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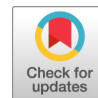

Molybdenum-Based Diazotrophy in a *Sphagnum* Peatland in Northern Minnesota

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ABSTRACT Microbial N_2 fixation (diazotrophy) represents an important nitrogen source to oligotrophic peatland ecosystems, which are important sinks for atmospheric CO_2 and are susceptible to the changing climate. The objectives of this study were (i) to determine the active microbial group and type of nitrogenase mediating diazotrophy in an ombrotrophic *Sphagnum*-dominated peat bog (the S1 peat bog, Marcell Experimental Forest, Minnesota, USA); and (ii) to determine the effect of environmental parameters (light, O_2, CO_2, and CH_4) on potential rates of diazotrophy measured by acetylene (C_2H_2) reduction and $^{15}N_2$ incorporation. A molecular analysis of metabolically active microbial communities suggested that diazotrophy in surface peat was primarily mediated by *Alphaproteobacteria* (*Bradyrhizobiaceae* and *Beijerinckiaceae*). Despite higher concentrations of dissolved vanadium ([V] 11 nM) than molybdenum ([Mo] 3 nM) in surface peat, a combination of metagenomic, amplicon sequencing, and activity measurements indicated that Mo-containing nitrogenases dominate over the V-containing form. Acetylene reduction was only detected in surface peat exposed to light, with the highest rates observed in peat collected from hollows with the highest water contents. Incorporation of $^{15}N_2$ was suppressed 90% by O_2 and 55% by C_2H_2 and was unaffected by CH_4 and CO_2 amendments. These results suggest that peatland diazotrophy is mediated by a combination of C_2H_2-sensitive and C_2H_2-insensitive microbes that are more active at low concentrations of O_2 and show similar activity at high and low concentrations of CH_4.

IMPORTANCE Previous studies indicate that diazotrophy provides an important nitrogen source and is linked to methanotrophy in *Sphagnum*-dominated peatlands. However, the environmental controls and enzymatic pathways of peatland diazotrophy, as well as the metabolically active microbial populations that catalyze this process, remain in question. Our findings indicate that oxygen levels and photosynthetic activity override low nutrient availability in limiting diazotrophy and that members of the *Alphaproteobacteria* (*Rhizobiales*) catalyze this process at the bog surface using the molybdenum-based form of the nitrogenase enzyme.

KEYWORDS *Alphaproteobacteria*, *Sphagnum*, acetylene, diazotrophy, methanotrophs, molybdenum, nitrogen cycle enzymes, nitrogen fixation, peatland, vanadium

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High-latitude peatlands store approximately one-third of global soil carbon and may pose a climatic threat if rising global temperatures accelerate the release of this stored carbon in gaseous forms, as either carbon dioxide or methane (1–3). Mineral-poor (ombrotrophic) peatlands receive most of their nutrient inputs from atmospheric

deposition and contain *Sphagnum* moss as their primary plant cover (2, 4). The peat moss *Sphagnum* is a keystone genus in these ecosystems and is responsible for much of the primary production and recalcitrant dead organic matter (5, 6). *Sphagnum* mosses also host complex microbiomes (7–10), including N_2 fixers (diazotrophs) that are significant nitrogen sources for peatland ecosystems (11).

Despite decades of research, there is still much debate on the identity of the dominant diazotrophs in ombrotrophic peatlands. Early work implicated *Cyanobacteria* (12–14) or heterotrophic bacteria (15) based primarily on microscopic studies, while more recent molecular analyses argue for the importance of methanotrophic *Beijerinckiaceae* (16) as major diazotrophs in *Sphagnum* peat bogs (17–20). Possible contributions from other potential diazotrophs, such as strictly anaerobic methanogenic *Euryarchaeota*, remain unknown. However, it is quite possible that diverse diazotrophs exist within defined niches of peatland environments (21).

Diazotrophy is catalyzed by the nitrogenase metalloenzyme, a complex of thee subunits (H, D, and K) that contains abundant iron as Fe-S clusters. This enzyme is extremely O_2 sensitive (22) and must be protected from exposure to O_2 for diazotrophy to occur (23). The most common form of nitrogenase, encoded by *nif* genes, contains molybdenum (Mo) as its cofactor. When Mo is scarce, some species of *Bacteria* and *Archaea* express nitrogenases containing vanadium ([V] *vnf* genes) or iron ([Fe] *anf* genes) in place of Mo, but these "alternative" nitrogenases are less efficient than the Mo form (24, 25). The most conserved nitrogenase gene, *nifH* (26), has become the marker gene of choice for environmental diazotrophy (27–29). Phylogenetic studies show five *nifH* clusters: aerobic bacteria (cluster I), alternative nitrogenases (cluster II), anaerobic bacteria and archaea (cluster III), uncharacterized sequences (cluster IV), and paralogs related to chlorophyll biosynthesis (cluster V) (30). Because *vnfH* and *anfH* genes in cluster II cannot be differentiated by sequence alone, the D subunit (*nifD-vnfD-anfD*) has become the preferred marker gene for studies of alternative nitrogenases (31). Consistent with higher concentrations of V than Mo in most rocks (32), microbes from diverse soils have been shown to contain *vnfD* genes (31, 33–37). Given that oligotrophic conditions dominate in peatlands, trace metals may limit diazotrophy. However, little is known about trace metal availability and the role of alternative nitrogenase pathways in ombrotrophic peatlands.

Similarly, methane monooxygenase ([MMO] the enzyme that catalyzes the first step of methane oxidation) occurs in particulate copper (Cu)-containing (pMMO) and soluble Fe-containing (sMMO) forms. While pMMO has more specific substrate requirements, pathways that employ sMMO can use a wider range of compounds (38). Both forms of MMO are inhibited by acetylene (C_2H_2) (39, 40). In organisms with both sets of genes, pMMO is expressed when Cu is abundant, whereas Cu limitation induces sMMO expression (41). The dominant peatland methanotrophs in *Alphaproteobacteria* and *Gammaproteobacteria* tend to possess both MMOs (42–46), although *Methylocella* and *Methyloferula* species containing solely sMMO have been isolated from peat bogs (47–49). While most studies have primarily targeted the *pmoA* gene (43, 45), *mmoX* genes and transcripts have also been reported from peatlands (46, 50, 51), raising questions about the relative importance of each form for peatland methane oxidation.

