Dear editor,

We would like to thank you for your comments on our revised manuscript.

Based on your recommendation, we further revised and improved the discussion. In addition to the changes that we had already made in response to the referees' comments, we made the following improvements to the discussion:

- In order to improve clarity, we changed the order of sections to a more logical sequence, discussing the effects of bicarbonate first;
- We merged the sections on phosphorus addition and nutrient stoichiometry;
- The section on the role of phosphorus availability may have been confusing, and the change in the order of paragraphs now leads to a more logical build up of our arguments;
- The last section on the importance of the symbiosis was shortened to improve the focus on our results.

Below, we added an itemized list of all changes we made since the publishing of the manuscript as a discussion paper. First you will find our responses to the comments of the referees as they were published online earlier, with adapted references to the lines of the new version of the manuscript. After this, we provided a list of the additional changes we made based on your decision for major revision.

Yours sincerely, on behalf of all authors,

Eva van den Elzen

General comments referee 1:

1a) This is an interesting contribution in which the authors demonstrate that nitrogen fixation and plant growth are not affected in the same way by phosphorus or bicarbonate. Their expectation was that increased N fixation, especially when coupled with additional phosphorus, would stimulate sphagnum performance. However, while both phosphorus and bicarbonate increased N fixation, they had neutral or negative effects on sphagnum photosynthesis, respectively. Based on this discrepancy between expected result and the actual result, the authors question the concept of a "direct mutualism". I am not sure what a "direct mutualism" is. This should be defined.

1b) We thank the referee for distinguishing this manuscript as an interesting contribution. With a direct mutualism we refer to a mutualism between microbial symbiont and a plant host, in which the host benefits from a direct transfer of nutrients from the symbiont (bacteria) to the host (Sphagnum), as explained by Ho & Bodelier (2015) (see references manuscript). This is in contrast to the uptake of nutrients after die-off of bacteria, which cannot be regarded as a real and direct mutualism, as this is the case for any plant taking up nutrients from decomposed bacterial biomass.

We agree that this term should be explained to readers and defined more thoroughly in the introduction of the manuscript. Therefore we added the following clarification to Page 3, lines 19-20 and lines 23-25: "...a process that we refer to as a direct mutualism, with reference to the direct transfer of chemicals between host and symbiont (Ho and Bodelier, 2015)" and "There may also be a different, indirect type of interaction in which Sphagnum receives a flow of nutrients from dead and lysed microorganisms."..."i.e. a direct mutualism or an indirect interaction,"

2a) I wonder whether the effect of P on plant performance is determinable in the time frame of this study. If, in response to P addition, N fixation increases, if this added N is retained by the microbes, they would have to turn over before the plant could have access to it. Thus, some more time may be necessary to see a P effect on plant performance.

2b) We thank the referee for bringing up this point, as we can indeed improve our manuscript by elaborating on this.

In various studies on peatlands the stimulating effect of phosphorus on Sphagnum growth was shown. During one growing season, phosphorus was shown to increase photosynthesis by 14% (Fritz et al 2001) and the length of photosynthetic material of Sphagnum *significantly by 42% (Carfrae et al., 2007), being 6-7 mm. Although our experiment lasted for 10 weeks, which is shorter than a growing season, it would still be sufficient to be able to see the effects of phosphorus addition on* Sphagnum *growth, being around 3 mm additional growth.*

The additional citations were added on Page 4, line 7: "...and lead to an increase in photosynthesis (by 14%) (Fritz et al., 2012) and moss growth (by 42%) (Carfrae et al., 2007)."

Besides, we expected the mutualistic interaction between Sphagnum and its diazotrophs to be of a direct nature (i.e. direct transfer of nutrients between symbiont and host, see previous point) and therefore an increase in N_2 fixation rates to result in a direct increase of N availability for Sphagnum, and thus an increase in growth rate. Such a mutualism is for instance known for Azolla *spp, were addition of P can in a short time (four weeks or less) greatly increase growth and also N content (Cheng et al., 2010). From our results such a direct symbiotic relationship between* Sphagnum *and its microbial community seems not to be the case, pointing towards an indirect interaction.*

To clarify this point, we added information on Azolla *spp to the introduction Page 4, lines 4-5: "...and in* Azolla *spp, a fern species with symbiotic cyanobacteria within its leaves, P was shown to drastically increase plant growth and N content (Cheng et al., 2010)."*
And to the discussion we added to Page 12, line 14-17: "This is in stark contrast to Azolla *spp, where P addition is known to directly increase the growth rate and N content of the host plant (direct mutualism) (Cheng et al., 2010). Under the present environmental conditions, the symbiosis between Sphagnum and its microbial community seems to be based on the indirect transfer of nutrients from microbial die-off (Ho and Bodelier, 2015)."*

Specific comments referee 1:
3a) The timing of events is not clear. How long were the treatments administered? How much time elapsed between treatment initiation and photosynthesis measurement or nutrient analysis?

3b) We agree that the timing was not stated clearly. The treatments were added during 10 weeks, after which nutrient content, nitrogen fixation and photosynthesis were measured. We added this information to the methods section on Page 6, line 1-2: "Treatment solutions were supplied during ten weeks, after which plant, microbial and abiotic measurements were conducted."

4a) The device used to measure photosynthesis is not designed specifically for measuring photosynthesis. One question is whether it is accurate and stable enough to actually detect small but significant differences in photosynthesis between treatments. Also, were the light levels used (not specified) representative of those expected in the field (as opposed to the mesocosm)? If not, the results from this measurement could be irrelevant.

4b) The device used is a small chamber connected in a closed loop to a near infra red spectroscopy (NIRS) gas analyzer with cavity ring down spectroscopy (CRDS), which is at present the most accurate instrument to measure CO_2 fluxes (and CH_4, N_2O fluxes), including decreased CO_2 levels as a result of photosynthesis. The set-up

is therefore exactly similar to, e.g., those using Infrared Spectroscopy Gas Analyzers (IRGA) to measure photosynthesis (Hunt, 2003). The laser-based NIRS-CRDS devices can, however, measure changes in CO_2 concentration much faster and with an extremely high resolution (Crosson, 2008). The light levels used were 150 µmol PAR m^{-2} s^{-1}, similar to mesocosm and field light levels.

In order to make this more clearly, we added more details on this method from Page 6, line 4 to line 9: "...fast greenhouse gas analyzer (NIRS) with cavity ringdown spectroscopy (CRD)" "at similar light conditions as used in the experimental set up (150 µmol m^{-2} s^{-1} PAR)" "...in a closed loop with the NIRS-CRDS gas analyzer capable of measuring concentration changes at a very high resolution (Crosson, 2008) and of accurately measuring photosynthesis (Hunt, 2003).

5a) The natural conditions of the site where the mosses were collected should be indicated (pH, bicarbonate concentration, phosphate concentration, etc.) in order to place in context the experimental treatments.

5b) We agree that this information would benefit our manuscript. Therefore we added an additional table, table 1A to Page 18 and referred to it in the method section on Page 5, line 5-6: "Field conditions of the site where the mosses were collected are shown in Table 1A."

6a) There appears to be a significant interaction between bicarbonate and P with respect to photosynthesis. That is P did not have a significant effect in the absence of bicarbonate, but it did in the presence of bicarbonate. So, when bicarbonate is present, P may be beneficial.

6b) We are very grateful that the referee noticed this error. Apparently, the wrong figure was added to the manuscript, which we very much regret and apologize for. We changed this figure for the correct one on Page 23, in which there is no appearance of an interaction effect, in agreement with the statistics, as stated on Page 7, line 27-28: "No interaction effects were found for any of the parameters".

General comments referee 2:

1a) This paper is of environmental importance as authors have discussed the symbiosis of peat plants and symbiotic microorganisms. They are of recent importance as they play vital role in carbon sequestration. It is an interesting paper as the outcomes obtained were not as obvious expected results. However, there are certain flaws in the approaches they have chosen and discussion made. Moreover, it does not have any broader impacts. Though the methodology is very meticulously designed; some pictures or a graphical abstract would make the approach more clear.

1b) We thank the referee for the interest and input with respect to the manuscript, and the statement that our paper is of environmental importance. We do indeed believe that our results have broader impacts, i.e. that the regulation of nitrogen

fixation by phosphorus is essential for our understanding of the nitrogen cycle, and how it influences the sequestration of carbon in peatlands. Besides, high additional doses of nitrogen by phosphorus-induced nitrogen fixation to already nitrogen-loaded peatlands can well be expected to lead to serious degradation of these important C storing systems. This is important in the context of ecosystem restoration in high nitrogen areas in which the input of phosphorus is simultaneously abundant and not able to offset nitrogen loads as could be expected.

We also thank the reviewer for the idea of adding a graphic figure of the experimental set up. We added both a picture and a graphical representation to new Figure 1 on Page 21. We referred to this picture in the methods section on Page 5, lines 26-27: "A graphic figure of the experimental set up and photo's can be found in Figure 1."

