Biogeochemistry (2014) 121:317–328
DOI 10.1007/s10533-014-0019-6

BIOGEOCHEMISTRY LETTERS

N_2-fixation by methanotrophs sustains carbon and nitrogen accumulation in pristine peatlands

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Received: 11 July 2014 / Accepted: 28 July 2014 / Published online: 14 August 2014

Abstract Symbiotic relationships between N_2-fixing prokaryotes and their autotrophic hosts are essential in nitrogen (N)-limited ecosystems, yet the importance of this association in pristine boreal peatlands, which store 25 % of the world's soil (C), has been overlooked. External inputs of N to bogs are predominantly atmospheric, and given that regions of boreal Canada anchor some of the lowest rates found globally ($\sim$1 kg N ha^{-1} $year^{-1}$), biomass production is thought to be limited primarily by N. Despite historically low N deposition, we show that boreal bogs have accumulated approximately 12–25 times more N than can be explained by atmospheric inputs. Here we demonstrate high rates of biological N_2-fixation in prokaryotes associated with *Sphagnum* mosses that can fully account for the missing input of N needed to sustain high rates of C sequestration. Additionally, N amendment experiments in the field did not increase *Sphagnum* production, indicating that mosses are not limited by N. Lastly, by examining the composition and abundance of N_2-fixing prokaryotes by quantifying gene expression of 16S rRNA and nitrogenase-encoding *nifH*, we show that rates of N_2-fixation are driven by the substantial contribution from methanotrophs, and not from cyanobacteria. We conclude biological N_2-fixation drives high sequestration of C in pristine peatlands, and may play an important role in moderating fluxes of methane, one of the most important greenhouse gases produced in peatlands. Understanding the mechanistic controls on biological N_2-fixation is crucial for assessing the fate

Responsible Editor: Matthew Wallenstein.

Electronic supplementary material The online version of this article (doi:10.1007/s10533-014-0019-6) contains supplementary material, which is available to authorized users.

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of peatland carbon stocks under scenarios of climate change and enhanced anthropogenic N deposition.

Keywords Biological N_2-fixation · Boreal peatlands · Carbon · Nitrogen · *Sphagnum* · Methanotrophs

Introduction

Globally, peatlands cover less than 3 % of the Earth's land surface, yet store an estimated 25 % of the world's soil carbon (C) (Yu 2012). More than 85 % of the global peatland area can be found in boreal regions of the northern hemisphere, with roughly 10 % of that total located in the boreal plain of western Canada (Vitt 2006). The substantial accumulation of organic C as peat results from a long-term excess of net primary production (NPP) of *Sphagnum* mosses over peat decomposition. In the northern hemisphere, boreal peatlands have accumulated more soil C during the Holocene than any other terrestrial ecosystem (Yu 2011). Peatlands also contain 9–16 % of the world's soil nitrogen (N) (Limpens et al. 2006). Accumulation of N in peat is a consequence of several factors, including low rates of N mineralization, exceptionally low concentrations of dissolved inorganic N in pore waters, negligible gaseous N fluxes, and low N losses by hydrologic export (Limpens et al. 2006; Bobbink et al. 2010).

Nitrogen availability controls many aspects of ecosystem productivity and therefore, the cycling of C and N is intrinsically linked (Vitousek et al. 2002; Houlton et al. 2008; Berg et al. 2013; Lindo et al. 2013). This linkage is especially true for ombrotrophic bogs—peatlands isolated from groundwater and surface water inputs, such that external supply of new nutrients is derived solely from the atmosphere (Damman 1988; Vitt 2006; Limpens et al. 2006; Wu and Blodau 2013). Bogs of boreal western Canada persist under some of the most extreme conditions known to peatlands, where mean annual temperature averages <2 °C, annual precipitation averages < 500 mm (Vile et al. 2011), which is nearly balanced by potential evapotranspiration (Wieder et al. 2009), and N inputs from atmospheric deposition are among the lowest found globally, typically <1 kg N ha^{-1} $year^{-1}$ (Vitt et al. 2003; Wieder et al. 2010). Despite these conditions, *Sphagnum* mosses are surprisingly productive with rates of NPP ~2500 kg ha^{-1} $year^{-1}$ (Vitt et al. 2003; Wieder et al. 2010), but can be much higher in considerably wet years (cf. Fig. 3). Here, we question how these purportedly nutrient-poor bog ecosystems are able to maintain such levels of NPP leading to substantial sequestration of both C and N in the face of exceptionally low atmospheric N deposition, which is the external input that has long been regarded as the primary source of new N to bogs.

Biogeochemical theory predicts that biological N_2-fixation, the conversion of atmospheric nitrogen (N_2) by diazotrophs (N_2-fixing prokaryotes) to ammonia, often represents the dominant input of new N to pristine ecosystems (Vitousek et al. 2002; Lindo et al. 2013), and ombrotrophic bogs of boreal Alberta, Canada should be no exception. Yet, both the acidity of bog porewaters (pH < 3.8), and low MAT have been thought to preclude high rates of N_2-fixation (Limpens et al. 2006; Houlton et al. 2008). Indeed, all earlier studies have indicated inconsequential contributions of N_2-fixation to the peatland N budget (cf. Limpens et al. 2006; Markham 2009).