The acetylene reduction assay (ARA) is commonly used as a proxy for diazotroph activity (52, 53). This assay is effective for capturing the potential activity of diazotrophs that are not inhibited by C_2H_2, such as *Cyanobacteria* and nonmethanotrophic *Proteobacteria* (e.g., *Bradyrhizobiaceae*) (54). However, a number of functional guilds of microorganisms, including methanotrophs, methanogens, sulfate reducers, and nitrifiers, are inhibited by C_2H_2 (55–60). If these or other C_2H_2-sensitive microbes perform diazotrophy and/or provide substrates to other diazotrophs (see Fulweiler et al. [61]), ARA may underestimate diazotrophy in that system. Thus, recent studies have shifted to tracking diazotrophy by incorporation of the stable isotope tracer, $^{15}N_2$ (20, 21, 62, 63).

In this study of the S1 peat bog at the Marcell Experimental Forest in northern Minnesota, USA, dissolved macronutrients (NH_4^+, NO_3^-, and PO_4^{3-}) and micronutrients (Fe, Cu, V, and Mo) were profiled along with the community composition and

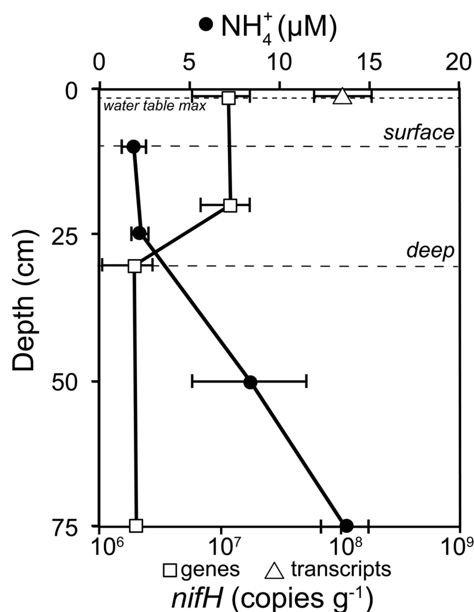

FIG 1 Depth profiles of NH_4^+ concentrations (black circles) and *nifH* gene copies (white squares) and transcripts (white triangle) in units of copies per gram (dry weight) for the S1 bog T3 middle site. Ammonium concentrations are means from measurements from May, June, and September 2014. *nifH* copy numbers are from July 2013. Error bars are standard errors. *nifH* transcripts were not detected at 20-, 30-, and 75-cm depths. Surface (0 to 10 cm) and deep (10 to 30 cm) peat depth intervals used for rate measurements are designated by dashed lines. The maximum water table depth at the S1 site in July 2013 was 2 cm below the hollow surface (dotted line) (97).

abundance of diazotrophic microorganisms. We also performed separate laboratory incubation experiments to measure potential rates of ARA and $^{15}N_2$ incorporation to (i) assess environmental controls (light, O_2, and CH_4) on diazotrophy; (ii) quantify the effect of C_2H_2 on rates of diazotrophy and methanotrophy; and (iii) search for diagnostic markers for alternative nitrogenase activity, such as a low conversion factor of ARA to $^{15}N_2$ incorporation (31) and C_2H_2 reduction to ethane (64). Finally, we make recommendations on universal *nifH* primers for amplicon sequencing and quantitative PCR based on our findings.

RESULTS

Macro- and micronutrient profiles. In S1 bog hollows, NH_4^+ was at a concentration of ~2 μM from the surface to the 25-cm depth and increased at greater depths (Fig. 1; see also Fig. S1a in the supplemental material). Nitrate was at a concentration of <1 μM in surface peat and decreased with depth (Fig. S1b). Phosphate was at a concentration of <0.1 μM from the surface to the 25-cm depth and then increased with depth (Fig. S1c). For the metal pairs of greatest interest to this study, V (at 5 to 21 nM) was consistently more abundant than Mo (at 1 to 7 nM) (Fig. 2), and the concentration of Fe (at 7 to 35 μM) was three orders of magnitude higher than that of Cu (7 to 38 nM) (see Fig. S3) at all three depth intervals (0 to 30, 30 to 50, and 100 to 150 cm) and at three sampling dates (September 2014, June 2015, and September 2015) (data for June 2015 are shown in Fig. 2 and also Fig. S2). Other trace nutrients were in the nano- to micromolar range: Co, 5 to 20 nM; Ni, 10 to 80 nM; Zn, 50 to 250 nM; and Mn, 60 to 2,220 nM (see Table S1). Essentially identical macro- and micronutrient profiles were obtained from the Zim bog, another ombrotrophic bog ~80 km southeast of the S1 bog, sampled in September 2014, with the exception of a lower Fe concentration at the surface (data not shown).

Nitrogenase expression and phylogeny. With the polF/polR primer pair, we measured 1.2×10^7 copies of *nifH* · g^{-1} at 1 and 20 cm and 0.2×10^7 copies · g^{-1} at 30 and 75 cm; *nifH* transcripts (12.2×10^7 copies · g^{-1}) were only detected at 1 cm (10:1

