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Stable isotope probing to investigate microbial function in soil

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Abstract

Most approaches for in situ phylogenetic characterization of soil microorganisms lack the ability to establish a causal relationship to function within the community. Recently, the use of stable isotopes to label phylogenetically informative biomolecules (phospholipid fatty acids, DNA, or RNA), typically referred to as stable isotope probing (SIP) has the advantage of providing more definitive evidence that a detected population is active in a specific process, if that process results in assimilation of C or N into cellular constituents. Carbon labeling is considerably more sensitive than N labeling, and is thus more generally

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useful. Application of SIP techniques to unsaturated soils should be treated with the same caution as other attempts to address function in soils. Of particular concern is the issue of bioavailability of the test substance, which is often neglected in ecology studies. Processes that appear to be amenable to SIP approaches include, but are not limited to, organic matter turnover, biodegradation of organic pollutants, nitrification, nitrogen fixation, and plant-microbe interactions.

Background

Microorganisms are responsible for many of the most important processes occurring in soils. To manage soils thus, is to manage microorganisms. Yet the vast majority of soil microorganisms remain unidentified and only a small fraction (perhaps 1%) have been successfully cultivated. This information gap may not be of practical consequence, as it has not been inherently necessary to know the identities of responsible organisms in order to manage various biological soil processes. A considerable amount of success has been realized in soil microbiology with the limited phylogenetic information available. However, applications of soil microbiology may have developed differently had more robust information been available about soil microbial communities, and in the future, such information could be useful in describing the causes of various observations made at the process level. As it becomes possible to know which organisms are responsible for activities of interest to humans, and how those organisms respond to manipulations of the soil environment, it may become possible to directly manage the causative agents (microorganisms) of the soil processes we seek to control.

Most of what is known about the impact of human activities on soil microbiology is either based on bulk scale measurements of impacted processes, or broad assessments of diversity. For example, reviews of the literature on the impact of pest management chemicals have shown widely varying responses, from no effect to both positive and negative effects of a particular compound on a targeted soil process (Lal and Lal, 1988; Sims, 1990). Numerous studies have been directed at determining the effects of agricultural management on soil microbial diversity (number of taxa and relative abundance). McCaig et al. (2001) used 16S rDNA clone libraries and PCR-DGGE to examine bacterial communities in grasslands under different intensities of management, with the DGGE profiles suggesting that greater inputs led to decreased diversity whereas the clone libraries revealed no differences. Bossio et al. (1998) observed changes in PLFA profiles among farms under a range of management approaches, though it is not certain what consequences might have resulted from these changes. Measurements at the process level provide little information about events at the scale of the organisms responsible for processes of interest. Changes in diversity or

community structure are expected as resource inputs or site specific conditions change, however, such changes may have little relationship to functions measured at the process level. It has been clear, even from very early work, that microbial populations can shift without affecting a variety of measurements at the bulk scale. Buddin (1914) showed that various solvents can result in increases in numbers of specific colony types, whether total numbers were increasing, decreasing, or relatively constant. Deployment of advanced molecular tools has provided similar information, showing that, for example the relative dominance of representatives of the ammonium oxidizing bacteria community (AOB) may not shift even in the presence of a significant change in the overall process (Avrahami, et al., 2002) or more profoundly, that the nature of the dominant nitrifying bacteria in soils may be Crenarchaeota (Archaea) rather than the bacterial AOB that are so familiar to most of us (Leininger et al., 2006). Such findings redefine which organisms we credit with “importance”, but moreover, show that the great phylogenetic diversity and functional redundancy of soil communities tend to insulate soils against major changes in critical processes. Lacking is information that links phylogeny to activity and how these activities (processes) may be sensitive to changing conditions that could be controlled through management.

A variety of improvements in molecular microbial ecology techniques have been developed to enhance the ability to examine microbial function *in situ*. The use of rRNA as a target for PCR has been said to reveal information about the active microbial community (Felske and Akkermans, 1998), and this has been coupled to real-time PCR to examine expression of genes in soil, such as those involved in atrazine degradation (Devers et al., 2004). Another approach for examining gene expression is the incorporation of 5-bromo-2'-deoxyuridine (BrdU) into DNA coupled to fingerprinting the active communities (Urback et al., 1999; Yin et al., 2000). Microarrays have been applied to environmental samples of various types with varying degrees of success (Cho and Tiedje, 2002; Zhou, 2003). Another recent advancement in ecological methods, the various applications of stable isotope probing, facilitates examining the identity of microorganisms involved in specific processes *in situ*, and is the subject of this review.

Overview of SIP

Stable isotopes are used for numerous purposes in biology for labeling of various biomolecules. *Stable isotope probing* could thus encompass many different kinds of research, however, recent developments have promoted specific connotations to the use of this term. Microbial ecologists presently use stable isotope probing (SIP) to refer to various techniques based on labeling certain types of microbial biomarkers (those that may be used for microbial identification) with stable isotopes (typically ^{13}C), followed by an

analysis of the labeled biomarker pools to identify the members of the microbial community active in assimilating the substance of interest. Though SIP is not the only tool that allows such analysis (quantitative dot blot and the combination of micro-autoradiography and fluorescent *in situ* hybridization have also been used), it is particularly well-suited for soils research. Wagner (2004) reviewed various molecular techniques that reveal microbial function and Dumont and Murrell (2005) recently provided a review of SIP and its potential uses.

