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Summer warming accelerates sub-arctic peatland nitrogen cycling without changing enzyme pools or microbial community structure

JAMES T. WEEDON*, GEORGE A. KOWALCHUK*†, RIEN AERTS*, JURGEN VAN HAL*, RICHARD VAN LOGTESTIJN*, NESLIHAN TAŞ‡, WILFRED F. M. RÖLING‡ and PETER M. VAN BODEGOM*

*Department of Systems Ecology, Institute of Ecological Science, VU University Amsterdam, De Boelelaan 1085, 1081 HV, Amsterdam The Netherlands, †Department of Microbial Ecology, Netherlands Institute of Ecology (NIOO-KNAW), Droevendaalsesteeg 10, 6708 PB, Wageningen The Netherlands, ‡Department of Molecular Cell Physiology, VU University Amsterdam, De Boelelaan 1085, 1081 HV, Amsterdam The Netherlands

Abstract

The balance of primary production and decomposition in northern peatlands may shift due to climate change, with potential feedbacks to atmospheric CO_2 concentrations. Nitrogen availability will modulate this shift, but little is known about the drivers of soil nitrogen dynamics in these environments. We used a long-term (9 years) open top chamber (OTC) experiment in an ombrotrophic *Sphagnum* peat bog in sub-arctic Sweden, to test for the interactive effects of spring warming, summer warming and winter snow addition on soil nitrogen fluxes, potential activities of nitrogen cycle enzymes, and soil microbial community composition. These simultaneous measurements allowed us to identify the level of organization at which climate change impacts are apparent, an important requirement for developing truly mechanistic understanding. Organic-N pools and fluxes were an order of magnitude higher than inorganic-N pools and fluxes. Summer warming approximately doubled fluxes of soil organic nitrogen and ammonia over the growing season. Such a large increase under 1 °C warming is unlikely to be due to kinetic effects, and we propose that it is linked to an observed seasonal decrease in microbial biomass, suggesting that N flux is driven by a substantial late-season dieback of microbes. This change in N cycle dynamics was not reflected in any of the measured potential peptidase activities. Moreover, the soil microbial community structure was apparently stable across treatments, suggesting a non-specific microbial dieback. Our results show that in these widespread peat bogs, where many plant species are capable of organic-N uptake, organic soil N dynamics are quantitatively far more important than the commonly studied inorganic-N dynamics. Understanding of climate change effects on organic soil N cycling in this system will be advanced by closer investigation of the seasonal dynamics of the microbial biomass and the input of substrates that maintain it.

Keywords: climate change, denaturing gradient gel electrophoresis, microbial community, nitrogen cycling, peatlands, peptidase enzymes, quantitative polymerase chain reaction

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Introduction

Northern peatlands are an important component of the global carbon cycle due to their dual role as a sink of atmospheric carbon dioxide and as a source of methane emissions (Limpens *et al.*, 2008). An accumulation of soil organic matter since the last glacial maximum (Adams *et al.*, 1990) has led to northern peatlands containing an estimated 15–30% of the global soil carbon pool (Gorham, 1991; Turunen *et al.*, 2002). This accumulation is due to a combination of low primary productivity and very low decomposition rates as a result of low temperatures, low oxygen levels, functionally limited decomposer communities and recalcitrant substrates with generally low N concentrations (Freeman *et al.*, 2001; Moore & Basiliko, 2006). As some or all of these constraints are likely to shift under a warmer or drier climate, climatic change may alter the current status of northern peatlands as a carbon sink, with potentially important feedbacks to atmospheric CO_2 concentrations and therefore climate (Davidson & Janssens, 2006; Luo, 2007). Understanding the mechanisms underlying the carbon balance of (sub-) arctic peatlands is thus crucial for resolving uncertainties in global climate models (Cox *et al.*, 2000; Heimann & Reichstein, 2008).

Correspondence: James T. Weedon, tel. + 31 20 598 7047, fax + 31 20 598 7123, e-mail: j.t.weedon@vu.nl

Nitrogen will strongly affect the complex responses determining changes to the terrestrial carbon balance under climatic change. This is especially the case in boreal, sub-arctic and arctic systems, where nitrogen supply has a strong influence on both decomposition (Robinson, 2002; Mack *et al*., 2004; Bragazza *et al*., 2006) and plant primary productivity (Chapin *et al*., 1995; Shaver & Chapin, 1995; van Wijk *et al*., 2004). Several recent modelling studies have already indicated that the dynamics of carbon storage under warming and CO_2 enrichment scenarios are sensitive to the incorporation of soil nitrogen dynamics (Sokolov *et al*., 2008; Gerber *et al*., 2010; Zaehle *et al*., 2010), although the different approaches vary in the predicted timescale over which C-N feedbacks are important. Accurate prediction of the role of nitrogen in modulating the carbon balance clearly requires both well-constrained estimates of process rates and mechanistic understanding of the response of the different components of the soil N cycle to direct and indirect consequences of climate change.

Some warming experiments have shown increases in nitrogen mineralization with increased temperature (Hobbie, 1996; Rustad *et al*., 2001), although such responses can be variable and may depend on local environmental conditions (Shaw & Harte, 2001; Jonasson *et al*., 2004; Aerts *et al*., 2006). However, studies that focus on nitrogen mineralization rates may not capture all the relevant dynamics since both plants and microbes are capable of assimilating small organic-N compounds in nitrogen-poor systems (Clemmensen *et al*., 2008; McFarland *et al*., 2010) bypassing the complete mineralization of organic nitrogen to ammonia (Nannipieri & Eldor, 2009). The critical step determining nitrogen supply rate is therefore depolymerization of complex organic forms of N (Schulten & Schnitzer, 1998; Schimel & Bennett, 2004), the estimation of which may provide a more relevant measure for nitrogen availability than net mineralization rates (Weintraub & Schimel, 2005). Furthermore, soil microorganisms often represent a strong sink for nitrogen, with the result that increased rates of soil nitrogen transformation in response to environmental changes may be masked by corresponding increases in immobilization by soil microbes (Jonasson *et al*., 1996).

