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Chemical and Molecular Approaches for Rapid Assessment of the Biological Status of Soils

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Introduction

Soil, although largely ignored by the general public, is a most precious resource. The sustainability of our civilization depends on its 'quality'/'health'. Soil health has been defined as the 'continued capacity of a soil to function as a vital living system within ecosystem and land-use boundaries to sustain biological productivity, promote the quality of air and water environments, and maintain plant, animal and human health' (see Doran and Safley, Chapter 1 this volume). Maintenance of soil health is the key to sustainable agriculture (Doran *et al.*, 1994) and the multitude of interacting soil physical, chemical, and biological properties that affect soil quality/health have been clearly related to the potential fitness or capacity to produce healthy and nutritious crops (Doran *et al.*, 1994). Is it possible to develop a rapid, automatable analytical system with a potential for field utilization to correlate the ecology of the soil microbiota to the soil quality/health?

Many soil biologists know in their hearts that the unseen microbes in their ecosystem must be very important. Microbes tend to be ignored because they are difficult to study. The classical methods of isolation and culture of microbes

 Biological Indicators of Soil Health and Sustainable Productivity (eds C. Pankhurst, B. Doube and V. Gupta)

that are taught in most microbiology courses have been enormously successful in clinical medicine where isolation of specific pathogens establishes the diagnosis of disease and the *in vitro* sensitivities to antimicrobials can often predict the success of treatments. The success of cultural isolation and subsequent characterization in public health microbiology has carried over into examinations of the soil microbiota. Well developed rapid methods of identification are now in widespread usage with isolated and culturable microbes.

Microbiologists have traditionally related the biomass of microbiota in a soil to the number of cells detected by viable count. While this is sufficient for monocultures of microorganisms which are readily grown in the laboratory, it is not satisfactory for soils and sediments where the viable counts often represent 0.1 to 10% of the cells detected using acridine orange direct counts (AODC) or biochemical measures (Olsen and Bakken, 1987; Albrechtsen and Winding, 1992; Bååth *et al.*, 1992). In soil and subsurface sediments, the problems are intensified as the heterogeneous distribution of the microbial community can significantly increase the variability of the accuracy of biomass determination by AODC. Direct microscopic counts present problems when autofluorescence of sediment and clay granules obscures detection of bacteria *in situ*. This problem is often ameliorated by inducing detachment of the microbes from soil granules with solutions containing multicharged ions such as polyphosphate followed by recovery on membrane filters for microscopic counting. However, based on signature lipid biomarker analysis, there is evidence that in subsurface sediments, the de-adherence of the microbes is selective and often not quantitative (White and Ringelberg, unpublished results). Also, with *in situ* direct counting of bacteria at densities of less than 10^4 cells g^{-2} dry weight of sediment, the accuracy of the counts is very low and the error large, even when counting 20 fields with adequate numbers of cells and replicate subsampling (Montagna, 1982). 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 from morphometrics.

Measurements of metabolic processes are complicated by the fact that most microbes in the soil are inactive, but poised for activity when nutrients appear. Adding labelled substrates to determine rates of metabolic activity induces major disturbance artefacts yielding much higher rates than those that actually exist in the environment. This is best exemplified in studies of the deep subsurface microbiota where oxygen and inorganic carbon are found in groundwater with a groundwater age of greater than 1.1×10^5 years (Phelps *et al.*, 1994). Measurements of metabolic activity based on isotope incorporation experiments in subsurface sediments were between 10^3 and 10^6 times greater than those that geochemical evidence would predict. The metabolic activities of the subsurface microbiota indicate growth rates on the order of centuries (Phelps *et al.*, 1994). Furthermore, microbes may be metabolically active even though they are not culturable by traditional methods. Classical microbial tests provide little indication of the nutritional status of microbes or the toxicity which can affect meta-

bolic activities and can be crucial to studies of the ecology of microbial communities.

This review will focus on four non-traditional methods for rapid assessment of the biological status of soils. These four methods are: (i) the Biolog system, which measures metabolic activities of isolates or the microbial community on a suite of substrates; (ii) the MIDI fatty acid pattern analysis of isolated culturable microbes; and (iii) two methods that focus on *in situ* analysis of components directly extracted from the soils. The two *in situ* methods are signature lipid biomarker analysis (SLB), and DNA based analyses.

Biolog

The Biolog system (Biolog, Inc., Hayward, California) correlates patterns of substrate utilization with identification of bacterial species. The system is comprised of a 95 well plate of which each well contains a different sole carbon source and nutrients. The 96th well contains neither carbon source nor nutrient and, as such, is a control. Oxidation of the carbon source is indicated by reduction of 2,3,5-triphenyl tetrazolium chloride into a coloured insoluble formazan. After a period of incubation, the plate is scanned colorimetrically with a reader and the resulting patterns compared to a comprehensive data base (Boucher and Savageau, 1977). Microbial communities have also been submitted to this form of sole-carbon source testing producing a community level physiological profile (CLPP) (Garland and Mills, 1991). Unfortunately, this form of analysis requires a transparent carbon-free inoculum. Although many groundwater samples can be assayed directly, soils and subsurface sediments need to be blended, extracted with sodium pyrophosphate, incubated without added carbon and nutrients for 24 hours with agitation, and have the supernatant flocculated with a mixture of calcium and magnesium salts before assay (Lehman *et al.*, 1995). With CLPP, colour development patterns are monitored daily for 7 days. Raw absorbance data is converted to net absorbance after subtraction of the control. An average value for each well is calculated by averaging all 95 responses for all samples and replicates and plotting against time. A classical sigmoid growth curve results. A reading corresponding to the maximum of the linear colour development is chosen and this value for each of the 95 components is normalized by division by the average well colour development for each plate reading.

Principal components and other statistical analyses have been performed on these responses (Garchow *et al.*, 1993; Garland and Mills, 1994; Zak *et al.*, 1994; Lehman *et al.*, 1995). The results provide both a pattern of the presence or absence of positive responses as well as a comparison of the same substrates used by different communities. Garland and Mills (1994) explored temporal trends within four sets of inoculated hydroponically grown plants (each with a different nutrient system), demonstrating consistent changes in the distribution of microbial communities. In particular, they showed a convergence in

community-level response and a decrease in the rate of change over time which suggested that the rhizosphere microbial community tended to approach a stable state. Zak *et al.* (1994) showed that by using the Biolog system it was possible to detect considerable variation in the substrate utilization of microbial communities of soils taken from six different plant communities whereby differences in functional diversity were found to be dependent on the class of carbon sources. By application of this methodology on subsurface sediments, Lehman *et al.* (1995) showed that subsurface microbial communities had a much stronger preference for acetate, Tween 40 and amino acids than did surface or groundwater microbial communities and that the subsurface community was clearly different from those communities present in drilling muds and surface sediments.