Specific comments referee 2:

2.1a) Word "symbiosis" in the title of paper is little ambiguous as the paper is only about the relation of P and N fixation and plant growth. Nowhere the microbial community had been addressed.

2.1b) We did indeed not assess the full microbial community, because that was not the purpose of our research. However, we did study the activity of nitrogen fixing microorganisms, and how this affects Sphagnum *growth. We choose to keep the word symbiosis in the title of the manuscript, because the interaction between the host* Sphagnum *and its diazotrophic microbiome is central to this manuscript.*

2.2a) Abstract is quite general; more specific results could have been included.

2.2b) In attempt to keep the abstract sufficiently concise, we decided to only include our main results. To be more specific, we have now added the exact rates of nitrogen fixation and the results of the light compared to dark incubations for nitrogen fixation to Page 2, line 7: "at a rate of 40 nmol N gDW^{-1} h^{-1}" and to Page 2, line 14-15: "In addition, nitrogen fixation was found to strongly depend on light, with rates 10 times higher in light conditions suggesting high reliance on phototrophic organisms for carbon."

2.3a) Actual field conditions should have been studied and mentioned in the paper. Possibly, few revelations could have been seen like for eg. presence of other growth promoting microorganisms in natural environment which could affect the P/N uptake and plant growth.

2.3b) We thank the reviewer for this remark and agree that the context of the field conditions would benefit our manuscript. Therefore, we added a table (Table 1A) with the abiotic conditions of the field site where the mosses were collected to Page

18 and referred to it in the method section on Page 5, line 5-6: "Field conditions of the site where the mosses were collected are shown in Table 1A."

However, additional information on the microbial community of peat soil we did not assess, since this would be out of our scope, very elaborate, and a different study by itself.

2.4a) Time course studies have not been well defined.

2.4b) We thank the reviewer for noticing this. The time course of the experiment was 10 weeks. We added this information to the methods section on Page 6, line 1-2: "Treatment solutions were supplied during ten weeks, after which plant, microbial and abiotic measurements were conducted."

2.5a) Three way ANOVA is the statistical technique used here using three independent variable (P, HCO3 and spp.) which is an appropriate technique. But, three way ANOVA is a technique in which dependent variables should be at continuous level. Here, some dependent variables do not come under this assumption. Moreover; the independent variable should have two or more categorical groups. Authors fail to do so. Authors can read: f Also, post-hoc analysis would make the scenario more clear as it would give precise idea of dependency of each of the independent variable.

2.5b) All dependent variables assessed with three-way ANOVA are at a continuous level, including nitrogen fixation rate, relative growth rate, number of capitula, length increment, pore water nutrients, alkalinity. Since there are only two groups for each variable, post-hoc analyses cannot be applied. All independent variables have two categorical groups: i.e. +/- phosphorus, +/- bicarbonate, S. palustre *or* S. squarrosum.

For clarification, we added this information to the method section on Page 7, line 22-25: "*...independent variables (fixed factors) with two categorical groups. All dependent variables were quantitative and at a continuous scale, i.e. nitrogen fixation rate, photosynthetic activity, relative growth rate, number of capitula, Sphagnum length increment, and pore water and tissue nutrient concentrations."*

Technical comments referee 2:

3a) Language used in the paper is pretty precise and clear.

3b) We thank the reviewer for this comment.

3.1a) Number of keywords can be reduced

3.1b) We removed 'ecophysiology' and changed 'nutrients' and 'nitrogen deposition' to 'nitrogen' on Page 2, line 26.

3.2a) Flow of introduction can be changed. Mention all the required introduction first and then mention your assumptions and reason for doing this study at the end.

3.2b) We have now made changes in order to move all hypotheses to the last paragraph of the introduction, as suggested by the reviewer.

On Page 4, lines 7-9 we adapted: "It is therefore expected that the addition of P can improve...N deposition areas". The next sentences of this paragraph "In addition...becomes limiting" we moved to Page 4, lines 25-28. In this last paragraph, we also made adjustments to lines 24-25 and 28-29. Leading to a changed last paragraph in lines 23-30: "Our prime research question was whether P availability and alkalinity were key regulators of both diazotrophic and Sphagnum *activity, with P increase having a positive effect on both partners, and alkalinity increase a negative effect. In addition, in view of a direct mutualistic relationship between the moss and its diazotrophs, as with* Azolla *spp and its cyanobacteria, we expect that higher N_2 fixation rates provide additional N. Combined with higher P availability, this may increase* Sphagnum *photosynthesis and growth even further, as long as no other resource or condition becomes limiting. By testing this hypotheses, we are able to explore the nature of the symbiotic interaction, i.e. which benefits or costs the diazotrophic microbial community experience through the close association with their host, and vice versa."*

3.3a) If your mentioning anything in your paper for first time mention it clearly. Like page 3, line 25, it was mentioned "our field sites"; as it was being mentioned for the first time it is better to mention the name.

3.3b) We added the specifics of our field site to this line, now on Page 3, line 31.

Additional changes based on referee reports

1) In order to make the abstract more specific, we changed on Page 2, line 22-24: "concept of a direct mutualism" to "...regulation of nitrogen fixation by Sphagnum *under these eutrophic conditions. The high N_2 fixation rates result in high additional nitrogen loading of 10 kg ha^{-1} y^{-1} on top of the high nitrogen deposition in these ecosystems."*

2) We further improved the introduction by adding to Page 4, line 28-29: "...we are able to not only investigate regulation of nitrogen fixation by these abiotic factors but also..."

*3) To improve the discussion, we moved the section "4.3 Both symbiotic partners...*Sphagnum *for nutrients" to Page 10, line 13 – Page 11, line 8 and changed its number to: "4.2". Besides, we made minor changes to Page 10, on line 30: from "more diazotrophs are present" to "diazotrophic communities are larger" and on line 32 from "N_2 fixation can be explained" to "N_2 fixation may be*

explained". On Page 11, line 1, we adapted: "...nutrient-rich conditions correlated with increased N_2 fixation rates..."

4) The section 'Role of P availability' was numbered '4.3' on Page 11, line 9. From its first paragraph we removed from Page 11, line 23 "Since the latter is unlikely given the different response in activity to increased P by Sphagnum *spp. compared to diazotrophs, the process of N_2 fixation, here, seems to depend on phototrophic microorganisms." And changed the last sentence to: "A high abundance of phototrophic organisms could be..."*

5) From Page 11, line 24 the title section "4.3 Nutrient stoichiometry" was removed and the first sentence "Both in light...performance was not." was replaced with: "P addition did, however, not increase Sphagnum *growth, raising the question which other factor may have been limiting its growth." on Page 11, Line 24. Besides the last part of the next sentence was removed: ", which is surprising given the high N loading rates."*

6) To improve the flow of the discussion, the last paragraph of section 4.3 "The low N:P ratios...input in the system" was inserted in the second paragraph on Page 11, line 27, after "P addition did...(Bragazza et al., 2004)". The first sentence of the inserted part was changed to: "However, under these eutrophic conditions with high N availability and high tissue N concentrations, low ratios rather seem to be an effect of high P concentrations (Jirousek et al., 2011)." And this new paragraph was divided after "...(Rydin and Jeglum, 2006; Gunnarsson, 2005)" on Page 12, line 3. The next sentence: "The increased N_2 fixation rates...N input in the system." was removed.

7) To shorten the discussion we removed from Page 11, line 27: "As stated before, the absolute N content of Sphagnum *is high, so N limitation seems unlikely." and from Page 11, line 29-30 we removed: "As N: K ratios higher than 3.3 were found to indicate K limitation". This was changed to: "N: K ratios of around 1.6 for the controls in our experiment did not support the idea of K limitation (Bragazza et al., 2004)." On Page 11, line 31 ",meaning that most important nutrients did not seem to be limiting* Sphagnum *growth here." was removed. Besides, for clarity "Mo" on page 11, line 31 was changed to "molybdenum" and the last sentence of the paragraph on Page 12, line 2-3 was changed to "Biomass production rates (based on the average growth rate...of 250 days) corresponded to around 300 g m^{-2} y^{-1}, which is indeed high (Rydin and Jeglum, 2006...)."*

8) To put the message more clearly the first sentences of the new last paragraph of section 4.3 on Page 12, line 5-6 were changed from "Although N_2 fixation rates doubled, the addition of P resulted in strong accumulation of P..." to: "With apparently no nutrient limitation for Sphagnum *growth, P addition led to accumulation in* Sphagnum*-microorganism tissue. This lowered the N: P ratio, pointing towards...". We also made small changes to Page 12, line 9 to "...different treatments, these can explain..." and removed from Page 12, line 15: "Still, growth rates remain stable even with increased uptake of P. This unbalanced uptake...", instead leaving only the second sentence, changed to: "The unbalanced uptake..."*

