Abstract

Sphagnum peat mosses play an important role in the global carbon cycle by sequestering carbon as biomass thorough photosynthesis. Biodegradation in the northern peatlands is slow in part due to acidic and nutrient-poor soil conditions, so biomass accumulates over time as peat. *Sphagnum* mosses partner with bacteria that fix atmospheric nitrogen to supplement their nitrogen intake in these nutrient-poor soils. Climate change is altering temperatures, precipitation, and nitrogen deposition rates in the northern peatlands. These changes are accompanied by an increased release of greenhouse gases and a decline in *Sphagnum* ground coverage. The nitrogen-fixing bacteria associated with *Sphagnum* mosses need to be studied in order to understand how their relationship will be impacted by climate change. In this study I show that higher pH levels and nutrient concentrations can negatively impact the growth of potential nitrogen-fixing bacteria associated with two species of *Sphagnum* mosses. I also demonstrate that bacteria associated with these mosses overall are adapted to low-nitrogen conditions and a wide pH range.

Introduction

Peatlands are a type of wetland characterized by a thick layer of dead plant matter. About 90% of the world's peatlands are located in in the northern hemisphere mostly in Russia, Canada, and the northern US covering roughly 3% Earth's terrestrial surface (Yu et al 2010). The northern peatlands are one of the largest stores of terrestrial carbon and have acted as net accumulators of atmospheric carbon for most of the past several millennia (Turunen et al 2002, 2004). Because of this they are extremely important to the global carbon cycle. Northern peatlands typically act as carbon sinks because cold, anoxic soil conditions inhibit microbial

degradation and allow dead biomass to accumulate. However, they can become significant sources of atmospheric carbon and contribute to global warming depending on factors like temperature and nutrient availability (Yu et al 2011). The northern wetlands are expected to release more greenhouse gases, like carbon dioxide and methane, in the future due to increases in temperature and precipitation (Olefeldt et al 2013).

Most of the northern peatlands are dominated by mosses in the *Sphagnum* genus (Daniels and Eddy 1985). *Sphagnum* mosses convert atmospheric carbon into biomass thorough photosynthesis. This biomass is rich in phenolic compounds which are difficult for microbes to break down, so carbon dioxide and methane release from microbial degradation are slowed (Verhoeven and Liefveld 1997). These mosses also slow microbial decomposition by making the surrounding soil more anoxic and acidic. In addition to slowing the growth of microbes, *Sphagnum* also inhibits the growth of vascular plants by impeding their access to mineralized nutrients (Malmer et al 2003). *Sphagnum* growth can be negatively impacted by increasing soil temperatures and nitrogen deposition which can result in the expansive growth of vascular plants (Bragazza et al 2006, Gerdol et al 2007). Increases in soil temperature can also increase the rate of nitrogen mineralization which may also cause *Sphagnum* coverage to decrease (Aerts et al 2004, Berendse et al 2001)

Sphagnum mosses are able to grow in the nitrogen-poor soils of the northern peatlands by partnering with diazotrophs, bacteria that can fix atmospheric nitrogen into compounds that plants can use (Opelt et al 2007). Some studies have found that *Burkholderia* comprise a significant portion of the *Sphagnum* microbial community and that most of these members belong to clades noted for their beneficial effects on plant health, such as nitrogen-fixation and protection from pathogenic fungi (Bragina et al 2013, Opelt et al 2007). Other findings suggest

the composition of microbial communities varies from one *Sphagnum* species to another since *S. magellanicum* is dominated by *Gammaproteobacteria* and *S. fallax* is dominated by *Verrucomicrobia* and *Planctomycetes* (Bragina et al 2012). Although the dependence of *Sphagnum* mosses on their bacterial endophytes is well-known, the impact of changing environmental factors, such as temperature, precipitation, and CO_2 concentrations, on their relationship is not yet fully understood (Weston et al 2014).

Studies have found that changing soil conditions can alter the endophytic communities of some plants (Kinkel et al 2000, Rasche et al 2006). It is unknown whether this applies to *Sphangum* mosses. If the environmental changes brought by climate change negatively impact bacteria beneficial to the mosses, such as diazotrophs, then mosses themselves could be negatively impacted as well. The microbiomes of *Sphagnum* mosses in northern peatlands need to be characterized in order to better predict how they interact with *Sphagnum* mosses and how these interactions may be affected by climate change. The goal of my research is to cultivate a variety of diazotrophs from the tissues of *S. fallax* and *S. magellanicum* and determine how they respond to different environmental conditions.

