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Natural abundance $\delta^{15}N$ and $\delta^{13}C$ of DNA extracted from soil

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Abstract

We report the first simultaneous measurements of $\delta^{15}N$ and $\delta^{13}C$ of DNA extracted from surface soils. The isotopic composition of DNA differed significantly among nine different soils. The $\delta^{13}C$ and $\delta^{15}N$ of DNA was correlated with $\delta^{13}C$ and $\delta^{15}N$ of soil, respectively, suggesting that the isotopic composition of DNA is strongly influenced by the isotopic composition of soil organic matter. However, in all samples DNA was enriched in ^{13}C relative to soil, indicating microorganisms fractionated C during assimilation or preferentially used ^{13}C enriched substrates. Enrichment of DNA in ^{15}N relative to soil was not consistently observed, but there were significant differences between $\delta^{15}N$ of DNA and $\delta^{15}N$ of soil for three different sites, suggesting microorganisms are fractionating N or preferentially using N substrates at different rates across these contrasting ecosystems. There was a strong linear correlation between $\delta^{15}N$ of DNA and $\delta^{15}N$ of the microbial biomass, which indicated DNA was depleted in ^{15}N relative to the microbial biomass by approximately 3.4‰. Our results show that accurate and precise isotopic measurements of C and N in DNA extracted from the soil are feasible, and that these analyses may provide powerful tools for elucidating C and N cycling processes through soil microorganisms.

Keywords: Natural abundance $\delta^{15}N$; Natural abundance $\delta^{13}C$; DNA; Soil microbial biomass

1. Introduction

Examining the natural abundances of stable isotopes of biologically important elements has substantially advanced ecosystem science (Lajtha and Michener, 1994). The stable isotopes of carbon (C) and nitrogen (N) can be quantified in a variety of soil pools, including various fractions of organic matter and inorganic species, but the most informative of these pools are those that are biologically active (Högberg, 1997). Microbial activity controls rates of many important ecosystem processes. The isotopic content of microorganisms in soil may provide insight into the metabolic pathways that are active in soil or the C and N sources that microorganisms are metabolizing. Several recent studies have focused on analyzing the isotopic composition of microbial molecules in the environment, including phospholipid fatty acids (Burke et al., 2003; Petsch et al., 2003; Ruess et al., 2004) and the total microbial biomass (Potthoff et al., 2003; Gregorich et al., 2000; Liang et al., 2002; Rochette et al., 1999; Staddon, 2004; Dijkstra et al., 2006a, b).

The isotopic composition of DNA extracted from soil has not been studied extensively, even though it is universally present in all microorganisms in soil (excepting RNA viruses). Carbon stable isotope composition of DNA has been studied in an incipient Paleosol (Jahren et al., 2006) and in estuarine bacterial concentrates (Coffin et al., 1990), but to date there have not been any reports on both C and N isotopic composition of DNA in modern surface soils.

Isotopic analysis of DNA extracted from soil provides several important advantages over using other biomolecules. It is a large molecule and therefore significant

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isotopic fractionation is unlikely to occur due to volatilization as the sample is prepared for isotope ratio mass spectrometry (IRMS) (Högberg, 1997). Unlike the total microbial biomass, DNA is a single molecule and therefore it is possible to measure its purity and to determine if other contaminating molecules are affecting the observed isotope value. DNA, in contrast to other biomolecules, does not turnover inside a cell but is made only at the beginning of a cell's life (Madigan et al., 2005). Therefore, it is likely to be one of the longer lived biomolecules in soil and will reflect process rates that controlled the isotopic composition of nutrient pools in soil when the cell was formed. Because cell growth in soil occurs continuously, DNA integrates these process rates over longer periods of time than do other biological molecules. Once a cell dies it is likely that a substantial fraction of DNA turns over rapidly because it is a good substrate for microbial growth. However, extracellular DNA can also sorb to clay minerals, protecting the nucleic acids from degradation and increasing turnover times (Ogram et al., 1987; Paget and Simonet, 1994).

