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New and Current Microbiological Tools for Ecosystem Ecologists: Towards a Goal of Linking Structure and Function

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ABSTRACT.—Interest in the relationships between soil microbial communities and ecosystem functions is growing with increasing recognition of the key roles microorganisms play in a variety of ecosystems. With a wealth of microbial methods now available, selecting the most appropriate method can be daunting, especially to those new to the field of microbial ecology. In this review, we highlight those methods currently used and most applicable to ecological studies, including assays to study various aspects of the carbon and nitrogen cycles (*e.g.*, pool dilution, acetylene reduction, enzyme analyses, among others), methods to assess microbial community composition (*e.g.*, phospholipid fatty acid analysis (PLFA), denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism analysis (TRFLP), quantitative polymerase chain reaction (qPCR)) and methods to directly link community structure to function (*e.g.*, stable isotope probing (SIP)). In our discussion of these methods, we describe the information each method provides, as well as some of their strengths and weaknesses. Using a case study, we illustrate how these methods can be applied to investigate relationships between microbial communities and the processes they perform in wetland ecosystems. We end our discussion with a series of questions to consider prior to designing experiments, in the hope that these questions will help guide ecologists in selecting the most appropriate method(s) for their research.

INTRODUCTION

Over the past 20 y, the variety of methods available to ecosystem and microbial ecologists has dramatically increased. At the same time, interest in soil microorganisms and the ecosystem processes they drive has expanded. Despite this interest, a serious disconnect exists between ecosystem and microbial ecology, with these disciplines approaching the same questions from very different scales. Ecosystem ecologists focus on the landscape and regional scales at which processes are manifested, whereas microbial ecologists focus on the pore or plot scale at which underlying mechanisms can be discerned and manipulated. For example, most studies of nutrient or carbon cycling, processes well known to be mediated by microorganisms, do not measure the diversity and structure of communities driving these functions (*e.g.*, Ruckauf *et al.*, 2004; Ström and Christensen, 2007). Similarly, numerous

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microbial community studies do not directly investigate their ecosystem roles (*e.g.*, Rinklebe and Langer, 2006; DeJournett *et al.*, 2007). Failure to connect these scales may limit our basic understanding of ecosystem functioning or impede identification and/or implementation of strategies for restoration or biodiversity preservation.

Historically, microbial communities and processes have been treated as a black box, in part due to methodological limitations. Current advances in microbiological techniques have overcome many of these constraints. Our goal is to introduce those unfamiliar with microbial ecology to various techniques that can probe ecosystems both functionally and structurally. A brief discussion of ecosystem function measurements is followed by descriptions of modern biochemical and molecular tools used to examine microbial community composition and to link ecosystem functions to microbial communities. Finally, a case study of a hypothetical wetland is used to illustrate how microbial methods can be applied to address ecosystem questions.

MICROBIAL COMMUNITY FUNCTION MEASUREMENTS

Microorganisms are intricately involved in global carbon and nitrogen cycles. Many methods familiar to ecologists quantify these microbially driven processes. Although these measurements provide information about microbial activity, most do not assess which microorganisms are responsible for cycling nitrogen and carbon. Here, we provide a sub-set of methods providing ecosystem-level function information; later sections will discuss means to characterize microbial communities driving these functions and how these approaches can be linked for greater understanding.

NITROGEN CYCLE

Each step of the nitrogen cycle is regulated by different bacterial functional groups. The main processes include nitrogen fixation (fixing atmospheric N_2 into NH_3), ammonia oxidation (conversion of NH_4^+ to NO_3^-), assimilatory nitrate reduction (conversion of NO_3^- to NH_4^+), nitrogen mineralization (conversion of organic N to inorganic N) and denitrification (conversion of NO_3^- to NO, N_2O and N_2). These processes, in addition to leaching, run-off and ammonia volatilization, regulate ecosystem nitrogen fluxes. To target specific portions of the nitrogen cycle, one can use acetylene reduction to measure nitrogen fixation rates (Hardy *et al.*, 1968), stable isotope techniques to follow nitrogen transformations and fluxes (*e.g.*, ammonia oxidation and nitrate reduction, denitrification, plant and microbial N uptake rates; Jackson *et al.*, 1989; Delaune *et al.*, 1998) and chloroform fumigation-incubation to determine potential net N mineralization (Jenkinson and Powlson, 1976).

CARBON CYCLE/DECOMPOSITION

Carbon cycle and decomposition processes are intimately linked. Carbon cycling can be assessed via enzyme activity, mass loss and respiration (Sinsabaugh *et al.*, 1993; Jackson *et al.*, 1995). By calculating mass loss from litter bags, decomposition rates can be determined and microbial activity can be measured as CO_2 evolution (*e.g.*, Jackson *et al.*, 1995). In wetlands, microbial methane production and consumption also are important components of the carbon cycle and typically are measured by gas chromatography (*e.g.*, Ström and Christensen, 2007).

UNDERSTANDING THE MICROBIAL SPECIES

Before we address means for characterizing communities, we should address the units that comprise these communities—the individual species present. Microbiologists agree that

species exist (Cohan, 2002), but how these species are defined explicitly depends on the species concept applied. Historically, microbial species were classified based on characteristics such as morphology and physiology (Rosselló-Mora and Amann, 2001; Johansen and Casamatta, 2005). With the advent of molecular techniques, genetic similarity began to play an increasingly important role in defining microbial species (Rosselló-Mora and Amann, 2001). Using these characteristics, a pragmatic definition of species can be applied in which species are clusters of phenotypically and genetically similar organisms (Cohan, 2002). However, this definition may yield a conservative estimate of species, as it does not recognize the unique ecological roles certain ecotypes play in the environment. Thus, applying Cohan's (2002) ecotypic species concept, we recognize that within a given named species, there are many ecotypes and, thus, a named species may be more similar to a genus than a species (as defined by macroecologists).

ASSESSING MICROBIAL COMMUNITY COMPOSITION

Many well established methods reveal information about microbially driven ecosystem processes; however, to fully understand why ecosystem function changes occur, functional measurements must be linked to microbial community assays. Microbial cells contain a wealth of information that can be exploited to determine community structure and function (Fig. 1, Table 1). Cell membrane lipids provide an estimate of total microbial biomass and community composition. DNA-based methodologies can identify organisms, determine evolutionary relationships and estimate potential function and abundance of individual species and microbial groups. In addition, by measuring rRNA or functional gene expression, microbial activity can be assessed. Numerous methods are available for extracting microbial DNA and RNA from a suite of environmental matrices (*i.e.*, soils, sediments, water, etc.) (Miller *et al.*, 1999). However, each of these methods carries its own bias, such that the specific nucleic acid extraction method can affect which members of a microbial community are detected (Martin-Laurent *et al.*, 2001; Luna *et al.*, 2006). Therefore, it is important to apply the same nucleic acid extraction method consistently during a given experiment to prevent unwanted variation between samples. Further, care should be given when choosing a nucleic acid extraction method to ensure that the method employed allows recovery of DNA/RNA from the target organisms and purifies it such that downstream analyses such as PCR are facilitated.