Materials and Methods

Obtaining isolates

S. fallax and *S. magellanicum* samples were taken from the Marcell Experimental Forest in Minnesota in June 2015. The samples were sealed in plastic bags, wrapped in aluminum foil, and stored at 2 °C. 2 g of each moss species were soaked in sterile deionized (DI) water, ground into a macerate with a sterile mortar and pestle, then mixed with 18 mL of phosphate-saline buffer (PBS) at pH 7.2. This suspension was serially diluted tenfold five times (10^{-1} to 10^{-5}) in

PBS pH 7.2 and citric acid-phosphate-saline buffer (CPBS) pH 4.5. 100 µL aliquots of each PBS or CPBS dilution were spread onto solid plates of Jensen's medium (HiMedia components) and R2A medium (HiMedia components) at pH 7.2 and 4.5. Platings were done in triplicate. The number of colonies on each plate were recorded and used to calculate CFU/g moss for all bacteria favoring pH 4.5 and pH 7.2 and for limited nitrogen adapted bacteria favoring pH 4.5 and pH 7.2. All dilutions were also plated on solid *Bradyrhizobium* selective medium without nitrogen (BJSM-N) and *Burkholderia* selective medium (BAz) (Tong and Sadowsky 1994, Estrada-De Los Santos 2001). The selective agents in BJSM-N are L-arabinose, sodium gluconate, and heavy metals Co^{2+} and Zn^{2+}. The selective agent in BAz is azelaic acid.

The previously described maceration, dilution, and plating methods were also conducted on *Sphagnum* samples that had been surface-sterilized. This was done by soaking the samples in sterile DI water followed by a 5 min wash in 20% H_2O_2 followed by three consecutive rinses in sterile DI water. CFU/g calculations were made using CFU counts from the Jensen's and R2A media at pH 4.5 and pH 7.2. The statistical significance of the differences between CFU/g for each treatment was tested using one-way ANOVA with Turkey's HSD post hoc analysis (http://vassarstats.net/anova1u.html).

Finding nitrogen-fixing isolates

Potential diazotrophs were screened by 50 cycles of polymerase chain reactions (PCR) targeting the *nifH* gene, a component of the nitrogenase-encoding operon and an indicator of nitrogen fixation capability. Each cycle consisted of a 1 min denaturation step at 95 °C, a 1 min annealing step at 52 °C, and a 1 min extension step at 72 °C as previously described (Gaby and Buckley 2012). Colonies were boil prepped in individual volumes of 10 µL DI water to lyse the

cells and 1 µL of each was added to 24 µL of reaction mix. reaction mix contained 2.5 µL 10x PCR buffer, 2.5 µL dNTPs (2 mM), 2.5 µL IGK3 (10 µM), 2.5 µL DVV (10 µM), 2.5 µL BSA (1%), 11.375 µL DI water, and 0.125 µL DreamTaq DNA polymerase (Thermo Fischer Scientific). The DVV primer sequence is ATIGCRAAICCICCRCAIACIACRTC and the IGK3 primer sequence is GCIWTHTAYGGIAARGGIGGIATHGGIAA (Gaby and Buckley 2012). *Azotobacter vinelandii* and *Paenibacillus* sp. and DI water was used as a positive controls and a blank/negative control respectively. The 400bp PCR product was visualized on a 1% agarose gel stained with SYBR Green gel stain (Thermo Fischer Scientific).

nifH PCR troubleshooting

nifH could not be amplified from colonies from glycerol stocks of originally *nifH*+ isolates. The same PCR protocol described above was rerun on colony matter boil preps from the original colonies and colonies from glycerol stocks. Semiquantitative PCR was also performed on boil preps and DNA extracts from the original colonies. In this protocol the reactions were prepared at double the original volume and run for 48 cycles. 5µL were removed from each reaction at four cycle intervals between 20 and 48 cycles. DNA extract from *Paenibacillus* sp. was used as a positive control. The PCR products were visualized on a 1% agarose gel stained with SYBR Green gel stain (Thermo Fischer Scientific).

Acetylene reduction assay (ARA)

Isolates that grew well on the Jensen's medium or showed amplification of the *nifH* gene were streaked onto Jensen's medium slants in 30 mL Balch tubes crimped with rubber stoppers in the mouth to create an air-tight chamber. 2 mL acetylene gas was injected into each tube to make up 10% of the head space (Capone 1993). *A. vinelandii* and *Paenibacillus* sp. were used as

positive controls. Samples were analyzed using flame ionization detection with a gas chromatograph to observe conversion of acetylene to ethylene.

