Effects of Nitrogen Deposition and Seasonality on Fungal and Bacterial Abundance in Alpine Soil

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Defense Date April 4th, 2016

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ABSTRACT

Increasing levels of reactive nitrogen (N) deposition have been shown to affect bacterial and fungal abundances in soils across a wide variety of terrestrial ecosystems, potentially influencing stabilization of excess inorganic N and associated detrimental ecological effects. However, few studies have investigated these changes in sensitive alpine systems or considered potential seasonal variation in microbial abundance. In the present study, soil samples were collected periodically throughout the course of a single growing season from plots subjected to long-term experimental N additions located in an alpine tundra dry meadow community on Niwot Ridge, Colorado. To examine recovery potential of plots impacted by long-term elevated N deposition, half of each experimental plot stopped receiving N treatment in 2007. Fungal to bacterial ratio (fungi:bacteria) was measured to estimate potential N leaching and destabilization of soil microbial communities due to increased N deposition. Quantitative polymerase chain reaction (qPCR) analysis was used on bulked soil samples to quantify both the effects of chronic N amendment on fungi:bacteria and recovery potential of fungi:bacteria in those same soils following cessation of N additions. Fungi:bacteria was found to change differently in response to N addition at different points during the growing season, driven primarily by changes in fungal abundance, with a decreased fungi:bacteria during the early growing season, no change to fungi:bacteria during the late growing season, and an increased fungi:bacteria during senescence. An overall decrease in microbial biomass with N addition was also observed, independent of growing season. The decrease in fungi:bacteria during the early season had a greater magnitude than the increase in fungi:bacteria during the late season, possibly inhibiting this community’s ability to act as an N sink overall. Additionally, there was no indication of recovery of microbial biomass from cessation of the N treatment after 7 years, indicating that the lowered capacity of the soil to stabilize inorganic N due to both the decrease in overall microbial biomass and fungi:bacteria may persist even if N deposition rates decrease to previous levels. These results suggest that increasing N deposition will be a significant factor driving changes in microbial abundance and fungi:bacteria in alpine tundra soils, possibly leading to long-term destabilization of alpine soil microbial communities and inorganic N storage.
INTRODUCTION

Fossil-fuel combustion, increased livestock production, and production of synthetic nitrogen fertilizers (Waldrop et al. 2004; Bouwman et al. 2002) have resulted in anthropogenic nitrogen (N) deposition increasing three to four fold over the past 150 years, with myriad impacts on the environment (Galloway and Cowling 2002; Howarth et al. 2002). These effects include increased concentrations of the potent greenhouse gas N₂O, acidification of soils, losses of soil nutrients such as potassium and calcium necessary for long-term maintenance and mobilization of toxic heavy metals such as aluminum, and eutrophication of lakes and streams (Vitousek et al. 1997). In many cases, each of these effects leads to loss of biological diversity, especially in ecosystems adapted for low soil N availability (Stevens et al. 2005; Suding et al. 2004). Exploration of plant and microbial community responses to increasing N deposition and their potential for serving as N sinks is thus becoming increasingly important.

Recent studies have shown alpine soils to be especially sensitive to N deposition. As generally oligotrophic systems adapted to low levels of nutrients, changes in these systems can serve as good early indicators for N deposition effects (Bowman et al. 2006). Alpine regions serve as watersheds for many large human population centers, and disrupting them may have considerable implications for water quality downstream. The responses of alpine areas to N deposition may differ significantly from those from forests and grasslands (Nemergut et al. 2008). Clarification of the microbial community compositional changes in different ecosystems is fundamental to understanding how anthropogenic N deposition will change the functioning of ecosystems. While Bowman et al. (2006) and other studies examining alpine soil response to N deposition have focused on plant ecology and soil responses, analysis of N deposition effects on the soil microbial community has not been thoroughly investigated, despite the importance of the microbial community to ecosystem functionality (Ninnipieri et al. 2003; Torsvik & Ovreas 2002; Griffiths et al. 2000; Finlay et al. 1997). Microbes play critical roles in soil
nutrient cycling, with fungi tending toward slow N cycling by immobilizing N in their extensive underground hyphal networks (Boddy 1999) and bacteria utilizing a more exploitative nutrient use strategy through rapid consumption of newly produced labile substrates (Bardgett et al. 2005). Consequently, an increase in soil fungi may lessen the effects of N deposition, while an increase in bacteria may exacerbate them, resulting in a positive feedback loop of adverse impacts within the system.