The community level physiological profile analyses were also found to agree with the phospholipid ester-linked fatty acid (PLFA) analyses in the differentiation of surface and drilling muds from subsurface microbial communities. Utilizing responses of isolates, model communities of known composition, and soils, Haack *et al.* (1995) showed that non-linear substrate oxidation rates were delayed by dilution of the inoculum. Patterns of substrate utilization were reproducible for model communities but the extent of substrate utilization was not. In addition, replicate soil communities from the same pots varied considerably.

MIDI

A highly successful bacterial identification system has been developed by Microbial ID, Inc., Newark, Delaware (known as the MIDI system). The MIDI system measures fatty acids (FAME) from both membrane lipids and lipopolysaccharide. It is most effectively applied to isolated microorganisms cultured with standard media under standardized conditions. 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 this MIDI database, which contains over 8000 bacteria (Welch, 1991). 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 25°C. Since many isolates are unable to grow at these restrictive growth conditions, the system does not lend itself to identification of many environmental organisms. The isolation and culture of individual organisms for MIDI analysis is somewhat time consuming and expensive so it is rarely applied to the analysis of soil microbiota (Haack *et al.*, 1994). Cavigelli *et al.* (1995) did apply this form of FAME analysis to hydrolysed lipid extracts in defining microbial biomass and community composition in soils. Since many of the FAMEs are common to different microbes, a straight forward interpretation of the results

from these soil extracts was complicated. With the application of a principal components analysis it was possible to define similarities and differences between differing soil communities. When a 'signature' FAME for a particular taxa was detected, the analysis became more specific. Comparisons with organisms which grew on R2A agar recovered from the same soils showed distinct differences indicating that many significant organisms were not cultured. However, the patterns of some of the isolates were recognizable in the soil community FAME profiles.

MIDI is most effective with analysis of cellular components on the relatively clean samples derived from isolated bacterial, fungal, and actinomycete cultures. Although the great majority of cellular fatty acids in bacteria are ester-linked in the phospholipids, some are ester-linked in the lipopolysaccharide within the Gram-negative classification of bacteria. Amide-linked fatty acids in the lipids or lipopolysaccharides will not be detected by the MIDI procedure. By applying the whole cell saponification approach used in this type of analysis, specificity is lost due to the lack of knowledge as to where the FAMEs reside in the microbial cell.

Phospholipid Ester-linked Fatty Acid

Increasing the specificity of the lipid analysis by extracting the intact lipids, fractionating them into major lipid classes (usually neutral lipids, glycolipids and polar lipids) and derivatizing by use of a mild alkaline transesterification has provided an analytical system for the direct analysis of soil lipid extracts with improved selectivity. When polar lipid fractions were examined for patterns of fatty acids it proved possible to readily detect differences in soil microbiota as a result of different agricultural practices (Zelles *et al.*, 1992), with exposure to alkaline dust (Bååth *et al.*, 1992), and with differences in and exposure to heavy metal dust (Frostegård *et al.*, 1993a,b). Shifts in viable microbial biomass and community composition as a result of exposure to pollutants in the subsurface (Smith *et al.*, 1986) were shown to reflect an increase in the viable biomass and in increased proportions of PLFA characteristic of Gram-negative heterotrophs, and in type II methane-oxidizing bacteria in soil columns gassed with methane and air (Nichols *et al.*, 1987). Addition of different fatty acid substrates to anaerobic sediment cores induced marked and expected changes in the bacterial community structure (Parkes *et al.*, 1992). Subsurface sediments perfused with methane, propane, and air showed shifts in community structure which correlated with trichloroethylene (TCE) biodegradation (Ringelberg *et al.*, 1988; Cox *et al.*, 1994). The polar lipid fatty acid analysis was not, however, sufficiently specific to reflect differences in agricultural soil management practices in a long-term farming system trial (Wander *et al.*, 1995). In this study, variation between sample replicates obscured any significant differences related to differing soil practices. Trends in the analysis did show that organic cover cropped

soils contained the largest biomass and most heterogeneous communities whereas organic-manure amended soils showed the least amount of heterogeneity but the highest level of metabolically active organisms. The phospholipid ester-linked fatty acid analyses (PLFA) of an inoculated soil has been compared to the FAME analyses (MIDI system) of the individual isolates that were used to inoculate the soil (Haack *et al.*, 1994). This study showed that not all the isolates in the community could be detected in the composite profile since there was overlap of 'signature' PLFA. Although the PLFA analysis did not allow for the detection of all of the species present in the soil, it did provide an overview of the community as a whole and did allow for detections based on specific physiological traits.

PLFA profiles of isolates have been shown to shift with temperature, substrate sources, and growth conditions (Lechevalier, 1977). However, when bacteria are grown in communities under natural conditions their cells contain a relatively constant proportion of their biomass as phospholipids (White *et al.*, 1979, 1980). Experience has shown that organisms added to communities, or that are induced to proliferate in that community by manipulations, can maintain a sufficiently characteristic PLFA profile to be recognized (White, 1988). The marked changes in PLFA induced in some microbes in monoculture by shifts in the environment apparently are not detected in the environment where competition with better adapted species restricts survival of specific strains to much narrower conditions of growth.

The specificity of the lipid analysis can be increased further by use of a more comprehensive examination, namely the signature lipid biomarker (SLB) analysis. This analysis involves the assay of a larger proportion of the cellular components of the microbiota. The cellular components chosen for assessment should be reasonably labile in the microbes so that dead 'fossil' organisms can be readily differentiated from viable cells. They should be sufficiently discriminatory so various subsets of the microbial community can be determined and, if possible, should provide indications of the nutritional/physiological status of the cells.