Identifying isolates

A portion of the 16S rRNA gene from each acetylene-reducing isolate was amplified using PCR with the 27F/1492r primer set. One 25 µL reaction mix contained 2.5 µL 10x buffer, 2.5 µL dNTPs (2 mM), 0.75 µL 27F (10 µM), 0.75 µL 1492r (10 µM), 2.5 µL BSA (1%), 15.375 µL DI water, and 0.125 µL DreamTaq DNA polymerase (Thermo Fischer Scientific). The 27F primer sequence is AGAGTTTGATCMTGGCTCAG and the 1492r primer sequence is TACCTTGTTACGACTT (Frank et al 2008). The samples then went through 25 cycles of PCR each cycle consisting of a 1 min denaturation step at 95 °C, a 1 min annealing step at 52 °C, and a 1 min extension step at 72 °C. The 1500bp.PCR product was visualized on a 1% agarose gel stained with SYBR Green gel stain. . The 16S PCR products were purified using the E.Z.N.A. Cycle Pure Kit and sent off site for Sanger sequencing. The 16S sequences were compared against the GenBank database using BLAST (http://blast.ncbi.nlm.nih.gov).

Physiological characterization

Five of the identified isolates were chosen and grown under three different concentrations of R2A (2x, 1x, 0.1x) and in 1x R2A with five different pH levels (3.5, 4.5, 5.5, 6.5, 7.5). *Bradyrhizobium* GV137 and *Rhizobium* UCT were chosen because they are potential nitrogen-fixers. *Granulicella pectinovorans* and Smag90 were chosen because Acidobacteria are abundant in peat. *Mucilaginibacter* sp. was chosen because it grew best on solid Jensen's medium. The different concentrations of R2A were made by preparing a volume of 2x R2A and then diluting portions down to 1x and 0.1x. Each of the five isolates was inoculated into three 5mL volumes of

each R2A concentration in 25mL test tubes. Optical density at 600nm was measured periodically with a spectrophotometer while the isolates were incubated at room temperature (25 °C) and shaken at 180rpm. 1x R2A was buffered at the five different pH levels by preparing CPBS with different ratios of citric acid and disodium phosphate. Each of the five isolates was inoculated into three 5mL volumes of each R2A concentration in 25mL test tubes. Optical density at 600nm was measured periodically with a spectrophotometer while the isolates were incubated at room temperature and shaken at 180rpm. The statistical significance of growth rate differences for each treatment was tested using one-way ANOVA with Turkey's HSD post hoc analysis (http://vassarstats.net/anova1u.html).

Results

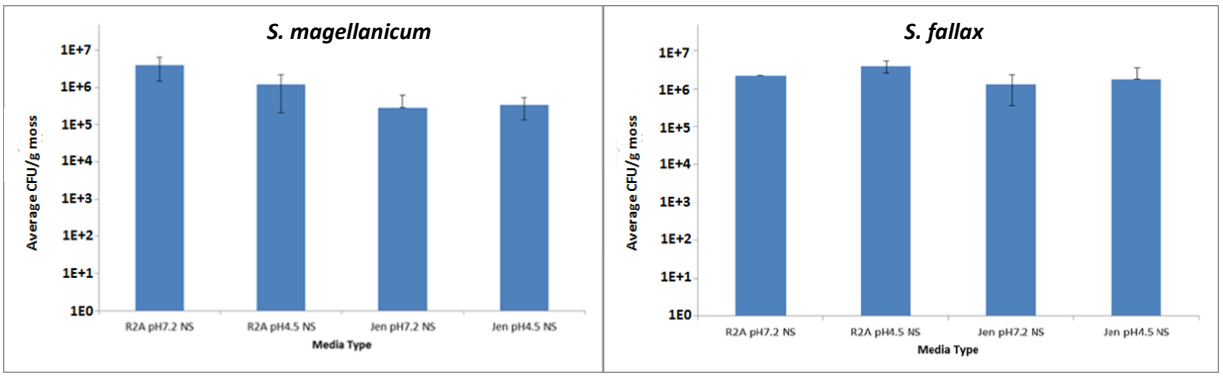

Figure 1. Calculated CFU/g from non-sterilized *S. magellanicum* (A) and *S. fallax* (B). Columns represent the average CFU/g calculated from the CFU counts on each media type (R2A or Jensen's at pH 7.2 or 4.5) from non-sterilized (NS) moss. The average CFU/g for each treatment is displayed, and error bars represent 1 SD.

There were no statistically significant differences between the CFU/g on any of the media types for either species (Figure 1). The differences between the CFU/g of a particular media type were also not statistically significant between moss species. There was often considerable variation among the calculated CFU/g. The calculated CFU/g from some plates differed from those from other plates of the same medium by orders of magnitude. The average calculated CFU/g values were all on the same order of magnitude for S. fallax, but for S. magellanicum the

values from the nitrogen-free medium (Jensen's) were an order of magnitude lower than those from the nitrogen-containing medium (R2A).