There are major experimental challenges in obtaining an accurate measurement of the natural abundance stable isotope content of DNA extracted from soil. IRMS analysis typically requires approximately 10 μg of N or C so that, depending on GC content of the DNA, between 62 and 69 μg or between 28 and 30 μg of DNA is required for the measurement of ^{15}N or ^{13}C content, respectively. These are larger amounts of DNA than commonly extracted from soil with commercially available kits (Luna et al., 2006). Furthermore, analysis of natural abundance stable isotope composition can easily be corrupted by the presence of contaminant N or C that can originate from the soil itself or from the reagents used in the DNA extraction.

The purpose of this study was to develop a method to extract large quantities of very clean DNA from soil that would be suitable for IRMS analysis and to compare the $\delta^{15}N$ and $\delta^{13}C$ of DNA extracted from surface soils across nine different ecosystems, spanning a broad range of climate conditions (temperate to tropical), vegetation types (grasslands, shrublands, and forests), and soil substrate ages (1000 to 3 million years old). The isotopic composition of DNA extracted from soil was compared to the isotopic values of total elemental soil pools and, in selected cases, of the soil microbial biomass, in order to test for isotopic enrichment or depletion of DNA in soil.

2. Materials and methods

2.1. Description of study sites

Samples were taken from the top 10 cm of mineral soil from four different ecosystems in Arizona—dry grassland, piñon–juniper woodland (*Pinus edulis–Juniperus monosperma*), ponderosa pine forest (*Pinus ponderosa*), and mixed conifer forest—all within 50 km of Flagstaff, Arizona, USA, and from five different sites along a soil chronosequence in Hawaii, USA (Table 1). The soil samples were stored at −45 °C until used for DNA extractions.

2.2. Extraction of DNA from soil

DNA was extracted from soil using a method modified from previously published procedures (Lee et al., 1996; Zhou et al., 1996). Approximately 40 g of frozen soil, 20 g of acid-washed glass beads (mesh size 150–212 μm), and 35 ml of lysis I solution (150 mM NaCl and 100 mM EDTA, adjusted to pH 8) were combined in a large bead beater (model 1107900, Biospec Industries, Bartlesville, OK, USA) and beaten for 1 min. The samples were poured into a 50 ml Falcon tube, 2 ml of 20% SDS were added and the samples were then incubated at 70 °C for 30 min. The tubes were centrifuged at 12,857 rcf for 10 min and the supernatant was transferred to a new Falcon tube. Five milliliters of 5 M NaCl and 5 ml of 10% Cetyl trimethylammonium bromide in 0.7 M NaCl were added to the tubes, which were then incubated at 70 °C for 15 min. After

Table 1
Selected characteristics of ecosystems and mineral soils (0–10 cm) used in this study

Ecosystem	Age (kyr)	Soil subgroup	MAT[a] (°C)	MAP[b] (mm)	Elevation (m)
Hawaii soil chronosequence[c]					
Thurston	0.3	Lithic Hhapludand	16	2500	1176
Laupahoehoe	20	Hydric Hapludand	16	2500	1170
Kohala	150	Hydric Hydruland	16	2500	1122
Kolekole	1400	Hydric Hydruland	16	2500	1210
Kokee	4100	Plinthic Kandiudox	16	2500	1134
Arizona elevation gradient[d]					
Mixed conifer		Pachic Udic Argiboroll	6	790	2640
Ponderosa pine		Mollic Eutroboralf	8	660	2260
Piñnion–Pinion Juniper		Calcic Haplustand	10	380	1975
Desert grassland		Typic Haplustoll	12	230	1750

[a]Mean annual air temperature.
[b]Mean annual precipitation.
[c]Crews et al. (1995).
[d]Dijkstra et al. (2006b).