A simple mass balance exercise revealed that rates of N accumulation in bogs of continental western Canada are in disequilibrium with inputs of N from atmospheric deposition. Using a C:N ratio for moss tissue of 46 ± 1 (Wieder et al. 2010), the amount of N required to support annual bog *S. fuscum* NPP can approach 40–50 kg ha^{-1} $year^{-1}$, an input far in excess of annual N deposition (even if wet deposition is doubled to roughly approximate and include dry deposition). Indeed, over a single growing season at four bogs in Alberta, the amount of accumulated N in *S. fuscum* NPP exceeded inputs from atmospheric N-deposition by 12–25-fold (Fig. 1). This disequilibrium between N input via deposition and N accumulation also exists when looking over longer time periods. Based on ^{210}Pb-dating of 15 bogs where N deposition ranges from 0.8 to 2.0 kg ha^{-1} $year^{-1}$, mean net N accumulation over the most recent 0–25 years averaged 19.7 kg ha^{-1} $year^{-1}$ (Online Resource 1). These high rates of N-accumulation and large moss N-demand can be explained neither by atmospheric deposition, nor by mechanisms of internal recycling. A ^{15}N tracer study showed that only 1.3–11.4 % of the annual N demand of growing *Sphagnum* is provided by upward retranslocation

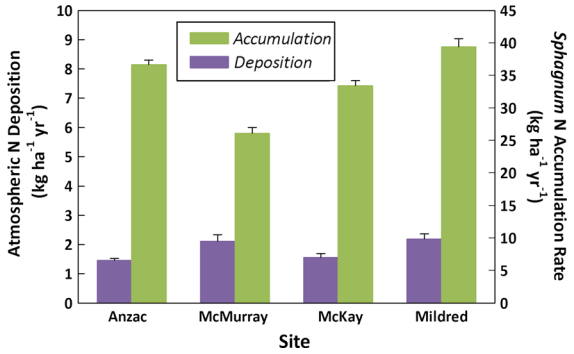

Fig. 1 N-deposition and accumulation rates. Atmospheric N-deposition (*purple*) and N-accumulation rates (*green*) in *S. fuscum* (kg ha^{-1} year^{-1}, mean ± one s.e.m.; $n = 5$) for the 2012 growing season as a function of 4 bog sites across northern Alberta (Anzac Bog, McMurray Bog, McKay Bog, and Utikuma Bog)

(Aldous 2002), which in Alberta bogs, would be equivalent to a maximum of 4.5 kg ha^{-1} year^{-1}. Similarly, N mineralization can provide recycled N to support new growth, but our measured rates in Alberta bogs are low, providing only 1.2 kg ha^{-1} year^{-1} (Online Resource 2).

We hypothesized that biological N_2-fixation is important in these bogs and is of sufficient magnitude to resolve the disequilibrium between the amount of N needed to support annual NPP of *Sphagnum* and the longer-term rate of N accumulation in peat. Moreover, we underscore that the reason that the peatland community overlooked the importance of biological N_2-fixation as a new source of N lies in the methodology. N_2-fixation routinely is measured using the acetylene reduction assay (ARA), with application of a conversion factor (CF) that was initially established for cyanobacteria by Hardy et al. (1968), of 3 moles of ethylene produced for every mole of N_2-fixed. Use of ARA is a low-cost alternative to direct measurement with $^{15}N_2$-incubations, and allows for greater spatial and temporal replication in large ecosystem-scale studies. Acetylene, however, can inhibit some diazotrophs (De Bont and Mulder 1976). If N_2-fixation is largely the result of cyanobacteria associations with moss, as has been found for feather mosses in upland boreal forests, the CF should be close to 3:1 (DeLuca et al. 2002; Leppänen et al. 2013). If, however, cyanobacteria do not dominate N_2-fixation, rates could be underestimated (e.g., Großkopf et al. 2012). Further, we hypothesize that if biological N_2-fixation represents an important input of N to these bogs, *Sphagnum* may not be as N-limited as previously thought, and will not exhibit a growth response to experimentally applied inorganic N additions. Lastly, we hypothesize that the diazotrophic community is not dominated by autotrophic cyanobacteria in these ombrotrophic bog ecosystems.

Materials and methods

N_2-fixation rates measured using the acetylene reduction assay (ARA)

We measured rates of N_2-fixation at 4 bogs, each receiving atmospheric N inputs of less than 2 kg ha^{-1} year^{-1}: McKay (57°13′N, 111°42′W), McMurray (56°37′N, 111°11′W), Anzac (56°28′N, 111°2′W) and Utikuma (55°54′N, 115°02′W) in boreal Alberta, Canada. Mean annual temperature is <2.0 °C, and mean annual precipitation is 465 mm (Vitt 2006). Cryptogams comprise 80–100 % of the ground cover for all bogs with *Sphagna* representing the largest component (53–84 %). We collected field-moist, intact *Sphagnum* cores (surface area of 32.8 cm^2) including all living moss material to approximately 5 cm below the surface. Cores were placed in 237-mL glass jars outfitted with gas-tight valves; 10 % of the headspace was replaced with acetylene (C_2H_2). Mosses were incubated in situ at ambient temperature (range 7–28 °C) over 24-h periods, capturing both autotrophic and heterotrophic N_2-fixation. Headspace samples were collected at 0 and 24 h [ethylene (C_2H_4) production was linear over a 48 h period], stored in gas-tight syringes, and transported to the University of Alberta's Meanook Biological Research Station. Concentrations of C_2H_4 were measured on a Shimadzu-2014 gas chromatograph equipped with an FID and Haysep® T column. Blank incubations (−moss, +C_2H_2) yielded consistently low C_2H_4 concentrations (<5 ppm) and there was no C_2H_4 production in control incubations (+moss, −C_2H_2). We conducted a total of 254 ARA incubations on 3 *Sphagnum* moss species (*S. fuscum, S. angustifolium, S. capillifolium*) across 4 sites during the 2009 and 2010 growing seasons.