Changes in both microbial community composition and the fungi to bacteria ratio (fungi:bacteria) are likely to be different for varying levels of N deposition (Fierer et al. 2011; Nemergut et al. 2008; Frey et al. 2004; Hogberg et al. 2007; De Vries et al. 2006; Demoling et al. 2008). Moreover, Fierer et al. (2011) showed that increasing N deposition levels shift microbial communities away from conservative to more active life history strategies. Finally, a recent study demonstrated non-mycorrhizal soil fungi as a possible N sink with fungi:bacteria as a reliable predictor of variation in N leaching from a terrestrial ecosystem (Hogberg et al. 2013), and N leaching often increasing with increased N deposition (Dise and Wright 1995).

Microbial communities often exhibit seasonal changes, with bacteria tending to dominate during the fall due to a large input of labile carbon during plant senescence, and fungi during the winter as only recalcitrant sources of carbon are available (Bardgett et al. 2005). Thus it is important to consider temporal variation when analyzing changes in fungi:bacteria. Unfortunately, very few, if any, studies looking at N deposition effects on microbial community composition have taken seasonality into account. This may help explain some conflicting results for effects of N deposition on fungi:bacteria for different ecosystems, particularly for those systems with strong seasonality like higher elevation alpine environments.

The goal of the present study was to evaluate how nitrogen deposition influences seasonal changes in the fungi:bacteria. The experiment utilized a N deposition manipulation in long-term plots
established in 1997 on Niwot Ridge (Bowman et al. 2006). Most previous studies of N deposition effects on alpine soil microbial communities used plots fertilized with high levels of urea-N, which does not allow the separation of carbon and nitrogen effects (Nemergut et al. 2008). The plots used in the present study were fertilized with ammonium nitrate, avoiding this complication of analyses. Soil samples were taken at three different times during the alpine growing season in order to evaluate seasonal variation of fungi:bacteria. Furthermore, N treatments ceased in half of the area in the plots after 2007, allowing examination of the legacy effects of N deposition following cessation of treatment.

The present study put forth the hypothesis that both fungal and bacterial abundance would decrease with N addition as has been found in previous studies of the alpine and other systems (Ramirez et al. 2012, Nemergut 2008, Treseder 2008, Wallenstein et al. 2006), providing less overall biomass to act as a N sink. Additionally, fungi:bacteria was predicted to decrease with N fertilization, showing yet a further destabilization of this ecosystem due to N deposition. The most pronounced effect of N fertilization on fungi:bacteria was anticipated during early growing season when both would normally be most inhibited by a lack of nitrogen in the soil, allowing more exploitative bacteria to increase more rapidly in response to addition of N at this time. Finally, temporal changes were expected in fungal and bacterial abundances, with fungal abundance dominating in the early season when mainly recalcitrant carbon sources are available, and bacteria peaking during senescence when more labile carbon becomes available from leaf litter.

MATERIALS AND METHODS

Site description and sampling technique. Soils were sampled from long-term N addition plots in an alpine tundra dry meadow site on Niwot Ridge established in 1997 (Bowman et al. 2006). To account for microsite variation, 5 experimental blocks were established. Each block included 4 1 m x 1 m plots, with plots receiving control and 3 N treatments (0, 20, 40, and 60 kgN/ha/yr). Each plot was further subdivided into continued treatment and recovery subplots in 2007, 7 years prior to samples
taken for the present study. The treatment halves each received the corresponding N treatment during the summer growing season and the recovery halves received no additional manipulation.