Often, microbial community composition is determined by examining an entire microbial community's "fingerprint". Here, a fingerprint is the presence/absence pattern, and sometimes abundance, of microbial groups in an environmental sample; fingerprint resolution can vary from species-level (DNA-based methods in conjunction with sequencing) to group-level (*e.g.*, bacteria, fungi; cell membrane-based methods). Microbial communities can be compared among environmental samples using the same multivariate statistics as community ecologists studying macro-organisms (*e.g.*, Feris *et al.*, 2003; Cordova-Kreylos *et al.*, 2006; Webster and Bourne, 2007). These methods can indicate if a treatment or perturbation changes overall community composition, specific groups or individual taxa. Frequently used methods include hierarchical classification measures such as cluster analysis; indirect gradient analysis methods such as principal components analysis, correspondence analysis and non-metric multidimensional scaling; and direct gradient analysis methods such as canonical correspondence analysis. Authors should be aware that different types of data are more or less appropriate for the various analyses based on model assumptions. For example, principal components analysis is not robust with datasets that are nonlinear and contain many zeroes (Ludwig and Reynolds, 1988). Additionally, proper interpretation of the output is not trivial. Thus, we recommend that authors consult the

various texts available discussing these methods before analyzing their data (see Jongman *et al.*, 1995; Legendre and Legendre, 1998; McCune and Grace, 2002; Lepš and Šmilauer, 2003). Three frequently used fingerprinting techniques that generate these multivariate datasets are phospholipid fatty acid analysis (PLFA), denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism analysis (TRFLP). Additionally, microbial abundance and identity can be determined by real-time PCR methods (qPCR).

Many fingerprinting approaches as well as other DNA and RNA-based analyses of microorganisms depend on the amplification of specific gene sequences via the polymerase chain reaction (PCR). PCR is a highly specific and robust enzymatic reaction that allows one to specifically amplify a gene target of interest from low amounts present in an environmental sample to quantities large enough to perform many laboratory-based analyses. PCR has been used widely to analyze microbial community responses to perturbation, assess the metabolic potential of a community by detecting and quantifying the presence of specific structural genes and to quantify levels of specific groups or species of microorganisms in situ (Feris *et al.*, 2003; Hristova *et al.*, 2003; and many others). Successful amplification of a target gene sequence requires the development or selection from the literature of an appropriate set of PCR primers. While there are a multitude of PCR primers described in the literature that target large phylogenetic groups (*e.g.*, Bacteria, Archaea, Eukarya), specific microbial groups/species (*e.g.*, β-proteobacteria, cyanobacteria, etc.) or functional genes (*e.g.*, genes necessary for nitrogen fixation, ammonia oxidation, etc.); the selection of the appropriate PCR primers for a given study may not be trivial. Some care should be given to ensure that the primer set chosen is either highly selective for the targeted group or general enough to capture all the organisms within a targeted group that are present in an environmental sample. The importance of proper primer choice is illustrated when we consider that PCR, while powerful and robust, can only detect genes of interest if those genes have some sequence homology with the PCR primers that are utilized in the PCR reaction.

In addition, for each of the approaches described here it is important to replicate each measure at a level appropriate for a given study to ensure that measured microbial responses can be related to ecosystem processes with a desired level of confidence. Many commercially available DNA extraction kits efficiently recover DNA from 0.5–1 g soil/sediment samples. However, it is unlikely that a single 1 g sample from a complex heterogeneous ecosystem will adequately describe the natural variation in the local microbial community. Thus, multiple samples of this size may need to be collected, extracted and possibly pooled to provide a sample that is representative of a given environment. To design an adequate sampling strategy it is important to consider the level of heterogeneity within a given environment at scales relevant to the organisms performing processes of interest. For example, scales of importance to microorganisms can range from the sub mm^3 to m^3 scales and greater (Ranjard *et al.*, 2003; Zhou *et al.*, 2004; Becker *et al.*, 2006; Kan *et al.*, 2007). Therefore, sampling at multiple scales and subsequently applying statistical analyses to determine at which scale microbial communities correlate with observed ecosystem functions may be necessary.

PHOSPHOLIPID FATTY ACID ANALYSIS (PLFA)

To analyze a community by PLFA, lipids are extracted directly from soil; then phospholipids are separated from other lipid fractions, methylated, and analyzed by gas chromatography (White *et al.*, 1979) (Fig. 2). PLFA should not be confused with total soil fatty acid methyl ester analysis (TSFAME), in which all soil lipids are extracted and analyzed