The proximate drivers of nitrogen depolymerization in soil are reactions catalyzed by enzymes, which are in turn produced by soil microorganisms (Allison *et al*., 2007; Wallenstein & Weintraub, 2008). It is therefore to be expected that effects of environmental change on nutrient fluxes will be mediated by changes in the structure of the soil microbial community and the enzymes they produce (Schimel & Gulledge, 1998; Bardgett *et al*., 2008). Molecular methods for describing microbial community structure provide powerful tools for detecting the response of the decomposer community to environmental changes, and the possibility to link these changes to ecosystem functions (Fierer *et al*., 2007; Reed & Martiny, 2007; Allison & Martiny, 2008). By combining such molecular methods with simultaneous measurements of soil enzyme activities and rates of soil processes, it should be possible to identify the levels of organization (community structure, enzyme pools or total fluxes) at which climatic changes are translated into altered ecosystem function.

A long-term (since 2000) climate manipulation experiment at an ombrotrophic peat bog at Abisko, northern Sweden (Dorrepaal *et al*., 2004), provides a suitable system to test for the effect of climatic changes on soil nitrogen flux and its underlying mechanisms. The experiment included treatments that simulate realistic warming scenarios during the growing season, as well as additional winter and spring treatments that simulate the predicted increase in winter snow accumulation and spring temperatures for high-latitude biomes (IPCC, 2007). The aims of the study reported here were to test the hypotheses that (i) seasonal climate manipulations increases soil nitrogen depolymerization and mineralization, in particular, that summer warming treatments would increase the rate of accumulation of (in-)organic nitrogen and (ii) that any changes in nitrogen cycling rates due to climate manipulations would be detectable in associated changes in the potential activity of soil enzymes related to organic nitrogen cycling, and/or profiles of the soil microbial community structure.

Materials and methods

Site description and sampling

The study site is situated on a gently sloping blanket bog about 800 m from the Abisko Scientific Research Station in Abisko, sub-arctic Sweden (68°21′N, 18°49′E, alt. 340 m). Mean annual rainfall in the area is 352 mm and mean monthly temperatures in January and July are −9.7 °C and 12.3 °C respectively (meteorological data 1999–2008, Abisko Scientific Research Station).

In June 2000, thirty experimental plots were established on this bog, which is dominated by *Sphagnum* sp. mosses (predominantly *Sphagnum fuscum*) and the dwarf shrubs *Empetrum hermaphroditum, Rubus chaemomorus, Betula nana* and *Vaccinium uliginosum*. The peat soil of this bog has a depth of approximately 60 cm. The area was divided into 5 contiguous blocks perpendicular to the slope. Within each block, plots were randomly assigned to one of six climate change treatments (i.e. $n = 5$ for each treatment). The treatments consist of factorial combinations of summer treatment (ambient or warming), and three winter/spring treatments (ambient, snow addition and snow addition + spring warming). For our analysis, we

sampled all plots with ambient summer and winter treatments (henceforth, AA), ambient summer with snow addition and spring warming (AS), summer warming and ambient winter/spring (WA) and summer warming with snow addition and spring warming (WS). Seasonal treatments are applied by means of open top chambers (OTCs): hexagonal, transparent polycarbonate structures 50 cm high, and with diameter 1.6–1.8 m at the top and 2.2–2.5 m at the base. The OTCs increase average daily mean air temperature by 0.3–1.0 °C in Spring (April–June) and by 0.2–0.9 °C in summer (June–October). During winter, snow passively accumulates, leading to approximately a doubling of the snow layer thickness and an increase of winter average soil temperature of 0.5–2.2 °C (Dorrepaal *et al.*, 2004, 2009). Previous studies at this site have shown that the climate manipulations alter the phenological responses of plants (Aerts *et al.*, 2006), and the growth of dominant moss species (Dorrepaal *et al.*, 2004) but have so far not significantly altered the plant species composition (Keuper *et al.*, 2011). In addition, the frequency and duration of soil freeze-thaw cycles are affected with potential implications for soil processes (Larsen *et al.*, 2002), as well as for the dynamics of nitrogen uptake and release by the microbial biomass (Brooks *et al.*, 1998; Lipson *et al.*, 1999). Summer and spring warming at this site have also been shown to increase soil respiration, with a large fraction of the increase resulting from mineralization of carbon in subsurface peat layers (Dorrepaal *et al.*, 2009).

Soil sampling was conducted during the summer of 2009, i.e. 9 years after the establishment of the experiment. For nitrogen flux measurements, a core incubation method was used to determine the changes in the sizes of inorganic, dissolved organic and microbial biomass nitrogen pools over the course of a growing season while excluding potential losses due to plant uptake and leaching through the peat profile. On June 4, two soil cores of 5 cm diameter and 15–20 cm depth were taken near the centre of each plot. One core was placed into a metal cylinder of the same diameter, capped with plastic closures and immediately replaced in the soil. The upper caps of the incubation cores were regularly opened during the summer to allow flushing of the headspace within the chamber. The other was immediately taken to the lab of the Abisko Scientific Research Station (800 m from the site) where we removed all coarse roots, homogenized and mixed the peat, took a sub-sample for water content determination and stored the remainder at −20 °C for further processing in subsequent molecular analyses and enzyme assays in Amsterdam (see below). On August 26, the cores were retrieved and treated in the same way for determination of end of season nitrogen pool sizes. The *in situ* community composition and enzyme activities for the 'August' time period (see below) were determined by sampling an additional core on August 16 as near as practical to the previous sampling location. This core was treated as above and used for DNA extraction and enzyme assays.

Nitrogen pool measurements

Nitrogen pools were measured on 5 g (fresh weight) subsamples from homogenized cores from both sampling occasions. Three separate subsamples were used. The first was fumigated with chloroform for 5 days and subsequently extracted by shaking for two hours in 25.0 mL 0.5 M K_2SO_4. The extract was digested in potassium persulfate (Cabrera & Beare, 1993) for determination of total (microbial + organic + inorganic) extractable nitrogen. A parallel non-fumigated sample was similarly extracted and digested for determination of non-microbial extractable N. Finally, the third sample was extracted for 2 h with 25.0 mL 1 M KCl for inorganic-N (ammonia and nitrate). Ammonia and nitrate in all extracts was measured using an SA-40 auto-analyzer (Skalar, Breda, The Netherlands), pool sizes calculated by the following formulae and expressed as mg N per g dry weight sample:

$$\mathrm{N_{inorganic}} = \mathrm{Ammonia_{Unfumigated,undigested}} + \mathrm{Nitrate_{Unfumigated,undigested}}$$

$$\mathrm{N_{organic}} = \mathrm{Nitrate_{Unfumigated,digested}} - \mathrm{N_{inorganic}}$$

$$\mathrm{N_{microbial}} = (\mathrm{Nitrate_{Fumigated,digested}} - \mathrm{N_{organic}} - \mathrm{N_{inorganic}})/K_{EN}$$

A correction factor K_{EN} = 0.45 was applied to the microbial N estimate as a standard correction for incomplete solubilisation of biomass N (Jenkinson *et al.*, 2004). We did not empirically determine K_{EN}, but seeing as variance in K_{EN} is related to soil type and pH only (Vance *et al.*, 1987; Jenkinson *et al.*, 2004), and our extractions were all performed on a single soil type, any error in the choice of K_{EN} will not have biased our results.

