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Soil warming and fertilization altered rates of nitrogen transformation processes and selected for adapted ammonia-oxidizing archaea in sub-arctic grassland soil

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ABSTRACT

The balance of microbial nitrogen (N) transformation processes in sub-arctic terrestrial ecosystems is most likely affected by global change, with potential feedbacks to greenhouse gas emissions and eutrophication. Soil temperature and N availability – their global increases being two of the most pressing global change features - will be prime drivers of N dynamics and microbial community structure, but little is known about their interactive effects in these ecosystems. We utilized geothermally warmed soils from Iceland as a natural experiment for assessing fertilization and warming effects on gross soil N transformation processes. Experimental incubations of these soils at different temperatures coupled with a dual ^{15}N-labelling/-tracing approach and pyrotag transcript-sequencing allowed for the analysis of independent and combined impacts of N fertilization and temperature shifts on gross N mineralisation, nitrification, and ammonium and nitrate immobilisation rates and archaeal ammonia-oxidizing (AOA) communities, being the key ammonia oxidizers in this soil. Gross nitrification in warmed soil was increased in relation to ambient temperature soil and exhibited a higher temperature optimum. Concomitantly, our results revealed a selection of AOA populations adapted to *in situ* soil temperatures. Phylogenetically distinct populations of actively ammonia-oxidizing archaea exhibited conserved temperature optima.

N mineralization and nitrification showed higher sensitivities in response to short-term temperature changes if the soils had been warmed. In part, the influence of short-term temperature changes could however be neutralized by the effects of N fertilization. Long-term N fertilization alone affected only gross N mineralization. However, all gross N transformation rates were significantly altered by the interactive effects of N fertilization and soil warming. We conclude that in order to reliably predict effects of global change on sub-arctic soil N transformation processes we need to consider multiple interactions among global change factors and to take into account the capacity of soil microbial populations to adapt to global change conditions.

1. Introduction

Nitrogen (N) availability and temperature are two of the most fundamental factors driving terrestrial N transformation processes. Both are dramatically increasing due to anthropogenic activities and are recognized as key features of global change. Annual inputs of reactive N to the earth's atmosphere, soils and water bodies from agricultural, industrial and transportation sources have increased tenfold in the last 150 years (UNEP and WHRC, 2007) and are predicted to double by 2050 including an increasing number of regions receiving potentially damaging levels of N inputs (Leff et al., 2015; Erisman et al., 2013; Phoenix et al., 2012). Until the end of the 21. century, the average global surface temperature is expected to

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rise by 1.5–4 °C (IPCC, 2013), but a predicted increase of intensity and frequency of short-term weather variations due to global change is of more immediate impact on the functioning of ecosystems (IPCC, 2013; Houghton, 2005). Changes in N-cycling are likely to result in substantial ecosystem-level changes in primary productivity (Melillo et al., 1993; Trumbore, 1997), plant species composition (Stevens et al., 2004; Suding et al., 2005; Wedin and Tilman, 1997), soil acidification (Noble et al., 2008), soil microbial respiration (Bradford et al., 2008), and N losses through nitrate-leaching and production of gaseous nitrogen species such as the potent greenhouse gas nitrous oxide (N_2O) (Guo and Gifford, 2002; IPCC, 2013; Magill et al., 1997; Mosier, 1998). In this context, soil nitrification is of specific importance since this process on the one hand provides biologically available N for assimilation and denitrification, but can on the other hand lead to eutrophicated waters and greenhouse gas emissions. Furthermore, changes in all N transformation processes can lead to C-cycling feedbacks to climate change. Hence, it is important to understand the response of terrestrial N transformation processes to altered temperatures and N deposition.

Several studies found elevated gross and net N fluxes in response to experimental soil warming (Contosta et al., 2011; Cookson et al., 2007; Macduff and White, 1985; Nadelhoffer et al., 1991; Schmidt et al., 1998; White et al., 2000). Additionally, Bai et al. (2013) could show in a meta-analysis including 528 observations from 51 publications that warming generally increased net N mineralization and net nitrification processes in soil by 52% and 32%, respectively, while net N immobilization was however usually not affected. A few studies report stimulated N mineralization and increased gross nitrification and N immobilization in response to elevated N deposition (Lee and Caporn, 1998; Goulding et al., 1998; Barnard et al., 2005). In contrast however, other studies report that increased available N levels in soil lead to a decrease in gross and net N mineralization rates as microbes invest less in production of exo-enzymes involved in the release of N from polymers (Bengtson et al., 2005; Craine et al., 2007; Ramirez et al., 2010). Perhaps these seemingly contrasting results can be explained by results of Gao et al. (2015) who report that effects of N deposition will vary depending on the state of N limitation of a soil and may in part be non-linear as for example pH changes with increased N deposition.

The few studies focusing on combined effects of global change factors on soil N transformation processes largely agree that interactive effects occur that single-factor studies cannot predict (Barnard et al., 2006; Rutting et al., 2010; Larsen et al., 2011; Björsne et al., 2014), even though there are indications that at least in some soil systems, global change factors can mainly have additive effects (Niboyet et al., 2011).

A little more is known about the community structure response of N-transforming microbes to elevated temperature and N deposition in natural systems. Both global change factors have been shown to select for subgroups of nitrifiers and cause gene abundance shifts as well as richness loss (Tourna et al., 2008; Freedman et al., 2013; Le Roux et al., 2016). Evidence is accumulating that soil AOA are not directly stimulated by the application of mineral N and are often inhibited by higher concentrations of ammonium, while bacterial ammonia oxidizers (AOB) show both stimulation and growth (Horz et al., 2004; Martens-Habbena et al., 2009; Stopnišek et al., 2010; Jung et al., 2011; Lehtovirta-Morley et al., 2011; Verhamme et al., 2011; Levicnik-Hofferle et al., 2012; Prosser and Nicol, 2012; Daebeler et al., 2015; Simonin et al., 2015).

