Do shifts in life strategies explain microbial community responses to increasing nitrogen in tundra soil?

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Dear Editors of Soil Biology & Biochemistry,

Please find attached a revised version of the manuscript SBB10882 “Do shifts in life strategies explain microbial community responses to increasing nitrogen in tundra soil?” submitted for publication in Soil Biology & Biochemistry.

The comments of the reviewers were again very constructive and helpful. We have carefully considered all comments and revised the manuscript accordingly. We hope that the manuscript is now suitable for publication.

On behalf of all authors,

Minna Männistö
Responses to reviewers comments

Reviewer #1: I have two concerns in the revised manuscript, but believe they can be corrected.

I believe that all the measurements (except respiration in Fig. 3) were made only at the end of six-week incubation. Therefore, the authors cannot explicitly say that "N addition significantly increased/decreased XX (e.g. L367)" by comparing results of N-amended and control soils, because the authors did not actually measure changes over time. A correct description should be "XX was significantly higher/lower (or not significantly different) in N-amended soils than the controls (e.g. L392-393)." These results MAY INDICATE (or IMPLY) that the N addition "changed" XX over time assuming that the control soils maintained their properties relatively unaffected by the 6-week incubation. Such descriptions are all over the manuscript, and I want the authors to thoroughly correct them. I point some of the examples below (I bet I miss a few).

L335 "reduced"
L367 "Increased" copy number
L374 "increased"
L390 "decreased"
L480 "shift"
L493 "Decreased"
L511 "increased"
L513 "increased"
L550 "decreasing"

Some "Highlights" as well.

RESPONSE:
We agree to this point and have made the following corrections:
L 339: PLFAs were lower
L351: LAP activity was lower
L355: NAG activity was higher
L370: gene copies were higher
L372: Higher copy numbers
L379-383: abundance was higher/lower
L402: diversity and species richness were lower, dominance higher
L485: higher copy numbers
L491: abundance was higher
L493: abundance was lower
L519: detected at high abundance
L555: lower microbial biomass

My second concern is in Discussion, 4.2. It looks like the authors cherry-picked references that are consistent with the results of this study, and ignored those inconsistent. For instance, Fiefer et al. (2007 Ecology) categorized Bacteroidetes as copiotrophic, but the authors found that N-amended soils had less Bacteroidetes than the controls. Fierer et al. (2007) also reported that beta-proteobacteria (Class, not Phylum, by the way) was copiotrophic - how about in this study? Table 4 shows a significant N effect for Betaproteobacteria. Is it consistent with Fierer et al. 2007? Maybe not based on Fig. 5. If so, do not ignore the inconsistency, but discuss it (e.g. L506-508).

Response:
We did in fact detect a negative response of Betaproteobacteria and Bacteroidetes to N addition. As
earlier noted (by reviewer 1 in the first round), copiotrophy was tested by adding labile carbon by Fierer et al. (2007) and it is likely that members of Bacteroidetes and Betaproteobacteria correlate positively with the fresh labile carbon and are not directly influenced by N.

We have added the following sentence in the discussion (Lines 498-504):
Contrary to what was observed in this study, field experiments have shown increased abundances of Betaproteobacteria and Bacteroidetes in N amended plots (Ramirez et al., 2010; Koyama et al., 2014). Both taxa responded positively to increased labile C and were categorized as copiotrophs by Fierer et al. (2007). Increased N availability may thus have little direct effect on these taxa but the observed positive responses in the field were likely mediated through increased primary production by plants as suggested by Ramirez et al. (2010).

I also have minor comments below.

Highlights
The fourth highlight is confusing. It should be gene copies per unit DNA, not just gene copies.
R: Corrected

L99-102. Awkward. Change it like "...to more copiotrophic species which have higher growth rates and carbon use efficiencies, and utilize simpler..."
R: Corrected

L109. Remover "-" between "animal" and "interactions".
R: Corrected

L115-116. Awkward. Rewrite it like", grazing also leaves significant temporal variation in soil nitrogen"
R: Corrected

L121. Replace "show" with "is associated with"
R: Corrected

L126. Change "nutrient" to "nitrogen"
R: changed to “N”

L138-139. "yearly" and "every year" are redundant.
R: We have revised the sentence: In a heavily grazed area, reindeer migration causes a drastic nitrogen pulse every year... (L136)

L143-151. I don't like the way that the authors set up the specific hypotheses. It looks like there are two hypotheses [with 1), and 2)], but the two predictions are no more than expected results from one hypothesis. Remove 1) and 2).
R: Corrected (L 140-148).

L 214. I believe these analyses were conducted for soils after six-week incubation. If so, it's better to mention to it here.
R: Corrected in line 213

L308. I want to make sure that the authors have the accession numbers by the time it's accepted.
R: Accession numbers have been added to lines 307-309.
L314. "Phyla" is still used throughout the manuscript, despite the authors are using some Classes in comparison (e.g. alpha-Proteobacteria, gamma-Proteobacteria). Taxa should be the correct term. I want the authors to correct them throughout the manuscript (e.g. L325, L386, L432, L484).

R: Throughout the manuscript, we have replaced “phyla” with “taxa” or in some cases (lines 327, 840) with “phyla or class” to avoid confusion to OTU-level data.

L333. Replace "proxies" with "indices"
R: Corrected (L 337)

L347. Confusing. The subject of the sentence should be "LAP calculated per SOM", NOT "Nitrogen addition"
R: Corrected to “When calculated per SOM LAP activity was lower and NAG activity higher in N amended microcosms compared to the controls.” (L351-352)

L356. Are "microbial metabolic potential" and "microbial metabolic quotient" the same? If so, be consistent. What is the unit of qCO2? Please provide the result of "microbial metabolic potential" either in figure/table in the text or that in appendix. ANOVA table is not good enough to see what was observed.
Response: Yes they are the same. We have corrected these for consistency and made the following changes:
- We added description of the calculation to the Methods section (lines 194-196)
- We added the qCO2 values in the Results section (lines 360-363)
- We changed the term “metabolic acticity” to microbial metabolic quotient in Discussion (line 554)

L362. Replace "changes" with "differences"
R: Corrected

L379. I believe the authors should provide figures of UniFrac results as supplemental materials. The results of statistical analyses are not enough for readers to tell what N amendment/grazing did on bacterial community structure.
Response:
We used CAP (constrained canonical analyses of principle coordinates) analysis of weighted and unweighted UniFrac distances to visualize the effects of N amendment and grazing history on the community structure. We have added a description of the analysis in the methods section (L 329-332), provided the ordination as a supplementary figure and added text in the Results section (L 395-400).

L383-385, and L392-393. This is a correct description - "indicated significant differences", NOT "N amendment"
R: Corrected

L384. "N" is used instead of "nitrogen". Be consistent throughout the manuscript.
R: For consistency we have now used N for nitrogen and C for carbon throughout the manuscript as suggested also by reviewer 2.

L409. Add "dominated by" between "be" and "more"
R: Corrected
L434-345. Delete "the N amended microcosms and" - the previous sentence was all about difference between grazed and non-grazed soils. The following sentence should focus on the topic, otherwise readers will be confused.

R: Corrected

L434. "N" is used here again.
R: We have now used "N" throughout the revised MS

L453. The authors are not supposed to use "&" for two authors but "and" - See page 11 in Author Information Pack (https://www.elsevier.com/wps/find/journaldescription.cws_home/332?generatepdf=true).
R: Corrected

L468-469. These fungal operon numbers need citations.
R: We have added the reference to Baldrian et al., 2013 and references therein

L549. Define "metabolic activity" and how it was calculated.
R: Done, see the earlier response for L356

L574-575. "in to" should be "into"
R: Corrected

L579. "C" is used, instead of "carbon". Be consistent throughout the manuscript.
R: All nitrogen and carbon abbreviated to N and C

L595. Add "respectively" after "grazing"
R: Corrected

Table 1.

Be consistent in units - for instance, ug/g SOM is used for microbial N in Table 1, but mg/g SOM is used for Fig. 1.

R: We have changed the unit for Fig 1.

In Table 1, OM is used, but SOM is used in Fig. 1 - are they the same? If so, be consistent.

R: yes they are the same, we have corrected the OM to SOM in Table 1

Microbial N was around 0.5 mg/g OM (i.e. 500 ug/g SOM) when soils were collected (Table 1), but 50 mg/g SOM at the end of experiment (Fig. 1). They are different in two orders of magnitude. Am I correct? If so, can you discuss it?

R: thank you for pointing this out. We had miscalculated values for Fig 1. These have now been corrected, microbial N was around 500 ug/g SOM
Reviewer #2: The revised manuscript focuses more directly on the N-amendments and does a much more convincing job of linking the results presented to existing knowledge of N and C cycling. This version has clarified the theoretical approach used and assumptions of the copiotroph vs. oligotroph dichotomy, and even provides a plausible explanation to reconcile previous conflicting results obtained for microbial activity in response to N amendments. I find this manuscript to be an interesting contribution and would like to see it published in SBB. I have only minor suggestions to improve the clarity and presentation of the manuscript.