Seasonal fluxes for ammonia, nitrate and organic nitrogen were calculated by subtracting the estimates for each N pool in the initial (June) soil samples from the soil samples taken from the incubated cores in August. The use of incubation cores necessarily introduces some potential artifacts. In particular, the exclusion of plant roots and leaching effects, while allowing a more complete accounting of changes in N pool sizes, may alter important N cycling processes that are controlled by plant-microbe interactions in the rhizosphere (Kowalchuk *et al.*, 2002; Chapman *et al.*, 2006). We therefore also calculated the changes in organic and microbial pools over the season using nitrogen measurements in soil samples taken on August 16 in undisturbed soil adjacent to the incubation cores.

Fluorometric enzyme assays

Soil enzyme assays use artificial fluorogenic model substrates to estimate the rate of hydrolytic processes in soil without substrate limitation, and thus estimate the size and functional potential of the soil enzyme pool (Wallenstein & Weintraub, 2008). *In situ* enzyme activities in the soil are controlled by the size of the soil enzyme pool, but are also affected by local conditions (e.g. pH, temperature, redox potential) and substrate supply rates. As such the data we collect and present here allow comparison of the effects of treatments on the potential (but not *in situ*) activity of soil enzymes. Assays were conducted according to the protocol of Steinweg and McMahon (http://enzymes.nrel.colostate.edu/) using substrates labelled with 7-amino-4-methylcoumarin (MUC). Specific substrates were chosen to measure the potential rate of peptidase activity, i.e. the hydrolysis of amino acids from the N-terminus of

polypeptides (Sinsabaugh *et al*., 2009). This is an intermediate step in the conversion of complex polymeric forms of N to forms suitable for biological uptake. We used L-leucine-7-amido-4-MUC (henceforth, Leu), L-alanine-7-amido-4-MUC (Ala), L-lysine-alanine-7-amido-4-MUC (Lys-Ala), and L-alanine-alanine-phenylalanine-7-amido-4-MUC (AAP) (all substrates supplied by Sigma-Aldrich). The protocol includes construction of unique seven-point calibration curves for each soil sample and provides better correction for non-linear fluorescence quenching dynamics than the usual single-point correction.

We followed the protocol described above using slurries created by homogenizing 4 g fresh weight of soil in 90 mL 0.5 M sodium acetate buffer (pH 5). This pH is higher than the *in situ* field pH ($pH_{KCl} \approx 3.0$, Lang *et al*., 2009) but allows direct comparison to the large enzyme dataset of Sinsabaugh *et al*. (2008). Given the previously measured lack of effect of the experimental treatment on *in situ* pH (Lang *et al*., 2009), our methods are adequate to study and compare the functional potential of enzyme pools across treatments within our study. Incubations were carried out at 4 °C (to reflect typical summer 5 cm soil depth temperatures in the field) for 22 h. Fluorometric measurements were made on a Spectramax Gemini XS microplate fluorometer (Molecular Devices, Sunnyvale, CA, USA) with excitation wavelength of 365 nm and emission detection at 450 nm. All measurements were converted to nanomols per gram dry weight per hour for statistical analysis.

DNA extraction

Community analysis based on DNA extracted from soils allows culture-independent characterization of the soil microbial community (Rappé & Giovannoni, 2003). DNA for molecular characterization of soil-borne microbial communities was extracted from 0.25 g (fresh weight) sub samples of previously homogenized peat cores from each plot × sampling date combination using the PowerSoil® DNA Isolation Kit (MoBio, Carlsbad, CA, USA) following manufacturer's instructions except for the use of a Fast Prep Bio101 bead-beater (Bio101, USA) for the cell disruption step. This method was shown in a pilot study to be optimal in terms of DNA yield, time efficiency, and removal of polymerase chain reaction (PCR)-inhibiting substances. Eluate containing DNA was checked for DNA concentration and quality by spectrophotometry (NanoDrop, Wilmington, NC, USA).

Real time quantitative polymerase chain reaction

Quantitative polymerase chain reaction (qPCR) uses real-time measurements of amplicon accumulation during a PCR to enumerate the number of target sequences in a mixed DNA sample (Smith *et al*., 2006). When assays using primers specific to particular microbial taxonomic groups are performed on the same environmental sample, a description of the relative abundance of different groups, and therefore microbial community structure is obtained (Fierer *et al*., 2005). We used primers designed to target the three main domains of soil microbial life (Bacteria, Archaea and Fungi) as well as those targeting the bacterial phyla known to dominate in acidic peatland soils: Acidobacteria, Actinobacteria, Alphaproteobacteria and Betaproteobacteria (Dedysh *et al*., 2006). The primer sets and standards for all qPCR assays are listed in Table 1. Standards were prepared by cloning target PCR amplicons from pure cultures into pGEM®-T vector (Promega, Madison, USA), picking colonies, amplifying the lysate with vector specific T7-Sp6 primers, purifying the resulting PCR product with DNA Clean & Concentrator™-5 kit (Zymo Research, Irvine, CA, USA) and quantifying the resulting concentration of amplicon. Dilutions for standard curves were made from 10^8 to 10^3 copies per μL. Reactions were performed in triplicate per standard or sample in 96-well plates using an AB 7300 Real-Time PCR System (Applied Biosystems, Branchburg, NJ, USA) with the following reaction mix (total volume per reaction 25 μL): 12.5 μL 2× DyNAmo HS SYBR Green qPCR mix (Finnzymes, Espoo, Finland), 1 μL each of forward and reverse primers (10 mM), 0.5 μL BSA (New England Biolabs, Ipswich, MA, USA), 0.625 μL 50× ROX internal standard dye and 4.375 μL sterile, nuclease-free water. Template was added as 5 μL of a ten times dilution of extracted DNA. Annealing temperatures are given in Table 1. The thermal cycling program was as follows: an initial warm-up period at 50 °C for 2 min, initial denaturation 95 °C for 15 min, then 40 cycles of 30 s at 94 °C, 30 s at the primer-specific annealing temperature (Table 1), and 45 s at 72 °C. The cycling was followed by a final extension step of 5 min at 72 °C and the construction of a melting curve to check for product integrity. Data was collected during the extension step of each cycle.