the samples were allowed to cool to room temperature, 10 ml of 24:1 (v/v) chloroform/isoamyl alcohol were added; after shaking the tube by hand, the tube was centrifuged for 5 min at 3214 rcf. Twenty-five milliliters of the supernatant were transferred to a new tube to which 25 ml of 13% PEG in 1.6 M NaCl were added. The tubes were incubated on ice for at least an hour or at 4 °C overnight and spun at 12,857 rcf for 20 min. The supernatant was removed and the pellet was washed once with 70% ethanol. The pellet was dissolved in 800 μl of TE buffer, pH 7.5, and transferred to a 1.5 ml Eppendorf tube. One hundred microliters of 10 M ammonium acetate were added to the samples, and these samples were then incubated at 4 °C overnight. The next day, the samples were centrifuged at 16,100 rcf for 10 min and 800 μl of the supernatant were transferred to a new tube. Eight hundred microliters of isopropanol were added to the tube and, after shaking vigorously, the tubes were centrifuged at 16,100 rcf for 10 min. The pellet was dissolved in 200 μl of TE buffer and stored in a −30 °C freezer.

2.3. Purification of extracted DNA

The DNA was further purified through ultra centrifugation on a cesium chloride gradient by combining 4 ml of saturated CsCl solution (1.9 g/ml), 300 μl of 2 × gradient buffer (200 mM Tris at pH 8.0, 200 mM KCl, and 2 mM EDTA), 50 μl of 1 mg/ml ethidium bromide and 200 μl of DNA extract. Water was added to completely fill the 4.7 ml ultracentrifuge tube, which, once inserted into a TLA-110 rotor (Beckmann Coulter Inc., Fullerton, CA, USA), was spun at 267,000 rcf for 48 h in an Optimax benchtop ultra centrifuge (Beckmann Coulter Inc., Fullerton, CA, USA). After centrifugation, 500 μl of DNA was removed from the tube and combined with 500 μl of NaCl saturated isopropanol. The tube was spun for 30 s at 2300 rcf and the upper pink phase that contained ethidium bromide was discarded. Five hundred microliters of water were added to the bottom DNA solution, 900 μl of isopropanol was added to the tube, and the samples were spun at 13,200 rpm for 10 min. The pellet was washed with 70% ethanol and subsequently air dried for 15 min. The pellet was resuspended in 50 μl of water. Absorbance of DNA at 230, 260 and 280 nm was measured to assess the purity of the extract with an Eppendorf Biophotometer (Eppendorf AG, Hamburg, Germany).

2.4. Microbial biomass and isotope analyses

Microbial biomass was measured in the soil samples from the Hawaiian soil chronosequence and its isotopic composition was analyzed using the chloroform fumigation extraction method (Horwath and Paul, 1994). The isotopic composition of the microbial biomass was determined, as described in Dijkstra et al. (2006b).

For isotopic analysis of DNA, 48 μl of DNA extract was added to 3.5 × 9 mm tin capsules (Costech Analytical Technologies Inc., Valencia, CA, USA) and the water was allowed to evaporate from the DNA solution overnight. To measure the ^{15}N and ^{13}C contents of soil, 40 mg of sieved (⩽2 mm), air-dried soil were added to 5 × 9 mm tin capsules (Costech Analytical Technologies Inc., Valencia, CA, USA). The natural abundances of ^{15}N and ^{13}C and N and C contents of microbial biomass, total soil, and dried DNA extracts were measured using a Carlo Erba NC 2100 Elemental Analyzer (CE Instruments, Milan, Italy) interfaced with a Thermo-Finnigan Delta Plus XL (Thermo-Electron Corp., Bremen, Germany) isotope ratio mass spectrometer at the Colorado Plateau Stable Isotope Laboratory (http://www.mpcer.nau.edu/isotopelab/). The ^{15}N and ^{13}C isotope composition were expressed in standard delta notation ($\delta^{15}N$, $\delta^{13}C$) in parts per thousand (‰) relative to air for nitrogen and VPDB (Vienna PeeDee Belemnite) for carbon, where $\delta = 1000[(R_{sample}/R_{standard})-1]$ and R is the molar ratio $^{15}N/^{14}N$ or $^{13}C/^{12}C$. The external precision on repeated measurements of an in-house laboratory working standard (National Institute of Standards and Technology, USA; NIST 1547—peach leaves) was always better than ±0.20‰ for $\delta^{15}N$ and ±0.10‰ for $\delta^{13}C$.