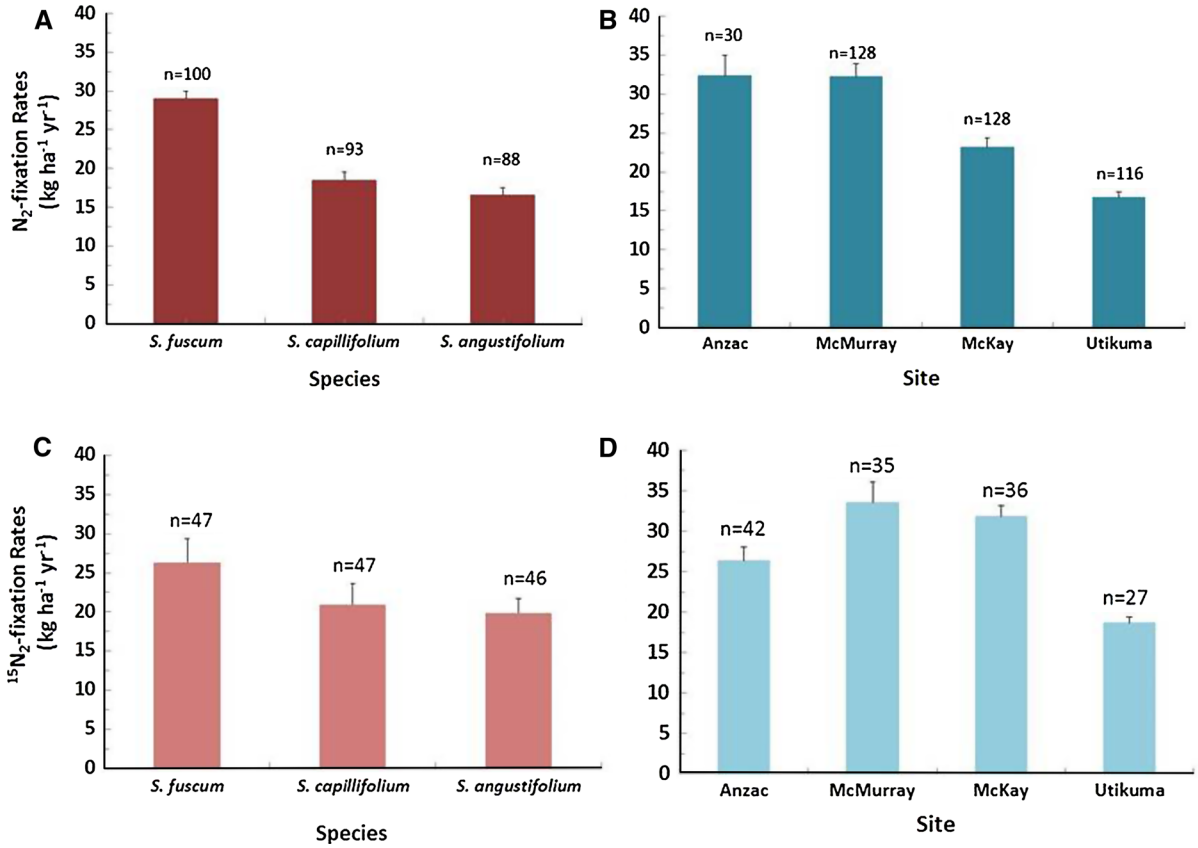

Fig. 2 Rates of N_2-fixation. (**a**) Mean rates (kg ha^{-1} yr^{-1}, ± one s.e.m.) of N_2-fixation in *Sphagnum* mosses (one-way ANOVA; $F_{2,251} = 8.17$, $p = 0.0004$, n = 254 (**b**), and site-scaled rates for 4 bogs in northern Alberta Canada (one-way ANOVA; $F_{3,250} = 6.63$, $p = 0.0003$, $n = 254$). Rates of $^{15}N_2$-fixation in *Sphagnum* mosses, $n = 140$ (**c**), and site-scaled $^{15}N_2$-fixation rates for 4 bogs in northern Alberta, $n = 140$ (**d**)

$^{15}N_2$–based rates of N_2-fixation

Additional paired (2 subsamples collected side-by-side) ARA/$^{15}N_2$ moss incubations were performed to capture the true rate of N_2-fixation in the absence of acetylene (De Bont and Mulder 1976). Moss samples used for calibration using $^{15}N_2$ were incubated as stated above with the exception that 10 % of the headspace was replaced with 99.8 % $^{15}N_2$ and incubated for 24-h in the field. At the end of each $^{15}N_2$-incubation, we removed mosses from their jars and immediately put them into a 65 °C oven to terminate fixation. Moss tissues were dried, homogenized and subsamples were sent to the stable isotope lab at The University of California-Davis, where ^{15}N-enrichment was measured on a PDZ Europa ANCA-GSL elemental-analyzer interfaced to a PDZ-Europa 20–20 isotope-ratio mass-spectrometer (Sercon Ltd., UK).

$^{15}N_2$-fixation calculations and scaling to annual values

Atom percent excess ^{15}N was determined by subtracting natural abundance ^{15}N (atom % $^{15}N_{natabu}$) in *Sphagnum* from the post-incubation enriched atom % $^{15}N_{enrich}$ to yield mg ^{15}N/per 100 mg total N, which we subsequently converted to mg ^{15}N per hour.

$$\frac{mg\ ^{15}N}{100\ mg\ TotN} * \frac{mgTotN}{g\ dry\ peat} * \frac{g\ dry\ peat}{24\ hr} = \frac{mg\ ^{15}N}{hr}$$

The amount of $^{15}N_2$ that was fixed was adjusted for the headspace volume $^{15}N_2$:$^{14}N_2$ratio, to quantify total $^{14}N_2 + {}^{15}N_2$ fixed. This quantity was scaled to units of

kg N ha^{-1} $year^{-1}$ using the surface area of the incubated moss samples (32.8 cm^2) and using 140 days per year as the average length of the growing season for our sites in northern Alberta. By October, rates of N_2-fixation are below detection until May.

We scaled to the site level by determining the percent cover of each moss at each site. We randomly selected 10, 2 × 2-m quadrats at each site which we then divided into smaller 50 × 50-cm quadrats. In total, 160 smaller quadrats per site were used to estimate percent cover of individual moss species. At Anzac, Utikuma, McKay, and McMurray bogs, 50, 70, 80 and 84 %, respectively, of the ground cover was comprised of *Sphagna*.