9) To conclude this new section we added after "associated microbial community." on Page 12, line 11: "In conclusion, either the fixed N was not directly available for Sphagnum*, or it could not be used due to physiological constraints. In both cases,* Sphagnum *could not profit from the additionally fixed N and seemed to be competing for nutrients with its symbionts rather than regulating their activity by supplying additional C." To the last sentence of this paragraph on Page 12, line 17, we added: "...rather than by a mutualistic interaction with* Sphagnum *directly benefitting from the additionally fixed N. More research is, however, needed to determine whether the symbiosis would change to a mutualistic interaction at low N conditions. At the ecosystem level, the increased N_2 fixation rates with the lack of additional biomass production of* Sphagnum *with added P, led to remarkably high amounts of 40 kg ha^{-1} y^{-1} of extra N input."*

10) Section 'Importance of the symbiosis' was numbered 4.4 on Page 12, line 22. To improve this section we added to Page 12, line 26: "or an increase of (micro)nutrients, other than P. This may well explain the differences in N_2 fixation rates between fens and bogs (Larmola et al., 2014)." Moreover, the two paragraphs of section 4.4 were merged and "However, this needs to be studied....by mineralization processes" was removed from Page 12, line 33. Besides, minor textual changes were made throughout the paragraph from Page 12, line 22 to Page 13, line 6, changing it to: "We showed that in these N rich fen systems, Sphagnum spp. still work as a filter monopolizing all N and their microbial community still experiences N limitation. With all N taken up by Sphagnum, diazotrophs fix N_2 at appreciable rates despite high N deposition. N_2 fixation rates are even more increased by addition of P and by a higher HCO_3^- concentration, as an effect of increased pH or an increase of (micro)nutrients, other than P. This may well explain the differences in N_2 fixation rates between fens and bogs (Larmola et al., 2014). The diazotrophic community seems to have different optimal environmental conditions than their host, and seem to trade off protection from herbivores inside Sphagnum hyaline cells against Sphagnums monopolization of N and active acidification. As peat mosses did not benefit from the fixed N, active control of the diazotrophic community (e.g. by additional organic compound supply) seems unlikely. Given the high N_2 fixation rates and accumulation of N in Sphagnum peat, we hypothesize that the fixed N is available by reabsorption from decaying and dead Sphagnum tissue and dead microbial biomass, rather than by the direct transfer between diazotrophs and Sphagnum. Ho and Bodelier (2015) also suggested this alternative pathway of N transfer between Sphagnum and N_2 fixing methanotrophs, and feather mosses were suggested not to depend on their cyanobacterial community for N (Rousk and Michelsen, 2016). Since N loads (25 kg ha^{-1} y^{-1}) were high here, and N_2 fixation added 10 kg ha^{-1} y^{-1} or more with high P loads, peat mosses can be expected to not be able to reabsorb the mineralized N, which then leaches deeper into the peat. Here, it may become available to vascular plants (Lamers et al., 2000). In this way, the high N_2 fixation rates may speed up decomposition rates and invasion of vascular plants by supplying additional N to an already N loaded system. As high P input still increases N_2 fixation rates, this will not be able to balance out the high N loads."

Symbiosis revisited: phosphorus and acid buffering stimulate N_2 fixation but not *Sphagnum* growth

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Abstract

In pristine *Sphagnum* dominated peatlands, (di)nitrogen (N_2) fixing (diazotrophic) microbial communities associated with *Sphagnum* mosses contribute substantially to the total nitrogen input, increasing carbon sequestration. The rates of symbiotic nitrogen fixation reported for *Sphagnum* peatlands, are, however, highly variable and experimental work on regulating factors that can mechanistically explain this variation is largely lacking. For two common fen species (*Sphagnum palustre* and *S. squarrosum*) from a high nitrogen deposition area (25 kg N ha^{-1} y^{-1}), we found that diazotrophic activity (as measured by $^{15\text{-}15}N_2$ labeling) was still present at a rate of 40 nmol N gDW^{-1} h^{-1}. This was surprising, given that nitrogen fixation is a costly process. We tested the effects of phosphorus availability and buffering capacity by bicarbonate rich water, mimicking a field situation in fens with stronger groundwater or surface water influence, as potential regulators of nitrogen fixation rates and *Sphagnum* performance. We expected that the addition of phosphorus, being a limiting nutrient, would stimulate both diazotrophic activity and *Sphagnum* growth. We indeed found that nitrogen fixation rates were doubled. Plant performance, in contrast, did not increase. Raised bicarbonate levels also enhanced nitrogen fixation, but had a strong negative impact on *Sphagnum* performance. These results explain the higher nitrogen fixation rates reported for minerotrophic and more nutrient-rich peatlands. In addition, nitrogen fixation was found to strongly depend on light, with rates 10 times higher in light conditions suggesting high reliance on phototrophic organisms for carbon. The contrasting effects of phosphorus and bicarbonate on *Sphagnum* spp and their diazotrophic communities reveal strong differences in optimal niche for both partners with respect to conditions and resources. This suggests a trade-off for the symbiosis of nitrogen fixing microorganisms with their *Sphagnum* hosts, in which a sheltered environment apparently outweighs the less favorable environmental conditions. We conclude that microbial activity is still nitrogen limited under eutrophic conditions because dissolved nitrogen is being monopolized by *Sphagnum*. Moreover, the fact that diazotrophic activity can significantly be upregulated by increased phosphorus addition and acid buffering, while *Sphagnum* spp do not benefit, reveals remarkable differences in optimal conditions for both symbiotic partners and questions the regulation of nitrogen fixation by *Sphagnum* under these eutrophic conditions. The high nitrogen fixation rates result in high additional nitrogen loading of 17 kg ha^{-1} y^{-1} on top of the high nitrogen deposition in these ecosystems.

Key-words: plant-microbiome interactions, symbiosis, diazotrophy, peatland, fen, bicarbonate, pH, nitrogen

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1. Introduction

Nitrogen (N) availability is considered to limit or co-limit primary production in pristine *Sphagnum*-dominated ecosystems (Aerts et al., 1992; Lamers et al., 2000; Limpens and Berendse, 2003). Peat mosses (*Sphagnum* spp.) function as a filter that very effectively absorbs particularly ammonium (NH_4^+) but also nitrate (NO_3^-) from atmospheric deposition, leading to N limitation in the rhizosphere of vascular plants (Lamers et al., 2000; Bragazza et al., 2004; Fritz et al., 2014). Since the

availability of N determines primary production, there appears to be a close link between the N and C cycles (Hungate et al., 2003; Vitousek et al., 2013). This link is especially important in peatlands, which, by storing substantial amounts of C, play an important role in global C cycling (Ruesch and Gibbs, 2008; Clymo and Hayward, 1982). Being ecosystem engineers in peatlands, *Sphagnum* spp. produce recalcitrant litter, rich in phenolic compounds (Verhoeven and Toth, 1995), and actively acidify their environment (Clymo and Hayward, 1982). This, combined with moist, anaerobic conditions results in the accumulation of peat with a high C content (Van Breemen, 1995). Recently, it has been shown that the high N_2 fixation activity of the *Sphagnum* microbiome could explain the discrepancy between low inputs of atmospheric N and high N accumulation rates in the peat of pristine *Sphagnum* peatlands (Vile et al., 2014), confirming the strong link between C and N accumulation. On the other end, high atmospheric N deposition may compromise the C sequestration function of peatlands by stimulating microbial processes such as overall decomposition (Bragazza et al., 2006) and denitrification (Gruber and Galloway, 2008).

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N_2 fixing microorganisms (diazotrophs) live on the surface and inside dead hyaline cells of *Sphagnum* (Opelt et al., 2007; Bragina et al., 2012; Larmola et al., 2014), forming a symbiosis with their host. A highly diverse microbial community, including Proteobacteria, Verrucomicrobia and Cyanobacteria has been found to colonize peat mosses (Bragina et al., 2014) and many of these microorganisms have the capacity to fix N_2 (Bragina et al., 2013; Kox et al., 2016). Also in other bryophytes, like *Hylocomiaceae* (feather mosses) such a symbiotic relationship can be found with N_2 fixing cyanobacteria, supplying up to 50% of the total N input in boreal forests (Rousk et al., 2013). These phototrophic diazotrophs provide N to their host in exchange for C compounds (Bay et al., 2013; Leppänen et al., 2013), a process that we refer to as a direct mutualism, with reference to the direct transfer of chemicals between host and symbiont (Ho and Bodelier, 2015). In these moss symbioses, as well as in vascular plant symbioses, application of high rates of inorganic N were found to decrease N_2 fixation rates, with the host plant shifting to the use of this readily available inorganic N source (Gundale et al., 2011; Zackrisson et al., 2004; Rousk et al., 2014). There may also be a different, indirect type of interaction in which *Sphagnum* receives a flow of nutrients from dead and lysed microorganisms. Although the exact nature of the *Sphagnum*-microorganism symbiosis remains unknown, i.e. a direct mutualism or an indirect interaction, N fixed by cyanobacteria associated with *Sphagnum* was found to enhance *Sphagnum* growth (Berg et al., 2013). A high variation in rates of N_2 fixation has not only been found for different species and different systems, but also for similar ecosystem types at different locations. To our knowledge, the mechanistic explanation for this high variation of symbiotic N_2 fixation rates in *Sphagnum* peatlands is still lacking.