2.5. Statistical analyses

Isotopic composition of DNA and soils were compared using analysis of variance. The differences between isotopic composition of DNA and total soil were compared among ecosystems using analysis of variance and a posthoc Tukey's test. Alpha values less than 0.05 were considered statistically significant in these analyses. Linear regression analysis was used to compare the isotopic composition of DNA to the total soil or the microbial biomass. All statistical analyses were performed using the SPSS version 14.0 statistical software package (Chicago, IL, USA).

3. Results

The UV light absorbance measurements of DNA extracted from soil indicate the DNA was not contaminated with an ingredient used in the DNA extraction procedure or a non-DNA component from the soil (Table 2). Pure DNA has a ratio of UV light absorbance at 260 to 280 nm (A_{260}/A_{280}) higher than 1.7, and a ratio of absorbance at 260 to 230 nm in excess of 2 (Sambrook et al., 1989). The absorbance measurements reported in this study (Table 2) also suggest the DNA was cleaner than what is reported for commercial kits (for instance, an $A_{260}/A_{280} = 1.57 \pm 0.14$ or 1.5 ± 0.3 was reported in Stres et al., 2004 or Luna et al., 2006, respectively). The carbon-to-nitrogen mass (C/N) ratios of extracted DNA also indicate that the majority of samples contained pure DNA. Based on the molecular formulas of deoxynucleotides, the C/N ratio of pure DNA is 2.860 if it consists entirely of deoxyadenosine and deoxythymidine, or 2.375 if the DNA contains only deoxyguanosine and deoxycytidine

(Rawn, 1989). With the exception of DNA extracted from the Kokee and Laupahoehoe soils of the Hawaiian soil chronosequence, the C/N ratio of extracted DNA was not significantly greater than 2.86. However, the C/N ratio of DNA extracted from Hawaiian soils was consistently higher than that for DNA extracted from Arizonan soils suggesting the Hawaiian DNA extracts were more susceptible to contamination by soil organic matter.

The ^{15}N isotope composition of DNA extracted from soil differed significantly between sites ($F(8,36) = 142.0$, $p < 0.001$) and correlated significantly with the $\delta^{15}N$ of soil total N (Fig. 1). Along the Arizona elevation gradient, the $\delta^{15}N$ values of DNA decreased with elevation (Tables 1 and 3). Overall, DNA was not enriched in ^{15}N relative to soil total N pools; however, $\delta^{15}N$ of DNA from the mixed conifer and Kokee soils were significantly lower than $\delta^{15}N$ of the soil total N, while the $\delta^{15}N$ of DNA from the ponderosa pine soil was significantly enriched in ^{15}N relative to soil total N pool (Fig. 2, Table 3).

Table 2
Mean values of spectroscopic properties and the carbon-to-nitrogen (C/N) mass ratio of DNA extracted from mineral soil of nine contrasting ecosystems

Ecosystem	A_{260}/A_{280}	A_{260}/A_{230}	C/N
Hawaii soil chronosequence			
Thurston	1.77 (0.03)	2.04 (0.08)	3.11 (0.20)
Laupahoehoe	1.74 (0.06)	1.90 (0.07)	4.27 (0.72)
Kohala	1.71 (0.02)	2.18 (0.10)	3.11 (0.23)
Kolekole	1.71 (0.03)	1.80 (0.08)	2.71 (0.06)
Kokee	1.78 (0.01)	1.91 (0.07)	4.90 (1.02)
Arizona elevation gradient			
Grassland	1.70 (0.03)	1.91 (0.06)	2.49 (0.05)
Piñon–Juniper	1.73 (0.02)	1.88 (0.02)	2.60 (0.06)
Ponderosa pine	1.76 (0.03)	1.88 (0.03)	2.66 (0.08)
Mixed conifer	1.70 (0.04)	1.94 (0.04)	2.72 (0.14)

Standard errors of the means are presented in parentheses. A_{260}/A_{280}: ratio of absorbance at 260 nm over absorbance at 280 nm. A_{260}/A_{230}: ratio of absorbance at 260 nm over absorbance at 230 nm.

