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# Variation in N<sub>2</sub> fixation in subarctic tundra in relation to landscape position and nitrogen pools and fluxes

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## ABSTRACT

Biological N<sub>2</sub> fixation in high-latitude ecosystems usually exhibits low rates but can significantly contribute to the local N budget. We studied N<sub>2</sub> fixation in three habitats of East European subarctic tundra differing in soil N stocks and fluxes: N-limited vegetated peat plateau (PP), frost formations of bare peat called “peat circles” (PC) with high availability of soil N, and vegetated upland tundra (UT) with low to intermediate N-availability. Nitrogen fixation was measured at field conditions twice during summer 2011 by acetylene reduction assay, and N<sub>2</sub> fixation rates were verified by <sup>15</sup>N<sub>2</sub> fixation assay. Response to variation in nutrients, carbon, and temperature was studied in complementary laboratory experiments. Further, we aimed to link N<sub>2</sub> fixation rates to N deposition and major N transformation rates (gross and net mineralization, plant N uptake) including high N<sub>2</sub>O emissions recently found from PC. We hypothesized that N<sub>2</sub>O emissions in PC were fueled partly by biologically fixed N. Contrary to that hypothesis, N<sub>2</sub> fixation was found solely in PP (0.01–0.76 mg N m<sup>-2</sup> d<sup>-1</sup>), where N<sub>2</sub> was fixed by moss-associated cyanobacteria and heterotrophic soil bacteria. The low N and high P availability corresponded with the occurrence of N<sub>2</sub> fixation in these soils. Nitrogen fixation represented only a small portion of plant N uptake in PP. Conversely, bare PC (as well as vegetated UT) lacked N<sub>2</sub> fixation and thus N<sub>2</sub>O efflux is most likely fueled by release of mineral N to the soil through internal nutrient cycling.

## INTRODUCTION

The productivity of high-latitude ecosystems is constrained by low availability of nutrients and by unfavorable climatic conditions for plant growth and microbial activity (Shaver and Chapin, 1986; Chapin et al., 1995; Mikan et al., 2002; Sistla et al., 2012). Nitrogen is frequently the nutrient limiting primary production in tundra. Thus, N input through biological N<sub>2</sub> fixation (i.e., the activity of nitrogenase enzyme) might be critical to the

N budget of Arctic ecosystems. Low nitrogenase activities have been reported from Arctic regions compared to other ecosystems due to the combined effect of low temperature, limited energy sources, and insufficient availability of other nutrients (Chapin and Bledsoe, 1992). Reported N<sub>2</sub> fixation rates from subarctic and Arctic tundra usually range from 0.15 to 6.45 mg N m<sup>-2</sup> d<sup>-1</sup> (Chapin and Bledsoe, 1992; Hobara et al., 2006; Sorensen and Michelsen, 2011; Stewart et al., 2011a; Stewart et al., 2011b), but can be as high as 1206 mg N m<sup>-2</sup> d<sup>-1</sup>

in biological soil crusts (Liengen, 1999a). Despite these generally low rates,  $N_2$  fixation can account for as much as 50%–90% of total annual N input to tundra ecosystem (Hobara et al., 2006; Solheim et al., 2006). However, information on  $N_2$  fixation as a portion of annual N turnover or local N stock is scarce in literature and little is known on the spatial variability and factors controlling  $N_2$  fixation rates in heterogeneous tundra landscapes.

Nitrogen fixing microorganisms (diazotrophs) in tundra habitats occur in various forms; biological soil crusts on bare surfaces, cyanobacteria as phycobionts in lichens, free-living or moss-associated cyanobacteria, and free-living or rhizosphere-associated heterotrophic bacteria. Leguminous symbiosis can also play a role in Arctic region (Bordeleau and Prévost, 1994). The energy needed for  $N_2$  fixation by heterotrophic diazotrophs is acquired from readily available carbon (C) provided via soil organic matter mineralization or by host plants. On the other hand, light has a direct positive effect on autotrophic diazotrophs. Photosynthesis constitutes the more abundant source of energy in tundra compared to easily decomposable organic compounds and therefore the majority of studies on  $N_2$  fixation in high-latitude ecosystems are focused on autotrophic  $N_2$  fixation by cyanobacteria (Liengen and Olsen, 1997; Liengen, 1999b; Solheim et al., 2002; Sorensen et al., 2006; Stewart et al., 2011a), while the contribution of heterotrophic bacteria has been considered insignificant. However, recent studies presented evidence that also diazotrophic soil bacteria can substantially contribute to the local soil N pool by fixing atmospheric  $N_2$  (Nosko et al., 1994; Hara et al., 2009; Zadorina et al., 2009). As such, there is a paucity of studies on  $N_2$  fixation in high-latitude ecosystems and our understanding of the role of diazotrophs for N budgets in tundra is poor.

The major components of East European subarctic tundra are represented by upland tundra (UT) and raised permafrost peat plateaus (PP) covering the most dominant parts of this area besides fens, water bodies, and forests (Virtanen and Ek, 2014). Patterned ground features called *peat circles* (PC), which are bare, round in shape, and characterized by relatively high water content and bulk density, occur on the peat plateaus (Repo et al., 2009; Biasi et al., 2014). The origin of the PCs is still unclear, but it is suggested that permafrost action and associated

uplifting of deeper soil layers to the surface are responsible for their development (Repo et al., 2009). They are characterized by different availabilities of C and nutrients and dissimilar C and N transformation pathways in soil compared to adjacent vegetated areas underlain by peat and mineral soils (Repo et al., 2009; Marushchak et al., 2011, 2013; Biasi et al., 2014). Large nitrous oxide ( $N_2O$ ) emissions were recently observed in PC (Repo et al., 2009). The discovery of these high  $N_2O$  fluxes in tundra was surprising since rates of denitrification, the main source of  $N_2O$ , are generally low in Arctic soils due to low nutrient status and high competition for N between plants and microbes (Ludwig et al., 2006; Siciliano et al., 2009). In PC lacking plant cover, however, a sufficient amount of mineral N is readily available for  $N_2O$  production (Repo et al., 2009; Marushchak et al., 2011). The origin of such abundant nitrate/nitrite sources in PC, the substrate for denitrification, has remained unrevealed. Even though  $N_2$  fixation is often inhibited by high N concentration (Chapin and Bledsoe, 1992), nitrogenase activity has never been measured in cold Arctic soils what exhibit  $N_2O$  emissions as high as tropical soils (Repo et al., 2009). It is likely that not only internal cycling supplies N for this process. Cleveland et al. (1999) and Stewart et al. (2013) suggested that high denitrification rates can be linked to active  $N_2$  fixation. Fixed N in the form of ammonia can enter a sequence of further biochemical transformations such as nitrification and denitrification and so can be eventually turned into  $N_2$  and  $N_2O$ . Peat circles with dark bare surfaces could provide favorable thermal environments for diazotrophs. Higher temperatures and high light levels are likely to support biological crust formation (Liengen and Olsen, 1997; Zielke et al., 2002; Stewart et al., 2011b). Indeed, thin moss layers were found on PC with varying coverage. Moss-crust surfaces are often inhabited with cyanobacteria responsible for  $N_2$  fixation (Evans and Johansen, 1999). In addition, PC are rich in available C (Biasi et al., 2014), and therefore we expect that also heterotrophic  $N_2$  fixation can be important to the N budget.

