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# Functional redundancy of the methane-oxidising and nitrogen-fixing microbial community associated with *Sphagnum fallax* and *Sphagnum palustre* in two Dutch fens

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## SUMMARY

*Sphagnum* mosses are adapted to thrive in cool, nutrient-poor acidic environments. Nitrogen (N<sub>2</sub>)-fixing and methane (CH<sub>4</sub>)-oxidising microorganisms living as epiphytes and endophytes on and in *Sphagnum* mosses are known to support *Sphagnum* growth. We explored how these processes combined with the microbial community link to different *Sphagnum* moss species, sites and moss segments (capitulum or shoot), using *Sphagnum palustre* and *Sphagnum fallax* sampled from two field sites and separated into upper (capitulum; 0–3 cm) and lower (shoot; 3–6 cm) segments. The results of 16S rRNA gene amplicon sequencing showed that the microbial community composition is both site- and moss-specific. The microbial activity, measured as <sup>15</sup>N-N<sub>2</sub> fixation ( $6.5 \pm 0.9 \mu\text{mol g}^{-1} \text{d}^{-1}$ ) and <sup>13</sup>C-CH<sub>4</sub> oxidation ( $0.5 \pm 0.1 \mu\text{mol g}^{-1} \text{d}^{-1}$ ), differed between field sites but not between moss species. The CH<sub>4</sub> oxidation was higher in the upper moss segments. On the basis of these results we hypothesise that the discrepancy between microbial activity and microbial community composition at moss-species level may be caused by functional redundancy; although microbial communities vary between *Sphagnum* species, they can function similarly when the mosses are growing in comparable environments.

**KEY WORDS:** methane oxidation, microbial community, nitrogen fixation, *Sphagnum* moss, wetland

## INTRODUCTION

The genus *Sphagnum* is an ancient and intriguing group of mosses, also known as the ‘peat mosses’. *Sphagnum* mosses are ecosystem engineers that can maintain harsh, wet, anaerobic, acidic conditions and accumulate partially decomposed organic matter *in situ* (Clymo 1963, Clymo & Hayward 1982, Rydin & Jeglum 2013). Thus, they are major contributors to the build-up of carbon in *Sphagnum*-dominated peatlands. They also rely partly on their microbiome for nutrition and protection (Kostka *et al.* 2016). Microorganisms are present as epiphytes on the outside of the *Sphagnum* plant and as endophytes living inside the water-holding hyaline cells. The *Sphagnum* microbiome is highly host-specific (Opelt *et al.* 2007) and remains so throughout the whole life cycle of the plant (Bragina *et al.* 2012a).

Most *Sphagnum*-dominated peatlands occur in the Northern Hemisphere and receive limited nutrient inputs (Rydin & Jeglum, 2013). Under these conditions, nitrogen (N) fixing microorganisms are an important source of biologically available N. Such microorganisms use the nitrogenase enzyme to fix

atmospheric N<sub>2</sub> as NH<sub>4</sub><sup>+</sup>, which they subsequently incorporate into amino acids. N<sub>2</sub> fixing microorganisms may associate with many species of moss, especially those living under nutrient-limited conditions (Holland-Moritz *et al.* 2018). In boreal forests dominated by feather mosses, cyanobacteria typically actively renew the N pool (Stewart *et al.* 2011, Leppänen *et al.* 2013, Rousk *et al.* 2013, Rousk *et al.* 2014). In *Sphagnum* mosses, N<sub>2</sub>-fixing methane (CH<sub>4</sub>)-oxidising microorganisms and heterotrophic diazotrophs, rather than cyanobacteria, are thought to be the most significant contributors to the N pool (Larmola *et al.* 2014, Vile *et al.* 2014, Ho & Bodelier, 2015). However, methanotrophy and N<sub>2</sub> fixation do not always seem to be correlated (Carrell *et al.* 2019, Kox *et al.* 2018, Leppänen *et al.* 2015, Warren *et al.* 2017).

While *Sphagnum*-dominated peatlands have an enormous potential to store carbon, they can also be sources of the greenhouse gas CH<sub>4</sub>. The emission of CH<sub>4</sub> is greatly reduced by the activity of CH<sub>4</sub>-oxidising microorganisms, which oxidise CH<sub>4</sub> to CO<sub>2</sub>. Methane-oxidising bacteria (MOB) use O<sub>2</sub> to convert CH<sub>4</sub> into CO<sub>2</sub>, and thereby also support the C

requirements of *Sphagnum* mosses, especially in submerged conditions where CO<sub>2</sub> is limiting (Raghoebarsing *et al.* 2005, Kip *et al.* 2010). MOB in and on *Sphagnum* mosses typically show higher activity immediately below the capitulum (Raghoebarsing *et al.* 2005, Kip *et al.* 2010, Van Winden *et al.* 2012). This often corresponds to the water table, which is an important factor controlling MOB activity because it partially dictates the availability of CH<sub>4</sub> and O<sub>2</sub>.

The location of N<sub>2</sub>-fixing microorganisms combined with their activity in and on *Sphagnum* mosses is less clear. The *Alphaproteobacteria* (of which many are capable of N<sub>2</sub> fixation) have been located in and on *Sphagnum* mosses, without an evident pattern, by using fluorescence *in situ* hybridisation (FISH) combined with confocal microscopy (Bragina *et al.* 2012a,b). In terms of preferred conditions for N<sub>2</sub>-fixing microorganisms, one study reported N<sub>2</sub> fixation to be higher in submerged conditions (Leppänen *et al.* 2015). This finding is not surprising, given the fact that O<sub>2</sub> is a notorious inhibitor of the nitrogenase enzyme. Subsequent studies have shown that the activity of the N<sub>2</sub>-fixing community associated with *Sphagnum* mosses is higher in O<sub>2</sub> depleted conditions (Warren *et al.* 2017, Kox *et al.*, 2018).

In addition to O<sub>2</sub> availability, other factors controlling N<sub>2</sub> fixation in *Sphagnum* mosses have been studied, but the results are ambiguous. In most cases, N<sub>2</sub> fixation activity in *Sphagnum* is negatively correlated with atmospheric N deposition (Kox *et al.* 2016, Leppänen *et al.* 2013, Rousk & Michelsen 2016) and influenced more by the environmental conditions at a specific site than by the species of *Sphagnum* moss (Bragina *et al.* 2012b, Leppänen *et al.* 2015, Gavazov *et al.* 2010). A recent study by Van den Elzen *et al.* (2017) postulated that *Sphagnum* mosses and their N<sub>2</sub>-fixing partners differ significantly in their optimal growth conditions. *Sphagnum* actively acidifies its surroundings, whereas N<sub>2</sub> fixation rates are higher in more neutral conditions (Van den Elzen *et al.* 2017). This discrepancy in optimal conditions for host and microbe might explain the ambiguity in factors controlling N<sub>2</sub> fixation. Furthermore, the comparison of N<sub>2</sub> fixation rates between different sites has proved problematic in most studies because different *Sphagnum* species were collected from different sites (Leppänen *et al.* 2015, Larmola *et al.* 2014).