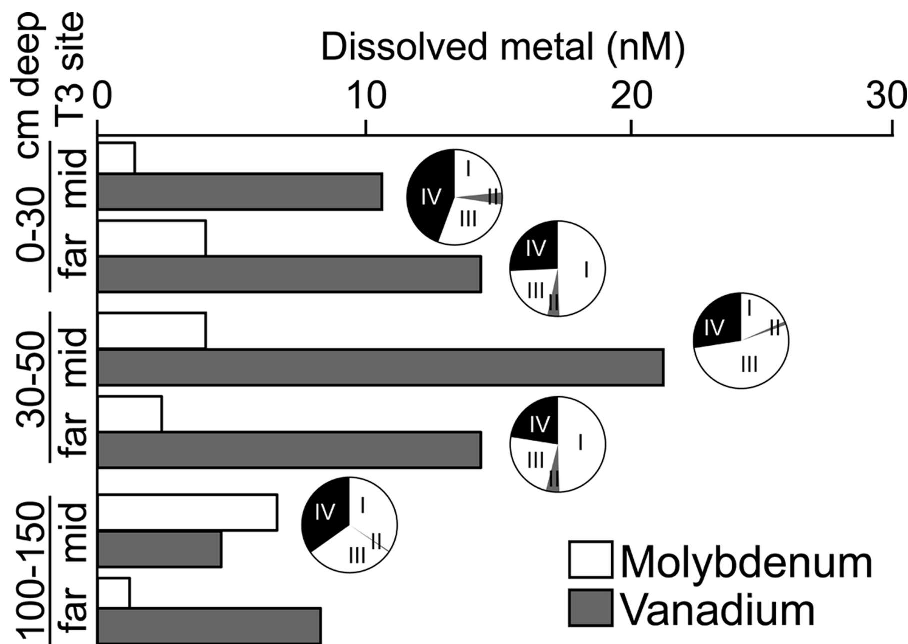

FIG 2 Dissolved molybdenum (white) and vanadium (gray) concentrations in porewater from three depths in S1 peat hollows (middle and far sites along T3 transect) from June 2015. Pie charts show the relative abundances of genes encoding the five nitrogenase H subunit clusters from metagenomes for each depth; clusters I and III encode Mo-Fe nitrogenases (*nifH*); cluster II encodes alternative (*vnfH*, *anfH*) nitrogenases; cluster IV encodes nitrogenase paralogs. Deepest metagenomes were from 75 cm; insufficient numbers of nitrogenase H subunit sequences were recovered from the far site for cluster analysis.

transcript-to-gene ratio) and not at deeper depths (Fig. 1; see also Fig. S3a). Sequencing of cDNA from surface peat amplified with polF/polR (for *nifH*) and nifD820F/nifD1331R (for *nifD*) primers showed that the majority of nitrogenase transcripts belonged to cluster I (*Alphaproteobacteria*), with *Beijerinckiaceae* dominating for the largest number of *nifH* sequences and *Bradyrhizobiaceae* dominating for *nifD* sequences (Fig. 3). Additional alphaproteobacterial *nif* transcripts matched to *Rhodospirillaceae*, *Rhizobiaceae*, *Rhodobacteraceae*, *Methylocystaceae*, and *Xanthobacteraceae* (Fig. 3). *Gammaproteobacteria*, *Cyanobacteria* (*Oscillatoriophycideae*), and *Nitrospira* were also observed at lower abundances in cDNA amplicon libraries (data not shown).

In metagenomes, *nifH* genes were roughly equally distributed between Mo-dependent clusters I and III and cluster IV/V paralogs (Fig. 3; see also Table S2).

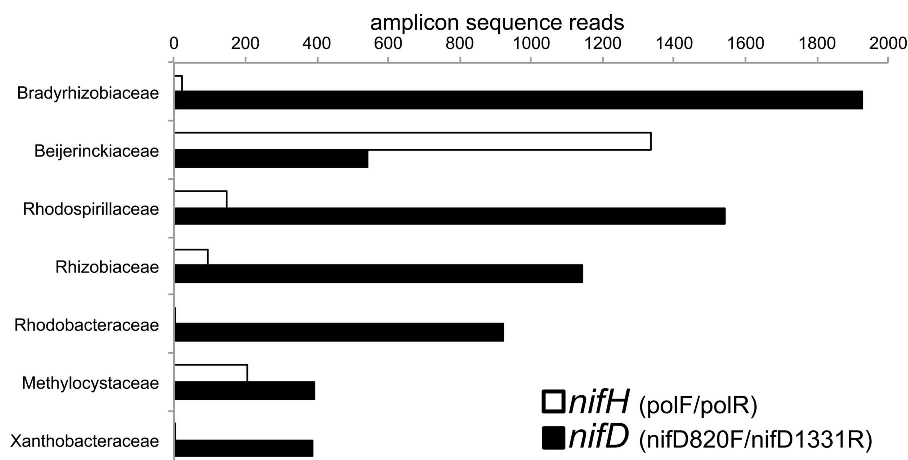

FIG 3 Numbers of cDNA amplicon sequence reads for *nifH* and *nifD* alphaproteobacterial transcripts. Primer sets were polF/polR and nifD820F/nifD1331R for *nifH* and *nifD*, respectively.

Sequences from cluster II (alternative nitrogenases, *vnfH* and *anfH*) were scarce at all depths (<5% overall); two *vnfD* genes from metagenomes showed phylogenetic similarity to those from soil *Proteobacteria* (see Fig. S4). Attempts to amplify *vnfD-anfD* from cDNA yielded few reads; those recovered were most similar to *Alphaproteobacteria anfD* from *Rhodopseudomonas* species (Fig. S4).

Methane-related gene expression and phylogeny. Like *nifH*, particulate methane monooxygenase (*pmoA*) and methyl coenzyme M reductase (*mcrA*) transcripts showed the highest abundances in surface peat (Fig. S3b and c). Surface *pmoA* transcripts mapped to *Methylocystaceae* (75%) and *Methylococcaceae* (25%). Surface *mcrA* transcripts mapped to *Methanosarcina* (58%), *Methanocella* (28%), and *Methanoregula* (11%). Attempts to amplify *mmoX* from cDNA were unsuccessful (data not shown). In metagenomes, genes for *pmoA* were dominant in surface peat, whereas the relative abundance of *mmoX* transcripts increased with depth (Fig. S2).

Rates of diazotrophy and methanotrophy. Potential rates of acetylene reduction were measured for peat collected from S1 bog hollows and hummocks in April, June, August and September of 2013 to 2015 and incubated for 1 week at 25°C. Acetylene reduction to ethylene was only detected in surface (0 to 10 cm) peat samples incubated in the light and not in deep (10 to 30 cm) peat or in surface peat incubated in the dark. Acetylene reduction to ethane was not detected in any incubation (data not shown). *Sphagnum* in peat incubations exposed to light became visibly greener over the course of the incubation (Fig. 4).