N fertilization experiment-field

To experimentally examine N limitation, we implemented a large-scale field-manipulation experiment at Mariana Bog (55°53′41″N 112°5′3″W) in 2011. We applied 7 N fertilization treatments ($n = 3$, 7.2-m^2 plots/treatment). Each plot was characterized by a complete cover of *Sphagnum*. Plots were fertilized eight times throughout each of the 2011 and 2012 growing-seasons (Fig. 4 show 2012 data), each N treatment received 205-L of synthetic rainfall; amendments increased annual rainfall by 50 % (684 mm·$year^{-1}$ total). Production in Alberta bogs is likely water-limited; therefore, we included a control (C) treatment (–N, $–H_2O$) and a water-only (0) treatment (–N, $+H_2O$). The seven treatments corresponded to C, 0, 5, 10, 15, 20, 25 kg N ha^{-1} $year^{-1}$, and were surface applied to mimic precipitation.

We determined *Sphagnum* growth responses in the field by measuring linear growth, bulk density, and NPP at our Mariana Bog site. In May 2012, 30-cranked wires were deployed in *S. fuscum* in each plot (Clymo 1970), and re-measured at the end of the growing season in October 2012. We assessed N-treatment responses with one-way ANOVAs for each *Sphagnum* species.

Molecular characterization of N_2-fixing communities

We isolated DNA from ~500 mg of capitula and stem leaves from either *S. angustifolium* or *S. fuscum* that we collected from the same 4 bogs where N_2-fixation was measured, with separate subsamples subjected to both light and dark conditions to ensure that cyanobacteria would not be light-limited. Post-treatment, samples were immediately placed on dry ice and shipped to Villanova University. DNA was isolated using a PowerPlant DNA Isolation kit from MoBio Laboratories (Carlsbad, CA, USA) and a 3 × 3 min treatment with a mini beadbeater-8 (Biospec Bartlesville, OK, USA). RNA was isolated with the PowerPlant RNA Isolation kit with a DNase I treatment and 3 × 3 min treatment with a mini beadbeater-8. We subjected various dilutions of DNA to PCR using a Taq/Pfu polymerase mixture and the following universal primers, which were ordered from Invitrogen:

nifH	5′-TAYGGIAARGGIGGIATYGGIAAR TC (position 25–50 called primer F1) 5′-GCCATCATYTCICCIGA (position 485–468 called primer R6) (Gaby and Buckley 2012; Marusina et al. 2001)
16S rDNA	5′-AGAGTTTGATYMTGGCTCAG (position 8–27 called 8F) 5′-GGTTACCTTGTTACGACTT (position 1510–1492 relative to *E. coli* called 1492R) 7 (Eden et al. 1991; Turner et al. 1999)

After confirmation of appropriate size by agarose gel electrophoresis, these PCR products were ligated into a pGEM-T vector (Promega, WI, USA) and ~48 bacterial clones containing PCR products were sequenced from *S. fuscum* and *S. angustifolium* using a T7 sequencing primer. We present data for *S. fuscum* (Online Resource 3); however, similar sequences were identified in *S. angustifolium*. While our goal was to examine *nifH* genes (Online Resource 5A), we used different primers to target 16S rDNA to estimate what organisms were present (Online Resource 5B, 7). We refer to 16S rDNA as the gene encoding 16S rRNA, and use this to clarify when we are amplifying from DNA vs. reverse transcribed RNA.

Purified RNA was quantified using a NanoDrop 2000, and 200 ng of RNA was reverse transcribed using the RevertAid First Strand cDNA Synthesis Kit using random hexamers from Thermo Scientific. The 20 μL reverse transcription reaction was diluted to a final volume of 80 μL. A mock reverse transcription reaction (water was substituted for reverse transcriptase) was performed with the same amount of RNA to determine the quantity of contaminating DNA. The

contaminating DNA was less than 5 % of the signal relative to the reverse transcribed sample, and in most cases less than 0.1 %, except in the dark treated *S. fuscum* samples. In these samples, there was so little *nifH* transcript that the contaminating DNA comprised up to 36 % of the RNA transcript abundance (most of these samples ranged between 5–20 % contaminating genomic DNA).

We used 3 μL of the reverse transcription reaction (and mock reaction) for each quantitative PCR (qPCR) reaction with 21.5 μL of SsoAdvanced SYBR Green Supermix (Bio-Rad) and 1 μM final concentration of each primer set such that the final volume of each qPCR reaction was 25 μL. Thus, all samples are normalized to the same amount of input RNA because we reverse transcribed equal amounts of RNA. We performed qPCR on a CFX connect real-time system from Bio-Rad (Hercules, CA, USA), with the following program: 95 °C-3 min, 39 (or 27 cycles) of 95 °C-5 s, 57 °C-10 s, 72 °C-30 s, 78 °C-10 s, plate read–followed by a melting curve of 1 °C every 10 s (78–95 °C). The number of cycles was reduced for the detection of 16S rRNA because of the high abundance of transcript. We set a threshold cycle [also called a C(t)] of 30.6 as 1 arbitrary unit of DNA. We designed five primer sets to amplify specific families of *nifH or* 16S rRNA based on alignments of sequences that were available from NCBI or that we sequenced (Online Resource 4, 9, & 11). The melting curves were uniform for the five qPCR primer sets indicating near homogeneity of PCR product formed in the reaction. Additionally, we confirmed that the primer sets were amplified appropriately by examining 4-fold dilutions of a subset of reverse transcribed RNA and confirming linearity of amplification on a log plot. In all cases, the R^2 values exceeded 0.9. Because the kinetics of increasing fluorescence with each primer set was similar, we chose a threshold of 750 relative fluorescence units (RFU) for determination of the C(t). We confirmed that data from replicates were reproducible from day to day and that our cut-off of 750 RFU was appropriate. We confirmed near homogeneity of PCR products by cloning at least 6 individual PCR products from a qPCR reaction with each primer set into pGEM-T vector and sequenced the products (Online Resource 5, 6, 9, 10, & 12). While we present data for the cyanobacterial variant 2 *nifH* gene (Online Resource 4 & 6), in nearly all samples, there was so little RNA transcript expressed that we disregarded it for analysis of cyanobacterial *nifH* transcript abundance.