To further investigate the functioning of the N<sub>2</sub>-fixing and CH<sub>4</sub>-oxidising microbial community associated with *Sphagnum* mosses, we studied N<sub>2</sub>-fixing and CH<sub>4</sub>-oxidising activity in two *Sphagnum* species (*S. fallax* and *S. palustre*) obtained from two

different sampling sites in the Netherlands. In addition, we analysed the microbial community of these mosses to investigate if the microbial composition of these mosses is site or species specific.

## METHODS

### Study sites

Fochteloërveen (53.005562 °N, 6.391979 °E; hereafter FV) is a restored bog system with wet depressions covered by dense *Molinia caerulea* that consist of an intermixed community of *S. fallax* and *S. palustre*. Ilperveld (hereafter IV) comprises a series of restored peatlands of which three were sampled (Ilperveld 1: 52.445126 °N, 4.925524 °E; Ilperveld 2: 52.442592 °N, 4.93250 °E; Ilperveld 3: 52.445675 °N, 4.944066 °E). All three subsites in Ilperveld were dominated by an intermixed vegetation of *S. fallax* and *S. palustre*. The first two sites were dominated by *Phragmites australis* growing with *Juncus effusus*, *Hydrocotyle vulgaris*, *Carex* spp. and *Polytrichum* species. The third site had multiple *Carex* species along with *Typha latifolia*, *Hydrocotyle vulgaris* and *Polytrichum* spp.

### *Sphagnum* sampling

*S. fallax* and *S. palustre* were sampled in mid-September 2015 from patches in a wet depression at FV and at IV 1, 2, and 3. The mosses from the three patches were combined into one sample (~100 g fresh weight) to reduce microhabitat specific variance and kept cool (4 °C) for three days in the dark until further handling. In the laboratory, mosses were washed three times with demineralised H<sub>2</sub>O and divided into different segments to be used for activity assays and DNA extraction: the chlorophyll-containing top segment (0–3 cm; hereafter referred to as capitulum (cap)) and the white-brown, lower shoot part and associated leaves of the moss (3–6 cm; hereafter referred to as shoot).

### pH and pore water measurements

Pore water sampling took place in mid-September 2015. Pore water and pH analyses were performed to assess the abiotic differences between the sites. Within each site the pH was measured, and pore water samples were taken using Rhizons (pore size 2 µm; 10 cm length). Pore water (10 ml) was acidified with HNO<sub>3</sub> and concentrations of Al, Ca, Fe, K, Mg, Mn, Na, P, S, Si, and Zn were measured using Inductively Coupled Plasma Emission Spectroscopy (ICP-OES iCAP 6000, Thermo Fisher Scientific). Concentrations of NH<sub>4</sub><sup>+</sup>, NO<sub>3</sub><sup>-</sup>, PO<sub>4</sub><sup>3-</sup> were analysed colorimetrically using an AutoAnalyzer 3

system (Bran & Luebbe GmbH, Germany) using hydrazine sulphate (Kamphake *et al.* 1967), salicylate (Grasshoff & Johannsen 1972) and ammonium molybdate (Henriksen 1965), respectively. Concentrations of  $\text{Cl}^-$  were also determined using the autoanalyser, with addition of a Sherwood 420 flame photometer (Bran & Luebbe, Norderstedt, Germany).

#### Activity assays for methane oxidation and nitrogen fixation

$\text{CH}_4$  oxidation and  $\text{N}_2$  fixation activity were assessed by measuring incorporation of  $^{13}\text{C}\text{-CH}_4$  and  $^{15}\text{N}\text{-N}_2$ . Assays were performed using a final concentration of 5%  $^{13}\text{C}\text{-CH}_4$  and 10%  $^{15}\text{N}\text{-N}_2$  in the headspace. Mosses were incubated in 40 ml flasks in triplicate, with capitula or shoot segments from four individual moss plants per assay. Natural isotope abundance determination ( $^{13}\text{C}$  and  $^{15}\text{N}$ ) was performed in duplicate. All incubations were performed at room temperature with 16 h light and 8 h dark. After 48 hours, the incubations were stopped and mosses were oven dried for 72 hours at 70 °C. The dried samples were grinded for 2.5 minutes at 30 Hz with a stainless-steel bead ( $\phi$  5 mm) using a Retsch MM200 ball mill (Retsch, Haan, Germany).

Next, samples were combusted by flash combustion (1800 °C) on a CNS analyzer (EA 1110, Carlo Erba Thermo Fisher Scientific, Waltham, MA, USA) coupled to an Isotopic Ratio Mass Spectrometer (Finnigan DeltaPlus, interface Conflo III, Thermo Electro GmbH, Bremen Germany). Background levels of  $^{15}\text{N}$  and  $^{13}\text{C}$  were uniform, average  $\Delta^{15}\text{N}/^{14}\text{N}$  (‰) was  $0.365 \pm 0.001$  and average  $\Delta^{13}\text{C}/^{12}\text{C}$  (‰) was  $1.076 \pm 0.003$ . All activity rates are expressed per gram dry weight plant material.

#### DNA isolation

*Sphagnum* samples were crushed in liquid nitrogen using pestle and mortar, after which DNA was extracted with two different methods to reduce DNA extraction bias. DNA was extracted using the FastDNA™ SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA, USA) following manufacturers protocol using 0.5 g of crushed biomass. In addition, DNA was also extracted from 1 g of crushed biomass using the phenol extraction method combined with ethanol precipitation, as described in Kip *et al.* (2010). DNA concentrations were determined using dsDNA HS Assay Kit of the Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA). Prior to PCR reactions, the obtained DNA was diluted to 0.5 ng/ $\mu\text{l}$  and afterwards DNA of each extraction method was pooled. The purity of the sample was assessed with the NanoDrop 1000 (Isogen Life Science, De Meern, the Netherlands).

#### Gene amplification and sequencing

The bacterial 16S rRNA V3-V4 region was amplified using a two-step PCR protocol and sequenced using Ion Torrent PGM™ technology (Thermo Fisher scientific, USA) as described by Kox *et al.* (2018). The amplicon libraries were loaded on either Ion 316™ v2 Chips or Ion 318™ v2 Chips and sequencing was performed to a minimum of 40,000 reads per sample according to the Ion PGM™ Hi-Q™ Sequencing Kit User Guide with an Ion Torrent Personal Genome Machine™ (Thermo Fisher Scientific, USA), for 850 nucleotide flows. In total 838,482 reads were obtained for 16 samples, with more than 40,000 reads per sample

#### Sequence analysis

The 16S rRNA gene sequences were analysed with the software Mothur (v.1.36.1; Schloss *et al.* 2009) and the Mothur 454 SOP (Schloss *et al.* 2011) as described in Kox *et al.* (2018). The quality filtering steps reduced the number of reads to a total of 383,450 prior to OTU clustering (see Table A1 in the Appendix for an overview of read processing). Using the average clustering algorithm, reads were clustered at 97% identity level and singletons were subsequently removed. Sequences were classified using the SILVA database v123 (Quast *et al.*, 2013), after which non-target sequences were removed (Chloroplasts, Mitochondria, unknown, Archaea and Eukaryota). All sequencing data have been deposited in the NCBI SRA database, project number PRJNA517516.