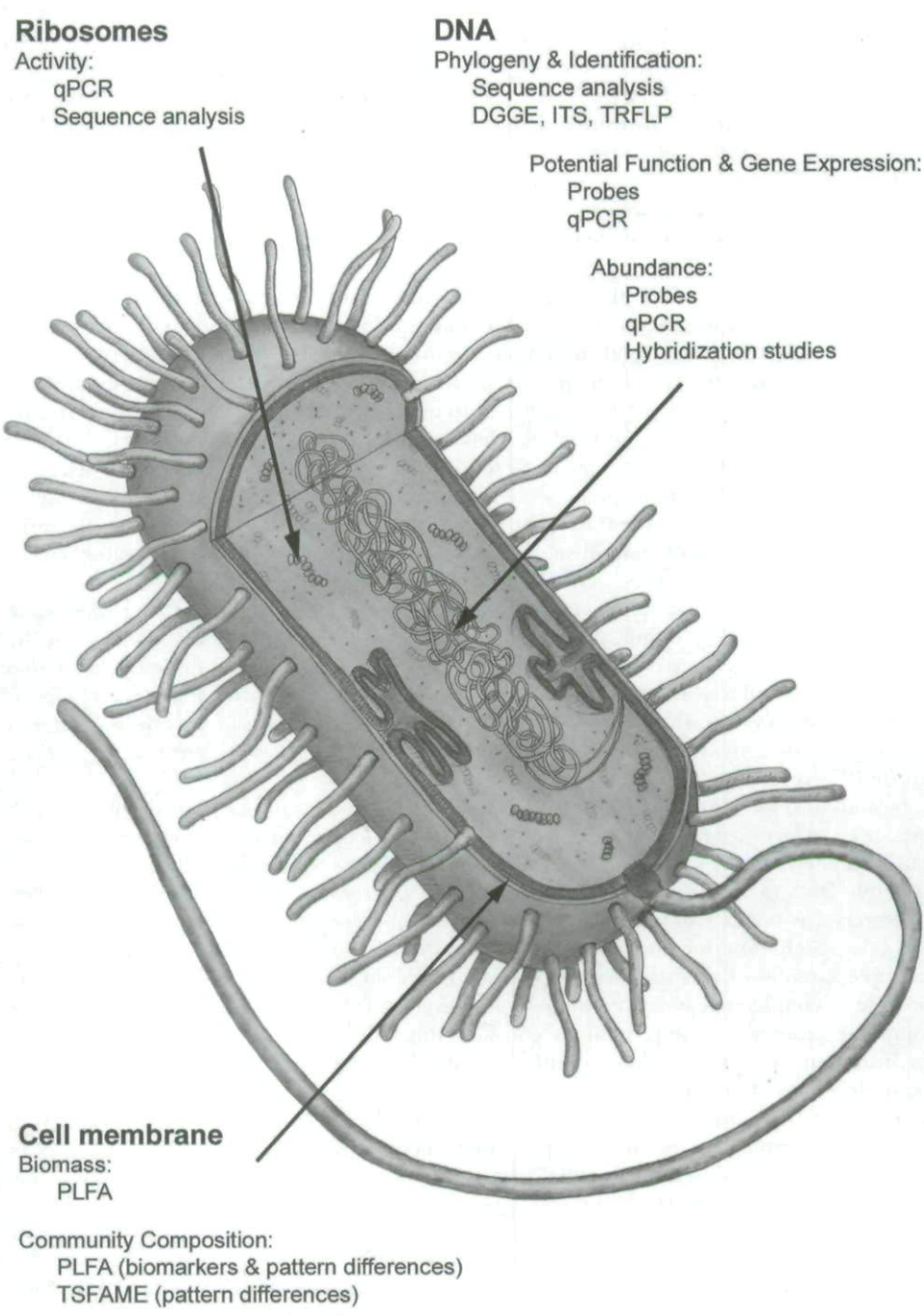

FIG. 1.—Illustration of generalized microbial cell indicating portions of organism which can be exploited to determine community composition, phylogenetic relationships, organism identity,

TABLE 1.—Comparison of methodologies for microbial community characterization

Method	Structure	Function	Pros	Cons
PLFA	X	X[1]	Quantitative (biomass and composition)	Community composition interpretation can be difficult (*e.g.*, lipids responsible for separating treatments are common among many organisms)
				Biomarker identity based on cultured organisms
			Standard protocol	Microorganisms within Archaea are not extracted
			Relatively rapid method	
All PCR-based	X	X[1]	Greater resolution than biochemical methods	DNA extraction efficiency can vary depending on properties of organisms and/or soil
			Can target activity (RNA) as well as presence (DNA) of community members	Amplification bias can occur; thus, results may not accurately represent community composition
				Primers design is limited by existing database and other sequences to which you have access
			Can target organisms involved in N cycle (in contrast to biochemical methods)	Not quantitative
Real-time qPCR	X	X[1]	High precision in quantification, dynamic range	Expensive reagents and equipment
			Eliminates post-PCR processing of PCR products, reducing possible contamination	
DGGE	X	X[1]	High level of resolution (species to sub-species) in conjunction with sequencing	Time-consuming
				Some skill required to pour gels
			Sequence recovery is straightforward as bands are physically cut from gel	Specialized software required to compare gels
TRFLP	X	X[1]	Highly reproducible	Lower resolution (genus level)
				Optimization of conditions and standardization of data is necessary
			Can be automated	Coarse level of phylogenetic information
				Optimization of conditions and standardization of data is necessary

[1] If used in conjunction with labeled substrate

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organism or functional group abundance, and/or physiological activity. Abbreviations are defined as follows: qPCR, quantitative PCR; DGGE, denaturing gradient gel electrophoresis; ITS, intergenic transcribed spacer analysis; TRFLP, terminal restriction fragment length polymorphism analysis; PLFA, phospholipid fatty acid analysis; TSFAME, total soil fatty acid methyl ester analysis

