediment SLB Applications: Despite the complexities of sedimentary microbiota, the SLB technique allows for *in situ* determinations into sedimentary processes. By sampling just ahead and just behind echinoderm sand dollars, *Mellita quinquiesperforata,* as they slowly moved through the sediments, it was possible to show with SLB that the feeding was selective for specific protozoans and bacteria [45] while diatoms passed through the sand dollar alimentary tract intact. By excluding top predators (fish and crabs) from an estuary, changes in the sedimentary microbiota were induced [46]. There was an immediate overgrowth of the opportunistic polychaete *Mediomastus ambiseta* and a marked decrease in polyenoic PLFA characteristic of nematodes and algae. There was also a concomitant increase in bacteria, especially anaerobic sulphate-reducing bacteria, as indicated by increases in specific PLFA which was closely followed by an increase in the proportion of linoleic acid which is characteristic of bacteriovorus protozoa. The SLB analysis provided a quantitative description of the following sedimentary process: the overgrowth of polychaetes which grazed on the nematodes and algae thus allowing the bacterial overgrowth to occur, in particular anaerobic growth resulting from the change in bioturbation which decreased the rate of aeration of the sediment.

Intrusions of pollutants into subsurface materials can result in remarkable shifts in microbial community compositions. The SLB analysis has been used to quantify these shifts in soil columns gassed with methane and air [47]. The viable biomass was shown to increase concomitant with an increase the proportions of PLFA characteristic of gram-negative heterotrophs and type II methane-oxidizing bacteria. Similar results were observed in an *in situ* study where subsurface sediments perfused with methane, propane, and air showed shifts in community composition that correlated with trichloroethylene (TCE) biodegradation [48, 49]. The active biodegradation of petroleum hydrocarbons in subsurface sediments also resulted in an increase in the total viable microbial biomass, but the community shift was toward only the aerobic heterotrophs. The community shift was also associated with decreased ratios of SLB biomarkers indicative of stationary phase and unbalanced growth and increases in the proportion of benzoquinone respiratory quinones indicating aerobic electron transport activity [50]. SLB analysis has also been successfully applied to microcosms isolated from contaminated environments which allow for greater manipulations.

Soil microbial biodiversity is still considered to be largely undefined [51] although the application of SLB has yet to be applied on a significant scale. Phospholipid ester-linked fatty acid (PLFA) analysis, a component of the SLB analysis, has been recognized as a method with powerful potential in soil quality determinations [52] and has been used in several investigations of effects of perturbations on the soil microbiota [53-57]. Community analysis by PLFA allows

for the detection of some but not all microbial species and some but not all physiological groups. Consequently, we have expanded the SLB analysis to include other classes of lipids and have insisted that all components analyzed be identified by mass spectrometry [1-5, 23].

Rhizosphere SLB applications: The roots of all the plants we have examined, thus far, have shown simple PLFA patterns. Thus, the detection of a rhizosphere microbiota by analysis of the steroid and PLFA profiles has been possible. The effects of adding bacteria isolated from the rhizosphere of the rape plant (*Brassica napus*) to surface sterilized seeds showed the bacteria attached to the roots after the plants had grown contained no evidence of starvation (increases in the ratio of cyclopropane PLFA to the monoenoic PLFA precursors) or toxicity (increases in the ratio of monoenoic PLFA in the *trans* configuration relative to PLFA with the *cis* configuration) [58]. The bacteria not associated with the roots showed high ratios of PHA/PLFA, indicating they were in a state of unbalanced growth. Wood chip compost can be manipulated so that it can either be conducive or suppressive to the growth of the damping off fungal pathogen, *Rhizoctania* [59]. The attached rhizosphere microbiota of cucumber seedlings (*Cucumis sativus* L.) in compost which suppressed the infection with *Rhizoctania* showed: i) a lower viable biomass (about 60% of the PLFA associated with the roots of conducive compost grown plants), ii) lower proportions of short chain terminally branched PLFA characteristic of gram-positive bacteria like *Arthrobacter*, iii) decreased proportions of tuberulostearic acid characteristic of *Actinomyces*, and iv) higher proportions of the monoenoic PLFA *cis*-vaccenic acid (16:1ω7c) and a branched 17 carbon monoenoic (i17:1ω8c) both of which are formed by a bacterial biosynthetic pathway characteristic of gram-negative bacteria. The root-associated gram-negative bacteria showed greater evidence of starvation (increased cyclopropane to precursor monoenoic PLFA ratio) in the suppressive compost as compared to the conducive compost. A series of experiments with long needle pines grown in sands and exposed to ambient and ambient + 720 μmol/mol CO_2 with either 40 or 400 kg/ha/yr reduced nitrogen (G. B. Runion and H. H. Rogers, Auburn University) showed significant increases in the viable rhizosphere biomass under the greater reduced nitrogen load. The greatest increase was observed when both high reduced nitrogen and elevated CO_2 were present.