#### Sequence data processing and statistical analysis

The output was analysed with R version 3.5.1 (R Core Team, 2018) and Rstudio v1.1.456 (RStudio Team, 2016) using the packages Phyloseq (McMurdie & Holmes, 2013) and vegan (Oksanen *et al.*, 2016). Read libraries were rarefied by random subsampling (seed: 12345) to 5,110 reads per sample (rarefaction curves, see Figure A1 in the Appendix). Statistical analyses were also performed in R version 3.5.1 (R Core Team, 2018) and Rstudio v1.1.456 (RStudio Team, 2016). Normality of the data was tested using a Shapiro-Wilk test (stats-package). Homogeneity of variances between groups was tested using Levene's test (car package). Data that were not normally distributed were transformed by reciprocal transformation ( $1/X_i$ ). Differences in pore water elemental composition between sites were tested using an independent t-test. For the activity of  $\text{CH}_4$  oxidation and  $\text{N}_2$  fixation, differences between *Sphagnum* species (*S. fallax*, *S. palustre*) and segments (capitulum, shoot) were tested using a two-way factorial ANOVA. A type III ANOVA was first

used to test for interactions, and if no interactions were found a type II ANOVA was applied. Differences in activity between sites (FV and IV) and *Sphagnum* species were tested using the same approach.

The microbial community diversity was studied by calculating a variety of alpha diversity metrics, namely species diversity (Shannon index), species richness (observed richness and Chao1) and community evenness (Pielou's J), using the vegan package. Dissimilarity between the microbial communities in different samples was explored by calculating a Bray-Curtis dissimilarity matrix and subsequently visualising by ordination based on Non-Metric Multidimensional Scaling (NMDS). Goodness of fit of the ordination was examined by inspecting the stress on the ordination via a Shepard plot. To determine which factor explained most of the observed dissimilarity, we used non-parametric permutational analysis of variance with 999 permutations.

## RESULTS

### Pore water element concentrations

We measured the concentrations of various elements in pore water from both sites. For IV and FV the concentrations of  $\text{PO}_4^{3-}$  (resp. 3.7 vs 0.2  $\mu\text{mol L}^{-1}$ ) and of  $\text{NO}_3^-$  (resp. 0.76 vs 0.02  $\mu\text{mol L}^{-1}$ ) were significantly higher in IV compared to FV (resp.  $\text{PO}_4^{3-}$   $t_{(2,78)} = -6.06$ ,  $p < 0.01$ ;  $\text{PO}_4$   $t_{(2,47)} = -9.54$ ,  $p < 0.01$ ;  $\text{NO}_3^-$   $t_{(2,19)} = -6.79$ ,  $p < 0.05$ ; see Tables A2 and A3). This trend was also observed for the concentrations of sodium ( $\text{Na}^+$  resp. 1365 vs 271  $\mu\text{mol L}^{-1}$ ), potassium ( $\text{K}^+$  resp. 21.4 vs 127  $\mu\text{mol L}^{-1}$ ;  $t_{(2,49)} = -2.83$ ,  $p = 0.08$ ) and ammonium ( $\text{NH}_4^+$ ; resp. 5 vs 1  $\mu\text{mol L}^{-1}$ ;  $t_{(2,97)} = -2.72$ ,  $p = 0.07$ ). The differences indicate that IV is more nutrient rich than the FV. Only the iron (Fe, FV vs IV resp. 198 vs. 8  $\mu\text{mol L}^{-1}$ ) and Zn concentrations (resp. 9.6 vs. 2.1  $\mu\text{mol L}^{-1}$ ) were higher in FV compared to IV.

### CH<sub>4</sub> oxidation activity

CH<sub>4</sub> oxidation rates were similar for the two investigated *Sphagnum* species. Within each species, CH<sub>4</sub> oxidation activity was higher in the capitula ( $0.53 \pm 0.1 \mu\text{mol g}^{-1} \text{day}^{-1}$  (mean  $\pm$  SEM), see Figure 1A) than in the shoots ( $0.18 \pm 0.1 \mu\text{mol g}^{-1} \text{day}^{-1}$ ;  $F_{(1,44)} = 20.87$ ,  $p < 0.001$ ). Between sites, the activity was higher at FV ( $0.65 \pm 0.1 \mu\text{mol g}^{-1} \text{d}^{-1}$ , see Figure 2A) than at IV ( $0.25 \pm 0.04 \mu\text{mol g}^{-1} \text{d}^{-1}$ ;  $F_{(1,44)} = 15.48$ ,  $p < 0.001$ ).

### N<sub>2</sub> fixation activity

Like CH<sub>4</sub> oxidation activity, N<sub>2</sub> fixation activity did not differ between *S. fallax* and *S. palustre*. The N<sub>2</sub> fixation activity was approximately ten times the CH<sub>4</sub> oxidation activity (Figures 1 and 2). In contrast to CH<sub>4</sub> oxidation activity, N<sub>2</sub> fixation activity did not differ between the different moss segments (see Figure 1B). N<sub>2</sub> fixation activity differed only between sites ( $F_{(1,44)} = 8.11$ ,  $p < 0.01$ ), being higher in FV ( $6.5 \pm 0.9 \mu\text{mol g}^{-1} \text{day}^{-1}$ , see Figure 2B) than in IV ( $4.4 \pm 0.2 \mu\text{mol g}^{-1} \text{day}^{-1}$ ).

### Microbial community composition

The microbial community was dominated by *Proteobacteria* and *Acidobacteria* with subpopulations of *Verrucomicrobia*, *Planctomycetes*, *Actinobacteria*, *Bacteroidetes*, *Chlamydiae*, *Cyanobacteria* and the candidate phyla *Patescibacteria* and *WPS-2* (Figure 3). *Verrucomicrobia*, *Actinobacteria* and *Planctomycetes* had relatively high abundance in the shoot segment compared to the capitulum. The relative abundance of *Proteobacteria* was highest in the capitulum. Most (~91%) of the *Proteobacteria* consisted of *Alphaproteobacteria* (see Figure 4). It is apparent that *Alphaproteobacteria* were more abundant in mosses from IV than in mosses from FV. The most abundant order within the *Alphaproteobacteria* was the mostly diazotrophic *Acetobacterales*.