Quantitative polymerase chain reaction efficiencies (Smith & Osborn, 2009) for the different targets ranged from 98% for Eubacteria to 81% for Acidobacteria, with a mean of 91% across all assay runs, and small (<4%) differences between plates for any given assay type. The R^2 of calibration curves was >0.99 for all assays. Before further data analysis, the copy number data for each target amplicon was scaled relative to the obtained value for eubacterial 16S rRNA gene copy number in the same sample. This normalizes across potential differences in sample DNA extract concentration and thus provides a measure of relative (not absolute) abundance of each target group within a sample (Fierer *et al*., 2005).

Denaturing gradient gel electrophoresis profiling and analysis

Denaturing gradient gel electrophoresis (DGGE) is a technique for fingerprinting environmental DNA samples based on the sequence diversity of amplicons produced by PCR with taxonomically informative primers (Muyzer *et al*., 1993; Kowalchuk *et al*., 1997; Boon *et al*., 2002). We targeted the three main groups identified by the qPCR analysis: fungi, Acidobacteria and Actinobacteria. Community profiles were generated for these three groups for all 40 DNA samples using the primer pairs Acid31 – Eub518 for Acidobacteria, Actino1175-1392R for Actinobacteria and ITS1f - 5.8s for fungi (see Table 1). Sequential PCR reactions were conducted for each sample using 2 μL template (ten times diluted DNA extract for the 1st

Table 1 Details of primer sets, annealing temperatures and standard organisms used for quantitative polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) analysis

Target group	Primers	Annealing temp	Standard organism
Eubacteria	Eub338 Eub518	53	*Acidobacterium capsulatum*
Archaea	Ar109f Ar915r	57	*Methanosarcina mazei*
Fungi	ITS1f 5.8s	52	*Cladosporium cladosporioides f. pisicola*
Acidobacteria	Acido31 Eub518	50	*Acidobacterium capsulatum*
Actinobacteria	Actino235 Eub518	60	*Rhodococcus opacus*
Actinobacteria (DGGE only)	Actino1175F Eub1392R	63	
Alphaproteobacteria	Eub338 Alf685	60	*Paracoccus denitrificans*
Betaproteobacteria	Eub338 Bet680	60	*Burkholderia plantarii*

Primer	Sequence	Reference
Eub338	ACTCCTACGGGAGGCAGCAG	(Muyzer *et al.*, 1993)
Eub518	ATTACCGCGGCTGCTGG	(Muyzer *et al.*, 1993)
Eub1392R	ACG GGC GGT GTG TAC A	(Lane, 1991)
Ar109f	ACKGCTCAGTAACACGT	(Lueders & Friedrich, 2003)
Ar915r	GTGCTCCCCCGCCAATTCCT	(Lueders & Friedrich, 2003)
ITS1f	TCCGTAGGTGAACCTGCGG	(Fierer *et al.*, 2005)
5.8s	CGCTGCGTTCTTCATCG	(Fierer *et al.*, 2005)
Acido31	GATCCTGGCTCAGAATC	(Fierer *et al.*, 2005)
Actino235	CGCGGCCTATCAGCTTGTTG	(Fierer *et al.*, 2005)
Actino1175F	GGT ACA GAG GGC TGC GAT AC	(DeAngelis *et al.*, 2009)
Alf685	TCTACGRATTTCACCYCTAC	(Fierer *et al.*, 2005)
Bet680	TCACTGCTACACGYG	(Fierer *et al.*, 2005)

reaction and the product of the 1st reaction in the 2nd) with GC clamps (5′– CGCCCGGGGCGCGCCCCGGGCGGGGCG GGGGCACGGGGGG – 3′) added to one of the primers in the second reaction. Reaction mixtures were as follows: 12.5 μL 2X Fidelitaq PCR premix (Affymetrix, Santa Clara, CA, USA), 1 μL of each primer (10 mM), 8.5 μL sterile, nuclease-free water. Annealing temperatures are given in Table 1. Samples were run in 6% (wt/vol) (Fungi and Acidobacteria) or 8% (Actinobacteria) polyacrylamide (37.5:1 acrylamide:bisacrylamide) gels with urea gradients of 25–40% for fungal fragments, and 35–55% for Acidobacteria and Actinobacteria. Gels were run at 200 V for 240 min in 1× TAE buffer maintained at a constant temperature of 60 °C using the DCode system (Biorad Laboratories, Hercules, CA, USA). After the run, gels were stained with 1× TAE buffer containing ethidium bromide for 60 min. Gel images were recorded with a DigiDoc camera system (Biorad Laboratories) and converted, normalized and analysed with GelCompar II software (Applied Maths, Kortrijk, Belgium). For each gel type we generated similarity matrices based on the calculation of Pearson product moment correlation coefficients from pair-wise comparisons of the densitometric curves of each sample (van Verseveld & Röling, 2004).

Statistical analysis

Data for seasonal change in soil nitrogen pool sizes were analysed using factorial ANOVA with summer and winter/spring treatment as fixed factors. Data for microbial biomass nitrogen, enzyme activities, and the relative abundance of each qPCR target group (standardized by total eubacterial 16S copy number per sample) were analysed with repeated measures ANOVA with summer and winter/spring treatments as fixed factors, and sampling date as the repeated measures factor. Diagnostic plots for all parametric models were checked to verify assumptions of homogeneity and independence of residual variance. qPCR data was also analysed in a multivariate model (to check for treatment effects on higher-order community structure). Relative abundances of each of the taxonomic groups were analysed with a permutational MANOVA model using the adonis function of the *vegan* package in R (Oksanen *et al.*, 2010) with the same repeated measures design as above. Distance matrices based on pairwise Pearson correlation coefficients from DGGE profiles for each primer set, as well as all three combined were also analysed using a permutational MANOVA with the same design. All analyses were conducted using R statistical software (R Development Core Team, 2010).

Results

Soil moisture and nitrogen fluxes

Soil moisture (% water by weight) significantly decreased between June and August sampling but the decline was small, from 88.0 ± 0.3% (mean ± S.E.) in June to 86.9 ± 0.5% in August, and was not significantly different from the decline in moisture calculated using soil samples taken outside the incubation cores in August (data not shown).