Both treatment and recovery subplots in the control (ambient N deposition) and the 60 kgN/ha/yr amendment plots were sampled within each of 5 blocks (n=5, 1 sample per treatment in each block). Two surface soil (0-5cm, A horizon) plugs were collected from each subplot using a sterile trowel and combined together to reduce the effect of in-plot variability. Samples were collected at 3 different times during the summer of 2014, representing early growing season (29 June), late growing season (13 August), and senescence (13 September) in order to assess the effects of seasonal variability.

**Quantitative PCR (qPCR).** Relative abundances of bacteria and fungi were quantified using quantitative polymerase chain reaction (qPCR) in accord with the most recent molecular techniques used in analyzing fungi:bacteria, following the protocol established and described by Fierer et al. (2005). All qPCR reactions were run in quadruplicate on 96-well qPCR plates, with bacterial (16S) and fungal (ITS) rRNA gene abundances determined from 100-fold dilutions of genomic DNA (gDNA) extracted from 0.25 g of each bulk soil sample using a standard MoBio Soil DNA extraction kit (Carlsbad, CA, USA). Standards for both 16S and ITS were made from plasmids containing full-length copies of the 16S and ITS rRNA gene from *Escherichia coli K-12* and *Aspergillus fumigatus*, respectively. As standards, the concentration of genomic DNA from *E.coli K-12* and *A. fumigatus* was assessed via a QuantiT PicoGreen dsDNA assay (Invitrogen Life Technologies, Grand Island, NY), and seven 10-fold dilutions were used to generate a standard curve, run in triplicate for each 96-well qPCR plate. The use of these standards means that results are conservatively reported in genome equivalents, but should be interpreted as estimates of the total number of fungal or bacterial cells. Universal primer sets for the bacteria (515F, 806R) and fungi (FF390, FR1) were those in standard use by Fierer et al. (2005). Each 20uL qPCR reaction well contained 12.5 uL of ABgene SYBR Master Mix (Rochester, NY, USA), 1.25 uL of each 10 mM forward and reverse primers, 5 uL of DNA-free water, and 5 uL of 1:100 sample gDNA. An Eppendorf Realplex 2 thermocycler was used to carry out the reactions with
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. Gene copy numbers were generated with a regression equation relating the threshold (Ct) value for each assay to the known number of copies in the standards as described by Nemergut et al. (2008) and Fierer et al. (2005).

**Statistical analyses.** Repeated measures ANOVA was conducted on fungal abundance (ITS copies), bacterial abundance (16S copies), and fungi:bacteria (ITS copies/16S copies) using all plot data for comparison of effect of N level (control vs. amendment), treatment history (continued treatment vs. recovery), and the interaction of N level and treatment history (N level x treatment history) on these metrics for samples taken during the three separate time periods. Along with N level and treatment history, block (1-5) and qPCR group (A-D) were also included as random model effects in order to take into account any variation introduced by those factors. All statistical analyses were conducted in JMP Pro (2015), unless otherwise indicated.

**RESULTS**

Fungi:bacteria showed a significant nitrogen treatment x seasonality interaction (Table 1), decreasing by 16% with N amendment in the early season, remaining neutral during the late growing season, and increasing by 14% with N amendment at senescence (Fig. 1). Furthermore, while both bacterial and fungal abundance were depressed with the addition of N throughout the growing season (Fig. 2, 3), differences in the fungi:bacteria at different points in the season (Fig. 1) were driven by differential decreases in fungal abundance (Fig. 2). During the early season there was a 31% decrease in fungal abundance with N amendment, which was less pronounced during the late season and at senescence, with only 4% and 6% decreases respectively. There was also a significant interaction between N amendment and time for the abundance of both bacteria and fungi (Table 1). The effect of N on bacterial abundance was consistent throughout the season, with 19%, 21%, and 19% decreases during early season, late season, and senescence, respectively (Fig. 3). N amendment resulted in significant effects, but no significant N level x time interaction was apparent (Table 1). There was no
significant interaction between the N treatment and treatment history (recovery) for the bacterial and fungal abundances and fungi:bacteria, indicating there has not been any significant recovery from the N treatment 7 years after cessation of the treatments (Table 1).

