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The effects of chronic nitrogen fertilization on alpine tundra soil microbial communities: implications for carbon and nitrogen cycling

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Summary

Many studies have shown that changes in nitrogen (N) availability affect primary productivity in a variety of terrestrial systems, but less is known about the effects of the changing N cycle on soil organic matter (SOM) decomposition. We used a variety of techniques to examine the effects of chronic N amendments on SOM chemistry and microbial community structure and function in an alpine tundra soil. We collected surface soil (0–5 cm) samples from five control and five long-term N-amended plots established and maintained at the Niwot Ridge Long-term Ecological Research (LTER) site. Samples were bulked by treatment and all analyses were conducted on composite samples. The fungal community shifted in response to N amendments, with a decrease in the relative abundance of basidiomycetes. Bacterial community composition also shifted in the fertilized soil, with increases in the relative abundance of sequences related to the *Bacteroidetes* and *Gemmatimonadetes*, and decreases in the relative abundance of the *Verrucomicrobia*. We did not uncover any bacterial sequences that were closely related to known nitrifiers in either soil, but sequences related to archaeal nitrifiers were found in control soils. The ratio of fungi to bacteria did not change in the N-amended soils, but the ratio of archaea to bacteria dropped from 20% to less than 1% in the N-amended plots. Comparisons of aliphatic and aromatic carbon compounds, two broad categories of soil carbon compounds, revealed no between treatment differences. However, G-lignins were found in higher relative abundance in the fertilized soils, while proteins were detected in lower relative abundance. Finally, the activities of two soil enzymes involved in N cycling changed in response to chronic N amendments. These results suggest that chronic N fertilization induces significant shifts in soil carbon dynamics that correspond to shifts in microbial community structure and function.

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Introduction

Over the last half century, human activity has dramatically altered the global nitrogen (N) cycle, and the anthropogenic creation of reactive N now likely exceeds all natural terrestrial sources combined (Vitousek *et al.*, 1997; Galloway *et al.*, 2004). This increase in fixed N inputs has a litany of consequences for the environment, including climate change, marine and freshwater eutrophication, air pollution, species loss and even human health threats (Vitousek *et al.*, 1997; NRC, 2000; Townsend *et al.*, 2003). Many of the effects on terrestrial ecosystems arise from increased N deposition rates, which in some parts of North America, Europe and East Asia are more than an order of magnitude greater than in pre-industrial times (Galloway *et al.*, 2004). The majority of temperate terrestrial ecosystems show clear signs of N limitation to net primary productivity (Vitousek and Howarth, 1991); thus, increases in N deposition can lead to increased carbon (C) storage in the form of plant biomass (Townsend *et al.*, 1996; Nadelhoffer *et al.*, 1999; Magnani *et al.*, 2007). Higher N inputs can also drive shifts in plant species composition (Bobbink *et al.*, 1998; Clark *et al.*, 2007), with growing evidence of a general trend towards a loss of diversity (Stevens *et al.*, 2004; Suding *et al.*, 2005).

However, changes in N availability also affect, and are affected by, the soil microbial community, as these organisms regulate many fundamental processes that determine ecosystem N availability (Paul and Clark, 1997). For example, deposition-driven increases in ammonium and nitrate represent larger substrate pools for nitrification and denitrification, both of which affect N loss to aquatic and atmospheric realms. As such, the ultimate fate of N from depositional processes, and the fraction that fuels primary production, is intimately linked to the underlying soil microbial community.

Nitrogen availability can also affect soil heterotrophic respiration, which comprises a yearly source of atmospheric CO_2 nearly an order of magnitude larger than fossil fuel emissions (Schimel *et al*., 2001). Yet our understanding of how increased N affects decomposition remains notably incomplete, with existing data suggesting a highly intricate set of interactions between changing N availability, biologically diverse soil microbial communities and chemically complex soil organic matter (SOM) (Neff *et al*., 2002; Zak *et al*., 2003). Resolving the links between N availability and SOM decomposition is of great importance, as SOM represents a C reservoir three to four times larger than that contained in either plant biomass or the atmosphere (Eswaran *et al*., 1993; Davidson and Janssens, 2006). Data from multiple N fertilization experiments show that N additions elicit a myriad of responses in soil heterotrophic processes, including changes in enzyme activity, respiration and decomposition (Carreiro *et al*., 2000; Saiya-Cork *et al*., 2002; Sinsabaugh *et al*., 2004; 2005; Waldrop *et al*., 2004a,b). However, the relative response varies by ecosystem type (Waldrop *et al*., 2004a), and can show significant seasonal and spatial variation within a given ecosystem (Bardgett *et al*., 1999; Schmidt *et al*., 2004), thus highlighting the complexity of the underlying mechanisms.

A recent study in an alpine tundra soil further emphasizes this complexity and offers some insight into how shifts in N availability may alter SOM turnover. Neff and colleagues (2002) used a set of long-term N fertilization plots in the Colorado Front Range to show that N inputs altered both the turnover time and the chemistry of different SOM fractions. Notably, this work showed that intermediate-aged (10- to 40-year-old) portions of the SOM pool displayed significant increases in decomposition following chronic N additions, and that much of this pool appeared to contain C-rich, N-poor compounds such as cellulose and its derivatives. At the same time, the results suggested the potential for the reduced decay of other SOM fractions. These findings were consistent with general theory suggesting that increases in N availability may spur the decay of specific fractions of the chemically complex SOM pool, especially those compounds that are N-poor, but may slow the decay of N-rich, chemically recalcitrant compounds such as lignin derivatives (Fog, 1988). Neff and colleagues (2002) also showed that traditional ecosystem metrics for assessing changes in SOM turnover – e.g. changes in total soil C pools – did not reveal what was clearly a dynamic but varied response in soil heterotrophic activity to changes in N availability.