SUMMARY

SLB analysis provides a quantitative and comprehensive basis for comparing the microbiota of different biomes. The ability to quantitatively define shifts in viable microbial biomass, community composition, and nutritional/physiological status as a result of disturbance and perturbation make the application of this technology ideal for monitoring *in situ* bioremediation and in providing a multi-species, multi-trophic level toxicity assessment.

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FIGURE LEGENDS

Figure 1. Signature Lipid Biomarker Analysis.

Figure 2. Viable biomass determined as total PLFA in sandy marine sediments. From left to right: Sand seagrass bed Moreton Bay, Queensland, Australia, top 2 cm of sediment at a 1 m depth, (n = 20) [29]; Tidal sea grass estuary in the Gulf of Mexico off Florida, top 1 cm of sediment at a 1 m depth (n = 23) [29]; Cape Evans East McMurdo Sound, Antarctica, top 1 cm of sediment at a 35 m depth + 1.5 m of ice (n = 3) [30, 31]; Arthur Harbor West McMurdo Sound Antarctica top 1 cm of sediment at a 26 m depth + 2.3 m of ice; Benthic sea bottom top 1 cm (n = 4) [30, 31]; Scotian Rise, North Atlantic 40° 27'N, 62° 20'W at 4820 m depth (n = 48) [32]; Scotian Rise, North Atlantic 40° 27'N, 62° 20'W at 4820 m depth, 9-10 cm below the sediment surface (n = 20) [32].