However the assessment of microbial responses on the level of community structure in combination with their gross activity rates is a promising, but vastly understudied approach to understanding the effects of global change on soil microbial N transformation processes. The objective of the present study was to investigate combined and single effects of short-term temperature changes and N fertilization on gross rates of nitrification, N mineralization, and ammonium and nitrate immobilization and on the structure of the active AOA community, since we found previously that nitrification in these soils is solely driven by AOA (Daebeler et al., 2012, 2014). We utilized sub-arctic soils with different *in situ* temperatures from Icelandic grasslands, which have been subjected to N fertilization since May 2005. Varying soil temperatures at the study site originate from local differences in geothermal activity. Geothermally warmed soil sites are warmed steadily and long-term. Therefore, N-cycling microorganisms at such sites may have a narrow and elevated range of temperature tolerance, or a wider temperature range encompassing these higher temperatures. N-cycling microorganisms at close-by ambient temperature sites experience seasonal temperature differences and are likely adapted to larger temperature fluctuations. Such adaptation to an elevated and stable or ambient but fluctuating temperature could be established from a diverse community of populations with narrow and wide temperature ranges via a) permanent and exclusive selection for N-cycling microorganisms, or b) temporal selection of populations, which will only be active at a time when their temperature tolerance range is met. The first scenario would lead to higher gross rates if the soil was incubated at *in situ* temperature in comparison to incubation at deviating temperatures, while the second scenario would show comparable rates over a larger temperature range.

We adopted a paired-site approach, with experimentally increased N input to the soil by fertilization with urea at sites with and without geothermal warming directly next to control sites. By incubating the soil at different temperatures in controlled microcosms we were able to test combined and single effects of short-term temperature shifts on gross rates of nitrification, N mineralization, and ammonium and nitrate immobilization, and on the structure of the active AOA community. At the same time, the incubations enabled the detection of long-term, single and combined effects of the global change factors temperature and N fertilization on N-converting processes and microbes in the grassland soils. Thereby, modes of temperature adaptation of N transformation processes could be assessed. Temperatures selected in this study partly exceed the predicted increase through global warming, but aid in identifying temperature relationships between processes and show the magnitude of adaptability of soil microbes.

In this study we specifically hypothesized that 1) gross rates of nitrification, N mineralization, and ammonium and nitrate immobilization in geothermally warmed soils will be higher and have a higher temperature optimum when incubated at the respective *in situ* temperature since microbial communities in these soils are selectively adapted to higher temperatures compared to microbial communities in soils with ambient temperature, 2) short-term changes in temperature will affect gross rates of nitrification, N mineralization, and ammonium and nitrate immobilization of geothermally warmed soils more than the corresponding rates of ambient temperature soils, since microbial communities in the latter soil experience a broader temperature range throughout the year and are therefore more accustomed to short-term temperature change, and 3) mineral N fertilization in the form of urea will decrease gross N mineralization rates due to repression of N-producing exo-enzymes and will not directly stimulate nitrification as we demonstrated before by quantification of the archaeal and bacterial *amoA* genes as well as by studying growing, nitrifying cells via a stable isotope labeling approach, that AOA dominate numerically and are the main ammonia oxidizers in these soils (Daebeler et al., 2012, 2014).

2. Materials and methods

2.1. Soil sampling and physico-chemical analyses

Sub-arctic soil from four geothermally warmed and four ambient temperature sites of an experimental research location in Grændalur valley, Iceland were sampled in August 2010. From May 2005 to July 2009, temperature data loggers had recorded the soil temperature at 10 cm depth six times a day and showed temperature ranges of 23–27 °C and 2–17 °C for the geothermally warmed and the ambient temperature soils, respectively (see Fig. S2 for mean annual temperatures determined from 2005 to 2009). The soil is a histic andosol with two tephra layers in the top 30 cm. All eight research sites consisted of a 1 m^2 N-fertilized part (10 g N m^{-2} yr^{-1} slow-release urea, Agroblen 35 + 0 + 0, Scotts International B.V., Geldermalsen, The Netherlands), which had been fertilized annually at the start of the growing season each May since 2005, and a 1 m^2 adjacent, non-fertilized control part. From each part three replicate soil cores of 10 cm depth were taken and pooled to account for spatial variability.

Soil clay and silt contents were analyzed from freeze-dried subsamples by a Master sizer (Malvern, model APA2000, serial number 34403/139). Water content and water-holding capacities of the soil samples were determined as described by Wilke (2005) immediately after sampling. Contents of total soil C and N were determined on an isotope ratio mass spectrometer (Delta V Advantage IRMS, Thermo Electron Corporation, Germany) coupled to a EuroVector elemental analyzer (EuroVector, Italy) after freeze-drying and milling. Table 1 gives an overview of the measured edaphic properties.

2.2. Microcosm incubations

To study the effects of short-term temperature changes on gross N transformation processes, we selected the three geothermally warmed and three ambient sites with the highest potential nitrification rate based on the results of the potential nitrification activity (PNA) assay. During transport soil samples were stored at their respective field temperature for one week before mixing and removal of roots. The soil samples were then incubated at their approximate *in situ* temperatures at time of sampling (15 and 25 °C for ambient and geothermally warmed sites, respectively) and at 10 °C above these temperatures (25 and 35 °C, respectively). In addition, for both sites, a more extreme temperature difference was chosen for incubation to test for differences in tolerance to larger temperature changes outside of the range that these soil experience. The ambient temperature soil samples were incubated at 35 °C (~20 °C higher than the *in situ* temperature) whereas the geothermally warmed soil samples were incubated at 5 °C (~20 °C lower than *in situ* temperature).

We applied a mirror-labeling, ^{15}N-tracing approach by adding either $^{15}NH_4^{14}NO_3$ or $^{14}NH_4^{15}NO_3$ to triplicate soil microcosms. By this, we were able to trace the ^{15}N signal of $^{15}NH_4^+$ to $^{15}NO_3^-$ and to further follow the dilution of the ^{15}N signal of the $^{15}NO_3^-$ pool through ^{14}N from inorganic N over a 6-day incubation period. 30 g of soil was transferred into sterile 250 mL bottles and 2 mL of either 0.1 mM of $^{15}NH_4NO_3$ solution or 0.1 mM of $NH_4^{15}NO_3$ solution was applied (both with 99% ^{15}N enrichment), resulting in an addition of 0.1 μg 15 N g^{-1} soil (i.e. 0.2 μmol N g^{-1} soil). A third bottle received 2 mL of a 0.1 mM non-labeled NH_4NO_3 solution serving as a blank control. Soil samples for determination of ^{15}N enrichment in inorganic N pools and molecular analyses were taken after 1, 3 and 6 days of incubation. Samples (approx. 4 g) for RNA extraction were immediately snap-frozen in liquid nitrogen and stored at −80 °C until further analysis.