In areas with high N deposition like in our field site in the Netherlands, the necessity for microorganisms with diazotrophic capacity to actually fix N_2 can be expected to diminish, as NH_4^+ availability usually leads to down-regulation of the expression of the nitrogenase enzyme responsible for N_2 fixation (Dixon and Kahn, 2004). Other nutrients than N have been suggested to influence N_2 fixation, especially phosphorus (P) (Vitousek and Field, 1999) which is generally the second

nutrient limiting primary production (Bieleski, 1973; Vance, 2001). P limitation has been shown to play an important role in biomass growth and functioning of peatlands (Larmola et al., 2013; Hill et al., 2014; Fritz et al., 2012) and appeared to control N_2 fixation rates (Toberman et al., 2015; Vitousek et al., 2002; Chapin et al., 1991). Besides, isolated cyanobacteria were shown to be directly stimulated by P (Mulholland and Bernhardt, 2005) and in *Azolla* spp, a fern species with symbiotic cyanobacteria within its leaves, P was shown to drastically increase plant growth and N content (Cheng et al., 2010). In peat mosses from N-rich sites, increased P availability can be expected to complement the high N supply (Limpens et al., 2004) and lead to an increase in photosynthesis (by 14%) (Fritz et al., 2012) and moss growth (by 42%) (Carfrae et al., 2007). It is therefore expected that the addition of P can improve the performance of the *Sphagnum*-microorganism association in high N deposition areas.

Next to nutrient availability, the alkalinity and pH of the environment is known to be a key biogeochemical factor affecting *Sphagnum* presence and performance in peatlands (mires). Higher concentrations of bicarbonate (HCO_3^-) and concomitantly higher pH values (from 7.5 and upwards), through the influence of minerotrophic groundwater or surface water in rich fens, have been shown to hamper *Sphagnum* growth (Clymo, 1973; Lamers et al., 1999). While the effect of environmental factors such as pH and nutrient availability on *Sphagnum* itself has been thoroughly studied (Clymo, 1973; Kooijman and Paulissen, 2006; Bragazza and Gerdol, 2002), it remains unknown how these environmental factors influence the activity of its diazotrophic community and how this in turn affects *Sphagnum* performance in peatlands. Information about the factors regulating the diazotrophic community is vital to understand the high variation in N_2 fixation rates in *Sphagnum* dominated wetlands that may strongly affect both nutrient and carbon cycling.

We therefore used a controlled, full-factorial set-up to experimentally test the effects of P and HCO_3 addition on N_2 fixation rates of the diazotrophic community and on photosynthesis and growth of two common fen species, *Sphagnum squarrosum* Crome and *S. palustre* L. from a Dutch rich fen. Our prime research question was whether P availability and alkalinity were key regulators of both diazotrophic and *Sphagnum* activity, with P increase having a positive effect on both partners, and alkalinity increase a negative effect. In addition, in view of a direct mutualistic relationship between the moss and its diazotrophs, as with *Azolla* spp and its cyanobacteria, we expect that higher N_2 fixation rates provide additional N. Combined with higher P availability, this may increase *Sphagnum* photosynthesis and growth even further, as long as no other resource or condition becomes limiting. By testing this hypotheses, we are able to not only investigate the regulation of N_2 fixation by these abiotic factors but also explore the nature of the symbiotic interaction, i.e. which benefits or costs the diazotrophic microbial community would experience through the close association with their host, and vice versa.

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2. Methods

2.1 Collection of *Sphagnum* and peat

Two common species of *Sphagnum*, *S. squarrosum* and *S. palustre* were chosen for their widespread occurrence (Europe, America, Asia, Australia), and their differences in habitat preference. While both are typical fen species, *S. squarrosum* is known to withstand slightly more buffered (higher pH) conditions (Clymo, 1973; Rydin and Jeglum, 2006). Field conditions of the site where the mosses were collected are shown in Table 1A. To mimic their natural habitat, including moist conditions and supply of substrate-derived CO_2 for *Sphagnum* development (Smolders et al., 2001), peat mosses were placed on *Sphagnum* peat monoliths. Both peat mosses and monoliths were collected from the peatland Ilperveld in the Netherlands (52°26'22.68"N; 4°56'54.81"E), where monoliths (25 x 12 x 20 cm depth) were placed in glass mesocosms (25 x 12 x 30 cm depth) and then transported to the lab. Soils were kept wet with demineralized water (1 cm above soil level) and allowed to acclimatize for 2 weeks. Patches of 70 (*S. palustre*) or 80 (*S. squarrosum*) capitula (top 2 cm of moss) representing similar fresh weights were placed on top of the monoliths. A total of 16 mesocosms were placed in a water bath maintained at 15° C (using a cryostat) with a light regime of 16h light using four 400 watt lamps (Hortilux Schreder HS2000, Monster, the Netherlands) and one growth lamp with 120 deep red/white LEDs (Philips, GreenPower LED, Poland), providing in total 150 µmol PAR m^{-2} s^{-1} and a temperature of 18° C at vegetation level. The light level was chosen to mimic realistic field conditions where *Phragmites australis* and sedges in these fens create low, but not limiting light levels for *Sphagnum* spp (Bonnett et al., 2010; Kotowski and Diggelen, 2004).

2.2 Experimental set up

After acclimatization, there was a constant flow of different treatment solutions through the mesocosms, at a flow rate of 5.4 L per week using peristaltic pumps (Masterflex L/S tubing pump; Cole-Parmer, Schiedam, the Netherlands) to create constant conditions in a 1 cm water layer over the soils. The lower 1 cm of *Sphagnum* spp. was flooded, while capitula were just above the water layer. Four different treatment solutions were applied (N=4 replicates per treatment), which were spatially distributed in a randomized block design. The treatments were applied in a full factorial design with a P treatment of 10 µmol L^{-1} P (as $Na_4P_2O_7$) and a HCO_3 treatment of 3 mmol L^{-1} $NaHCO_3$. Also 5 mg L^{-1} of sea salt with small amounts of trace elements (Tropic Marine, aQua united LTD, Wartenberg, Germany) was added to all treatment solutions (including control) to mimic rainwater quality and to prevent osmotic stress. A graphic figure of the experimental set up and pictures can be found in Figure 1. Furthermore, each mesocosm was provided with an amount of rainwater equivalent to the mean annual rainfall in the Netherlands (750 mm) and with an N concentration equivalent to the Dutch atmospheric deposition of 25 kg N ha^{-1} y^{-1}. Three times a week, 150 ml of artificial rainwater was sprayed on the peat mosses, containing 5 mg L^{-1} sea salt (Tropic Marine, aQua united LTD, Wartenberg, Germany), 19 µmol L^{-1} KCl, 10 µmol L^{-1} $CaCl_2$, 10 µmol L^{-1} Fe-EDTA, 1 µmol L^{-1} KH_2PO_4, 0.7 µmol L^{-1} $ZnSO_4$, 0.8 µmol L^{-1} $MnCl_2$, 0.2 µmol L^{-1} $CuSO_4$, 0.8 µmol L^{-1} H_3BO_3, 8 nmol L^{-1}

$(NH_4)_6Mo_7O_{24}$ and 91 µmol L^{-1} NH_4NO_3. Treatment solutions were supplied during ten weeks, after which plant, microbial and abiotic measurements were conducted.

2.3 Plant performance

Photosynthetic rates of the mosses were determined using a fast greenhouse gas analyzer (NIRS) with cavity ringdown spectroscopy (CRD) (GGA-24EP; Los Gatos Research, USA). From each mesocosm one individual of each moss species was taken and placed in a closed glass vile (100 ml) at similar light conditions as used in the experimental set up (150 µmol m^{-2} s^{-1} PAR), connected to the gas analyzer. Changes in CO_2 concentrations were measured over a time period of 5 minutes, in a closed loop with the NIRS-CRDS gas analyzer capable of measuring concentration changes at a very high resolution (Crosson, 2008) and of accurately measuring photosynthesis (Hunt, 2003). Additionally, dark measurements were carried out for each sample, and gross photosynthetic rates were calculated by correcting the slope of CO_2 decrease in light with the slope of the CO_2 increase in dark. Also, capitula were counted and average lengths of *Sphagnum* individuals determined. Total fresh weight (FW) of *Sphagnum* biomass was measured, after which material was dried at 70° C for 48 hours to determine dry weight (DW) in order to calculate relative growth rates.