However, the role of the soil microbial community in these complex interactions is poorly understood. There are a suite of molecular phylogenetic tools to examine how changes in N availability affect the community structure of specific types of N cycling organisms, including nitrifiers (e.g. He *et al*., 2007), denitrifiers (e.g. Enwall *et al*., 2005) and N fixers (e.g. Tan *et al*., 2003). Parallel investigations into the effects of N availability on soil microbial C cycling are more challenging because of the lack of a single, conserved gene for decomposition. Thus, our aim was to use the same plots sampled by Neff and colleagues (2002) and ask if the microbial community composition shifted in the long-term N-amended plots. We integrate a molecular phylogenetic study of the abundance and diversity of bacterial, archaeal and fungal members of the soil microbial community with a high-resolution characterization of the commensurate changes in soil C pools. Our results show that soil C chemistry and microbial community composition undergo shifts, but the activity of most C-cycling enzymes did not change significantly in a composite soil sample derived from N-amended plots.

Results

Microbial community composition

Surface soil (0–5 cm) samples were collected from five control and five long-term N-amended plots established and maintained through the Niwot Ridge Long-term Ecological Research (LTER) site in the fall of 2003. Samples were bulked by treatment in the field and all subsequent analyses were conducted on composite samples, hereafter referred to as control and N-amended soils. We used molecular phylogenetic techniques to analyse the bacterial community structure in composites of both control and N-amended soils. Community composition shifted in response to N amendments (Fig. 1). For example, 16S rRNA genes related to the *Verrucomicrobia* comprised 10% of the control library, but were not detected in the +N library. Additionally, sequences related to the *Bacteroidetes* and the *Gemmatimonadetes* were present in the unamended soils (5% and 4% respectively), but increased in relative abundance in the fertilized soils (19% and 10% respectively).

Both the P test (Martin, 2002) and the UniFrac metric (Lozupone and Knight, 2005), statistical techniques that compare the overall phylogenetic diversity within micro-

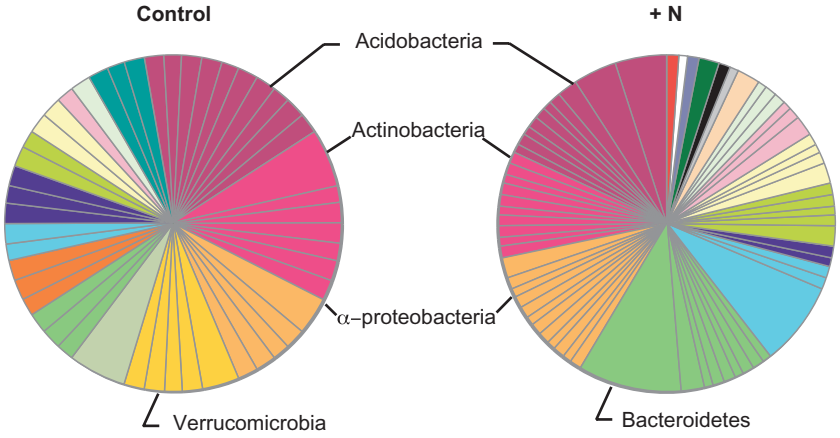

Fig. 1. Relative abundance of 16S rRNA gene phylotypes in clone libraries in the control and N-amended soils. Colours represent phyla- or subphyla-level clades and black lines represent individual phylotypes (> 3% different from all other sequences). Number of sequences = 54 (control) and 99 (+N). ■, *Acidobacteria*; ■, *Actinobacteria*; ■, α-*Proteobacteria*; ■, *Verrucomicrobia*; ■, *Cyanobacteria*; ■, *Bacteroidetes*; ■, δ-*Proteobacteria*; ■, *Gemmatimonadetes*; ■, candidate division TM7; ■, candidate division OP10; ■, *Planctomycetes*; ■, β-*Proteobacteria*; ■, *Chloroflexi*; ■, candidate division TG1; ■, *Firmicutes*; ■, γ-*Proteobacteria*; ■, candidate division OD2; ■, *Viridiplantae*; ■, candidate division OP11; □, candidate division SC3; ■, candidate division AD3.

bial communities, revealed significant differences in the control and the +N bacterial 16S rRNA gene libraries (Table 1). However, the same three phyla – the *Acidobacteria*, the *Actinobacteria* and the α-*Proteobacteria* – dominated in both soils (Fig. 1). Thus, we applied UniFrac and the P test to compare the diversity of sequences from within these three phyla in the two soil types. These tests demonstrated that the acidobacterial sequences from the N-amended soils were different from those recovered from control soils, while the sequences related to the *Actinobacteria* and the α-*Proteobacteria* were more similar across treatments (Table 1). Therefore, although the relative abundance of sequences related to the *Acidobacteria* was identical in both soils, the actual types of *Acidobacteria* were different in the control and +N treatments (Table 1).

Long-term N amendments also resulted in a significant shift in the phylogenetic diversity of the soil fungal community (Table 1, Fig. 2). The relative abundance of *Ascomycota* LSU rRNA genes increased by 30% in response to fertilization, while the fraction of sequences related to the *Basidiomycota* and a novel, unclassified fungal group decreased in relative abundance. LSU rRNA genes related to other groups, including the *Mucoromycotina*, *Chytridiomycota* and *Blastocladiomycota*, were also detected in the fungal libraries, but were found in low abundance and showed no obvious response to N amendments.

We also used molecular methods to assess the archaeal community structure in the control and N-amended soils. Here, we used primers that were designed to amplify a wide diversity of archaea, including the highly divergent nanoarchaeal clade (Costello and Schmidt, 2006). As a consequence, these primers also target bacterial and eukaryotic SSU rRNA gene sequences. In the control soils, 43% of the library was archaeal, while only one sequence from the +N library was archaeal (data not shown). Although this precluded a direct comparison of archaeal diversity in control and fertilized soils, it suggested an overall change in the relative abundance of eukaryotic, bacterial and/or archaeal communities in the +N soils. Thus, we applied domain level quantitative PCR (qPCR) techniques to further explore this result. The relative abundance of fungi to bacteria did

Table 1. Phylogenetic comparison of SSU gene (bacteria) and LSU gene (fungi) clone libraries for control and N-amended soils.

