Methanotrophy induces nitrogen fixation during peatland development

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Nitrogen (N) accumulation rates in peatland ecosystems indicate significant biological atmospheric N₂ fixation associated with Sphagnum mosses. Here, we show that the linkage between methanotrophic carbon cycling and N₂ fixation may constitute an important mechanism in the rapid accumulation of N during the primary succession of peatlands. In our experimental stable isotope enrichment study, previously overlooked methane-induced N₂ fixation explained more than one-third of the new N input in the younger peatland stages, where the highest N₂ fixation rates and highest methane oxidation activities co-occurred in the water-submerged moss vegetation.

CH₄ | diazotrophy | mire | peat | phosphorus

Peat-accumulating wetlands, i.e., peatlands, store approximately 30% of the global soil carbon (C) (1), and this value is even higher if the permafrost regions in the Northern Hemisphere are also taken into account (2). As peat accumulates, the ecosystem becomes independent of the groundwater influence and the vegetation becomes more nutrient limited. This gradual succession from minerotrophic fen to Sphagnum-dominated ombrotrophic bog ecosystem is the general peatland development pattern. Because the growth and decomposition rates of Sphagnum mosses are greatly responsible for the C biosequestration in peatlands, the ecology of Sphagnum mosses is of particular interest. Recent studies have shown that Sphagnum mosses have an association with methanotrophic bacteria that leads to a reduction in methane (CH₄) emissions to the atmosphere and the provision of additional carbon dioxide (CO₂) source for the host plants (3, 4). However, the growth of Sphagnum mosses in peatlands is often N limited, at least under low atmospheric N deposition (5), so biological fixation of atmospheric N₂, i.e., the biological conversion of dinitrogen to plant-available ammonium, may stimulate moss growth (6). Under low atmospheric N deposition, moss-associated cyanobacteria have been shown to play an important role in the N budget in forests (7, 8) and peatlands (9), where N₂ fixation is favored by moist conditions. In peatlands, however, molecular analyses of genes that encode nitrogenase reductase proteins (nifH) in Sphagnum mosses have indicated that moss-associated N₂ fixers (diazotrophs) belong mainly to the metabolically diverse class Alphaproteobacteria (10), which includes phototrophic, heterotrophic, and methanotrophic genera.

In previous studies, N₂ fixation rates in peatlands have been found to correlate with the minerotrophy of peatlands, in particular with the level of phosphorus (P) (11), but the nutrient controls of N₂ fixation have not been linked to peatland succession toward the bog stage. Further, in these studies, N₂ fixation has been measured by using acetylene reduction assay (12), which does not provide a quantitative measure of N added to the system, because it inhibits the activity of many noncyanobacterial diazotrophs, but specifically methanotrophic bacteria by inactivating the essential methane monooxygenase enzyme (13). This inhibition is a serious drawback, because a broad range of methanotrophic bacteria contains genes that code for the N₂ fixation pathway and shows nitrogenase activity (11, 14, 15). Because of this methodological problem, the role of methanotrophic N₂ fixation and the relationship of N₂ fixation with C cycling have not previously been evaluated at an ecosystem scale. The elucidation of the linkage between methanotrophy and the overall N cycle in peatlands becomes feasible by the application of stable isotopes (¹⁵N) techniques (8, 16).

We studied N₂ fixation and CH₄ oxidation in the dominant flark and hummock vegetation of 12 pristine peatlands, which varied in age from 200 to 2,500 y due to still ongoing postglacial rebound on the coast of Bothnian Bay, Finland (Fig. 1 and Table S1). Together with the Hudson Bay Lowlands in Canada, the Bothnian Bay of the Baltic Sea between Finland and Sweden is the region where the rebound after the pressure of ice mass and the consequent formation of new land from the sea is most rapid. This chronosequence of peatlands offers an exceptional opportunity to study the links between N and C cycling over an undisturbed peatland gradient.