2.4 N_2 fixation rates and elemental composition of *Sphagnum*

Two subsamples (the top 2 cm of two individuals) of *S. squarrosum* and *S. palustre* from each mesocosm were placed separately in 30 ml glass serum bottles with rubber stoppers. 6 ml of headspace was removed with an injection needle and replaced with $^{15-15}N_2$ gas (98 atom% ^{15}N, Sigma-Aldrich, Germany), leading to 20% $^{15}N_2$ labeling. Samples were incubated for 48 hours with a light regime of 16 hours of light (150 µmol m^2 s^{-1} PAR) at 18° C. They were then dried at 70° C for 48 hours and ground using a mixer mill (MM301, Retsch, Germany) for 2 minutes at 30 rotations s^{-1}. Total N concentrations and isotopic ratios were determined using an elemental analyzer (Type NA 1500 Carlo Erba, Thermo Fisher Scientific Inc., USA) coupled online via an interface (Finnigan Conflo III) to a mass-spectrometer (Thermo Finnigan DeltaPlus, USA). For every control and P-treated sample an additional incubation was carried out under similar but dark conditions. For every incubated subsample a control sample was taken that had not been incubated with $^{15-15}N_2$, to correct for background isotopic composition as influenced by the different treatments. The corrected increases in ^{15}N labeling were converted to N_2 fixation rates (nmol N_2 gDW^{-1} h^{-1}), using the average of both labeled subsamples. These N_2 fixation rates were also converted to rates of N fixed per unit area with bulk density data from the field (dry weight of upper 2 cm of each species in a 10 cm^2 plot (N=4 replicates)). Fixation rates per hectare per year were calculated assuming N_2 fixation activity throughout the growing season (Rousk et al., 2015) during a growing season of around 250 days for peatlands in the northern hemisphere with mild winters (Helfter et al., 2015; Zhu et al., 2012) and corrected for an average seasonal temperature of 13° C, assuming a Q10 of three (Kravchenko and Doroshenko, 2003; Granhall and Selander, 1973; Alexander and Schell, 1973).

Total P and potassium (K) concentrations were determined in digestates of dried and ground *Sphagnum*-microorganism tissue. Digestates were prepared by heating in 500 µl HNO_3 (65%) and 200 µl H_2O_2 (30%) for 16 min in a microwave (mls 1200 Mega, Milestone Inc., Sorisole, Italy). After dilution with demineralized water, P and K concentrations were measured by inductively-coupled plasma emission spectrometry (IRIS Intrepid II, Thermo Electron corporation, Franklin, MA, USA).

2.5 Soil and water chemistry

At the end of the experiment, two soil subsamples of a fixed volume were taken from each mesocosm. Homogenized subsamples were dried at 70° C for 72 hours and weighted to determine bulk densities. Organic matter concentrations were determined through loss on ignition at 550° C for 3 hours. Dried soils were digested with 4 ml HNO_3 (65%) and 1 ml H_2O_2 (30%) using a microwave and measured by inductively-coupled plasma emission spectrometry as described above. C and N contents of dried soil were measured using an elemental analyzer (see above). Soil properties can be found in Table 1B.

The pH of surface water was measured with a standard Ag/AgCl electrode (Orion Research, Beverly, USA) combined with a pH meter (Tim840 titration manager; Radiometer analytical, Lyon, France). Alkalinity was determined by titrating down to pH 4.2 with 0.1 N HCl using an auto burette (ABU901 Radiometer, Copenhagen, Denmark). Concentrations of PO_4^{3-}, NO_3^- and NH_4^+ were measured colorimetrically with a 3 Auto Analyzer system (Bran & Luebbe, Norderstedt, Germany), using ammonium molybdate (Henriksen, 1965), hydrazine sulfate (Kamphake et al., 1967) or salicylate (Grasshoff and Johannsen, 1972), Cl was determined with a Technicon Flame Photometer IV Control (Bran & Luebbe, Norderstedt, Germany). Concentrations of Al, Ca, Fe, S, Mg, Mn, Na, P and K were analyzed by inductively coupled plasma spectrometry (see above).

2.6 Statistical analyses

Values displayed in bar graphs are means ± standard error (SEM) (N=4). To test for the effect of P, HCO_3^- and different species on different parameters three-way ANOVA's were used, using P, HCO_3^- and species as independent variables (fixed factors) with two categorical groups. All dependent variables were quantitative and at a continuous scale, i.e. nitrogen fixation rate, photosynthetic activity, relative growth rate, number of capitula, *Sphagnum* length increment, and pore water and tissue nutrient concentrations. Normality was tested with a Shapiro-Wilk test on the residuals of the ANOVA and data that were not normally distributed were log-transformed prior to analysis to meet conditions of parametric tests. Homogeneity of the data was checked with Levene's test of equality of variances. No interaction effects were found for any of the parameters and significance was accepted at a confidence level of $P < 0.05$. Statistical tests were performed using IBM SPSS Statistics 21.0 (IBM Corporation, 2012).

3 Results

From our full factorial experiment with additions of P and/or HCO_3 we took measurements on surface water (water quality changes) and on *Sphagnum*-microorganism tissue: N_2 fixation activity, plant performance parameters and nutrient contents.

3.1 Water quality changes

The addition of P (10 µmol L^{-1}) resulted in an increase in total P in the surface water ($F = 6.044$; $P < 0.05$) from 0.7 µmol L^{-1} to a concentration of 6.0 µmol L^{-1}, indicating net uptake and/or binding of P. Supply of HCO_3^- increased pH (from 4.3 to 8.0) and alkalinity (from 0.1 to 2.8 meq L^{-1}) in the surface water ($F=2780.292$; $P<0.001$). Furthermore, upon addition of HCO_3^- the concentrations of NH_4, Ca, Mg, Cl, S, Fe and Al in the water increased two to five times, and K concentration was increased by a factor 1.4 (Table 2).

3.2 N_2 fixation

Under light conditions, diazotrophic activity was similar for both *Sphagnum* spp. Control incubations showed high average N_2 fixation rates of around 40 nmol N gDW^{-1} h^{-1}, translating to high area-based rates of around 10 kg N ha^{-1} y^{-1}. When treated with HCO_3^- and/or P, however, *S. squarrosum* showed 40% higher fixation rates compared to *S. palustre*, ($F=4.510$; $P<0.05$) (Figure 2). Addition of P positively affected N_2 fixation for both *Sphagnum* species ($F=12.639$; $P<0.005$), leading to at least two times higher fixation rates compared to their controls (Figure 2). HCO_3^- addition had an even greater effect, and resulted in around four times higher N_2 fixation rates ($F=32.103$; $P<0.001$) (Figure 2). The combined P and HCO_3^- treatment increased N_2 fixation rate to 300 nmol N gDW^{-1} h^{-1} in *S. squarrosum*.

In general, N_2 fixation rates were highest in light incubations and around 10 times lower under dark conditions ($F=65.642$; $P<0.001$) (Figure 3). However, a similar increase (1.5 times higher) in fixation rates upon P addition was found under both light and dark conditions ($F=18.588$; $P<0.001$).

3.3 Plant performance

S. squarrosum and *S. palustre*, had similar photosynthetic rates of around 65 µmol CO_2 gDW^{-1} h^{-1} and showed a strong negative response to HCO_3^--rich water ($F=21.468$; $P<0.001$), resulting in approximately 50% lower photosynthetic rates (Figure 4). HCO_3^- also resulted in 50-70% lower relative growth rates ($F=29.339$; $P<0.001$), relative decrease in the number of capitula ($F=86.090$; $P<0.001$) and average length ($F=268.846$; $P<0.001$) of both species (results not shown). Final biomass of HCO_3 treated mosses was around 10% lower than that of the control group. Controls of both species ended up with a final dry weight of around 3 g per *Sphagnum* patch, containing around 86 capitula with a length of around 73 mm per moss. This corresponds to a growth rate of 8.5 mg gDW^{-1} d^{-1}. In contrast, P treatment did not show an effect on any of the measured plant performance variables of the *Sphagnum* mosses.

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3.4 Nutrient contents of *Sphagnum*-microorganism association

Concentrations of N, P and K in *Sphagnum* tissue including their microbial community were clearly influenced by surface water treatments (Table 2). Addition of P-rich surface water increased the P content in *Sphagnum*-microorganism tissue by 75% for both *Sphagnum* species ($F=11.549$; $P<0.005$), while N and K concentrations remained unchanged. In treatments with HCO_3^--rich water the N concentration increased by around 20% ($F=6.955$; $P<0.05$), and the concentration of K in the tissue decreased by around 25% ($F=140.343$; $P<0.001$), without affecting P concentrations, indicating K leakage. Individual N contents did not correlate with N_2 fixation rates (results not shown).

N: P ratios differed between the two *Sphagnum* species ($F=4.673$; $P<0.05$), with overall slightly higher ratios for *S. palustre* (mean of controls: 11.8), compared with *S. squarrosum* (mean controls: 7.9) (Figure 5). These ratios decreased by 57-73% after addition of P ($F=8.656$; $P<0.01$) to 6.7 and 5.8 respectively, while HCO_3^- addition did not influence ratios at all. N: K ratios did not differ between the two *Sphagnum* species and were unaffected by addition of P. Addition of HCO_3^- however, increased N: K ratios by 80% ($F=143.049$; $P<0.001$), due to leaking of K from *Sphagnum* tissue. Therefore the HCO_3 treatments were not included in Figure 5.