	Fungi	Bacteria	*Acidobacteria*	*Actinobacteria*	α-*Proteobacteria*
Per cent of sequences in control soil	NA	NA	18.2%	16.4%	10.9%
Per cent of sequences in +N soil	NA	NA	18.2%	10.1%	13.1%
P test[a]	**	*	*	NS	NS
UniFrac[b]	**	*	*	NS	*

a. Phylogenetic test, as in Martin (2002), results were similar for distance and parsimony-derived trees.
b. UniFrac, as in Lozupone and Knight (2005), results were similar for distance and parsimony-derived trees.
*$P < 0.05$; **$P < 0.01$.
NA, not applicable; NS, not significant.

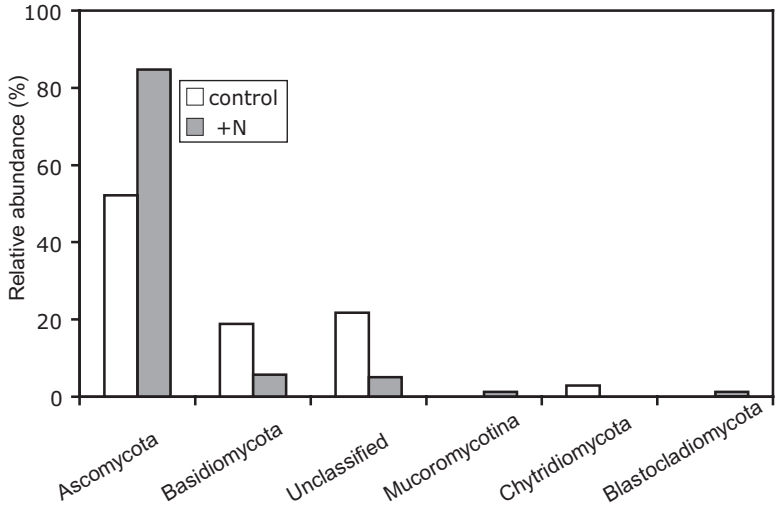

Fig. 2. Relative abundance of fungal LSU rRNA gene phylotypes in clone libraries in the control and N-amended soils. Bars represent the abundance of fungal phyla or subphyla in the libraries. Number of sequences = 69 (control) and 157 (+N).

not change in fertilized soils, but the ratio of archaea to bacteria dropped dramatically, falling from 20% in control soils to less than 1% in the N-amended soils (Fig. 3).

SOM chemistry and soil enzyme activities

We used pyrolysis-gas chromatography-mass spectrometry (Py-GC-MS; Gleixner *et al.* 1999) to examine the response of specific soil C fractions to chronic N amendments. Py-GC-MS analyses have the advantage of allowing a relatively comprehensive look at how different classes of soil organic compounds change in response to environmental forcings (Gleixner *et al.*, 1999). Neff and colleagues (2002) first applied this technique to the investigation of SOM responses to N fertilization. Subsequent developments in the analytical procedures allowed us to take a more detailed look at SOM pools. Comparisons of aliphatic and aromatic C compounds, two broad categories of soil C compounds, revealed no difference in the N-amended and control soils (Fig. 4A). In contrast, G-lignins were found in higher relative abundance in the fertilized soils, while proteins were detected in lower relative abundance (Fig. 4B). Other SOM fractions showed high analytical variability so it is difficult to infer what effect, if any, long-term N amendments have had on their abundance (Fig. 4B).

Finally, we investigated extracellular soil enzyme activity to examine the functional response of the soil microbial community to fertilization. Two enzymes directly related to N transformations changed significantly: leucine amino peptidase (LAP), which is associated with microbial N acquisition from proteins, declined, while urease, which is involved in urea mineralization, increased (Fig. 5). Notably, there was a lack of a significant shift in the activity of a broad suite of soil enzymes involved in many steps of C cycling (Fig. 5).

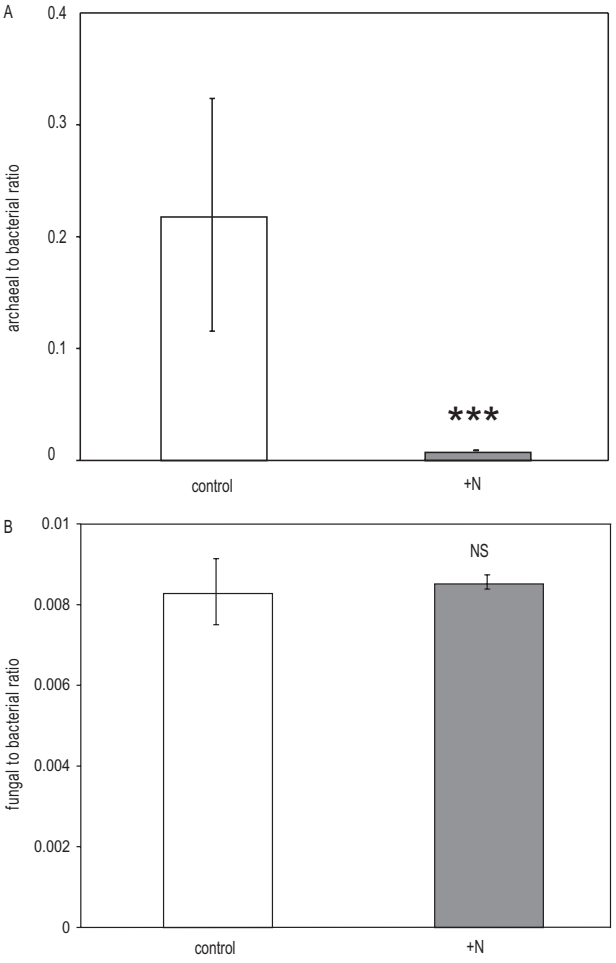

Fig. 3. Relative abundances of bacterial, archaeal and fungal SSU rRNA genes in control and N-amended soils, as estimated using qPCR assays. Error bars are the standard errors of the mean for four independent qPCR reactions for both archaea and bacteria (A) or bacteria and fungi (B). Thus, the standard error of the ratios reflects the error in the quantification of both sets of genes. ***$P < 0.001$; NS, not significant.