In young peatlands, unstable hydrological conditions are likely to result in low CH₄ emissions and, thereby, small methanotrophic carbon cycling and N accumulation in peatlands becomes feasible by the application of stable isotope (¹⁵N) techniques (8, 16).

Significance

In peatlands, the external sources of nitrogen are mainly atmospheric, but the atmospheric nitrogen deposition alone cannot explain the long-term annual nitrogen accumulation rates to these ecosystems. Because of methodological problems, methane-induced fixation of atmospheric dinitrogen gas has been previously overlooked as an additional nitrogen input mechanism. We found that the activity of methane-oxidizing bacteria provides not only carbon but also nitrogen to peat mosses and, thus, contributes to carbon and nitrogen accumulation in peatlands, which store approximately one-third of the global soil carbon pool. Our results imply that nitrogen fixation in wetlands may be strongly underestimated when methods inhibiting methane oxidizers are used.

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CH₄ oxidation rates in minerotrophic fens and ombrotrophic bogs have been found to overlap (4). It has been hypothesized that despite the low inorganic N concentration in bogs, CH₄ oxidation is not N limited because methanotrophic N₂ fixation may compensate for the N requirement of methanotrophic bacteria (20). With reference to these factors, we hypothesized that (i) CH₄ induces moss-associated N₂ fixation, which would be most pronounced in the late stages of peatland succession and (ii) the overall N₂ fixation rates along the peatland succession gradient are governed by gradually changing environmental factors, such as water table depth, availability of CH₄ in the pore water, and concentrations of P, iron (Fe), or molybdenum (Mo). The rationale for studying the role of Fe and Mo was that two methane monoxygenases (e.g., ref. 21) and all three nitrogenase isoenzymes contain Fe, and one of the nitrogenase isoenzymes contains Mo as a necessary cofactor (22). We tested the two hypotheses in a ¹⁵N₂ and ¹³CH₄ pulse-labeling experiment, where moss samples collected from flark and hummock habitats of four successional peatland stages were incubated in situ with and without CH₄ addition, each under prevailing light conditions and in the dark. These treatments were used to reveal whether N₂ fixation was attributed to photosynthetic, heterotrophic, or methanotrophic activity.

