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DNA buoyant density shifts during ^{15}N -DNA stable isotope probing

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Summary

DNA-based stable isotope probing (SIP) is a novel technique for the identification of organisms actively assimilating isotopically labeled compounds. Herein, we define the limitations to using ^{15}N -labeled substrates for SIP and propose modifications to compensate for these shortcomings. Changes in DNA buoyant density (BD) resulting from ^{15}N incorporation were determined using cultures of disparate GC content (*Escherichia coli* and *Micrococcus luteus*). Incorporation of ^{15}N into DNA increased BD by $0.015 \pm 0.002 \text{ g mL}^{-1}$ for *E. coli* and $0.013 \pm 0.002 \text{ g mL}^{-1}$ for *M. luteus*. The DNA BD shift was greatly increased (0.045 g mL^{-1}) when dual isotope (^{13}C plus ^{15}N) labeling was employed. Despite the limited DNA BD shift following ^{15}N enrichment, we found the use of gradient fractionation, followed by a comparison of T-RFLP profiles from fractions of labeled and control treatments, facilitated detection of enrichment in DNA samples from either cultures or soil.

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Introduction

Nucleic-acid-based stable isotope probing (SIP) is a novel method that facilitates the examination of links

between metabolic function and taxonomic identity, and precludes the need for culture isolation. SIP entails the exposure of microbes to labeled target compounds, and the recovered pool of cellular nucleic acid is separated by density gradient centrifugation. A resulting increase in the buoyant density (BD) of isotopically enriched nucleic acids corresponds to cells that assimilated the target compound. The recovered nucleic acid fractions can then be characterized by a number of available

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molecular-based methods. Though SIP might be possible using substrates containing isotopically labeled C, H, O or N, to date, the technique has been applied almost exclusively to C-labeled compounds (Radajewski et al., 2003). Clearly our investigative opportunities could be greatly expanded with the use of other isotope labels (^{15}N , ^2H , ^{18}O).

The use of ^{15}N -DNA-based SIP is particularly attractive because of its potential for investigating microbial processes such as environmental nitrogen cycling, or the biodegradation of environmentally important nitrogen-containing compounds. For example, ^{15}N -DNA SIP could be utilized to investigate the biodegradation of the nitrogen-based pollutants 2,4,6-trinitrotoluene (TNT) and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) (Keith and Telliard, 1979; Van Aken et al., 2004) or to expand on previous research addressing the fate of nitrogen from agrochemicals (Bichat et al., 1999). The primary concern for using ^{15}N -DNA SIP, compared to the already established method of ^{13}C -DNA SIP, is the nitrogen content of DNA, which limits the potential BD increase following ^{15}N incorporation into target DNA. Owing to differences in GC content among bacterial taxa, the range in BD of unlabeled DNA is significant (Lueders et al., 2004a) compared to the change in BD expected from ^{15}N enrichment (Meselson and Stahl, 1958). Based partly on theoretical values, one group of researchers (Cadisch et al., 2005) concluded that it should be possible for ^{15}N SIP to resolve labeled from unlabeled DNA among organisms of differing guanine–cytosine (% GC) contents, though this remains to be proven empirically.

In this study, we quantify the DNA BD shifts associated with ^{15}N -DNA SIP using two organisms (*Escherichia coli* and *Micrococcus luteus*) of disparate (medium and high) GC content ($\sim 50\%$ and $\sim 72\%$ for *E. coli* and *M. luteus*, respectively (Orskov, 1984; Stackebrandt et al., 1995)) using a simple substrate ($^{15}\text{NH}_4\text{Cl}$) to maximize enrichment. Additionally, we report for the first time the effect of dual labels (^{15}N and ^{13}C) on the separation of light and heavy DNA. We also examine the effectiveness mixed DNA separation (^{14}N - and ^{15}N -DNA from *E. coli* and *M. luteus*) using terminal restriction fragment length polymorphism (T-RFLP) and quantitative real-time PCR. Finally, we investigate the feasibility of ^{15}N -DNA SIP with mixed soil communities.