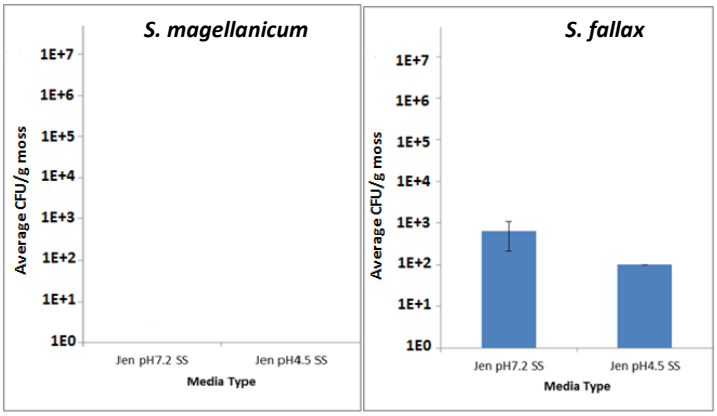

Figure 2. Calculated CFU/g from surface-sterilized *S. magellanicum* and *S. fallax*. Columns represent the average CFU/g calculated from the CFU counts on each media type (R2A or Jensen's at pH 7.2 or 4.5) from surface-sterilized (SS) moss. The average CFU/g for each treatment is displayed, and error bars represent 1 SD.

Growth occurred on Jensen's inoculated with surface-sterilized *S. fallax* but not with surface-sterilized *S. magellanicum*. The average CFU/g from pH 7.2 Jensen's for surface-sterilized *S. fallax* was significantly higher than the average CFU/g from Jensen's for surface-sterilized *S. magellanicum* ($P<0.01$). The average CFU/g from pH 7.2 Jensen's for surface-sterilized *S. fallax* was also significantly lower than the average CFU/g from pH 7.2 Jensen's for non-sterilized *S. fallax* ($P<0.05$). Statistical significance could not be calculated for *S. fallax* from pH 4.5 Jensen's because only one plate had colonies. CFU/g could not be calculated from pH 4.5 R2A inoculated with surface-sterilized moss because the plates only had lawn growth with no distinct colonies.

S. fallax			*S. magellanicum*		
Phylum	Genus, Species	Identity Match	Phylum	Genus, Species	Identity Match
Proteobacteria	*Janthinobacterium* S5C1	100%	Proteobacteria	*Burkholderia* Y	100%
Proteobacteria	*Pseudomonas fluorescens*	100%	Proteobacteria	*Burkholderia* PB1	100%
Bacteriodetes	*Mucilaginibacter* sp.	99%	Proteobacteria	*Burkholderia* HC87	100%
Bacteriodetes	*Mucilaginibacter* MDB2-30	98%	Proteobacteria	*Burkholderia sordidicola*	100%
Acidobacteria	*Granulicella pectinivorans*	100%	Proteobacteria	*Bradyrhizobium* GV137	100%
			Proteobacteria	*Rhizobium* UCT	100%
			Acidobacteria	*Granulicella pectinivorans*	100%
			Proteobacteria	*Pseudomonas* 230	99%
			Acidobacteria	n/a*	98%

Table 1. Phylum, genus, and species names of identified isolates. Percent identity matches to Genbank sequences displayed to the right. Cell marked with * indicates a match without a genus or species name.

Most of the identified isolates belong to the phylum Proteobacteria (Table 1). The other two phyla represented are Acidobacteria and Bacteriodetes. One isolate (Smag90) matched with an OTU that has never been cultured. This OTU is most closely related to Acidobacteria. The majority of isolates from *S. magellanicum* were Proteobacteria. The Acidobacterium *Granulicella pectinovorans* was found in both moss species. Bacteriodetes species were only found in *S. fallax*.

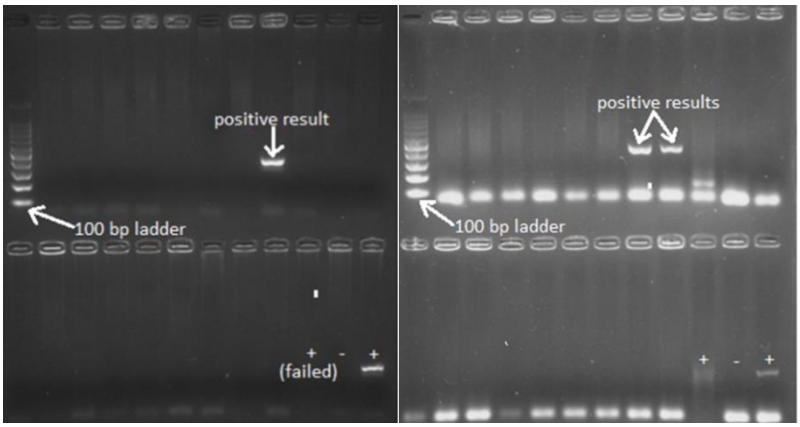

Figure 3. *nifH*+ PCR Results. Bands indicating presence of the *nifH* gene are labeled "positive result." Positive controls are indicated by pluses (+) and the negative controls are indicated by minuses (-). Each gel contains a 100bp ladder which is labeled.