Results and Discussion

Fixation of N₂ was found in the live parts of the Sphagnum mosses in all of the studied moss patches. Although Sphagnum-associated N₂ fixation was observed in all of the study peatlands of different successional stages, the process was significantly higher (five- to ninefold) in the wet depressions (flarks) of the midsuccessional minerotrophic fens compared with the other successional stages, which consisted of younger meadows, older oligotrophic fens and fen-bog transitions (Fig. 2A and Tables S2 and S3). When the summertime N₂ fixation rates (0–126 mmol g⁻¹ of moss biomass h⁻¹) were converted to annual areal values (0.1–2.9 g of N m⁻² y⁻¹; Fig. 2B), they were up to 10 times greater than the current inorganic N deposition rates (0.3 g m⁻² y⁻¹) for the region. These annual values indicate that Sphagnum-associated N₂ fixation is a major N input to boreal peatlands and, in conjunction with atmospheric N deposition, explains the long-term annual N accumulation rate (0.6–1.3 g m⁻²; ref. 23) in the studied peatland ecosystems (Fig. 2B). Our areal estimations for fen stages and fen-bog transition are within the wide range of the admittedly few estimates available for Sphagnum-associated N₂ fixation in temperate, boreal, and subarctic ecosystems. These range from 0.1–6.4 g of N m⁻² y⁻¹ for fens (11, 16, 24) and 0.1–1 g m⁻² y⁻¹ for bogs (9, 25). Nevertheless, our estimates may be rough because of the large SEs and the extrapolation over time: We assume a 6-mo active season, but substantial heterotrophic microbial activity in addition to phototrophic activity could occur during winter. At these sites, 30–65% of annual moss growth occurs during the October–April period, outside the traditionally defined growing season (26). In all studied peatland stages except wet meadows, incubation under prevailing light conditions resulted in enhanced N₂ fixation, on average, threefold in comparison with the dark treatments, which suggests that phototrophic organisms may be the most active N₂ fixers or that photosynthesis provides carbohydrates to fuel heterotrophic N₂ fixers (Fig. 2A). Methane-induced N₂ fixation contributed approximately 40% (33–47%) of the N₂ fixation in the three younger peatland stages, but was negligible in the fen-bog transition stage (Fig. 2A and B). Thus, the hypothesis of a larger methanotrophic contribution to N₂ fixation in late successional stages was not supported. The rate of the biomass incorporation of ¹³CH₄-derived C indicated that moss-associated methanotrophy was also highest in mesotrophic fens and continued at moderate rates in the flarks of the older stages (Fig. 2C), where CH₄ addition did not enhance N₂ fixation. The meadows showed significantly lower CH₄ oxidation rates compared with the other stages (Table S2). A comparison of the successional patterns of N accumulation and N₂ fixation (Fig. 2B) provides further evidence of the ecological and biogeochemical importance of N₂ fixation and methanotrophic N₂ fixation during peatland development. Based on peat profiles from our sites, 70% of N and 40% of C accumulated during the first 1,000 y of the 2,500-y period (23), a period during which N₂ fixation peaked and had the strongest response to CH₄ addition. This pattern implies that methanotrophic N₂ fixation contributes to rapid N accumulation in the fen stages. The predicted warming conditions in the northern latitudes (27) may impact on boreal peatland development in two ways: At the southern limit of permafrost, melting is promoting a reverse succession from ombrotrophic bog to fen ecosystem, whereas at the southern border of the fen region (aapamires, wet fen-dominated peatland complexes) drainage due to increased evapotranspiration may accelerate ombrotrophication, i.e., succession toward bog ecosystem (28). Our results indicate that these successional changes are likely to lead to changes in N₂ dynamics.

Based on the growth rates of Sphagnum species at our sites (26) and site-specific N content of the Sphagnum species (Table S4), we estimate that N obtained by N₂ fixation could correspond to an average 37 ± 18% (mean ± SEM, range 4–58%, n = 6) of the moss biomass N increment. This proportion is in agreement with the recent estimate of 35% for Sphagnum riparium, inferred from N content and growth rate in a 2-mo laboratory experiment (6). Despite the low absolute areal rate of N₂ fixation in the older mainly rainwater-fed stages (fen-bog transitions), where moss growth is more nutrient limited, the comparison of Sphagnum...
growth rates (26) and N contents further suggests that the proportion of fixed N of the new biomass N increment may increase to 58%. The rest of the N is, we presume, being taken up as inorganic ions and organic N, or recycled to new growth from older parts of the moss shoot (29). The time scale at which N fixed in the moss becomes available for the moss host and for other plants may range from fast exchange over a time span of days (6, 11) to slower nutrient release from decomposing Sphagnum litter over a period of years.

Our results showed faster CH$_4$-C biomass incorporation in light than in dark (Fig. 2C), which suggests that mosses fixed additional CH$_4$-derived CO$_2$ during photosynthesis. The contribution of CH$_4$-derived C was 26% in light, but 10% in dark (calculated as above for N) for Sphagnum C in the flarks. In hummocks, where CH$_4$ and CO$_2$ concentrations are at atmospheric levels, CH$_4$-derived C contributed to only 0–3% of the incorporated C in light and dark. These findings indicate that CH$_4$ can be a significant C source for submerged Sphagnum, supporting the results of previous studies (3, 4).