Materials and methods

Strains and culture conditions

E. coli (ATCC 15524) was grown overnight (shaking at 37°C) on LB (Fisher Chemicals) and

transferred (4% v/v) twice successively to a phosphate-buffered (pH 7) minimal medium (Fries et al., 1994) modified to contain glucose (1 g L^{-1}) as the sole carbon source, and $^{15}\text{NH}_4\text{Cl}$ (98+ at%, Isotec, Miamisburg, OH) or unlabeled NH_4Cl (0.35 g L^{-1}) as the sole source of assimilable nitrogen. *M. luteus* (ATCC 49442) was grown (shaking at 25°C) on ATCC medium 1780 modified by substituting pyridine with glucose (1 g L^{-1}) and NH_4Cl (0.35 g L^{-1}) and transferred (10% v/v) twice successively to minimal medium containing glucose and unlabeled NH_4Cl or $^{15}\text{NH}_4\text{Cl}$ (as described above) but supplemented with thiamine ($2\text{ }\mu\text{M}$ (Sims et al., 1986)). To investigate SIP with dual label, *E. coli*, following growth on LB, was transferred (4% v/v) twice successively on minimal medium modified to contain DL-lactate (1.3 g L^{-1}) as the sole carbon source and NH_4Cl (0.35 g L^{-1}) as the sole nitrogen source as follows: unlabeled lactate (Sigma) with unlabeled NH_4Cl (0.35 g L^{-1}); unlabeled lactate with $^{15}\text{NH}_4\text{Cl}$ (0.35 g L^{-1}); $^{\text{L}}\text{-}^{13}\text{C}$ -lactate ($^{13}\text{C}_3$, 98+ at%, Cambridge Isotope Laboratories, Inc., Andover, MA) with unlabeled NH_4Cl ; or ^{13}C -lactate with $^{15}\text{NH}_4\text{Cl}$. Following growth on minimal media, DNA was extracted from cultures as described below. Additionally, washed cell pellets were subject to isotopic analyses by the ^{15}N Analysis Service at the University of Illinois, as previously described (Khan et al., 1997).

Soil experiment

Glucose (Sigma) ($12.5\text{ mg g soil}^{-1}$), $(^{15}\text{NH}_4)_2\text{SO}_4$ (^{15}N , 99.4 atom %, Isotec) or unlabeled $(\text{NH}_4)_2\text{SO}_4$ (Fisher Scientific) ($0.68\text{ mg g soil}^{-1}$) and water (50% water holding capacity) were added to samples (0.5 g, dry weight) of a Westola soil (coarse-loamy, mixed, superactive, calcareous, thermic Typic Ustifluvents), and following incubation (30°C) for 1 week, DNA was extracted with the UltraClean Soil DNA Kit (MoBio Laboratories, Inc.), following the manufacturer's instructions.

DNA extraction and CsCl density gradient ultracentrifugation

Cells were harvested during late exponential growth and DNA was extracted from the pure cultures with a DNeasy tissue system (Qiagen, Inc, Valencia, CA), following the manufacturer's instructions (Gram-positive protocol for *M. luteus*). Throughout this study, DNA was quantified by fluorometry with the PicoGreen nucleic acid quantification dye (Molecular Probes),

according to the manufacturer's instructions, on an Opticon 2 Real Time Thermal Cycler (MJ Research, Bio-Rad Laboratories, Hercules, CA) as previously described for the ABI Prism 7700 sequence detection system (Tian and Edenberg, 2004).