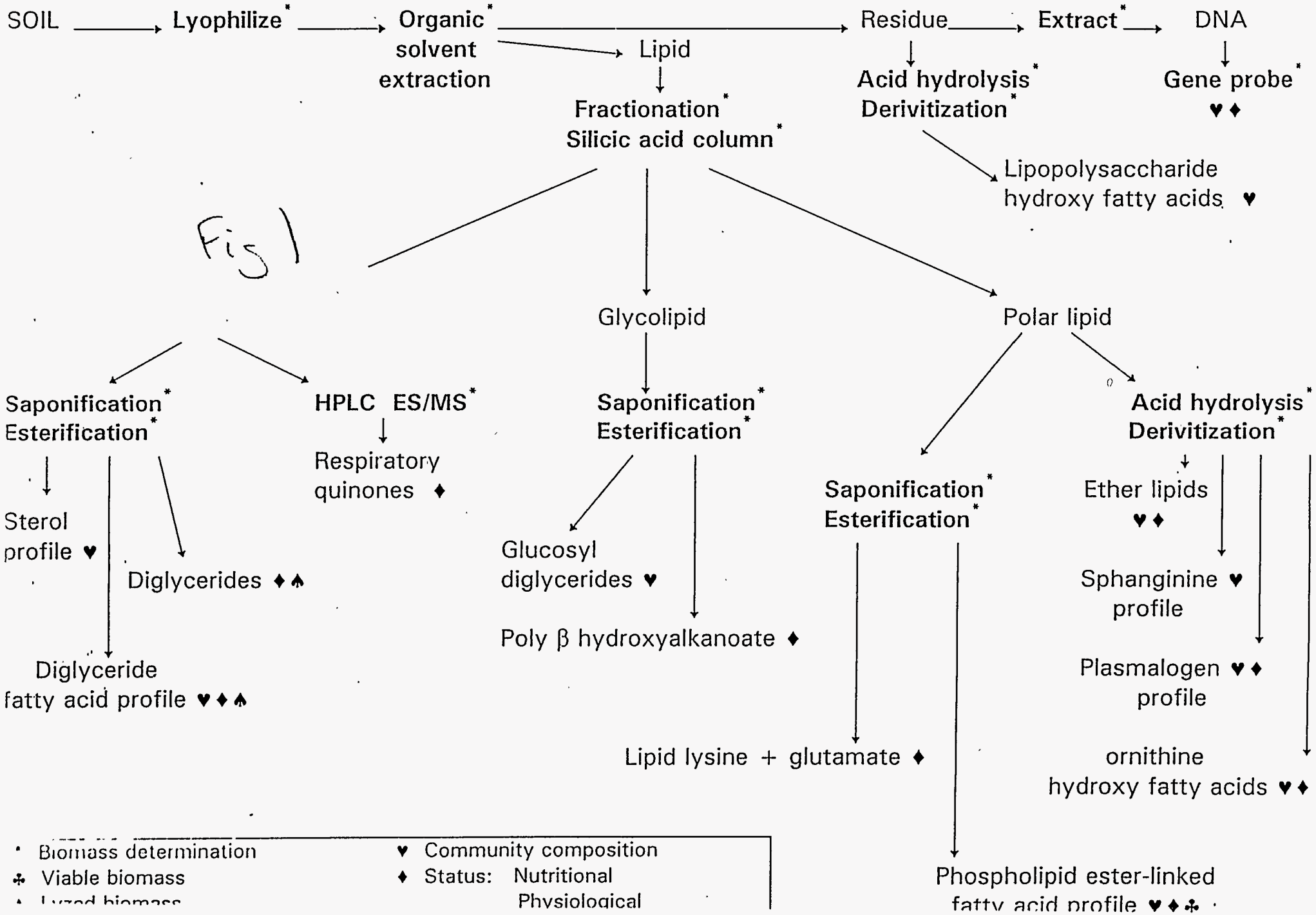
Figure 3. Community composition of the sandy marine sediments of Figure 2 based on the proportions of terminal branched PLFA (found largely in gram-positive and sulfate-reducing gram-negative bacteria), polyenoic PLFA (found primarily in the photosynthetic and heterotrophic bacteria), the monoenoic PLFA (found in gram-negative heterotrophic bacteria), and the saturated PLFA (found in all organisms) [29-32].

Figure 4. Viable biomass and community composition from the SLB analyses of surface soils across the USA. Surface soils were recovered from Aiken, SC [33]; Decatur, GA; Oak Ridge, TN [34]; Upper Peninisula, MI [60]; Parachute Creek, CO; Cerro Negro, NM; Idaho Falls, ID; and Richland, WA.

Figure 5. Viable biomass and community composition from the SLB analysis of selected biomes. Indoor air biocomtaminants collected on a glass fiber from 10^3 m^3 of indoor air; Forest leaf litter from Oak Ridge, TN; Fine root rhizosphere from longneedle pines grown in sand with 400 kg/ha/yr nitrogen and 720 ppm CO_2 (+) compared to the rhizosphere of pines grown with 40 kg/ha/yr nitrogen and ambient CO_2 (-) [34]; Surface soils supporting annual and perennial weeds (A/P) versus soils supporting a dimorphic community of sedges (D) near Decatur, GA; Tidal Salt marsh in England [35], Clam *Potamocorbula amurensis* gills feeding on a phytoplankton bloom (+) versus no bloom (-) [36]; Periphyton from freshwater streams (-) compared to the same system impacted by hypochlorite after 30 days of exposure (+) [37]; Marine deep sea sediment near Easter Island (208°E at the Equator); Endeavor Ridge hydrothermal vent sediment at 47°57'N, 129° 06' W in the Pacific ocean; and deep subsurface lacustrine sediment 186 m below the surface at Richmond, WA.