2.3. Determination of inorganic N and isotopic signatures

Samples for analysis of inorganic N (approx. 5 g) were shaken in 20 mL 1 M KCl solution for 1 h and the supernatants were stored at −20 °C until determination of NH_4^+ and $NO_2^- + NO_3^-$ using a continuous flow auto analyzer (Skalar SA-40, The Netherlands, data provided in Supplementary Fig. S3) and analysis of the isotopic signature in inorganic N pools, making use of a microdiffusion technique modified from Stark and Hart (1996).

In brief, glass fiber micro filters (Whatman, GF/A) of 6 mm diameter were spiked with 13 μL of 1 M $KHSO_4$ and packed in Teflon tape (19 mm width) to seal the filter from liquid while enabling diffusion of NH_3. Approximately 10 mL of the 1 M KCl soil extract were given into 250 mL plastic bottles and contents of total NH_4^+ - N were adjusted to 100 μg by adding NH_4^+ from a standard solution if needed to meet the detection limit of the elemental analyzer. To have comparable headspaces in all the bottles, the volume of the solution was brought to 20 mL with 1 M KCl. The pH was raised to approximately 10 with ~0.4 g of ashed MgO. One packed filter was added per bottle, after which the lid was closed. Containers were left at 25 °C and shaken every other day. After 7 days, the packed filters for ^{15}N-NH_4^+ determination were collected, washed in demineralized water, unpacked, and dried. According to Stark and Hart (1996) 6 days at room temperature are sufficient to remove >92% of the ammonium from the solution. After removal of the filters for ^{15}N-NH_4^+ determination, 0.4 g of Devarda's alloy was added to the solution to convert all NO_3^- into NH_4^+. Another filter was added to each bottle, after which the bottle was closed and left at 25 °C with intermittent shaking for another 7 days.

Then the filters for determination of ^{15}N from NO_3^- were collected and treated as described above. All filters were placed in tin capsules for analyses of the isotopic signature of N on the same

Table 1
Characteristics of soil samples collected in August 2010 from ambient temperature and geothermally warmed sites of Icelandic grasslands with and without N fertilization. Values are averages (n = 3) ± standard error. Lower case letters indicate significant differences between soil groups ($p < 0.05$).

	Amblent, Without N deposition	Amblent, with N deposition	Warmed, without N deposition	Warmed, with N deposition
Temperature at sampling [°C]	17.63 ± 1.01[a]	17.06 ± 0.73[a]	31.56 ± 5.79[b]	29.62 ± 3.36[b]
Mean annual Temperature [°C]	10.66 ± 0.88[a]	10.66 ± 0.88[a]	21.46 ± 1.31[b]	21.46 ± 1.31[b]
Moisture [%]	72.82 ± 2.98[a]	72.42 ± 3.73[a]	71.32 ± 5.08[a]	67.47 ± 6.80[a]
pH	7.40 ± 0.14[a]	7.29 ± 0.12[a]	6.83 ± 0.07[b]	6.87 ± 0.15[b]
N [mg/g dry soil]	12.34 ± 2.74[a]	16.85 ± 2.33[a]	17.76 ± 2.37[a]	19.74 ± 2.14[a]
C [mg/g dry soil]	117.04 ± 21.59[a]	150.03 ± 27.26[a]	193.97 ± 30.11[a]	211.30 ± 53.52[a]
C:N	21.39 ± 2.23[a]	20.47 ±1.80[a]	19.72 ± 3.99[a]	17.80 ± 1.43[a]
Clay [%] (<2 μm)	0.74 ± 0.63[a]	0.61 ± 0.47[a]	0.45 ± 0.43[a]	0.55 ± 0.38[a]
Silt [%] (2-63 μm)	19.87 ± 11.12[a]	17.69 ± 7.24[a]	12.93 ± 4.78[a]	15.75 ± 3.55[a]
Fine sand [%] (63-200 μm)	13.77 ± 2.24[a]	16.22 ± 1.80[a]	15.91 ± 0.41[a]	16.10 ± 1.74[a]
Coarse sand [%] (>200 μm)	15.44 ± 3.64[a]	19.50 ± 3.21[a]	24.80 ± 3.21[a]	22.33 ± 0.64[a]

EA-IRMS as above. To be able to perform blank corrections for the determination of isotopic ratios of N, ten bottles with 20 mL 1 M KCl without soil extracts were prepared and treated like the samples. In order to determine the isotope ratio of N in a sample with correction for the addition of standard solution containing ^{14}N, the following formula was applied:

$$R_{sample} = \frac{R_{measured} \times \left(N_{sample} + N_{added}\right) - (N_{added} \times R_{added})}{N_{sample}}$$

Were $R_{measured}$ is the isotope ratio measured in the sample, N_{sample} is the total N content of the sample, N_{added} is the amount of ^{14}N added to the sample and R_{added} is the isotopic ratio of the added N.

To further correct for dilution of the isotopic signal by background contamination we applied the following formula:

$$R_{corrected} = \frac{R_{sample} \times N_{sample} - R_{blank} \times N_{blank}}{N_{sample} + N_{blank}}$$

Where R_{sample} is the isotopic ratio of N in a sample corrected for addition of standard, R_{blank} is the average isotopic ratio of N in the methodological blanks and N_{blank} is the total average amount of N in the methodological blanks.

2.4. Quantification of gross N transformation rates

Gross N transformation rates were determined with the numerical ^{15}N-tracing model *Ntrace* (Fig. S1) applying a Monte Carlo sampling technique (Müller et al., 2007). The advantage of this technique over the commonly used ^{15}N dilution technique is that process-specific N transformation rates can be quantified and that interactions between processes are accounted for (Rütting and Müller, 2007). A range of model setups varying in the number of N transformations and N pools were tested. The one with the least N transformations but which was still adequate to simulate the data was chosen (Müller et al., 2007). The final model depicted in Fig. 1 consisted of five N transformations: M_{Norg}, mineralization of organic N to NH_4^+; O_{Norg}, oxidation of organic N to NO_3^-; I_{NH4}, immobilization of NH_4^+ to organic N; O_{NH4}, oxidation of NH_4^+ to NO_3^- and I_{NO3}, immobilization of NO_3^- to organic N. All transformations were calculated by first-order kinetics, except M_{Norg}, which was calculated by zero-order kinetics.