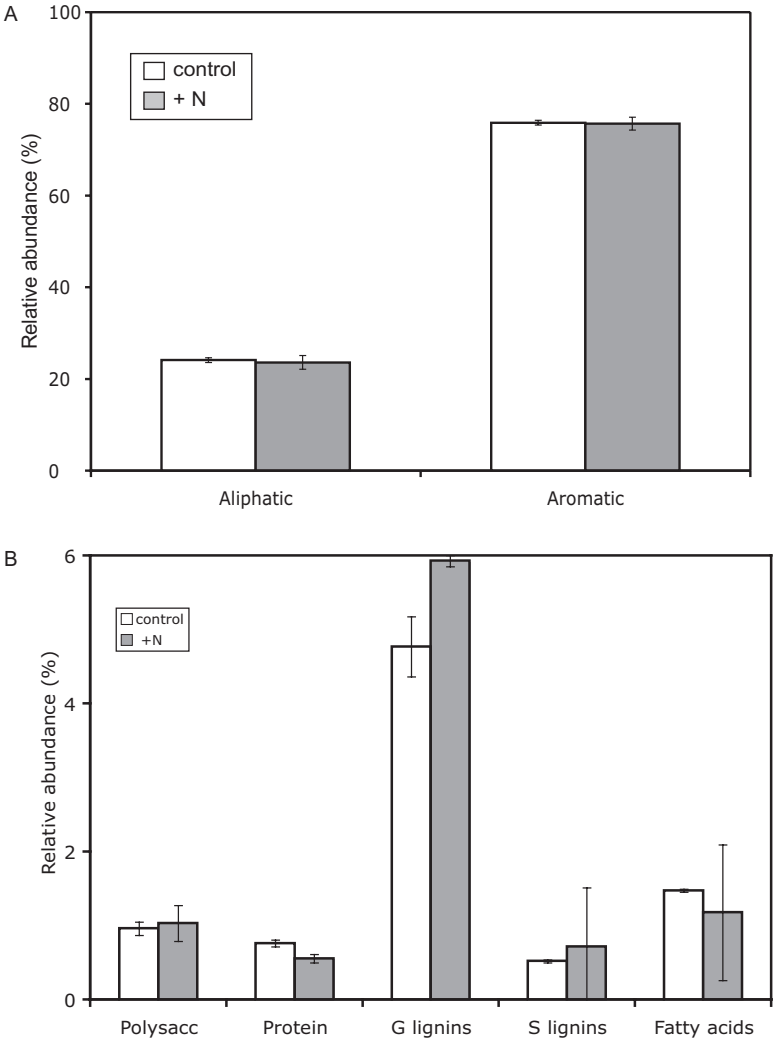

Fig. 4. Effect of chronic N amendments on SOM.
A. Bars show the relative abundance of aliphatic and aromatic compounds in the control and +N soils.
B. Bars show the relative abundance of polysaccharides (polysacc), proteins, G- and S-lignins, and fatty acids in the control and +N soils.
The compound classes shown were developed from compound identifications from the published literature. Lignin derivatives were based primarily on identifications in Dijkstra and colleagues (1998), and cellulose-related peaks were based on information in Steinbeiss and colleagues (2006). Additional compound-type identifications were supplemented by information from Chefetz and colleagues (2002) and Buurman and colleagues (2007).

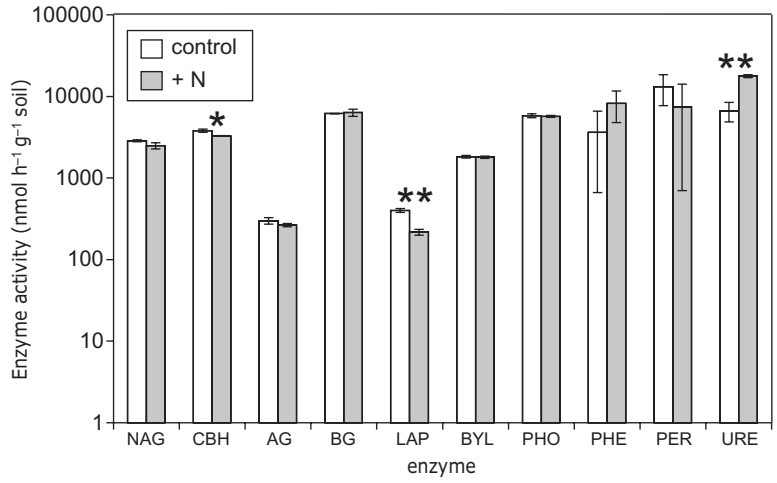

Fig. 5. Effect of chronic N amendments on the activity of soil enzymes. Bars show the specific activity of β-1,4-*N*-acetyl-glucosaminidase (NAG), β-D-1,4-cellobiosidase (CBH), α-1,4-glucosidase (AG), β-1,4-glucosidase (BG), leucine amino peptidase (LAP), β-1,4-xylosidase (BYL), acid phosphatase (PHO), phenol oxidase (PHE), peroxidase (PER), urease (URE). $^{*}P < 0.10$; $^{**}P < 0.05$.