Please indicate significant differences among treatments in Fig. 1.
R: Done

My feeling is that for simplicity sake Table 3 could be relegated to supplementary materials if significant effects were noted in the upper right hand corner of Fig 3 (move legend to left hand panel).
R: we agree and have added the significant effects in Fig 3 and moved Table 3 to supplementary materials.

Remove results for rRNA gene copy numbers from Table 2 and instead indicate significant effects in Figure 4.
R: Done

Indicate significant differences among treatments in Fig. 6.
R: Done

After first use please abbreviate nitrogen as N and carbon as C consistently.
R: Corrected throughout the MS

L30 "...nutrient pulses in the soils located along migratory routes."
R: Corrected

L110 "...high temporal and spatial variation..."
R: Corrected (L 109)

Add accession numbers to L308.
R: Done

The first and second paragraphs of the discussion are quite repetitive. As the first paragraph provides only a brief summary, which is explored in greater detail in subsequent subsections, I think it could simply be deleted. However if retained please revise the first sentence for clarity. It is far too long and convoluted. I suggest:

L408 "We predicted that microorganisms from soils under heavy grazing, experiencing associated pulses in nitrogen, would be more copiotrophic..."

L411"...grazing history had little impact on microbial activities..."

L413 "...depending on grazing history. Instead N addition..."

R: We agree to the comment and have removed the first paragraph.

L505 "...SOM under N limitation..."
R: Corrected

L564 "variation in N availability." (punctuation missing)
R: Corrected
Highlights

Similar bacterial community structure in soils under different grazing intensities.

N amendment decreased respiration in tundra soil.

N amendment decreased biomass but increased rRNA copy numbers per unit DNA.

Copiotrophic taxa were more abundant in N amended soils.
Do shifts in life strategies explain microbial community responses to increasing nitrogen in tundra soil?

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Key words: tundra, nitrogen, bacterial communities, PLFA, enzyme activities, qPCR, next generation sequencing
ABSTRACT

Subarctic tundra soils store large quantities of the global organic carbon (C) pool as the decomposition of plant litter and soil organic matter is limited by low temperatures and limiting nutrients. Mechanisms that drive organic matter decomposition are still poorly understood due to our limited knowledge of microbial communities and their responses to changing conditions. In subarctic tundra large grazers, in particular reindeer, exert a strong effect on vegetation and nutrient availability causing drastic nutrient pulses in the soils located along the migratory routes. Here we studied the effect of increased nitrogen (N) availability on microbial community structure and activities by laboratory incubations of soil collected from two sites with contrasting grazing intensities. We hypothesized that heavily grazed soil experiencing nutrient pulses harbor more copiotrophic taxa that are able to respond positively to increases in available N leading to increased enzyme activities and respiration. Contrary to our hypothesis, there were only minor differences in the microbial community composition between the lightly and heavily grazed soils. N amendment shifted the bacterial community composition drastically, but the changes were similar at both grazing intensities. The relative abundance of diverse *Actinobacteria* and *Rhodanobacter*-affiliated *Gammaproteobacteria* increased in the N amended microcosms, while the abundance of *Acidobacteria*, *Alphaproteobacteria*, *Deltaproteobacteria*, *Verrucomicrobia* and *Bacteroidetes* decreased. Contrary to our hypotheses, increased N availability decreased respiration and microbial biomass at both grazing intensities, while increased N availability had little influence on the extracellular enzyme activities. We propose that similar to what has been reported in other systems, elevated N availability suppressed microbial respiration and biomass by favoring copiotrophic species with faster growth rates and with limited capabilities to decompose recalcitrant organic matter. Similar responses in soils from contrasting vegetation types, soil organic matter (SOM) quality and N availabilities in response to grazing intensity indicate that nutrient pulses may have a strong direct impact on the microbial communities. Responses detected using laboratory incubations
are likely amplified in the field where the direct effect of increased N availability is combined with increase in labile C through changes in plant production and species composition.

1. Introduction

As subarctic tundra soils store a significant proportion of the global soil C stock, there is a considerable interest in understanding how the C stored in these systems will respond to changes in environmental conditions. In addition to low temperatures as a factor that limits primary production and soil microbial activity, the role of N availability for soil C decomposition is considered to be a key factor in the responses of soil C stocks to climate change (Hobbie et al., 2002; Robinson, 2002; Mack et al., 2004). Accelerated SOM decomposition and nutrient mineralization in response to climate warming may lead to increased nutrient availability, which can in turn further increase decomposition rates. However, the role of increased N availability on soil microbial biomass and activities remains under dispute due to the fact that there are two contrasting lines of evidence regarding the direction of the N effect on soil microbial biomass and activities. The first line of evidence suggests a negative effect of increased N, as fertilization experiments across biomes have indicated that increased N availability reduces microbial biomass and activities (Treseder, 2008; Ramirez et al., 2010, 2012). The negative effect of N enrichment on respiration has been attributed to inhibitory effects of added N on ligninolytic enzymes (e.g. Sinsabaugh 2010), reduced need of the microorganisms to decompose organic matter for N acquisition (Craine et al., 2007), and switching of the microbial community from overflow metabolism to utilization of C for growth (Schimel & Weintraub, 2003). In overflow metabolism microorganism respire the excess C that cannot be utilized for growth due to nutrient limitations. Thus adding N to nitrogen limited systems may reduce respiration as it allows the microbes to utilize the excess C for growth (Schimel & Weintraub, 2003). The “nitrogen mining hypothesis” (Craine et al., 2007) suggests that SOM decomposition may be
driven by N limitation whereby microbes utilize complex organic matter as a N source rather than a C source.

Especially in Arctic tundra, another line of evidence suggests a positive effect of increased N. In these extremely N-limited tundra soils, low N availability may limit the production of extracellular enzymes and, hence, increased N availability may accelerate SOM decomposition by enhancing microbial enzyme production (Schimel & Weintraub, 2003; Wallenstein et al., 2009; Sistla et al., 2012), especially enzymes that hydrolyze C-rich compounds (Koyama et al., 2013; Stark et al., 2014). Long-term fertilization experiments in Alaskan tundra have shown increased C losses due to increased N availability in the soil organic layer (Mack et al., 2004), suggesting that increased N availability subjects centuries old SOM to microbial decomposition (Nowinski et al., 2008).

The divergent results on the effects of N on microbial biomass and activities reflect the close and complex inter-linkages between soil N and C cycles. The mechanisms by which microbial communities influence the nutrient cycles are still poorly understood, but the community structure is considered to be a key determinant of the functions (Fontaine et al. 2003; Fierer et al., 2012; Chen et al., 2014; Leff et al., 2015). Both field and laboratory experiments have identified major shifts in microbial community structure after fertilization treatment (Nemer gut et al., 2008; Ramirez et al., 2010; 2012; Fierer et al., 2012; Koyama et al., 2014; Leff et al., 2015) which are considered to reflect concurrent changes in microbial biomass (Fierer et al., 2012), enzymatic activities (Koyama et al., 2014) or soil respiration (Ramirez et al., 2010; 2012). It has been suggested that N availability is an important determinant for the dominant life strategy of the soil microbial community (Fontaine et al., 2003; Fierer et al., 2012; Chen et al., 2014; Leff et al., 2015). The priming effect theory predicts that under N limitations, microbial communities are dominated by slow growing K-strategists (oligotrophic species) that are able to mine nutrients in SOM whereas under abundant N concentrations, microbial communities are dominated by fast-growing r-strategists (copiotrophic species) that utilize mineral N and more labile C (Fontaine et al., 2003; Chen et al, 2014). A decrease
in respiration and enzyme activities through increased N availability has been linked to a shift in the microbial community to more copiotrophic species which have higher growth rates and carbon use efficiencies, and utilize simpler C sources with reduced need for extracellular enzyme production (Fierer et al., 2012; Ramirez et al., 2012; Leff et al., 2015). The abundance of Actinobacteria, Proteobacteria and Bacteroidetes has been shown to increase with increased N availability and these are suggested to represent copiotrophic taxa while members of the Acidobacteria and Verrucomicrobia have been considered to represent oligotrophic taxa (Fierer et al., 2007, 2012; Ramirez et al., 2012; Leff et al., 2015).