We confirmed that primers were equally efficient at amplifying products (allowing us to compare different abundances with different primer sets), by purifying plasmid DNA from each of the above 4 major products, quantifying plasmid DNA using a NanoDrop 2000, and mixing them in the following ratios: 1:1:1:1, 1:0:1:1, 1:1:0:1, and 1:1:1:0 (Beijerinckiaceae 16S: Beijerinckiaceae *nifH*: cyanobacterial 16S: cyanobacterial variant 1 *nifH*). We then made 8-fold dilutions of these mixtures and performed qPCR with the four sets of primers. When appropriate, primer sets reached the C(t) within 1.5 cycle of one another in all dilutions sets and did not cross-amplify, indicating that each primer set amplifies with roughly equal efficiency. All primers are reported (Online Resource 13) and all sequences have been deposited at the NCBI (Accession #s: KF377964-KF377976) (Online Resource 14). Data are reported as transcript abundance of *nifH* and 16S rRNA for Nostocaceae and Beijerinckiaceae associated with two *Sphagnum* moss species, *S. fuscum* and *S. angustifolium,* incubated in both light and dark conditions.

Phylogenetic tree

We constructed a phylogenetic tree by aligning *nifH* genes from the Beijerinckiaceae family relative to the two highlighted variants identified in this study (the "peat_*nifH*" is from Online Resource 3 and the variant_*nifH*_qPCR is from our qPCR studies—see above). Sequences in the Beijerinckiaceae family were downloaded from NCBI with the accession number reported on the tree. All sequences were trimmed to ignore primer sequences and aligned in Geneious (version 6.16) using a ClustalW alignment and an IUB cost matrix. The phylogenetic tree was generated in Geneious using PhyML with a GTR substitution model, estimated proportion of invariable sites and gamma distribution, and optimized for topology/length/rate and BEST topology search.

Results

Biological N_2-fixation

To provide a robust estimates of the ARA:$^{15}N_2$ conversion factor (CF), we calibrated ARA rates with 140 paired incubations spanning seven bogs, on 13 sampling

dates between May and September, over three growing seasons (2011, 2012, 2013) to obtain estimates of species-, season-, and site-specific ARA:$^{15}N_2$ CFs. These CFs were then applied to the 254 ARA measurements made in 2009–2010. The mean CF (± s.e.m.) across all sites, species, and dates was 0.32 ± 0.05., ranging from 0.05 to 2.92, $n = 140$. Of the 140 CFs, only 15 measurements had CFs > 1.0, with only 6 of those 15 having CFs > 2; the higher CFs corresponded to early or late season and/or low moisture conditions. Mean conversion factors (±s.e.m.) by species were: 0.29 ± 0.09 *(S. fuscum)*, 0.35 ± 0.12 (*S. capillifolium)*, and 0.46 ± 0.09 (*S. angustifolium)*.

When averaged across sites and sampling dates for both years, we found substantial rates of $^{15}N_2$-calibrated biological N_2-fixation, with a mean rate of 25.8 ± 2.4 kg N ha^{-1} year^{-1} (Fig. 2), ranging from 4.8 to 62.3 kg N ha^{-1} year^{-1} for individual samples. N_2-fixation rates were highest in *S. fuscum*, the dominant peat moss in Alberta bogs (Fig. 2a). There were significant site differences, as well, with the lowest rates found at Utikuma bog and the highest rates at McMurray bog (Fig. 2b). Site differences were largely due to composition of the moss ground-cover, which is related to bog age as defined as time since the most recent fire (cf. Wieder et al. 2009).

To demonstrate the utility of the ARA technique when adequately paired with $^{15}N_2$-incubations, we calculated rates of $^{15}N_2$-fixation based only on the 140 independently determined $^{15}N_2$-incubations for each *Sphagnum* species and for 4 sites (Fig. 2c, d). The $^{15}N_2$-derived fixation rates confirm the converted ARA rates (Fig. 2c, d). As a result, with adequate calibration using $^{15}N_2$, ARA can extend the level of replication at a lower cost than $^{15}N_2$-based measurements.

Effect of N amendments on *Sphagnum* growth

To experimentally examine N-limitation, we performed a large-scale field N-amendment experiment. In the field, N additions (up to 25 kg N ha^{-1} year^{-1}) did not lead to a significant increase in NPP of either *S. angustifolium* or *S. fuscum* (Fig. 3).

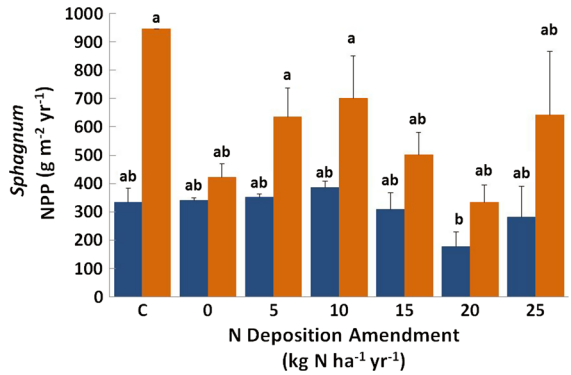

Fig. 3 Rates of *Sphagnum* net primary production (NPP) with N-fertilization in the field. Analyzing *Sphagnum* species separately, mean rates (g m^{-2} year^{-1} ± s.e.m., $n = 3$ for each bar; except for *S. angustifolium* C, 0, 5, and 15 kg ha^{-1} year^{-1} where $n = 2$) of NPP did not differ significantly with N-treatment for either *S. fuscum* (*blue*; one-way ANOVA; $F_{6,20} = 1.508$, $p = 0.246$) or *S. angustifolium* (*orange*; one-way ANOVA; $F_{5,13} = 1.987$, $p = 0.185$) over the 2012 growing season at the Mariana Lakes Peatland, Alberta, Canada