The major aim of this study was to investigate the magnitude and spatial patterns of  $N_2$  fixation rates in variable tundra habitats differing in environmental conditions, C and N stocks, as well as transformation rates. Specifically, we wanted to assess the impact of environmental variables (C and

nutrient concentration in soil, temperature) on nitrogenase activity and to determine the role of autotrophs and heterotrophs in  $N_2$  fixation. Our particular objective was to relate  $N_2$  fixation rates to major N transformation processes—for example, gross and net N mineralization, plant N uptake, and  $N_2O$  emissions (the latter only relevant for PC where  $N_2O$  emissions occur)—and compare to atmospheric N deposition. These data were concurrently measured or available from the site. Such a survey is rare in the literature on N cycling in Arctic ecosystems. We hypothesized that  $N_2$ -fixing biological crust boosts high denitrification rates in PC by supplying additional N, resulting in high soil-N concentration and high  $N_2O$  emissions. Thereby, we aimed to test the traditional view that  $N_2$  fixation is limited in N-rich soils. Nitrogen fixation rates were studied in three habitats of East European tundra: PP, PC, and UT. We conducted field measurements to characterize nitrogenase activity under natural light conditions and under dark, which included dark  $N_2$  fixation by cyanobacteria and heterotrophic diazotrophs. In laboratory experiments, we examined factors controlling  $N_2$  fixation by heterotrophic soil bacteria to better understand the factors that control the process. The acetylene reduction assay (ARA) used to detect  $N_2$  fixation rates was calibrated with a  $^{15}N_2$  assay. Seasonal N input via  $N_2$  fixation was estimated for each habitat using observed temperature variation.

## MATERIALS AND METHODS

### Study Site

The study site is located 70 km southwest of the city of Vorkuta, Komi Republic, Russia (67°03'N, 62°57'E, 100 m a.s.l.) and falls within the discontinuous permafrost zone of subarctic tundra. Prevailing habitats are UT on mineral soil with thin organic layer (<10 cm) and raised PP with deep organic deposits spreading over 50% and 16% of the study site area, respectively (Hugelius et al., 2011). Upland tundra has typical tundra heath vegetation (*Betula nana*, *Salix* sp., *V. uliginosum*, and *Vaccinium vitis-idaea*), while PP is covered by tundra bog vegetation (*Ledum decumbens*, *Rubus chamaemorus*, *Vaccinium uliginosum*, and *Sphagnum* mosses). Distinct dark spots of bare peat soil, referred to as PC (round in shape, about

20 m diameter on average), cover about 4% of the PP (Repo et al., 2009). Detailed description of the study site, including climate, topography, vegetation composition, and general soil properties are given in previous studies conducted at this site (Repo et al., 2009; Marushchak et al., 2011). All examined soils were highly acidic with pH values in a range from 3.1 to 3.5. No legumes were present in the vegetation cover of both PP and MT, possibly due to the low pH (Bordeleau and Prévost, 1994). Ambient air and soil temperatures were monitored continuously over the vegetation season of 2011, when this study was conducted, using S-THA-M006 and S-TMB-M006 sensors (Onset Computer Corporation, Bourne, Massachusetts, U.S.A.), respectively, in conjunction with a HOBO Micro Station Data Logger H21-002 (Onset Computer Corporation, Bourne, Massachusetts, U.S.A.).

### Experimental Design

Nitrogen fixation assays were divided into measurements with intact soil cores performed immediately after sampling, under field conditions (later referred to as *field measurements*), and experiments with homogenized soil conducted in the laboratory under controlled environmental conditions (*laboratory experiments*).

Total  $N_2$  fixation under field conditions was measured twice during the 2011 summer season; the first sampling period took place on 20 July when the day length was 21 h 35 min and included measurements in all three habitats (PP, PC, UT). The second sampling period was carried out on 18 August 2011 with day length 16 h 55 min. The number of replicates were doubled for the PP site because of high variability among samples in July. Due to negligible nitrogenase activity in the first measurement, UT was not included again. In order to better understand the spatial variation in  $N_2$  fixation, PC with differing amounts of small moss-species cover were chosen for the second measurement. Compared to PC selected in the first measurement, they represented early succession on the bare surface with more pronounced biological soil crust, which was potentially favorable for associated diazotrophs. Intact soil cores were collected immediately before start of the field measurements. Each of the examined habitats was represented by three field replicates (unless noted otherwise). The

sampling sites were chosen as typical of the respective habitat. Four intact soil samples of the 5-cm-thick top layer were collected using a polypropylene soil core of 5 cm diameter from each sampling site. Vascular plants were cut from the surface of soil cores, but the moss layer remained intact.

Laboratory experiments were designed to examine heterotrophic  $N_2$  fixation and the potential controlling factors; response to carbon availability, effect of N and phosphorus (P) addition and temperature dependence were assayed. Thereafter, a stable isotope technique using  $^{15}N_2$  was used to convert nitrogenase activity measured as acetylene reduction into amount of fixed N. Soil samples for laboratory experiments were collected in the same manner as for field measurements on 23 August 2011. Soil cores were kept in dark at 4 °C until processing (for a few weeks). Storage conditions were unfavorable for autotrophic organisms, and so we assumed that contribution of cyanobacteria to nitrogenase activity was negligible here. Prior to laboratory experiments, soil samples for each habitat were mixed and homogenized.

## Nitrogen Fixation

### Field Measurements

Nitrogen fixation rates in field measurements were assayed using acetylene reduction assay (ARA) modified from Černá et al. (2009). Intact cores were inserted into glass jars with final headspace of 350 mL. Jars were closed by screw-lids equipped with three-way stopcock for gas sampling, and soil cores were incubated in ambient air with 10% (v/v) acetylene ( $C_2H_2$ ). At the end of a 48-hour incubation, 10 mL of headspace was withdrawn by airtight syringe from incubation flasks and the ethylene concentration was measured (see Analytical Methods). Control samples without acetylene and several blanks were also incubated and analyzed for endogenous ethylene production and ethylene traces in the acetylene gas used. The difference between the sample and blank ethylene concentrations in headspace was used to calculate the field acetylene reduction rate.