The ^{13}C composition of DNA extracted from soils differed significantly among sites ($F(8,36) = 248.3$, $p < 0.001$) and correlated well with the $\delta^{13}C$ of soil total C pool (Fig. 1). DNA was enriched in ^{13}C on average by 4.5‰ relative to the soil, though the degree of enrichment varied between sites and ranged from 2.4 to 5.2‰ (Fig. 2, Table 3). The degree of ^{13}C enrichment of DNA relative to the soil total C pool was not significantly different among sites with the exception of the arid grassland soil in Arizona, where DNA was significantly less enriched than the other sites (Fig. 2).

The ^{15}N composition of DNA extracted from Hawaiian soil samples correlated significantly with the ^{15}N composition of microbial biomass in soil (Dijkstra et al., 2006a, b). DNA was consistently depleted in ^{15}N relative to the total microbial biomass on average by 3.4‰ (Fig. 3).

4. Discussion

The ^{13}C values of DNA followed the $\delta^{13}C$ of the total soil C pool but were on average 4.5‰ (± 0.02) higher (Fig. 1). The correlation between the $\delta^{13}C$ values of DNA and $\delta^{13}C$ values of soil total C pool suggests that the isotopic composition of C in DNA is controlled by the ^{13}C composition of organic matter in soil, and that enrichment of DNA relative to total soil occurs through fractionation or preferential substrate utilization that is consistent among different ecosystems. The variation in $\delta^{13}C$ values of soil in our data set was caused presumably by differences in abundance of C_3 plants, which are more depleted in ^{13}C, compared to C_4 plants (Smith and Epstein, 1971; Farquhar et al., 1989). Like soil DNA, DNA from plants is also enriched in ^{13}C relative to the total plant biomass (Jahren et al., 2004). However, DNA extracted from soil appears to be more enriched in ^{13}C relative to total soil organic matter (≈ 4.5‰ on average) than plant DNA is relative to total plant biomass (≈ 1.3‰). The DNA extracted from soil likely originated from soil microorganisms such as bacteria,

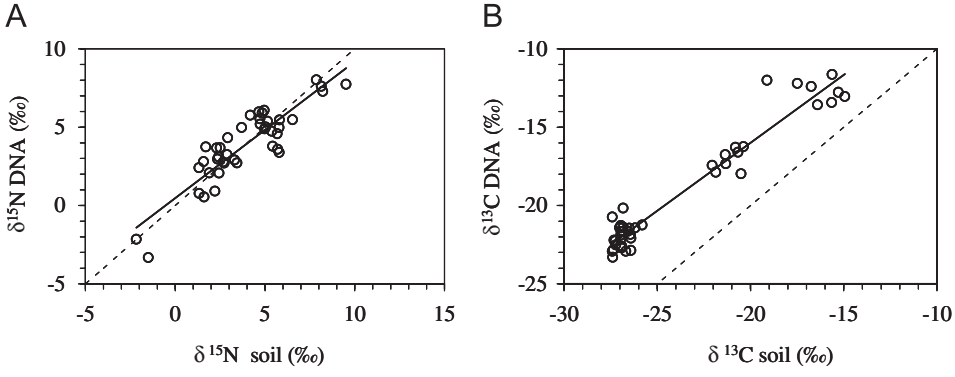

Fig. 1. Comparison of isotopic values of DNA extracted from nine different surface mineral soils with those of total elemental pools. Figure shows data taken from field replicates. Variation in isotopic values of DNA between field replicates is reported in Table 3. Panel A shows $\delta^{15}N$ of DNA vs. $\delta^{15}N$ of soil total N ($r = 0.892$, $n = 42$, $p < 0.001$). Panel B shows $\delta^{13}C$ of DNA vs. $\delta^{13}C$ of soil total C ($r = 0.971$, $n = 43$, $p < 0.001$). Dashed line in each panel shows the 1:1 relationship.