In hollows, where surface peat was dominantly covered by a mix of *Sphagnum fallax* and *S. angustifolium*, ARA rates were higher and more variable in degassed versus oxic incubations (0 to 163 versus 2 to 23 μmol $C_2H_4 \cdot g^{-1} \cdot h^{-1}$, respectively) and were unaffected by the presence or absence of 20% CO_2. The ARA rates in hollows incubated with degassed headspace were positively correlated ($P < 0.0001$) with peat water content (93 to 96%). In both hollows and hummocks, ARA rates were not affected by the addition of 1% CH_4. In hummocks with lower water contents (90 to 91%), surface peat was dominantly covered by *S. magellanicum*, and oxic and degassed treatments had similarly low ARA rates (0 to 8 μmol $C_2H_4 \cdot g^{-1} \cdot h^{-1}$). *nifH* transcripts in surface peat from hollows incubated under degassed headspace with 1% C_2H_2, with or without 1% CH_4, ranged from 10^4 to 10^7 copies $\cdot$ g^{-1} (July 2014) (data not shown), which were 1 to 4 orders of magnitude lower than those from field samples from the previous summer (10^8 copies $\cdot$ g^{-1}) (Fig. 1) and higher and more variable in hollows than *nifH* transcripts from hummock incubations (see Fig. S5).

$^{15}N_2$ incorporation showed overall trends similar to those of ARA rates (e.g., 90% higher rates in degassed versus oxic conditions and no significant CH_4 effect) (Fig. 5). In degassed treatments, 1% C_2H_2 inhibited $^{15}N_2$ incorporation by 55% but had no effect on oxic treatments. In oxic treatments, CH_4 consumption rates were 100 times higher than $^{15}N_2$ incorporation rates, and 1% C_2H_2 addition suppressed CH_4 oxidation rates by 95% (Fig. 5). Using the four sites measured with both methods, a conversion factor of 3.9 for $^{15}N_2$ to ARA was calculated (see Fig. S6). In sum, laboratory incubations of native peats revealed that diazotrophy was stimulated by light, suppressed by O_2, and minimally affected by CH_4 and CO_2.

DISCUSSION

Diazotrophs are active in surface peat. By definition, the only source of nutrients to ombrotrophic peat bogs is the atmosphere (65). Scarce (low μM concentrations) dissolved nitrogen and phosphorus in S1 peat porewater suggests oligotrophic conditions (65–67), consistent with low rates of atmospheric deposition in Minnesota peat bogs (68). Low nutrient concentrations add further evidence to previous suggestions that these nutrients may limit *Sphagnum* productivity (21) and/or complex mechanisms may exist for nutrient scavenging at ultralow concentrations. Diazotroph activity (ARA and *nifH* transcription) was solely detected in surface peat samples incubated in the light. The apparent absence of diazotrophy at greater depths is consistent with previous reports (69) and may be due to light limitation and/or remineralization of

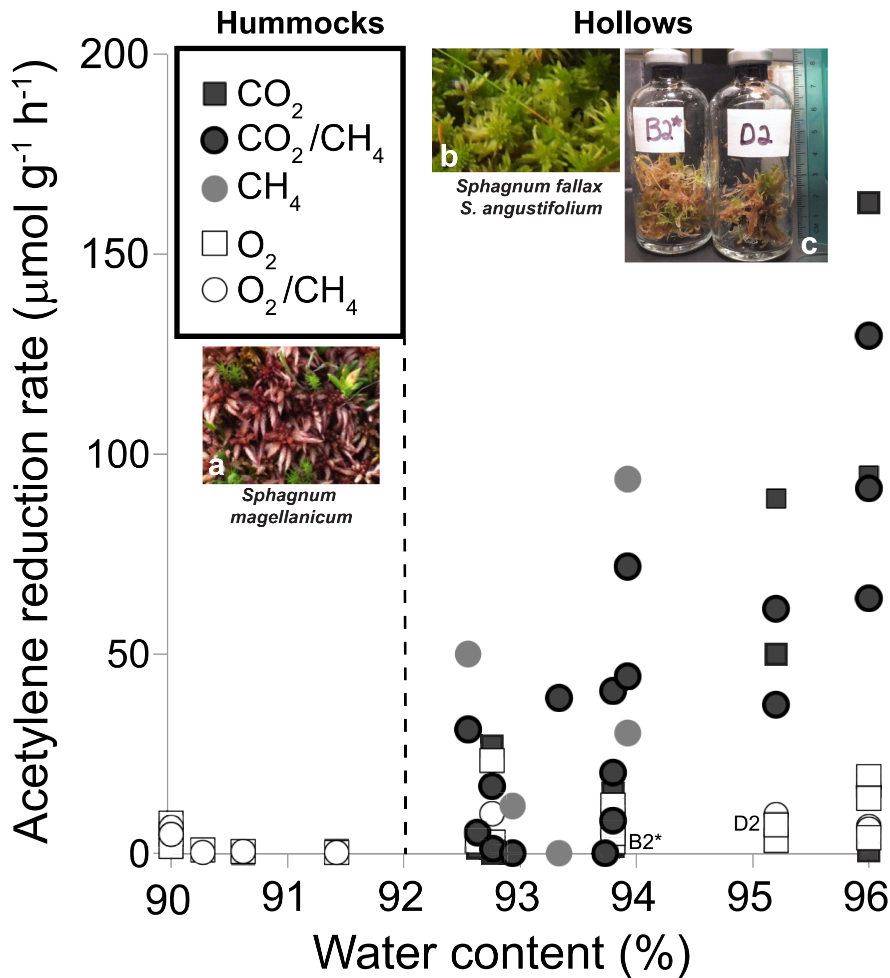

FIG 4 Acetylene reduction rates for hummocks (90 to 91% water content) and hollows (93 to 96% water content) at the S1 bog, T3 transect (0- to 10-cm depth incubated in the light at 25°C for 7 days). ARA units are μmol ethylene produced per gram (dry weight) per hour. Photo insets show dominant *Sphagnum species* in hummocks (*S. magellanicum*) (a) and hollows (*S. fallax* and *S. angustifolium*) (b). Photo inset (c) shows *Sphagnum* greening after incubation of hollow samples in the light for 7 days at 25°C; bottle B2* (April 2014) received air headspace with 1% CH_4 and D2 (Sept 2013) received air headspace without CH_4. The vertical dotted line divides the hummock samples (dominated by S. *magellanicum*) from the hollow samples (dominated by *S. fallax/angustifolium*) in terms of water content (92%).

organic nitrogen to ammonium, which is preferentially used as a nitrogen source by microbes.