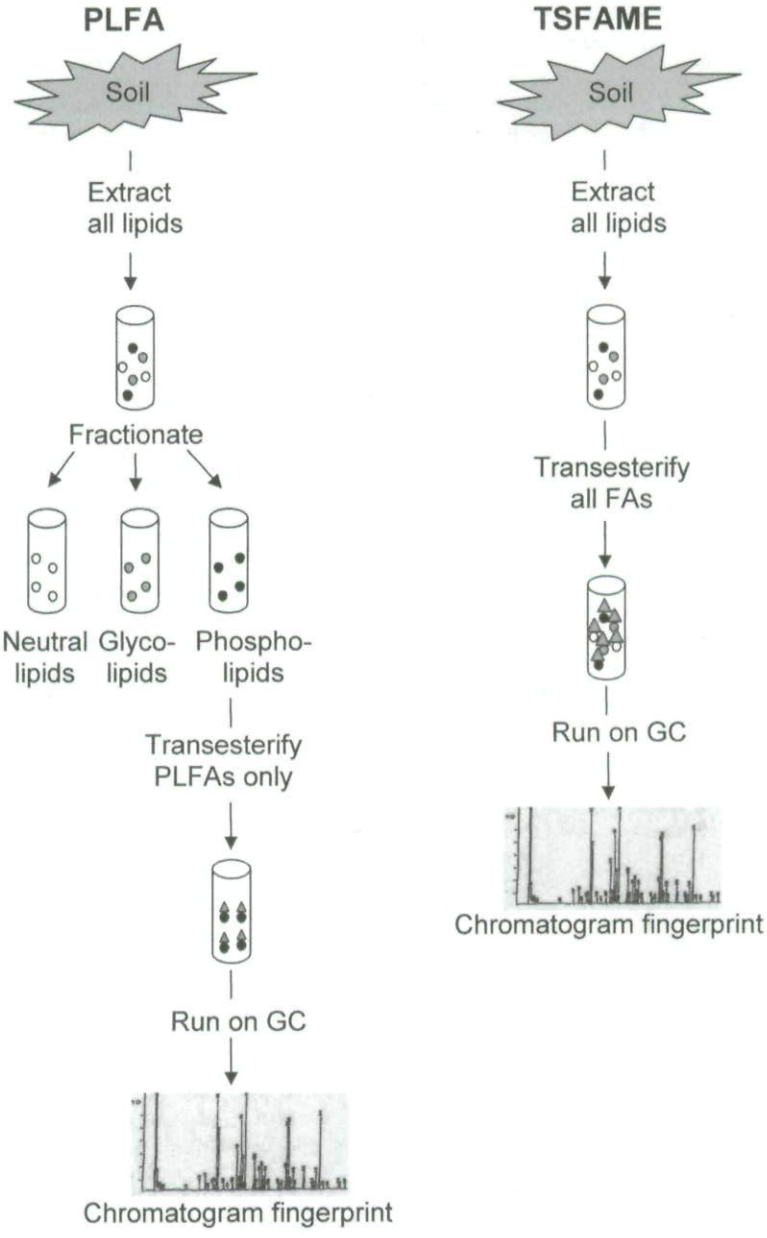


FIG. 2.—Diagrammatic example of soil extraction for PLFA and TSFAME analysis

(Fig. 2; *see* Drenovsky *et al.*, 2004 for a detailed comparison). In contrast to TSFAME, PLFA extracts contain only cellular membrane lipids, which decompose rapidly in the environment, and thus, more accurately represent the living microbial community (White *et al.*, 1979; Pinkart *et al.*, 2001).

PLFA provides information regarding microbial community composition, biomass and diversity. Since microbial groups vary in cellular membrane composition, changes in extracted PLFA can indicate shifts in community structure (Vestal and White, 1989). Although some fatty acids are considered biomarkers for certain microbial groups (*e.g.*, fungi: 18:2 ω6,9c), many PLFAs are shared among organisms; thus, biomarkers must be interpreted with caution and with consideration of sample environmental conditions (Bossio and Scow, 1998). Although PLFA analysis can provide biomarker information regarding organisms involved in the carbon cycle (*e.g.*, methanotrophs, fungi), widely accepted biomarkers for the N cycle have yet to be determined. Additionally, PLFA cannot describe the archaeal community (*e.g.*, methanogens), as these organisms have ether-linked, not ester-linked, membrane lipids. Summing all fatty acid concentrations in an environmental sample estimates total microbial biomass (less the archaeal biomass), and the number of fatty acids detected in a sample provides a diversity proxy (*e.g.*, Ravit *et al.*, 2003). Although there is not a one-to-one relationship between fatty acids detected and number of microbial taxa present, large differences between samples may indicate differences in microbial diversity, especially when entire groups of fatty acids are missing or in low abundance.

DENATURING GRADIENT GEL ELECTROPHORESIS (DGGE) AND TERMINAL RESTRICTION FRAGMENT LENGTH POLYMORPHISM ANALYSIS (TRFLP)

Compared to lipid-based approaches, DNA- and RNA-based methods provide high resolution (*i.e.*, species-specific) microbial community characterization. Here we focus on two common DNA- and RNA-based fingerprinting methods, DGGE and TRFLP analysis. Both methods exploit gene sequence variation within and between microbial species to assess community composition and diversity. Both are PCR-based, requiring nucleic acid extraction from environmental samples and subsequent PCR amplification of target genes (Fig. 3) (Muyzer *et al.*, 1993; Clement *et al.*, 1998). Based on the PCR primers employed, DGGE and/or TRFLP can assess entire bacterial, archaeal or eukaryotic communities (Feris *et al.*, 2003; Feris *et al.*, 2004; Cookson *et al.*, 2007; Yergeau *et al.*, 2007), target specific phylogenetic groups (Perez-Jimenez and Kerkhof, 2005), detect individual species (Hristova *et al.*, 2003) or identify specific functional groups (Burgmann *et al.*, 2005). Often, ribosomal DNA (rDNA) genes are targeted because they contain both highly conserved and more variable regions useful in species-level assessments of community composition (Amann *et al.*, 1995).

Although there are many molecular tools available for characterizing microbial communities, including microarrays, metagenomic and metaproteomic approaches, we have chosen to focus our review on DGGE and TRFLP. These methods require less optimization and technical expertise and less expensive instrumentation, and yet they still provide adequate resolution for characterizing microbial community responses to environmental perturbations. Additionally, although clone library creation for species-level characterization of microbial communities is a well-accepted method and can provide a great deal of information about community structure, this approach is difficult to apply to multiple samples collected across large scales, as would be of interest to ecosystem ecologists. Thus, we present approaches that can be used to characterize microbial communities at larger spatial scales and provide information to guide subsequent high resolution characterizations of specific samples, as deemed important by the initial higher throughput screening/analytical tests we describe.

With DGGE, PCR products are separated based on sequence content via polyacrylamide gel electrophoresis (Muyzer *et al.*, 1998). The PCR products partially denature as they travel through a gradient of chemical denaturants incorporated in the gel matrix. Specifically, the