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4. Discussion

4.1 Diazotrophic activity under high N conditions

Surprisingly, the diazotrophic communities of *S. squarrosum* and *S. palustre* showed appreciable N_2 fixation rates of around 40 nmol N_2 gDW^{-1} h^{-1}, even though they had been subjected to high (25 kg ha^{-1} y^{-1}) historical and experimental airborne N input. These rates are well in the range of N_2 fixation rates reported by Larmola et al. (2014) for *Sphagnum* spp in Finnish peatlands (0-126 nmol gDW^{-1} h^{-1}) and equal to the rates they found for mesotrophic fens, even though atmospheric N inputs were significantly lower in Finland (3 kg ha^{-1} y^{-1}; Mustajärva et al 2008). On an areal basis, N_2 fixation rates of our controls translated to an average N input of 17 kg N ha^{-1} y^{-1} in the upper 2 cm of peat moss for a 250 day growing season (at an average temperature of 13° C). This is in the same order of magnitude as the range of 12-25 kg ha^{-1} y^{-1} reported for pristine boreal bogs, although their growing season only lasts 140 days per year (Vile et al., 2014). Furthermore, similar to Markham (2009), we found *Sphagnum*-associated N_2 fixation rates to be at least 5 times higher than those found in feather mosses, which are around 1.5-3 kg ha^{-1} yr^{-1} (Rousk et al., 2014; DeLuca et al., 2002; Zackrisson et al., 2009; Leppänen et al., 2013). This could be due to morphological differences between the moss species (including hyaline cells of *Sphagnum* providing additional space and protection to microorganisms) and differences in microbial communities resulting from differences in habitat conditions and resources, i.e. availability of inorganic and organic nitrogen and carbon compounds, moisture content and presence of oxygen.

The tissue N concentration of around 11.8 mg g^{-1} in *Sphagnum* spp. appears to be high compared to a range of *Sphagnum* N contents for different N deposition sites (Lamers et al., 2000). Optimal growth conditions for *Sphagnum balticum* were found at an N content of 12.9 mg g^{-1} (Granath et al., 2009), suggesting that *Sphagnum* in our experiment is around the saturation point. Indeed high amounts of inorganic N were still taken up from rainwater by *Sphagnum* spp., leaving the surface water nearly depleted of N (Table 2). These high N uptake rates, especially for NH_4^+, from surface water or rainwater are indeed typical for *Sphagnum* spp. (Fritz et al., 2014). Simultaneously, the associated diazotrophs were still fixing N_2 at appreciable rates under these N-rich conditions, even though N_2 fixation is an energy demanding process (Vitousek et al., 2002). The fact that N_2 fixation rates were high and all N present as NH_4^+ in rainwater was taken up by the moss therefore suggests that dissolved inorganic N was not or hardly available for the microbial community and diazotrophs were still experiencing N limitation. Next to this absolute limitation, the relative lack of N was also great, given the high concentrations of all other (micro)nutrients present in the surface water. So, even the high supply of 25 kg N ha^{-1} y^{-1} by rainwater was rapidly taken up by *Sphagnum*, leaving insufficient N for the microbial community that, in this way, still experienced N limitation.

~~4.~~2 Both symbiotic partners strongly differ in optimal abiotic conditions

As expected, an increase in HCO_3^- concentration, resulting in a higher alkalinity and related higher pH, decreased *Sphagnum* performance. Photosynthetic rates and relative growth rates decreased by around 50% for both species. Furthermore, HCO_3^- addition led to slightly higher surface water NH_4^+ concentrations (Table 2), which most likely resulted from leakage from *Sphagnum* tissue. Increased N: K ratios indicated that K was also leaking from tissue, both pointing towards cell die-off. This is in accordance with earlier studies that showed sensitivity of *Sphagnum* spp. to buffered conditions (Clymo, 1973; Lamers et al., 1999), although the fen species used in this study are known to be more tolerant than typical bog species (Harpenslager et al., 2015). Here, we showed that direct infiltration of HCO_3^- from mineral-rich surface waters or groundwater into the moss layer negatively affects fen *Sphagnum* spp performance, rather than Ca^+, which does not directly affect pH (Lamers et al., 2015).

To our surprise, the response of the diazotrophic community to high HCO_3^- levels was completely opposite to that of *Sphagnum*. Although *Sphagnum* biomass decreased by 10% after treatment with HCO_3^-, the diazotrophic community was stimulated and showed around 4 times higher N_2 fixation rates. The increase of N_2 fixation may, therefore, have been a direct effect of leakage of C or other compounds from deteriorating *Sphagnum* tissue. However, a second plausible explanation for the increase in N_2 fixation is a direct beneficial effect of the increase in pH in the surface water on microbial growth rates and diazotrophic activity. It is indeed known for aquatic systems that dominant diazotrophs can be inhibited by a decrease in pH (Shi et al., 2012) and from agricultural soils that diazotrophic communities are larger in higher pH soils (Silva et al., 2013). In addition, the stimulated N_2 fixation may be explained by an indirect effect of increased decomposition rates as a result of buffering (Smolders et al., 2002), leading to the mobilization of additional organic compounds and nutrients from the soil to the surface water. This was also shown in a field gradient analysis at lower atmospheric N-input, where nutrient-

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rich conditions correlated with increased N_2 fixation rates (Larmola et al., 2014). Since nutrient concentrations in surface water increased 2 to 5 fold in this study, increased N_2 fixation by increased decomposition is a likely third possibility.

Regardless of the effect of HCO_3^- being direct, indirect or both, it is still surprising that diazotrophic microorganisms associated with *Sphagnum*, a genus that requires a low pH and actively acidifies its environment, would thrive under more alkaline conditions. This strongly suggests that for the diazotrophic community the symbiosis with *Sphagnum* seems to be a trade-off, where a sheltered environment (including prevention of drought and predation (Jassey et al., 2013; Andersen et al., 2013) in hyaline cells outweighs the sub-optimal, acidic conditions and the competition with *Sphagnum* for nutrients.

4.3 Role of P availability

Sphagnum spp. and their diazotrophic microorganisms were found to respond in a remarkably different way to the addition of P. As hypothesized, based on N_2 fixation being a P demanding process (Vitousek et al., 2002), higher P availability doubled the N_2 fixation rates. This increase in N_2 fixation by P addition was 75% higher in *Sphagnum squarrosum* compared to *S. palustre*, pointing out differences in response of the microbiomes of both species. Even more surprising, however, was that *Sphagnum* performance of both species was not at all affected by increased P availability. This implies that diazotrophs were stimulated directly by higher availability of P, rather than indirectly by additional supply of compounds obtained from the moss. This is also shown by the similar increase of N_2 fixation activity with P addition under dark conditions that we found (Figure 3). Most of the diazotrophic activity in both *Sphagnum* species appeared to be light related, as N_2 fixation rates went down by 90% under dark conditions. This may have different reasons: 1. most of the diazotrophs are photoautotrophs; 2. most diazotrophs rely on other phototrophic microorganisms for their energy supply; or 3. most diazotrophs depend directly on products of *Sphagnum* photosynthesis. A high abundance of phototrophic microorganisms could be explained by the high availability of nutrients, since mutualistic interactions can be altered by nutrient loading in favor of phototrophic partners (Shantz et al., 2016).

P addition did, however, not increase *Sphagnum* growth, raising the question which other factor may have been limiting its growth. The low N: P ratios of *Sphagnum* tissue of controls (around 10) indicate relative N limitation (Wang and Moore, 2014; Bragazza et al., 2004). However, under these eutrophic conditions with high N availability and high tissue N concentrations, low ratios rather seem to be an effect of high P concentrations (Jiroušek et al., 2011). Concentrations of N, P and K in *Sphagnum* tissue (including their microbial community) were all high or on the high end for *Sphagnum* in minerotrophic peatlands, particularly for P (Aerts et al., 1999; Lamers et al., 2000; Bragazza et al., 2004) (Table 3). N: K ratios of around 1.6 for the controls in our experiment did not support the idea of K limitation (Bragazza et al., 2004). Other (micro)nutrients, like molybdenum were also readily available from the surface water. Since light conditions provided in the experiment resulted in at least 80-90% of saturation of the *Sphagnum* photosystem (Harley et al., 1989) and drought was avoided, growth limitation by light or water also seem unlikely. The lack of additional growth with added P and additionally

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fixed N can therefore most likely be explained by the fact that control peatmosses were already at their physiological maximum. Biomass production rates (based on the average growth rate of 8.5 mg gDW^{-1} d^{-1} and a growth season of 250 days) corresponded to around 300 g m^{-2} y^{-1}, which is indeed high (Rydin and Jeglum, 2006; Gunnarsson, 2005).