Nitrogenase sequences from *Alphaproteobacteria* (*Rhizobiales*) dominate in peatland. Diazotrophic methanotrophs have the potential to serve as a methane biofilter and a nitrogen source for peatland ecosystems. Previous work showed that *Alphaproteobacteria* (*Rhizobiales*), including type II methanotrophs (20, 50), were the dominant diazotrophs in *Sphagnum*-dominated peatlands and may provide the unaccounted nitrogen input resulting from an imbalance in atmospheric nitrogen deposition and an accumulation in *Sphagnum* mosses (19–21, 70, 71). However, the carbon metabolism of peatland diazotrophs remains unclear, because the two dominant *Rhizobiales* families grow on both complex organics (*Bradyrhizobiaceae* [72]) and simple alkanes and C_1 compounds (*Beijerinckiaceae* [16]). Consistent with results from previous studies, *Rhizobiales* showed the highest relative abundance in transcript libraries in this study. *nifH* and *nifD* amplicons from *Beijerinckiaceae* and *Bradyrhizobiaceae*, respectively, dominated. However, the complex taxonomic classification of *Rhizobiales nifH* and *nifD* genes (18) prevented distinguishing methanotrophic *Beijerinckiaceae* from heterotrophic *Beijerinckia indica* and *Bradyrhizobiaceae* solely on the basis of *nifH* and *nifD* phylogenies.

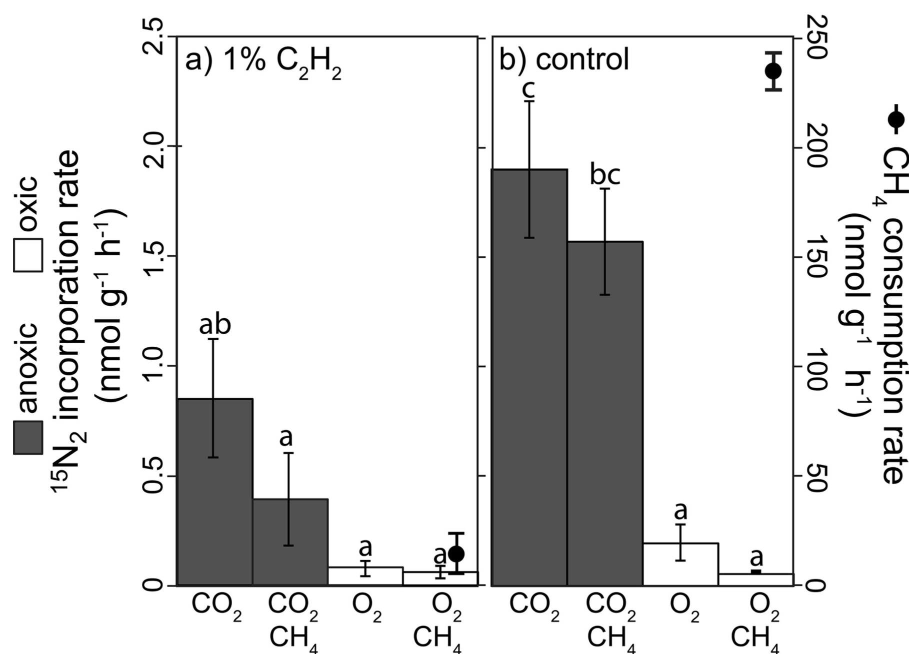

FIG 5 Effects of 1% C_2H_2 on $^{15}N_2$ incorporation and CH_4 consumption for S1 bog surface peat. Rates were measured for samples collected from the northwest (NW) S1 bog transect in September 2014. $^{15}N_2$ incorporation treatment conditions were 80% N_2 plus 20% CO_2 with and without 1% CH_4 (shaded bars) or 80% N_2 plus 20% O_2 with and without 1% CH_4 (white bars) with (a) and without (b) 1% C_2H_2; units are nmol $^{15}N_2$ incorporated per gram (dry weight) per hour. CH_4 consumption treatments were 80% N_2 plus 20% CO_2 plus 1% CH_4 (black circles) with (a) and without (b) 1% C_2H_2; units are nmol CH_4 consumed per gram (dry weight) per hour. Error bars are standard errors. Lowercase letters indicate statistically different elemental contents ($P < 0.05$ based on Tukey-Kramer HSD test).

Diazotrophy is catalyzed by the molybdenum form of nitrogenase. Peatland conditions such as low pH, nitrogen, and temperature would be expected to favor diazotrophy by alternative nitrogenases. Molybdenum sorption to peat is enhanced at low pH (73), biological requirements for Mo are higher when bacteria are fixing N_2 than when growing on other nitrogen compounds (74), and alternative nitrogenases have higher activity and expression at lower temperatures (24, 25). Below 10 nM Mo, diazotrophy is limited in laboratory cultures (75–79), and alternative nitrogenases, if present, are expressed (80). Transcription of alternative nitrogenase genes has been reported for *Peltigera* cyanolichens (35) but, to our knowledge, has not previously been investigated for *Sphagnum* peatlands.

Molybdenum concentrations in S1 bog porewaters were 1 to 7 nM, which are within the same range as Mo in other oligotrophic freshwaters (74) and lower than those of V (5 to 21 nM) and Fe (7 to 35 μM). Similar metal concentrations among (i) sampling dates in September 2014, June 2015, and September 2015, (ii) those in another ombrotrophic Minnesota peatland, Zim bog, ~80 km from the S1 bog, and (iii) those in acidic peatlands in Northern Europe (81) suggest that the values we measured are spatially and temporally representative for diverse northern peatlands.