Investigations on the effects of increased N availability on soil microbial activity and community composition have considered mainly steady increases in soil N availability due to long-term fertilization, while little attention has been paid to plant-animal interactions, which may result in high temporal and spatial variation in soil N availability. Large migratory grazers have a substantial effect on vegetation across the world’s biomes, with important effects on soil C storage as well as N availability (Tanentzap and Coomes 2012). In subarctic tundra, grazing by reindeer (Rangifer tarandus L., same species as caribou in the northern parts of the American continent) causes important changes in vegetation (Zamin and Grogan 2012; Bernes et al., 2015), N availability (Olofsson et al., 2004), and soil microclimate (Stark et al., 2015), but grazing also leaves significant temporal variation in soil N. Reindeer migrate annually between summer and winter ranges depending on the availability of suitable forage, and in sites located along the migration routes reindeer herds cause substantial N pulses with drastically increasing N concentrations repeated each year (Stark and Väisänen 2014). Migratory grazing is often associated with a shift in the dominant vegetation from evergreen and deciduous dwarf shrubs to a grassland that is associated with enhanced plant productivity, soil N availability, and microbial respiration (Olofsson et al., 2004). Tundra systems along migration routes thus differ from the surrounding systems in several ways; while lightly grazed systems experience continuously low N concentrations and are dominated by
slow-growing dwarf shrub plant species, systems along the migration route experience drastic N pulses each year during the reindeer migration and are dominated by graminoids. This difference in the natural patterns of soil N concentrations could provide interesting opportunities for investigating the soil microbial responses to increasing N, and offer novel insights into the capacity of microbial communities for adapting to different patterns of soil nutrient availability.

Here, we hypothesized that – resulting from community-level adaptations to prevailing N levels – soil microbial responses to increasing N availability depend on natural patterns in soil N concentrations. Understanding this relationship was expected to improve our understanding on the basic mechanisms by which microbial communities influence tundra soil C and N cycles. We tested the hypothesis through laboratory incubations with experimental N additions to soils from two sites with contrasting reindeer grazing intensities. In a lightly grazed area, the access of reindeer during migration is prevented by a fence built in the 1960’s, the vegetation is dominated by dwarf shrubs and the soil contains low N concentrations with little temporal variation (Stark and Väisänen 2014).

In a heavily grazed area, reindeer migration causes a drastic N pulse every year, the vegetation is dominated by graminoids and the soil shows higher N concentrations as well as extreme temporal variation during the growing season. We hypothesized that parallel to shifts in vegetation and soil N, grazing has induced shifts in the microbial community composition towards higher functional adaptation of the soil microbial community to sudden increases in N. More specifically, we predicted that bacterial communities and decomposition under lightly grazed N-poor soils are driven by more oligotrophic, slow growing K-strategist bacteria that utilize organic N by decomposing the recalcitrant C pools (a.k.a nitrogen mining). Consequently increased N availability triggers only weak responses in the microbial activities. On the other hand, we predicted that bacterial communities in the heavily grazed soils with higher N availability are dominated by more copiotrophic r-strategist species that respond more strongly to increased nutrient availability which in
turn is detected as increased respiration, enzyme activities for C acquisition and significant shifts in microbial community structure reflecting growth of the faster growing r-strategist taxa.

2. Materials and methods

2.1 Study site, sampling and laboratory incubation

Soil for the laboratory incubation was obtained from a mesic tundra heath (Raisduoddar, Norway [69°39’N, 27°30’E]) located in the suboceanic section of northernmost Fennoscandia (Oksanen and Virtanen, 1995). Owing to a pasture rotation fence built in the 1960s, one sub-section in Raisduoddar is used by the reindeer only briefly for passage (hereafter referred to as light grazing). Vegetation under light grazing is dominated by evergreen and deciduous dwarf shrubs (Empetrum nigrum ssp. hermaphroditum, Betula nana, Vaccinium vitis-idaea), and the soil is N poor (Table 1; Stark et al. 2002; Olofsson et al. 2004). Another sub-section has been subjected to intensive grazing for the past 50 years during the annual reindeer migration period during the first half of August (hereafter referred to as heavy grazing). Owing to the repeated disturbance, vegetation under heavy grazing is dominated by graminoids (Carex spp.), plant productivity is high, and the soil is N rich (Table 1; Stark et al., 2002; Olofsson et al., 2004). Soil N concentrations are at highest during the reindeer migration, when soil NH$_4$-N concentration under heavy grazing are approx. 270 µg g$^{-1}$ SOM relative to values of approx. 24 µg g$^{-1}$ SOM under light grazing (Stark and Väisänen 2014).

Five blocks were established along the reindeer fence that separates lightly and heavily grazed sub-sections (distance between blocks > 20 m). Within each block, we selected plots of about 5 × 5 m at both lightly and heavily grazed sides of the reindeer fence (distance between plots with differing grazing intensity < 20 m). Soil material was collected by coring approx. 2 kg of fresh soil, which corresponded to 10-15 soil cores (diameter 7.5 cm) to approx. 5 cm depth in the soil organic
layer (Stark et al., 2015). Prior to the experiment, soils were pre-incubated for two months at 4°C in order to deplete soils of plant-derived labile C substances with rapid turnover rate (e.g. root exudates). After the pre-incubation, soils were sieved (mesh 2 mm) and soil moisture (drying at 105°C, 12 h), organic matter content (loss on ignition at 475°C, 4 h), and water-holding capacity (WHC) determined. Sub-samples were also taken for analysis of soil N concentrations.

Soil microcosms were established by weighing 30 g of soil obtained from each of the five blocks per grazing treatment in 500 ml glass bottles capped with rubber stoppers. Soil moisture was adjusted to 30% of the WHC. N was added as NH₄NO₃ to the N amended (+N) microcosms from a 100 g⁻¹ stock solution to a final concentration of 12.5 mg NH₄NO₃/g SOM, corresponding to 8.75 mg of N per gram of soil C (Hartley et al. 2010). Controls were amended with the same volume of milli-Q water. We used the same dosage of NH₄NO₃ as in Hartley et al. (2010), because these soils constituted the most similar soils in the literature compared with our site and thus created an interesting comparison. Soils were incubated for six weeks at 9°C after which soil was sampled for microbial activity, community composition and soil chemical analyses. The effect of NH₄NO₃ on soil pH was tested in a separate experiment by adding NH₄NO₃ and water to 10 g of soil in the same ratio. Soil pH was measured immediately and after 4 week incubation in 3:5 v/v soil:water suspensions (Denver Instrument Model 220).

2.2 Microbial respiration and extracellular enzyme activities

Microbial respiration (total CO₂-C release) was analyzed at selected time points from the headspace of the incubation bottles using an Agilent 6890N GC equipped with a ShinCarbon ST micropacked column (Restek) and thermal conductivity detector. Microbial metabolic quotient (qCO₂, respiration per biomass) was calculated after 6 weeks of incubation by dividing the respiration rate (mg CO₂ h⁻¹ g⁻¹ SOM) by total PLFA concentration (µmol PLFA g⁻¹ SOM).
The effect of N addition on potential extracellular enzyme activities (EEAs) was tested after the 6 week incubation as described earlier (Stark et al., 2015). Briefly, betaglucosidase (BG), β-N-acetylglucosaminidase (NAG), acid-phosphatase (AP) and leucine aminopeptidase (LAP) activities were analyzed using para-nitrophenyl(pNP)-β-glucopyranoside (5 mM), pNP-β-N-acetylglucosaminide (3 mM), pNP-phosphate (5 mM) and leucine p-nitroanilide (5 mM) as substrates, respectively. Three g sub-samples of soil were suspended in 60 ml sodium acetate buffer (50 mM, pH 5.0), after which 100 µl of the sample was mixed with 200 µl of enzyme substrate in a 96-well plate. Blanks with 100 µl of the sample mixed with 200 µl of sodium acetate were prepared to account for the color in the soil slurry and the background absorbance of the enzyme substrate was analyzed using blanks with 100 µl of sodium acetate mixed with 200 µl of enzyme substrate. Following incubations at room temperature, samples were centrifuged and 100 µl of the supernatant transferred into another 96-well plate, 5 µl of 1.0 M NaOH added, and the absorbance was measured at 410 nm using a Multiscan FC microplate reader (Thermo Scientific).

2.3 Soil N, microbial biomass and community composition

Soil and microbial biomass N were analyzed after the 6 week incubation. A ~3 g subsample of soil was extracted with 50 mL of 0.5 M K₂SO₄ while another subsample was extracted using the same method after chloroform fumigation for 18 h (Brookes et al., 1985). The concentration of NH₄-N was determined from soil extracts according to the standard protocol (SFS 3032) using a Shimadzu UV-1700 spectrophotometer. NO₃-N was determined from soil extracts via flow analysis (FIA Perstorp). The total extractable N in both soil and fumigated extracts was oxidized to NO₃ (Williams et al., 1995) and then analyzed as NO₃-N (FIA, Perstorp). Microbial N was calculated by subtracting the total extractable N of the soil extracts from that of the fumigated extracts.
Phospholipid fatty acid (PLFA) and lipid phosphate (PO$_4$) concentrations were used as proxies for soil microbial biomass (Findlay et al., 1989; Frostegård et al., 1991; Frostegård and Bååth, 1996). Lipids for both analyses were extracted from 1 g (wet weight) of freeze-dried soil using 10 ml of a one-phase mixture (1:2:0.8 v/v/v) of chloroform, methanol and 0.15 M citrate buffer (pH 4.0) for 2 hours after which the tubes were centrifuged at 1500 x g for 10 min. The supernatant was transferred to a new tube and the soil was re-extracted with 5 ml extraction solvent for 1 h, centrifuged and supernatants from the first and second extraction combined. The phases were separated by adding 4 ml of chloroform and 4 ml of citrate buffer. After overnight separation, the lipids were separated into neutral lipids, glycolipids and phospholipids in silicic acid columns as described by Frostegård et al. (1991). The phospholipid fraction was then subjected to mild alkaline methanolysis (White et al. 1979) after which the fatty acid methyl esters were analyzed as described by Männistö and Häggblom (2006). PLFA 18:2ω6c was used to indicate fungal biomass [including saprotrophic, ectomycorrhizal and ericoid mycorrhizal fungi (Olsson 1999; Ruess et al. 2002)], while the sum of PLFAs i15:0, a15:0, 15:0, i16:0, 16:1ω9c, i17:0, a17:0, 17:0, cyclo-17:0, 18:1ω7c and cyclo-19:0 was used to indicate bacterial biomass (Frostegård and Bååth 1996). For the lipid-PO$_4$ analysis, 0.2 ml of the lipid phase (containing all lipid fractions) was pipetted to a new test tube and dried under a stream of N. Lipids were digested with 1.8 ml saturated potassium persulfate by incubating the samples at 95°C for 50 h. PO$_4$ was measured by the malachite green method as described by Findlay et al. (1989) using a Shimadzu UV-1700 spectrophotometer.