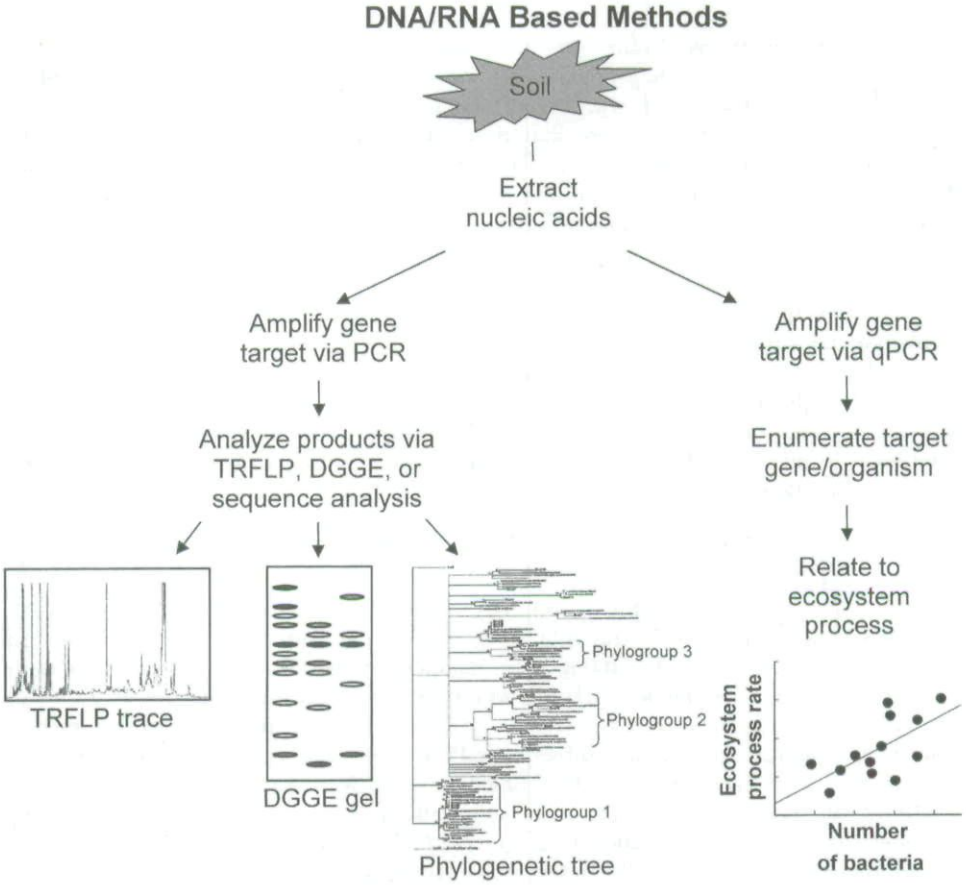


FIG. 3.—Diagrammatic example of soil extraction of DNA/RNA for nucleic acid-based community analyses

separation of PCR fragments on a DGGE gel is dependent on the GC content of the DNA fragment, such that PCR products with higher GC contents are more difficult to denature. This difference is due to the three hydrogen bonds between a GC base pair relative to the two hydrogen bonds between an AT base pair. Thus, PCR products with higher GC contents will migrate farther in a DGGE gel before denaturing and slowing their migration rate. Denaturation of the PCR products retards further movement, resulting in DNA "band" formation. By separating PCR product mixtures from environmental samples, a community fingerprint is created (Fig. 4). The number of DNA bands, their positions and in some cases their relative intensities can be compared by standard diversity analyses. When more specific information is required, bands can be cut from the gel, sequenced and compared to sequences of cultured organisms or other environmental samples in databases such as Ribosomal Database Project II (RDPII) (http://rdp.cme.msu.edu/) or NCBI Blast (http://www.ncbi.nlm.nih.gov/BLAST/)

TRFLP rapidly is becoming the community analytical method of choice due to its high resolution, reproducibility and throughput. In TRFLP, one or both PCR primers contain a

5′-terminal fluorescent tag that becomes incorporated into the PCR product. Amplification of community DNA produces PCR products with varying sequence content. Digestion (*i.e.*, cutting) of such PCR products with sequence-specific restriction enzymes forms a mixture of terminal restriction fragments (TRFs); their sizes and amounts then are determined using an automated DNA sequencer (Clement *et al.*, 1998). Similar to DGGE band patterns, the resulting TRF pattern can be used to characterize communities and identify the presence of unique microbial species/groups. However, to obtain precise species-specific identification requires matching TRFs of interest with TRFs in a clone library of known DNA sequences.

Commonly, each DGGE band or TRF is thought to represent a single sequence type and, thus, an individual "species". However, a single DGGE band or TRF can contain multiple sequences, leading to conservative estimates of species richness (Klappenbach *et al.*, 2000). Also, PCR artifacts can affect band and TRF peak intensity; thus, intensity does not necessarily imply species abundance and must be used with caution in comparative analyses.

In contrast to DNA-based approaches, which characterize community composition, RNA-based measures indicate microbial activity (Burgmann *et al.*, 2005; Holmes *et al.*, 2005). Actively growing and dividing cells maintain higher intracellular rRNA concentrations; thus, rRNA presence indicates cellular activity (Wagner, 1994). By comparing rRNA and rDNA patterns via DGGE or TRFLP, metabolically active and inactive community members can be detected (Duineveld *et al.*, 2001; Norris *et al.*, 2002). However, RNA is degraded rapidly by native RNAses present in most environments. Thus, precautions must be taken during sample collection, transportation and processing to prevent RNA degradation for accurate in situ assessment of microbial activity.

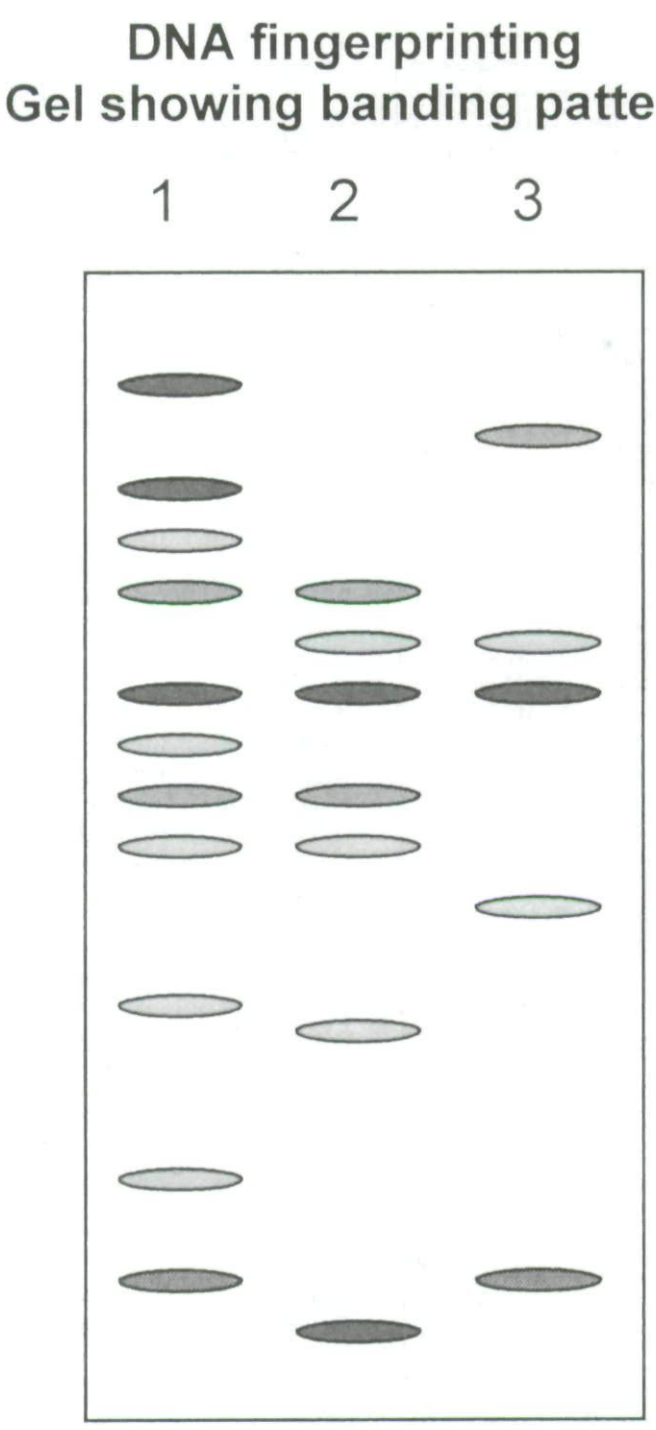
REAL-TIME PCR QUANTIFICATION OF SPECIFIC MICROBIAL GROUPS

One drawback of traditional molecular methods is that they provide qualitative, but not quantitative, information. Development of quantitative techniques such as fluorescent in situ hybridization (FISH), competitive reverse-transcriptase (RT)-PCR and real-time PCR has improved our ability to garner quantitative information about microbial populations in situ. Here we focus on real-time PCR, which rapidly is becoming the most commonly used quantitative molecular method in microbial ecology.