It is now widely accepted that many ecologically important microorganisms are uncultured. For example, examination of microbial communities of various Arizona soils concluded that of the organisms detected, seven bacterial divisions were represented among the uncultured organisms versus three for cultured isolates (Dunbar et al., 1999). The focus of much SIP work has been on the prospect of discovering the role of heretofore uncultivated organisms in important processes, however, it is likely that some of the organisms we already know about (or their close relatives) are also important in key functions in the environment. SIP can thus be just as useful in examining the behavior of organisms (regardless of novelty) in response to various stimuli, which may provide practical insight into key microbial functions, perhaps leading to improved environmental or agricultural management. This review examines the most common forms of SIP used in soil microbiology, describes some of the findings that have been obtained using these approaches, and suggests fruitful directions for the application of SIP to soils research.

Targeted biomarkers

As noted above, SIP methods in general entail the exposure of microbes to labeled compounds followed by the isolation of a target compound from the community, that compound having been preferentially enriched in the organisms directly involved in the process of interest. To identify organisms using this approach, the recovered constituent must provide unique taxonomic information about the organism. Typical target compounds thus include phospholipid fatty acids, DNA, and RNA. Since techniques similar to SIP are already well established in the field of proteomics (Gruhler et al., 2005), it is likely that environmental SIP targeting proteins will eventually be realized as well. Of the established SIP methods, nucleic acid SIP is generally thought to be the most informative for taxonomic purposes, particularly among closely related organisms, in part owing to the extensive 16S rRNA database (some 120,000 sequences) that has been developed. Nucleic acid SIP has the inherent disadvantage of relying on a much less sensitive approach (separation by ultracentrifugation) for detecting isotopic enrichment when compared to PFLA analysis using isotope ratio mass spectrometry (IRMS). It is thus likely the

sensitivity of PFLA-SIP will promote its continued use despite the limited taxonomic information provided.

PLFA-SIP overview

In practice, PLFA-SIP consists of amending environmental samples with a ^{13}C -labeled substrate, extracting lipids and analyzing them via GC, GCMS or IRMS. Lipid extracts may be fractionated (by elution with different solvents from silicic acid) to yield several pools of lipids based on polarity. PLFAs elute with the most polar solvent (typically methanol). In preparation for GC analysis, PLFAs are derivatized (to yield fatty acid methyl esters, or FAME) to make them volatile in the GC injector. PFLA extracts from known organisms may be used as reference standards for identification purposes. Identification based on PFLA analysis is limited to previously cultivated organisms for which a PFLA database or authentic standard is available. PLFAs are much more amenable to strictly instrument analysis than nucleic acids, thus targeting this pool is not only more sensitive, but less labor intensive, and more compatible with laboratories outfitted primarily for process measurement rather than molecular ecology. Identification of the organism is dependent on the quality of identification of the PFLA molecules, thus mass spectrometry techniques are preferred (over capillary GC), which may be cost prohibitive for many laboratories.

PLFA-SIP applications

PLFA-SIP was applied before nucleic acid-SIP, and was perhaps first reported by Boshchker et al., (1998), who used ^{13}C -labeled acetate and methane to identify sulfate reducers and methanotrophs in aquatic sediments. This work showed that ^{13}C -acetate was predominantly consumed by sulphate-reducing bacteria (SRB) that were related to the less well documented Gram-positive *Desulfotomaculum acetoxidans* as opposed to the more well studied Gram-negative *Desulfobacter* spp. The use of ^{13}C -methane revealed that type I methanotrophic bacteria were the dominant methane oxidizers at the site. The approach has since been applied to various environmental samples. Lu et al (2004) pulse-labeled rice plants with $^{13}\text{CO}_2$ and examined PLFA patterns over the rice growing season. Relative abundance of PLFAs was observed to vary as the plants matured, which has been attributed to shifts in which members of the community were actively assimilating rice root exudates. These findings were consistent with the earlier observation that bacterial nitrogenase activity associated with rice roots exhibits both seasonal and diurnal variation that was closely coupled to the growth stage of the plants and the availability of photosynthate required for exudate production (Sims and Dunigan, 1984). Lu et al. (2006) applied 49 pulses of $^{13}\text{CO}_2$ to rice plants in a microcosm during a

7-day incubation. The degree of incorporation of ^{13}C into PLFAs decreased markedly outside the rhizosphere zone and the PLFA patterns differed between rhizosphere and bulk soil. Gram-negative bacteria and eukaryotic microorganisms appeared to be most active in assimilating exudates, whereas Gram-positive microorganisms appeared to be more important in the bulk soil. These results are consistent with microscopic and culture-based examination of the rhizosphere in experiments dating back to the 1970s (Brown, 1975). Mohanty et al., 2006 incubated rice field and forest soils with N fertilizers and ^{13}C methane. Type I methanotrophs (Methylococcaceae) were stimulated by fertilizer application while type II methanotrophs (Methylocystaceae) were apparently inhibited. Results also supported differences between forest soils and rice fields in the dominant active methanotroph populations.

Macgregor et al., 2006 incubated North Sea sediment cores with ^{13}C -labeled acetate, propionate, amino acids, or glucose and detected incorporation of label into bacterial rRNA and PLFAs, with more label being detected in the latter pool. Chang et al. (2005) introduced ^{13}C -acetate into wells in porous activated carbon beads. PLFA profiling demonstrated incorporation of ^{13}C into bacterial lipids the results of which compared favorably with ^{13}C -DNA SIP indicating activity of a member of the sigma-proteobacteria. Geochemical data, including Fe(III), U(VI), and SO_4^{2-} reduction were in agreement with the SIP results as well. Geyer et al. (2005) also used baiting (biotraps) and PFLA-SIP to examine biodegradation of pollutants (benzene and toluene) *in situ*. Taxonomic identification of the degraders proved not to be successful with PFLA in this study.