Total genomic DNA was extracted from 0.25 g of soil using a modified phenol-chloroform-isoamylalcohol (PCI) protocol after Griffiths et al. (2000). In brief, 0.5 g of acid-washed and baked glass beads (0.1 mm) together with 500 µl PCI (ratio 25:24:1) and 500 µl cetrimonium bromide buffer (CTAB) were added to the soil sample followed by bead beating on a Precellys 24 Dual homogenizer for 30 s at 5500 rpm. Samples were centrifuged (16000 x g, 5 min) and the extraction repeated with PCI followed by a final extraction with chloroform-isoamyl alcohol (CI;
DNA was precipitated with 30% PEG solution for 1 h on ice, washed with ice-cold 70% ethanol and the DNA pellet was re-suspended in 1x TE buffer and stored at -80°C for further processing.

Amplification of the V1-V3 region of the 16S rRNA gene was done using the primer pair 27F (5'-AGAGAGTTTGATCMTGGCTCAG- 3', Lane, 1991) and 518R (5'-ATTACCGCGGCTGCTGG- 3; Muyzer et al., 1993). A 25 μl PCR reaction contained 5 μl 5x Phusion HF buffer (Thermo Scientific), 0.5 μl dNTPs (10 mM), 0.5 μl bovine serum albumin (20 mg/ml), 1.25 μl of each primer (10 μM), 1 U Phusion High Fidelity polymerase (Thermo Scientific) and ~50 ng of the DNA extract, filled up with PCR grade water. PCR amplification was carried out on a Biometra TPProfessional Basic cycler (Biometra, Germany) with 98°C for 30 s, followed by 30 cycles of 98°C, 10 s; 52°C, 30 s; and 72 °C for 180 seconds, and a final elongation step of 5 min at 72°C. Successful PCR products were re-amplified for 6 cycles with the same primer pair, but including adaptor A (5’-CCATCTCATCCCTGCGTGTCTCCGAC- 3’) and unique 10-12 bp long barcodes in the beginning of the forward primer to allow Ion Torrent sequencing and assignment to specific samples. PCR products were cleaned using the Agencourt AMPure XP magnetic beads purification system (Beckman Coulter) and quantified using the Qubit dsDNA HS Assay Kit (Invitrogen). Amplicons were then combined in equimolar concentrations for sequencing. Next, pooled samples were re-amplified using the PGM-specific sequencing adaptor A (5’-CCATCTCATCCCTGCGTGTCTCCGAC 3’) and P1_338r (5’–CCTCTCTATGGGCAGTCGGTGAT TGCTGCCTCCCGTAGGAGT–3’) for 6 cycles using the Platinum PCR SuperMix (Life Technologies) to reduce the size of the template suitable for Ion Torrent sequencing (region V1–V2) and to add adapter P1 to the end of the product. The product was then purified using the Agencourt AMPure XP beads and seeded into an Ion PGM Template OT2 reaction following manufacturer’s instructions (Life Technologies). Templated beads were enriched
using the Ion OneTouch ES system and sequencing libraries were loaded on Ion 316 Chips and
sequenced using the Ion PGM Sequencing 400 Kit.

Quantitative PCR (qPCR) was performed in triplicate to evaluate bacterial 16S rRNA and
fungal 18S rRNA gene copy numbers using the CFX96 Real-time PCR detection system (Bio Rad).
qPCR for bacteria was done with the SsoFast Kit (BioRad) and the primer pair Eub341f (3’-CCT
ACG GGA GGC AGC AG-5’) and Eub534r (3’-ATT ACC GCG GCT GCT GG-5’) (Muyzer et al.,
1993). Each 15 µl reaction mixture contained 7.5 µl 2x SsoFast EvaGreen qPCR Supermix (Biorad),
0.375 µl of each primer (10 µM), 4.25 µl PCR grade water and 2.5 µl template in a 1000-fold
dilution. PCR conditions were 98°C for 2 min followed by 40 cycles of 98°C, 5 s; 56°C, 20 s
(following a plate read). Genomic DNA from *Granulicella tundricola* MPACTX9 was used as a
standard. qPCR for fungi was done with the KAPA Sybr Fast qPCR Kit (KAPA Biosystems) and
the primer pair FF390 (3’-CGA TAA CGA ACG AGA CCT-5’) and FR1 (3’-AIC CAT TCA ATC
GGT AIT-5’) (Vainio and Hantula, 2000). Each 15 µl reaction mixture contained 7.5 µl KAPA Sybr
Fast qPCR Mix (KAPA Biosystems), 0.375 µl of each primer (10 µM), 4.25 µl PCR grade water and
2.5 µl template in a 1000-fold dilution. PCR conditions were 97°C for 2 min followed by 40 cycles
of 95°C, 20 s; 50°C, 30 s and 72°C, 30 s (following a plate read). A plasmid containing the target
sequence amplified from *Phialocephala fortinii* was used as a standard for the fungal qPCR. A melt
curve read for each qPCR run was done after the last cycle from 65°C to 95°C in 0.5°C increments
for 5 s. In addition, qPCR products were checked on a 1% agarose gel for the correct size or the
appearance of additional bands.

Sequences were analyzed using Mothur (Schloss et al., 2009) and QIIME (Caporaso et al.,
2010a) software packages. Sequences shorter than 250 bp or which contained ambiguities and
homopolymer stretches of more than 8 bases were removed. Chimera check was performed using
UCHIME (Edgar et al., 2011) and singleton sequences were removed. After quality filtering 78403
reads were obtained from all samples, with an average of 3920 reads per sample (min = 2206, max =
For downstream analyses all samples were rarefied to an equal sequence number to avoid heterogeneity in sequencing depth which would affect calculations for α- and β-diversity. Sequences were then clustered into operational taxonomic units (OTU) using UCLUST (Edgar, 2010) with a sequence similarity value of 97%, and representative sequences were randomly chosen from each cluster. Taxonomy assignment of the OTUs was based on the naïve bayesian RDP classifier (Wang et al., 2007) using a SILVA-based reference database (www.mothur.org/wiki/Silva_reference_files) with a confidence threshold of 60%. Alpha diversity indices (Faith’s phylogenetic diversity, Chao1, dominance and observed OTUs) were calculated using a script available in QIIME. Dominance is here Dominance=1-Simpson index. For β-diversity analyses, representative OTU sequences were aligned to the SILVA reference database using PyNAST (Caporaso et al. 2010b) and a phylogenetic tree was build using FastTree (Price et al., 2009), followed by calculation of weighted and unweighted UniFrac distance metrics (Lozupone et al., 2006; 2011).

Sequences were deposited in the Sequence Read Archive of NCBI under accession number SRP069050 (runs SRR3133443, SRR3133444, SRR3133446 - SRR3133455, SRR3133457 - SRR3133464).

2.4 Statistical analyses

The effects of N and grazing intensity on EEAs, soil and microbial N, lipid-PO₄, total-PLFA, bacterial and fungal copy numbers, bacterial diversity indices and the relative abundances of dominant bacterial taxa were tested using the linear mixed effects model (PASW 12.0 Statistical software) with N addition (control, N+) and grazing intensity (light grazing, heavy grazing) as fixed factors and block as a random factor nested within grazing intensity. The effects of N and grazing intensity on microbial respiration were tested with the same model but incubation time was added to
the model as a repeated factor. Logarithmic transformations were used to meet the assumptions of
  
  The effect of N and grazing intensity on bacterial community structure was tested using
  PERMANOVA (Anderson, 2001) with N and grazing intensity as fixed factors and block as a
  random factor nested within grazing intensity. Weighted and unweighted UniFrac distances of 16S
  rRNA gene sequences were calculated using Qiime (as described above) and used as the distance
  measure for PERMANOVA procedure using 999 permutations for the probability tests. To test shifts
  within the dominant bacterial taxa, the abundance (number of sequences) in OTUs classified to
  specific phylum or class were used. The OTU data was square root normalized, Bray-Curtis
  similarities were calculated using PRIMER 6 software (Clarke and Gorley, 2006) and
  PERMANOVA tests performed as described for the UniFrac distances. Canonical analysis of
  principal coordinates (CAP, Anderson and Willis 2003) was used to visualize shifts in the bacterial
  community composition. Weighted and unweighted UniFrac distances were used as the metric and N
  as the grouping factor for CAP.