Signature Lipid Biomarkers

The rationale for this analysis is based on the hypothesis that soil microbial community composition is a faithful reflection of the soils recent history and provides predictable effects on sustainable crop yields and other measures of soil quality/health (Tunlid and White, 1992). Waxman (1927) made a prediction that quantitative analysis of soil microorganisms could indicate the actual or potential fertility of a particular soil. Visser and Parkinson (1992) indicated that soil quality correlations be tested at three levels: the population of individual species, the community level, and at the soil ecosystem level through measures of organic matter decomposition rates, soil respiration as CO_2 efflux, and microbial biomass carbon. The community level assessment seemed most problematic

as methods based on classical microbiology with microscopic or cultural methods were wholly inadequate. The great majority of soil microbes are not culturable and their direct microscopic analysis in the soil or after attempts at quantitative release from the soil matrix are time consuming and not very effective. Domsch *et al.* (1983) summarized results of many studies using these methods and suggested various culturable microbes could be classified into those with high, medium, and low sensitivities to side-effects from agrochemicals. Nitrifiers, *Rhizobium*, actinomycetes, and rates of organic matter degradation showed the highest sensitivity to the side-effects; *Azotobacter*, total culturable microbes, ammonifiers, and aerobic nitrogen fixing capacity were the least sensitive predictors; algae, bacteria, fungi, soil respiration, CO_2 evolution, oxygen uptake, denitrification and ammonification were of medium sensitivity in predicting the side effects. None of these tests were sufficiently comprehensive in themselves to serve as the sole quantitative analysis of soil microbes needed to test Waxman's predictions. A powerful quantitative assessment method for soil microbiota has been developed over the past 20 years in our laboratory which defines the viable biomass, community composition and nutritional/physiological status of environmental microbial communities. This is based on the application of SLB analysis (White, 1988, 1993; Vestal and White, 1989; Tunlid and White, 1992).

The SLB analysis can provide *in situ* indications of starvation, exposure to toxicity, unbalanced growth, phosphate availability, microniche pH, moisture, aerobic/anaerobic metabolic balance, growth rate, and the proportion of the recently lysed (dead) microorganisms all of which provide significant insight into the phenotypic activity of the microbial community (White, 1995a). Soil and rhizosphere microbial biodiversity is still considered to be largely undefined (Aldhous, 1994), although an application of SLB analysis with its increased specificity has yet to be utilized on a significant scale. Since the SLB analysis includes other classes of lipids in addition to the PLFA, it is also very important that each of the components being analysed be verified through the use of mass spectrometry (White, 1988, 1993; Vestal and White, 1989; Tunlid and White, 1992). We have found that it is critical that each component be structurally verified and that this often entails forming different derivatives, such as that used in the confirmation of the position and configuration of double bonds (Nichols *et al.*, 1986). Often, analysis of fragmentation patterns at the front and tail of a chromatographic peak will confirm the presence of multiple components which, without mass spectrometry, would go undetected.

SLB analysis provides quantitative insight into three important attributes of microbial communities.

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 which contains PLFA. The cellular enzymes hydrolyse the phosphate group within minutes to hours of cell death (White *et al.*, 1979). The remaining lipid is diglyceride (DG). The resulting DG contains the same signature fatty acids as the phospholipids, allowing for a comparison of the ratio of phospholipid fatty acids to diglyceride fatty acids (viable to non-viable microbes). The ratio of diglyceride fatty acids/PLFA was shown to generally increase with depth in the soils and subsurface sediments of the semi arid northwest of the USA (White and Ringelberg, 1996). A careful study of subsurface sediments showed that the 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 done acridine orange direct counts (AODC) (Balkwill *et al.*, 1988). Conversion factors between lipid analysis and cell numbers have been discussed (White *et al.*, 1995).

Community structure

The SLB analysis provides a quantitative definition of the microbial community structure. Specific groups of microbes often contain unusual lipids (White, 1988; 1993; Vestal and White, 1989; Tunlid and White, 1992). For example, specific PLFA are prominent in the hydrogenase-containing *Desulfovibrio* sulphate-reducing bacteria, whereas the *Desulfobacter* type of sulphate-reducing bacteria contain distinctly different PLFA (Edlund *et al.*, 1985; Dowling *et al.*, 1986). Hierarchical cluster analysis of PLFA patterns have shown that relationships between species of isolated methane-oxidizing and sulphate-reducing bacteria almost exactly parallel the phylogenetic relationships based on sequence similarities of the 16S rRNA (Guckert *et al.*, 1991b; Kohring *et al.*, 1994). Hierarchical cluster analyses of PLFA patterns of total microbial communities can be used to quantitatively define relatedness between different microbial communities. This has been done with deep subsurface sediments in which the microbial communities of permeable strata were shown to be distinct from surface soil, clay aqualude, and drilling fluid communities (White *et al.*, 1991, 1996; White and Ringelberg, 1995, 1996).

The analysis of other lipids such as sterols (for the microeukaryotes such as fungi, algae and protozoa) (White *et al.*, 1980), glycolipids (phototrophs, Gram-positive bacteria), or hydroxy fatty acids from the LPS Lipid A (Gram-negative bacteria) (Parker *et al.*, 1982; Walket *et al.*, 1993; Ringelberg *et al.*, 1994) can provide an even more detailed community structure analysis.

Nutritional status

The formation of poly β-hydroxyalkanoic acid (PHA) in bacteria (Nickels *et al.*, 1979; Findlay and White, 1983) or triglyceride in microeukaryotes (Gehron and

White, 1982) relative to the PLFA provides a measure of nutritional status. For example, 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 (Guckert *et al.*, 1986). Exposure to toxic environments can lead to minicell formation and a relative increase in specific *trans* monoenoic PLFA compared to the *cis* homologues. It has also been shown that for increasing concentrations of phenol toxicants, the bacteria *Pseudomonas pudita* forms increasing proportions of *trans* PLFA (Heipieper *et al.*, 1992). Some useful insights come from the analysis of organisms like the *Pseudomonas* species which have been shown to form acyl-ornithine lipids when grown with limited bioavailable phosphate (Minnikin and Abdolrahimzadeh, 1974). Some Gram-positive bacteria form increased levels of acylamino acid phosphatidylglycerols when grown at sub-optimal acidic pH levels (Lennarz, 1970). Aerobic growth conditions with high-potential electron donors induce facultative Gram-negative heterotrophic bacteria to form respiratory benzoquinones (White, 1988, 1993; Vestal and White, 1989; Tunlid and White, 1992). Prolonged exposure to conditions sustaining stationary growth phase induce the formation of cyclopropane PLFA (Guckert *et al.*, 1986; Tunlid and White, 1992). The distribution of organisms showing these specific responses in most soils is sufficiently universal that they can be utilized to define the conditions in the microniches they occupy. This has been established by manipulating environments and then subjecting them to SLB analysis (White, 1988).