Figure 1. Relative abundances of fungal (ITS) and bacterial (16S) small subunit (SSU) rRNA gene copies in control (0 gN/m²/yr) and N-amended (6 gN/m²/yr) soils for both continued treatment and recovery subplots over a single growing season, separated by treatment history into continued treatment (treatment) and recovery subplots, as estimated using qPCR assays. For control plots, early season (DoY 180) mean = 0.04255, se = 0.002327; late season (DoY 225) mean = 0.04672, se = 0.004509; senescence (DoY 256) mean = 0.03500, se = 0.002172. For N-amended plots, early season mean = 0.03554, se = 0.001714; late season mean = 0.004655, se = 0.003032; senescence mean = 0.04145, se = 0.002238. Error bars represent 95% confidence interval.

Figure 2. Abundances of fungal (ITS) small subunit (SSU) rRNA gene copies in control (0 gN/m²/yr) and N-amended (6 gN/m²/yr) soils for both continued treatment and recovery subplots over a single growing season, separated by treatment history into continued treatment (treatment) and recovery subplots, as estimated using qPCR assays. For control plots, early season (DoY 180) mean = 7039.80, se = 535.32; late season (DoY 225) mean = 7360.00, se = 556.22; senescence (DoY 256) mean = 6851.25, se = 397.10. For N-amended plots, early season mean = 4822.72, se = 452.80; late season mean = 7040.55, se = 575.72; senescence mean = 6465.25, se = 379.90. Error bars represent 95% confidence interval.
Figure 3. Abundances of bacterial (16S) small subunit (SSU) rRNA gene copies in control (0 gN/m^2/yr) and N-amended (6 gN/m^2/yr) soils for both continued treatment and recovery subplots over a single growing season, separated by treatment history into continued treatment (treatment) and recovery subplots, as estimated using qPCR assays. For control plots, early season (DoY 180) mean = 175403, se = 12846; late season (DoY 225) mean = 205705, se = 18473; senescence (DoY 256) mean = 200050, se = 8244. For N-amended plots, early season mean = 141767, se = 11884; late season mean = 162535, se = 13246; senescence mean = 161085, se = 7309. Error bars represent 95% confidence interval.

Table 1. Statistical results for repeated measures ANOVA for fungal abundance (16S copies), bacterial abundance (ITS copies), and fungi:bacteria (ITS copies/16S copies) using all all plot data. N level (control 0 gN/m^2/yr, amendment 6 gN/m^2/yr), treatment history (continued treatment T, recovery R), interaction of N level and treatment history (N level*T,R), block(1-5), and qPCR group (A-D) included as model effects. *significant to the 95% confidence interval

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DISCUSSION

The findings of the present study on fungi:bacteria show that soil microbial responses to elevated N deposition levels are strongly linked to seasonality and may have important ecological implications. The results supported the hypothesis that fungi:bacteria shift seasonally under ambient conditions (Bardgett et al. 2005; Schadt et al. 2003) and that both fungal and bacterial abundance decreased with N addition (Ramirez et al. 2012, Nemergut et al. 2008, Treseder 2008, Wallenstein et al. 2006). N addition was also found to cause differential seasonal changes in bacterial versus fungal abundance, in parallel with previously described changes in microbial functional groups due to N addition with seasonality in tundra soils (Schmidt et al. 2004). As a result, fungi:bacteria changed in opposite directions between the ambient and N fertilized plots during the beginning and end of the growing season, with fungi:bacteria decreasing with N amendment during the early season, and increasing with N amendment during senescence. Unexpectedly, this change was driven primarily by a differential effect of N addition on fungal abundance for different points in the growing season rather than changes in bacterial abundance.