3. Results

3.1 Effect of grazing intensity and N addition on microbial biomass and soil nitrogen

After 6 weeks of incubation all indices for microbial biomass were significantly lower in N-amended
microcosms compared to the controls (Fig 1, Table 2). PLFA analysis indicated that especially
bacteria responded negatively to the N addition, as bacterial PLFAs were lower in the N amended
microcosms. There were no statistically significant differences in the fungal PLFAs between N
amended and control soils. Grazing intensity of the site did not influence the total biomass indicators
(total PLFAs, lipid-PO₄ or microbial-N) or bacterial PLFAs, but the abundance of fungal PLFAs was
significantly higher in the lightly compared to heavily grazed soils (Fig. 1, Table 2). The
concentrations of total extractable N was significantly increased by N addition, being 0.11 mg g\(^{-1}\) SOM and 2.77 mg g\(^{-1}\) SOM in control and N-amended soils under light grazing, respectively, and 0.24 mg g\(^{-1}\) SOM and 3.29 mg g\(^{-1}\) in control and N-amended soils under heavy grazing, respectively (Table 2). Soil pH at the beginning of the experiment was between 5.0 and 5.2, NH\(_4\)NO\(_3\) addition decreased pH on average by 0.67 units.

### 3.2 Effect of grazing intensity and N addition on microbial activities

When calculated per SOM LAP activity was lower and NAG activity higher in N amended microcosms compared to the controls. When the enzyme activities were calculated per total PLFA concentration (as a proxy for total biomass), there were no statistically significant differences in LAP, BG or AP activities between the N amended and control microcosms or in lightly and heavily grazed soils, but NAG activity was significantly higher in the N amended microcosms. Grazing intensity affected only NAG activity (calculated per organic matter) which was lower in the heavily grazed soil (Table 2; Fig. 2).

Microbial respiration declined over the 6 week incubation in both N amended and control microcosms regardless of previous grazing intensity (Fig. 3, Table S1). However, respiration declined more rapidly in the N amended microcosms. N addition resulted in a lower microbial metabolic quotient (respiration per biomass) when calculated per total PLFA concentrations. In the lightly grazed soils qCO\(_2\) was 5.79 vs. 4.23 CO\(_2\) h\(^{-1}\) mmol\(^{-1}\) PLFA for control vs. N amended microcosms, respectively, compared to 5.78 vs. 4.25 mg CO\(_2\) h\(^{-1}\) mmol\(^{-1}\) PLFA, in the heavily grazed soils, indicating that decreased respiration was not solely caused by the decline in microbial biomass.

### 3.3 Effect of N addition on microbial community structure in soils of different grazing intensity

Quantitative PCR (qPCR) analysis was used to detect changes in the bacterial and fungal ribosomal RNA gene copy numbers. Previous grazing intensity had no significant effect on bacterial copy.
numbers, but fungal copy numbers were higher in the heavily than in the lightly grazed soil (Table 2, Fig. 4). Both bacterial 16S rRNA and fungal 18S rRNA gene copies were higher in the DNA extracted from the N amended microcosms compared to the control microcosms (Table 2, Fig. 4). Higher copy numbers per ng DNA indicate that the composition of the bacterial and fungal communities shifted in the N amended microcosms to species with a higher number of 16S/18S rRNA gene copies. On the other hand, in soils under intensive grazing the fungal community appeared to consist of species with higher 18S rRNA gene copy numbers while there was no significant grazing effect on bacterial rRNA gene copy numbers (Fig. 4).

Bacterial communities were further compared by 16S rRNA gene sequence analysis. Classification of sequences indicated minor differences in the abundance of different taxa in the lightly and heavily grazed soils. However, the abundance of Actinobacteria was higher in N amended soils of both grazing intensities and the abundance of Gammaproteobacteria was higher especially in the N amended heavily grazed soils, while the abundance of members of Acidobacteria, Bacteroidetes, Alpha-, Beta- and Deltaproteobacteria, Planctomycetes and Verrucomicrobia were lower in N amended soils of both grazing intensities (Table 3, Fig. 5).

PERMANOVA analysis of the weighted UniFrac distances further indicated that after 6 week incubation the soil bacterial community structures were not significantly different in the heavily and lightly grazed soils, but N addition shifted the communities under both grazing intensities (Table 4). Shifts in the bacterial communities tended to differ in soils with different grazing history (grazing x N interaction, p=0.062). PERMANOVA analysis of the unweighted UniFrac distances indicated significant differences in the N amended vs. control microcosms but no interaction with grazing intensity. To detect shifts in the community structure within the dominant taxa, we tested the OTU data (abundance of sequences in different OTUs) of each phylum and in addition different Proteobacteria and Acidobacteria classes separately. PERMANOVA analysis indicated significant shifts within all major taxa in the N amended microcosms, while grazing
intensity affected only \textit{Gammaproteobacteria} and no grazing x N interactions were detected (Table 4). Canonical analysis of principal coordinates (CAP, Anderson and Willis, 2003) was used to visualize the grouping of samples based on the weighted and unweighted UniFrac distances. Similarly as the other analyses, CAP indicated that the bacterial community structure was controlled by N addition more than grazing history. Moreover, Spearman correlation of the CAP axes with dominant bacterial taxa supported the strong correlation of \textit{Gammaproteobacteria} and \textit{Actinobacteria} with the N amended soils (Fig. S1 in the supplementary materials). Comparison of $\alpha$-diversity indices indicated that the diversity (Faith’s phylogenetic diversity) and species richness (Chao1, observed species) were lower, but the dominance was higher in N amended microcosms of soils from both grazing intensities (Fig. 6, Table S2 in the supplementary materials). There were no significant differences in the species richness between the heavily and lightly grazed soils, but dominance was significantly higher in the heavily grazed soils. Moreover, N amendment decreased species richness more in the heavily than lightly grazed soil. These results indicate that while grazing intensity had no effect on the bacterial diversity, there were more dominant groups in the heavily grazed soils. N amendment increased the dominance in soils of both grazing intensities which decreased the diversity and species richness especially in the heavily grazed soils.

Comparison of the relative abundance of the 50 most abundant OTUs in the N amended microcosms identified several dominant OTUs that responded to the N amendment. The most abundant OTUs that increased in the N amended microcosms were generally members of \textit{Actinobacteria} (orders Actinomycetales, Acidimicrobiales and Solirubrobacterales) and \textit{Gammaproteobacteria} (\textit{Rhodanobacter} spp.) while those that responded negatively to N amendment included members of \textit{Alphaproteobacteria} (\textit{Rhizobiales}), \textit{Acidobacteria} and \textit{Gammaproteobacteria} (\textit{Sinobacteraceae}) (Table S3 in the supplementary materials).

\textbf{4. Discussion}
4.1 Differences in microbial community composition in response to grazing intensity

We predicted that the bacterial community composition should be more copiotrophic in microcosms with soil from the heavily grazed site with higher mineral N concentrations and stronger seasonal fluctuation. Higher N concentrations in the soil solutions have been linked to a higher dominance of r-selected microorganisms which utilize simpler organic compounds and are not able to mine N by degrading recalcitrant SOM (Fontaine et al., 2003; Fierer et al., 2012; Chen et al., 2014; Leff et al., 2015). However, there were only minor differences in the bacterial community structures between the grazing intensities as evidenced by both qPCR analysis which indicated no shifts in the bacterial copy numbers and sequence analysis which indicated no significant differences in the bacterial community structure (weighted or unweighted UniFrac distances), diversity or abundance of most of the dominating taxa. Of the dominating taxa, only the abundance and diversity of Gammaproteobacteria differed between the lightly and heavily grazed soils. These differences resulted mainly from the higher abundance of Rhodanobacter affiliated OTUs in the soil from heavy grazing pressure. The minimal effects of grazing on bacterial community composition were surprising given the large differences in the plant community structures and N availability between soils under light and heavy grazing. In other soil biomes, members of Acidobacteria and Verrucomicrobia have been reported to correlate negatively, while Actinobacteria, Bacteroidetes, Alpha-, Beta- and Gammaproteobacteria have been found to correlate mostly positively with increased N availability in field studies (Nemergut et al., 2008; Cambell et al., 2010; Ramirez et al., 2010; Fierer et al., 2012; Koyama et al., 2014; Leff et al., 2015). Vegetation type and shifts in plant community composition after fertilization have been reported to influence bacterial community structure in the Alaskan tundra (Chu et al., 2011) and grasslands across the globe (Leff et al., 2015).