Ultracentrifugation was performed in Quick-Seal polyallomer tubes (13 × 51 mm, 5.1 mL, Beckman Coulter, Fullerton, CA) in an Optima LE-80K Preparative Ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA) equipped with a VTi 65.2 vertical tube rotor for 30 h, 228 000 g_{av} (20 °C). BDs were measured with a AR200 digital hand-held refractometer (Leica Microsystems Inc., Buffalo, NY). Ultracentrifugation conditions for all experiments were the same except for the type and amount of DNA added, and the BD of the initial Tris-EDTA (TE, pH 8.0)/CsCl solution. The CsCl/TE starting BD was adjusted for optimal gradient formation depending on the target DNA BD. Following centrifugation, water was pumped into the top of the centrifuge tube and fractions were collected at the bottom (150 μ L) as previously described (Lueders et al., 2004a), the BD of each fraction was determined, CsCl was removed (Griffiths et al., 2000; Lueders et al., 2004a) and DNA in each fraction was quantified as described above.

PCR and T-RFLP analyses

Fractions containing pure culture, mixed isotope DNA (14 N- and 15 N-DNA) and soil DNA were analyzed by 16S rDNA T-RFLP following standard procedures (Liu et al., 1997). PCR primers (Operon Biotechnologies) utilized were 27F-FAM (5'-AGAGTTT-GATCMTGGCTCAG, 5' end-labeled with carboxy-fluorescein) and 534R (5'-ATTACCGCGGCTGCTGG) for pure culture DNA or 1492R (5'-GGTTACCTTG-TACGACTT) for soil DNA. PCR mixtures (100 μ L) included the TaKaRa Ex Taq mixture (Takara Bio), primers (45 pmol each) and 5 μ L DNA sample. The PCR program was: 94 °C (5 min); 94 °C, 55 °C, 72 °C (1.5 min) (25 or 29 cycles); 72 °C (10 min). PCR products were purified with the QIAquick[®] PCR purification kit (Qiagen Inc.), following the manufacturer's instructions. The purified PCR product was digested with *Hae*III according to the recommended protocol (New England Biolabs). DNA fragments were separated by capillary electrophoresis (model 3730xl Genetic Analyzer, Applied Biosystems) at the W.M. Keck Center (UIUC). Data were analyzed with GeneMapper V3.7 software (Applied Biosystems).

Analyses of *E. coli* DNA by quantitative real-time PCR

Quantification of the *E. coli*-specific 16S rDNA was performed by real-time PCR amplification (Opticon 2 Real Time Thermal Cycler) using previously described primers (Huijsdens et al., 2002) and QuantiTect SYBR[®] Green PCR Master Mix (Qiagen), following the manufacturer's instructions. PCR conditions were: 95 °C (15 min); 94 °C (10 s), 60 °C (20 s), 72 °C (30 s) (26 cycles); then 72 °C (7 min). Melting curves were performed from 50 °C to 95 °C (read every 1 °C). Standard curves were generated using serial dilutions of genomic *E. coli* DNA.

Results and discussion

14 N- and 15 N-DNA separation when centrifuged individually

The distributions of *E. coli* and *M. luteus* DNA following ultracentrifugation over a range of BD values, both with and without 15 N incorporation, are illustrated in Fig. 1. As expected, based on the GC contents of *E. coli* (~50%) and *M. luteus* (~72%) (Orskov, 1984; Stackebrandt et al., 1995), unlabeled *E. coli* DNA was found at lower BD values (1.701 ± 0.001 g mL⁻¹, average peak DNA mass, $n = 3$) than unlabeled *M. luteus* DNA (1.715 ± 0.001 g mL⁻¹, $n = 3$). Similarly, 15 N-labeled *E. coli* DNA was present in fractions with lower BD values (1.715 ± 0.002 g mL⁻¹, $n = 3$) compared to 15 N-labeled *M. luteus* DNA (1.727 ± 0.002 g mL⁻¹, $n = 3$). The DNA BD increase following 15 N incorporation was 0.015 ± 0.002 g mL⁻¹ ($n = 3$) for *E. coli* and 0.013 ± 0.002 g mL⁻¹ ($n = 3$) for *M. luteus*. Isotopic analyses indicated *M. luteus* and *E. coli* cells contained 87.7 and 88.0 at% 15 N, respectively.