Diazotrophic composition and transcript abundance of *nifH* and 16S rRNA

Transcript abundance of 16S rRNA was significantly greater for cyanobacteria than for Beijerinckiaceae indicating that cyanobacteria were more metabolically active (Fig. 4a). Transcript abundance of *nifH,* however, was significantly greater for Beijerinckiaceae than for cyanobacteria in both light and dark conditions (ANOVA, $p < 0.0001$, Fig. 4b). We used relative units converted from threshold cycle (C(t)) measured at equal fluorescence intensities as an indicator of abundance; for comparison, background contaminating DNA is ~1 relative unit. To verify repeatability of data in Fig. 4, we performed the above DNA and RNA protocols on a second set of *Sphagnum* samples collected in late May/early June of 2013 to bracket the beginning of the growing season (this complements the data in Fig. 4, which represent peak season in July/early August 2012). The early season data show the same result, namely that mean methanotrophic *nifH* expression (119 relative units ± 26, values range from 59 to 264, $n = 15$) is roughly ten times higher than cyanobacteria *nifH* expression (13 relative units ±5, values range from 1 to 43, $n = 15$).

Phylogenetic tree

With such a high level of expression of *nifH* in species that are generally associated with methanotrophs, we generated a phylogenetic tree to determine whether the *nifH* was most closely related to the true methane

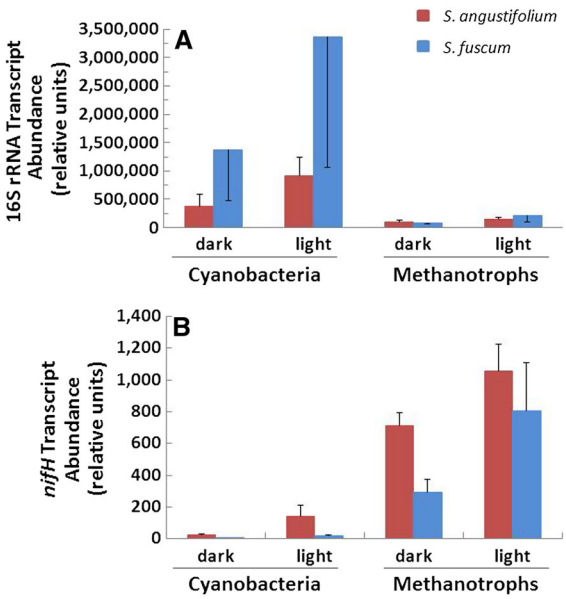

Fig. 4 Transcript abundance of 16S rRNA and *nifH*. Mean (± one s.e.m., $n = 5$) transcript abundance of 16S rRNA (**a**) and *nifH* (**b**) and for Nostocaceae and Beijerinckiaceae associated with two *Sphagnum* moss species, *S. fuscum* and *S. angustifolium,* in both light and dark incubations to ensure that cyanobacteria would not be light-limited, and therefore have adequate photosynthetic capability. *nifH* transcript abundance is significantly greater for Beijerinckiaceae than for cyanobacteria in both light and dark conditions (ANOVA, $p < 0.0001$). Abundance is relative units converted from threshold cycle (C(t)) measured at equal fluorescence intensities; for comparison, background contaminating DNA is ~1 relative unit

oxidizing bacteria, or to the one bacterium in this clade that appears to only utilize methanol (Fig. 5). Species examined were *Methylocapsa acidiphila*, *Methyloferula stellata*, *Methylocella palustris* (or *silvestris*), and the species that are described on the tree. We included the *Beijerinckia* species names as *B. mobilis* and *B. derxii* are known methanotrophs, while *B. indica* appears to be the only species in this bacterial family that is not methanotrophic, and this appears to be a derived trait (Tamas et al. 2014). Our sequences correspond most closely in a number of trees supported by a bootstrap of 78.2 % to the obligate methanotrophs.

Discussion

Recently, cryptogamic covers were shown to play a significant role in the global C and N cycle, producing 7 % of land-plant NPP, and almost half of the biological N_2-fixation in terrestrial ecosystems (Elbert et al. 2012). In our bogs, cryptogamic mosses represent ~90 % of the ground cover, contribute up to 65 % of NEP (Wieder et al. 2009), and host diazotrophs that contribute 85–96 % of the bog N-input from N_2-fixation. These exceptionally high rates of *Sphagnum*-associated N_2-fixation are the first to be reported for boreal bogs. While earlier studies in boreal biomes have indicated inconsequential contributions of N_2-fixation to the peatland N budget (e.g., Basilier 1979; Limpens et al. 2006), more recently, Deluca et al. (2002) demonstrated a modicum of fixed N (1.5–2.0 kg N ha^{-1} $year^{-1}$) in the feather moss, *Pleurozium schreberi*, in boreal upland forests. Lindo et al. (2013) recently published a review paper that encompasses rates of bryophyte-associated N_2-fixation in high latitude terrestrial systems. Of the 35 published studies covered in this review, 15 were conducted in the boreal forest, with only one conducted in a boreal peatland (Markham 2009). Rates of N_2-fixation were found to be low (0.2–1.9 kg N ha^{-1} $year^{-1}$) quite possibly due to use of ARA without a paired $^{15}N_2$-incubation (Markham (2009) assumed a 3:1 conversion factor). Other studies of *Sphagnum*-associated N_2-fixation have taken place in the arctic tundra (Lindo et al. 2013), but it is important to note that both boreal upland forests and arctic tundra are functionally and structurally very different ecosystems than bogs. We are aware of no other published studies on N_2-fixation in boreal bogs. The recent study by Larmola et al. (2014) demonstrated high rates of N_2-fixation (~29 kg N ha^{-1} $year^{-1}$), but in a mesotrophic fen, which is also structurally and functionally distinct from a bog. Further, while Larmola et al. (2014) coupled rates of N_2-fixation to methane oxidation, the study did not examine the diazotrophic community. Our data are the first to demonstrate the dominant role of methanotrophs in providing newly fixed N to pristine bogs.