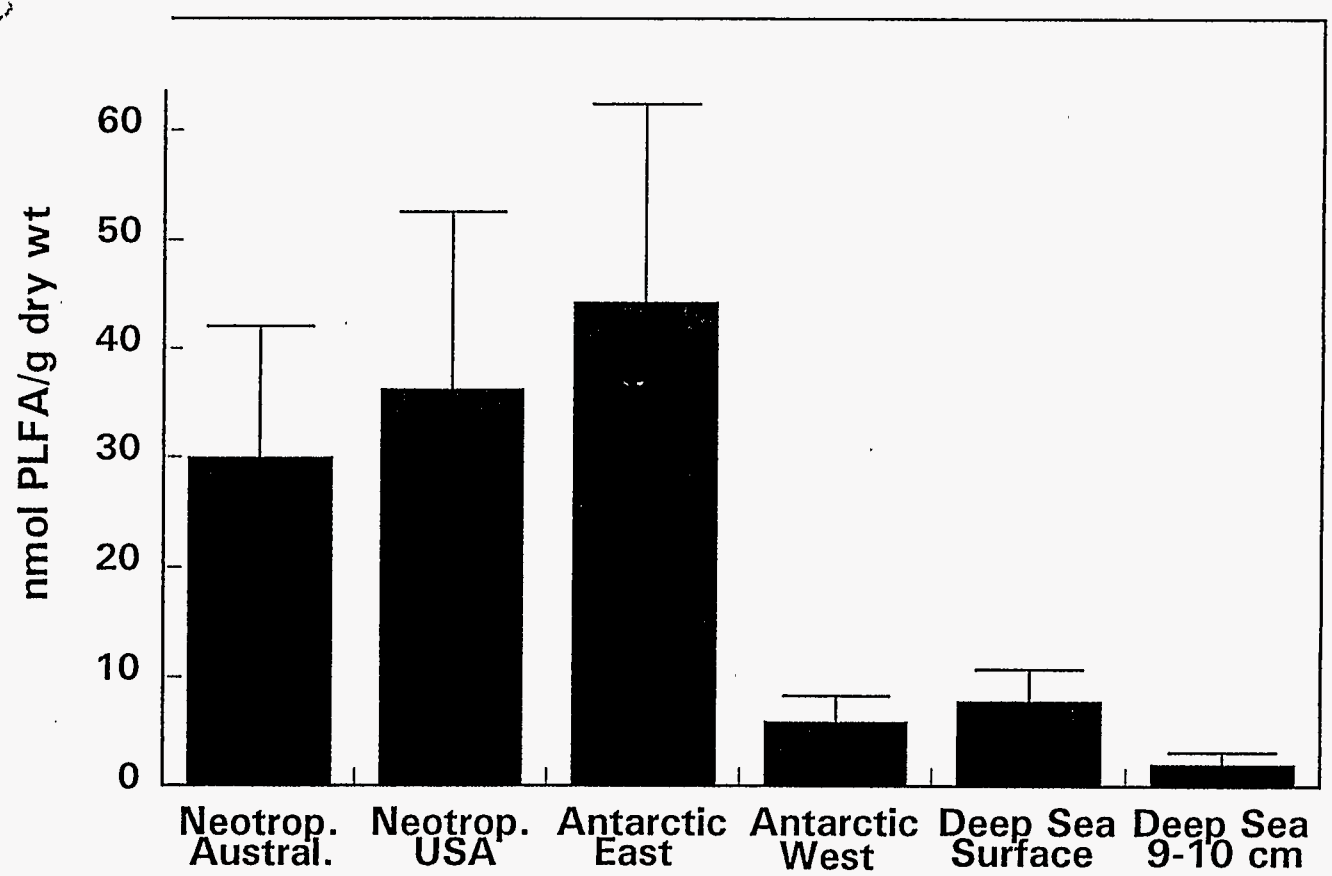
DISCLAIMER

Figure 1. SLB Analysis

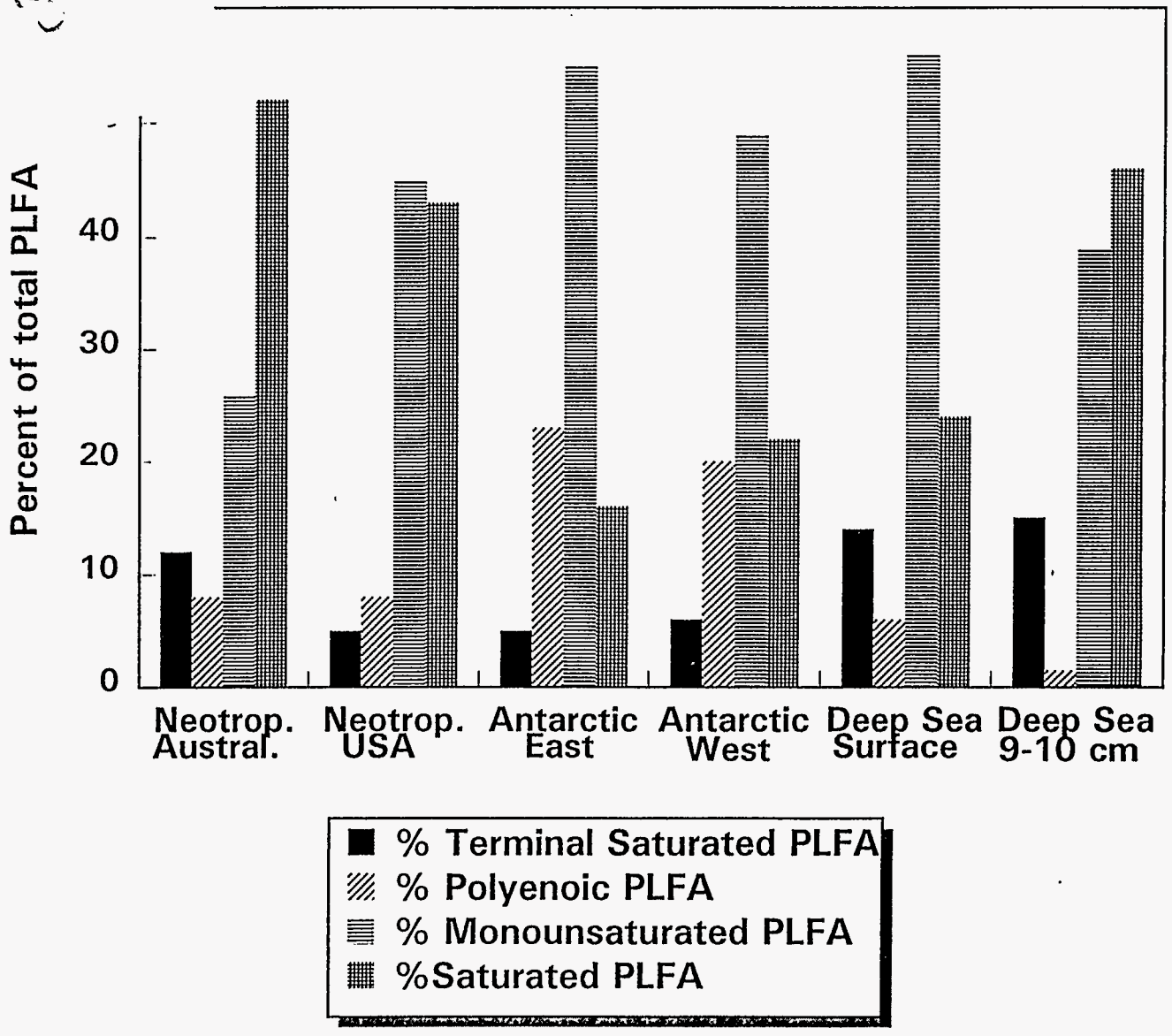