Real-time quantitative PCR (qPCR) has been used to quantify abundance of specific microbial groups and/or species in environmental samples by enumerating 16S rDNA or functional gene copy numbers (Suzuki *et al.*, 2000; Hristova *et al.*, 2003; Okano *et al.*, 2004). This technique is based on either 5′-nuclease chemistry (*e.g.*, TaqMan assay; Holland *et al.*, 1991) or incorporation of a DNA-binding fluorescent dye (*e.g.*, SYBRGreenI; Feris *et al.*, 2003; Cavagnaro *et al.*, 2007). Both approaches can provide precise quantitative measures of a specific DNA or RNA sequence (*e.g.*, species, functional group or phylogenetic group) in an environmental sample and, depending on the standard used, absolute microbial cell densities or relative densities can be determined. Some advantages of real-time qPCR include sensitivity, a broad linear quantification range, amplification and detection homogeneity and potential for high throughput.

COSTS ASSOCIATED WITH COMMUNITY PROFILING TECHNIQUES

One pragmatic concern regarding these techniques is the associated cost. Instrumentation costs can be substantial. For example, a new gas chromatograph and autosampler for analyzing PLFA samples can be well over $50,000, and a capillary system for TRFLP an analysis can cost over $100,000. In addition to instrumentation costs, the cost of reagents and supplies, as well as labor costs can quickly accumulate. Those new to the field will benefit from collaborating with researchers who routinely do these analyses and, thus,


DNA fingerprinting
Gel showing banding pattern
1
2
3

currently have the appropriate analytical instrumentation and reagents. Additionally, this type of interaction should foster cross-discipline interactions between microbiologists and ecosystem ecologists.

DIRECTLY LINKING STRUCTURE TO FUNCTION IN MICROBIAL COMMUNITIES

Traditionally, links between microbial community structure and function have been limited to correlative approaches. Whereas these techniques provide some insight into structure-function relationships, they offer no direct evidence linking particular microbial community member(s) to specific processes. In this section, we describe how stable isotope probing (SIP) techniques can be used to directly link microbial community structure to a specific function.

SIP applies labeled substrates (*e.g.*, ^{13}C methane) to environmental samples and monitors their incorporation into phylogenetically significant bio-molecules (PLFAs, DNA and RNA) (Radajewski, 2003; Osaka *et al.*, 2006; Cébron *et al.*, 2007; Neufeld *et al.*, 2007). If the substrate is consumed and assimilated, the label is incorporated into new microbial biomass, which can be extracted and its fingerprint analyzed. Thus, it is possible to directly link a measurable function (*e.g.*, methane oxidation, carbon consumption/oxidation) with specific microorganisms in an environment. These techniques, in some cases, are most applicable to questions regarding incorporation of carbon-based compounds into microbial biomass, as some nitrogen cycle transformations (*e.g.*, nitrification and denitrification) do not incorporate N into microbial biomass. However, SIP techniques for identifying organisms involved in other aspects of the N cycle (*e.g.*, nitrogen fixation and other N-assimilation pathways) have been developed (Buckley *et al.*, 2007a, b).

PLFA-SIP relies on gas chromatography-combustion-isotope ratio mass spectrometry (GC-c-IRMS) to study isotopic composition of individual PLFA. Although PLFA-SIP is extremely sensitive and provides quantitative information about soil microbial communities, species-level identification is not possible, unlike DNA-based SIP, which can resolve structure-function relationships at genus and species levels. DNA-SIP relies on separation of heavy (labeled) and light (unlabeled) DNA by density gradient centrifugation (Radajewski, 2003; Cébron *et al.*, 2007). However, clear separation of labeled and unlabeled DNA depends on substrate isotopic enrichment, limiting this method to compounds highly enriched with a rare stable isotope (^{2}H, ^{13}C, or ^{15}N). DNA-SIP mainly has been demonstrated using single carbon compounds (*e.g.*, methane or CO_2); however, examples using more complicated ^{13}C labeled substrates exist (Jeon *et al.*, 2003; Park *et al.*, 2006).

Although DNA-SIP provides an enhanced level of resolution relative to PLFA-SIP, a number of factors may influence ^{13}C enrichment in DNA. These include: whether the assimilated substrate is used by a few or many community members, whether the operative anabolic pathway prevents incorporation in an unevenly labeled substrate and how long the ^{13}C substrate pulse duration is relative to how rapidly the labeled carbon is turned over, converted, and made available to secondary consumers. Thus, incubation times often must be empirically determined to maximize label incorporation while minimizing the likelihood of secondary consumption. Other considerations include relative abundance of naturally

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FIG. 4.—Diagrammatic example of DGGE gel showing banding patterns for three soil samples containing unique microbial communities. Banding patterns can be compared between samples to determine similarity and diversity. Additionally, by sequencing and cloning specific bands, the identity of microorganisms present in the sample can be determined

TABLE 2.—Major questions used to probe ecosystem function and microbial community structure of our case study wetlands and examples of techniques that could be used to address them

Question	Method(s)
Does the effluent change overall community composition?	PLFA, DGGE, TRFLP
Is the effluent reducing or increasing diversity/number of methanogens/methanotrophs, nitrifiers/denitrifiers?	DGGE, TRFLP, qPCR
Do gas fluxes (N_2O, CH_4) change with effluent addition?	Gas chromatography (GC), stable isotopes
How does effluent loading influence the N cycle?	^{15}N pool dilution, enzyme assays
Which organisms are metabolizing the effluent pollution?	DGGE or TRFLP in combination with SIP
Which microbial groups are metabolizing the effluent pollution?	PLFA, DGGE or TRFLP in combination with SIP
What is the population size of organisms that are actively metabolizing the effluent pollution?	qPCR in combination with SIP

occurring unlabeled substrate, and the active microbial population's DNA synthesis rate (Lueders *et al.*, 2004).