Samples from each sampling site were incubated under the natural light conditions as well as in the dark. Two mL of 0.05 M sucrose solution were added only into dark samples in order to detect potential light-independent  $N_2$  fixation by cyanobacteria

and heterotrophic soil bacteria. The temperature inside three dark and three light jars was monitored by inserting small temperature data loggers into the jars (i-button DS1921G, Maxim, Sunnyvale, California, U.S.A.). Mosses and liverworts covering the soil cores were identified, and cyanobacteria and their abundance in the uppermost layer of soil cores were determined after incubation. The soil cores were dried and  $N_2$  fixation was expressed on dry weight basis ( $gdw^{-1}$ ). Dry weight ranged between 0.02 and 0.50  $gdw\ gfw^{-1}$ .

### Laboratory Experiments

For each of the laboratory experiments, 40 mL glass vials were filled with 2.5 g of fresh soil. Samples were preincubated for 24 hours at relevant incubation temperature. All laboratory experiments were carried out in the dark and each treatment was performed in triplicate ( $n = 3$ ). The ARA followed the same protocol described above except that gas samples were taken three times over an incubation period of six days (on the 2nd, 3rd, and 6th day).

- I. *Kinetic of nitrogenase activity (C substrate addition).* Soils were amended with different amounts of a labile C source (sucrose) simulating plant root exudates, in order to determine the dependence of nitrogenase activity on additional energy source (Černá et al., 2009). Sucrose was added in 1.0 mL of solution; final carbon addition accounted for 0, 0.5, 1, 2, 5, 10, 20, and 30 mg C  $gdw^{-1}$ . Samples were incubated at 12 °C.
- II. *Carbon and nutrient manipulation.* The responses of nitrogenase activity to increased concentrations of available P and N were examined at 20 °C, at optimal temperature, based on results of a temperature dependence experiment (see below). Soil samples were amended with 1.0 mL of a solution that contained optimal C concentrations (30 mg C  $gdw^{-1}$ ) in one of three different nutrient treatments; carbon-only addition (C), carbon-phosphorus (C+P), and carbon-nitrogen (C+N) addition. Control samples with the same amount of distilled water were included. Phosphorus was added in the form of  $K_2HPO_4$  solution and N as  $NH_4NO_3$  solution, with the final concentration of added nutrient

in soil 40  $\mu\text{g PO}_4\text{-P gdw}^{-1}$ , 30  $\mu\text{g NH}_4\text{-N gdw}^{-1}$  and 30  $\mu\text{g NO}_3\text{-N gdw}^{-1}$ , respectively, which corresponded to the highest measured nutrient levels in sampling area.

III. *Temperature dependence.* Nitrogenase activity was measured at four temperatures within a range of natural local conditions during the vegetation period (4, 12, 20, and 25 °C). Optimal C concentration, evaluated in the first experiment (30 mg C  $\text{gdw}^{-1}$ ), was applied to all samples.

Acetylene reduction rates were calculated over the incubation period as a slope of the linear regression. The starting point was always represented by blanks. All data based on ARA were recalculated to  $\text{N}_2$  fixation rates based on the results from the laboratory experiment with  $^{15}\text{N}_2$  fixation (see below).

## Additional Experiments

Occurrence of cyanobacteria was assessed in the soil cores used in field ARA measurements to verify contribution of autotrophic diazotrophs to  $\text{N}_2$  fixation. Cyanobacteria were isolated from the uppermost 2 cm layer and cultivated in liquid BG-11 medium for three weeks at 20 °C, day-night mode 10/14 h with light intensity  $\text{PAR} = 100 \mu\text{mol m}^{-2} \text{s}^{-1}$ . Species of cyanobacteria present were then determined under a microscope Nikon Eclipse 80i at magnifications  $\times 300$ , 675, or 1000. Sample scanning was repeated five times.

Net N mineralization was assayed under field conditions to quantify potentially plant available inorganic N in the soil. The buried bag technique was used (Knoepp and Swank, 1995; Holub and Záhora, 2008) and field triplicates were established for each habitat in July 2011. The uppermost soil layer of 5 cm excluding vegetation was sampled from PP and PC while organic horizon was taken from UT using a soil corer of 5 cm diameter; always in field triplicates. Soil cores were closed in polypropylene bags and reburied in the soil for 25 days to incubate at ambient temperatures. Ammonium ( $\text{NH}_4^+$ ) and nitrates ( $\text{NO}_3^-$ ) were extracted immediately at the beginning of the experiment and right after the in situ 25-days incubation, and the soil extracts were kept frozen until analysis. Extractions and analyses followed the protocol described below. Net N mineralization rates were calculated

from the difference in  $\text{N-NH}_4^+$  plus  $\text{N-NO}_3^-$  concentrations during the incubation period. Gross N mineralization was determined monthly during a period June–August 2008 ( $n = 3$ ) using pool dilution method (for more details see Marushchak et al., 2011).

The  $^{15}\text{N}_2$  fixation assay was conducted in the laboratory to calibrate fixation rates acquired by the ARA method. Two nutrient manipulations (C and C+P similar to experiment III) were included to test the sensitivity of both methods to changes in nitrogenase activity. Soil samples for the  $^{15}\text{N}_2$  fixation assay were incubated in a similar way to the ARA laboratory experiments. However, the incubation vials were filled with an artificial aerobic atmosphere consisting of 80%  $^{15}\text{N}_2$  (98 at%, Cambridge Isotope Laboratories, Andover, Massachusetts, U.S.A.) and 20% pure  $\text{O}_2$  (Messer Technogas, Prague, Czech Republic) and incubated for six days at 20 °C to achieve sufficient amounts of  $^{15}\text{N}$  label in soil (Šantrůčková et al., 2010). Enrichment of soil labeled by  $^{15}\text{N}_2$  was elevated by 20‰ to 70‰. Control samples with the same nutrient treatment assayed by ARA (experiment II) provided data on acetylene reduction rates and natural abundance of  $^{15}\text{N}$ . At the end of the experiment, soils from  $^{15}\text{N}$ -labeled samples and controls were frozen, freeze-dried, and ground by a ball mill (Mixer Mill MM 200, Retsch, Germany). The ratio of stable isotopes  $^{15}\text{N}:^{14}\text{N}$  in soil samples related to a standard (atmospheric  $\text{N}_2$ ), denoted as  $\delta^{15}\text{N}$ , was then analyzed (see below) and the final  $\text{N}_2$  fixation rate was calculated using the equation