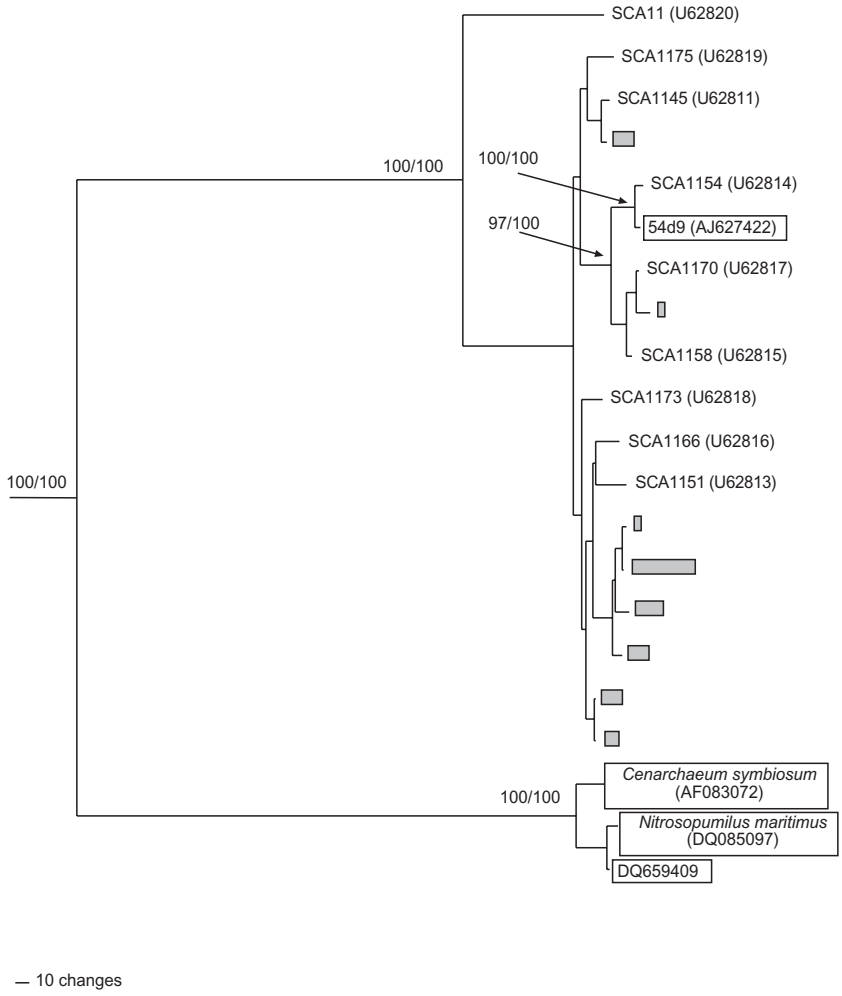

Fig. 6. Neighbour-joining phylogenetic tree showing representative sequences from the clones related to the archaea in the control plots. Grey rectangles represent sequences from this study, and the relative size of the rectangle symbolizes the number of sequences. SCA sequences are from the Bintrim and colleagues (1997) study. Sequences in boxes are nitrifiers. Tree is rooted with four bacterial 16S rRNA gene sequences (not shown). Values indicate distance and parsimony bootstrap values respectively.

Discussion

Previous work at this site documented accelerated decomposition of intermediate-aged portions of the SOM pool following chronic N additions (Neff *et al*., 2002). Here, we asked if these dynamic changes in SOM (Fig. 4B) are matched – and perhaps partially explained – by shifts in microbial composition. We collected soils in the fall, an important season for decomposition in the alpine tundra (Schmidt *et al*., 2004). Our results revealed significant changes in all fractions of the soil microbial community. Bacteria and fungi shifted in diversity (Figs 1 and 2, Table 1), and the overall abundance of archaea decreased dramatically in the N-amended soils (Fig. 3). The activities of two soil enzymes involved in N cycling changed in response to chronic N amendments, but seven other enzymes involved in a variety of C decomposition steps showed no response to fertilization (Fig. 5). Below, we propose several hypotheses to relate these changes in C and N cycling processes and the shifts in the soil microbial community composition.

In a much earlier study of these plots, Fisk and Schmidt (1996) showed that nitrification potentials in N-amended soils were significantly higher than control soils during the fall season. While our approach does not allow us to make direct links to changes in functional groups, we did not uncover any bacterial sequences that were closely related to known nitrifiers in either the control or +N soils (Fig. 1). Archaeal nitrifiers (Könneke *et al*., 2005) are also important in soil and water environments (Francis *et al*., 2005; Leininger *et al*., 2006); thus, we performed a detailed phylogenetic analysis of the archaeal community from control soils (as mentioned above, only one archaeal sequence was obtained from N-amended plots). We detected a number of 16S rRNA genes that fall between the crenarchaeal Groups 1.1a and 1.1b (Fig. 6), which contain marine and terrestrial archaeal nitrifiers (Nicol and Schleper, 2006). Although it is difficult to assign functions to the organisms represented by these sequences, it is possible that they may be important in ammonium oxidation in the unamended dry meadow soils. Interestingly, archaeal abundance declined precipitously in N-amended

plots (Fig. 3). Little is known about the physiology of the archaea, and it is possible that they are sensitive to changes in pH or C sources in N-amended soils, or that they are simply out-competed under more eutrophic conditions. This response of archaea to N fertilization may be a widespread phenomenon, as others have reported decreases in abundance (He *et al*., 2007) and diversity (Gattinger *et al*., 2007) of archaea in response to N amendments.

The increase in G-lignin-related compounds (Fig. 4B) is in agreement with other studies supporting decreases in lignin decomposition in response to N amendments (Carreiro *et al*., 2000; Waldrop *et al*., 2004a). In general, lignin-related compounds are viewed as chemically recalcitrant, because substantial energy investment is required for their breakdown, and the net caloric return is small (Kirk and Farrell, 1987). The recalcitrance of these molecules also relates to their structural uniqueness. Thus, one school of thought has been that in N-poor environments, investment in lignin decay may be partly driven by demand for its relatively high N content (Craine *et al*., 2007). According to this hypothesis, if N availability is enhanced, lignin decay should decline. Our pyrolysis data (Fig. 4B) provide a time-integrated picture that suggests a general decrease in lignin degradation over the course of the multi-year fertilization. Other mechanisms for the increase in lignin pools are possible as well, such as N-fuelled increases in the lignin content of plant tissues (Knops *et al*., 2007), or a change in lignin inputs because of the plant community shifts in these plots (Bowman *et al*., 1993).