Besides soil N availability, the 50-year difference in grazing intensity had also significantly altered
the chemical quality of accumulated soil organic matter in our study site. Characterization of soil C using $^{13}$C-NMR spectroscopy showed higher proportion of carbohydrates under light grazing and higher proportion of aliphatic-not-O-substituted C under heavy grazing (Väisänen et al., 2015). We suggest that the similarity of bacterial community composition in soils from different grazing intensities may partially result from the fact that we used pre-incubated soils that are depleted in labile C by e.g. plant root exudates. We found no effects of grazing on respiration and EEAs in the present laboratory incubation, but under field conditions and fresh soil samples, respiration, BG and LAP activities are found to be consistently higher under heavy than light grazing (Stark and Väisänen, 2014). In pre-incubated soils, bacteria associated with plant exudates may decrease and consequently, bacterial community composition to a large extent depicts the influence of the chemical quality of accumulated soil organic matter. By contrast, under field conditions the supply of plant-derived labile C may be a strong driver of the bacterial communities as well as microbial respiration rates. This notion is supported by our findings that the effect of grazing on bacterial community composition was slightly stronger in fresh soils collected from the same study site in the following year (manuscript in preparation).

Total PLFA, lipid phosphate and microbial-N concentrations were similar at both grazing intensities indicating no clear differences in microbial biomass. However, the fungal:bacterial ratio and the concentration of the fungal fatty acid 18:2ω6,8 indicated higher fungal abundance in soils under light grazing, which likely reflects the dominance of ecto- and ericoid mycorrhizal vegetation ($B$. $nana$, $Empetrum$ and $Vaccinium$ species) in the lightly grazed area as oppose to the graminoid-dominated vegetation in the heavily grazed area. Moreover, contrary to fungal PLFA concentrations, fungal 18S rRNA gene copy numbers were higher in soils under heavy than light grazing indicating that the fungal community structures differed between the grazing intensities. The ribosomal RNA gene copy numbers of fungi vary widely and numbers between 20 and 200 copies per genome have been reported (Baldrian et al., 2013 and references therein), while
forest soil fungal isolates were reported to contain $5 \times 10^5$ to more than $1 \times 10^7$ copies ng$^{-1}$ DNA (Baldrian et al., 2013). The high variability in copy numbers between different species, or even strains within the same species, limits the applicability of qPCR as a marker for fungal biomass while the fungal PLFA 18:2ω6 has been proposed as a more reliable estimate for fungal biomass (Frostegård and Bååth, 1996; Baldrian et al., 2013). Taken together, fungal PLFA concentrations indicated that a long history of heavy grazing has reduced the fungal biomass while simultaneously shifting the community composition to species with considerable higher ribosomal RNA gene copy numbers.

4.2 Effects of N addition on bacterial community composition

Experimental manipulation of soil N resulted in significant differences in the soil bacterial community composition between N amended and control soils. Nearly all measures, i.e. 16S rRNA gene copy numbers, UniFrac distances, and abundance and composition of different taxa indicated significantly different bacterial community structures in the N amended and control soils after 6 week incubations. We hypothesized that N amendment would increase the proportion of copiotrophic to oligotrophic bacterial taxa as suggested by Fierer et al. (2012). Higher copy numbers in the N amended microcosms, as indicated by the qPCR analysis, was in line with this hypothesis as the ribosomal RNA gene copy numbers have been linked to bacterial life strategy. Low copy numbers are attributed to oligotrophic life style (K-strategists) while high copy numbers reflect ability to respond dynamically to favorable growth conditions (Klappenbach et al., 2000). Sequence analysis indicated that the abundance of Actinobacteria and Gammaproteobacteria members was higher in N amended soils especially of the high grazing intensity, while the relative abundance of e.g. members of Acidobacteria, Alpha-, Beta- and Deltaproteobacteria, Bacteroidetes, Planctomycetes and Verrucomicrobia were lower in N amended microcosms. The negative response
to N in the relative abundance of *Acidobacteria, Planctomycetes* and *Verrucomicrobia* is in line with a N induced shift from oligotrophic to copiotrophic taxa as these are considered to represent oligotrophic bacteria (Fierer et al., 2007) and have been shown to respond negatively to increased nutrient availability earlier both in field (Fierer et al., 2012; Leff et al., 2015) and laboratory incubation studies (Ramirez et al., 2012). Contrary to what was observed in this study, field experiments have shown increased abundances of *Betaproteobacteria* and *Bacteroidetes* in N amended plots (Ramirez et al., 2010; Koyama et al., 2014). Both taxa responded positively to increased labile C and were categorized as copiotrophs by Fierer et al. (2007). Increased N availability may thus have little direct effect on these taxa but the observed positive responses in the field were likely mediated through increased primary production by plants as suggested by Ramirez et al. (2010). Similar to this study, *Actinobacteria* and *Gammaproteobacteria* have been reported to respond positively to increased N availability in field (Ramirez et al., 2010; Campbell et al., 2010; Leff et al., 2015) and laboratory studies (Ramirez et al., 2012). *Actinobacteria* and *Proteobacteria* are considered to include copiotrophic taxa that may benefit from increased N availability either directly or indirectly as an increase in labile C through increased plant primary production (Fierer et al., 2012; Eilers et al., 2010). Moreover, copiotrophic r-strategist taxa are considered to out-compete oligotrophic K-strategists at higher N availability because of their faster growth compared to the K-strategists that benefit from their ability to mine N from SOM under N limitation (Fontaine et al., 2003; Chen et al., 2014). However, studies indicate that within both phyla, there are members with different life–strategies and categorizing taxa in the different life history strategies remains challenging.

In this study we detected two distinctly different groups among the order *Xanthomonadales* (*Gammaproteobacteria*). *Rhodanobacter* related sequences were more abundant in the heavily grazed soil and N amended soils, while members of the family *Sinobacteraceae* were more abundant in the control soils and responded negatively to N amendment. *Rhodanobacter*
related sequences were detected at high abundance in fertilized plots of a field study at the same site (Männistö, unpublished data) and in a long-term fertilized tundra soil in Alaska (Campbell et al., 2010) indicating that N availability has a strong and direct effect on members of this taxon. OTUs related to the Sinobacteraceae have been abundantly detected in other, generally N-poor tundra soils where the abundance shifts similarly as the oligotrophic Acidobacteria (Männistö et al., 2013; Männistö, unpublished). Opposite shifts to nutrient and substrate concentrations are thus detected within taxa of the same order, highlighting the importance of analyzing the communities within fine taxonomic resolution and questioning the use of phylum level identification to classify the sequences into K- and r-strategists.

Similarly, while diverse members of Actinomycetales were shown to respond to labile C and were linked to copiotrophic, fast growing bacteria (Goldfarb et al., 2011), also other mechanisms could underlie the effects of N amendment on the abundance and community composition Actinobacteria. Some Actinobacteria have been linked to utilization of recalcitrant substrates such as lignocellulolytic C (Goodfellow & Williams, 1983; 1987; Ball et al., 1989; Větrovský et al., 2014) and known to possess a wide diversity of enzyme activities for decomposition of lignin-derived substrates (Bugg et al., 2011; le Roes-Hill et al., 2011). Actinobacteria may upregulate the lignocellulolytic activity under increased N availability (Barder & Crawford, 1981) and increased N availability has been shown to affect the structure of Actinobacteria community (Eisenlord & Zak, 2010) and richness of genes involved in the depolymerization of lignin and other plant polysaccharides (Eisenlord et al., 2013). Increase in Actinobacteria in the N amended microcosms may thus be linked to their ability to degrade recalcitrant C in addition or instead of preference to labile C. Several Actinobacteria phylotypes, including those related to the orders Actinomycetales and Acidimicrobineae, were reported to increase in warmed plots of a field experiment in Alaska. Increase of these Actinobacteria was linked to a decrease in labile C availability in warmed plots and these phylotypes were considered K-
strategists capable of utilizing the more recalcitrant C pools (Deslippe et al., 2012). N induced increases in a wide diversity of Actinobacteria likely reflects different life-strategies within this phylum with a wide diversity of functions related to C cycling.

4.3 Effect of increased N availability on microbial activity – a potential link to changes in bacterial life strategy?

Contrasting with our hypothesis, N addition did not increase microbial activities for C acquisition, but instead respiration and microbial biomass declined with N addition irrespective of grazing intensity. Microbial respiration was lower in N amended microcosms both when calculated per SOM and per microbial biomass, indicating decreased microbial metabolic quotient (qCO2) which in combination with lower microbial biomass led to a considerable decrease in microbial release of CO2. A decrease in respiration and microbial biomass after an increase in N availability has been reported in many field and laboratory studies (Treseder, 2008). Importantly, decreased qCO2 would be consistent with the copiotrophy theory stating that enhanced N availability increases rapidly growing copiotrophic microbial species with higher carbon use efficiencies and faster turnover rates (Fierer et al. 2012). The more copiotrophic community is less likely to decompose more recalcitrant SOM which further reduces respiration and EEAs (Fierer et al., 2012; Ramirez et al., 2012). Reduced respiration in the N amended microcosms is in line also with the priming effect theory predicting that under higher N availability, r-strategist members of the decomposers utilize the less recalcitrant C without the need of priming the decomposition of the recalcitrant SOM for N acquisition (Fontaine et al., 2003; Chen et al., 2014). Changes in the community structure together with increased ribosomal RNA copy numbers indicate that the decrease in microbial respiration in the N amended microcosms is linked to an increase in r-strategist taxa that compete better at higher N concentrations.
Unexpectedly, this mechanism functioned similarly at both systems with stable N concentrations and systems experiencing high seasonal variation in N availability.