With apparently no nutrient limitation for *Sphagnum* growth, P addition led to accumulation in *Sphagnum*-microorganism tissue. This lowered the N: P ratio pointing towards unbalanced uptake of P or luxury consumption (increased nutrient accumulation without any gain in *Sphagnum* biomass). The amount of N fixed by diazotrophs under light conditions correlates with the N content of *Sphagnum* including its microbiome tissue (Figure 6). When we use the rate of N_2 fixation to calculate theoretical increases in N content for different treatments, these can explain the increase in N content (result not shown). The unbalanced uptake of P, relative to N, therefore questions a direct role of the high diazotrophic N_2 fixation rates we found here for *Sphagnum* growth, and rather suggests N accumulation in the associated microbial community. In conclusion, either the fixed N was not directly available for *Sphagnum,* or it could not be used due to physiological constraints. In both cases, *Sphagnum* could not profit from the additionally fixed N and seemed to be competing for nutrients with its symbionts rather than regulating their activity by supplying additional C. This is in stark contrast to *Azolla* spp, where P addition is known to directly increase the growth rate and N content of the host plant (direct mutualism) (Cheng et al., 2010). Under the present environmental conditions, the symbiosis between *Sphagnum* and its microbial community seems to be based on the indirect transfer of nutrients after microbial die-off (Ho and Bodelier, 2015) rather than by a mutualistic interaction with *Sphagnum* directly benefitting from the additionally fixed N. More research is, however, needed to determine whether the symbiosis would change to a mutualistic interaction at low N conditions. At the ecosystem level, the increased N_2 fixation rates with the lack of additional biomass production of *Sphagnum* with added P, led to remarkably high amounts of 40 kg ha^{-1} y^{-1} of extra N input.

4.4 Importance of the symbiosis

We showed that in these N rich fen systems, *Sphagnum* spp. still work as a filter monopolizing N and their microbial community still experiences N limitation. With all N taken up by *Sphagnum*, diazotrophs fix N_2 at appreciable rates despite high N deposition. N_2 fixation rates are even more increased by addition of P and by a higher HCO_3^- concentration, as an effect of increased pH or an increase of (micro)nutrients, other than P. This may well explain the differences in N_2 fixation rates between fens and bogs (Larmola et al., 2014). The diazotrophic community seems to have different optimal environmental conditions than their host, and seem to trade off protection from herbivores inside *Sphagnum* hyaline cells against *Sphagnums* monopolization of N and active acidification. As peat mosses did not benefit from the fixed N, active control of the diazotrophic community (e.g. by additional organic compound supply) seems unlikely. Given the high N_2 fixation rates and accumulation of N in *Sphagnum* peat, we hypothesize that the fixed N is available by reabsorption from decaying and dead *Sphagnum* tissue and dead microbial biomass, rather than by the direct transfer between diazotrophs and *Sphagnum*. Ho and Bodelier (2015) also suggested this alternative pathway of N transfer between *Sphagnum* and N_2 fixing

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Moved up [3]: The low N: P ratios seem to be an effect of high P concentrations rather than an indication of N limitation (Jiroušek et al., 2011). As stated before, the absolute N content of *Sphagnum* is high, so N limitation seems unlikely. Concentrations of N, P and K in *Sphagnum* tissue (including their microbial community) were all high or on the high end for *Sphagnum* in minerotrophic peatlands, particularly for P (Aerts et al., 1999; Lamers et al., 2000; Bragazza et al., 2004) (Table 3). As N: K ratios higher than 3.3 were found to indicate K limitation (Bragazza et al., 2004), the N: K ratios of around 1.6 for the controls in our experiment did not support the idea of K limitation either. Other (micro)nutrients, like Mo were also readily available from the surface water, meaning that most important nutrients did not seem to be limiting *Sphagnum* growth here. Since light conditions provided in the experiment resulted in at least 80-90% of saturation of the *Sphagnum* photosystem (Harley et al., 1989) and drought was avoided, growth limitation by light or water also seem unlikely. The lack of additional growth with added P and additionally fixed N can therefore most likely be explained by the fact that control peatmosses were already at their physiological maximum. Biomass production, calculated with the average growth rate of 8.5 mg gDW^{-1} d^{-1} and a growth season of 250 days, corresponding to around 300 g m^{-2} y^{-1}, was indeed on the high end of production rates found in literature (250-300 g m^{-2} y^{-1} for various *Sphagnum* species) (Rydin and Jeglum, 2006; Gunnarsson, 2005). The increased N_2 fixation rates with the lack of additional biomass production of *Sphagnum* with added P, led to remarkably high amounts of 40 kg ha^{-1} y^{-1} of extra N input in the syste ... [4]

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methanotrophs, and feather mosses were suggested not to depend on their cyanobacterial community for N (Rousk and Michelsen, 2016). Since N loads (25 kg ha^{-1} y^{-1}) were high here, and N_2 fixation added 17 kg N ha^{-1} y^{-1} or more with high P loads, peat mosses can be expected to not be able to reabsorb the mineralized N, which then leaches deeper into the peat. Here, it may become available to vascular plants (Lamers et al., 2000). In this way, the high N_2 fixation rates may speed up decomposition rates and invasion of vascular plants by supplying additional N to an already N loaded system. As high P input still increases N_2 fixation rates, this will not be able to balance out the high N loads.

5. Conclusions

1. In N saturated fens with an N deposition of 25 kg ha^{-1} y^{-1} the activity of diazotrophs can still be unexpectedly high (40 nmol N gDW^{-1} h^{-1}). Since *Sphagnum* spp. monopolize all N in surface water, its microbial community still experiences N limitation.
2. Diazotrophs are stimulated by addition of P and HCO_3^- (buffer capacity), benefitting from additional organic compounds, nutrients and/or an increase in pH, which explains variations in N_2 fixation rates reported for peatlands differing in nutrient supply or buffering.
3. *Sphagnum* growth is -in stark contrast- hampered by the high HCO_3^- concentrations. This questions the concept of a direct mutualism and seems to point to a compromise for the diazotrophic community between a sheltered environment on the one hand and a sub-optimal pH and competition for nutrients with their host on the other.
4. Appreciable N_2 fixation rates in *Sphagnum* in high N deposition sites result in a very high total N input, which may speed up decomposition and stimulate the invasion of vascular plants, affecting C sequestration.

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Tables

Table 1A. Field conditions of pore water in *Sphagnum* layer at collection site (N=4).

	S. palustre		*S. squarrosum*	
	Mean	S.E.M.	Mean	S.E.M.
pH	4.57	0.09	5.25	0.17
Alkalinity (meq L^{-1})	0.24	0.03	0.39	0.04
P (μmol L^{-1})	10.49	6.47	1.47	0.03
NH_4^+ (μmol L^{-1})	41.64	26.77	3.17	1.55
NO_3^- (μmol L^{-1})	0.04	0.04	0	0
K (mg g^{-1})	198.01	84.07	24.64	10.12

Table 1B. Properties of peat monoliths in the experiment (N=16).

	Mean	S.E.M.
Bulk density (kg DW L^{-1})	0.27	0.01
Organic matter (mg g^{-1})	573.33	28.60
C (mg g^{-1})	294.75	14.54
N (mg g^{-1})	18.02	0.60
P (mg g^{-1})	0.80	0.04
K (mg g^{-1})	2.00	0.16

Table 2. Surface water characteristics for the different treatments: control (C), addition of P (P) or HCO_3^- (HCO_3), or both (P + HCO_3). Displayed are means ± standard error of the mean (N=4). Unit for alkalinity (alk) is meq L^{-1}, for all elements concentrations are expressed as µmol L^{-1}. In the effect row, significant differences of P or HCO_3 treatment are indicated by asterisks, where * represents P≤0.05, ** represents P≤0.01 and *** represents P≤0.001.

	pH	alk	NO_3	NH_4	P	K	S
C	4.37	0.06	0.00	0.83	0.74	10.42	36.32
	± 0.09	± 0.03	± 0.00	± 0.06	± 0.36	± 1.06	± 7.38
P	4.31	0.09	0.46	0.66	5.97	9.72	30.32
	± 0.03	± 0.04	± 0.27	± 0.20	± 0.41	± 0.30	± 8.54
HCO_3	7.59	2.76	0.00	3.10	3.86	11.37	102.93
	± 0.10	± 0.04	± 0.00	± 0.54	± 2.24	± 1.10	± 57.05
HCO_3 + P	8.40	2.86	0.03	4.15	5.24	16.45	67.81
	± 0.38	± 0.08	± 0.03	± 0.39	± 1.38	± 2.18	± 15.45
P effect					*		
HCO_3 effect	***	***		***		*	

	Al	Ca	Fe	Mg	Mn	Na	Cl
C	6.08	25.25	7.17	16.00	0.29	113.09	26.96
	± 1.92	± 5.40	± 3.53	± 2.02	± 0.06	± 3.31	± 2.30
P	4.86	19.28	10.94	12.54	0.23	130.32	16.02
	± 0.50	± 6.45	± 5.92	± 4.36	± 0.05	± 8.80	± 10.73
HCO_3	14.65	54.99	60.32	34.16	0.54	2819.60	66.00
	± 2.22	± 20.32	± 6.02	± 10.89	± 0.18	± 72.70	± 16.87
HCO_3 + P	14.92	43.03	31.18	27.52	0.39	2900.83	102.35
	± 0.87	± 11.33	± 9.27	± 3.47	± 0.03	± 94.27	± 18.36
P effect							
HCO3 effect	***	*	***	**		***	***

Table 3. Concentrations of N, P and K (mg g^{-1}) in Sphagnum for different treatments. Since no significant differences between species were found, data of both species were combined to display mean ± standard error (N=8). In effect row, significant differences of P or HCO_3 treatment are indicated by asterisks: * P≤0.05, ** P≤0.01 and *** P≤0.001.