The shift in fungal community composition may support the hypothesis that lignin decay is suppressed in the N-amended soils. Basidiomycetes possess a suite of lignin oxidase enzymes (Hammel, 1997), and these organisms appear to decrease in relative abundance in fertilized soils (Fig. 2). However, other explanations for this community shift are possible. For example, many basidiomycetes are ectomycorrhizal, and declines in both the diversity and abundance of mychorrizal infections have been seen in response to N amendments (reviewed in Treseder, 2004). Indeed, the increase in graminoid species cover in response to N fertilization suggests a decline in the importance of ectomycorrhizal associations in the tundra (Bowman *et al*., 1993). Thus, a shift to fewer basidiomycetes could be due to both N inhibition of lignin decay and a decreased prevalence of ectomycorrhizal infections because of increased N availability.

Although the function of organisms represented by specific sequences is difficult to predict, some hypotheses can be generated about the bacteria responsible for the changes in C cycling in the N-amended soils. For example, fertilized soils appeared to have higher relative abundances of *Bacteroidetes* and *Gemmatimonadetes*, two bacteria phyla which may be important in the decomposition of recalcitrant C compounds (Zhang *et al*., 2003; Lipson and Schmidt, 2004), perhaps suggesting a mechanism for the increase in the decomposition of 10- to 40-year-old C compounds revealed by Neff and colleagues (2002). Additionally, the *Verrucomicrobia*, which have been shown to be an abundant component of the summer microbial community in similar soils (Lipson and Schmidt, 2004), comprised 10% of the control library, but were not found in the +N library. Relatives of these organisms have been shown to ferment plant C compounds and to thrive under oligotrophic conditions (Albrecht *et al*., 1987; Janssen *et al*., 1997; 2002), which may be a more common feature of the lower productivity control soils (Bowman *et al*., 1993). More recent work shows that some *Verrucomicrobia* can also oxidize methane in acidic soils (Dunfield *et al*., 2007). N fertilization has been shown to significantly decrease methane oxidation at this site (Neff *et al*., 1994); thus, the decrease in the relative abundance of these organisms may be linked to decreased methane cycling in N-amended soil. However, it is important to note that few organisms have been studied in pure culture for each of these divisions. Thus, future approaches should include cultivation-based work as well as stable isotope probing to allow more direct links between changes in community composition and C-cycling processes.

The lack of changes in C-related enzymes may suggest that enzyme analyses are not suitable for distinguishing more subtle, long-term changes in SOM turnover. They may also be due to the notable seasonality of the communities in these soils, and their strong responses to the annual dynamics of plant C inputs to the soil (Nemergut *et al*., 2005; Schmidt *et al*., 2007). In other ecosystems, changes in soil enzyme activities in response to N enrichment vary both spatially and seasonally (DeForest *et al*., 2004; Lucas *et al*., 2007). Thus, it is possible that other seasons may reveal differences in the activities of C-degrading soil enzymes in fertilized and control soils. The similarity in enzyme activities among treatments despite the presence of different microbial communities may also suggest that there is considerable redundancy of function when it comes to C decay (e.g. Kemmitt *et al*., 2008).

Finally, we note that our emphasis in this study was on a thorough characterization of the soil microbial community, which prevented us from assessing spatial or seasonal variability within or across treatment types. We believe the differences between control and +N plots are robust for our fall sampling period, not only based on the analyses performed here, but because other work at this site has demonstrated comparatively low spatial heterogeneity in microbial community structure (Lipson and Schmidt, 2004). However, seasonal shifts in the microbial

community can be profound in alpine tundra systems (Lipson and Schmidt, 2004; Schmidt *et al*., 2007). Thus, while we chose to sample in the fall because it is a key time for heterotrophic activity in the alpine (Schmidt *et al*., 2004), we stress that additional study of seasonal differences across nutrient treatments is a logical next step.

Conclusions

The results of this study demonstrate that long-term, high-level N fertilization caused significant changes both in the chemical composition of SOM pools and in the structure of the microbial community. Moreover, some of the microbial community shifts could provide at least a partial explanation for changes in SOM structure. Most notably, steep declines in basidiomycetes (Fig. 2) make sense in light of the higher lignin concentrations of the N-amended plots (Fig. 4B). Additionally, the observed increase in the bacterial groups *Bacteroidetes* and *Gemmatimonadetes* (Fig. 1) may reflect earlier observations from these sites suggesting that the decay of 10- to 40-year-old C compounds is enhanced following N additions (Neff *et al*., 2002).

However, while these results provide potential links between microbial community structure and ecosystem function, we stress that unravelling the biological controls over SOM turnover will be a significant challenge. Multiple factors can alter SOM pools following increases in N availability, including shifts in both plant productivity and species composition. Likewise, changes in plant dynamics may alter the microbial community in ways that do not necessarily have implications for SOM turnover. In addition, microbial communities at this site are extraordinarily dynamic across seasons (Schadt *et al*., 2003; Schmidt *et al*., 2007), while the changes in SOM pool chemistry reflect the integrated activity of the microbial community over annual to decadal time scales. Thus, drawing inferences from SOM–microbial community comparisons for a single time point must be done with caution.

Last, the lack of significant changes in C-degrading enzyme activity between treatments may hint at a broader debate on the role of microbial communities in ecosystem function: under what conditions and/or for what processes will community shifts result in detectable changes in ecosystem-relevant functions? In the case of processes driven by groups of organisms with relatively low phylogenetic diversity, such as methanogenesis and nitrification, one might expect to see a community shift frequently coincide with a change in process. However, SOM decomposition is carried out by an astonishing variety of organisms interacting with an equally daunting array of chemical compounds; thus, one might expect much greater redundancy of function when investigating a trait like heterotrophy. A suite of methods, including ones that can reflect microbial activity over different time scales, are likely to be required to clearly establish links between soil community structure and SOM turnover.