Consistent with the results of previous studies, a decrease was shown in both bacterial and fungal abundance due to N deposition, indicating an overall decline in alpine soil microbial biomass due to long-term increased N deposition. This result may signal an overall decline in the ability of the microbial community to act as an N sink. This decrease in microbial abundance is congruous with findings of decreased microbial biomass in both alpine (Nemergut et al. 2008) and a wide range of other ecosystems (Treseder 2008, Wallenstein et al. 2006). In an experimental study, Ramirez et al. (2012) found that microbial biomass decreased by 35% on average with N addition over a year-long incubation period of soils from a wide range of climate and soil characteristics, suggesting that this may be a general response. Further evidence of this response of biomass to N addition is presented in a meta-analysis by Treseder (2008) where it was found that total microbial biomass declined on average
by 15%. The present study demonstrates bacterial abundance declining on average by 20% and fungal abundance by as much as 31%, depending on the time of season. These findings are consistent with the amount of decrease found in the above mentioned studies, further bolstering the idea that increased N deposition results in a decrease in microbial biomass across biome types.

Contrary to expectations based on previous studies (Boddy 1999; Bardgett et al. 2005), findings of the present study suggest that changes in fungal abundance, rather than bacterial abundance, drive the differences in the fungi:bacteria response to N amendment. Because bacteria are generally thought to be faster cycling than fungi (Bardgett et al. 2005), they were expected to respond more quickly to increased nutrient input and seasonal variation, with these changes being the main driver for differences in fungi:bacteria. Results indicate, however, that a differential depressive effect of N addition on fungal abundance actually changed fungi:bacteria more than bacterial abundance in response to the interaction of seasonal change and N amendment, with the depressive effect of increased N on bacterial abundance remaining fairly stable throughout the season. N input affected fungal abundance by a greater amount early in the growing season than it did during the late season and senescence when resources were probably more plentiful, with fungal abundance only slightly depressed with N addition outside of the early growing season. Several factors may help explain this unexpected result including higher resistance of fungi to the effects of toxic heavy metals in general (Rajapaksha et al. 2004, Hiroki 1992). Another contributing factor may be that fungal populations have a greater ability to increase biomass in response to increased N deposition over the long-term (Singh et al. 2013; Orwin et al. 2011; Rillig et al. 2007; Treseder et al. 2006; Boddy et al. 1999). The relatively high level of C pools in alpine soils (Ni 2001), may thus allow fungi to respond more quickly to increased N availability than expected. Moreover, fungal and bacterial abundance did not change seasonally as expected. According to previous studies (Bardgett et al. 2005), there should be a relatively higher fungi:bacteria in winter and the early growing season due to fungi’s ability to immobilize and store nutrients and access more recalcitrant carbon and other nutrient sources and lower
fungi:bacteria during senescence due to an increase of exploitative bacteria when more labile carbon and nutrients are available from new leaf litter (Boddy 1999). Instead, the present study found that the fungi:bacteria increased through the growing season and then decreased slightly after senescence. Fungi:bacteria does not appear to decrease due to an increase of bacterial activity, as bacterial abundance showed no increase during aboveground plant senescence, with or without N addition.