$$\text{nmol N fixed gdw}^{-1} \text{ h}^{-1} = (\text{nmol N}_{\text{tot}} \text{gdw}^{-1} \times \text{tracer fraction}) t^{-1} \quad (1)$$

where total N amount in  $^{15}\text{N}$ -labeled soil sample ( $\text{nmol N}_{\text{tot}} \text{gdw}^{-1}$ ) was calculated with respect to a mass ratio  $^{15}\text{N}:^{14}\text{N}$  in the sample and  $t$  was incubation time (hours). *Tracer fraction* expressed the distribution of the  $^{15}\text{N}$  label between the gas phase and soil in incubation vial of  $^{15}\text{N}$ -labeled sample according to the equation

$$\text{tracer fraction} = (\text{at}\% \text{ } ^{15}\text{N}_{\text{soil-labeled}} - \text{at}\% \text{ } ^{15}\text{N}_{\text{soil-control}}) (\text{at}\% \text{ } ^{15}\text{N}_{\text{air-labeled}} - \text{at}\% \text{ } ^{15}\text{N}_{\text{air-control}})^{-1} \quad (2)$$

where *at%* indicates molar ratio of  $^{15}\text{N}$  to total N. Further,  $\text{C}_2\text{H}_2:\text{N}_2$  ratio (i.e., conversion factor) was

expressed as nmol C<sub>2</sub>H<sub>2</sub> reduced to nmol N<sub>2</sub> fixed in corresponding samples.

## Analytical Methods

Ethylene concentrations from gas samples taken in ARA measurements were determined using an Agilent 6890N gas chromatograph (Agilent Technologies, U.S.A.) with a flame ionization detector and a 0.53 × 30 m GS-Alumina column at 45 °C. Total N and δ<sup>15</sup>N of soil samples were analyzed using an elemental analyzer (Vario micro cube, Elementar Analysensystem GmbH, Germany) coupled to isotope ratio mass spectrometer (IR-MS DELTA plus XL, Finnigan, Germany). Nitrate and phosphate (PO<sub>4</sub><sup>3-</sup>) concentrations were determined in distilled H<sub>2</sub>O soil extracts by an ion chromatograph (DX 120, Dionex Corporation, U.S.A.). Ammonium ions were extracted by 1 M KCl from soil and extracts were analyzed using spectrophotometer (Ultrospec 300 Pro, Biochrom, U.K.) according to Maljanen et al. (2009). Dissolved organic carbon (DOC) and dissolved nitrogen (DN) in water extracts were measured on DOC/DN analyzer (LiquicTOC II, Elementar, Germany). Since the studied soils were highly acidic, available P for C:N:P ratio calculation was determined in oxalate extracts (P<sub>ox</sub>, Kopáček et al., 2001).

## Data Analyses

For recalculation of acetylene reduction rates to N<sub>2</sub> fixation rates, we used a conversion factor of 7.54 determined in our calibration experiment using <sup>15</sup>N<sub>2</sub> (see above). The factor was not affected by nutrient manipulation ( $t = 0.33$ ,  $df = 6$ ,  $P = 0.75$ ). The molar ratio of C<sub>2</sub>H<sub>2</sub>:N<sub>2</sub> reduced by nitrogenase enzyme in excess of acetylene is between 3 and 4 (Zechmeister-Boltenstern and Kinzel, 1990; Tate, 2000, respectively). However, a wide span of values ranging between 0.56 and 22 has been reported in studies from terrestrial ecosystems (summarized in Liengen, 1999a). Studies on N<sub>2</sub> fixation in northern ecosystems reported ratios 0.022–4.9 (Liengen, 1999a; DeLuca et al., 2002; Sorensen et al., 2006). The reason for the relatively high conversion factor in our soils can be explained by low nitrogenase activity, but remains inconclusive.

Temperature dependence of heterotrophic N<sub>2</sub> fixation measured under laboratory conditions

(experiment III) was described using the Arrhenius equation:

$$k = A \times e^{\frac{-E_a}{R \times (T + 273.15)}} \quad (3)$$

where  $k$  indicates N<sub>2</sub> fixation rate (ng N gdw<sup>-1</sup> h<sup>-1</sup>),  $A$  and  $E_a$  (J·mol<sup>-1</sup>) were estimated equation parameters,  $R$  represents gas constant (8.314 J·K<sup>-1</sup>·mol<sup>-1</sup>), and  $T$  is temperature (°C). Parameters of the Arrhenius equation were acquired using GraphPad Prism 4 for Windows (GraphPad Software, 2003).

We attempted to assess the importance of N<sub>2</sub> fixation relative to other N transformations in the overall N budget at the sampling sites including also the amount of N bound annually in NPP (net primary production). Data on N<sub>2</sub> fixation and net N mineralization from the present study and data on gross N mineralization rates determined in summer 2008 under laboratory conditions at 15 °C (Marushchak et al., 2011) were used. Based on aboveground biomass data from Seida site presented by Hugelius et al. (2011), we approximated total plant biomass; moss biomass was subtracted from the total aboveground biomass to express the aboveground biomass of vascular plants. Belowground biomass of vascular plants was estimated using the root:shoot ratio specific to each land cover type (Hogan, Crittenden, and Virtanen; unpublished data). Total plant biomass was a sum of moss, vascular plant aboveground, and belowground biomass. Shaver and Chapin (1991) concluded that the productivity:biomass ratio and C:N in NPP were relatively stable in different tundra habitats. Accordingly, we estimated the annual N allocation in NPP using C:N ratios calculated from the data given by Shaver and Chapin (1991). Using these assumptions, the average daily N requirement of NPP over a 60-day growing season was calculated. The total wet atmospheric N deposition in our sampling region was estimated based on data provided by Walker (2003).

The difference in N<sub>2</sub> fixation between two sampling campaigns and the effect of light/dark treatment on nitrogenase activity under field conditions were tested by a factorial ANOVA. The effect of nutrient addition in laboratory experiments was examined using one-way ANOVA with Tukey honest significant difference (HSD) post-hoc test. Nitrogen fixation rates from field measurements

were log-transformed in order to meet the assumptions of the ANOVA. Linear regression was applied to test for an effect of moisture on the magnitude of  $N_2$  fixation in field measurements and also to examine the dependence of nitrogenase activity on C availability under laboratory conditions.