Some field experiments in tundra ecosystems have shown increased microbial biomass, respiration and enzyme activities after N fertilization (Koyama et al., 2013, Stark et al., 2014), while other experiments have shown decreased microbial activity in response to N addition (Stark and Grellmann, 2002). Furthermore, using the exactly same dosage of N addition in laboratory incubations in sub-arctic soils, Hartley et al. (2010) found no effects of N addition on microbial respiration. The reason why N addition leads to such differing consequences in different experiments remains unknown. It is possible that soil microbial responses in field experiments are largely mediated by changes in the C availability due to increasing plant biomass and shifts in plant species composition, which affects litter and root exudate quality and quantity, rather than directly from increased N availability. Moreover, laboratory incubations with sieved soil do not take into account the plant-mycorrhiza interactions that may be important in determining the effects of increased N availability (Leff et al., 2015). The effect of N fertilization on microbial activities and community structure may also depend on the duration of the fertilization treatment (Koyama et al., 2013; 2014; Campbell et al., 2010). High N concentrations may inhibit soil microorganisms directly by increasing osmotic potential in soil solution or indirectly by decreasing pH or altering C availability (Treseder 2008). Similar to what was detected by Hartley et al. (2010), addition of NH\textsubscript{4}NO\textsubscript{3} decreased soil pH, which could be a possible explanation for the reduced respiration. However, the shift in the bacterial community composition does not support this mechanism since the taxa that increased in abundance in the N amended microcosms (Actinobacteria, Gammaproteobacteria) were ones that have been reported to respond negatively to reduced pH, while Acidobacteria that decreased in abundance in the N amended microcosms, have been correlated with lower pH (Lauber et al., 2009, Männistö et al., 2007). Moreover, earlier studies have indicated that N induced decline in respiration is neither linked to decrease in pH nor to the form of N utilized in the experiment (Ramirez et al., 2010).
5. Conclusions

This study showed drastic shifts in the tundra soil microbial community structure after N addition. However, contrary to our hypothesis, the responses of the bacterial communities to increased N availability were highly similar in N-poor and N-rich soils collected from habitats under light and heavy grazing, respectively. Increased abundance of members of Actinobacteria and Gammaproteobacteria in response to N addition with a concurrent decrease in Acidobacteria and respiration would support the theory of the increase of copiotrophic taxa, introduced by Fierer et al. (2012). However, another possible mechanism explaining the decrease in respiration and increase of the abundance of Actinobacteria would be the depletion of the labile C in the pre-incubated soils. Thus, while the community shift to a higher share of copiotrophic species may partially explain changes in the microbial activities, other mechanisms, such as the capacity to degrade recalcitrant C substances, are also likely. Changes in N availability likely affects the communities by multiple mechanisms, which shift the community structure to maintain the ecosystem functioning.

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References


Goldfarb, K.C., Karaoz, U., Hanson, C.A., Santee, C.A., Bradford, M.A., Treseder, K.K.,


Figure legends

Fig. 1. The effect of N amendment on the microbial biomass measures lipid phosphate, microbial N, total PLFA, bacterial PLFA and fungal PLFA concentrations after 6 week incubation in soils collected from lightly (LG) and heavily grazed (HG) tundra. Values are means ± S.E., N=5. Effect of N amendment and grazing intensity are indicated by significance levels *, p<0.05 and **, p<0.01.

Fig. 2. Potential N-acetyl-glucosaminidase (NAG) and leucine aminopeptidase (LAP) activities after 6 week incubation in N amended and control soils from lightly and heavily grazed tundra. Effect of N amendment and grazing intensity are indicated by significance levels *, p<0.05 and **, p<0.01.

Fig. 3. Effect of N amendment on the microbial respiration during the 6-week incubation in soils collected from lightly and heavily grazed tundra. Effect of N amendment, grazing intensity and incubation time are indicated by significance levels *, p<0.05 and **, p<0.01.

Fig. 4. Effect of N amendment on bacterial 16S rRNA gene and fungal 18S rRNA gene copies in the DNA of lightly and heavily grazed soils after 6 week incubation. Effect of N amendment and grazing intensity are indicated by significance levels *, p<0.05 and **, p<0.01.

Fig. 5. The relative abundance of bacterial sequences related to dominant taxa after 6 week incubation of N amended and control soils from lightly and heavily grazed tundra. Values are means ± S.E., N=5.

Fig. 6. Faith’s phylogenetic diversity (PD), species richness (Chao 1), dominance and observed OTUs in N amended and control soils from lightly and heavily grazed tundra after 6 week incubation. Effect of N amendment and grazing intensity are indicated by significance levels *, p<0.05 and **, p<0.01.
Supplementary information

Table S1. The effects of nitrogen addition, grazing, incubation time, and their interactions on the rates of microbial respiration of CO₂ during a 6-week laboratory incubation. F- and P-values are obtained by the Linear Mixed Effects Model (LME). Significance levels $P < 0.05$ are indicated in bold.

Table S2. The effects of N addition and grazing and their interactions on bacterial diversity indices. F- and P-values are obtained by the Linear Mixed Effects Model (LME). Logarithmic (*) transformations were used when necessary to meet the assumptions of LME. Significance levels $P < 0.05$ are indicated in bold.

Table S3. Average number of 50 most abundant OTUs in the lightly and heavily grazed soils with or without N amendment. Significant effects of grazing intensity and N amendment were tested using Linear Mixed Effects Model (LME). Logarithmic transformations (*) were used when necessary to meet the assumptions of LME. Significance levels *, $p < 0.1$; **, $p < 0.05$; ***, $p < 0.01$

Figure S1. Canonical analysis of principal coordinates (CAP) of weighted and unweighted UniFrac distances before (T0) and after 6-week laboratory incubation with (N) or without (C) nitrogen amendment. LG and HG soils were collected from lightly and heavily grazed tundra, respectively. Vectors show Spearman correlations of the dominant phyla or class with the CAP axes (only those with lengths >0.3 are shown).
Table 1. Soil properties under light and heavy grazing in Raisduoddar study site. Values are mean and S.E. in parentheses, N = 5.

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<thead>
<tr>
<th>Soil properties</th>
<th>Light grazing</th>
<th>Heavy grazing</th>
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<tbody>
<tr>
<td>SOM (%)</td>
<td>75.6 (2.3)</td>
<td>68.4 (8.0)</td>
</tr>
<tr>
<td>pH</td>
<td>5.1 (0.1)</td>
<td>5.2 (0.1)</td>
</tr>
<tr>
<td>NH$_4$-N (µg g$^{-1}$ SOM)</td>
<td>15.0 (5.6)</td>
<td>61.7 (29.1)</td>
</tr>
<tr>
<td>NO$_3$-N (µg g$^{-1}$ SOM)</td>
<td>3.1 (0.2)</td>
<td>9.8 (6.3)</td>
</tr>
<tr>
<td>Extractable organic N (µg g$^{-1}$ SOM)</td>
<td>102.9 (13.4)</td>
<td>105.6 (17.2)</td>
</tr>
<tr>
<td>Microbial N (µg g$^{-1}$ SOM)</td>
<td>495.5 (64.6)</td>
<td>504.8 (41.5)</td>
</tr>
</tbody>
</table>
**Table 2.** The effects of nitrogen addition and grazing and their interactions on microbial metabolic quotient (qCO$_2$), the potential activities of β-glucosidase (BG), N-acetylglucosamidase (NAG), acid-phosphatase (AP), and leucine-aminopeptidase (LAP), the concentration of lipid phosphate (lipid-PO$_4$), total, bacterial, and fungal PLFAs, microbial and inorganic N, and bacterial and fungal rRNA gene copy numbers after a 6-week laboratory incubation. F- and P-values are obtained by the Linear Mixed Effects Model (LME). Logarithmic (*) and square root (†) transformations were used when necessary to meet the assumptions of LME. Significance levels $P < 0.10$ are indicated by underline and $P < 0.05$ in bold.