Looking at microbial relative abundance overall (Figure 3), the proposed Patescibacterial phylum and the *Bacteroidetes* were more abundant in *S. palustre* than in *S. fallax*. Finally, at site level, the microbial community associated with mosses from FV showed high relative abundance of *Verrucomicrobia* and *Planctomycetes* when compared to the community from IV (Figure 3).

### Microbial diversity

Microbial diversity was calculated for the rarefied dataset using the Good's coverage estimator, which showed that this dataset covered the original diversity well (>94% in all samples; Tables A4 and A5). The microbial community associated with *S. palustre* was marginally more diverse in terms of species than the microbial community associated with *S. fallax* (Shannon index, see Table A4). Additionally, the microbial community of *S. palustre* was more even in composition. For both *Sphagnum* species a more diverse microbial community was associated with shoot segments than with capitula (Shannon diversity index *S. fallax* shoot  $4.2 \pm 0.5$ ; capitulum  $3.8 \pm 0.5$ ; *S. palustre* shoot  $4.8 \pm 0.3$ ; capitulum was  $4.3 \pm 0.3$ ; Table A4). Also, microbial species richness was

higher in shoot segments (observed richness for *S. fallax* shoot  $510 \pm 101$  and *S. palustre* shoot  $578 \pm 59$ ; Table A4) than in capitula (resp.  $393 \pm 93$  and  $475 \pm 50$ ).

Comparing microbial diversity between the two sites, it was evident that the microbial community associated with mosses from FV had higher microbial diversity and the most even microbial community composition (see Table A5).

### Community similarity

The low stress of the NMDS ordination ( $<0.05$ ; see Figure 5) indicated that it is a good representation of the dissimilarity originally observed between the samples. The main factors explaining microbial

community dissimilarity between the different samples were site and *Sphagnum* species (non-parametric permutational analysis of variance for Site  $F_{(1,8)} = 8.78$ ,  $p < 0.01$  and *Sphagnum* spp.  $F_{(1,8)} = 3.50$   $p < 0.01$ , see supplementary Table A6). Moss segments appeared to have a minor effect on the observed dissimilarities.

## DISCUSSION

### Site-specific microbial community composition and activity

Analysis of the microbial community composition based on 16S rRNA gene sequencing revealed a

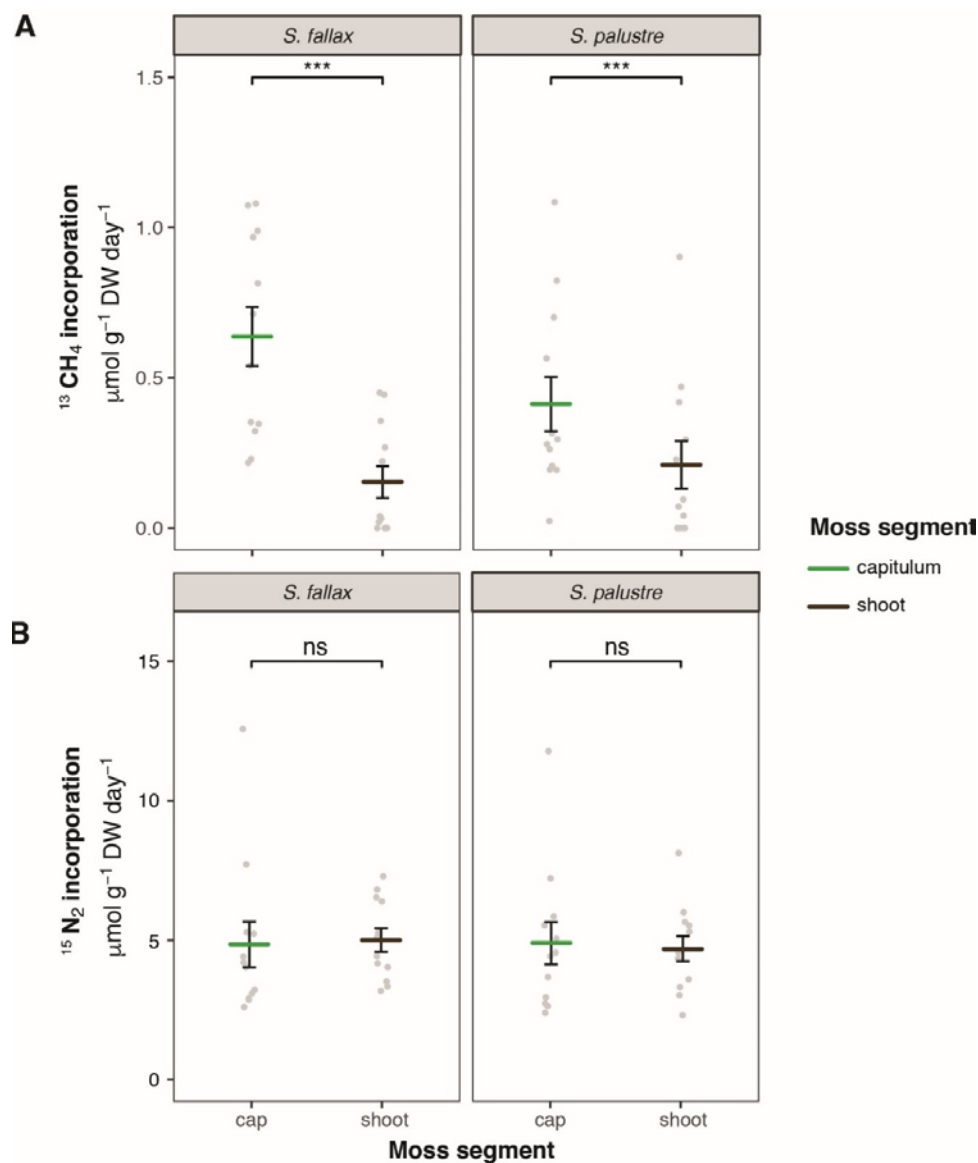


Figure 1. A: Methane oxidation activity ( $^{13}\text{C}$ - $\text{CH}_4$  incorporation rate); and B: Nitrogen fixation ( $^{15}\text{N}$ - $\text{N}_2$  incorporation rate) for both *Sphagnum fallax* and *S. palustre* segments capitulum (green) and stem (brown). Significant differences are indicated as follows \*\*\*  $p < 0.001$ ; \*\*  $p < 0.01$ ; \*  $P < 0.05$ ; ns = not significant.

typical microbial community profile associated with *Sphagnum* mosses as observed by others (Bragina *et al.* 2012a, Bragina *et al.* 2014, Holland-Moritz *et al.* 2018, Kox *et al.*, 2018, Carrell *et al.* 2019). Overall, site was the dominant factor determining the differences in both N<sub>2</sub> fixation and CH<sub>4</sub> oxidation activity as well as in microbial community composition. The sites were mainly characterised by differences in nutrient availability and pH. The high activity of both processes as well as the higher microbial diversity at FV could be explained by the limited amount of nutrients available there (Bragazza *et al.* 2005, Bodelier 2011, Leppänen *et al.* 2013, Kox *et al.* 2018). The abiotic factors pH and pore water

elemental concentration also differed greatly between the sites. As abiotic factors contribute greatly to energy constraints encountered by microorganisms in peatlands (Andersen *et al.* 2013), it is most likely that the differences in abiotic factors contributed strongly to the differences in both microbial activity and community composition between the two study sites.