## RESULTS

### Moss Species and Abundance of Cyanobacteria

No cyanobacteria were detected on the mostly bare peat soil of PC as well as on the vegetated surface of mineral UT (Table 1). In contrast, cyanobacteria were found at PP surface. Heterocystous cyanobacteria of genus *Nostoc* or *Anabaena* were observed in all samples collected in July while they were absent in samples from August. Also, representatives of nonheterocystous genera (*Chroococcus*, *Gleotheca*, and *Microcystis*) were found in PP, with more frequent occurrence in July than in August. The surface of PP was dominated by *Sphagnum* sp. with a low occurrence of other mosses and different field replicates had highly variable composition of species. Various bryophyte species, namely liverwort *Gymnocola inflata* and moss *Dicranella cerviculata*, were found on PC in the early succession stage (Table 1).

### Nitrogen Fixation—Field Measurements

No nitrogenase activity was found in the PC and UT soils lacking cyanobacteria, either in natural light conditions or after C addition in the dark (Fig. 1). Both soils displayed higher gross and net N mineralization rates than PP (Table 2). PC soil was rich in mineral N and poor in P while UT soil was poor in N and P (Table 3). In contrast, nitrogenase activity was detected in PP that exhibited low N mineralization rates and was poor in mineral N and rich in P (Tables 2 and 3). Nitrogenase activity significantly diverged between the two sampling campaigns ( $F = 19.73$ ,  $df = 1$ ,  $P < 0.001$ ). The highest rates were detected on 20 July (124 and 44 ng N  $gdw^{-1} day^{-1}$  in light and dark treatment, respectively; Fig. 1), while much lower temperatures on 18 August were associated with lower  $N_2$  fixation rates (5.0 and 2.0 ng N  $gdw^{-1} day^{-1}$  in light and dark, respectively; Fig. 1). The large spatial

heterogeneity of PP vegetation cover represented by moss species composition and associated cyanobacterial assemblage (Table 1) was mirrored by the high variability of nitrogenase activity. No statistical difference in nitrogenase activity between the light and dark treatment was found either in July or in August ( $F = 0.27$ ,  $df = 1$ ,  $P = 0.61$ ) because each replicate exhibited a different response to the light/dark treatment. The water content of the PP sampling subsites ranged from 0.56 to 0.98 g  $H_2O$   $gdw^{-1}$  and had no effect on nitrogenase activity ( $F = 0.53$ ,  $df = 1$ ,  $P = 0.48$ ).

### Nitrogen Fixation—Laboratory Experiments

Nitrogen fixation in laboratory conditions was detected exclusively in the PP soil and only these data are referred to in this section.

- I. *Kinetic of nitrogenase activity (C substrate addition)*. Addition of C substrate positively affected diazotrophic activity in PP soil (Fig. 2). Nitrogenase activity increased linearly and doubled following C addition of 30 mg C  $gdw^{-1}$ . This addition was used in further experiments.
- II. *Carbon and nutrient manipulation*.  $N_2$  fixation in PP soil was significantly affected by nutrient manipulations ( $F = 13.24$ ,  $df = 3$ ,  $P < 0.01$ ), which changed C:nutrient stoichiometry in the soil. Ratios of C:N and C:P substantially increased in all treatments (Table 4). Samples amended with C only (C-treatment) showed increased nitrogenase activity by 111% compared to control samples without C and nutrient addition. Nitrogen amendment as ammonium nitrate suppressed nitrogenase activity by 26% on average compared to the C-treatment, while P addition in form of monopotassium phosphate increased nitrogenase activity by 26% compared to the C-treatment.
- III. *Temperature dependence*. Nitrogenase activity in laboratory conditions increased exponentially with increasing temperature and fit the Arrhenius model well ( $R^2 = 0.904$ ,  $df = 10$ ). Temperature dependence derived from the laboratory experiments was within the range of field data (Fig. 3). Therefore, the equation was used to estimate cumulative  $N_2$  fixation in the field.



TABLE 1

Occurrence of mosses and cyanobacteria species in the uppermost layer of soil samples taken from subarctic tundra.

Sampling site	Sampling campaign	Replicate	Moss species	Cyanobacteria species	
PP	20 July	1	<i>Sphagnum russowii</i>	<i>Nostoc</i> cf. <i>paludosum</i> * <i>Chroococcus</i> sp. <i>Microcystis</i> sp.	
			<i>Mylia anomala</i>		
		2	<i>Sphagnum fuscum</i>	<i>Nostoc paludosum</i> * <i>Anabaena oscillarioides</i> * <i>Chroococcus minimus</i> <i>Gloeothece</i> sp. <i>Microcystis</i> sp.	
			<i>Mylia anomala</i>		
		3	<i>Dicranum leioneuron</i>	<i>Nostoc</i> sp.*	
			<i>Sphagnum</i> sp. (Sect. <i>Cuspidata</i> )		
			<i>Barbilophozia binsteadii</i> <i>Cephalozia</i> cf. <i>pleniceps</i>		
	18 August	1	<i>Sphagnum russowii</i>	—	
			<i>Mylia anomala</i>		
		2	<i>Sphagnum fuscum</i>	—	
			<i>Mylia anomala</i>		
3	<i>Warnstorfia fluitans</i>	—			
	<i>Pleurozium schreberi</i>	—			
5	<i>Dicranum leioneuron</i>	n.d.			
	<i>Barbilophozia binsteadii</i> <i>Polytrichum strictum</i> <i>Mylia anomala</i>				
6	<i>Cladonia</i> sp.	<i>Chroococcus minutus</i>			
	<i>Lophozia ventricosa</i> s.l.				
PC	20 July	1	—	n.d.	
		2	—	—	
		3	—	—	
	18 August	1	cf. <i>Dicranella cerviculata</i> (juv.)	—	
		2	<i>Gymnocolea inflata</i>	—	
		3	<i>Dicranella cerviculata</i>	—	
	UT	20 July	1	n.d.	—
			2	n.d.	—
			3	n.d.	—
18 August			n.d.	n.d.	

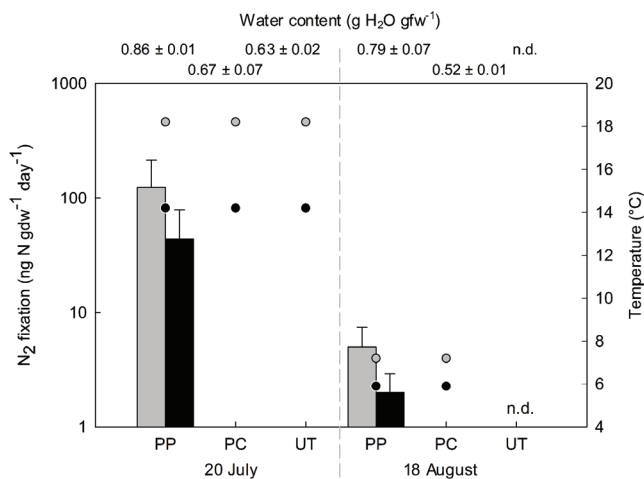
\*Cyanobacterium species with heterocysts.