RNA-based SIP avoids some of these complications. In active cells RNA synthesis rates are high, and labeling can occur without DNA synthesis or organism replication (*i.e.*, the organism can be metabolically active but not growing/dividing). Therefore, labeled RNA may be detected more quickly than labeled DNA and, thus, may be more appropriate for environmental studies, if RNA can be extracted reliably from the environment in question (Lueders *et al.*, 2004; Schwarz *et al.*, 2007).

CASE STUDY: APPLYING MICROBIAL TECHNIQUES TO ASSESS WETLAND SYSTEMS

Methane production and denitrification are two ecosystem functions affected by the specific types and activities of microorganisms present (Sylvia *et al.*, 2005). Here, we use approaches presented above to measure how pollutant loading could impact these functions. We compare two hypothetical wetlands, 20 km apart, with similar geology and hydrology but different pollutant loadings. The first is relatively pristine, with limited inputs from urban and industrial settings. The second is impacted heavily by anthropogenic activity, annually receiving significant sewage effluent rich in labile C and N. With these inputs, methane emissions and denitrification rates are five and ten times higher in the polluted wetland, respectively. In our study, we want to: (1) understand how the effluent is changing microbial community composition, (2) determine if community changes correlate with alterations in methane efflux and denitrification rates and (3) link observed functional changes to specific organisms or functional groups (Table 2).

Using both lipid and DNA-based approaches, we can examine community changes in response to sewage input at two levels of resolution while simultaneously taking advantage of these approaches' strengths. Since PLFA is relatively rapid and standardized, we can sample intensively, quickly gaining quantitative, landscape-level information about microbial groups associated with particular site characteristics. From our mock PLFA chromatograms, it is apparent that the pristine site has lower total PLFA number (a proxy for diversity) and lower total PLFA concentration (*i.e.*, total microbial biomass) (Fig. 5). Analyzing our data using correspondence analysis (CA), we can see that the soil samples from the two sites group separately from one another, indicating that they differ in fatty acid composition (Fig. 6). In New Jersey salt marshes, PLFA revealed significantly fewer fatty acids in physically disturbed and chemically polluted soils compared to undisturbed, relatively

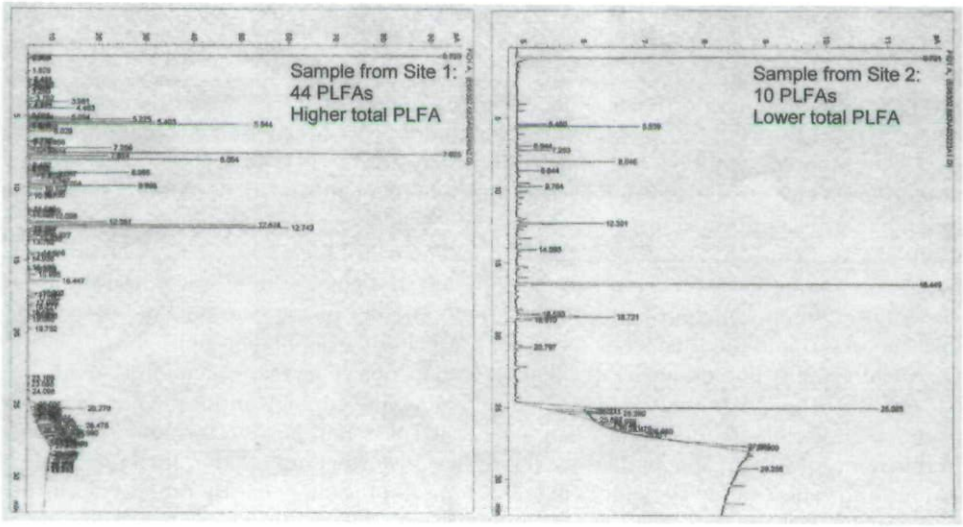

FIG. 5.—Mock PLFA chromatograms for soil samples from the two case study sites (Site 1, pristine site; Site 2, impacted site). From these chromatograms, we can observe large differences in total number of PLFA present (a proxy for diversity) and total PLFA concentration (*i.e.*, total microbial biomass). Note the difference in scale for the y-axis (*i.e.*, the peaks from site 2 are smaller, indicating less biomass)

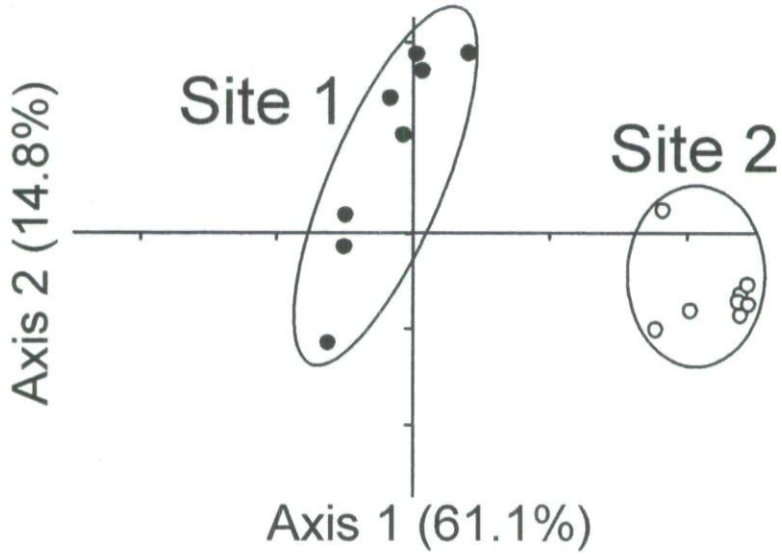

FIG. 6.—Correspondence analysis (CA) plot of PLFA data from the two sampling sites. From the plot, we can see that the two samples from the two sites group separately, indicating that they have different microbial communities

pristine soils, suggesting chemical and physical disturbance may decrease microbial diversity (Ravit *et al.*, 2003). Likewise, in floodplain soils from Germany, differences in lipid profiles between sites suggested that permanent flooding, compared to intermittent flooding, prevented establishment of fungi (Rinklebe and Langer, 2006).