### Moss segments

Higher microbial diversity was observed in the capitula. The uppermost 3 cm of the *Sphagnum* moss carpet are often the most dynamic zone, which may explain the high microbial diversity.

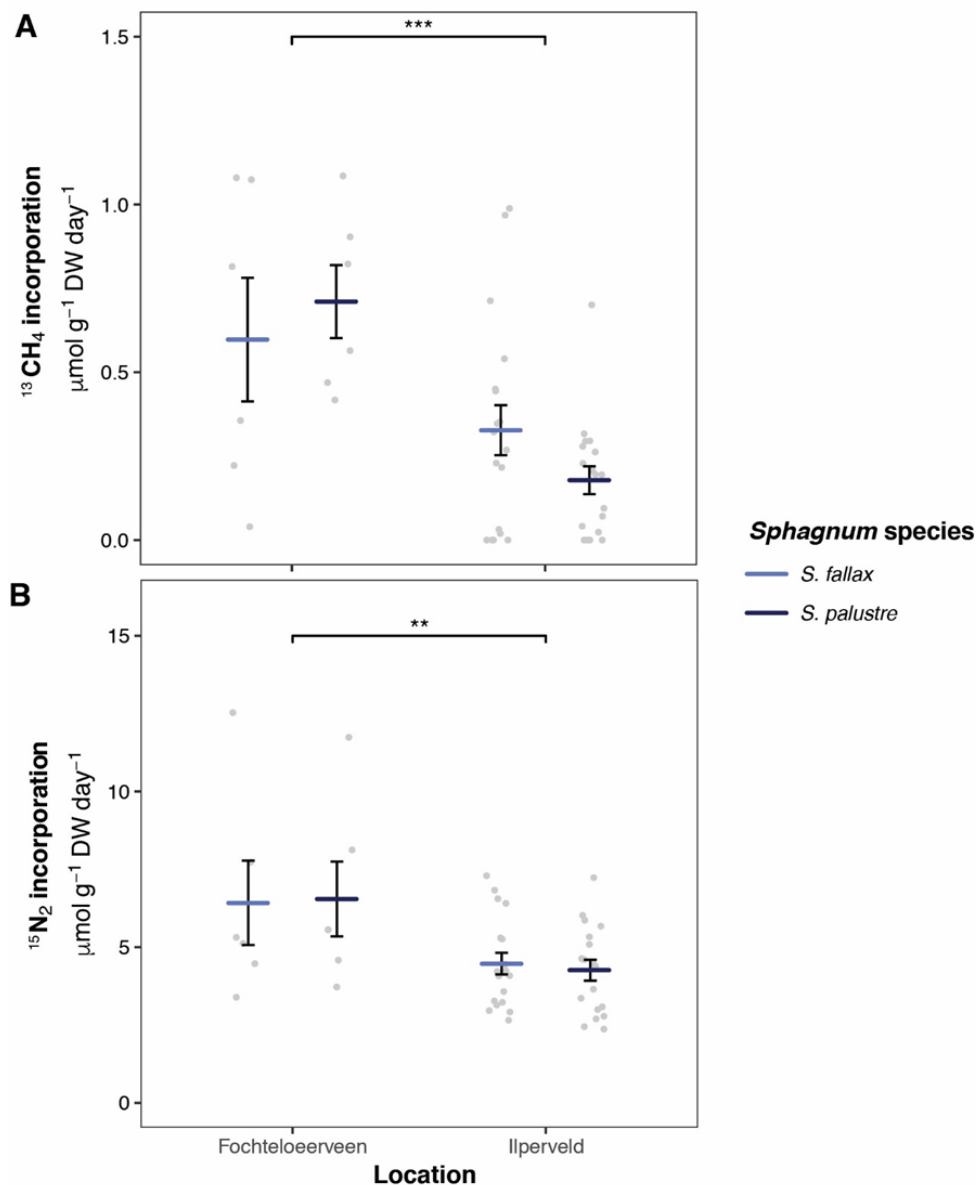


Figure 2. A: Methane oxidation activity (<sup>13</sup>C-CH<sub>4</sub> incorporation rate); and B: Nitrogen fixation (<sup>15</sup>N-N<sub>2</sub> incorporation rate) for *Sphagnum fallax* (light blue) and *S. palustre* (dark blue) indicated per site (FV and IV). Significant differences are indicated as follows \*\*\* p<0.001; \*\* p<0.01; \* P<0.05; ns = not significant.

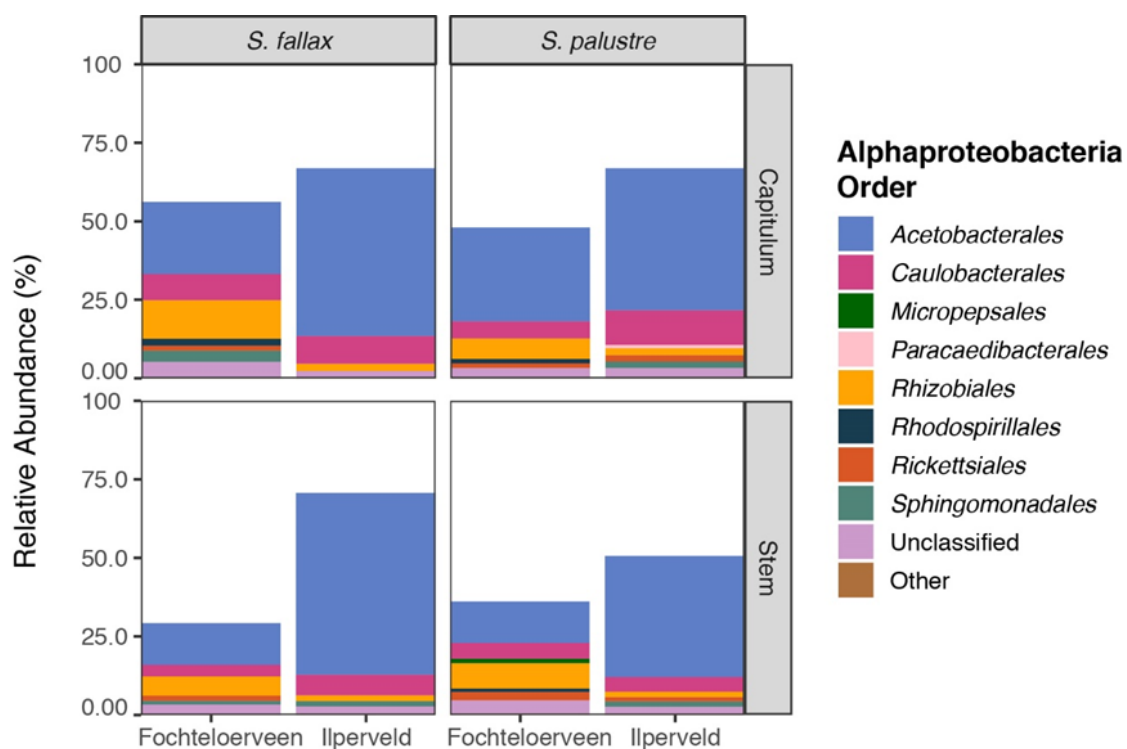


Figure 3. The relative abundance of all phyla (only phyla that make up >1% of the total community are shown) in the microbial community associated with the different *Sphagnum* segments capitulum (upper 2 panels) and stem (lower 2 panels) from the two *Sphagnum* species studied; *S. fallax* (left) and *S. palustre* (right) in the different sites (FV and IV).