To date, only one report has addressed the use of 15 N for DNA SIP. These investigators (Cadisch et al., 2005) quantified the width (mm) of separation of unlabeled and 15 N-DNA bands from *Pseudomonas putida* by ultracentrifugation using ethidium bromide staining to visualize DNA bands. However, without quantitative data on the increase in DNA BD caused by 15 N incorporation, these researchers were unable to compare their results to the range of DNA BD values found in natural communities (difference between highest and lowest DNA BD of ~ 0.03 g mL⁻¹ (Lueders et al., 2004a)). Our results suggest the limited increase in DNA BD caused by 15 N incorporation could result in unlabeled, heavy DNA (high GC content) co-eluting with lower BD

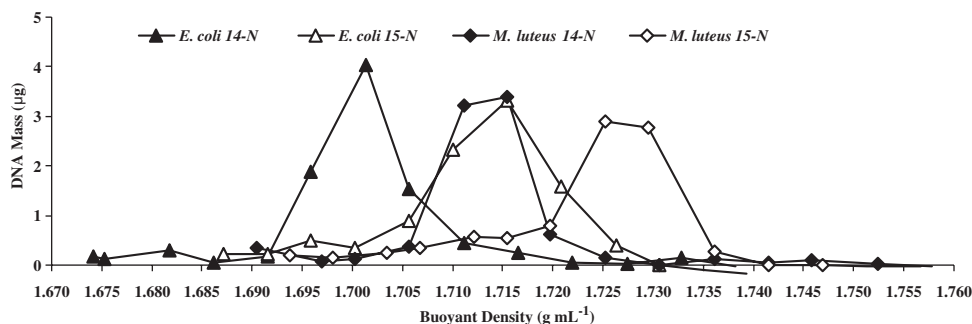


Figure 1. Distribution of unlabeled (^{14}N) (closed triangles) and labeled (^{15}N) (open triangles) *E. coli* DNA and unlabeled (^{14}N) (closed diamonds) and labeled (^{15}N) (open diamonds) *M. luteus* DNA over a range of BD values. Each DNA type was centrifuged independently and data from one replicate of each are represented above. Replicate samples had similar distributions (data not shown).

^{15}N -DNA regions. This concern is illustrated in Fig. 1, where unlabeled *M. luteus* DNA (high GC content) is found at a similar BD as ^{15}N -labeled *E. coli* DNA. Therefore, previously reported methods for ^{13}C -DNA SIP involving removal of the well separated, heavy band may not be appropriate for ^{15}N -DNA SIP.

Separation of mixed DNA by T-RFLP and qPCR

For ^{13}C -DNA SIP researchers have used community analyses methods such as T-RFLP or denaturing gradient gel electrophoresis (DGGE) to compare microbial communities in unexposed samples (no label added) with those from exposed samples (label added) in different BD fractions to identify organisms incorporating the labeled substrate (Manefield et al., 2002; Lueders et al., 2004b). Here, we examined the effectiveness of mixed DNA (^{14}N - and ^{15}N -DNA from *E. coli* and *M. luteus*) separation using T-RFLP. We expected T-RFLP analyses would demonstrate that the various DNA types could be separated and thus found in the BD fractions illustrated in Fig. 1. Although T-RFLP indicated DNA from both organisms was distributed throughout all fractions, the signals were concentrated in the appropriate fractions, i.e., *E. coli* and *M. luteus* signals were dominant in the lighter and heavier fractions, respectively (Fig. 2A). These results indicate that community analysis profiles must be carefully interpreted, as even small DNA residues when coupled with the sensitivity of PCR can mislead identification of target organisms. Quantitative PCR enabled a more precise target DNA BD determination (Fig. 2B), illustrating that *E. coli* DNA was proportionally dominant in fractions corresponding to *E. coli* DNA BD values illustrated in Fig. 1.