Only three out of ~200 isolates from both moss species were found to possess *nifH*, Smag16, Smag26, and Smag27 (Figure 3). All three of these isolates came from *S. magellanicum* and were identified as *Bradyrhizobium* GV137.

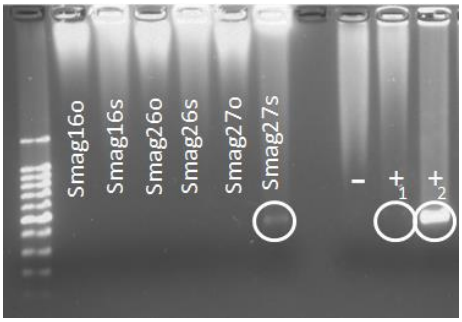

Figure 4. *nifH* PCR of Original and Stored *nifH*+ Isolates. PCR products from original colonies are indicated by the "o" following the isolate ID. PCR products from colonies from glycerol stocks are indicated by the "s" following the isolate ID. *nifH*+ results are circled. The positive controls are indicated by pluses (+), and the negative control is indicated by a minus (-).

Although the amplification of *nifH* from the original colonies was initially strong, the gene could not be amplified from glycerol stocks of these isolates. When the same PCR protocol was conducted on the original colonies and colonies from glycerol stocks, only the Smag27 glycerol stock produced a *nifH*+ band which was faint (Figure 4). All of the gel lanes produced smearing, but the moss samples displayed the most which was concentrated around the wells.

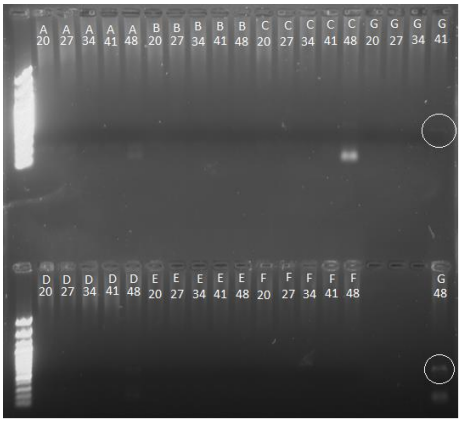

Figure 5. Semiquantitative *nifH* PCR. A: Smag16 colony, B: Smag16 DNA extract, C: Smag26 colony, D: Smag26 DNA extract, E: Smag27 colony, F: Smag27 DNA extract, G: Purified *nifH* from *Paenibacillus* sp. (+ control). The number below each letter indicates during which PCR cycle the sample was taken. *nifH*+ results are circled. 100bp ladders are in the leftmost wells.

When colony matter and DNA extracts from the three initially *nifH*+ isolates were used in *nifH* PCR, none produced a distinct band after any number of cycles (Figure 5). There may be an extremely faint *nifH*+ band in lane D-48 which is Smag26 DNA extract after 48 cycles, but it's hard to tell.

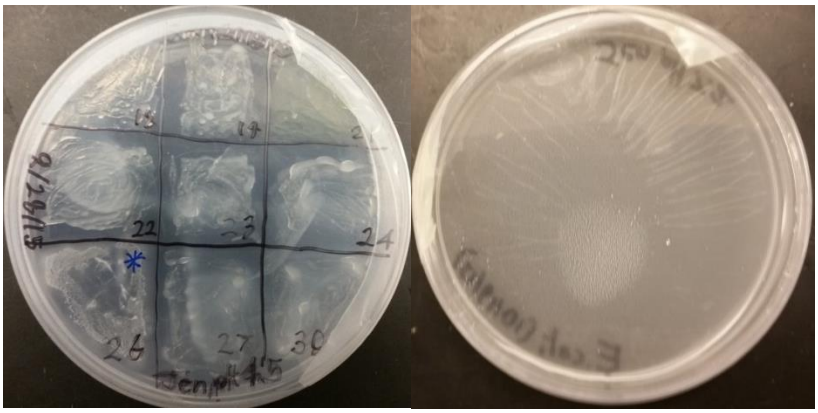

Figure 6. *Sphagnum* Isolates (A) and *Escherichia coli* (B) on Jensen's. The left plate of Jensen's has various *Sphagnum* isolates growing on it. The right plate of Jensen's was streaked with *E. coli*.

Although many of the *Sphagnum* isolates grew robustly on Jensen's medium (Figure 6), none of them were able to reduce acetylene during ARA when they were grown on solid Jensen's medium. Both positive controls, *A. vinelandii* and *Paenibacillus* sp., reduced acetylene while grown on solid Jensen's medium. *E. coli*, a non-nitrogen fixer, was streaked on solid Jensen's medium and was unable to grow at all.