Table 3
Mean isotopic composition of total soil or DNA extracted from surface mineral soils from nine contrasting ecosystems

Ecosystem	δ^{15}N soil (‰)	δ^{15}N DNA (‰)	δ^{13}C soil (‰)	δ^{13}C DNA (‰)
Hawaii soil chronosequence				
Thurston	−1.8 (0.2)	−2.4 (0.3)	−27.4 (0.1)	−22.9 (0.2)*
Laupahoehoe	4.9 (0.1)	5.4 (0.2)	−27.1 (0.1)	−22.3 (0.2)*
Kohala	2.2 (0.2)	2.7 (0.2)	−26.8 (0.1)	−21.7 (0.3)*
Kolekole	2.6 (0.2)	3.3 (0.2)	−26.6 (0.2)	−21.4 (0.1)*
Kokee	1.5 (0.3)	0.7 (0.1)*	−26.9 (0.1)	−22.7 (0.1)*
Arizona elevation gradient				
Grassland	8.4 (0.4)	7.7 (0.2)	−15.6 (0.3)	−13.2 (0.2)*
Piñon–Juniper	5.8 (0.3)	5.3 (0.1)	−17.2 (0.7)	−12.1 (0.2)*
Ponderosa pine	4.4 (0.3)	5.4 (0.2)*	−21.5 (0.3)	−17.2 (0.3)*
Mixed conifer	5.6 (0.1)	3.8 (0.3)*	−20.8 (0.2)	−17.0 (0.4)*

Standard errors of the means are presented in parentheses. For each isotope, DNA values denoted with an asterisk (*) are significantly different ($p \ll 0.05$) from corresponding total soil isotopic values.

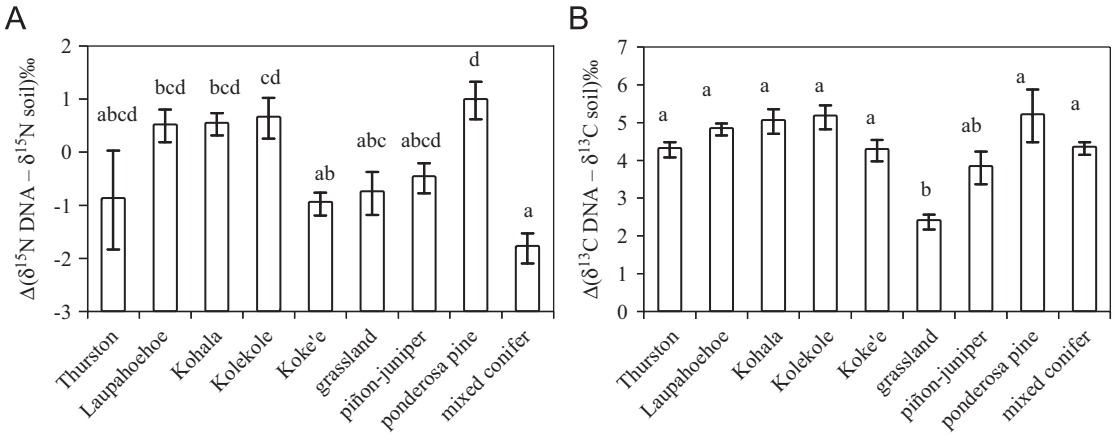

Fig. 2. Isotopic enrichment (Δ) of DNA extracted from soil relative to the soil total elemental pool for (A) ^{15}N and (B) ^{13}C. Vertical lines represent ±1 SEM. For each figure panel, different letters above bars denote significant differences between mean values (Tukey's test, $\alpha = 0.05$).

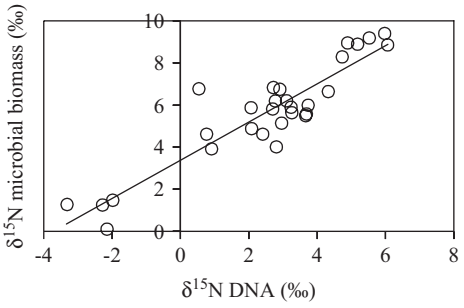

Fig. 3. Relationship between δ^{15}N of the microbial biomass and the δ^{15}N of DNA extracted from soil for five sites along a soil chronosequence in Hawaii ($r = 0.916$, $n = 28$, $p < 0.001$; δ^{15}N of microbial biomass $= 0.91 \times (\delta^{15}$N of DNA$) + 3.4$‰. Figure shows data taken from field replicates.