Mixed isotope DNA separation

^{15}N -DNA SIP was compared to the more established method of ^{13}C -DNA SIP by examining the BD increase in *E. coli* DNA resulting from the following: ^{15}N incorporation; ^{13}C incorporation; and ^{15}N , ^{13}C incorporation combined. The increase in *E. coli* DNA BD following ^{15}N incorporation (0.012 g mL^{-1}) was less than that resulting from ^{13}C incorporation (0.038 g mL^{-1}) or from both (0.045 g mL^{-1}) combined (Fig. 3). In agreement with our results, previous research with *Methylobacterium extorquens* (Lueders et al., 2004a) found the DNA BD increase following ^{13}C incorporation was 0.04 g mL^{-1} . To date, there are no reports of combining heavy isotopes for SIP; however, our results suggest this would provide a significant advantage (large increase in BD) to the method. Dual isotope SIP should be particularly useful for investigating compounds that could be used as both a nitrogen and carbon source, as this should guarantee a large increase in BD. Conversely, the approach may not be as beneficial if each compound contains only one heavy isotope, as the same microorganism may not be able to use both compounds.

^{15}N -DNA SIP in soil communities

We applied ^{15}N -DNA SIP to mixed community DNA (soil samples), comparing BD profiles from soils exposed to ^{15}N -labeled or unlabeled $(\text{NH}_4)_2\text{SO}_4$, and found DNA BD increased in soils exposed to the label (Fig. 4A). This limited increase (0.008 g mL^{-1}) demonstrates the need to compare unlabeled controls for differentiating candidate taxa exhibiting BD increases due to ^{15}N enrichment. The soil amendments (C, N and water) stimulated a flush in