Intriguingly, despite the presence of conditions that would be expected to favor alternative nitrogenase expression (e.g., low pH, long winters, and [Fe] > [V] > [Mo]), our collective evidence suggests that diazotrophy at the S1 bog was catalyzed by the Mo-containing form of the nitrogenase enzyme. The majority of *nifH* genes retrieved from metagenomes belonged to Mo-containing clusters I and III. A significant number of sequences came from the uncharacterized cluster IV, recently shown to contain a functional nitrogenase (82) that likely binds a Mo-Fe cofactor (83). The metagenomes contained very few cluster II *vnfD-anfD* genes, and minimal numbers of *vnfD-anfD* transcripts were amplified from peat cDNA. Ethane, a biomarker of alternative nitrogenase, was undetectable in ARA incubations. Finally, the $^{15}N_2$-to-ARA conversion

factor (3.9) was within the same range (31, 80) as other peat bogs (20, 52, 84) and matched that of Mo-nitrogenase in pure culture experiments as opposed to the lower values measured for alternative nitrogenases (31). The question of how peatland diazotrophs access scarce Mo remains uncertain; it is possible that Mo bound to peat organic matter can be scavenged by diazotrophs as is the case in forest soils (85, 86).

Methanotrophs are active in incubations and inhibited by acetylene. We performed bottle experiments to test whether methanotrophs were active. Complete CH_4 consumption in air amended with 1% CH_4 showed that methanotrophs were active in our incubations and that the black bromobutyl stoppers we used were not toxic to peatland methanotrophs, whereas nonhalogenated stoppers are toxic to aquatic aerobic methanotrophs (87). Acetylene fully inhibited CH_4 consumption, demonstrating that the methanotrophs in our incubations were C_2H_2 sensitive, similar to laboratory strains tested in previous studies (55, 60). Like diazotrophy, methanotrophy was apparently also mediated by the enzyme requiring the scarcer metal; concentrations of dissolved Fe were consistently orders of magnitude higher than those of Cu in peat porewater, yet *pmoA* sequences were more abundant than *mmoX* sequences in surface peat where the highest CH_4 consumption was observed (43, 46). This finding is also consistent with a higher transcription of *pmoA* versus *mmoX* in other acidic peatlands (50, 81, 88). In laboratory studies, the "copper switch" from sMMO- to pMMO-supported growth occurs when the concentration of Cu is >1 μM (41), which is several orders of magnitude higher than Cu concentrations measured at the S1 bog (7 to 38 nM). It is possible that the copper switch occurs at a lower threshold in peat bog or that there are other factors controlling the type of MMO expressed, such as CH_4 or O_2 availability. Additionally, the inherent nature of the peat matrix, with characteristically high levels of particulate and dissolved organic matter, likely also affects metal bioavailability in complex ways (89) not addressed in our study.

Surface peatland diazotrophy is sensitive to oxygen and acetylene. If methanotrophs were the dominant diazotrophs in peatlands, as previously proposed (20, 21), CH_4 addition should have stimulated $^{15}N_2$ incorporation in our bottle experiments, and C_2H_2 should have inhibited it. Instead, this and previous (63) works found that $^{15}N_2$ incorporation was not affected by CH_4 addition. Acetylene partially inhibited $^{15}N_2$ incorporation under degassed conditions (as reported by Kox et al. [90]) and had a minimal effect on oxic $^{15}N_2$ incorporation, suggesting that C_2H_2- and O_2-sensitive microbial clades contributed approximately half of the diazotrophic activity in our incubations. This finding highlights the importance of quantifying peatland diazotrophy by $^{15}N_2$ incorporation instead of, or in addition to, ARAs.

Based on *nif*, *pmoA*, and *mcrA* phylogenies from native peat, the O_2- and C_2H_2-sensitive diazotrophs were likely methanotrophic *Beijerinckiaceae* that can only fix N_2 under microoxic conditions and/or strict anaerobes in cluster III, such as methanogenic *Euryarchaeota*. These two families are the most active in surface peat based on the numbers of transcripts and amplicon sequences. Since the numbers of *nifH* transcripts were 1 to 4 orders of magnitude lower in our incubations than in native peat, it is likely that diazotrophs were stressed, possibly due to O_2 exposure during the sampling process. Indeed, we observed the highest ARA rates in hollows where the water table was typically at the bog surface, limiting O_2 penetration into the peat. The other half of the O_2-sensitive diazotrophic activity was likely performed by C_2H_2-insensitive heterotrophic *Bradyrhizobiaceae* and/or *Beijerinckiaceae* (91, 92) or C_2H_2-insensitive methanotrophic *Methylocystaceae*, which can adapt to a wide range of CH_4 concentrations (93). Less-O_2-sensitive microbes, such as heterotrophic *Beijerinckiaceae* (92) and photosynthetic *Oscillatoriophycideae* (23), likely contributed to the minor amount of ARA activity in the presence of O_2.

Molecular markers for diazotrophy. We end with a word of caution with regard to the molecular detection of diazotrophs. The majority of studies in peatlands have employed PCR amplification and sequencing of the *nifH* marker gene for studying the dynamics of diazotrophs in peatlands. A wide range of *nifH* primer sets exist, with

varied universality (28). Peat bog sequencing efforts have used polF/polR (this study and reference 50), F1/R6 (20), FGPH19-polF/polR-AQER (63), and 19F/nifH3 plus nifH1(1)/nifH2 (12) with nested PCR (17, 90). *In silico* evaluation predicts that the polF/polR primer set will not amplify the majority of *Proteobacteria* and/or *Cyanobacteria* and group III *nifH* sequences (28); however, this primer set yields the highest efficiency for qPCR (94). Of the *nifH* primer sets used previously, F1/R6, 19F/nifH3, and nifH1(1)/nifH2 are predicted to have the highest coverage for soils (>80% predicted primer binding for sequences from soil ecosystems). However, it is important to be aware that the F1/R6 primer set contains a number of mismatches with sequences from cluster III in peatlands, including of methanogenic *Euryarchaeota* (see Fig. S7). To maximize sequence coverage, we suggest using primer sets that can amplify *nifH* from cluster III, such as IGK(3)/DVV (28), for future studies.