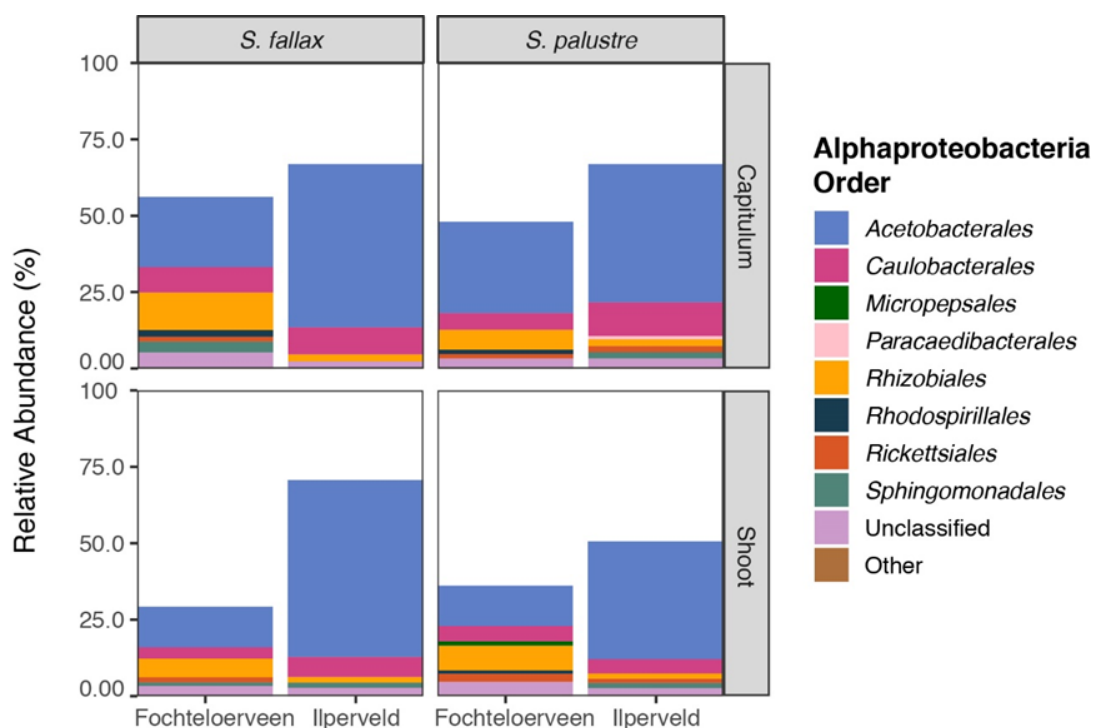


Figure 4. The relative abundance of all Alphaproteobacteria (only order levels that make up >1% of the total community are shown) in the microbial community associated with the different *Sphagnum* segments capitulum (upper 2 panels) and stem (lower 2 panels) from the two *Sphagnum* species studied; *S. fallax* (left) and *S. palustre* (right) in the different sites (FV and IV).



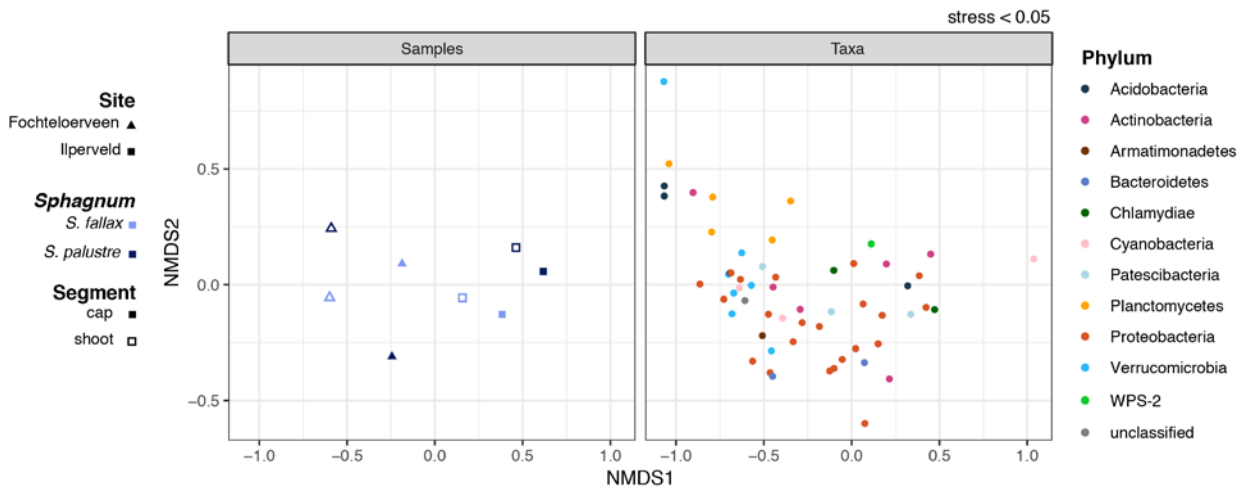


Figure 5. Split plot of the NMDS ordination (based on Bray-Curtis dissimilarity matrix; stress <math>< 0.05</math>) of the samples (left) and the corresponding taxa at phylum level (right).

The  $\text{CH}_4$  oxidation activity differed between *Sphagnum* segments, with higher activity in the capitula. This is in line with previous studies, where higher  $\text{CH}_4$  oxidation rates were observed just below the capitulum, at the level of the water table (Raghoebarsing *et al.* 2005, Kip *et al.* 2010, Van Winden *et al.* 2012). The location of methanotrophic activity in the uppermost 3 cm of *Sphagnum* shoots is beneficial to the plants, as *Sphagnum* has only a capillary system for transport of water and nutrients (poikilohydric character) (Rydin & Jeglum 2013) and, thus, limited capability for transport of nutrients in comparison with vascular plants. Most of the transport is directed upwards towards the capitulum (Rydin & Clymo 1989). As the top 3 cm of *Sphagnum* is the most actively growing segment (Clymo 1970), the production of additional  $\text{CO}_2$  by methanotrophs is most beneficial here. This is especially important in submerged conditions where diffusion of  $\text{CO}_2$  from the atmosphere into the water column limits  $\text{CO}_2$  availability (Raghoebarsing *et al.* 2005, Kip *et al.* 2010).