It is likely that PFLA-SIP will often work (e.g. label will be detected) when nucleic acid –SIP fails, however, the taxonomic information obtained will have more limited utility. Examples where PFLA-SIP may be the better choice include screening studies in which the research goal is to detect whether the members of a community responsible for a particular function are changing (rather than identification of specific organisms) and experiments where small quantities of labeled material are likely to be assimilated.

Nucleic acid-SIP overview

Radajewski et al. (2000) published their DNA-SIP technique only six years prior to this review. The approach shares with PLFA-SIP the feature that only active populations are targeted. Since the development of DNA-SIP, RNA has become the other important biomarker for SIP. In practice, DNA- and RNA-based SIP are accomplished with the same basic protocol (see Figure 1). After exposure of a community to a substrate labeled with ^{13}C or ^{15}N (typically at the highest enrichment possible), the environmental sample is then extracted to recover the pool of cellular nucleic acid. Extraction approaches for DNA and RNA may differ as considerable difficulties have been

encountered in the recovery of RNA from soils (Saleh-Lakha, et al., 2005). The extracted nucleic acids are then separated by density gradient centrifugation. The simplest approach for recovering the enriched nucleic acid pools is to incorporate a visualizing agent, such as ethidium bromide in the gradient and locating the heavy band using uv illumination. This approach is dependent upon a strong separation of enriched versus un-enriched nucleic acids, and has only been successful for ^{13}C labeling. Elution of the tube contents coupled to fraction collection (Lueders et al., 2004a) has provided the greatest sensitivity, and has yielded the only success to date for ^{15}N enrichment with soil samples (Cupples et al., 2006), however the approach is quite tedious and labor intensive. In some cases, separated heavy DNA has been further purified by additional ultracentrifugation (Radajewski and Murrell, 2002). The presence of background DNA (unlabeled DNA distributed throughout the gradient) can be a serious problem, especially if target DNA is in low abundance or poorly enriched (Lueders et al., 2004a). Figure 2 illustrates the

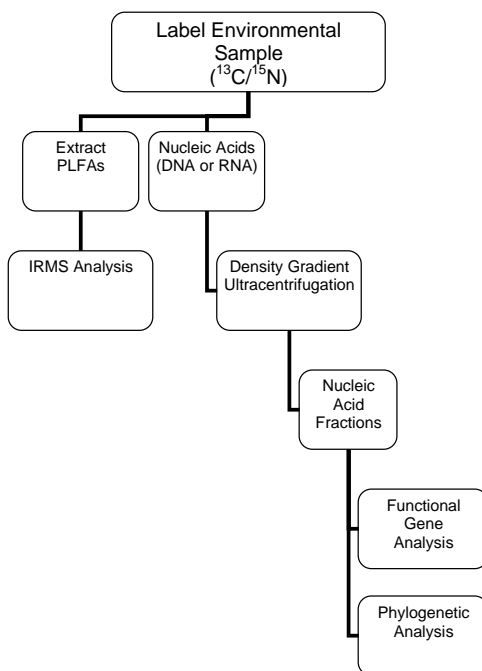


Figure 1. Flow chart for basic steps in stable isotope probing. In practice, all of these schemes will result in production of substantial data sets, the interpretation of which may become a greater task than the laboratory work.

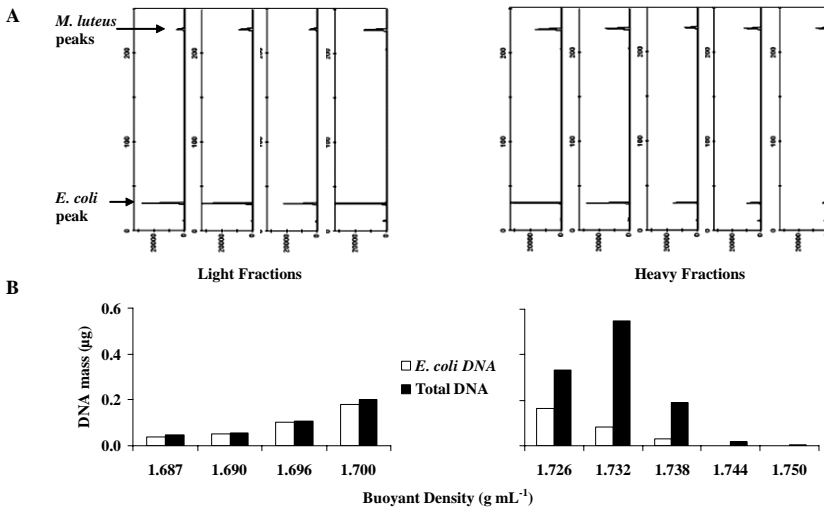


Figure 2. (A) TRFLP electropherograms of HaeIII digests of DNA from lightest and heaviest buoyant density fractions containing both unlabeled (^{14}N) and labeled (^{15}N) *E. coli* (31 bp) and *M. luteus* DNA (227 and 228 bp). All four DNA types were centrifuged together. (B) The proportional quantity of *E. coli* DNA measured by real time quantitative PCR present in corresponding fraction.

broad distribution of both labeled and unlabeled DNA from two organisms centrifuged in the same gradient. Also shown is the utility of real time PCR for revealing the dominant form of DNA in a particular fraction. Inclusion of an internal standard to assist in finding the appropriate DNA pool can also be used to deal with background (Singleton et al., 2005). It is possible to enhance the recovery of trace quantities of bacterial ^{13}C -DNA by including ^{13}C -archaeal “carrier” DNA in the gradient (Gallagher, et al., 2005). The latter approach may also be possible with ^{15}N .