Conclusions. This study revealed that peatland diazotrophs preferentially transcribed the Mo-based, rather than the V-based, form of the nitrogenase enzyme, despite the dominance of V over Mo in the environment. It also highlighted the sensitivity of diazotrophic peatland communities to O_2 exposure during sample collection and quantified the inhibitory effect of C_2H_2 addition on peatland diazotrophy. Under our experimental conditions in lab incubations, we did not observe CH_4-stimulated diazotrophy. However, the quantification of the relative contributions of methanotrophic and heterotrophic diazotrophy *in situ* awaits further investigation.

MATERIALS AND METHODS

Site description and sample collection. Samples were collected from the S1 (black spruce *Sphagnum* spp.) peat bog at Marcell Experimental Forest (MEF; 47°30.476′N, 93°27.162′W), the site of the DOE SPRUCE (Spruce and Peatland Responses Under Climatic and Environmental Change) experiment in northern Minnesota, USA (95). The S1 bog is ombrotrophic and acidic (average pH, 3.5 to 4 [66, 96]). Over the summer months, the water table is ±5 cm from the hollow surface (66, 97). Dissolved O_2 levels decrease to below detection (~20 ppb) within the top 5 cm of the bog. Three locations were sampled along S1 bog transect 3 (T3) at near, middle, and far sites (see Lin et al. [98] for further details). Surface (0- to 10-cm depth) peat was collected from hollows dominated by a mixture of *Sphagnum fallax* and *S. angustifolium* and from hummocks dominated by *S. magellanicum*. Peat depth cores (0 to 200 cm) were sampled from hollows where the water level reached the surface of the *Sphagnum* layer.

Macronutrients. Peat porewater was collected using piezometers from depths of 0, 10, 25, 50, 75, 150, and 200 cm. Piezometers were recharged the same day as collection, and porewater was pumped to the surface, filtered through sterile 0.2-μm polyethersulfone membrane filters, and stored frozen until analysis. Nitrate (NO_3^-) and nitrite (NO_2^-) were analyzed using the spectrophotometric assay described by García-Robledo et al. (99). Ammonium (NH_4^+) concentrations were determined with the indophenol blue assay (100). Phosphate concentrations were measured with the molybdate-antimony ascorbic acid colorimetric assay (101).

Micronutrients. Peat porewater was collected from two locations in the S1 bog from cores at depths of 0 to 30 cm, 30 to 50 cm, and 100 to 150 cm by filtration through 0.15-μm Rhizon soil samplers (Rhizosphere Research Products). All plastics were washed with HCl prior to sampling; Rhizon soil samplers were cleaned by pumping 10 ml of 1 N HCl through them, followed by rinsing with ultrapure water until the pH returned to neutral (~100 ml/filter). After collection, samples were acidified with 0.32 M HNO_3 (Fisher Optima) and analyzed using a Thermo Element 2 high-resolution inductively coupled plasma mass spectrometer (HR-ICP-MS; National High Magnetic Field Laboratory, Florida State University). Initial analyses resulted in the frequent clogging of the nebulizer, likely due to the abundance of dissolved organic carbon. Therefore, samples were diluted 1:10 to minimize interruptions from nebulizer clogs. Concentrations were quantified with a 7-point external calibration using standards prepared in 0.32 N HNO_3 from a multi-element standard mix (High-Purity Standards).

To generate an organic-free sample matrix suitable for ICP-MS analysis without contaminating or diluting the sample, subsequent samples were digested as follows: 1-ml aliquots of the porewater samples were heated in 15-ml Teflon beakers (Savillex) with 1 ml of 16 N HNO_3 (Ultrex II, JT Baker) and 100 μl of 30% H_2O_2 (Ultrex II, JT Baker) for 36 h at 230°C in a trace-metal-clean polypropylene exhaust hood. The HNO_3-H_2O_2 mixture oxidizes any dissolved organic matter (DOM) to CO_2, but the resulting matrix is too acidic for direct ICP-MS introduction. Therefore, samples were evaporated to near dryness, resuspended in a 0.32 N HNO_3 matrix suitable for ICP-MS analysis, and analyzed using the Element2 ICP-MS along with parallel blank solutions.

Quantification and sequencing of gene and transcript amplicons. Peat was frozen on dry ice at the field site in July 2013 or in liquid N_2 after 7-day incubations at 25°C in the light under a degassed (80% N_2 plus 20% CO_2) headspace with 1% C_2H_2, with or without 1% CH_4, for June 2014 incubations (see "Acetylene reduction and methane consumption rates" below). DNA and RNA were extracted with Mo Bio PowerSoil DNA and total RNA extraction kits, respectively, as described by Lin et al. (46). RNA was cleaned with a Turbo DNA-free kit (Ambion). Nucleic acid purity was analyzed for the 260/280 absor-

TABLE 1 qPCR and sequencing primers used in this study[a]

Gene(s)	Primer set(s)	Reference
mmoX	mmoX-206f/mmoX-886r	107
pmoA	A189f/Mb661r	108
mcrA	Mlas/mcrA-rev	109
nifH	polF/polR	110
nifD	nifD820F/nifD1389R and nifD820F/nifD1331R	31
vnfD-anfD	vnfD_anfD548F/vnfD_anfD1337R and vnfD_anfD548F/vnfD_anfD1291R	31

[a]PCR conditions were based on those reported in references 43, 50, 109, 28, and 31 for *pmoA*, *mmoX*, *mcrA*, *nifH*, and *nifD-vnfD-anfD*, respectively.

bance ratio (of 1.8 to 2.0) on a NanoDrop spectrophotometer. cDNA was synthesized using the GoScript reverse transcription system (Promega) according to the manufacturer's protocol.

Plasmid standards for qPCR were constructed according to the method of Lin et al. (102). Primer pairs are given in Table 1. The gene fragments of *nifH*, *pmoA*, and *mcrA* for constructing plasmid standards for qPCR were amplified from genomic DNA of *Rhodobacter sphaeroides*, *Methylococcus capsulatus* strain Bath, and S1 peat bog peat soil, respectively. To prepare cDNA standards, plasmid DNA with a positive gene insert was linearized with NcoI restriction enzyme following the manufacturer's protocol (Promega) and purified by using a MinElute PCR purification kit (Qiagen). RNA was synthesized from the linearized plasmid DNA by using the Riboprobe *in vitro* transcription system (Promega) followed by cDNA synthesis using the GoScript reverse transcription system (Promega) according to the manufacturer's protocols.