In determining both the composition and abundance of the diazotroph community by quantifying gene expression of 16S rRNA and *nifH,* a gene encoding a subunit of nitrogenase, the key enzyme involved in N_2-fixation we elucidated microbial controls on biological N_2-fixation. N_2-fixing prokaryotes associated with *Sphagnum* comprise autotrophic cyanobacteria and proteobacterial methanotrophs; the

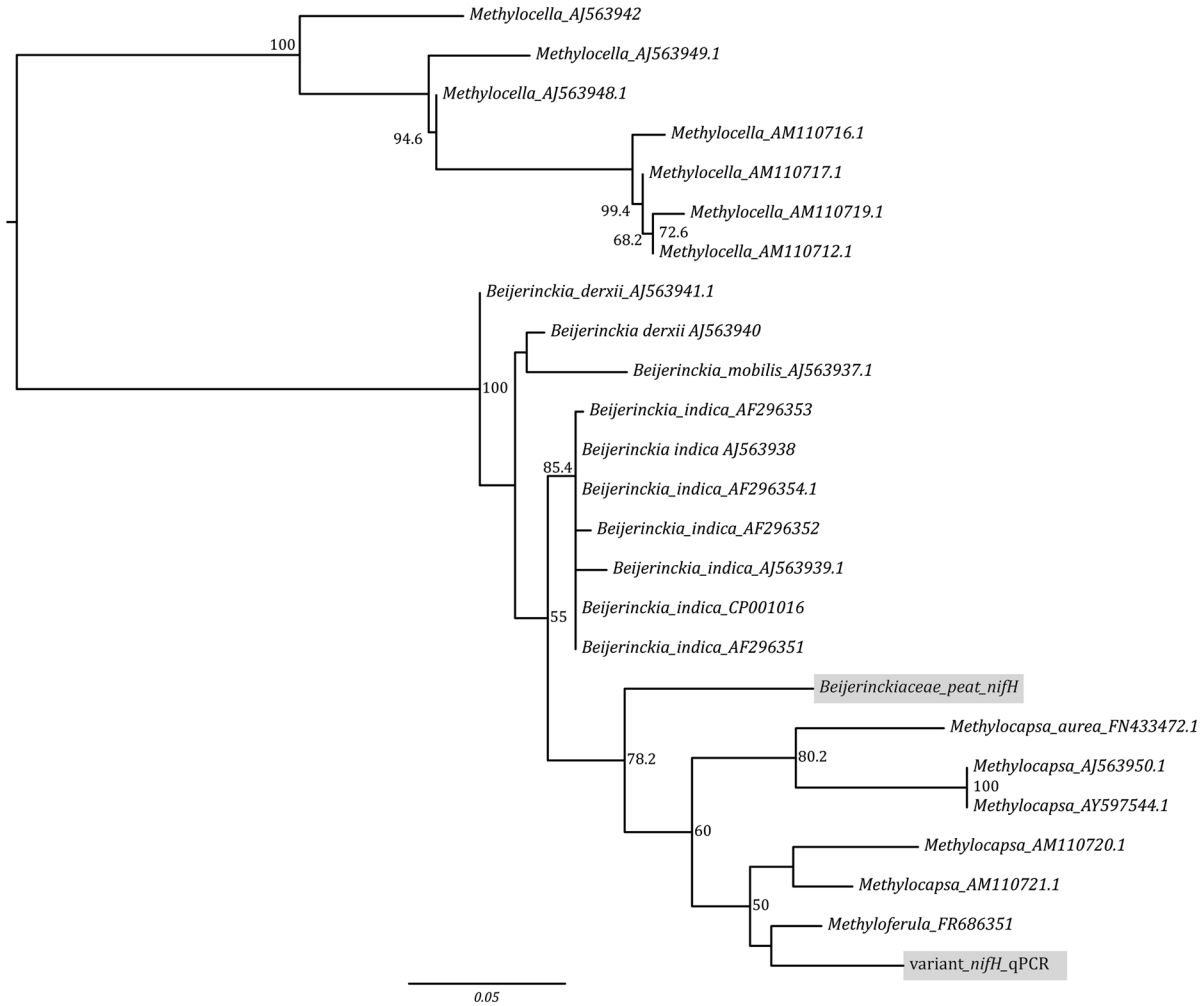

Fig. 5 Alignment of *nifH* genes from the Beijerinckiaceae family relative to the two highlighted variants identified in this study. The "peat_*nifH*" is from Online Resource 3 and the variant_*nifH*_qPCR is from our qPCR studies. The numbers at the node indicate bootstrap support with 500 bootstraps. Where there are no numbers, the bootstrapping was below 50 %. It is worth noting that using neighbor joining, as well as trees generated using amino acid sequences, gave similar results

latter also oxidize methane to carbon dioxide (Khadem et al. 2010). Given that N_2-fixation is energetically costly (16 ATP for every mole of N_2 fixed), autotrophic cyanobacteria should have a competitive advantage in oligotrophic ecosystems (Houlton et al. 2008; Lindo et al. 2013), yet this pattern does not hold for ombrotrophic bogs. We demonstrated that methanotrophs express significantly greater amounts of *nifH* transcript than cyanobacteria in both light and dark conditions, even though cyanobacteria were more metabolically active as evidenced by 16S rRNA expression. Based on these findings, the magnitude of previously underestimated N_2-fixation is in part a consequence of using ARA without paired $^{15}N_2$-incubations when methanotrophs, whose activity is inhibited by acetylene (De Bont and Mulder 1976; Kip et al. 2010; Khadem et al. 2010), dominate the diazotrophic community, as is the case in bogs of Alberta, Canada.