An increase in the buoyant density (BD) of isotopically-enriched nucleic acids is assumed to correspond to cells that assimilated the target compound. Recovered DNA fractions can then be characterized by a number of available approaches, most commonly using PCR-based methods that amplify rRNA genes or functional genes, in some cases including T-RFLP analysis (terminal restriction fragment length polymorphism) in which DNA is cut with restriction enzymes and the resulting fragments separated and quantified by capillary electrophoresis (Clement et al., 1998; Liu, et al., 1997; Osborn et al., 2000). Individual organisms will have characteristic T-RFLP patterns when several enzymes are used. This has been used to complement SIP by performing T-RFLP analysis on whole soil DNA (without the density gradient

separation) and searching for substrate response in the relative abundance of the fragments that had been found to be enriched using SIP. Cupples and Sims (2006) were able to use this approach to show that an organism detected using SIP was apparently growing in the soil in response to added substrate. The organism can be identified by cloning the heavy DNA (into *Escherichia coli*), sequencing the insertions and comparing them to a sequence database (e.g., GenBank). Other techniques such as microarrays have also been used. Analysis of RNA may require examination of numerous fractions as the labeled RNA will tend to be dispersed through the gradient. This has been accomplished using RT-PCR and DGGE (Manefield et al., 2002). In RT-PCR (reverse transcriptase-PCR), cDNA (complementary DNA) is synthesized from RNA by reverse transcription and the cDNA is amplified by PCR. DGGE (denaturing gradient gel electrophoresis), is used to separate DNA fragments of the same length but different sequences based on decreased electrophoretic mobility of a partially melted double-stranded DNA molecule in a polyacrylamide gel containing a gradient of DNA denaturants. Some of these analysis techniques produce very large data sets which may pose a serious challenge for interpreting results.

Available isotopes for nucleic acid-SIP

Though SIP might be possible using substrates containing isotopically-labeled C, H, O, or N (all are present in nucleic acids), it appears that C is by far the most practical, with very little work done with N-labeled substrates (Cadisch et al, 2005; Cupples et al., 2006) and none reported for either H or O, likely due to sensitivity limitations (Radajewski et al., 2003). Figure 3 (Cupples et al., 2006) illustrates the relative shifts in buoyant density observed when ^{13}C , ^{15}N , or both labels are incorporated into DNA at a very high level of enrichment. Figure 4, also taken from Cupples et al. (2006) shows the shifts in BD from ^{15}N enrichment of *E. coli* and *Micrococcus luteus* (GC contents ~50 % and ~72 % respectively). Though the approach used in the study has been subsequently refined to improve sensitivity, it is apparent that ^{15}N labeling is inherently insensitive owing to the rather small change in DNA buoyant density. Nucleic acids from a mixed bacterial community exhibit a range of BD, owing largely to differences in the relative proportions of AT versus GC, with GC contents of chromosomal DNA ranging from 25 to 80 mol% among species. This natural range of BD for nucleic acids is larger than the change in BD achieved by ^{15}N enrichment (Cupples et al., 2006; Meselson and Stahl, 1958) thus requiring careful use of unlabeled controls to tease out the effects of labeling, particularly for organisms with relatively low GC content (and thus lower buoyant density). Though inherently operating at or near the detection limit, ^{15}N -SIP should not be discounted as the benefits from its successful application will justify the risk of failure for many researchers. The primary

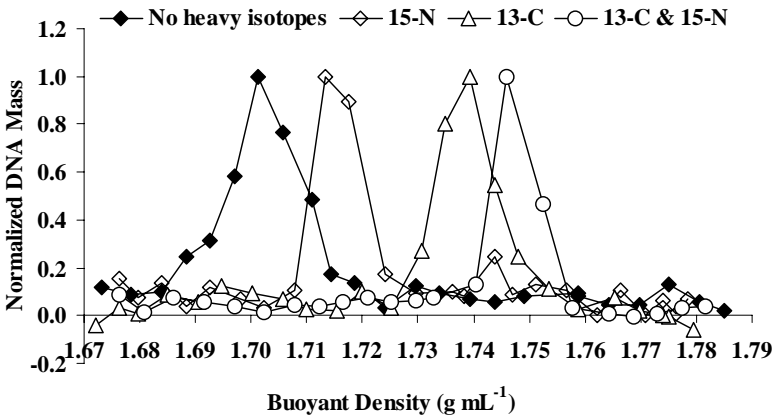


Figure 3. Distribution of *E. coli* DNA over a range of BD values following single or dual isotope enrichment treatments. Each DNA type was centrifuged independently. DNA mass is normalized to highest value for each DNA type due to variability in DNA recovery (reproduced from Cupples et al., 2006).