A lower fungi:bacteria has been shown to correlate with greater N leaching (Hogberg et al. 2013) due to a longer fungal lifespan and increased ability to immobilize N in hyphal networks (Boddy 1999). Results of the present study showing variable responses of fungi:bacteria imply differential effects of N addition on N stabilization over the growing season in alpine soils, with N leaching increasing during the early growing season and decreasing during senescence. More specifically, lower fungi:bacteria during early growing season due to increasing N deposition may act to exacerbate the detrimental effects of increased N deposition with a relatively lower number of fungi to immobilize N in their hyphal networks, thus lowering the capacity to prevent leaching during the time of year when major run-off occurs in the alpine (Hogberg et al. 2013). On the other hand, the higher fungi:bacteria during senescence may decrease N leaching and lower N availability in soil during the winter with bacterial die-off after plant senescence. Since senescence generally occurs after the peak of precipitation during the summer, and results of the present study for both recovery and continued plots taken together show the increase in fungi:bacteria is slightly (2%) lower than the prior decrease, this possible natural buffering mechanism may not be enough to make up for the more prominent fungi:bacteria decrease occurring during the early season. Soil samples collected early in the season, prior to the initiation of snowmelt, would help verify whether the decrease in fungi:bacteria occurs before or after peak run-off. The decrease in fungi:bacteria with increased N deposition before peak run-off in the spring may be a negative feedback to the detrimental effects of increasing N deposition. This decrease in fungi:bacteria co-occurs with the largest seasonal decrease in microbial abundance before peak plant biomass, leaving a gap in ecosystem N sinks at a critical point of peak N deposition.
In essence, the unbalanced change in fungi:bacteria with the decrease during the early season, having a greater magnitude than the increase during senescence especially if it occurs before the bulk of spring run-off, will lower the ecosystem’s N sinks and therefore lead to a greater potential for environmental impacts of excess inorganic N in alpine soils.

This time-of-season-dependent response of fungi:bacteria to N addition may help explain why previous findings in alpine systems contrast with results from studies conducted in other ecosystems. Across different communities, fungi:bacteria tends to decrease with increasing levels of N deposition (Frey et al. 2004 in temperate hardwood and pine forests, Hogberg et al. 2007 in boreal forests, De Vries et al. 2006 in grasslands, and Demoling et al. 2008 in coniferous forests). On the other hand, Nemergut et al. (2008) did not find a decrease in fungi:bacteria with increasing N levels in alpine soils, likely due to missing the effect of seasonal changes in fungi:bacteria. For example, since the present study noted a near complete masking of the effect of N level on fungi:bacteria during the late growing season, samples taken only during this time would produce results suggesting no effect of N deposition on fungi:bacteria in alpine soils. In any case, with some conflicting results, resolving for which biomes a decrease in fungi:bacteria is generally correlated with increasing N deposition is still important as a building block for further study.

The present study found no evidence that bacterial and fungal abundance recovered 7 years after ceasing treatments; No significant differences were found in fungi:bacteria response in subplots receiving continuous treatment when compared to the recovery subplots that stopped receiving nitrogen amendments in 2007. This suggests that the capacity of alpine soil microbial communities to act as N sinks may remain compromised for long periods as a result of impacts from increased N deposition. As a result, increases to inorganic N leaching due to the decrease in overall biomass and lowered fungi:bacteria may continue even after N deposition rates decrease as a result of better management of N emissions.
In summary, the results of the present study support the hypothesis that fungi:bacteria will respond strongly to increasing N deposition in alpine soils, increasing during the early season and decreasing during senescence, lowering the capacity of the microbial pool to act as a sink for inorganic N. This trend is consistent with the responses of soils in other ecosystems to N deposition (Demoling et al. 2008; Hogberg et al. 2007; De Vries et al. 2006; Frey et al. 2004). The present results also confirm that seasonality is an important factor influencing the response of the microbial community composition to changes in N deposition (Nemergut 2008; Schadt 2003). Further, it is worth noting for future studies in this field that in some cases, the generalization of fungi and bacteria as conservative and exploitative, respectively, (Bardgett et al. 2005) may need to be re-evaluated. Lastly, the results of the present study indicate that changes in fungi:bacteria due to elevated levels of N deposition, and the associated decreased capacity to serve as a sink for inorganic N, cannot be reversed by lowering N deposition levels after a period of prolonged high N deposition, indicating legacy impacts on inorganic N stabilization in alpine soils.
Sources Cited