Abbreviations: PP—vegetated peat plateau; PC—peat circles, i.e. circular cryogenic formations of bare peat soil; UT—upland tundra; n.d.—not determined.

## N Transformations and Estimated N Input

The input of N via heterotrophic N<sub>2</sub> fixation was estimated using the soil temperature records

of the subsites and the temperature functions established during the laboratory experiments (Fig. 3). Because the N<sub>2</sub> fixation rates during laboratory measurements were lower than the field rates,



**FIGURE 1.** Nitrogen fixation rates of three main tundra habitats assayed in field measurements (20 July and 18 August 2011); mean incubation temperatures and water content of soil cores: N<sub>2</sub> fixation rates plotted as columns, temperature as points; gray—natural light conditions, black—dark treatment. N<sub>2</sub> fixation rates are means with standard errors ( $n = 3$ , except of PP-Aug where  $n = 6$ ); water content given in numbers above the graph. For abbreviations, see legend of Table 1

our estimates are considered conservative. Estimated N input to PP via N<sub>2</sub> fixation over a 60-day growing season (July and August 2011) was 0.54  $\mu\text{g N gdw}^{-1}$  (equivalent to 1.36  $\text{mg N m}^{-2}$ ). More N<sub>2</sub> was fixed during the warmer month of July (0.37  $\mu\text{g N gdw}^{-1}$ ) compared to the colder August (0.24  $\mu\text{g N gdw}^{-1}$ ).

Net N mineralization rates were generally the lowest in PP, intermediate in UT, and the highest in PC (Table 2). When daily rates were calculated for July, net N mineralization in PP was somewhat lower than N<sub>2</sub> fixation. Gross N mineralization was three orders of magnitude higher than net N mineralization in PP, while this difference was two orders of magnitude in UT and one order of magnitude in PC. Net N mineralization rates were roughly equivalent to the N in N<sub>2</sub>O emissions in PC. Because the polyethylene bags are permeable for gases, denitrification should have occurred in the net mineralization experiment; thus, actual net mineralization rates should be at least two times larger in PC. The region received on average 0.69  $\text{mg N m}^{-2} \text{day}^{-1}$  from atmospheric deposition (Walker, 2003), which was the only external input of N for UT and PC soils. The N<sub>2</sub> fixation rate in PP soil was in the same order of magnitude as N deposition (Table 2).

Mean plant N demand in UT (N in NPP, Table 2) was about three times lower than the mineral N delivered through gross N mineralization but much higher than net N mineralization. The N requirement for NPP in PP matched gross N mineralization and greatly exceeded net N mineralization. Similar as for UT, negligible N losses via N<sub>2</sub>O emissions were observed in PP. PC soil with no vegetation cover exhibited high gross and net N mineralization, which occurred concurrently with high N<sub>2</sub>O emissions (Table 2).

**TABLE 2**

**A comparison of various N transformation processes in the peak season of a subarctic tundra landscape; in situ detected total N<sub>2</sub> fixation, an estimate of atmospheric N deposition, net and gross N mineralization in the uppermost 5 cm layer of soil, an estimate of N bound in NPP and N<sub>2</sub>O emissions from surface.**

Sampling site	N transformation processes					
	mg N m <sup>-2</sup> d <sup>-1</sup>					
	total N <sub>2</sub> fixation <sup>a</sup>	atmospheric N deposition <sup>b</sup>	net N mineralization <sup>a</sup>	gross N mineralization <sup>c</sup>	N in NPP <sup>d</sup>	N <sub>2</sub> O emissions <sup>c</sup>
PP	0.30 ± 0.23	0.43–0.94	0.05 ± 0.07	50 ± 6	51	0.04 ± 0.03
PC	0.00 ± 0.00	0.43–0.94	9.25 ± 2.59	295 ± 73	0	5.16 ± 1.37
UT	0.00 ± 0.00	0.43–0.94	0.63 ± 0.33	160 ± 84	58	-0.01 ± 0.00

<sup>a</sup>Rates determined under natural field conditions in July 2011.

<sup>b</sup>Calculated from Walker (2003).

<sup>c</sup>Data from season 2008 published by Marushchak et al. (2011).

<sup>d</sup>Estimate based on biomass data from the study site provided by Hugelius et al. (2011).

Actual measured data (<sup>a</sup> and <sup>c</sup>) are given as means with standard errors ( $n = 3$ ). For abbreviations, refer to the legend of Table 1.

TABLE 3

The concentration of nitrogen inorganic forms (DIN), phosphates, dissolved organic matter (DOC), and DOC:DIN ratio in tundra soils in July 2011 (concentration values are means with standard errors,  $n = 3$ ). For abbreviations, see the legend of Table 1.

Sampling site	N-NO <sub>3</sub> <sup>-</sup> (μg gdw <sup>-1</sup> )	N-NH <sub>4</sub> <sup>+</sup> (μg gdw <sup>-1</sup> )	P-PO <sub>4</sub> <sup>3-</sup> (μg gdw <sup>-1</sup> )	DOC (μg gdw <sup>-1</sup> )	DOC : DIN (mol mol <sup>-1</sup> )
PP	0.08 ± 0.13	0.88 ± 1.53	25.1 ± 22.0	613 ± 152	636
PC	15.9 ± 11.9	4.55 ± 3.29	0.33 ± 0.57	849 ± 234	42
UT	0.00 ± 0.00	1.38 ± 2.39	5.36 ± 1.30	630 ± 357	457

## DISCUSSION

We detected N<sub>2</sub> fixation only in the vegetated peat soil of PP. Corresponding with that finding, cyanobacteria were found solely in PP soil. Contrary to our hypothesis, the bare peat soil of PC did not show any detectable N<sub>2</sub> fixing activity. Nitrogen fixation by pioneer autotrophic organisms facilitates the early succession stage when plants are absent and nutrients are lacking (Hodkinson et al., 2003; Nemerugut et al., 2007). Nevertheless, the bare soil of PC had higher mineral N concentration and much lower DOC:DIN ratio compared to the adjacent PP (Table 3). Therefore, N<sub>2</sub> fixation did not provide any advantage for colonizing species in PC. In addition to autotrophic N<sub>2</sub> fixation, heterotrophic diazotrophs

can play an important role. However, nitrogenase activity in the PC soil was triggered neither by C addition under field conditions nor by C and P addition in laboratory experiments (results not shown), which indicates a lack of heterotrophic diazotrophs. The development of diazotrophs in the bare PC soil may have been restricted by combined effect of high mineral N and low P availability. According to our data, the traditional view that N<sub>2</sub> fixation is limited by high N availability holds true also for Arctic soils exhibiting high N<sub>2</sub>O emissions. Therefore, the large N<sub>2</sub>O emissions and high N availability in PC are not linked to high inputs of N via biological N<sub>2</sub> fixation.