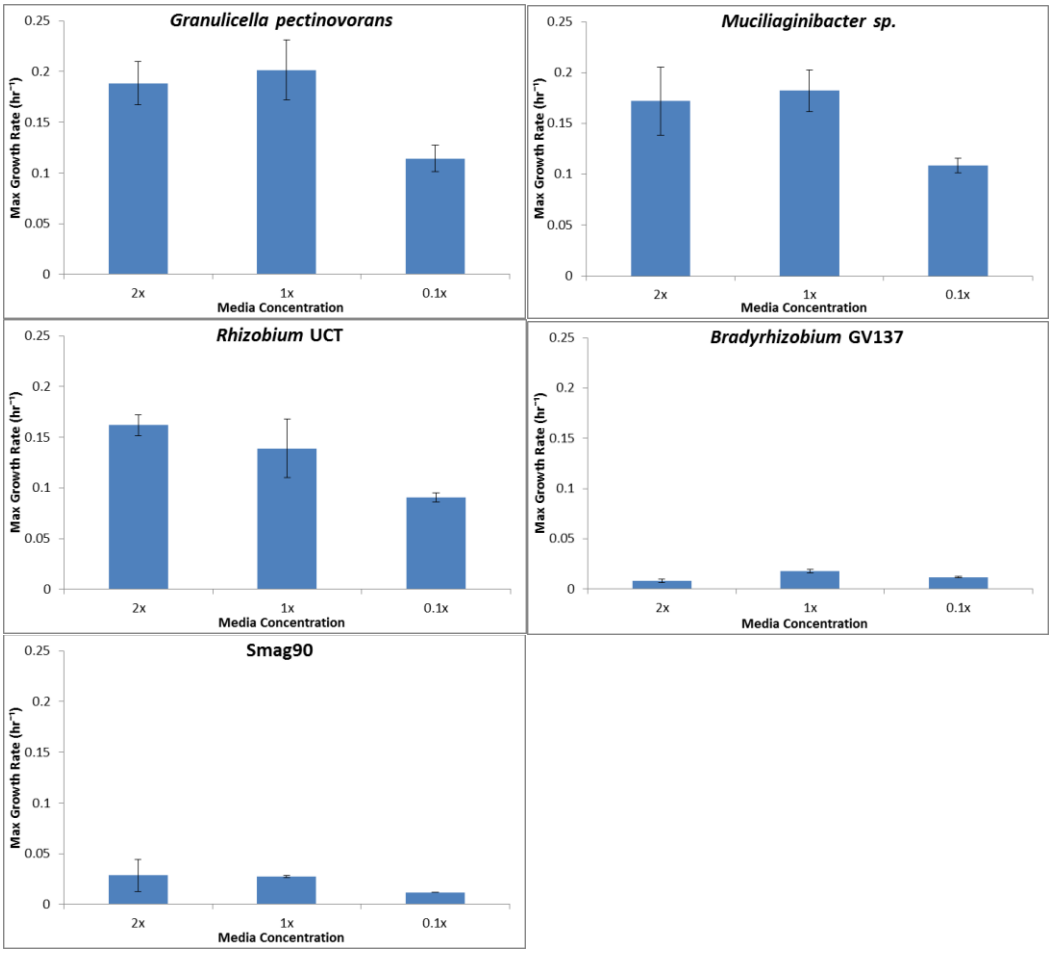

Figure 7. Maximum Growth Rates of *Sphagnum* Isolates in 2x, 1x, and 0.1x R2A. *Granulicella pectinovorans* (A), *Mucilaginibacter* sp. (B), *Rhizobium* UCT (C), *Bradyrhizobium* GV137 (D), Smag90 (E). Growth rate is on the y-axis in hours^{-1}.

Rhizobium UCT was the only isolate that grew fastest in 2x R2A ($P<0.05$, $P<0.01$). Two isolates grew significantly beter at 1x R2A: *Granulicella pectinovorans* ($P<0.01$, $P<0.05$) and *Mucilaginibacter* sp. ($P<0.05$, $P<0.05$). *Bradyrhizobium* GV137 grew fastest in 1x R2A too, but not significantly. *Bradyrhizobium* GV137 was also the only isolate that grew faster in 0.1x than in 2x, but the difference wasn't significant. Smag90 grew about the same in 2x and 1x and worst in 0.1x, but the difference was not statistically significant. The Smag90 culture in 2x was also much clumpier than at 1x. Smag90 and *Bradyrhizobium* GV137 grew much more slowly than the other three isolates.