Experimental procedures

Site description and sampling

The plots are in a well-characterized high alpine tundra dry meadow site, part of the Niwot Ridge LTER site in the Front Range of the Colorado Rocky Mountains, USA (reviewed in Bowman and Seastedt, 2001). In late October of 2003, we used a sterile trowel to remove the top 5 cm of soil from 10 different 2×2 m plots, five control and five N-amended plots. Soil samples were bulked in the field; thus, within-plot variability was not assessed. Fertilized plots had received 25 kg N ha^{-1} $year^{-1}$ in 1991, and 10 kg N ha^{-1} $year^{-1}$ in years 1992–2000 in the form of urea (Bowman *et al*., 1993; Neff *et al*., 2002). Detailed site descriptions have been previously presented (Bowman *et al*., 1993; Fisk and Schmidt, 1996).

Pyrolysis-gas chromatography-mass spectrometry (Py-GC-MS)

We examined the chemical structure of the light and heavy C fractions using previously described methods (Neff *et al*., 2006). Three replicates of each pooled soil (control and +N) were pyrolysed at 590°C in pyrofoils (Pyrofoil F590, Japan Analytical Company, Tokyo, Japan) within a Curie-Point pyrolyser (Pyromat, Brechbühler Scientific Analytical Solutions, Houston, TX, USA). Pyrolysis products were transferred online to a gas chromatograph (ThermoQuest Trace GC, Thermo Finnigan, San Jose, CA, USA). We used an interface temperature of 250°C with a split injection (split ratio 50:1, He flow rate 1.0 ml min^{-1}). Separation of pyrolysis products was performed on a BPX 5 column (60 m $\times$ 0.25 mm, film thickness 0.25 μm) using a temperature programme of 40°C for 5 min, 5°C min^{-1} to 270°C followed by a jump (30°C min^{-1}) to a final temperature of 300°C with a 10 min hold. The column outlet was coupled to a Thermo Polaris-Q ion-trap mass spectrometer (Polaris Q, Thermo Finnigan) operated at 70 eV in the EI mode. The transfer line was heated to 270°C and the source temperature was held at 200°C.

Peaks corresponding to pyrolysis products were compared to reference spectra after deconvolution and extraction using AMDIS v. 2.64 and National Institute of Standards and Technology mass spectral libraries and published literature (Pouwels *et al*., 1989; Schulten and Schnitzer, 1997).

DNA extraction and clone library construction

DNA was extracted from pooled +N and control soil samples using a modification of the protocol described by Moré and colleagues (1994). Half gram of soil was added to 0.3 g each of 0.1 mM, 0.5 mM and 1 mM sized glass beads (Biospec Products, Bartlesville, OK, USA) with 1 ml of phosphate extraction buffer and agitated for 2 min with a bead beater (Mini-bead-beater 8, Biospec Products). Samples were then centrifuged at 14 000 r.p.m. for 10 min and the supernatant

was removed to a fresh tube. Two hundred millilitres of 7.5 M ammonium acetate was added and samples were incubated on ice for 5 min. Extracts were centrifuged at 14 000 r.p.m. for 3 min and the supernatant was again removed to a fresh tube. Samples were then extracted with an equal volume of phenol : chloroform : isoamyl alcohol (25:24:1). DNA was precipitated with 200 ml of isopropanol overnight at –80°C. Samples were then centrifuged at 14 000 r.p.m. for 20 min and DNA pellets were washed with 1 ml of 70% ethanol. For each soil type (control and +N), eight separate DNA extractions were pooled to form composite extracts and purified over Sepharose 4B (Sigma, St Louis, MO, USA) packed columns as described in Jackson and colleagues (1997).

Approximately 30 ng of DNA was amplified by using the primers 8f and 1492r (bacterial; Lane, 1991), ITS9 and nLSU1221R (fungal; Schadt *et al*., 2003) and 8fa (Costello and Schmidt, 2006) and 1492 (archaeal; Lane, 1991) using reaction conditions described elsewhere (Schadt *et al*., 2003; Costello and Schmidt, 2006; Nemergut *et al*., 2007) and optimizing for the fewest possible number of cycles to attain visible product on an agarose gel. For bacterial 16S rRNA genes, we used 25 cycles, and, to maximize the diversity of genes targeted (Dahllof, 2002), a gradient of annealing temperatures ranging from 48°C to 68°C was employed. For archaeal SSU rRNA genes, we amplified for 30 cycles, and used annealing temperatures ranging from 45°C to 65°C. For fungal LSU genes, we used 35 cycles and annealing temperatures between 50°C and 70°C. The same PCR conditions were employed for both control and N-amended soil DNA samples. PCR products from four separate reactions with different annealing temperatures were pooled and gel-purified using QIAquick gel purification columns (Qiagen, Valencia, CA, USA). Purified products were ligated into the vector TOPO 2.1 (Invitrogen, Carlsbad, CA, USA) and transformed into *Escherichia coli* following the manufacturer's instructions.

Inserted 16S rRNA genes were PCR-amplified from the plasmids using the primers M13F and M13R (Invitrogen) and sequenced with the T7 promoter primer and the M13-9 primer using the BigDye Terminator Cycle Sequencing kit v. 3.0 (PE Biosystems, Foster City, CA, USA) following the manufacturer's directions. Fungal LSU rRNA genes clones were sequenced as described by Schadt and colleagues (2003). Sequencing products were analysed at the Iowa State University DNA Sequencing Facility and Functional Biosciences (Madison, WI, USA). Sequences were deposited in GenBank with the Accession No. EU861594–EU861972.