Viable biomass from several worldwide marine sediments

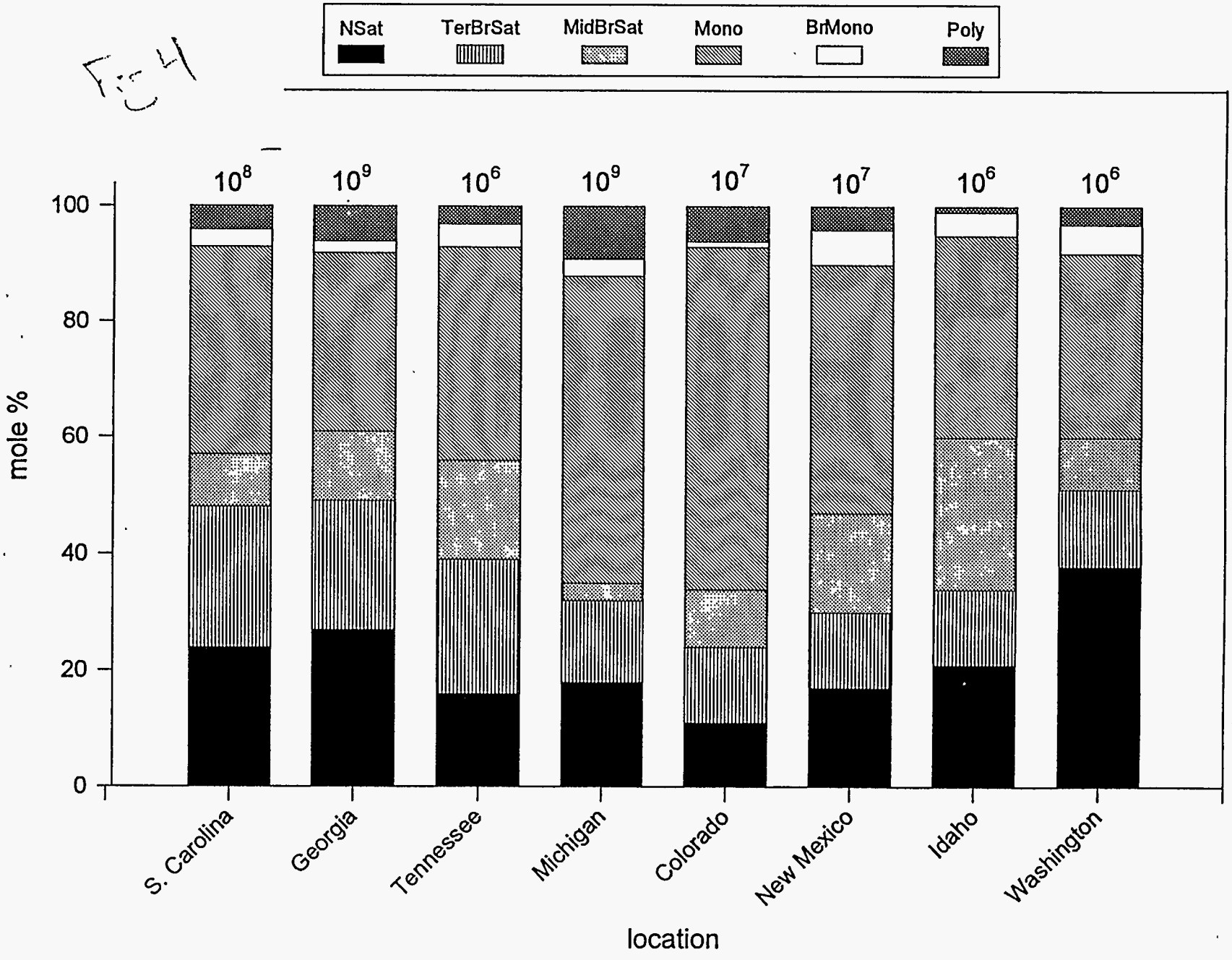
Fig 2



Community Structure



ırface Soil Microbial Community Composition



Biome Microbial Community Composition