Nitrogen fixation was also lacking in vegetated mineral soil of UT where no cyanobacteria were found. An active N<sub>2</sub>-fixing moss-associated community has been reported from similar habitats of subarctic heath (Sorensen and Michelsen, 2011). It is possible that climatic conditions at our site were more favorable for soil microbial activity, resulting in a faster internal cycling of nutrients

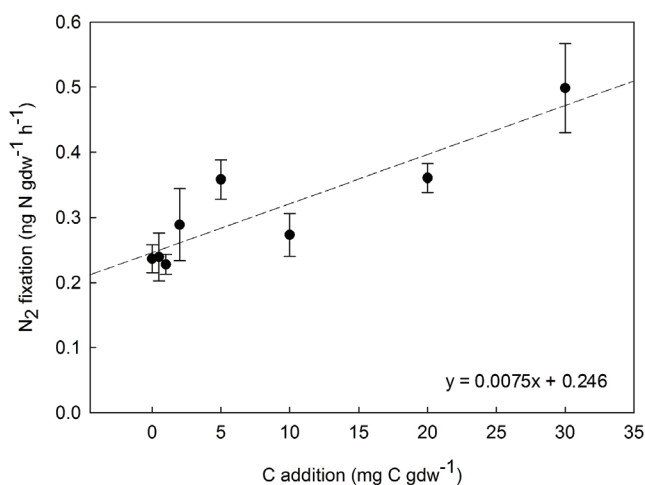
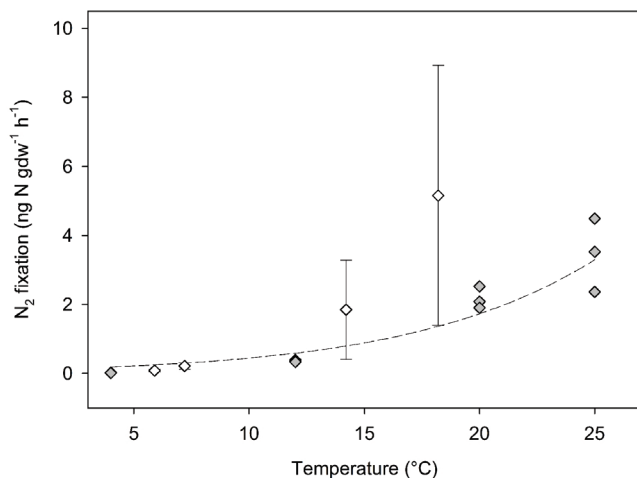


FIGURE 2. The response of heterotrophic nitrogenase activity in PP soil to C source addition (means with standard errors,  $n = 3$ ). The correlation between N<sub>2</sub> fixation and C addition was significant ( $R^2 = 0.774$ ,  $P = 0.002$ ). The linear regression equation (dashed line) is shown in lower right-hand corner

TABLE 4

Nitrogen fixation rates in PP after different nutrient additions and the final C:N:P ratio expressed as DOC:DN:P<sub>ox</sub>; N<sub>2</sub> fixation rates as means with standard errors ( $n = 3$ ). Different letters denote statistically significant differences in nitrogenase activity among treatments ( $P < 0.05$ ).

Nutrient treatment	DOC : DN : P <sub>ox</sub>	N <sub>2</sub> fixation (ng N gdw <sup>-1</sup> h <sup>-1</sup> )	
Control	13 : 0.2 : 1	1.03 ± 0.11	a
C	168 : 0.2 : 1	2.17 ± 0.19	cb
C+P	138 : 0.1 : 1	2.74 ± 0.11	b
C+N	168 : 0.5 : 1	1.60 ± 0.32	ca



**FIGURE 3.** The temperature dependence of nitrogenase activity in PP soil acquired from data measured under laboratory conditions.  $N_2$  fixation rates measured under field conditions are also depicted: gray symbols—individual laboratory measurements, solid line—Arrhenius T-dependence fit through laboratory data [ $k = 9.42 \cdot 10^{16} e^{-9.37 \cdot 10^4 / R \cdot (T + 273.15)}$ ,  $R^2 = 0.880$ ,  $df = 10$ ], white symbols—field data as mean for each incubation temperature with standard errors ( $n = 3$  for July measurements at 14.2 and 18.2 °C, and  $n = 6$  for August measurements at 5.9 and 7.2 °C).

and thus sufficient delivery of N to plants and microbes. Indeed, relatively high gross N mineralization rates suggested fast N turnover, and significant net N mineralization rates implied that microbial community had a surplus of available N (Table 2).

Only vegetated PP showed  $N_2$  fixation among the studied habitats. There were favorable conditions for diazotrophic microorganisms due to low available N in soil and the highest DOC:DIN ratio and P availability among other habitats (Table 3). The regulating role of nutrients was confirmed in laboratory experiments (Table 4) and was significant within a short time period. These trends were consistent with other studies evaluating long-term fertilization experiments (Liengen, 1999b; Weiss et al., 2005; DeLuca et al., 2007; Šantrůčková et al., 2010). High soil concentration of ammonium as an end product of the enzymatic reaction process suppresses nitrogenase activity (Paul and Clark, 1996). Phosphorus is an essential element in ATP production for energy-demanding metabolism of diazotrophs, and P deficiency within soil can limit their activity (Chapin et al., 2002). Nitrogen fixation is

related not only to the total amounts of individual nutrients, but mostly the ratios of nutrients regulate the process (Reed et al., 2007). High C:N and low N:P favored nitrogenase activity in PP soil in both field and laboratory experiments. With respect to the very low N:P ratio during the laboratory experiment (Table 4), it was remarkable that further addition of P still had positive effect.

Heterotrophic  $N_2$ -fixing bacteria require, besides available P, a labile source of C as a substrate for efficient respiratory metabolism—that is, simple sugars or organic acids (Burgmann et al., 2005). Heterotrophic  $N_2$  fixation linearly increased with added C up to the highest addition rate of 30 mg C gdw<sup>-1</sup> (Fig. 2). Neither substrate saturation nor inhibition was reached in the investigated range of C addition, unlike results reported by several other studies (Černá et al., 2009; Hara et al., 2010). Our results suggest that heterotrophic  $N_2$  fixation in PP was at least partly limited by the source of available C.