There are additional insights that can be gained by further extending the SLB analysis but it is important to differentiate between free fatty acids, ester-linked fatty acids, amide linked fatty acids and vinyl ether linked fatty components. The alkaline hydrolysis used in the analysis of FAME or PLFA does not liberate the amide linked fatty acids that could be present in lipopolysaccharide or other extractable lipids. The use of acid hydrolysis is almost always necessary for the quantitative recovery of amide linked fatty acids.

DNA

Two approaches to DNA extraction from soil have been utilized, namely direct extraction of the whole soil and indirect extraction of a crude bacterial fraction of the soil. Of these two methods the direct approach yields approximately one order of magnitude more DNA than the indirect approach (Ogram *et al.*, 1987; Steffan *et al.*, 1988). Torsvik *et al.* (1994) determined that soils contained thousands of species of microbes by determining the heterogeneity of DNA. Ritz and Griffiths (1994) applied a total DNA community hybridization to the analysis of shifts in the soil community structure whereby similarity was determined by measuring the relative extent to which DNA from one community

cross-hybridized with that from another (Lee and Fuhrman, 1990). The method showed great potential for the analysis of shifts in the isolated bacterial fraction of the soil although the more heterogeneous DNA isolated from the whole soil proved too complex to analyse using this technique (Ritz and Griffiths, 1994).

Once protocols were established for the recovery of DNA from soils (Ogram *et al.* 1987; Steffan *et al.*, 1988), the technology of gene probing was applied toward the detection of specific genes in the environment (Ogram and Sayler, 1988; Steffan *et al.*, 1989; Sayler and Layton, 1990; Sayler, 1991; Sanserverino *et al.*, 1994). Specific genes for specific enzymes can be detected by DNA hybridization, although the presence of a gene does not necessarily indicate its activity. Theoretically, it is possible to detect one gene in a sample (if no inhibitors are present and all controls are negative) by enhancing the sensitivity of nucleic acid analysis via the use of the polymerase chain reaction (PCR). Specific mRNA can often be recovered from environmental samples as again pioneered in the Sayler laboratory (Flemming and Sayler, 1993) and the community structure can be determined by recovery of the gene coding for ribosomal RNA (rDNA) (Ward *et al.*, 1992). Highly conserved portions of the small and large subunit rRNA sequences can be used as universal probes with kingdom specific sequences used to determine the proportions of the eukaryotes, Archaea and Bacteria, and more variable sequences used as species specific probes (Ward *et al.*, 1992).

One of the major problems encountered when utilizing these powerful molecular techniques for analysis of environmental samples is the lack of quantitative recovery of nucleic acids from various complex environmental matrices. DNA extraction depends on quantitative lysis and it has been repeatedly demonstrated that components of the soil microbiota are differentially lysed by chemical and physical methods (Moré *et al.*, 1994). Gram-positive bacteria such as actinomycetes, various cocci, spores and yeasts may be particularly difficult to lyse and subsequently extract (Johnson, 1991). In addition, fractionation of the microbial biomass prior to DNA extraction introduces a bias towards those organisms which are easily dislodged from the soil resulting in non-representative sampling. Polyphenols, tannins, iron chelates and clays make the recovery, amplification, and detection of DNA (by DNA:DNA hybridization) difficult. Where enzyme action is required in the analysis, as in the amplification by Tac polymerase in the PCR or restriction digestion, the concurrent extraction of enzyme inhibitors is a serious problem. For example, although PCR is theoretically capable of detecting one target molecule in a sample, impurities present with DNA extracted from soil can lower the sensitivity of PCR by 10^4–10^8 cells g^{-1} of soil (Picard *et al.*, 1992). Despite the large efforts of several laboratories, quantitative recoveries have been restricted to environments lacking in tannins, clays or enzyme inhibitors such as thermal spring microbial mats or pelagic seawater communities (Ward *et al.*, 1992). Where RNA (either mRNA or rRNA) is the target molecule, the recovery problems are magnified due to the presence of RNA degrading enzymes (Flemming *et al.*, 1992).

The DNA probe analysis offers powerful insights because of its exquisite

specificity for the detection of functional genes for enzyme processes and their control, and for the rDNA (coding for the bacterial small and large subunit rRNA and microeukaryote small and large subunit rRNA) for organism identification at the kingdom, family, genus or species levels. However, at present there is a lack of knowledge regarding the effects of all parameters involved in DNA analysis which precludes obtaining truly quantitative results. As such, care should be taken when interpreting results of community structure analysis solely based on nucleic acid analysis. Although several methods have been developed for DNA extraction there is no guarantee that they extract all of the naturally occurring DNA or RNA. For example, sampling bias can result from cell recovery procedures which are selective against specific types of microorganism (Hahn *et al.*, 1990), while failure to lyse all members of a microbial community also negatively impacts the DNA extraction (Ward *et al.*, 1992). Furthermore, during enzymic processes such as PCR other restrictions such as primer choice (Cariello *et al.*, 1991), PCR conditions (Mayerhans *et al.*, 1990; Cariello *et al.*, 1991; Weller *et al.*, 1991; Reysenbach *et al.*, 1992), PCR drift and selection (Wagner *et al.*, 1994) and cloning (Cariello *et al.*, 1991) are introduced again biasing results. PCR selection occurs when a reaction favours certain members of a gene family (i.e. separate reactions produce results skewed towards the same genes) while PCR drift is a bias that occurs as a result of random events in the early cycles of the reaction. PCR drift can be countered by carrying out several reactions and pooling the products to reduce any skewness of the results caused by the independent random PCR events (Wagner *et al.*, 1994). In addition, PCR selection can be countered by carrying out the reaction using the smallest possible number of amplification cycles (Wagner *et al.*, 1994). Recent evidence has shown that genome size and the number of rRNA genes (rDNA) present per cell will impact on the species represented in clonal libraries of environmental samples (Farrelly *et al.*, 1995). Unless these variables are known for the species present it will be impossible to truly quantify the number of species present in a sample from such libraries.

Another major disadvantage of PCR when applied to community structure analysis and organism identification is the formation of chimeric sequences, that is hybrid molecules made up of two different 16S rDNA types (Leisack *et al.*, 1991). The formation of chimera introduces risk when using PCR to examine microbial community structures. As such, critical analysis of sequences using a computer program such as Check Chimera (Olsen *et al.*, 1992) is vital to any interpretation of the data. These factors should all be taken into account when interpreting nucleic acid data in the absence of corroborating analyses.