The summer warming treatment had a significant positive effect on the seasonal accumulation of dissolved organic nitrogen in the incubation cores, with summer warmed plots (WA and WS) accumulating 165% and 79% more organic nitrogen, respectively, compared to control (AA) plots (Fig. 1, summer $F_{1,16} = 10.03$, $P < 0.01$). There was also a significant interaction between summer and winter/spring treatment caused by opposite effects of spring/winter treatments under the different levels of the summer treatment (summer:winter, $F_{1,16} = 5.0$, $P < 0.05$). There was no treatment effect on seasonal organic nitrogen accumulation based on measurements taken in undisturbed soil cores (Appendix S1, Supplementary Information).

There was a positive effect of summer warming on net nitrogen mineralization in the incubation cores over the same period – although the absolute values were an order of magnitude lower than for organic nitrogen (Fig. 1, summer $F_{1,16} = 14.65$, $P < 0.01$). Summer warmed plots accumulated on average 110% more ammonia than summer ambient plots and there was also an additional significant negative effect of winter snow accumulation and spring warming (winter $F_{1,16} = 13.11$, $P < 0.01$) – but no significant interaction between the two treatments. Nitrate accumulation, on a similar order of magnitude as ammonia accumulation, was not affected by any of the treatments (Fig. 1).

Enzyme activities

Mean enzymatic potentials without substrate limitation at 4 °C for the measured peptidase enzymes ranged from 4.5 nmol g^{-1} DW soil $hour^{-1}$ for Lys-Ala to 134.3 nmol g^{-1} DW soil $hour^{-1}$ for Ala-Ala-Phe (AAP). Potential activities of three of the four enzymes (Leu, Ala and AAP) were significantly lower in August compared to June (Fig. 2, month $P < 0.05$) with reductions equivalent to 50%, 30% and 32% (respectively) of June activity levels. Additionally there was an interaction with the winter snow addition and spring warming treatment for Leu activity (winter × month, $F_{1,16} = 4.77$, $P < 0.05$) due to a greater activity reduction in the WA and AA treatments (54% of June level) than in the WS and AS treatments (41%). Summer warming had no effect on mean enzymatic potentials on its own, nor in interaction with the other treatments and time of measurement.

Microbial biomass

The seasonal change in the size of the microbial biomass nitrogen pool was significantly affected by the summer warming treatment (Fig. 3, summer × month $F_{1,16} = 4.82$, $P < 0.05$). Summer warmed plots showed a mean 21% decrease in the size of this pool from June to August, compared to a 1.5% decrease in summer ambient plots. In absolute terms these decreases are very similar to the observed accumulation of organic-N in the same plots: WA plots lost 0.14 mg N g^{-1} DW soil from the microbial N pool, and accumulated 0.16 mg N g^{-1} DW in the organic-N pool. For WS plots the corresponding values were 0.11 and 0.12 mg N. The winter/spring treatment had no significant effects on either the absolute size of the microbial N pool or the seasonal change. Parallel measurements in undisturbed

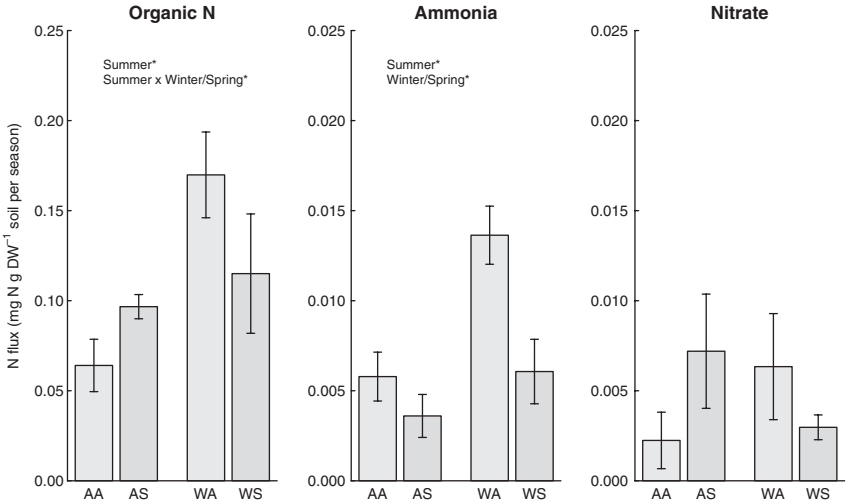

Fig. 1 Seasonal accumulation (June – August) of different soil nitrogen forms expressed as milligrams of N per gram dry weight of soil. Treatment codes – first letter: A = summer ambient, W = summer warming; second letter: A = spring/winter ambient, S = spring warming, winter snow accumulation. Named factors indicate significant *F* values in ANOVA ($P < 0.05$). Error bars indicate ± standard error of mean in each treatment.

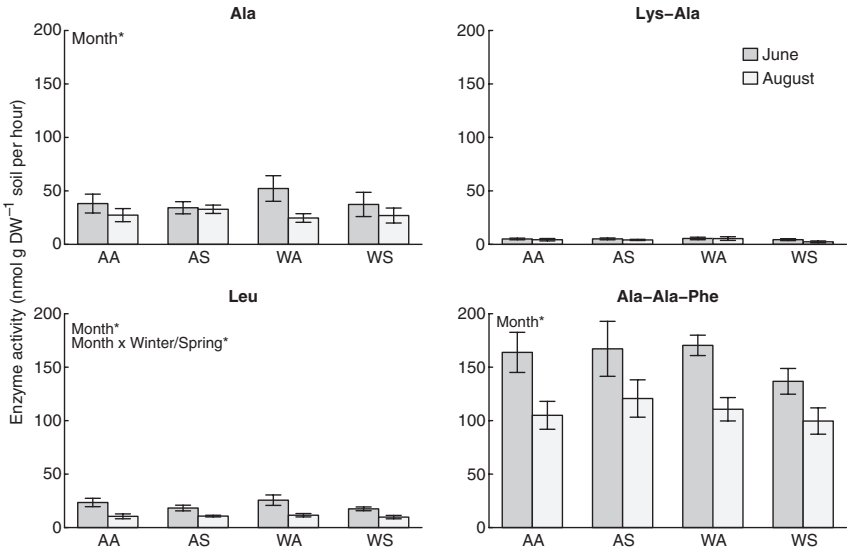

Fig. 2 Effect of climate treatments on potential peptidase activities in June and August. Treatment codes – first letter: A = summer ambient, W = summer warming; second letter: A = spring/winter ambient, S = spring warming, winter snow accumulation. Named factors indicate significant *F* values in repeated measures ANOVA ($P < 0.05$). Error bars indicate ± standard error of mean in each treatment × month combination.

soil samples (i.e. taken adjacent to the incubation cores) showed a seasonal decrease in the microbial N pool in all treatments, but this was significantly greater (45% decrease versus 22%) in the summer warmed plots (Appendix S1, Supplementary Information).