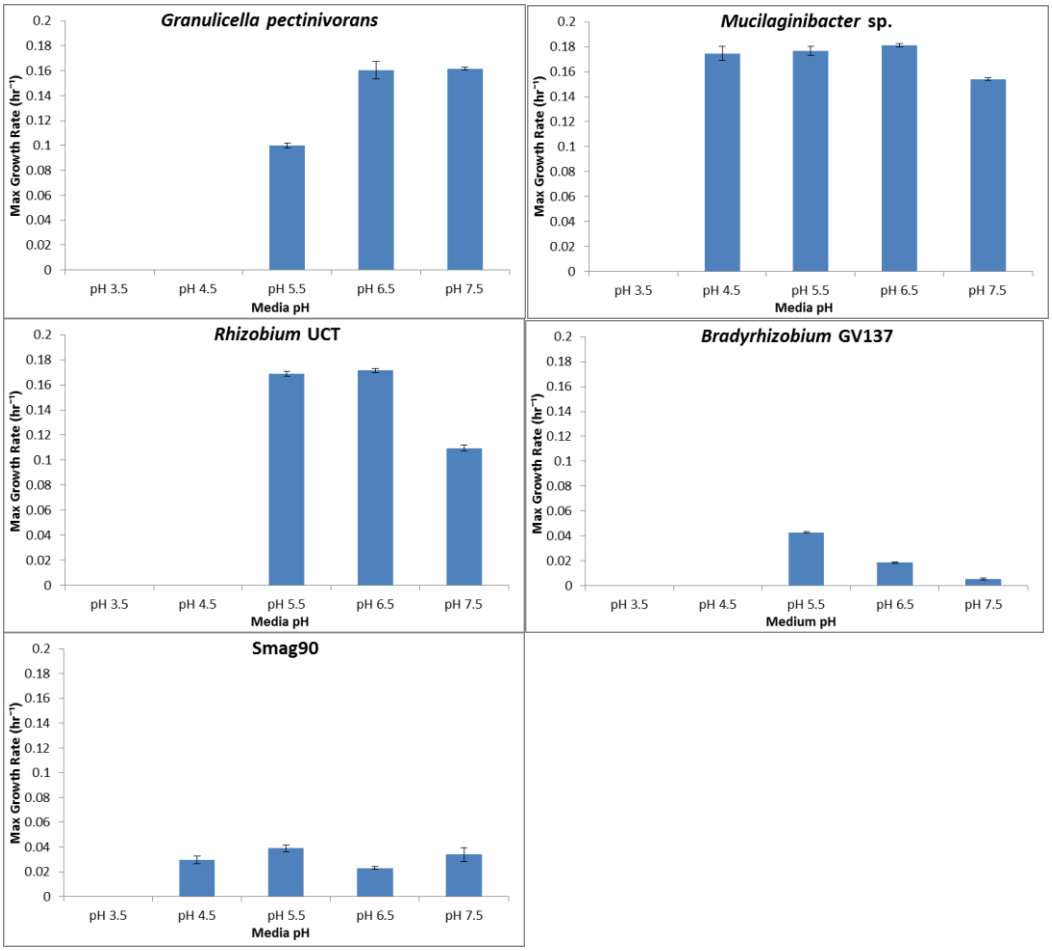

Figure 8. Maximum Growth Rates of *Sphagnum* Isolates at pH 3.5, 4.5, 5.5, 6.5, and 7.5. *Granulicella pectinovorans* (A), *Mucilaginibacter* sp. (B), *Rhizobium* UCT (C), *Bradyrhizobium* GV137 (D), Smag90 (E). Growth rate is on the y-axis in hours^{-1}.

None of the isolates grew at pH 3.5, and only *Muciliaginibacter* sp. and Smag90 grew at pH 4.5. *Granulicella pectinovorans* grew better at pH 6.5 and pH 7.5 than at pH 5.5 ($P<0.01$, $P<0.01$). *Mucilaginibacter* grew equally better at pH 4.5, pH 5.5, and pH 6.5 than at pH 7.5 ($P<0.01$, $P<0.01$, $P<0.01$). *Rhizobium* UCT grew equally better at pH 5.5 and pH 6.5 than at pH 7.5 ($P<0.01$, $P<0.01$). *Bradyrhizobium* GV137's optimum pH was 5.5 by a significant amount ($P<0.01$. $P<0.01$) and barely grew at pH 7.5. Smag90 grew better at pH 5.5 and pH 7.5 than at pH 4.5 and pH 6.5, but the rates were only significantly higher than at pH 6.5 ($P<0.01$, $P<0.01$). Most isolates grew best at pH 5.5.

Discussion

The insignificance of the difference between colony counts on acidic (pH 4.5) and neutral (pH 7.2) media for both *S. magellanicum* and *S. fallax* suggests that their bacterial communities are tolerant of acidic and neutral pH levels. This is to be expected because *Sphagnum* tissues and peat bog soil are acidic (Yu et al 2011). Another study also found colony counts on acidic (pH 5.5) and neutral (pH 7.2) media from *S. magellanicum* and *S. fallax* to be practically identical (Opelt et al 2007). This suggests that the bacterial communities of both mosses contain roughly the same number of cultivatable bacteria. The CFU/g calculated for both mosses in the study were also on the same order of magnitude (10^6) (Opelt et al 2007).