Recovery of DNA from Lipid Extraction

Recent evidence indicates that the lipid extraction used in the SLB analysis also liberates cellular DNA (Kehrmeyer *et al.*, 1995). Over 99% of ^{32}P-labelled DNA added to soil was recovered in the residue and aqueous phase of the lipid

extraction. Over 50% of the gene *nahH-lux* added in intact *Pseudomonas fluorescens* to the soil recovered by the standard techniques utilized in the Sayler Laboratory (Ogram *et al.*, 1987) was recovered in the aqueous phase of the lipid extraction (Kehrmeyer *et al.*, 1996). The DNA recovered from the lipid extraction was amplified successfully by PCR. The SLB analysis can now be expanded to also provide DNA for gene probing and enzymatic amplification on the same samples. The powerful, quantitative assessment method developed over the past 20 years by our laboratories to define the viable biomass, community structure and nutritional/physiological status of environmental microbial communities based on signature lipid analysis can be expanded to include defining the proportions of specific genes or microbial species based on the extracted DNA.

Concomitant DNA/lipid analysis readily provides quantitative recoveries independent of the ability to isolate or culture the microbes. Whereas the actual biomass can be calculated from the lipid analysis, the gene probes enable comparisons of specific proportions of the community to the more general universal, bacterial, archaeal and 'eukaryote' sequences in the rDNA. The second advantage of the combined SLB/DNA analysis comes from the fact that detection of a functional gene shows only the potential for its activity. The lipid analysis provides an indication of physiological status reflective of the activity indicated in the detection of the enzyme(s) by gene probe analysis. Most genes are not expressed (White, 1995a). Since the SLB analysis provides *in situ* indications of a number of physiological traits, the analysis provides a basis by which a better prediction of the metabolic activity of the genes detected by probe analysis can be made. The concomitant SLB and DNA gene probe analysis of soils and rhizospheres should provide a powerful new analysis system for determining the ecology and community dynamics of soil microbiota. The signature lipid biomarker analysis is both quantitative and provides insights into the nutritional/physiologic status of a microbial community. The addition of a DNA extraction into the technique greatly extends its specificity which will make the utility in toxicity assessment and the determination of 'how clean is clean' much more rational and scientifically defensible. An analysis of currently available signature biomarkers for soil microbiota is illustrated in a diagram of the extractions, fractionations, acid, and alkaline hydrolysates with detection/identification by GC/LC/MS in Fig. 15.1.

Application of SLB Analysis to the Rhizosphere Microbiota

One application of the SLB analysis compared the effects of adding bacteria isolated from the rhizosphere of the rape plant (*Brassica napus*) to surface sterilized seeds of the same plant, and then compared the resulting root systems post germination and growth (Tunlid *et al.*, 1985). From these studies it was shown that the PLFA of sterile roots are very simple which allows for the presence of bacteria to be easily determined. When incubated in sterile sand, elaborations of

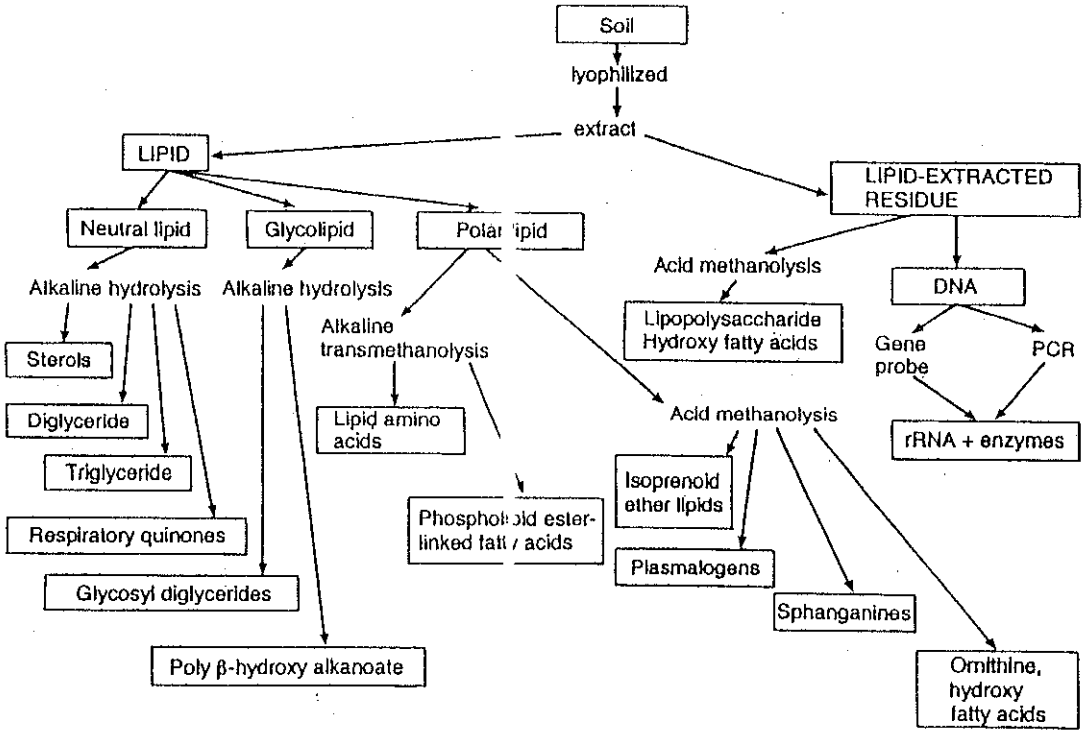

Fig. 15.1. Diagram of the combined SLB and DNA analysis method. The lipid is extracted and fractionated into neutral lipids, glycolipids and phospholipids on silicic acid columns. After acid of alkaline hydrolysis (or methanolysis) the components are derivatized and analysed by GC/MS. The lipid-extracted residue is extracted for DNA recovery and acid hydrolysed and extracted to recover covalently bound lipids.

the roots greatly stimulates the growth of bacteria. A comparison of the nutritional status of the bacteria attached to the roots with those not associated with the roots showed that the rhizosphere bacteria 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 in the *cis* configuration) whereas the non-rhizosphere associated bacteria showed high ratios of PHA/PLFA indicating a state of unbalanced growth (growth without cell division). The effects of exudates, like those from roots, on the nutritional status of attached and unattached bacteria has been demonstrated in a model with a filter apparatus which supplies nutrients through the filter. The stimulation of a biofilm on the membrane surface with starving bacteria in the bulk phase was demonstrated (Odham *et al.*, 1986).