Interestingly,  $\text{N}_2$  fixation activity was similar in both moss segments. Previous studies have shown that part of the active  $\text{N}_2$ -fixing community consists of light-dependent cyanobacteria (Stewart *et al.* 2011, Berg *et al.* 2012) and  $\text{CH}_4$ -oxidizing microorganisms (Larmola *et al.* 2014, Vile *et al.* 2014). Therefore, we hypothesised that  $\text{N}_2$  fixation rates would be highest in the capitulum segment, corresponding with both the higher  $\text{CH}_4$  oxidation rates found there and the energy demand of cyanobacteria (the capitulum being the segment that receives most light). One explanation for the similarity of  $\text{N}_2$  fixation rates in both moss segments

might be that  $\text{N}_2$  fixation was limited by the ambient oxygen levels used in the incorporation assays. Oxygen has been shown to limit  $\text{N}_2$  fixation in previous studies (Warren *et al.* 2017, Kox *et al.* 2018). In the field, the moss capitula form a tight carpet that might result in lower  $\text{O}_2$  levels just below the capitulum. Furthermore, light was limiting for the moss shoots in their natural habitats but not in our laboratory incubations, so cyanobacteria on the shoots may have been more active in the laboratory than they would be under natural conditions.

The  $\text{N}_2$  fixation and  $\text{CH}_4$  oxidation rates we observed were comparable to rates found in other studies (Larmola *et al.* 2014, Kox *et al.* 2016, Van den Elzen *et al.* 2017, Warren *et al.* 2017, Putkinen 2018, Van den Elzen *et al.* 2018), although the  $\text{CH}_4$  oxidation rates were at the lower end of the range measured by Kip *et al.* (2010). Previous work has shown a tight link between  $\text{CH}_4$  and nitrogen cycling in *Sphagnum*-dominated peatlands (Larmola *et al.* 2014; Vile *et al.* 2014), but  $\text{N}_2$  fixation was approximately ten times the  $\text{CH}_4$  oxidation in this study.  $\text{N}_2$  fixation is an energetically costly process (Houlton *et al.* 2008, Vitousek & Field 1999) that often becomes inactive as soon as the N:P ratio in the environment increases (Vitousek *et al.* 2002, Larmola *et al.* 2014, Kox *et al.* 2016, Van den Elzen *et al.* 2017). A methanotroph requires fairly high  $\text{CH}_4$  oxidation activity to sustain an  $\text{N}_2$  fixing lifestyle, and only then can methanotrophy and diazotrophy co-occur. The comparatively low  $\text{CH}_4$  oxidation rates measured in our study make it highly questionable whether methanotrophic diazotrophs were the main contributors to  $\text{N}_2$  fixation in this case.

### Moss-specificity and functional redundancy

The microbial community was not only site-specific, but also host-specific. *S. palustre* had a more diverse microbial community than *S. fallax*. This is in line with previous studies, in which the *Sphagnum*-associated microbial community was found to be host-specific independently of geographical location (site). These studies used various techniques (SSCP fingerprinting and amplicon sequencing) and targeted the total microbial community (16S rRNA gene) as well as specific groups (*nifH*) (Opelt *et al.* 2007, Bragina *et al.* 2011a, Bragina *et al.* 2013). However, none of these studies combined the analysis of microbial community composition with activity assays, as in our study.

We found that microbial activity for N<sub>2</sub> fixation and CH<sub>4</sub> oxidation was non-specific for moss species. In contrast, microbial community composition was moss-specific. The diversity of the microbial community associated with *S. palustre* was more even, compared to the community of *S. fallax*. Both *Patescibacteria* and *Bacteroidetes* were more abundant in *S. palustre*. Although the abiotic factors mentioned above might explain site-specificity, they do not explain host-specificity. Since the mosses were sampled from the same intermixed communities, abiotic factors were highly similar and thus variance was kept to a minimum, providing ideal conditions for identifying moss-specific traits affecting microbial activity and microbial community composition.

Therefore, we hypothesise that the discrepancy in host specificity between microbial community composition (specific) and microbial activity (non-specific) is due to functional redundancy in *Sphagnum*-associated N<sub>2</sub> fixation and CH<sub>4</sub> oxidation. In other words, different organisms may perform the same functions (Louca *et al.* 2018). Hence, the microbial community can differ taxonomically between the *Sphagnum* hosts without affecting its functions (N<sub>2</sub> fixation and CH<sub>4</sub> oxidation). Our hypothesis is supported by previous work reporting functional redundancy as a potentially important controlling mechanism for the diversity and functioning of microbial communities in peatlands (Andersen *et al.* 2013). Follow-up studies with intensified sampling would be required to determine the degree to which the processes investigated here are functionally redundant. In particular, more research on functional redundancy in *Sphagnum*-associated CH<sub>4</sub> oxidation could improve our understanding of the sensitivity of the *Sphagnum* CH<sub>4</sub> biofilter of peatlands to climate change (Putkinen 2018).

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### AUTHOR CONTRIBUTIONS

Martine Kox's role in the manuscript was conceptualisation, investigation, methodology, formal analysis, data curation, visualisation, writing and editing original draft. The role of Linnea Kop and Eva van den Elzen was conceptualisation, investigation, methodology and formal analysis and editing original draft. The contribution of Theo van Alen was on methodology and formal analysis and editing the original draft. Leon Lamers participated in conceptualisation, resources, editing and reviewing and supervision. Mike Jetten's role was in conceptualisation, funding acquisition and resources, editing and reviewing and supervision. The role of Maartje van Kessel was conceptualisation, editing and reviewing and supervision. None of the authors have competing interests in the manuscript.

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## Appendix

Table A1. Step-by-step overview of sequence read processing.