The gross N transformation rates were calculated by simultaneously optimizing the kinetic parameters for the various N transformations. The modeled data were fitted against observed values of NH_4^+, NO_3^- and their respective ^{15}N enrichments simultaneously for the three ^{15}N treatments (Müller et al., 2004). Rates for each treatment and replicate were quantified separately as the variation between pools of NH_4^+ and NO_3^- in replicate geothermally warmed and ambient temperature soil samples was too large to reliably quantify average rates. To examine the dependencies of gross transformation rates on each other, a correlation matrix was calculated from the parameter vector of iterations after the 'burn-in time' as described previously using the corrcoef function in Matlab (Müller et al., 2007). Parameter optimization was carried out with a Markov chain Monte Carlo Metropolis algorithm (MCMC-MA), which performs a random walk in model parameter space and is very robust against local minima (Müller et al., 2007). Initial pool sizes of NH_4^+ and NO_3^- were estimated by extrapolating the concentrations from the first two soil extractions to the time point zero, separately for each soil sample. As O_{Norg} could not be detected in any of the soil incubations we assume all NO_3^- to be produced via NH_4^+ oxidation (i.e. chemolithotrophic nitrification; O_{NH4}).

Q_{10} temperature coefficients, which show the rate of change of gross N transformation processes per 10 °C, were calculated as:

$$Q_{10} = \left(\frac{R_2}{R_1}\right)^{10/(T_2 - T_1)}$$

where R is the N transformation rate and T is the incubation temperature in °C.

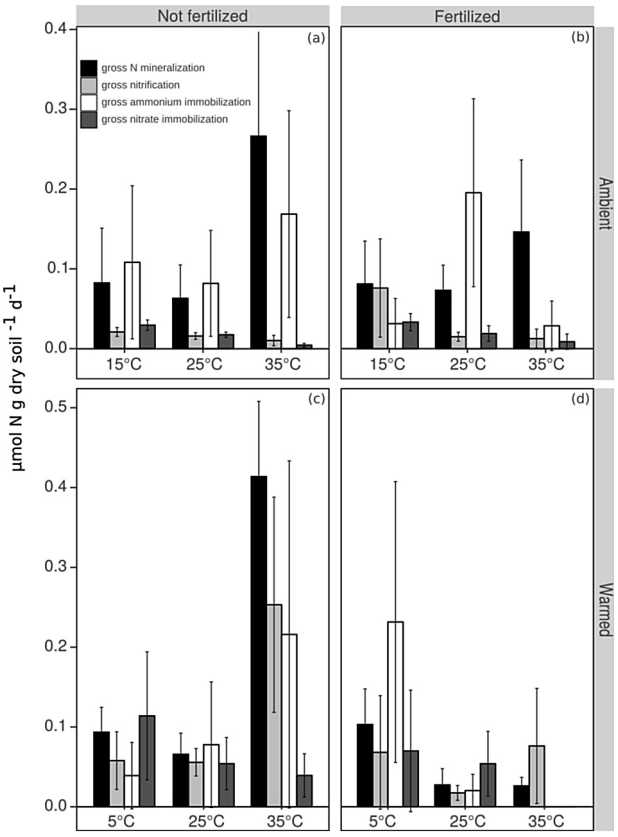

Fig. 1. Gross N transformation rates in Icelandic grassland soil during an incubation at different temperatures of 6 days. Data is sorted to present rates in soils originating from ambient temperature field conditions (a, b), warmed field conditions (c, d), not fertilized sites (a, c) and fertilized sites (b, d). Values are averages (n = 3; with two exceptions in panel d were n = 2 due to partially missing data) ± standard error.

2.5. RNA extraction, cDNA synthesis, sequencing and analysis of amoA transcript sequences

Actively ammonia-oxidizing AOA were determined by analyzing archaeal *amoA* transcripts of a representative soil sample of ambient temperature and geothermally warmed origin each. Of these, all samples from all time points and incubation temperatures of the microcosm experiment were analyzed. Total soil nucleic acids were extracted according to Angel and Conrad (2009, http://www.molmeth.org/node/92/revisions/231/view). Briefly, approx. 0.4 g of wet soil were subjected to phenol-/SDS-based acid extraction with two bead-beating steps followed by purification via chloroform/isoamylalcohol, polyethylene glycol 8000 and ethanol. Immediately afterwards 10 µl sample were treated with DNA-free DNase (Qiagen) followed by RNA purification (RNAeasy Mini Kit, Qiagen), exonuclease treatment (mRNA-ONLY Prokaryotic mRNA Isolation Kit, Epicentre Technologies) and another purification step (RNAeasy Mini Kit, Qiagen) according to manufacturers' instructions. Samples were checked for DNA contamination by attempting to amplify the archaeal *amoA* gene by PCR and

subsequent gel electrophoresis as described below. Only samples that gave a negative result were used for reverse transcription of the archaeal *amoA* gene. Reverse transcription was performed using the primer ArchamoA-2R (5′-GCGGCCATCCATCTGTATGT-3′; (Francis et al., 2005) and cycling conditions were as follows: 60 °C for 1 h followed by 85 °C for 5 min cDNA products of the reverse transcription PCR were checked for quality and correct length by PCR targeting the archaeal *amoA* gene as described in Daebeler et al. (2012) and cDNA was stored at −20 °C until used for 454 pyrosequencing as described in Daebeler et al. (2014) using a Roche 454 GS FLX Titanium sequencer. The raw, demultiplexed SFF files were deposited into NCBI SRA database (https://www.ncbi.nlm.nih.gov/sra) and can be found under study accession PRJNA343494.

Raw flowgrams were denoised using AmpliconNoise and checked for chimeras using Perseus (Quince et al., 2011) as implemented in Mothur (Schloss et al., 2009). Denoised reads were dereplicated in Mothur and exported for further clustering. Reads were preclustered at 98% identity using the cluster_smallmem command in USEARCH (Edgar, 2010) and OTU representative sequences were defined using UPARSE (Edgar, 2013). Reads were mapped to OTU representatives at 85% sequence divergence using the usearch_global command in USEARCH with maxaccepts = 0 and maxrejects = 0 and OTU tables were created using the uc2otutab.py script available from USEARCH. Representative sequences for the created OTUs were searched against the NCBI database using BLAST and sequences not mapping to archaeal *amoA* were removed from the analysis. Remaining representative sequences were aligned to a reference *amoA* alignment using SINA (Pruesse et al., 2012) version 1.2.11 and manually curated using ARB (Ludwig et al., 2004). The average number of sequences per sample remaining after curation was 1617 and library sizes were rarefied to 859 for comparative analysis. Analysis of *amoA* transcript richness and diversity was performed in Mothur (Schloss et al., 2009). For phylogenetic analysis representative sequences for each OTU (341 bp) and their closest NCBI Blast hits were placed into the *amoA* reference tree as published by Pester et al. (2011) using the evolutionary placement algorithm (EPA, Berger and Stamatakis, 2011).