The abundance of functional gene transcripts was quantified in samples run in duplicates on a StepOnePlus real-time PCR system (ABI) using Power SYBR green PCR master mix. Reaction mixtures of 20 μl comprised 2 μl of template cDNA (10 to 100 ng/μl) added to 10 μl of SYBR green master mix, 0.5 to 1.6 μl of each forward and reverse primer (0.3 to 0.8 μM final concentration; Table 1), and 4.8 to 6.5 μl of PCR-grade water. Samples were run against a cDNA standard curve (10^1 to 10^7 copies of plasmid gene fragment) on a StepOnePlus qPCR instrument with 96 wells with an initial denaturation step of 2 to 5 min at 95°C and 40 cycles of denaturation at 95°C for 15 to 30 s, annealing at 55 to 64°C for 30 to 45 s, extension at 72°C for 30 to 45 s, and data acquisition at 83 to 86.5°C for 16 to 30 s. To minimize the effects of inhibitors in assays, peat DNA was diluted to 1/40 of the original concentrations, and duplicate 20-μl reaction mixtures, each containing 2 μl of diluted DNA, were run for each sample. Functional gene and transcript copy numbers were normalized to the dry weight of peat or 16S rRNA transcript copies for incubation samples. Amplicons were sent to the University of Illinois at Chicago for DNA sequencing using a 454 platform. Raw sequences were demultiplexed, trimmed, and quality filtered in CLC bio software. The phylogenies of *vnfD-anfD* sequences were inferred using the maximum likelihood method based on the Kimura 2-parameter model in MEGA5 (103).

Acetylene reduction and methane consumption rates. Samples of bulk peat (*Sphagnum* spp. and surrounding soil) were collected from 0- to 10- and 10- to 30-cm depths in September 2013, April 2014, June 2014, September 2014, and August 2015 and stored at 4°C until the start of laboratory incubations. Samples from the 0- to 10-cm depth were gently homogenized so as not to rupture *Sphagnum* sp. tissues, while peat samples from the 10- to 30-cm depth were fully homogenized. For each sample, 5 g of bulk peat was placed in 70-ml glass serum bottles, stoppered with black bromobutyl stoppers (Geo-Microbial Technologies) (pretreated by boiling 3 times in 0.1 M NaOH), and sealed with an aluminum crimp seal. Headspaces were oxic (room air, 80% N_2 plus 20% O_2) or degassed (100% N_2 or 80% N_2 plus 20% CO_2) with or without 1% C_2H_2 or 1% CH_4. Treatments were incubated for 1 week at 25°C in the light or dark. A gas chromatograph with a flame ionization detector (SRI Instruments) equipped with a HayeSep N column was used to quantify CH_4, C_2H_2, and C_2H_4. Samples were measured for C_2H_4 production daily until C_2H_4 production was linear (~7 days). Controls not amended with C_2H_2 did not produce ethylene (C_2H_4). Incubations of hollow peat from June 2014 incubated under an oxic headspace with and without 1% C_2H_2 were also monitored for the consumption of 1% CH_4. Statistical analysis was performed with JMP Pro (v. 12.1.0) using the Tukey-Kramer honestly significant difference (HSD) comparison of all means.

$^{15}N_2$ incorporation rates. In September 2014, samples were quantified for N_2 fixation rates by $^{15}N_2$ incorporation in parallel with ARA measurements. Incubations were set up as described above and supplemented with 7 ml of 98% $^{15}N_2$ (Cambridge Isotope Laboratories, Tewksbury, MA, USA). After 7 days, samples were dried at 80°C, homogenized into a fine powder, and analyzed for N content and $\delta^{15}N$ by isotope ratio mass spectrometry (IRMS) with a MICRO cube elemental analyzer and IsoPrime100 IRMS (Elementar) at the University of California, Berkeley, corrected relative to National Institute of Standards and Technology (Gaithersburg, MD, USA) standards.

Metagenomic analyses. Metagenomes were generated in a previous study (104). Diazotrophic and methanotrophic pathways were investigated using the following bioinformatics approaches. Briefly, Illumina reads were filtered by quality (Phred33 score threshold of Q25) using Trim Galore (Babraham Bioinformatics) and a minimum sequence length cutoff of 100 bp. The sequences were then queried using RAPSearch2 (105) against the NCBI nr database of nonredundant protein sequences as of November 2013. Sequences with bit scores of 50 and higher were retained to determine the total number of functional genes for normalization across the different samples. The taxonomic composition of protein-coding sequences was determined based on the taxonomic annotation of each gene accord-

ing to the NCBI nr taxonomy in MEGAN5 (106; minimum score, 50; maximum expected, 0.01; top percent, 10; minimum complexity, 0.3).

To classify sequences by nitrogenase cluster type, genes were analyzed using BLASTX (E value, 0.1; bit score, 50) versus a custom *nifH* database that includes a phylogenetic tree to distinguish the principal clusters (I, V, and III) in the *nifH* phylogeny, as well as paralogous cluster IV *nifH*-like sequences (27). Abundances of *nifH* genes from the four clusters were normalized to those of total protein-coding genes from RAPSearch2 output sequences. The relative abundance of particulate (*pmoA*) versus soluble (*mmoX*) methane monooxygenase was based on previous analyses reported by Lin et al. (46).

Accession number(s). Metagenomes were reported in a previous study (104) and deposited in BioProject PRJNA382698 (SAMN06712535-06712540). *pmoA* cDNA amplicons were reported in a previous study (43) and deposited in BioProject PRJNA311735. *nifH*, *mcrA*, *nifD*, and *vnfD-anfD* cDNA amplicons were deposited in BioProjects PRJNA382268, PRJNA382282, PRJNA382288, and PRJNA382295, respectively.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01174-17.

SUPPLEMENTAL FILE 1, PDF file, 9.5 MB.

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