Step		Total read count	# unique sequences	Mean read length (bp)
1	Unprocessed reads	838482		
2	Screen seqs	383450	383450	
3	Unique sequences	383450	356835	350.4
4	Align sequences	383450	356835	341.2
5	Screen seqs	356320	331510	349.6
6	Filter positions in alignment	356320	164517	217.8
7	Pre-clustering	356320	77336	217.7
8	Chimera removal	347783	71790	217.7
9	Unwanted lineage removal	347783	71790	217.7

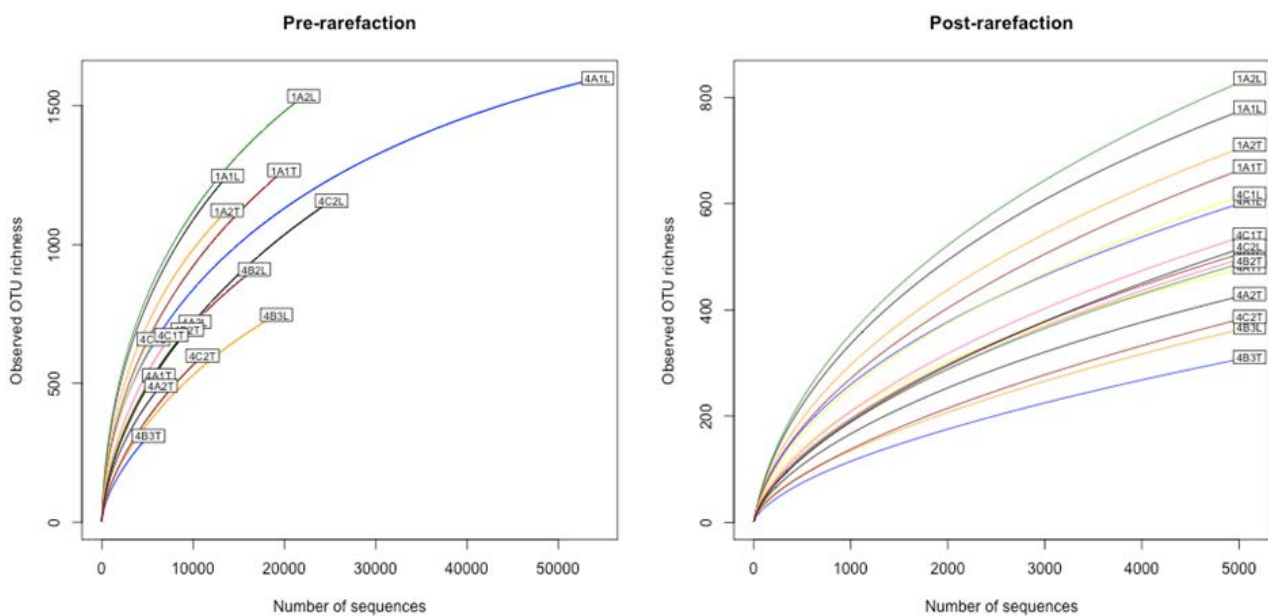


Figure A1. Observed OTU richness pre-rarefaction (left panel) and post rarefaction (right panel)

Table A2. Results of the elemental analysis of the pore water using the ICP-OES. Differences between sites were tested with independent t-test. Significant differences are indicated in the title using \*.

Site	pH			Alkalinity (eq ml <sup>-1</sup> )			Al (μmol L <sup>-1</sup> )			Ca (μmol L <sup>-1</sup> )			Fe (μmol L <sup>-1</sup> )		
	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n
Fochteloërveen	4.4	0.4	2	0.00	0.0	2	35.3	27	2	59	28	2	198	192	2
Ilperveld	4.8	0.3	3	0.16	0.07	3	8.0	3	3	132	79	3	7.9	2.5	3

Site	K (μmol L <sup>-1</sup> )			Mg (μmol L <sup>-1</sup> )			Mn (μmol L <sup>-1</sup> )			Na (μmol L <sup>-1</sup> )			P* (μmol L <sup>-1</sup> )		
	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n
Fochteloërveen	21.4	13	2	41.6	13	2	1.02	0.64	2	271	45	2	0.58	0.33	2
Ilperveld	127	35	3	171	107	3	2.08	0.34	3	1365	931	3	5.00	0.65	3

Site	S (μmol L <sup>-1</sup> )			Si (μmol L <sup>-1</sup> )			Zn (μmol L <sup>-1</sup> )		
	Mean	SE	n	Mean	SE	n	Mean	SE	n
Fochteloërveen	53.2	36	2	63.0	41	2	9.6	6.7	2
Ilperveld	75.0	17	3	30.1	6.4	3	2.1	1.1	3

Table A3. Results of the elemental analysis of the pore water with the autoanalyzer. Differences between sites were tested with independent t-test. Significant differences are indicated in the column header using \*.

Site	PO <sub>4</sub> <sup>3-</sup> ** (μmol L <sup>-1</sup> )			NO <sub>3</sub> <sup>-</sup> * (μmol L <sup>-1</sup> )			NH <sub>4</sub> <sup>+</sup> (μmol L <sup>-1</sup> )			Cl <sup>-</sup> (μmol L <sup>-1</sup> )		
	Mean	SE	n	Mean	SE	n	Mean	SE	n	Mean	SE	n
Fochteloërveen	0.20	0.1	2	0.02	0.02	2	1.0	1.0	2	53	38	2
Ilperveld	3.7	0.3	3	0.76	0.1	3	5.1	1.2	3	931	530	3

Table A4. Alpha diversity per *Sphagnum* species and segment (capitulum (cap) or stem).

<i>Sphagnum</i> species	Segment	Good's sampling coverage	Shannon diversity index	Species richness		Pielou's evenness
				Observed richness	Chao1 estimator	
<i>S. fallax</i>	cap	96.9 ± 0.79	3.75 ± 0.49	393 ± 93	567 ± 136	0.63 ± 0.06
	shoot	95.3 ± 0.73	4.17 ± 0.51	510 ± 101	828 ± 138	0.67 ± 0.06
<i>S. palustre</i>	cap	96.2 ± 0.63	4.29 ± 0.25	475 ± 50	689 ± 94	0.70 ± 0.03
	shoot	95.3 ± 0.70	4.79 ± 0.28	578 ± 59	870 ± 123	0.75 ± 0.03

Table A5. Alpha diversity indices by site.

Site	Good's sampling coverage	Shannon diversity index	Species richness		Pielou's evenness
			Observed richness	Chao1 estimator	
Fochteloërveen	94.1 ± 0.32	5.28 ± 0.14	704 ± 39.1	1052 ± 69.0	0.81 ± 0.02
Ilperveld	96.5 ± 0.31	3.91 ± 0.17	417 ± 28.1	634 ± 54.3	0.65 ± 0.02

Table A6. Analysis of similarity between groups using permutations (999) on Bray-Curtis dissimilarity matrix (DM), Formula: DM by Site + *Sphagnum* spp. \* Segment.

Factors	Df	Sum of squares	R <sup>2</sup>	F	p	
Site	1	0.77	0.32	8.72	0.001	***
<i>Sphagnum</i> spp.	1	0.31	0.13	3.50	0.008	**
Segment	1	0.18	0.08	2.10	0.064	.
Site * <i>Sphagnum</i> spp.	1	0.17	0.07	1.93	0.074	.
Site * Segment	1	0.11	0.05	1.27	0.204	
<i>Sphagnum</i> spp. * Segment	1	0.05	0.02	0.61	0.769	
Site * <i>Sphagnum</i> spp. * Segment	1	0.08	0.04	0.95	0.431	
Residual	8	0.70	0.30			
<b>Total</b>	15	2.38	1.00			