The effects of increased temperature induced shifts in the rhizosphere microbiota (Zac *et al.*, 1995). In addition, it has been shown that exposure of plants to increased CO_2 increased the total rhizosphere microbiota associated with the fine root mass without affecting the community composition in white oaks exposed for 3 years in nitrogen limited forest soils in open topped enclosures (Ringelberg *et al.*, 1996). With SLB we showed wood chip compost can be manipulated so that it can either be conducive or suppressive to the growth of the damping off fungal pathogen *Rhizoctonia* (Tunlid *et al.*, 1989a). The attached rhizosphere microbiota of cucumber seedlings (*Cucumis sativus* L.) in compost which suppressed the infection with *Rhizoctonia* showed a lower viable biomass (about 60% of the PLFA associated with the roots of conducive compost grown plants), lower proportions of the short terminal branched PLFA characteristic of Gram-positive bacteria like *Arthrobacter*, decreased proportions of tuberculostearic acid PLFA characteristic of *Actinomycetes*, and higher proportions of the monoenoic PLFA *cis*-vaccenic acid, (16:1ω7c), including a branched 17 carbon monoenoic PLFA, (i17:1ω8c) all formed by a bacterial biosynthetic pathway characteristic of Gram-negative bacteria. These 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. The root-associated microbiota had at least a 5-fold greater biomass than the substrate in both suppressive and conducive composts. It proved possible to generate suppressive conditions in conducive compost by growing cucumbers with seeds dipped in cultures of the bacterium *Flavobacterium balustinum* 299. Rhizosphere microbiota recovered from the plants grown in conducive compost exposed as seeds to the *F. balustinum* 299 showed a similar pattern to those in suppressive compost by exhibiting high levels of *cis*-vaccenic acid and the iso-branched 17 carbon monoenoic PLFA characteristic of the bacterium. We have shown differences in sterols and PLFA profiles between 3 and 4 year old oaks grown in the same forest soil. The 4 year old oaks were grown from acorns and inoculated with *Pisolithus tinctorius* in soil-free mix and then planted in the enclosures and 3 year old trees were transplanted as seedlings into the enclosures as nursery stock.

A series of experiments with long needle pines grown in sand and exposed to ambient, ambient + 360 μmol mol^{-1}, and ambient + 720 μmol mol^{-1} CO_2, with either 40 or 400 kg ha^{-1} $year^{-1}$ reduced nitrogen show significant increases in the viable rhizosphere biomass when grown with high reduced nitrogen (G.B. Runion and H.H. Rogers, Auburn University). The greatest increase was observed when both high reduced nitrogen and elevated CO_2 were present. Changes in the rhizosphere microbial community structure were detected. The principal components most influential in the separation between treatments are those associated with the fungal mycorrhiza (White, 1995b).

Detection of Disturbance with SLB Analysis

Since the SLB technique involves the separation and assay of distinctive lipid biomarkers, rates of formation from precursors or turnover during growth may be determined (White and Tucker, 1969). Radioactive or mass labelled precursors are added to the sediment and the incorporation or metabolic activity determined at timed intervals after isolation of the product. Adding the labelled precursor to sediments in slurries, or by injection with various degrees of disturbance resulted in progressively greater apparent metabolic rates (Findlay *et al.*, 1990a,b).

Natural disturbances by benthic sediment processing invertebrates, sting ray feeding, or wave action on tidal flats can be detected both as increased rates of incorporation of labelled precursors and/or shifts in PHA/PLFA ratios if the gentlest methods of labelled precursor application are utilized (Findlay *et al.*, 1990a,b). With these methods, the sedimentary microbiota have been shown to be remarkably responsive to disturbances that allow metabolic activity. Microbes in sediments exist like coiled springs awaiting metabolic opportunities thereby creating opportunities for determinations of activity that are much greater than the actual basal rates. Measurements at the surface with recovered subsurface sediment slurries gave metabolic rates 5 orders of magnitude higher than could possibly maintain the oxygen measured in the ground water at that depth and at the known minimal recharge from the surface (Phelps *et al.*, 1994). Since estimates of carbon dioxide and methane production by soil and benthic microbes are important in calculations of greenhouse effects, these disturbance artefacts have possibly introduced serious errors.

Despite the complexities of the sedimentary microbiota, the SLB technique allows *in situ* determinations that provide insights into sedimentary processes. By sampling just ahead and just behind echinoderm sand dollars *Mellita quinquiesperforata* as they slowly move through the sediments processing the sand, it was possible to show with the SLB that the feeding was selective for protozoans and bacteria to some extent (Findlay and White, 1983). Diatoms passed through the sand dollar alimentary tract intact. Excluding the top predators (fish and crabs) from an estuary by caging induced changes in the sedimentary

microbiota (Federle *et al*,. 1983). There was an immediate overgrowth of the opportunistic polychaete *Mediomastus ambiseta* with markedly decreased polyenoic PLFA characteristic of nematodes and algae. There was a concomitant increase in bacteria, especially anaerobic sulphate-reducing bacteria, as detected in specific PLFA patterns. Shortly after the increase in bacteria the proportions of linoleic acid characteristic of bacteriovorous protozoa increased. The overgrowth of the polychaetes grazed the nematodes and algae which allowed the bacterial overgrowth. The change in bioturbation decreased the aeration of the sediment with an increase in the proportions of anaerobes.

Microcosms isolated from the environment they are designed to mimic are created for assessing the toxicity of xenobiotics on benthic biota. The quantitative definition of the sedimentary microbial community structure in these microcosms can be compared directly with that in the field by using the SLB technique. Experiments showed microbial biomass and community structure were detectably different. However, the degree of difference was not large and did not increase with time when compared with microcosms from a shallow, turbid, highly disturbed bay enriched with riparian runoff that is characterized by low macroscopic species diversity and high biomass (Federle *et al.*, 1986). Microcosms prepared from a more stable, higher salinity system with much greater diversity that is controlled by epibenthic predators showed great differences between replicate microcosms themselves as well as to the field, moreover, the divergence's between microcosm's increased with time (Federle *et al.*, 1986).