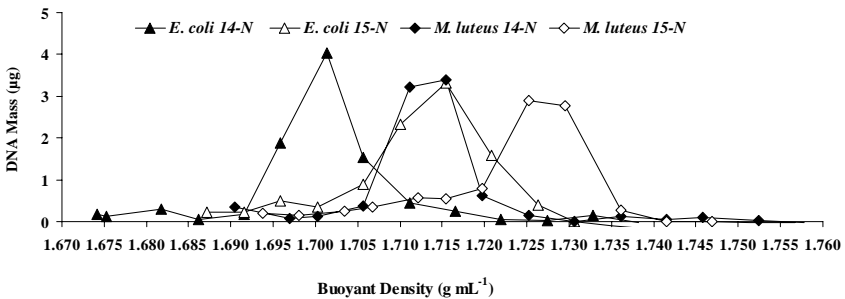


Figure 4. Distribution of unlabeled (^{14}N) and labeled (^{15}N) *E. coli* DNA and unlabeled (^{14}N) and labeled (^{15}N) *M. luteus* DNA over a range of BD values. Each DNA type was centrifuged independently and data from one replicate of each is represented above. Replicate samples had similar distributions (data not shown).

concern for using ^{15}N -DNA SIP, compared to ^{13}C -DNA SIP, is the nitrogen content of DNA, which limits the potential BD increase following ^{15}N incorporation into target DNA.

Applications of nucleic acid SIP

Numerous applications of DNA-SIP are now available to provide an assessment of the utility of this approach. To date, the method has been

applied to assimilation of a variety of compounds, such as glucose, caffeine, (Padmanabhan et al., 2003), naphthalene (Padmanabhan et al., 2003; Yu and Chu, 2005), phenol (DeRito et al., 2005; Manefield et al., 2002; Padmanabhan et al., 2003), methanol (Lueders et al., 2004c), methane (Morris et al., 2002), propionate (Lueders et al., 2004a) methyl bromide, methyl chloride (Miller et al., 2004), pentachlorophenol (Mahmood et al., 2005), ammonium (Cupples et al., 2006) and 2,4-D (Cupples and Sims, 2006).

Lin et al. (2004) applied DNA-SIP to identify the active members of the methanotroph community in a soda lake. This study provided results that were precisely what SIP was meant to accomplish. Though a large diversity of methanotrophs, representing Methylococcaceae and Methylocystaceae had been detected in the sediments using PCR, only a small fraction of these organisms, primarily Methylococcaceae, proved to be active when ^{13}C -DNA-SIP was used. The use of $^{13}\text{CO}_2$ to label rice plants provided a useful substrate to examine CH_4 production from plant-derived carbon (Lu and Conrad, 2005). In this study RNA-SIP detected enriched Archaea belonging to a group that has not yet been isolated. Several other DNA-SIP studies have focused on C_1 compounds and methylotrophs, which can use these substrates as sole sources of carbon (Borodina, et al., 2005; Ginige, et al., 2004; Hutchens, et al., 2004; Leuders et al., 2004; Miller et al., 2004; Radajweski and Murrell, 2002). Webster et al (2006) enriched sediments with ^{13}C -glucose and acetate to study anaerobic sulfate-reducing (prokaryotic) communities. This was the first study to compare PLFA-SIP and DNA-SIP directly, and found that the techniques were complementary and amenable to use with low concentrations of labeled substrate.

SIP has also been used with some success in examining the ecology of biodegradation of various organics pollutants as noted above. DeRito et al (2005) applied ^{13}C -SIP to examine the flow of phenol carbon through a soil microbial community. This study included the novel approach of imaging the ^{13}C -treated soil with secondary ion mass spectrometry to demonstrate labeling of bacterial cells. Kasai et al. (2006) examined organisms responsible for benzene degradation in a gasoline-contaminated aquifer with ^{13}C (benzene)-SIP using DGGE to examine RT-PCR amplified extracts. The authors reported activity of an organism related to *Azoarcus* only in the presence of nitrate. Padmanabhan et al. (2003) applied ^{13}C -SIP in the field to study the fate of phenol and naphthalene. Singleton et al. (2005) examined bacteria involved in degradation of salicylate, naphthalene, and phenanthrene during soil remediation in a bioreactor. Jeon et al (2003) demonstrated the use of SIP for studying biodegradation in the field and were able to identify a novel naphthalene-degrading isolate (*Polaromonas naphthalenivorans* sp. nov.) (Jeon et al., 2004). Perhaps the first use of SIP to examine biodegradation of a herbicide involved the use of ^{13}C -labeled 2,4-D (Cupples and Sims, 2006), one

of the few herbicides for which a considerable amount of information is available on organisms involved in its degradation. In this study, it was observed that the T-RFLP profiles of soil DNA revealed the presence of at least one organism that appeared to be responding (based on relative abundance) to the presence of the herbicide. When ^{13}C -labeled 2,4-D was introduced, this same terminal restriction fragment was shown to become labeled, indicating assimilation of the herbicide (Figure 5). The organism appeared to be a member of the β subdivision of the Proteobacteria, representatives of which have been previously shown to degrade 2,4-D (Cavalca, 1999). Cupples et al. (2006) observed labeling of several operational taxonomic units (OTUs) during T-RFLP analysis of heavy DNA from an ^{15}N -ammonium SIP study (Figure 6).