One possible explanation for the prevailing view that biological N_2-fixation is not important in boreal bogs could be that the ARA assay may grossly underestimate true rates of fixation, especially when a CF of 3:1 is assumed. Using a CF of 3:1 rather than our overall CF of 0.32 would underestimate true N_2 fixation by 10-fold. It is likely that a CF of 3:1 is

reasonable in boreal upland forests given the aerobic nature of the soils in these systems, which as a result, tend not to produce methane. Bogs, however, typically have a deep anaerobic layer that can be several meters thick overlain by a much thinner aerobic layer. As a result, bogs in particular provide conditions suitable for both methanogens and methanotrophs. Given that acetylene can be inhibitory to some methanotrophs, our CFs deviate from the theoretical and established set of CFs for cyanobacteria in upland ecosystems. Low CFs are rare in the literature, but not unprecedented. We know of two other studies with CFs less than 1. One reported a CF of 0.25 for bryophytes in New Zealand (Menge and Hedin 2009), and a second reported a CF of 0.85 for *Sphagnum* mosses in arctic tundra (Stewart et al. 2011).

Biological N_2-fixation not only explains the high rates of N and C accumulations in our peats, but also challenges the long-standing assumption that *Sphagnum* mosses in boreal bogs are predominantly limited by N (Damman 1988; Aerts et al. 1992; Vitt 2006). To experimentally examine N limitation, we performed a large-scale field N-amendment experiment that did not lead to a significant increase in *Sphagnum* NPP (Fig. 3), indicating that while the vascular plant community of ombrotrophic Alberta bogs may be limited by N, *Sphagnum* growth is not limited. From an evolutionary standpoint, this finding makes sense. For bogs in low N deposition environments, *Sphagnum* mosses and other bryophytes maintain a symbiotic relationship with N_2-fixing bacteria, affording these diazotrophs the ability to adapt their metabolism to meet not only their own N demands, but also the requirements of growing mosses. The microbes, in exchange, receive organic compounds, and shelter against predation (Opelt et al. 2007; Lindo et al. 2013). For bryophytes, this association is even more vital than for vascular plants, as mosses lack roots and associated mycorrhizae, restricting their ability to scavenge N (Vitt 2006; Limpens et al. 2006).

In addition to evolving solutions for overcoming N deficiency, symbiotic methanotrophic bacteria can increase *Sphagnum* photosynthesis by more than 10–30 % by providing additional C in the form of CO_2 (Raghoebarsing et al. 2005), thus further aiding in the C-sequestration potential of these peatlands. Once N is transferred from diazotrophs to mosses, N is used to fuel *Sphagnum* NPP. It is likely that these symbiotic adaptations have fueled high C- and N-sequestration rates under pristine ombrotrophic conditions in boreal bogs since the last glaciation 8–10,000 years ago (Yu 2011). In tropical montane forests, it has been suggested that atmospheric deposition and N_2-fixation "conspire" to generate widespread N richness (Brookshire et al. 2012). Given the coupling of low atmospheric N-inputs with large N stores in pristine bogs, we provide another example of an N richness phenomenon not previously documented for ecosystems in the boreal zone.

Our high rates of N_2-fixation have implications for the biogeochemical functioning of bog ecosystems. First, biological N_2-fixation is the most parsimonious explanation for the observed high rates of N accumulation in bog peat confirming our hypothesis that biological N_2-fixation is of sufficient magnitude to resolve the disequilibrium between the amount of N needed to support annual NPP of *S. fuscum* and the longer-term rate of N-accumulation in peat bogs. Second, the magnitude of N_2-fixation fuels *Sphagnum* NPP allowing for high rates of C accumulation and this fixation is carried out primarily by methanotrophs. Third, our findings also call into question the long-standing assumption that *Sphagnum* NPP in boreal bogs is predominantly limited by N (Damman 1988; Aerts et al. 1992; Vitt 2006). Fourth, the abundance of methanotrophic N_2-fixation in these bogs provides a mechanistic explanation for the exceptionally low ARA:^{15}N CFs, and has direct implications for the regulation of CH_4 fluxes from bogs. Peatlands currently function as a net source of atmospheric CH_4, yet measured fluxes from western Canadian bogs are consistently low (Vile et al. 2003). The activity of N_2-fixing methanotrophs may, in part, explain these low fluxes (Kip et al. 2010; Larmola et al. 2010).

Conclusions

High rates of biological N_2-fixation in pristine Alberta bogs are primarily responsible for large stocks of both N and C. This unexpected large N-input can fully account for the disequilibrium in these bog budgets, leading to levels of N-accumulation that enable high rates of C sequestration. Previous underestimation of the importance of N_2-fixaton is directly linked to the composition of the diazotrophic community. These results have implications for the utility of measuring rates of N_2-fixation solely with ARA when non-

cyanobacteria diazotrophs dominate. Globally, these results also have implications for the balance between methane production and consumption. Because methane is a powerful greenhouse gas, any mechanistic alteration in fluxes from peatlands could have consequences for global climate change. In contrast to peatlands of eastern North America and parts of Europe where elevated N deposition has prevailed for decades, peatlands in the Fort McMurray region of Alberta are just beginning to receive elevated N deposition related to development of the oil sands resource (Hazewinkel et al. 2008). Because all N_2-fixation scenarios involve substantial C-costs, we expect upon exposure to enhanced N deposition, biotic feedbacks will likely down-regulate biological N_2-fixation, with potentially important implications for the vulnerability of global peatland C and N stores, as well as for potential changes in methane fluxes.

Acknowledgments This work was supported by grants from The Wood Buffalo Environmental Association, The Cumulative Environmental Management Association, and the NSF to M.A.V., R.K.W. & D.H.V, and funding for aspects of the molecular work was provided by the Dennis M. Cook Endowed Gregor Mendel Chair in Genetics Fund to D.D.W. We thank J. Graham for retranslocation data and discussion, N. Weston for questioning the low CF ratio, and A. Langley for comments on an earlier version of the manuscript.

Conflict of interest The authors declare no competing financial interests.

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