	N (mg g^{-1})	P (mg g^{-1})	K (mg g^{-1})
C	11.80	1.36	7.56
	± 0.53	± 0.22	± 0.71
P	12.38	2.36	9.41
	± 1.06	± 0.38	± 1.17
HCO_3	13.50	1.73	2.31
	± 1.19	± 0.22	± 0.20
HCO_3 + P	16.05	2.82	2.10
	± 1.11	± 0.31	± 0.11
P effect	*	**	
HCO3 effect	**		***

Figures

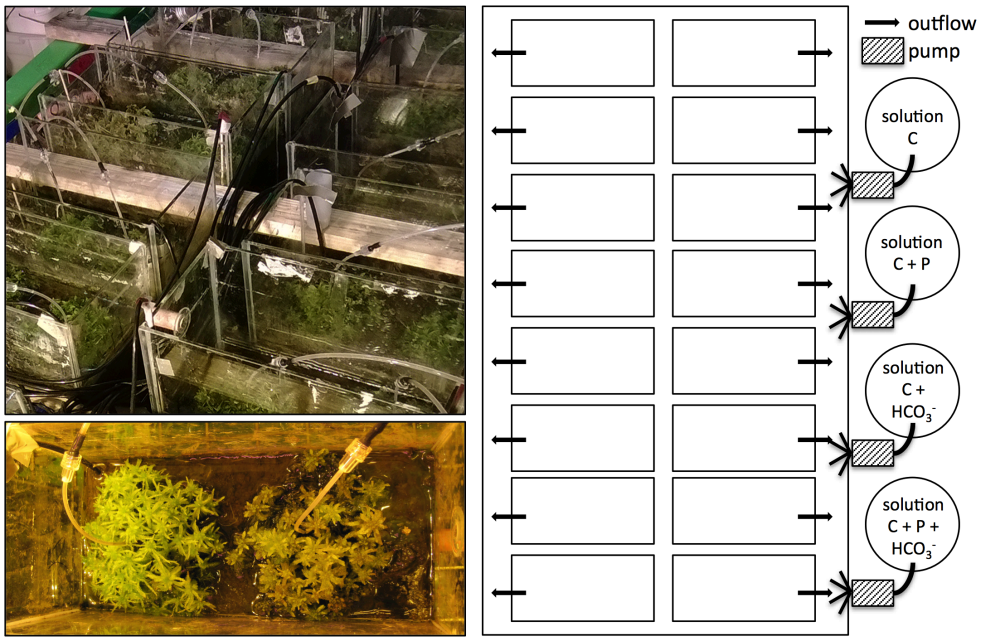

Figure 1. Picture of the mesocosms with *Sphagnum* layer and rhizons, placed in a temperature controlled water bath (left, up), close-up of one mesocosm (left, down), and the experimental design (right) showing the 16 mesocosms with water outflows and 4 treatment solution inflows: C (control), P addition (C+P), bicarbonate addition (C + HCO_3^-), and P plus bicarbonate addition (C + P + HCO_3^-), each randomly assigned to 4 mesocosms.

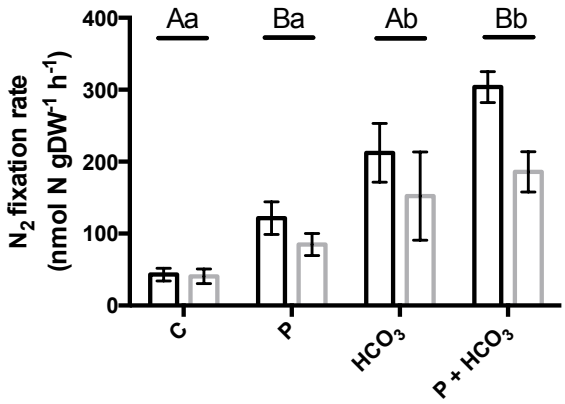

Figure 2. Rates of N_2-fixation of the diazotrophic communities of *Sphagnum squarrosum* (dark bars) and *S. palustre* (grey bars), under different treatments. Both P and HCO_3 treatment significantly increased N_2 fixation in both species, shown by letter combinations: P treatment (capital letter) and HCO_3^- treatment (lower case).

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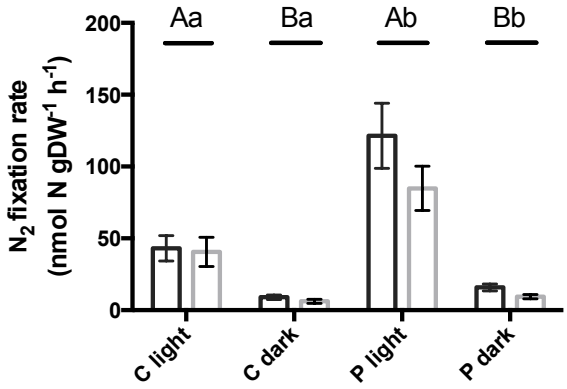

Figure 3. N_2 fixation rates of diazotrophic communities of *Sphagnum squarrosum* (dark bars) and *S. palustre* (grey bars), under light or dark conditions. Displayed is the mean ± standard error (N=4) of the control and P treatment (see text). Dark conditions significantly decreased N_2 fixation rates (shown by capital letter) and P treatment significantly increased rates (shown by lower case).

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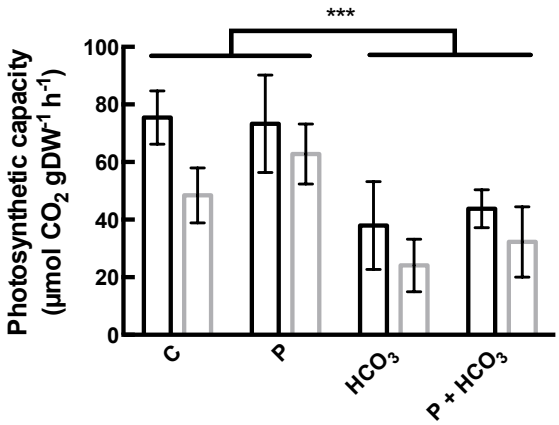

Figure 4. Photosynthetic rates of *Sphagnum squarrosum* (dark bars) and *S. palustre* (grey bars) under different surface water treatments. Displayed is the mean ± standard error (N=4). HCO_3^- significantly decreased rates, shown by *** ($P<0.001$).

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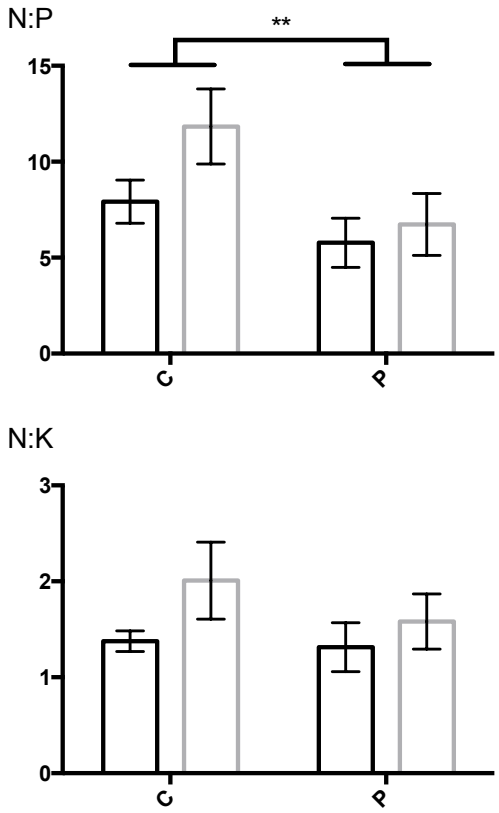

Figure 5. Means of N:P ratio and N:K ratios for *Sphagnum squarrosum* (dark bars) and *S. palustre* (grey bars), displayed for control (C) and addition of P (P) to surface water. Given is the mean ± standard error of the mean (N=4). HCO3 treatments were not included, because of leaking of nutrients from tissue (see text). Significant differences between treatments are shown with ** (P<0.01) in graph.

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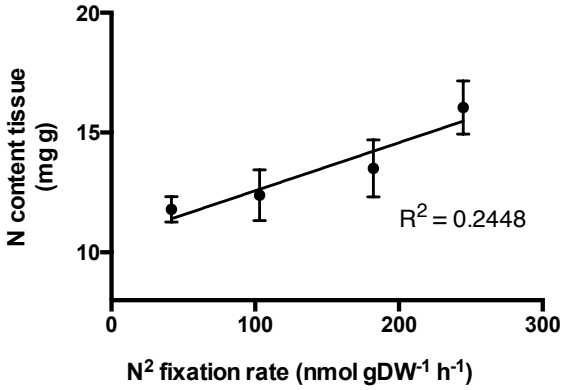

Figure 6. Linear regression of average N content of *Sphagnum* including its microbiome against average N_2 fixation rates of both species.

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