Microbial community structure

Of the four bacterial taxonomic groups quantified, Acidobacteria were by far the most abundant with a mean of 39% (all bacterial 16S rRNA gene copy data analysed as % of Eubacterial 16S rRNA gene copies), followed by Actinobacteria (8.8%), Alphaproteoabacteria (2.4%) and Betaproteobacteria (1.1%). The mean ratio of fungal 18S rRNA gene copies to eubacterial 16S gene copy numbers was 0.64:1 with a range from 0.36:1 to 1.4:1. The mean ratio of eubacterial 16S rRNA gene copies to archaeal 16S rRNA gene copies was approximately 3500:1. Note that this is at the lower end of known soil archaeal relative to eubacterial abundance data across three continents (Bates *et al.*, 2011).

Only Alphaproteobacteria and Betaproteobacteria relative abundances were affected by the experimental treatments. Plots with winter snow addition and spring warming had lower proportions of these two classes independent of sampling date and summer treatment (Fig. 4, winter (alpha) $F_{1,16} = 11.02$, $P < 0.01$, winter (beta) $F_{1,16} = 7.25$, $P < 0.05$). There was no significant seasonal change in the relative abundances of any of the target groups when considered separately. When all six taxonomic groups were considered together in a permutational MANOVA, neither sampling time, nor experimental treatments significantly explained the variation in the community composition (all terms $P > 0.05$).

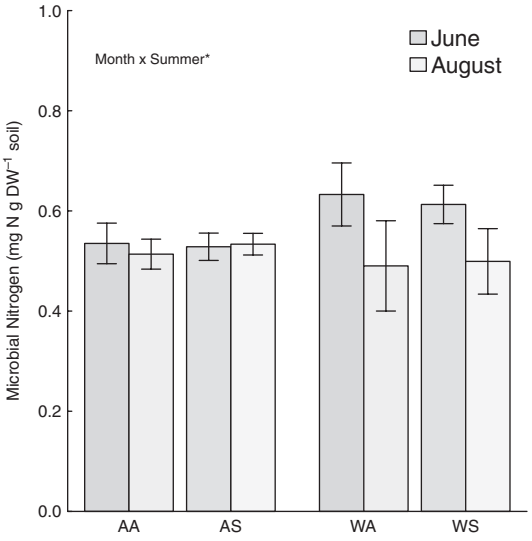

Fig. 3 Effects of climate treatment on microbial biomass nitrogen at the beginning (June) and end (August) of growing season. Named factors indicate significant *F* values in repeated measures ANOVA ($P < 0.05$). Treatment codes – first letter: A = summer ambient, W = summer warming; second letter: A = spring/winter ambient, S = spring warming, winter snow accumulation. Error bars indicate ± standard error of mean in each treatment × month combination.

Treatments and time of sampling also had no effect on the DGGE profiles of the three most abundant taxonomic groups (Acidobacteria, Actinobacteria and Fungi) based on permutational MANOVA conducted on distance matrices derived from all three profiles combined, as well as from each target group separately (all models, all terms $P > 0.05$). The profiles for Fungi were highly variable, with a mean Pearson correlation coefficient of 0.41 (all pairwise comparisons, $n = 780$). Profiles for acido- and actinobacteria were less variable with mean Pearson coefficient values of 0.70 and 0.62 respectively. Non-metric multidimensional scaling (NMDS) plots visualizing the relative similarity of DGGE profiles according to treatment are provided in Supplementary Information (Appendix S2).

Discussion

Our study utilized a unique experimental setup that simultaneously examines the effects of summer warming, spring warming and winter snow addition on soil nitrogen fluxes, potential peptidase activities and soil microbial community structure over a growing season. Although other studies have looked at individual aspects of this system (e.g. Jonasson & Michelsen, 1996; Rinnan *et al*., 2007) our integrated approach allowed for the identification of responses of soil N dynamics to seasonal climate manipulations across different levels of ecological organization.

Experimental climate change leads to doubled N-accumulation rates

Experimental warming approximately doubled the summer accumulation of both organic nitrogen and ammonia in incubation cores relative to ambient conditions. Organic nitrogen is an important source of plant nutrition in N-poor systems (Lipson & Näsholm, 2001; Clemmensen *et al*., 2008), and the strong effects of warming we observed are likely to have contributed to the sustained positive effect of summer warming treatment on *Sphagnum* moss and *Betula nana* (dwarf birch) growth in the same plots (Keuper *et al*., 2011). The results for ammonia echo previous observations of a positive effect of warming on nitrogen mineralization across a range of systems (Schmidt *et al*., 1999; Rustad *et al*., 2001). More surprising was the detection of nitrate accumulation in our incubation cores. Nitrate pathways are usually assumed to be negligible in peatland systems (Rosswall & Granhall, 1980; Limpens *et al*., 2006), so this observation merits closer examination in the future. In any case, in absolute terms the