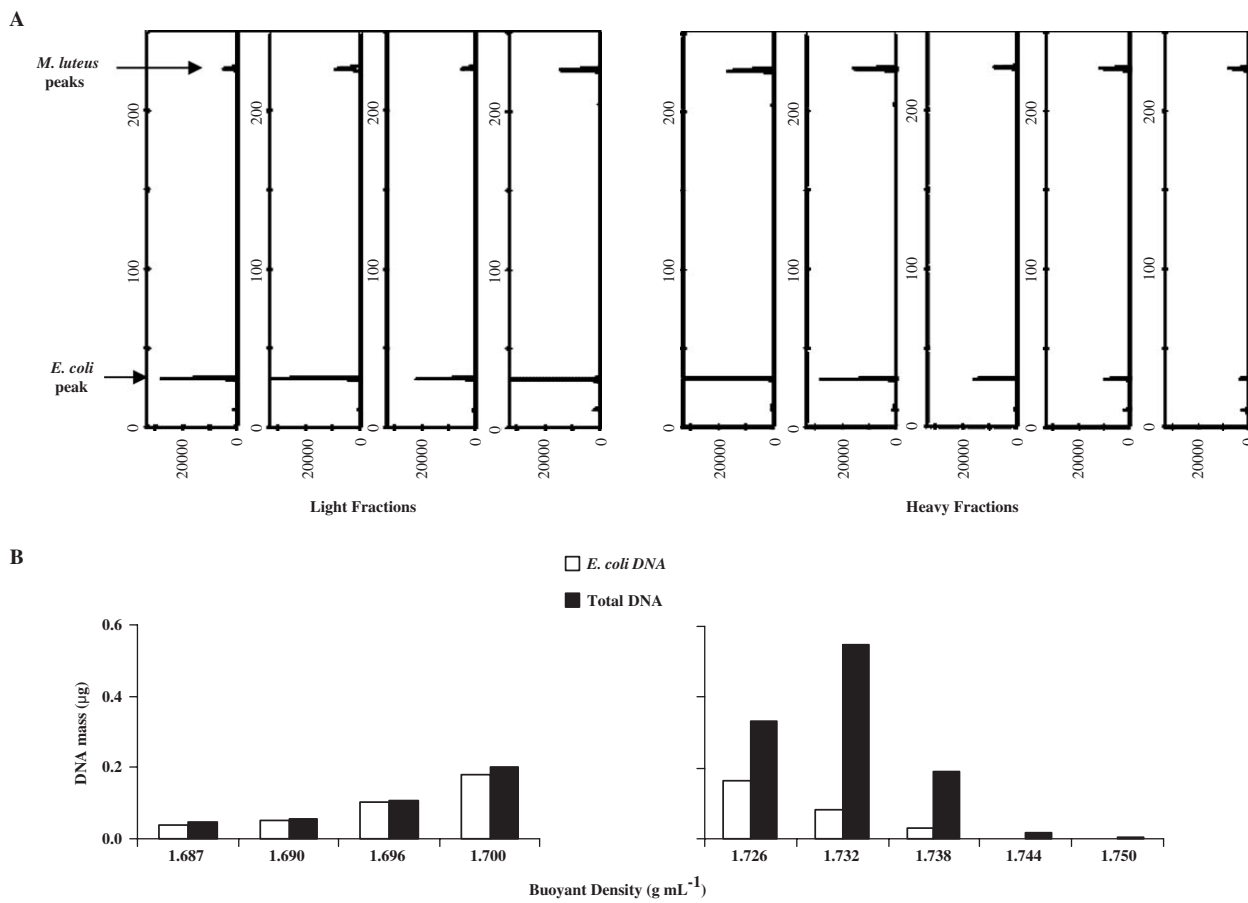


Figure 2. (A) TRFLP electropherograms of HaeIII digests of DNA from lightest and heaviest buoyant density fractions containing both unlabeled (^{14}N) and labeled (^{15}N) *E. coli* (31 bp) and *M. luteus* DNA (227 and 228 bp). All four DNA types were centrifuged together. (B) The proportional quantity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

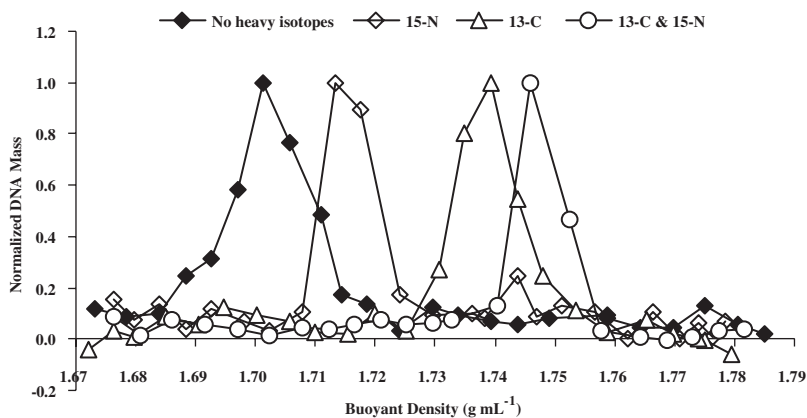


Figure 3. Distribution of *E. coli* DNA over a range of BD values following no (closed diamonds), single (^{15}N open diamonds, ^{13}C open triangles) or dual isotope (open circles) enrichment treatments. Each DNA type was centrifuged independently. DNA mass is normalized to highest value for each DNA type due to variability in DNA recovery.

microbial activity, resulting in the emergence of 9 dominant operational taxonomic unit (OTU) fragments compared to the unamended profiles (Fig. 4B). These organisms exploited the relatively

universal substrates, ammonium and glucose, and were dominant in both labeled and control populations, demonstrating the effect of ^{15}N enrichment on DNA BD.