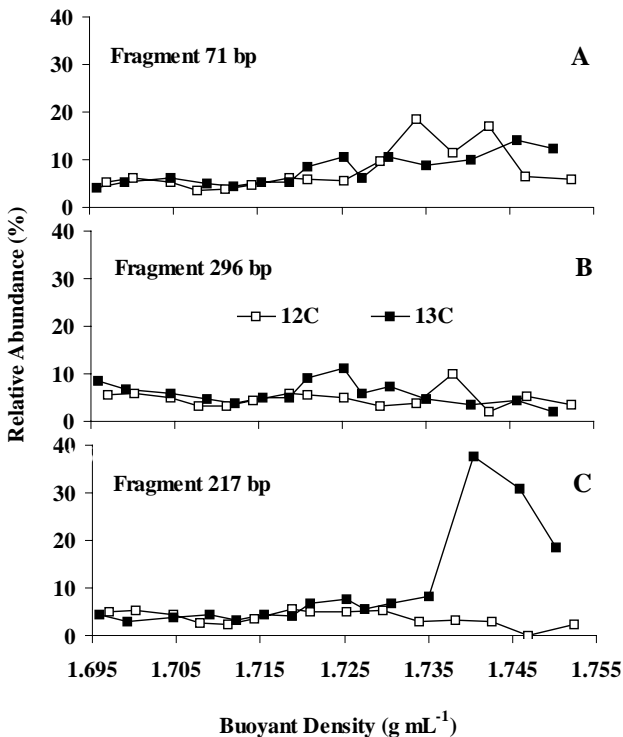


Figure 5. Comparison of relative abundance of the three dominant fragments over a range of buoyant density (BD) from DNA extracted (day 17) from soil amended with either labeled (^{13}C) or unlabeled (^{12}C) 2,4-D. Replicate soil TRFLP data from day 17 and 7 illustrated the same trend (reproduced from Cupples and Sims, 2006).

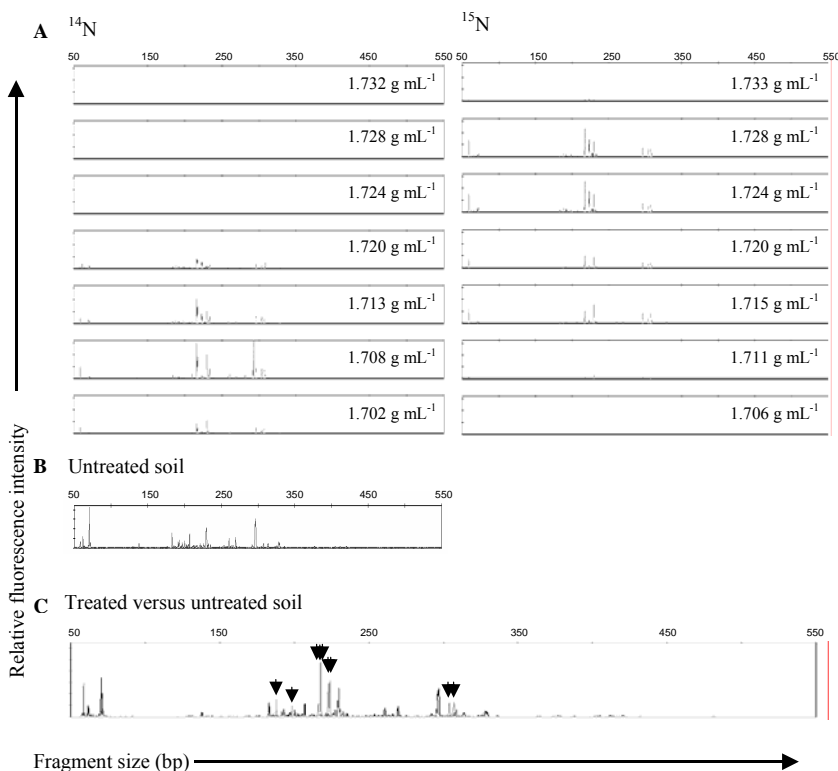


Figure 6. DNA T-RFLP profiles from soils supplied with $(^{14}\text{NH}_4)_2\text{SO}_4$ (control) or $(^{15}\text{NH}_4)_2\text{SO}_4$ over a gradient of buoyant densities (A). The replicate treatment illustrated the same trend. DNA T-RFLP profile from unamended soil (B). Overlay of profiles from unamended (black) and amended soils (gray), illustrating the 9 dominant OTU fragments (arrows) resulting from the amendments (C).

Limitations of nucleic acid-SIP

Nucleic acid based SIP methods are subject to the various weaknesses of molecular methods, such as PCR bias and difficulties in recovering suitable quality nucleic acids from soil samples. In addition, timing of nucleic acid extraction relative to introduction of label must be experimentally determined. Sufficient incubation time must be allowed in order to achieve the minimum enrichment for detecting a BD shift, however too long an incubation may result in dispersal of label among organisms not directly involved in the process of interest (cross feeding), though this may also be an experimental

goal. The use of a kinetic curve seems an obvious approach in this regard, however if each potentially labeled fraction from each replicate in every time point is subjected to analysis, such as T-RFLP, the resulting labor costs and data analysis rapidly become unmanageable. Various controls have been used to facilitate detection of enrichment, with unlabeled controls being the most common approach used. One of the primary advantages of RNA-SIP over DNA-SIP is the potentially short incubation times possible (RNA synthesis occurs at a faster rate). However, for soil incubations, extremely short incubation times may result in problems with insufficient substrate distribution, particularly in unsaturated soils.

Combining SIP with process measurement

SIP is amenable to incorporation into label studies primarily designed for process measurement. The key concerns for combining SIP with other measurements are the labor requirement for SIP (particularly nucleic acid-SIP) and the use of highly enriched substrates (typically 99 atom %), which can pose a risk for contamination in analytical labs specializing in low-level isotope work (as is common in process analysis). The addition of small quantities of ^{14}C into ^{13}C SIP studies will not affect the resulting DNA buoyant density or PLFA signatures, but can have advantages for tracing material flow through the experimental system, and has potential for locating the labeled DNA fractions. We have found this approach particularly useful for combining SIP with biodegradation studies. Since our group is interested in bioavailability questions, the addition of a ^{14}C label facilitates a variety of measurements useful in determining bioavailability (e.g. whether the material is in solution, in a labile sorbed phase, or an irreversibly sorbed phase).