Quantitative PCR

Relative abundances of bacteria, archaea and fungi were quantified following the qPCR protocol described in Fierer and colleagues (2005). Bacterial and archaeal 16S rRNA gene abundances were determined from a 10-fold serial dilution of a plasmid containing a full-length copy of the 16S rRNA gene from *E. coli* and *Sulfolobus acidocaldarius* respectively. Likewise, fungal gene abundance was quantified using a plasmid containing the *Saccharomyces cerevisiae* SSU rRNA gene. Standard curves were run in triplicate with five non-zero concentrations per assay. Primer sets for the bacterial (Eub338, Eub518) and fungal (ITS1f, 5.8s) qPCR assays are described in Fierer and colleagues (2005) and we used the archaeal–primer set (ARCH967f, ARCH1060r) whose specificity has been demonstrated previously (Cadillo-Quiroz *et al*., 2006). Each 25 μl qPCR reaction contained 12.5 μl of ABgene SYBR Master Mix (Rochester, NY, USA), 0.5 μl of each 10 μM forward and reverse primers and 10.5 μl of sterile, DNA-free water. Standard and environmental DNA samples were added at 1.0 μl per reaction after normalizing soil DNA concentrations to 10 ng μl^{-1}. The reaction was carried out on a Eppendorf Realplex 2 thermocycler using the following conditions: 94°C for 3 min followed by 40 cycles of 94°C for 30 s, 50°C for 45 s and 72°C for 30 s. Melting curve and gel electrophoresis analysis were performed to confirm that the amplified products were the appropriate size. Ratios of gene copy numbers (bacterial to fungal and bacterial to archaeal) were generated with a regression equation for each assay relating the threshold (Ct) value to the known number of copies in the standards. Reporting the results as ratios of gene copy numbers limits the effects of differential amplification efficiencies on estimates of relative taxon abundances (Fierer *et al*., 2005). All qPCR reactions were run in quadruplicate with pooled DNA samples from each plot.

Phylogenetic analysis and comparisons

Bacterial SSU gene sequences were edited in Sequencher (Gene Codes, Ann Arbor, MI, USA) and aligned to the ARB database (Ludwig *et al*., 2004) using the Greengenes NAST aligner (DeSantis *et al*., 2006). Alignments were subjected to Bellerophon (Huber *et al*., 2004) and all putative chimeras were removed from further analyses. Taxonomic affiliations were assigned based on both BLAST (Altschul *et al*., 1990) matches and ARB alignments. We obtained 54 and 99 non-chimeric bacterial 16S rRNA genes for the control and N-amended samples respectively. We obtained 69 and 157 non-chimeric fungal LSU genes for the control and N-fertilized samples respectively. For archaeal 16S rRNA gene libraries, many of the genes that we sequenced were actually bacterial and eukaryotic SSU rRNA genes. Indeed, despite obtaining ~75 sequences from each of these libraries, only 29 (control) and 1 (+N) were archaeal 16S rRNA genes. OTU assignments were made by calculating a distance matrix in ARB and importing it into DOTUR (Schloss and Handelsman, 2005). Fungal LSU gene sequences were assembled and edited in Sequencher. Each sequence was then assigned a higher-order phylogenetic classification based on the top 10 matches from BLAST scores using the PLAN interface (http://bioinfo.noble.org/plan/) and manual annotation of phylogenetic lineages based on the GenBank taxonomy. Those clones grouping only with environmental sequences and those displaying only short (< 50% or query length) or divergent matches (< than 80%) homology were grouped as unclassified.

Phylogenetic trees were constructed by exporting alignments from ARB with the appropriate sequence mask. All alignments were subjected to Modeltest (Posada and Crandall, 1998) and the best model was used to construct a neighbour-joining tree. Parsimony trees were constructed using the heuristic search algorithm. Our crenarchaeal phylogenetic reconstruction was subjected to 100 bootstrap rep-

licates, using both the distance and parsimony optimality criteria. UniFrac (Lozupone and Knight, 2005) and P test phylogenetic comparisons (Martin, 2002) were made using the UniFrac web interface (Lozupone *et al*., 2006).

Enzyme assays

We analysed the activity of 10 enzymes involved in C and nutrient cycling (see Weintraub *et al*., 2007 for descriptions of the reactions that these enzymes catalyse) using the methods reported in Saiya-Cork and colleagues (2002). Sample suspensions were prepared by homogenizing 1.0 g of soil in 125 ml of 50 mM sodium acetate buffer adjusted to the mean pH of the soil samples. We created 16 replicate wells for each enzyme assay and sample, and all samples were incubated in the dark at 13 $\pm$ 2°C for up to 24 h.

For fluorometric assays, we added 50 μl of a 200 mM substrate solution to each sample well. Blanks, quench controls, substrate controls and reference standards were included in each microtitre plate. Fluorometric assays were terminated by adding 10 μl of 1.0 M NaOH to each well. We measured fluorescence with 365 nm excitation and 460 nm emission filters using a microplate fluorometer (Fluoroskan II, Thermo Labsystems, Franklin, MA, USA).

For the phenol oxidase and peroxidase assays, we combined 200 μl of sample suspension and 50 μl of a 25 mM L-3,4-dihydroxyphenylalanine (L-DOPA) solution. We created the phenol oxidase-negative control wells by combining 200 μl of acetate buffer and 50 μl of L-DOPA; blank wells contained 200 μl of sample suspension and 50 μl of acetate buffer. All of the wells in the peroxidase assays were identical to the phenol oxidase assays except they received 10 μl of 0.3% H_2O_2. Phenol oxidase and peroxidase activity was quantified spectrophotometrically by measuring absorbance at 460 nm (Spectramax Microplate Spectrophotometer, Molecular Devices, Sunnyvale, CA, USA). We subtracted phenol oxidase activity from peroxidase activity to calculate the net peroxidase activities reported here.

We set up the urease microplates in the same manner as the phenol oxidase assay plates except that 10 μl of 400 mM urea was used as the substrate. To quantify urease activity, ammonium concentrations were measured using colorimetric ammonium assay reagent packets (Sinsabaugh *et al*., 2000).

We analysed the enzyme data using one-way multivariate analysis of variance (MANOVA; DataDesk version 6.1, Ithaca, NY, USA) with N addition as the factor. To account for the possibility of finding significant differences simply by chance, which is a concern when several statistical comparisons are made together, the Bonferroni correction was used with all of the MANOVA tests, and all of the *P*-values reported here are Bonferroni corrected.

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