archaea, fungi and protozoa but also from other eukaryotic organisms including plants. Unlike DNA of microbial origin the isotopic composition of DNA from plants was controlled by plant metabolism and not decomposition of organic matter in soil. It remains very difficult to determine what fraction of DNA extracted from soil originated from plants because there are not many suitable target genes, their copy number in the plant genome is often unknown and the total size of the plant genome may vary substantially among different species of plants. A study of estuarine bacteria in culture found that DNA was not enriched in ^{13}C relative to the entire cell but did find that nucleic acids were enriched by 2.4‰ relative to the substrate (Coffin et al., 1990). The degree of enrichment of DNA in ^{13}C relative to total soil may reflect the number of times C atoms in biomass have turned over. Phospholipid fatty acids were also enriched in ^{13}C in a study of tropical soils (Burke et al., 2003) but depleted in ^{13}C in a study of shale weathering (Petsch et al, 2003) relative to soil organic matter. Studies of ^{13}C composition of the soil microbial biomass (Gregorich et al., 2000; Liang et al., 2002; Rochette et al., 1999; Staddon, 2004) also have generally found that microorganisms were enriched in ^{13}C relative to total soil C. However, one previous study reported that the soil microbial biomass was depleted by an average of 1.60‰ relative to the soil total C, which was

ascribed to the recent dominance of C_3 plants in a C_4 soil (Dijkstra et al., 2006b).

Similar to ^{13}C, the ^{15}N content of DNA reflected the isotopic composition of soil organic matter. However, in contrast to ^{13}C, $\delta^{15}N$ of DNA was not consistently higher than $\delta^{15}N$ of soil organic matter. The soil N cycle is more complex than the C cycle and includes several processes known to fractionate N pools in soil (Robinson, 2001). The ^{15}N composition of DNA relative to soil organic matter is likely to be more variable because specific N cycling processes occur at variable rates among soils. As proposed in previous reports for ^{15}N natural abundance estimates of the soil microbial biomass (Dijkstra et al., 2006a, b), the degree of ^{15}N enrichment of soil DNA relative to the soil organic matter may provide additional information about soil N cycling processes that cannot be obtained by measuring the ^{15}N content of the soil organic matter pool alone.

The $\delta^{15}N$ of the soil microbial biomass is consistently higher than $\delta^{15}N$ of the soil total N pool (Dijkstra et al., 2006a, b). In a study conducted at the northern Arizona elevation gradient sites, the microbial biomass was enriched in ^{15}N by an average of 3.10‰ relative to the soil total N (Dijkstra et al., 2006b). In a second study along a cattle manure gradient, the $\delta^{15}N$ values of the soil microbial biomass was between 0 and 9‰ higher than the $\delta^{15}N$ values of soil total N (Dijkstra et al., 2006a). In the current study, we found the $\delta^{15}N$ of microbial biomass and DNA extracted from soils taken along the Hawaiian soil chronosequence were highly correlated, but that DNA is depleted in ^{15}N relative to the microbial biomass by approximately 3.4‰ (Fig. 3). This result suggests that different components of the microbial biomass have contrasting $\delta^{15}N$ values, but that these biomolecules are all likely correlated with the ^{15}N values of the soil total N pool. There are still very few studies of the $\delta^{15}N$ of bacteria in culture or environmental samples, but studies of fungi show biomass can be either enriched or depleted in ^{15}N relative to their substrates. In laboratory cultures, Henn and Chapela (2004) found that during initial growth both saprotrophic and ectomycorrhizal fungi are strongly depleted in ^{15}N relative to their substrates. In contrast, Hobbie et al. (1999) observed that ectomycorrhizal fungi are enriched in ^{15}N relative to the soil NH_4^+ pool and relative to saprotrophic fungi. In spite of the complexity of interpreting natural abundance signals of ^{15}N in ecosystems (Robinson, 2001), we posit that identifying the mechanisms that lead to enrichment or depletion of ^{15}N in microbial biomass, as well as specific biomolecules of microbial cells such as DNA, may provide important insights into the soil N cycle.

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