The differences between the colony counts on Jensen's and R2A were also not significant for either moss species when not sterilized. This suggests that the bacterial communities of both moss species are adapted to nitrogen-limiting conditions. However, in the case of *S. magellanicum*, the colony counts on Jensen's medium were an order of magnitude lower than the colony counts on R2A, so it's likely this difference would have been statistically significant if quality of the data was better. If the endophytes are adapted to low-nitrogen conditions, then it could be difficult to isolate diazotrophs by inoculating solid nitrogen-free media. The colony counts on Jensen's medium were significantly higher for *S. fallax* than *S. magellanicum* when the mosses were surface-sterilized. This could suggest that *S. fallax* contains more endophytic diazotrophs than *S. magellanicum*. This would be surprising since *S. magellanicum* occupies more nutrient-poor areas than *S. fallax*, so one would expect it to harbor more nitrogen-fixing species to compensate (Daniels and Eddy 1985). An alternative explanation is that *S. fallax* has more cultivatable endophytic diazotrophs than *S. magellanicum*.

Even though isolates Smag16, Smag26, and Smag27 were originally *nifH*+, they did not produce the expected bands during several subsequent *nifH* PCR reactions. The species the isolates were identified as, *Bradyrhizobium* GV137, has previously been demonstrated to possess *nifH*, so it's unlikely these results were false positives (Cobo-Díaz et al 2014). A reason for this could have been that the original colonies carried the *nif* operon on a plasmid that was lost between storage and replating (Banfalvi et al 1981). However, the original colonies also did not produce the expected band when tested again with *nifH* PCR, but one of the glycerol stocks did. This implies that an error was occurring during PCR. Excessive cycling during PCR can result in loss of product and large smears on the gel which occurred during PCR of the original colonies and glycerol stocks (Bell and DeMarini 1991). The results from semiquantitative *nifH* PCR dispute this since no bands were produced after any number of cycles. Both colony matter and DNA extracts were used during semiquantitative PCR, so inhibition from cellular debris is also not the source of the issue. It's possible that the DVV and IGK3 primers anneal poorly to the *nifH* gene of these isolates and only produced product before because the conditions were just right by chance. The probable failure of the DVV/IGK3 primer set could mean that there were other *nifH*+ isolates that were not detected. Other *nifH* primer sets should be used to try to amplify the gene from these isolates.

The failure of any of the samples to reduce acetylene during ARA could mean than none of the isolates are diazotrophs. It's also possible that some of them are diazotrophs, but the conditions weren't appropriate to induce nitrogen fixation. Low-oxygen conditions should be used in the future to encourage nitrogen fixation (Fischer 1994).

Some of the isolates had slower growth rates at higher concentrations of R2A which could indicate that their growth is inhibited by higher nutrient concentrations. If important

nitrogen fixers and other plant growth promoting bacteria for *Sphagnum* mosses are inhibited by higher concentrations of nutrients, then it's possible that increased rates of nitrogen deposition in Northern peatlands could negatively impact the growth of these bacteria and as a result *Sphagnum* mosses (Gerdol et al 2007). Some of the isolates also had slower growth rates at more neutral pH levels. This was most pronounced with the potential diazotroph *Bradyrhizobium* GV137. If bacteria beneficial to mosses are also sensitive to neutral pH levels, then a rising soil pH in the peatlands caused by decreased *Sphagnum* coverage could exacerbate the loss of *Sphagnum* by affecting these beneficial species. Knowing the nutrient and pH sensitivity of the isolates will also be useful when further characterizing the isolates and their relationship with *Sphagnum*.

Only a very small subset of the *Sphagnum* microbiome was cultivated in this study and only one species with *nifH* was isolated and partially characterized. This species (*Bradyrhizobium* GV137) and another isolate (*Rhizobium* UCT) are classified under the order Rhizobiales which most *nifH*-carrying species in *Sphagnum* appear to belong to (Leppänen et al 2015). Although nutrient and pH sensitivity were demonstrated in some of these isolates, a broader, more representative set of bacteria needs to be isolated and studied in order to predict how *Sphagnum* microbiomes will respond to changing environmental conditions. Since much of the *Sphagnum* microbiome has adapted to nitrogen limited conditions, special care needs to be taken to prepare media for isolating diazotrophs to eliminate as much residual nitrogen as possible. As mentioned previously, a suitable *nifH* primer set must be found and rigorously tested before attempting to locate any more diazotrophs.

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