Use in undisturbed soil systems

The failings of most techniques become apparent when they are applied to “undisturbed” environmental samples. SIP has primarily been promoted as a tool for examination of processes *in situ*, thus minimal disturbance has been encouraged. For a liquid culture, wastewater treatment system, bioreactor, or aquatic habitat, the key to success will be introducing the substance and taking samples without changing the chemistry of the test system. The problem becomes much more complex when the approach is applied to structured soil. Though unsaturated soils have been examined with SIP (see above), the technique has been primarily employed with saturated sediments or aqueous systems, in which relatively uniform introduction of the labeled substrate may be possible without extensively disturbing the experimental system. Use in unsaturated soils, however, is subject to the same issues that have always

plagued soils research, not the least of which is the effect of the strategy used to introduce the substrate on substrate bioavailability or microbial activity.

The enormous diversity of microorganisms present in soils is due, in part, to the extensive compartmentalization of the soil system (Zhou et al., 2004). Compartmentalization is greatest in structured soils, in which various hierarchies of organization (intra-aggregate, inter-aggregate, etc.) impose limits on the potential for introducing a test substance into all of the inhabited soil compartments without destroying soil structure. Many substances of environmental interest exhibit limited solubility, and thus if required at relatively high concentrations (as may be the case for herbicides), must be introduced either with an organic solvent or very large volumes of water. Either approach may alter the experimental system. Obviously, organic solvents may prove to be toxic to some organisms present, or conversely, may prove to be a better substrate than the test substance. Tor et al. (2000), faced with the necessity of introducing trifluralin in a solvent (the herbicide is only soluble in water at 0.3 mg/L), determined the effect of the added solvent on the experimental system (anaerobic) and found significant stimulation of iron reduction, a process that was found to be linked to trifluralin fate. Substances introduced into undisturbed soils as aqueous solutions will likely be concentrated in a subset of the micropore space if application volumes are small enough to avoid flow into macropores, whereas materials carried into soil columns by saturated flow move preferentially through a very small portion of the soil macropore volume. For example, Watson and Luxmoore indicated that 96% percent of the flow through a forest watershed occurred through 0.32% of the pore volume via preferential flow paths. This raises serious concerns about the distribution of introduced substances with respect to the distribution of microorganisms.

For many substances, bioavailability is the key factor limiting biodegradation (Sims and Cupples, 1999), thus water content, which dictates the mean aqueous cross section available for transport, may determine the proportion of the total diversity of organisms that are exposed to the test substrate. Substances moving primarily through solution tend to permeate the pore space more quickly at higher water contents, whereas movement through the vapor phase is facilitated by lower water content. As researchers move to short-term incubations, as recommended for RNA-SIP, the kinetics of distributing the test substance in the experimental system will become more of an issue. Figure 7 provides some insight into the nature of redistribution of a substance introduced into a structured soil. Fifty aggregates from a Drummer silty clay loam (Fine-silty, mixed, superactive, mesic Typic Endoaquolls), were placed into each of five replicate glass vials and treated with 15 drops (130 μ L) of a radiolabeled test substance dissolved in methanol. At each sample time point (up to 101 hours), all of the aggregates from a particular vial

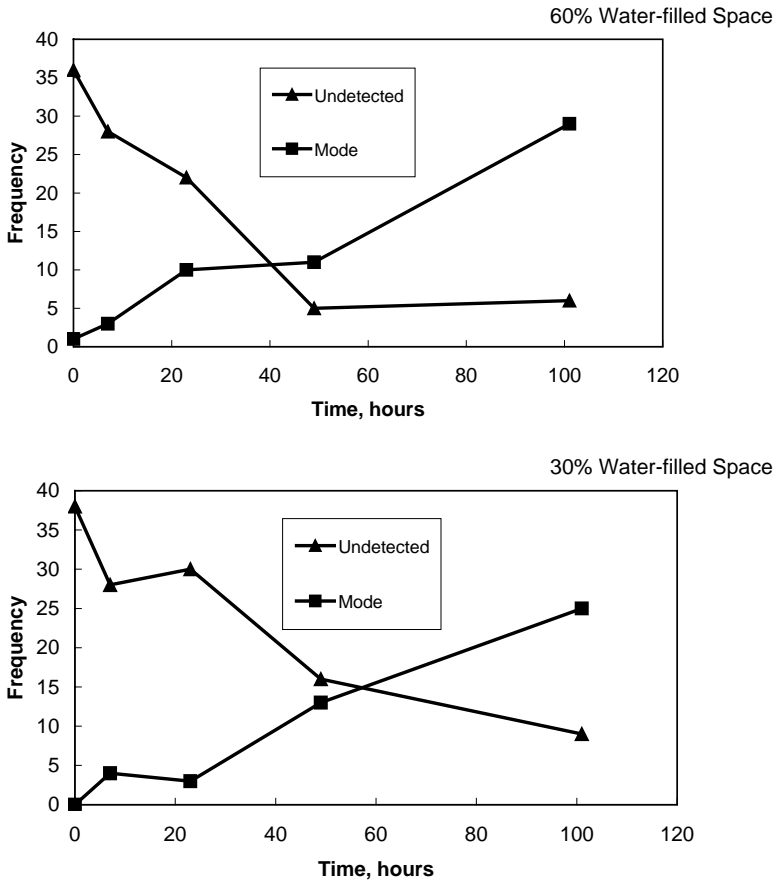


Figure 7. Flow chart for basic steps in stable isotope probing. In practice, all of these schemes will result in production of substantial data sets, the interpretation of which may become a greater task than the laboratory work.