We found that both autotrophic and heterotrophic diazotrophs were contributing to  $N_2$  fixation in PP. The light/dark treatment indicated neither of the two groups dominated the  $N_2$  fixation in the field sites (Fig. 1). It is presumed that cyanobacteria with heterocysts, the main autotrophic diazotrophs, fix  $N_2$  during the day because of spatial separation of oxygen-sensitive nitrogenase and oxygenic photosynthesis (Fay, 1992; Liengen, 1999b), while nitrogenase activity at night is assumed in the case of nonheterocystous species (Fay, 1992; Bergman et al., 1997; Misra, 1999; Compaore and Stal, 2010). However, there is evidence that heterocystous cyanobacteria can also fix  $N_2$  at night (Schell and Alexander, 1973; Liengen, 1999b). The heterocyst of the cyanobacteria has an incomplete photosynthetic pathway, so its respiratory metabolism is dependent on organic assimilates transported from neighboring green cells. Thus, heterocystous cyanobacteria can fix  $N_2$  as long as available assimilated C is transported into the heterocysts (Fay, 1992; Bothe et al., 2010). Therefore, it is possible that they contributed to nitrogenase activity in both light and dark treatments during our 48-hour incubation under field conditions. This implies that  $N_2$  fixation by autotrophs can take place not only during daytime but also during short nights in Arctic summers. However, the results do not exclude

participation of heterotrophs in this process. In fact, when we compared total  $N_2$  fixation values measured under field conditions and values of potential heterotrophic  $N_2$  fixation from laboratory experiments (Fig. 3), it was evident that heterotrophic diazotrophs could account for a considerable part of total  $N_2$  fixation in PP.

In our study, viable heterocystous cyanobacteria were detected on the PP surface in July, when generally higher nitrogenase activity was evident. However, they were completely absent in samples collected in August, which could have been a response to low temperatures and a shorter day period in the diurnal cycle (Fig. 1). No macroscopic cyanobacterial colonies were observed in PP, but cyanobacteria occurred in association with mosses. All soil samples where cyanobacteria were found were dominated by *Sphagnum* mosses, similar to a trend observed in a comparable habitat by Sorensen and Michelsen (2011). Many studies also documented an association of *Nostoc* sp. with *Pleurozium schreberi* (DeLuca et al., 2002; Houle et al., 2006; Gundale et al., 2012). However, soil cores covered with this moss species were the only ones from vegetated PP that did not exhibit any nitrogenase activity in our study.

Low pH and moisture can limit nitrogenase activity (Chapin and Bledsoe, 1992). Nevertheless,  $N_2$  fixation was operating here even at a pH as low as 3.2–3.4. Furthermore, we do not assume moisture controls  $N_2$  fixation in PP given that a sufficient amount of water was available in this tundra area over the whole vegetation season. Nitrogen fixation differed significantly between July and August as the latter month was colder by 8–11 °C (Fig. 1) and the day length shorter by 4 h 20 min. The air temperature in the summer months in this region frequently ranges from 5 to 25 °C. Heterotrophic nitrogenase activity determined within this temperature range in the laboratory showed an exponential increase with rising temperature and  $N_2$  fixation rates were negligible at close-to-zero temperatures (Fig. 3). Temperature dependence measured under field conditions followed the same pattern, which suggested that the summer season remained the only period when there was a significant contribution of diazotrophs to the local N budget.

The  $N_2$  fixation detected in PP (0.01–0.76 mg  $N\ m^{-2}\ day^{-1}$ ) was lower than rates reported from

subarctic and Arctic tundra (Chapin and Bledsoe, 1992; Hobara et al., 2006; Sorensen and Michelsen, 2011; Stewart et al., 2011a; Stewart et al., 2011b). Zielke et al. (2002) reported  $N_2$  fixation rates by moss-associated cyanobacteria in tundra mires that were similar to those reported in this study. Net and gross N mineralization determined at our Seida study site were in good agreement with other data from the scientific literature on N mineralization rate (Schmidt et al., 2002; Schimel et al., 2004; Kaiser et al., 2005; Pare and Bedard-Haughn, 2012; Wild et al., 2013).

In order to relate  $N_2$  fixation rates to other N transformation processes in this ecosystem, we used measurements of gross N mineralization already conducted at this study site, estimates of N deposition, and estimates of N uptake in plant biomass. Because these data were collected at different times and estimated using laboratory measurements, the estimated N budget should be considered with caution. Nitrogen fixation rates measured in PP were in the same order of magnitude as N deposition and higher than net N mineralization rates. The low net mineralization rates were probably associated with high microbial immobilization rates (Schimel and Bennett, 2004). The estimated N demand by plants in PP was about two orders of magnitude higher than  $N_2$  fixation rates. Nitrogen fixation, N deposition, and net N mineralization together (about 2% of N in NPP, Table 2) could not meet the annual plant N demand. This result agrees with previously published data from the Arctic (Atkin, 1996; Stieglitz et al., 2000). However, ARA method cannot quantify  $N_2$  fixation by methanotrophs (Flett et al., 1975), which was recently shown to be of great importance in N budget of boreal peatlands (Larmola et al., 2014; Vile et al., 2014). This so-far omitted process would need further attention also in subarctic mires.

Relatively high net N mineralization rates in PC together with other favorable environmental conditions (high water content, higher temperature, low DOC:DIN) resulted most likely in high  $N_2O$  emissions despite low pH (Palmer et al., 2011). According to our approximation, a large portion of mineralized N in excess (net N mineralization) could be denitrified as  $N_2O$  in PC (Table 2). Thus, internal N cycling can fully fuel the large emissions of this strong greenhouse gas.

To sum up, N<sub>2</sub> fixation was a significant part of the N cycle solely in N-limited vegetated PP, while no nitrogenase activity was detected in bare PC and vegetated UT. We conclude that the N abundance in PC soil originated from internal N cycling (N mineralization) and currently external N inputs such as biological N<sub>2</sub> fixation do not play a significant role. Also, N deposition could not account for N emitted as N<sub>2</sub>O. Thus, no relation between N<sub>2</sub>O emissions and N inputs into soil was found, while the absence of plants and the low C:N ratio of the soil appeared to be the main reason for high N availability and supply in PC. The concentration and proportion of nutrients in soil as well as occurrence of diazotrophs explained, at least partly, the differences in nitrogenase activity in three habitats of subarctic tundra. Both autotrophic and heterotrophic N<sub>2</sub> fixation was proven present in PP. The diazotrophic community fixed N<sub>2</sub> under light as well as dark conditions, and activity varied with changing temperature during the season. Nitrogen fixation represented only a small portion of N built into NPP in PP, but was comparable to other inputs into the soil N pool such as N deposition and net N mineralization.

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