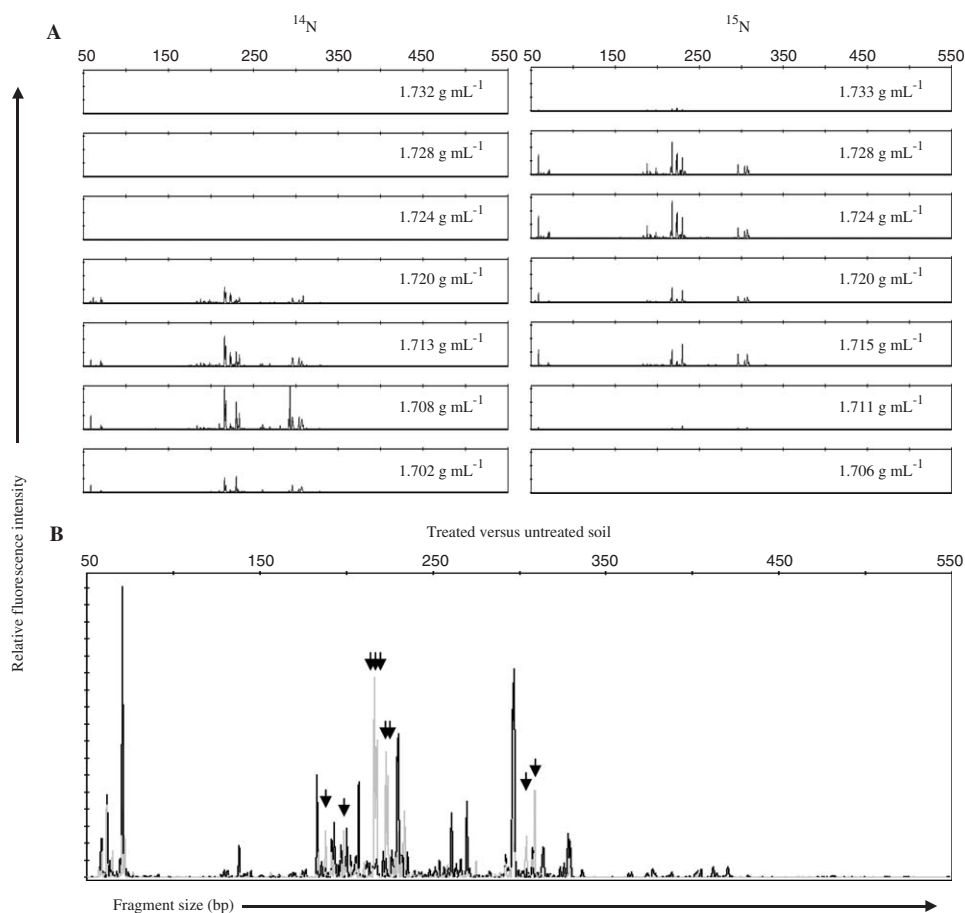


Figure 4. DNA T-RFLP profiles from soils supplied with $(^{14}\text{NH}_4)_2\text{SO}_4$ (control) or $(^{15}\text{NH}_4)_2\text{SO}_4$ over a gradient of buoyant densities (A). The replicate treatment illustrated the same trend. Overlay of profiles from unamended (black) and amended soils (gray), illustrating the 9 dominant OTU fragments (arrows) resulting from the amendments (B).

In summary, for the first time, we quantified DNA BD increases from ^{15}N enrichment, and from dual label enrichment. Our data illustrate dual labels will significantly increase BD shifts, enabling easier identification of enriched organisms. Although ^{15}N incorporation resulted in a limited increase in DNA BD, gradient fractionation, followed by a comparison of T-RFLP from fractions of labeled and control treatments, facilitated detection of ^{15}N enrichment with both pure cultures and soil DNA samples. Thus, despite its inherent limitations, ^{15}N SIP is a promising technique that will enable researchers to better understand the ecology of organisms involved in transformation of nitrogenous compounds.

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