were destructively analyzed for total radioactivity. Results, expressed as a percentage of total applied radioactivity were binned as whole percentages and the frequencies of particular outcomes plotted as a function of time. It is clear that the number of aggregates devoid of radioactivity (undetected) decreases over time while the number of aggregates most resembling the final outcome (mode) increases. The time required to achieve an experimental system in which the distribution of material is suitable for taking measurements indicative of microbial activity for the function of interest appears to be on the order of days rather than hours or minutes. While the question of distribution

in an unsaturated soil provides some challenges for SIP in the early stages of incubation, other issues related to bioavailability will persist throughout the experiment. For example, sorption controls the availability of many materials in soils, and depending on the mechanism of sorption, release of sorbed material back to the solution phase may be instantaneous or perhaps rate limiting for the overall process. As a result, knowledge of the physicochemical characteristics of the experimental system and the behavior of the test substance in soil may be critical to interpreting results. Owing to the serious bioavailability restrictions that are possible in soils, the experimental question at hand may ultimately drive the choices made in introducing a test material.

Based on the observations above, it seems appropriate to use SIP as part of an experimental approach that provides insight into the bioavailability of the substrate. Unfortunately, SIP can be relatively tedious to employ, and the data obtained may be difficult to interpret, thus when combined with other measurements, SIP studies have the potential to rapidly become unwieldy. Addressing bioavailability may thus be a hard sell for practitioners of SIP. Moreover, even simple biodegradation experiments seldom include data necessary to assess the extent to which the test substrate was available to soil microorganisms. While water content is typically indicated, water availability (water potential or fraction of water filled pore space) is not often characterized. The physical condition of the soil aggregates after various manipulations and wetting are generally not reported. Similarly, the portion of substrate present in solution, or at least its sorption properties (K_d) in the test soil are rarely known when microbial ecology is the focal point of a biodegradation experiment. In order for SIP to reflect which organisms are active, rather than just which ones were exposed to the substrate, some attention should be paid to identifying convenient approaches for assessing bioavailability.

Future directions

Isotopic enrichment of cellular constituents is not a new approach, and has long been used for examining the ecology of soil microbial communities. Bichat et al., 1999 used $^{15}\text{N}/^{14}\text{C}$ -dual labeled atrazine to examine the use of the atrazine ring as a nitrogen source in soil. The use of the two labels revealed sorting of N and C from the atrazine ring, using incorporation into soil amino acids as an indicator of microbial assimilation. This work was an extrapolation to soil communities of basic findings obtained from pure culture studies. Marsh et al., (2003) developed a method for detecting $^{14}\text{C}/^{15}\text{N}$ from amino acids isolated from bulk soil and subsequently applied this method to demonstrate utilization of urea carbon for biomass production (Marsh et al., 2005), presumably by ammonia oxidizing bacteria (AOB). The ratio of incorporation of ^{14}C and ^{15}N labels into amino acids was consistent with the

nutritional/energy requirements of AOB. This study was also founded on culture studies that established the precedent for the research hypothesis using pure cultures of the organisms of interest. Both of the above studies are examples of research questions that have recently been addressed with conventional approaches that may have been particularly well-suited to SIP.

At present, the SIP literature still contains a preponderance of methods development work, with a relatively limited number of applications of the technique to real samples. Whereas the many improvements recommended in the various methods papers and other reviews of SIP methodology certainly should be acted upon, it seems at least as important that more results become available using SIP as is practiced at present. It will be difficult to judge the utility of SIP if the bulk of the literature in which the technique is employed remains focused on improvements in the method or cataloging additional techniques with which it might be paired. The future direction for SIP thus should be primarily in the application of the technique to address research questions about microbial function. One fruitful area for application of SIP to soils research may be examining whether agricultural inputs (pesticides, fertilizers) impact which organisms are responsible for particular functions and if there is potential to influence these function through such changes in microbiology. SIP might also be used to help gain insight into the role of microorganisms in soil fertility and how that role might be measured for predicting nutrient availability to plants.

Conclusions

Most approaches for *in situ* phylogenetic characterization of soil microorganisms lack the ability to establish a causal relationship to function within the community. Stable isotope probing has arrived as an important technique for achieving this goal, and can be used in soils. The technique is not universally applicable, nor is it compatible with every laboratory infrastructure, and there are definite limitations to the sensitivity of nucleic acid-based SIP methods. Given those caveats, SIP will obviously not prove to be a panacea for soil microbiologists, however, the primary limitation is not with the utility of the technique, but with the limited number of studies that have employed SIP to test ecological hypotheses. Carbon labeling is considerably more sensitive than N labeling, and is thus more generally useful. Application of SIP techniques to unsaturated soils should be treated with the same caution as other attempts to address function in soils. Of particular concern is the issue of bioavailability of the test substance, which is often neglected in ecological studies. SIP has proven to be useful for a variety of processes, such as organic matter turnover, biodegradation of organic pollutants, nitrification, nitrogen fixation, and plant-microbe interactions.

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