The moss ratios of N:P, C:N, and C:P, integrated indices of nutrient availability, showed decreasing ratios with increasing N$_2$ fixation activity (Spearman $\rho = -0.32$, $-0.61$, and $-0.54$, for N:P, C:N, and C:P, respectively, $P < 0.003$). The successional pattern in N$_2$ fixation rates was best explained by P availability (Fig. 3 and Tables S4 and S5). The highest rates of N$_2$ fixation were associated with the lowest moss N:P ratios, being lower than the threshold for N limitation in soils (N:P < 7:1; ref. 30). Thus, N$_2$ fixers were able to respond to N demand relative to P supply, as shown (e.g., ref. 31). In the older stages, N:P ratios indicated that plant growth was limited by P (32), yet active moss associated N$_2$ fixation persisted there as well (Figs. 2 and 3).

Although the moss P content was the sole significant predictor of N$_2$ fixation in the light treatment without CH$_4$, the contribution of CH$_4$-induced N$_2$ fixation was best explained by the combination of the moss P and Fe contents (Table S5). Among the sampled microhabitats, moss Fe content correlated strongly with the water table depth below the moss capitula and pH (Table S4). Thus, provided that P supply was sufficient, CH$_4$-induced N$_2$ fixation correlated with CH$_4$ oxidation (hypothesis 2; Fig. 3), but the rate of CH$_4$ oxidation as such was not the primary factor controlling CH$_4$-induced N$_2$ fixation in these sites. The CH$_4$ oxidation was, in turn, best explained by the moss Fe content that depended on the water table level (Fig. S1). Concurrent CH$_4$ concentration in pore water did not significantly correlate with N$_2$ fixation (Spearman rank correlation $P = 0.96$, $n = 80$). Thus, CH$_4$ oxidation can be a significant C source for submerged Sphagnum, supporting the results of previous studies (3, 4).

Overall, our results demonstrate that methanotrophy and N$_2$ fixation are tightly linked in the wet fen depressions and that methanotrophic activity enhances N$_2$ fixation (hypothesis 1). However, because the interactions within the endosymbiotic microbial communities complicate the analysis, we do not directly interpret the experimentally observed dark, light-induced, and CH$_4$-induced rates as heterotrophic, phototrophic, and methanotrophic N$_2$ fixation. The experimental approach cannot distinguish whether the CH$_4$-induced N$_2$ fixation equals methanotrophic N$_2$

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Data are averages of light and dark incubations in $^{15}$N$_2$ and $^{15}$N$_2$+$^{13}$CH$_4$ treatments, respectively, weighted with the proportions of flark and hummock microhabitats in each successional stage. Long-term average peat N accumulation rates for the sites (total accumulation divided by the year since peatland initiation; ref. 23) is shown with filled circles. (C) Incorporation of $^{13}$CH$_4$-C into the biomass (moss + microbes) based on the weighted averages of flark and hummock microhabitats in each successional stage. In each bar, the filled area indicates the incorporation of $^{13}$CH$_4$-derived C in the dark (i.e., incorporation of CH$_4$ into methanotroph biomass) and the open area indicates the average additional incorporation of $^{13}$CH$_4$-derived C under prevailing light conditions (i.e., incorporation of CO$_2$ emitted by methanotrophs into autotrophic plant or microbial biomass via photosynthesis).
induction may be, at least partly, indirect. The higher CO₂ concentrations that result from CH₄ oxidation inside the Sphagnum cells would enhance photosynthesis, as well as for the moss itself. Both Sphagnum fixation and CH₄ oxidation activities of the plant-associated bacteria. In conclusion, moss-associated microbes may explain the dominance of Sphagnum mosses in nutrient-poor peatlands: besides providing an additional in-cell CO₂ source for photosynthesis, these microbial communities provide new N in the cold and acidic environment where decomposition and nutrient recycling are otherwise slow.

Materials and Methods

Field Site. The Siikajoki peatland chronosequence is a replicated primary successional series of peatland ecosystems in the land-uplift coast of Bothnian Bay, Finland (64° 45' N, 24° 42' E; refs. 23 and 34). Our study sites at Siikajoki consisted of 12 peatlands that represent four successional stages, which vary in age from 200 to 2,500 y (Fig. 1). Each stage had three replicates (Table S1). The sites were located within 8 km on the coast of Bothnian Bay, where the mean annual temperature is 2.3 °C, and the mean annual precipitation 521 mm. The inorganic N deposition rate in the region is 0.3 g m⁻² y⁻¹ (36).