<table>
<thead>
<tr>
<th></th>
<th>Nitrogen</th>
<th>Grazing</th>
<th>Nitrogen × Grazing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>$P$</td>
<td>F</td>
</tr>
<tr>
<td>qCO$_2$</td>
<td>25.1</td>
<td>$&lt; 0.001$</td>
<td>0.1</td>
</tr>
<tr>
<td>BG</td>
<td>0.5</td>
<td>0.498</td>
<td>0.1</td>
</tr>
<tr>
<td>PLFA$_{tot}$</td>
<td>1.2</td>
<td>0.316</td>
<td>0.1</td>
</tr>
<tr>
<td>NAG</td>
<td>4.0</td>
<td>0.079</td>
<td>4.3</td>
</tr>
<tr>
<td>PLFA$_{tot}$</td>
<td>17.4</td>
<td><strong>0.004</strong></td>
<td>0.6</td>
</tr>
<tr>
<td>AP</td>
<td>0.4</td>
<td>0.524</td>
<td>0.0</td>
</tr>
<tr>
<td>PLFA$_{tot}$</td>
<td>2.0</td>
<td>0.202</td>
<td>0.2</td>
</tr>
<tr>
<td>LAP</td>
<td>3.5</td>
<td>0.079</td>
<td>1.5</td>
</tr>
<tr>
<td>PLFA$_{tot}$</td>
<td>0.1</td>
<td>0.711</td>
<td>0.0</td>
</tr>
<tr>
<td>NH$_4$-N (log)</td>
<td>290.2</td>
<td>$&lt; 0.001$</td>
<td>6.8</td>
</tr>
<tr>
<td>NO$_3$-N (log)</td>
<td>329.6</td>
<td>$&lt; 0.001$</td>
<td>2.5</td>
</tr>
<tr>
<td>Microbial N (log)</td>
<td>9.8</td>
<td><strong>0.014</strong></td>
<td>0.2</td>
</tr>
<tr>
<td>Lipid-PO$_4$</td>
<td>11.3</td>
<td><strong>0.011</strong></td>
<td>1.1</td>
</tr>
<tr>
<td>Category</td>
<td>Value 1</td>
<td>Value 2</td>
<td>Value 3</td>
</tr>
<tr>
<td>------------------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
</tr>
<tr>
<td>Total PLFAs</td>
<td>23.5</td>
<td>0.002</td>
<td>2.5</td>
</tr>
<tr>
<td>Bacterial PLFAs</td>
<td>18.2</td>
<td>0.004</td>
<td>0.2</td>
</tr>
<tr>
<td>Fungal PLFAs</td>
<td>0.6</td>
<td>0.475</td>
<td>8.4</td>
</tr>
<tr>
<td>F:B ratio</td>
<td>4.8</td>
<td>0.065</td>
<td>5.7</td>
</tr>
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</table>
Table 3. The effects of nitrogen addition and grazing and their interactions on the abundance of the main bacterial phyla after a 6-week laboratory incubation. F- and P-values are obtained by the Linear Mixed Effects Model (LME). Logarithmic (*) and square root (†) transformations were used when necessary to meet the assumptions of LME. Significance levels $P < 0.10$ are indicated by underline and $P < 0.05$ in bold.

<table>
<thead>
<tr>
<th>Phylum</th>
<th>Nitrogen</th>
<th>Grazing</th>
<th>Nitrogen × Grazing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F</td>
<td>P</td>
<td>F</td>
</tr>
<tr>
<td>Acidobacteria all</td>
<td>17.4</td>
<td>$&lt; 0.001$</td>
<td>2.2</td>
</tr>
<tr>
<td>Acidobacteria SD1</td>
<td>5.54</td>
<td>0.032</td>
<td>2.498</td>
</tr>
<tr>
<td>Acidobacteria SD2</td>
<td>15.36</td>
<td>0.004</td>
<td>1.41</td>
</tr>
<tr>
<td>Acidobacteria SD3</td>
<td>27.83</td>
<td>$&lt; 0.001$</td>
<td>7.144</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>17.5</td>
<td>$&lt; 0.001$</td>
<td>0.0</td>
</tr>
<tr>
<td>Alphaproteobacteria</td>
<td>42.7</td>
<td>$&lt; 0.001$</td>
<td>0.05</td>
</tr>
<tr>
<td>Betaproteobacteria</td>
<td>16.1</td>
<td>0.004</td>
<td>2.3</td>
</tr>
<tr>
<td>Deltaproteobacteria</td>
<td>86.3</td>
<td>$&lt; 0.001$</td>
<td>0.17</td>
</tr>
<tr>
<td>Gammaproteobacteria</td>
<td>5.1</td>
<td>0.054</td>
<td>5.1</td>
</tr>
<tr>
<td>Bacteroidetes</td>
<td>16.6</td>
<td>$&lt; 0.001$</td>
<td>0.48</td>
</tr>
<tr>
<td>Planctomycetes</td>
<td>117.3</td>
<td>$&lt; 0.001$</td>
<td>1.5</td>
</tr>
<tr>
<td>Verrucomicrobia</td>
<td>59.4</td>
<td>$&lt; 0.001$</td>
<td>3.1</td>
</tr>
<tr>
<td>Gemmatimonadetes</td>
<td>15.0</td>
<td>0.055</td>
<td>2.0</td>
</tr>
<tr>
<td>Chloroflexi</td>
<td>0.03</td>
<td>0.879</td>
<td>5.9</td>
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</table>
Table 4. The effects of nitrogen addition and grazing and their interactions on bacterial community composition after a 6-week laboratory incubation. Pseudo-F- and $P$-values were obtained by Permanova analyses of weighted and unweighted UniFrac distances or OTUs within the dominant phyla. Significance levels $P < 0.10$ are indicated by underline and $P < 0.05$ in bold.

<table>
<thead>
<tr>
<th></th>
<th>Nitrogen</th>
<th></th>
<th>Grazing</th>
<th></th>
<th>Nitrogen × Grazing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pseudo-F</td>
<td>$P$</td>
<td>Pseudo-F</td>
<td>$P$</td>
<td>Pseudo-F</td>
</tr>
<tr>
<td><strong>Whole community</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weighted UniFrac</td>
<td>8.439</td>
<td><strong>0.004</strong></td>
<td>1.334</td>
<td>0.207</td>
<td>2.783</td>
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<tr>
<td>Unweighted UniFrac</td>
<td>1.652</td>
<td><strong>0.074</strong></td>
<td>1.061</td>
<td>0.285</td>
<td>1.147</td>
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<tr>
<td>OTUs</td>
<td>4.326</td>
<td><strong>0.002</strong></td>
<td>1.693</td>
<td>0.124</td>
<td>1.184</td>
</tr>
<tr>
<td><strong>Phyla</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acidobacteria</td>
<td>3.111</td>
<td><strong>0.018</strong></td>
<td>1.652</td>
<td>0.114</td>
<td>1.084</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>4.050</td>
<td><strong>0.003</strong></td>
<td>1.674</td>
<td>0.144</td>
<td>1.179</td>
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<tr>
<td>Bacteroidetes</td>
<td>5.500</td>
<td><strong>0.004</strong></td>
<td>1.491</td>
<td>0.150</td>
<td>0.786</td>
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<tr>
<td>Alphaproteobacteria</td>
<td>1.724</td>
<td><strong>0.076</strong></td>
<td>1.669</td>
<td>0.122</td>
<td>0.954</td>
</tr>
<tr>
<td>Betaproteobacteria</td>
<td>4.742</td>
<td><strong>0.005</strong></td>
<td>1.569</td>
<td>0.177</td>
<td>1.324</td>
</tr>
<tr>
<td>Deltaproteobacteria</td>
<td>7.283</td>
<td><strong>0.001</strong></td>
<td>1.049</td>
<td>0.344</td>
<td>1.480</td>
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<tr>
<td>Gammaproteobacteria</td>
<td>2.989</td>
<td><strong>0.015</strong></td>
<td>1.740</td>
<td><strong>0.041</strong></td>
<td>1.624</td>
</tr>
<tr>
<td>Planctomycetes</td>
<td>3.834</td>
<td><strong>0.007</strong></td>
<td>1.520</td>
<td>0.105</td>
<td>1.369</td>
</tr>
<tr>
<td>Verrucomicrobia</td>
<td>3.237</td>
<td><strong>0.017</strong></td>
<td>1.172</td>
<td>0.337</td>
<td>1.202</td>
</tr>
</tbody>
</table>
Figure 1

Microbial N

Lipid PO₄

Total PLFAs

Bacterial PLFAs

Fungal PLFAs

Control +N

µg g⁻¹ SOM

µmol g⁻¹ SOM

N* N*

N** N**

G*
Figure 2
Figure 3

The figure shows the measurement of CO$_2$ emissions (mg CO$_2$ h$^{-1}$ g$^{-1}$ SOM) over the course of laboratory incubation days for lightly and heavily grazed samples. Two conditions are compared: Control and +N. The y-axis represents the CO$_2$ emissions, while the x-axis indicates the days of laboratory incubation. The data points for lightly grazed samples are shown on the left, and for heavily grazed samples on the right. Each condition is marked with distinct symbols: Control (filled circles) and +N (open circles). Error bars indicate the variability of the measurements.
Figure 4

16S rRNA gene copies ng⁻¹ DNA

Control

+ N

18S rRNA gene copies ng⁻¹ DNA

N**
Figure 5

Lightly grazed

Heavily grazed

Number of sequences
Click here to download Supplementary Material for online publication only: Table S1.docx
Click here to download Supplementary Material for online publication only: Table S3.xlsx