Using DGGE or TRFLP, we can focus on variation in broad functional groups (*e.g.*, methanotrophs, denitrifiers) and target specific organisms (*e.g.*, *Methylocystis* or *Paracoccus denitrificans*). This greater taxonomic resolution complements the quantitative biomass and functional group information gained by our PLFA analyses. For instance, in a microcosm study of rice field soil, DGGE and TRFLP were used to assess how changes in protistan bacteriovore abundance changed bacterial diversity and composition over time (Murase *et al.*, 2006). After applying our fingerprinting methods, we can augment our studies by using qPCR to determine whether sewage additions are altering population densities of specific microbial groups. For example, qPCR was used to detect increased ammonia oxidizing bacteria (AOB) population densities in marine sediments with high anthropogenic N inputs from wastewater plants and other urban systems in Tokyo Bay, Japan (Urakawa *et al.*, 2006). In this study, qPCR was able to detect cell numbers near the limit of other methods (*e.g.*, in situ hybridization and slot blot hybridization), indicating its detection sensitivity. Additionally, cell numbers determined by qPCR and immunofluorescence staining were well-correlated, suggesting that although DNA may be lost during purification and amplification, the results from this assay are still quite robust.

Our second objective is to link greenhouse gas production to changes in community composition. Using gas chromatography and stable isotope techniques, we could monitor wetland methane emission and denitrification rates. Ultimately, we want to understand direct relationships between community composition and ecosystem function; therefore, a first step would be to test whether community composition differences correlate with measured process rates (*e.g.*, denitrification rates and methane fluxes). Understanding how fluxes of greenhouse gases change with conditions can give us important information linking community structure to community function. Using canonical correspondence analysis (CCA), we could determine how microbial community composition (as assessed by PLFA, DGGE, or TRFLP) is related to these measured abiotic variables (Fig. 7). From the CCA biplot we can see that samples from the two sites group separately, indicating that they have different microbial communities. Additionally, samples from the impacted site are associated with higher methane efflux, N_2O efflux and soil nitrate concentrations, as the vectors for these environmental variables are increasing in the direction of the samples from the impacted site. In a study of California salt marsh sediments, it was determined that heavy metal concentrations were stronger drivers of microbial community composition than organic pollutants using partial canonical correspondence analysis (pCCA) (*i.e.*, effects of spatial variables were removed) (Cordova-Kreylos *et al.*, 2006).

Once a link between composition and process rates is established, we can explore which specific community members are involved in observed changes in ecosystem function. To target organisms responsible for elevated methane fluxes in the contaminated wetland, a ^{13}C labeled low molecular weight substrate (*e.g.*, acetate) could be added to wetland sediments in situ or in microcosms. After short incubation times, ^{13}C levels in CO_2 and CH_4 pools could be monitored and methane production and consumption rates calculated via pool-dilution by GC/MS. Following DNA or RNA extraction and ^{13}C DNA/RNA fraction isolation, DGGE or TRFLP could be used to identify organisms that have incorporated the ^{13}C label. By simultaneously measuring methane efflux, characterizing CH_4 ^{13}C enrichment and tracking ^{13}C flow into microbial biomass we can directly link microbial community

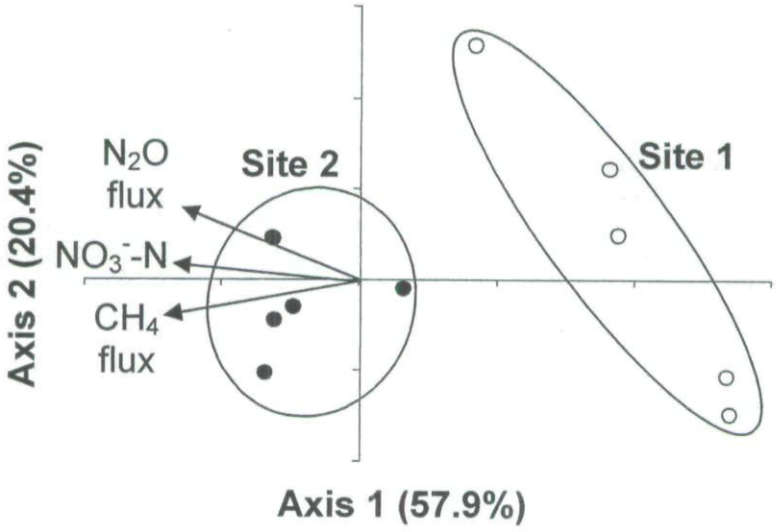

FIG. 7.—Canonical correspondence analysis (CCA) biplot of PLFA and environmental data from the two sampling sites. From the biplot, we can see that the microbial communities from the impacted site are associated with higher methane efflux, N_2O efflux and soil nitrate concentrations

structure to an observed ecosystem function. Using a similar approach, Padmanabhan *et al.* (2003) characterized the soil microbial communities involved in phenol and naphthalene biodegradation via GC/MS and SIP.

Combining GC/MS and SIP, we could track how anthropogenic disturbance alters carbon flow and conversion through wetland food webs. Lueders *et al.* (2004) combined RNA and DNA-based SIP with real-time PCR, TRFLP and comparative sequence analysis to trace carbon flow from methylotrophs into secondary eukaryotic consumers in rice field soil. By observing enrichment of specific methylotrophic nucleic acids over time, they identified methylotrophs actively consuming the applied ^{13}C-methanol and then tracked ^{13}C enrichment in various RNA sequences from eukaryotic organisms unable to consume methanol directly. Thus, SIP provided information about both the primary methanol oxidizers and their possible interactions with fungi and predators in a complex food web.

CONCLUSIONS

Recent advances in microbial ecology make it possible to ask more specific questions about how microbial community composition is linked to ecosystem function. However, with the diversity of new methods, choosing the best method to answer a particular question can be difficult. Considering the following questions may help guide this decision and develop experimental design: (1) Is the study targeting specific organisms or broad, functional group changes in microbial community composition? If the study is aimed at detecting landscape-level patterns, methods such as PLFA, which highlight changes in broad microbial groups, may be most appropriate. However, if the goal is to detect specific microorganisms, methods such as DGGE may be more appropriate. (2) What are the methodological limitations? Recognizing a technique's positive and negative aspects can guide interpretation and application of microbial data (Table 1). (3) At what scale is the question being asked, and how will soil heterogeneity affect the outcome? If the research

questions focus on the role of soil microorganisms on a landscape/ecosystem scale, the sampling strategy employed must encompass the sampled system's heterogeneity as perceived on a microbial scale. As with other soil measurements, compositing sub-samples can help represent this heterogeneity. (4) How will seasonal fluctuations in precipitation, temperature, and day length influence the focal microorganisms? Seasonal changes can affect both microbial community structure and function and, thus, should be taken into consideration when developing sampling protocols.

Advancements in microbial ecology provide new avenues for exploring linkages between environmental processes and microbial communities, allowing both ecosystem and microbial ecologists to answer questions that previously were intractable. Demystifying recent methodological developments in microbiology should spark new avenues of investigation and stimulate collaboration between microbial and ecosystem ecologists, two groups now recognized to share common goals and offer complementary perspectives in ecosystem studies.

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