The peatlands, 0.5-1.5 ha in size, have developed in depressions between sand dune formations with similar underlying soil (18, 37, 38). Along the chronosequence, the vegetation changes from flooded Carex nigra and Agrostis canina dominated meadows in the early stages to Carex-dominated mesotrophic and oligotrophic fens and to fen-bog transitions where the bog vegetation is characterized by an increasing cover of dwarf shrubs in hummock surfaces. The moss layer in the meadows is sparse (total moss cover 20-70%), and the N:P ratio is typically 15-40, indicating P limitation (32). The moss layer in the meadows is sparse (total moss cover 20-70%), and the N:P ratio is typically 15-40, indicating P limitation (32).

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In all, our findings imply that interlinks of N₂ fixation and CH₄ cycling constitute an important mechanism that fulfils the need of new N and explains the rapid peat and N accumulation rates during the fen stages of peatland development. Our results further support the idea that N₂ fixation is the primary mechanism linking N and P availability, as suggested for terrestrial and aquatic ecosystems (e.g., refs. 31 and 33). Both the N₂ fixation rates and the methanotrophic contribution to N₂ fixation along the peatland succession gradient were primarily governed by P availability. The successional patterns of different microbial groups involved in N₂ fixation were further regulated by water table level and Fe content, which controlled both the N₂ fixation and CH₄ oxidation activities of the plant-associated bacteria. In conclusion, moss-associated microbes may explain the dominance of Sphagnum mosses in nutrient-poor peatlands: besides providing an additional in-cell CO₂ source for photosynthesis, these microbial communities provide new N in the cold and acidic environment where decomposition and nutrient recycling are otherwise slow.

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Field Experiment. In early June 2010, we collected the dominant Sphagnum mosses from both flark and hummock habitats at each site. Moss sampling was based on vegetation maps at which the Sphagnum were dried at 60 °C for 48 h and weighed to convert the incubation results to N₂ fixation and CH₄-C biomass incorporation (CH₄ oxidation) rates per peatland surface area (m²). A subset of plots (n = 1 in each stage) was surveyed for the relative proportions of flark and hummock habitats by measuring the water table depth in a network of 8–12 wells. Phosphorus, Fe, and Mo (below detection limit) contents in the tissue of the incubated Sphagnum samples were extracted with NH₄OH by using wet digestion (EPA-3051) and measured by using a plasma emission spectrometer (ICP-OES, IRIS Intrepid II XSP).

Data Analyses. The rates of N₂ fixation and CH₄ oxidation measured as CH₄-C incorporation in to the most biomass were calculated as enrichment values and thereafter as fixation rates (nmol g⁻¹ of dry moss-1 h⁻¹) by following ref. 8. The values represent the actual ¹⁵N and ¹³C uptake in each sample during the 45-h incubation. The rates of N₂ fixation, CH₄-induced N₂ fixation, and CH₄-C biomass incorporation were natural-log (ln) transformed to meet the requirements of normality. The effects of terrestrial age and treatments on N₂ fixation rates were analyzed with four-way nested analysis of covariance (ANCOVA) with successional stage, site nested within the successional stage, light treatment, and CH₄ treatment as the four factors. Water table depth was used as a covariate to take into account microtopography. The effects of terrestrial age and light on the rates of biomass incorporation of CH₄-derived C were analyzed with three-way nested ANCOVA with successional stage, site nested within the successional stage and light treatment as the three factors and water table depth as a covariate. Pair-wise differences in successional stages were tested by using Bonferroni post hoc tests. The relationships between environmental variables (nutrient contents, water table depth, water pH, and CH₄ concentration) and N₂ fixation, CH₄-induced N₂ fixation, and the biomass incorporation of CH₄-derived C were analyzed by using Spearman rank correlations and stepwise regressions.

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