2.6. Statistical analysis

All analyses were performed using the statistical software R (R Core Team, 2013) version 3.0.2. Significant differences in Q_{10} values were determined by Tukey's HSD test. Linear mixed models (LMM) generated with the function lmer of the package lme4 (Bates et al., 2015) were used to evaluate the influence of soil properties, field temperature, N deposition and incubation temperature on gross N transformation rates. Since such analysis takes hierarchically structured data and random effects into consideration when assessing significance, it results in different conclusions then when performing a non-mixed linear analysis (e.g. Tukey's HSD test or ANOVA). Co-linearity was observed between C:N ratio and soil moisture, between C:N ratio and clay content and between soil moisture and nitrate concentration ($r = 0.55, -0.58, -0.59$, respectively). To avoid distortion of predictor effect sizes caused by the observed co-linearity, the soil properties C:N ratio and moisture content were excluded from further analysis. Main effects of the centered soil properties (pH, clay content, ammonium and nitrate concentrations), N fertilization, field temperature regime and incubation temperature were included in the analysis. Additionally, the interaction between N fertilization and the field temperature, between the field temperature and incubation temperature as well as the interaction between N fertilization and the incubation temperature were included. Models further included the site in the field from which a soil sample originated as a random effect. Models were selected according to Zuur et al. (2009) choosing only factors with a significant correlation to the gross N transformation rates to be incorporated in the final model (be it precursors or products of the modeled gross N transformation processes). Prediction intervals describing possible future observations according to the selected final LMMs were generated by drawing 2000 random values from the joint posterior distribution of the model parameters with the function sim of the package arm (Gelman and Su, 2015).

Differences in OTU richness and diversity of active AOA communities were analyzed by ANOVA utilizing the observed number of OTUs for richness and the inverse Simpson index as a proxy for diversity. Differences in relative abundance of active AOA OTU phylotypes were determined by Tukey's HSD test. Principal coordinate analysis (PCoA) of active AOA communities in the samples was performed using the cmdscale function. Environmental factors and the relative abundance of OTUs were fitted to the ordination using the command envfit. Only significant fits with the ordination were plotted. To asses significant differences between AOA community structures we performed an analysis of variance using Bray-Curtis distance matrices with the adonis function. All three commands are part of the package vegan (Oksanen et al., 2010). Hierarchical clustering of active AOA communities was performed utilizing Ward's method, which calculates minimal variances between clusters.

3. Results

3.1. Gross N transformation rates

Absolute values of the determined gross N transformation rates can be found in Fig. 1. To understand main and interactive effects of field and incubation conditions on the gross N transformation rates we performed a linear mixed modeling (LMM) analyses with field temperature at the time of sampling, N fertilization, soil properties and incubation temperature as explanatory factors and the gross N transformation rates as dependent variables. By this analysis we determined that gross N mineralization was significantly, positively related to incubation temperature and ammonium concentration and had significant negative relationships with N fertilization as well as with clay content of the soil (Table 2). Furthermore, N fertilization resulted in significantly higher gross N mineralization rates in ambient soils, but caused lower gross N mineralization rates in warmed soils.

We did not detect any oxidation of organic N to NO_3^- in any of the samples and therefore assume that the process of nitrification was entirely chemolithotrophic. Gross nitrification rates (*i.e.* chemolithotrophic NH_4^+ oxidation) of soil from geothermally warmed sites were significantly higher than those of soil from ambient temperature sites and had a significant, positive relationship with incubation temperature (Table 2). Additionally, interactions of N fertilization with field temperature, of N fertilization with incubation temperature and of field temperature with incubation temperature significantly related to the gross nitrification rates. N fertilization alone as a main factor however, had no effect on gross nitrification rates. To demonstrate the extent and direction of effects as found by the LMM analysis we predicted gross nitrification rates taking the correlation among the model parameters into account (Fig. S4). This analysis shows the negative effect of elevated incubation temperature on gross nitrification rates of ambient temperature, fertilized and non-fertilized soil as determined by the LMM. Rates in unfertilized, geothermally warmed soil however were predicted to be positively affected by incubation temperature, while rates of the corresponding fertilized soil were predicted to be not affected.

Table 2
Fixed effects of the best fitting, reduced, linear mixed models of gross rates of N mineralization, nitrification, ammonium immobilization and nitrate immobilization in relation to field temperature at the time of sampling, N fertilization, incubation temperature and soil properties. Df_{num}, degrees of freedom in the numerator; Df_{den}, degrees of freedom in the denominator; F, F-statistic; p, p-value; Ef_{dir}, direction of effects; / not included in final, reduced model as result of the analysis; NA, not applicable. Bold font indicates significance.

	Gross N mineralization					Gross nitrification					Gross ammonium immobilization					Gross nitrate immobilization				
	Dfnum	Dfden	F	p	Efdir	Dfnum	Dfden	F	p	Efdir	Dfnum	Dfden	F	p	Efdir	Dfnum	Dfden	F	p	Efdir
Field temperature	1	4	0.063	0.815	NA	1	4	13.325	**0.022**	+	1	4	0.001	0.980	NA	1	4	21.090	**0.010**	+
N fertilization	1	86	25.340	**<0.0001**	-	1	89	3.901	0.051	NA	1	88	2.404	0.125	NA	1	92	1.493	0.225	NA
Inc. temperature	1	86	36.660	**<0.0001**	+	1	89	4.703	**0.033**	+	1	88	1.629	0.205	NA	1	92	22.837	**<0.0001**	-
Field temperature : N fertilization	1	86	12.786	**0.001**	NA	1	89	20.589	**<0.0001**	NA	1	88	11.880	**0.001**	NA	1	92	8.612	**0.004**	NA
Field temperature : Inc. temperature	1	86	0.027	0.871	NA	1	89	13.889	**0.000**	NA	1	88	0.006	0.937	NA	/	/	/	/	NA
N fertilization : Inc. temperature	1	86	21.009	**<0.0001**	NA	1	89	15.447	**0.000**	NA	1	88	21.500	**<0.0001**	NA	/	/	/	/	NA
Field temperature : N fertilization : Inc. temperature	1	86	2.026	0.158	NA	1	89	2.116	0.149	NA	1	88	6.790	**0.001**	NA	/	/	/	/	NA
NH4	1	86	19.842	**<0.0001**	+	/	/	/	/	NA	/	/	/	/	NA	/	/	/	/	NA
NO3	/	/	/	/	NA	1	89	2.693	0.104	NA	1	88	11.482	**0.001**	-	1	92	13.471	**0.0004**	+
pH	1	86	0.028	0.867	NA	/	/	/	/	NA	1	88	3.190	0.078	NA	/	/	/	/	NA
Clay	1	86	7.742	**0.007**	-	/	/	/	/	NA	/	/	/	/	NA	/	/	/	/	NA