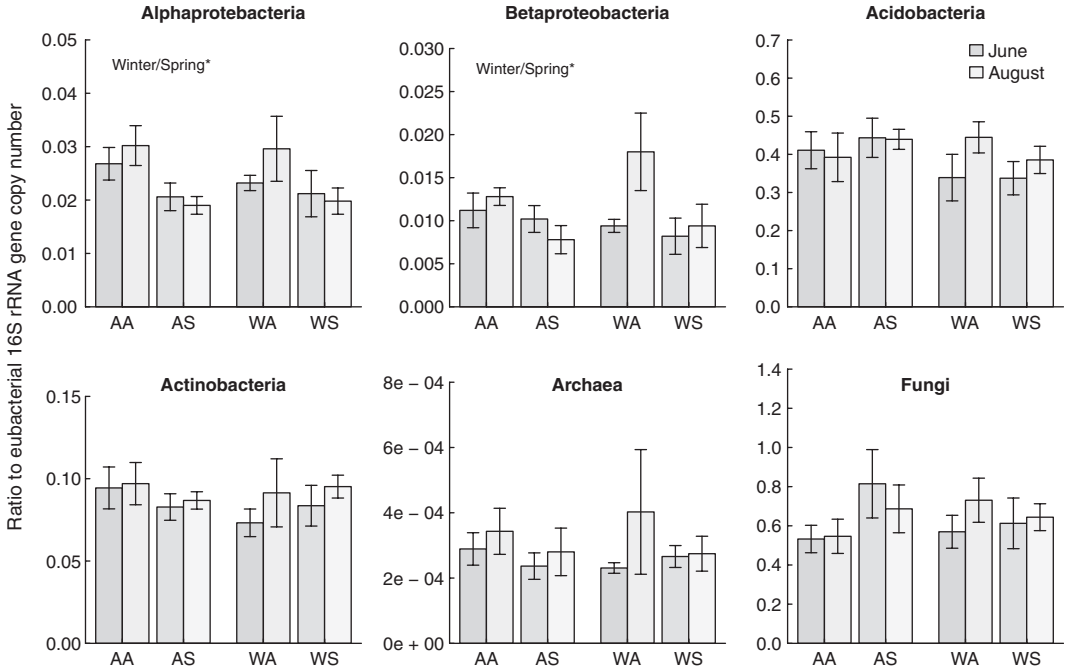

Fig. 4 Effect of climate treatment and sampling data on 16S rRNA gene (bacterial phyla and archaeal) or 18S rRNA gene (fungi) copy number as determined by quantitative PCR expressed as a ratio with total Eubacterial 16S copy number (see Table 1 for details of primer sets). Treatment codes – first letter: A = summer ambient, W = summer warming; second letter: A = spring/winter ambient, S = spring warming, winter snow accumulation. Named factors indicate significant F values in repeated measures ANOVA ($P < 0.05$). Error bars indicate ± standard error of mean in each treatment × month combination.

accumulation of ammonia and nitrate was an order of magnitude less than organic-N. This reinforces the idea that measuring organic nitrogen turnover in these systems is more informative than only measuring nitrogen mineralization (Jonasson & Shaver, 1999; Schimel & Bennett, 2004; Nannipieri & Eldor, 2009).

Importantly, our results demonstrate that the strong effect of spring and summer warming on carbon flux previously observed at the same study site (Dorrepaal *et al*., 2009) is also observed for soil nitrogen fluxes. The magnitude of the effect is also comparable – an approximately 100% increase in both cases, which is higher than previously found general increases of 46% and 20% for N mineralization and soil respiration respectively, in warming experiments in temperate and (sub-) arctic biomes (Rustad *et al*., 2001). Based on the records over the study period from soil temperature loggers in our plots (10 cm depth, temperature range over experimental period: 3.1–18.1 °C; data not shown) we calculated that the process Q_{10} (i.e. the multiplication of process rates with a 10 °C increase in temperature) that is required to produce the observed difference in ammonia accumulation between WA and AA plots over the summer season would be approximately 30. Q_{10} values for biological processes are often assumed to be around 2–3, implying that it is unlikely that the temperature response is solely due to temperature effects on enzyme reaction rates (Chapman & Thurlow, 1998). The change in soil moisture over the season was on the order of 1% by weight, and thus also unlikely to explain the observed patterns. We therefore suggest that the much larger effect of warming that we observed is mostly a consequence of indirect effects of temperature, including the inherently non-linear dynamics of soil processes close to the freezing point (Jefferies *et al*., 2010), possible indirect effects of temperature via changes to substrate input and microbial biomass dynamics (see below), and the potential for positive feedback action of increased N mobilization on N-limited decomposition processes.

The strong amplification of nitrogen fluxes potentially has important implications for the long-term status of northern peatlands as a carbon sink. Nitrogen availability limits decomposition in these environments (Robinson, 2002; Mack *et al*., 2004; Bragazza *et al*., 2006). So there is potential for a warming-induced acceleration of decomposition processes to mobilize the large pools of organic nitrogen sequestered in relatively recalcitrant soil organic matter fractions (Rosswall & Granhall, 1980; Limpens *et al*., 2006), and thus further accelerate decomposition in a positive-feedback loop. On the other hand, plant productivity is also N-limited (Chapin *et al*., 1995; Shaver & Chapin, 1995; van Wijk *et al*., 2004), so increased N supply rates may lead to increased C storage in vegetation and litter (Melillo *et al*., 2002), and/or eventual changes to the structure of the plant community (Aerts, 2010). Our data do not allow for the discrimination between these two scenarios, but we note that microorganisms are routinely identified as the stronger competitor for increased soil nitrogen (Zak *et al*., 1990; Schmidt *et al*., 1999; McFarland *et al*., 2010).

Experimental climate change has no detectable effects on microbial community structure and enzymatic potential

Given that there is a strong, consistent effect of summer warming on the rates of nitrogen fluxes in our experiment, we attempted to investigate the underlying mechanisms behind these patterns. We did so by examining simultaneous measurements of the activities of soil enzymes and the structure of the resident microbial communities that produce them. Perhaps surprisingly, neither the potential activity of four peptidase enzymes nor a broad-scale DNA-based characterization of the soil microbial community showed corresponding effects of the treatments on the structure and functioning of the soil community.

Despite measuring idealized, potential and not actual *in situ* activities, standardized assays of potential peptidase activity are widely used as an indicator for the links between microbial metabolism and nitrogen cycling (Sinsabaugh *et al*., 2009), and other studies in systems similar to ours have shown that potential enzyme activities are sensitive indicators of differences in environmental conditions across small-scale topographic and plant community gradients (Wallenstein *et al*., 2009), or in response to alterations in mineral nutrient supply (Nemergut *et al*., 2008; Enowashu *et al*., 2009; Currey *et al*., 2010). Our study showed that enzymatic potentials of peptidases are insensitive to changes in spring and summer temperature, and depth of winter snow cover. Studies in temperate ombrotrophic bogs did not find a strong association between seasonal changes in soil temperature and extra-cellular enzyme activities (Bonnett *et al*., 2006), and no significant effects of 3 °C warming on phenol oxidase and β-glucosidase activities (Fenner *et al*., 2007). In these systems, enzyme activity seems to be controlled more by changes in soil oxygen content, ionic concentrations and the presence of suppressive compounds (Freeman *et al*., 2001; Bonnett *et al*., 2006). Similarly, evidence from studies measuring soil enzyme pool responses to experimental warming in other environments seems to suggest that activities, or at least potential activities, are either insensitive to small increases in soil temperature (Bell *et al*., 2010; Bell & Henry, 2011), or limited to certain enzymes (Allison *et al*., 2010), or that the size of the response is