Utility of SLB as a Toxicity Assessment Monitor

The responsiveness of the microbiota to manipulations of the environment can provide a multi-species multi-trophic level toxicity assessment assay. At Oak Ridge National Laboratory, this multi-trophic level, multi-species assay system based on periphyton (the slime covering rocks in streams) to monitor the disturbance involved with pollution abatement in streams was used (Guckert *et al.*, 1991a). Unglazed tiles or rocks were incubated in an unpolluted stream for a month and then transferred to three sites in East Fork Poplar Creek which had different levels of toxicity as estimated by the responses to *Ceriodaphnia* and *Pimephales promelas* larval assays. After a month the tiles were recovered and a portion incubated in ^{14}C-acetate for an hour with the rest of the tiles subjected to SLB analysis. The ratio of rates of PLFA synthesis (membrane) to PHA (storage lipid) synthesis showed an increase as the system was more highly impacted. Toxicity increased the formation of membrane lipids without much effect on the storage lipids. Principal components analysis of the PLFA showed three distinct clusters in which signature PLFA of diatoms were associated with the least impacted cluster and signature PLFA of green algae were associated with the most impacted cluster. The intermediate site was intermediate. The experiment was repeated three times at different seasons with identical results.

The periphyton analysis of wastewater abatement was repeated in a different stream with identical results (Napolitano *et al.*, 1994). Outdoor artificial streams made from plastic rain gutters, seeded with tiles and over which water was pumped from ponds and the toxicant added showed a similar response (Kohring, 1994). We have proposed applying the same system of monitoring the environmental health in the subsurface by analysis of shifts in the subsurface microbes in studies sponsored by the EPA (White and Wilson, 1989). Studies are underway to test natural attenuation and the response of the subsurface microbiota in a deliberate implantation of petroleum hydrocarbons at an Air Force Base in Mississippi (Stapleton *et al.*, 1994). Active biodegradation of petroleum hydrocarbons in subsurface sediments results in increases in viable biomass, shifts to largely aerobic Gram-negative heterotrophic bacterial communities, decrease in biomarkers indicative of stationary phase growth, decrease-in PHA/PLFA ratio, and increases in the proportion of benzoquinone respiratory quinones indicative of aerobic electron transport activity (Ringelberg and White, 1992). The degree of these shifts parallel the effectiveness of the bioremediation in many cases.

These results indicate that the SLB analysis of soil microbiota is a reasonable target for defining effects of manipulations on soil quality/health and the subsequent predictions of productivity. There is ample evidence that agricultural practices such as crop rotations, residue management, fertilization, cultural and other management practices can significantly affect soil quality by changing the soil physical, chemical and biological parameters. These changes are reflected in changes in the soil microbiota (Eash *et al.*, 1994; Fauci and Dick, 1995; Karlen *et al.*, 1994; Rice and Garcia, 1994).

Application of SLB/DNA Analysis

If the SLB analysis, particularly with the added specificity of concomitant DNA gene probing is so comprehensive and useful why is it not universally applied? The principal reasons why the SLB methodology is not widely utilized for microbial characterizations are because lipid extraction, fractionation and derivatization procedures are time consuming and labour intensive. The 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 extensive understanding of a widely dispersed data-base. These factors have prevented the wide usage of this quantitative analytical definition of the soil and sediment microbiota in long term ecological studies. Signature lipid biomarker analytical techniques are just too difficult for graduate students or investigators interested in ecological interactions involving the soil or sediment microbiology to take up as a task secondary to their primary ecological interest.

Microbial Insights, Inc. (Knoxville, Tennessee, USA) has made the SLB and selected gene probe analysis commercially available to users. Currently,

intense research efforts are being aimed at producing an automated SLB analytical system to lower the cost, increase its sensitivity, selectivity and specificity by creating derivatives (Tunlid *et al.*, 1989b), and speed up the analysis with supercritical fluid extraction (Anderson *et al.*, 1993, Hawthorne *et al.*, 1992).

Acknowledgements

This work has been supported by grant DE-FG05-90ER60988 from the Subsurface Science program, administered by F.W. Wobber, grant 94UOT001S from the National Institute for Global Environmental Change, South East Regional Center, from the US Department of Energy and grants N00014-94-1-0961, N00014-94-1-0765 and N00014-93-1-1317 from the Office of Naval Research, US Department of Defense, and grants HRA 699-510-94 and WQI 699-524-94 from the National Water Research Institute, Fountain City, California.

References

Albrechtsen, H.-J. and Winding, A. (1992) Microbial biomass and activity in subsurface sediments from Vejen, Denmark. *Microbial Ecology* 23, 303–317.

Aldhous, P. (1994) Biodiversity: Ecologists draft plan to dig in the dirt. *Science* 264, 1521.

Anderson, T.R., Nivens, D.E. and White, D.C. (1993) Use of supercritical fluid extraction to obtain bacterial polar lipid fatty acids and neutral lipids from environmental samples. *93rd American Society for Microbiology General Meeting*. Atlanta, Georgia., p. 391 (Abstract).

Bååth E., Frostegård, Å. and Fritze, H. (1992) Soil bacterial biomass, activity, phospholipid fatty acid pattern, and pH tolerance in an area polluted with alkaline dust deposition. *Applied and Environmental Microbiology* 58, 4026–4031.

Balkwill, D.L., Leach, F.R., Wilson, J.T., McNabb, J.F. and White, D.C. (1988) Equivalence of microbial biomass measures based on membrane lipid and cell wall components, adenosine triphosphate, and direct counts in subsurface sediments. *Microbial Ecology* 16, 73–84.

Boucher, B.R. and Savageau, M. (1977) Generalized indicator plate for genetic, metabolic, and taxonomic studies with microorganisms. *Applied and Environmental Microbioliology* 33, 434–444.

Cariello, N.F., Thilly, W.G., Swenberg, J.A. and Skopek, T.R. (1991) Deletion mutagenesis during polymerase chain reaction: Dependence on DNA polymerase. *Gene* 99, 105–108.

Cavigelli, M.A., Robertson, G.P. and Klug, M.J. (1995) Fatty acid methyl ester (FAME) profiles as measures of soil microbial community structure. In: Collins, H.P., Robertson, G.P. and Klug, M.J. (eds) *The Significance and Regulation of Soil Biodiversity*. Kluwer Academic Publishers, Boston, Massechusetts, pp. 97–113.

Cox, E.E., Major, D.W., Acton, D.W., Phelps, T.J. and White, D.C. (1994) Evaluating

trichloroethyene biodegradation by measuring the *in situ* status and activities of microbial populations. In: Hinchee, R.E., Leeson, A., Semprini, L. and Ong, S.K. (eds) *Bioremediation of Chlorinated Polycyclic Aromatic Compounds.* Lewis Publishers, Ann Arbor, Michigan, pp. 37–49.