Gross ammonium immobilization rates were similar in soil from ambient and geothermally warmed sites and exhibited no pattern with incubation temperature or N fertilization treatment alone (Fig. 1; Table 2). Soils from geothermally warmed sites showed significantly higher rates than from the ambient temperature, but the relationship of gross ammonium immobilization with incubation temperature was significantly negative (Table 2). Even though the gross nitrate immobilization rates were comparable between sites with and without increased N fertilization, we found higher rates in ambient soils that had been fertilized, but lower rates in warmed soils with fertilization.

To examine the sensitivity of gross nitrogen transformation rates to short-term temperature changes we calculated the Q_{10} temperature coefficients and found Q_{10} values of gross nitrification to be significantly higher (Tukey's HSD test, $p = 0.027$) in geothermally warmed than in ambient temperature soils (Fig. 2). There was no significant effect of N fertilization on Q_{10} values for any of the determined gross N transformation processes.

3.2. Active archaeal ammonia oxidizers

The communities of actively ammonia-oxidizing AOA were composed of a total of 17 OTUs spanning the major known diversity of AOA in soils. They were phylogenetically affiliated with the *Nitrosopumilus*, the *Nitrosotalea*, and the *Nitrososphaera* clusters and with a *Nitrososphaera*-sister cluster (see Fig. S5). There were no significant differences in OTU richness or diversity between incubation temperature, time points or N fertilization (ANOVA, $p_{richness} = 0.62$, 0.89, 0.47 and $p_{diversity} = 0.28$, 0.56, 0.98, respectively). Active AOA communities from warmed sites did however display significantly higher OTU richness, but not diversity (ANOVA, $p_{richness} = 0.001$; $p_{diversity} = 0.08$).

Community structures of active AOA from samples with similar gross nitrification rates were found to be more similar to each other than to communities from samples with higher or lower gross nitrification rates (Fig. 3, ADONIS, $p = 0.03$). Furthermore, active AOA communities from the same field temperature (*i.e.* ambient temperature or geothermally warmed) were more similar to each other than to communities from the other field temperature (ADONIS, $p = 0.04$). However, the different incubation temperatures, time points, or the fertilization treatment did not lead to significant differences between active AOA community structures (ADONIS, $p = 0.52$, 0.46, 0.78 respectively). We did observe significant shifts in relative phylotype abundances however. Phylotypes OTU5 and OTU51 had a significantly higher relative abundance in warmed soils (Tukey's HSD test, $p = 0.05$ and 0.02 respectively) and the relative abundance of OTU51 was also positively related to incubation temperature in warmed but not in ambient temperature soils (Tukey's HSD test, $p = 0.01$ and 0.76 respectively).

4. Discussion

4.1. Long-term temperature effects on N transformation rates and microbial communities

In our first hypothesis, we assumed that through the long-term temperature elevation at geothermally warmed sites a selection for microbial communities with higher temperature optima has taken place resulting in higher temperature optima of N transformation processes. Indeed, our results on the community of actively ammonia-oxidizing archaea and on related gross nitrification and support this assumption. Gross nitrification rates in soils from ambient temperature sites were negatively related to increasing incubation temperatures, while in contrast increased temperature