small relative to interannual variation (Gutknecht *et al.*, 2010). Combining these results with large effect of warming on inorganic and organic nitrogen fluxes in the current study suggest the following (non-exclusive) interpretations: (i) increased fluxes are due to increased supply of peptide substrate due to e.g. warming-related increases in the concentrations and activities of soil proteases (Weintraub & Schimel, 2005), and/or enhanced input from other sources of organic nitrogen such as plant litter and root exudation/turnover, and microbial biomass (see further discussion below), (ii) changes in organic-N fluxes are due to changes in the concentrations of non-peptide organic-N components, such as heterocyclic N, which may make up around 35% of soil organic-N pools (Schulten & Schnitzer, 1998). Such compounds would be processed by enzymatic pathways which we have not examined, and a detailed examination of the chemical composition of the organic-N fraction in our system would be required to properly assess this interpretation. Finally, despite the lack of difference in the size of the peptidase pools, realized *in situ* activities might have been increased in warming plots due to direct treatment effects on temperature and/or pH and therefore enzyme activity. However, we have discussed above that the large flux amplification over an approximately 1.5 °C temperature difference with insignificant changes in pH are unlikely to be purely due to enzyme kinetics. These effects, although potentially present, do thus not seem to be sufficient to account for the observed treatment effects.

The structure of the soil microbial community also appeared to be unaffected by over 9 years of experimental climate manipulations – and all the changes in temperature regime, vegetation phenology, moisture dynamics and soil freeze-thaw frequency that they bring about. This is despite the fact that we used techniques (taxonomic qPCR and DGGE) that have been shown to be sensitive to differences in microbial communities along large-scale gradients in ecosystem type and soil properties (Muyzer & Smalla, 1998; Fierer *et al.*, 2005, 2007, 2009; Yergeau *et al.*, 2007). Studies with similar temperature manipulations at a nearby heath tundra site only detected warming effects on microbial community structure after 15 years of experimental manipulations, suggesting that there is a long time lag (possibly mediated by decade-scale vegetation dynamics) between climate changes and detectable responses in the microbial community (Rinnan *et al.*, 2007). Although we cannot rule out the possibility that higher resolution methods may have revealed treatment effects on the microbial community structure (for example on taxonomic groups we did not measure), our enzyme results indicate that any undetected alteration in the community would nevertheless imply functional redundancy with respect to peptidase production. We therefore conclude that the effect of summer warming on fluxes cannot be explained with reference to microbial community structure at the level of domain to sub-phyla, nor to peptidase pools.

Non-specific microbial mortality drives doubled N-accumulation rates

The question remains as to how large effects on both organic and inorganic nitrogen fluxes can be observed without corresponding changes in the pool of enzymes responsible for these fluxes, or the microbial community that produces them? The answer appears to reside in the effect of warming on the seasonal change in the size of the microbial biomass N pool. The seasonal increase in organic nitrogen under summer warming is mirrored by a decrease in the size of the microbial biomass pool of an almost identical magnitude, a pattern not observed in the summer ambient plots. A very similar pattern is observed in soil samples taken outside the incubation cores (Appendix S1, supplementary information) which suggests that the observed microbial biomass decline is not an artefact caused by the exclusion of rhizosphere effects. Microbes in summer warmed plots thus attain a higher peak biomass early in the season, which decreases through microbial mortality resulting in a biomass equivalent to ambient conditions at the end of the growing season. Importantly, this mortality seems to be generally non-specific as it is not associated with detectable changes to the microbial community structure in the major taxonomic groups we examined (see above). Earlier studies have established that the nitrogen economy of annually frozen soils can be greatly influenced by the dynamics of microbial biomass in late-winter and immediately following spring thaw (Zak *et al.*, 1990; Deluca *et al.*, 1992; Brooks *et al.*, 1998), although in our case the experimental manipulation of winter snow depth and spring temperature were less important than summer warming. We propose that indirect effects of summer warming on the overwintering microbial biomass via e.g. increased plant litter input (Brooks *et al.*, 2005), or increased growing season root exudation (Nguyen, 2003) translates into a greater flux of labile carbon and nitrogen following spring thaw (Lipson *et al.*, 1999; Jefferies *et al.*, 2010). This flux facilitates a higher summer peak microbial biomass, which declines later in the season through non-specific mortality, resulting in the observed enhancement of nitrogen fluxes.

Our interpretation of the processes leading to increased N-accumulation under summer warming has two implications. First, it suggests a mechanism for the

commonly observed warming effect on soil respiration and nitrogen mineralization related to altered seasonal behaviour of the microbial biomass, rather than the result of direct temperature effects, or the mobilization of older, recalcitrant soil organic matter pools (Dorrepaal *et al.*, 2009). Second, in contrast to the common view that global change effects on soil ecosystem function will be mediated by changes in microbial community structure (Schimel & Gulledge, 1998; Allison & Martiny, 2008), our data suggest that microbial communities and enzyme pools that are unaffected by climate change treatments can nevertheless produce a large range in magnitudes of biogeochemical fluxes, apparently as a result of those same treatments. Our results suggest that a focus on the effects of climate change drivers on the quantity, timing and composition of the inputs of substrates (litter, root exudates and turnover in the microbial biomass) will be particularly fruitful for resolving some of these issues. Furthermore, *in situ* experiments using labelled N compounds (for which there have been recent, exciting developments, e.g. Whiteside *et al.*, 2009; Rütting *et al.*, 2011) would provide a more rigorous test of our hypothesized relationship between seasonal microbial biomass die-off and organic nitrogen fluxes, and allow measurements in the presence of rhizosphere influences such as plant uptake and rhizodeposition (Jonasson *et al.*, 2004).

Conclusions

Nine years of manipulation of spring and summer air and soil temperature led to doubled rates of organic and inorganic nitrogen release in a sub-arctic *Sphagnum* peat bog with strong implications for the role of these systems as a carbon sink. These large spring and summer warming effects on nitrogen transformation could not be explained by changes in the microbial community structure or peptidase pools, but seemed to be related to altered seasonal dynamics of the microbial biomass as a whole. Future studies of climate change effects on nitrogen cycling in this globally important carbon store should focus on the effects of warming on the seasonal dynamics of the microbial biomass, the quantity, timing and composition of the substrate inputs that maintain it, and their interplay with soil enzyme activities and microbial community structure.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Comparison of organic and microbial N pool changes in incubated and undisturbed soil cores.
Appendix S2. NMDS plots of DGGE profiles for Acidobacteria, Actinobacteria, Fungi and all three profile types combined.

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