Domsch, K.H., Jagnow, G. and Anderson, T.-H. (1983) An ecological concept for the assessment of side-effects of agrochemicals on soil microorganisms. *Residue Reviews* 86, 65–105.

Doran, J.W. and Parkin, T.B. (1994) Defining and assessing soil quality. In: Doran, J.W., Coleman, D.C. and Bezdicek, D.F. (eds) *Defining Soil Quality for a Sustainable Environment.* Serial Publications Number 35, Soil Science Society of America, Madison, Wisconsin, pp. 221–228.

Dowling, N.J.E., Widdel, F. and White, D.C. (1986) Phospholipid ester-linked fatty acid biomarkers of acetate-oxidizing sulfate reducers and other sulfide forming bacteria. *Journal of General Microbiology* 132, 1815–1825.

Eash, N.S., Karlen, D.L. and Parkin, T.B. (1994) Fungal contributions to soil aggregation and soil quality. In: Doran, J.W., Coleman, D. C. and Bezdicek, D.F. (eds) *Defining Soil Quality for a Sustainable Environment.* Serial Publications Number 35, Soil Science Society of America, Madison, Wisconsin, pp. 221–228.

Edlund, A., Nichols, P.D., Roffey, R. and White, D.C. (1985) Extractable and lipopolysaccharide fatty acid and hydroxy acid profiles from *Desulfovibrio* species. *Journal of Lipid Research* 26, 982–988.

Farrelly, V., Rainey, F.A. and Stackebrandt, E. (1995) Effect of genome size and *rrn* gene copy number on PCR amplification of 16S rRNA genes from a mixture of bacterial species. *Applied and Environmental Microbioliology* 61, 2798–2801.

Fauci, M. and Dick, R.P. (1994) Microbial biomass as an indicator of soil quality: Effects of long-term management and recent soil amendments. In: Doran, J.W., Coleman, D.C. and Bezdicek, D.F. (eds) *Defining Soil Quality for a Sustainable Environment.* Serial Publication Number 35, Soil Science of America, Madison, Wisconsin, pp. 229–234.

Federle, T., Livingston, R.J., Meeter, D.A and White, D.C. (1983) Modification of estuarine sedimentary microbiota by exclusion of epibenthic predators. *Journal of Experimental Marine Biology and Ecology* 73, 81–94.

Federle, T.W., Livingston, R.J., Wolfe, L.E. and White, D.C. (1986) A quantitative comparison of microbial community structure of estuarine sediments from microcosms and the field. *Canadian Journal of Microbiology* 32, 319–325.

Findlay, R.H. and White, D.C. (1983) The effects of feeding by the sand dollar *Mellita quinquiesperforata* on the benthic microbial community. *Journal of Experimental Marine Biology and Ecology* 72, 1068–1074.

Findlay, R.H. and White, D.C. (1993) Polymeric beta-hydroxyalkanoates from environmental samples of *Bacillus megaterium. Applied and Environmental Microbioliogy* 45, 71–78.

Findlay, R.H., Trexler, M.B., Guckert, J.B. and White, D.C. (1990a) Laboratory study of disturbance in marine sediments: Response of a microbial community. *Marine Ecological Progress Series* 61, 121–133.

Findlay, R.H., Trexler, M.B. and White, D.C. (1990b) Response of a benthic microbial community to biotic disturbance. *Marine Ecology Progress Series* 61, 135–148.

Flemming, J. and Sayler, G.S. (1993) Quantitative analysis of naphthalene dioxygenase mRNA in polyaromatic hydrocarbon contaminated soils. *Environmental Science and Technology* 27, 1068–1074.

Flemming, J., Sanseverino, J. and Sayler, G.S. (1993) Quantitative relationship between napthalene catabolic frequency and expression in predicting PAH degradation at town gas manufacturing sites. *Environmental Science and Technology* 27, 1068–1074.

Frostegård Å., Tunlid, A. and Bååth E. (1993a) Phospholipid fatty acid composition, biomass and activity of microbial communities from two soil types experimentally exposed to different heavy metals. *Applied and Environmental Microbiology* 59, 3605–3617.

Frostegård Å., Bååth E. and Tunlid, A. (1993b) Shifts in the structure of soil microbial communities in limed forests as revealed by phospholipid fatty acid analysis. *Soil Biology and Biochemistry* 25, 723–730.

Garchow, H.L., Collins, H.P. and Klug, M.J. (1993) Characterization of whole soil microbial communities using Biolog GN microplates. *Third Annual Center for Microbiology Ecology Forum,* Michigan State University, East Lancing, Michigan, September 1993.

Garland, J.L and Mills, A.L. (1991) Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilization. *Applied and Environmental Microbiology* 57, 2351–2359.

Garland, J.L. and Mills, A.L. (1994) A community level physiological approach for studying microbial communities. In: Dritz, K., Dighton, J. and Giller, K.E. (eds). *Beyond the Biomass, Compositional and Functional Analysis of Soil Microbial Communities.* John Wiley & Sons, New York, pp. 77–83.

Gehron, M.J. and White, D.C. (1982) Quantitative determination of the nutritional status of detrital microbiota and the grazing fauna by triglyceride glycerol analysis. *Journal of Experimental Marine Biology* 64, 145–158.

Guckert, J.B., Hood, M.A. and White, D.C. (1986) Phospholipid, ester-linked fatty acid profile changes during nutrient deprivation of *Vibrio cholerae*: Increases in the *trans/cis* ratio and proportions of cyclopropyl fatty acids. *Applied and Environmental Microbiology* 52, 794–801.

Guckert, J.B., Ringelberg, D.B., White, D.C., Henson, R.S. and Bratina, B.J. (1991a) Membrane fatty acids as phenotypic markers in the polyphasic taxonomy of methylotrophs within the proteobacteria. *Journal of General Microbiology* 137, 2631–2641.

Guckert, J.B., Nold, S.C., Boston, H.L. and White, D.C. (1991b) Periphyton response along an industrial effect gradient: lipid-based physiological stress analysis and pattern recognition of microbial community structure. *Canadian Journal of Fish Aquatic Science* 49, 2579–2597.

Haack, S.K., Garchow, H., Odelson, D.A., Forney, L.J. and Klug, M.J. (1994) Accuracy, reproducibility and interpretation of fatty methyl ester profiles of model bacterial communities. *Applied and Environmental Microbiology* 60, 2483–2493.

Haack, S.K., Garchow, H., Klug, M.J. and Forney, L.J. (1995) Analysis of factors affecting the accuracy, reproducibility, and interpretation of microbial com-