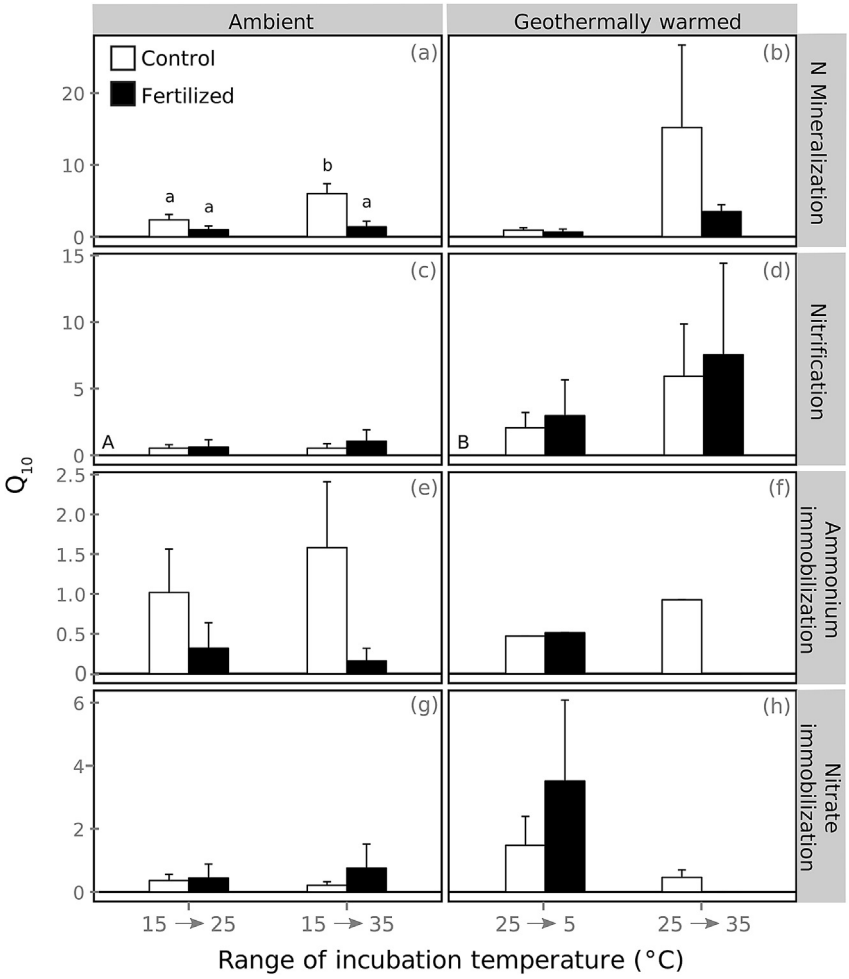

Fig. 2. Q_{10} temperature coefficients of gross N transformation processes in 6-day incubations of soil samples from ambient temperature (a, c, e, g) and geothermally warmed (b, d, f, h) sites of Icelandic grassland with and without N fertilization calculated from four different incubation temperatures. Values are averages (n = 3) ± standard error, except for the rate of ammonium immobilization in geothermally warmed soils where n = 1 due to missing data. Lower case letters indicate significant ($p < 0.05$) differences between bars within a panel; upper case letters in lower-left corners indicate significant differences between horizontal panels. Absence of letters indicates no significant difference.

led to increased gross nitrification rates in soils from geothermally warmed sites. At the same time, warmed soils were more sensitive to temperature changes than ambient temperature soils as shown by the Q_{10} analysis. Therefore, these results support our expectations of lower temperature optima of gross N transformations in ambient temperature soils as compared to warmed soils in the case of gross nitrification rates. Several studies (Birgander et al., 2013; Björsne et al., 2014; Zhao et al., 2014; Rousk et al., 2012) reported increased temperature optima of gross nitrification, carbon mineralization or bacterial growth in response to long-term elevated soil temperatures suggesting an analogous effect of temperature on these microbial processes across different soil types.

Interestingly, gross nitrification rates were significantly higher in geothermally warmed than in ambient soil. Concomitantly, active AOA communities were richer in OTU number and differed significantly in structure. Moreover, two phylotypes belonging to the *Nitrosopumilus* subcluster 5.2 and the *Nitrososphaera* subcluster 8.2 were significantly enriched in relative abundance in geothermally warmed soils. The *Nitrosopumilus*-like phylotype even increased in relative abundance with rising incubation temperature. These shifts in relative abundance, community structure and richness of active AOA with the elevated gross nitrification rates in warmed soils hints at a selection for different AOA phylotypes by temperature. Thereby our results support the findings of previous studies conducted with various soil systems that dissimilar AOA populations with distinct ammonia monooxygenase proteins possess different nitrifying potentials (Tourna et al., 2008; Alves et al., 2013; Zhao et al., 2015; Oton et al., 2016). This means that the phylogenetic diversity of soil AOA is probably not functionally redundant with regards to ammonia oxidation.

The ammonia-oxidizing community in this soil is AOA-dominated and other classical ammonia oxidizers do not play a role in nitrification as we could demonstrate before through quantification of the bacterial and archaeal *amoA* gene and identification of solely AOA as active ammonia oxidizers via a stable isotope labeling approach (Daebeler et al., 2014, 2015). While we cannot exclude that the detected archaeal *amoA* genes were to some extent constitutively expressed as recently described for a freshwater AOA strain (French and Bollmann, 2015), strong evidence for differential transcriptional responses of soil AOA to different conditions of pH and temperature (Nicol et al., 2008; Tourna et al., 2008) rather suggest that archaeal *amoA* mRNA detected in soil can be linked to actively nitrifying AOA. We therefore conclude that in response to long-term elevation of soil temperature gross nitrification rates have increased. Likely, this increase has taken place via selection for specialized archaeal ammonia-oxidizing populations with either higher temperature optima or wider temperature ranges.

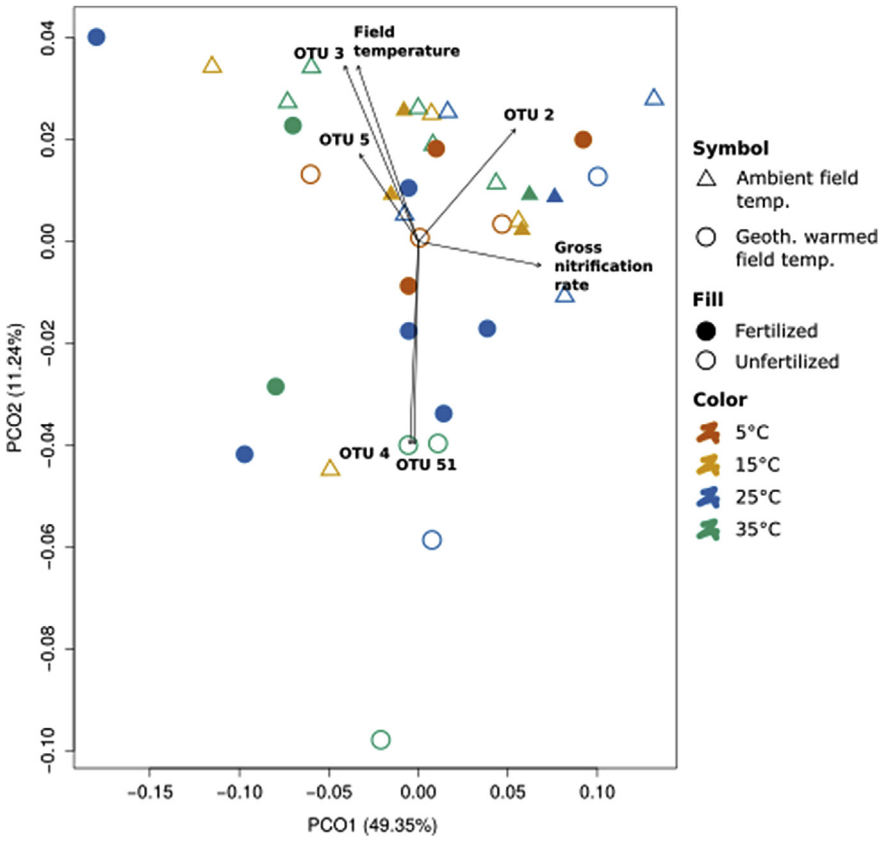

Fig. 3. Biplot showing the first two axes of a principal components analysis of active AOA communities in samples from soil microcosms incubated for 0 and 6 days at 5 °C (red), 15 °C (yellow), 25 °C (blue) or 35 °C (green) with samples originating from ambient temperature (triangles) or geothermally warmed (circles) as well as unfertilized (open symbols) and N-fertilized (filled symbols) sites. The axes cumulatively explain 83.59% of the observed variation. Active AOA communities were assessed by *amoA* transcript OTUs that clustered at 85% sequence similarity. Arrows indicate significant correlations of gross nitrification rate, field temperature and relative abundance of single OTUs with the presented ordination space. OTU codes correspond to Fig. S5. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4.2. Short-term temperature effects on N transformation in ambient and warmed soils

With our second hypothesis we proposed that gross N transformation rates in ambient temperature soil would be less affected by moderate short-term temperature changes than the gross rates geothermally warmed soil. This was assumed because the latter soil experiences a smaller temperature range throughout the year and thus likely selected for microbial populations that are exclusively adapted to the elevated *in situ* temperature. Our results on the gross nitrification rates partially support this hypothesis. Gross nitrification rates of geothermally warmed soil were more sensitive to short-term temperature changes as shown by the linear mixed model analysis and the Q_{10} values. Thereby our results indicate that long-term, stably elevated temperature in sub-arctic soils of Iceland has caused the processes of nitrification to be more sensitive to short-term temperature changes than in the neighboring sites without warming. Since the microbial communities from warmed soils were exposed to lower and the communities from ambient temperature soils to elevated temperatures however, it has to be considered that the difference in temperature sensitivity could also reflect microbial responses to temperature drops in comparison to temperature rises.

In a study on temperature adaptation of soil microbial communities along an Antarctic climate gradient, Rinnan et al. (2009) have reported bacterial communities in soil from warmer regions to be more sensitive to temperature changes than communities from colder regions. Furthermore, Rousk et al. (2012) could show that bacterial communities had a higher temperature sensitivity in an artificially warmed soil. We cannot exclude indirect effects of long-term warming on gross nitrification, but possibly such higher sensitivity of microbial nitrifiers is the underlying reason for our observation. Indeed, the short-term temperature deviations from the *in situ* soil temperature during our incubation did not activate genotypically different AOA populations. Considering the short time frame of our incubations, the differences in gross nitrification rates were therefore likely due to less activity per cell and not to growth or death of well and less well adapted species.

Q_{10} values for gross ammonium immobilization were significantly lower than two but larger than one, which possibly points at near temperature independence and maximum performance of ammonium immobilization at the investigated temperature range. Likely the immobilization of ammonium was to a considerable extent abiotic, since about 70% of the ^{15}N signal could be retrieved from samples after incubation for one day indicating a rather high cation exchange capacity of this soil as is typical for Andosols. Therefore, temperature independence and Q_{10} values falling out of the 'usual' range of biological systems for gross ammonium immobilization is not unexpected.

4.3. Long-term effects of N fertilization on N transformation rates

As shown by our linear mixed model, N fertilization had a significant, negative effect on the rate of gross N mineralization. This supports our third hypothesis of decreased gross N mineralization in response to N deposition. Further, it confirms the proposition of

Bengtson et al. (2005) that microorganisms will most likely not invest in the generation of N-mineralizing exo-enzymes when inorganic N is abundant. There was however not significantly more N detectable in the soil at fertilized sites. Because we extracted total N via cation exchange from the soil, the additional N supplied via fertilization must have either been immobilized into biomass of the grassland plants, as well as into soil micro- and macro fauna or lost via nitrification causing NO_3^- leaching and/or loss through gaseous N forms. Confirming our third hypothesis, however, gross nitrification rates were not positively related to N fertilization and also not to ammonium content. This finding adds to the body of evidence that both fertilization with mineral N and higher NH_4^+ concentrations do not stimulate or may even inhibit soil AOA activity (Stopnišek et al., 2010; Levicnik-Hofferle et al., 2012; Verhamme et al., 2011; Pratscher et al., 2011) since archaea are the main ammonia oxidizers in this soil (Daebeler et al., 2012, 2014). As gross nitrification was not increased by N fertilization, an increased loss of inorganic N via NO_3^- leaching or gases is not a likely explanation for the absence of raised N concentrations at fertilized sites. Hence, two alternative explanations remain. Either, the additional N was primarily consumed by plants, leading to more root biomass and N-rich organic root exudates. Or N-mineralizing microorganisms were efficient in competing for the additional N with the plants and used it for assimilation. In both cases mineralizers would be less limited in N and therefore mineralize less organic N.

4.4. Combined long-term effects of warming and N fertilization on soil N transformations

We found significant interactive relationships of long-term warming and N fertilization with all gross N transformation rates even though single factors were never both significantly related to gross N transformation process. In the case of gross nitrification, predictions from the LMM analysis showed that N fertilization compensated for the positive effect of incubation temperature in geothermally warmed, but not in ambient temperature soil. Effects of short-term temperature increases were however not be mitigated by long-term N deposition. Similarly, N fertilization compensated an increased sensitivity of gross N mineralization to elevated temperature in ambient temperature soils.

Our results and previous work (Björsne et al., 2014; Brown et al., 2011; Larsen et al., 2011; Leuzinger et al., 2011; Auyeung et al., 2013; Arndal et al., 2013; Liu et al., 2015) demonstrate the importance of multifactor studies for research on environmental N-cycling in a changing world. They show that global change factors can counteract each other through interactive relationships and allow for more realistic predictions because environmental change cannot be understood through single-factor responses alone.

4.5. Conclusions

By studying interactive effects of global change features we found that stable, long-term warming of sub-arctic grassland soil resulted in increased gross nitrification with a higher temperature optimum. Possibly the observed increase in nitrification was the result of alleviated N limitation under conditions of long-term warming. N mineralization and nitrification in sub-arctic grasslands showed higher sensitivities in response to short-term temperature changes if the soils had been warmed. By analyzing the active, archaeal ammonia-oxidizing community we were able to relate these findings to a temperature-induced selection for adapted AOA populations with elevated, narrower temperature optima and phylogenetically distinct ammonia-monooxygenase enzymes.

Our results further suggest that enhanced deposition of nitrogen to sub-arctic grassland soils can be expected to slow down gross N mineralization and may not directly affect nitrification rates. Considering the interplay of global change factors however showed that raised nitrification rates and a higher temperature sensitivity in response to short-term temperature changes could be compensated by the effects of N fertilization. Thereby we provide support for the notion that global change will not only impact N-cycling in the long-term, but also through immediate and interactive effects of short-term weather variations, which are increasing in intensity and frequency.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.soilbio.2016.12.013.

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