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Research Article

RESEARCH ARTICLE

Iron limitation effects on nitrogen-fixing organisms with possible implications for cyanobacterial blooms

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One sentence summary: Phosphorus-iron co-limitation influenced the relative abundance of stream nitrogen-fixing organisms.

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ABSTRACT

Cyanobacteria-dominated harmful algal blooms are increasing in occurrence. Many of the taxa contributing to these blooms are capable of fixing atmospheric nitrogen and should be favored under conditions of low nitrogen availability. Yet, synthesizing nitrogenase, the enzyme responsible for nitrogen fixation, is energetically expensive and requires substantial concentrations of iron. Phosphorus addition to nitrogen poor streams should promote nitrogen fixation, but experimental results so far have been inconclusive, suggesting that other factors may be involved in controlling this process. With iron potentially limited in many streams, we examined the influence of phosphorus-iron colimitation on the community structure of nitrogen-fixing organisms. In stream microcosms, using microscopic and molecular sequence data, we observed: (i) the greatest abundance of heterocyst forming nitrogen-fixing cyanobacteria in low nitrogen treatments with high phosphorus and iron and (ii) greater abundance of non-photosynthetic nitrogen-fixing bacteria in treatments with nitrogen compared to those without it. We also found that comparisons between molecular results and those obtained from microscopic identification provided complementary information about cyanobacterial communities. Our investigation indicates the potential for phosphorus-iron colimitation of stream nitrogen-fixing organisms.

Keywords: cyanobacteria; nifH; nitrogen-fixers; community structure; phosphorus-iron colimitation; cyanoHABs

INTRODUCTION

Dramatic increases in both the geographic distribution and frequency of harmful algal blooms (HABs) are occurring worldwide (Lopez *et al.* 2008; Pearl, Hall and Calandrino 2011). Many of these blooms comprise naturally occurring species of cyanobacteria that reach nuisance levels under the right sets of conditions and are collectively referred to as CyanoHABs (Lopez *et al.* 2008; Pearl, Hall and Calandrino 2011). CyanoHABs can produce toxic secondary metabolites or cyanotoxins, with potentially detrimental impacts on ecosystem function as well as human and animal health (Mur, Skulberg and Utkilen 1999; Jonasson *et al.* 2010). Increased nutrient availability, typically abundant phosphorus

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concentrations, can trigger CyanoHABs, although it is increasingly evident that the problem is not merely limited to eutrophic systems, as reports of blooms in oligotrophic systems are also on the rise (Sorichetti, Creed and Trick 2014). Additionally, many of the cyanobacteria taxa contributing to CyanoHABs are species capable of diazotrophy or nitrogen fixation.

Organisms having the capacity to fix atmospheric nitrogen are favored under conditions of low nitrogen availability (Schindler 1977; Fairchild, Lowe and Richardson 1985; Marcarelli and Wurtsbaugh 2006; Schindler *et al.* 2008; Larson, Liu and Passy 2015). However, synthesizing the nitrogenase enzyme, the catalyst involved in nitrogen fixation, is an energetically expensive process (Howarth, Marino and Cole 1988), making nitrogen fixation advantageous only under conditions of prolonged periods of low nitrogen availability (Kunza and Hall 2013). Increased phosphorus concentrations should promote nitrogen fixation in nitrogen-limited systems (Schindler *et al.* 2008). However, experimental manipulations of phosphorus levels in streams with low nitrogen have produced mixed results, showing both positive (Marcarelli and Wurtsbaugh 2006; Marcarelli and Wurtsbaugh 2007) and non-significant effects (Scott *et al.* 2009) on nitrogen fixation. This suggests the possibility that nitrogen fixation may also depend on other nutrients.

Nitrogenase is an iron-rich enzyme, which imposes a high demand for iron on diazotrophs (Paerl, Prufertbebout and Guo 1994; Berman-Frank, Lundgren and Falkowski 2003; Mills *et al.* 2004; Moore *et al.* 2009). Consequently, iron was shown to limit nitrogen fixers in lakes and marine systems (Wurtsbaugh and Horne 1983; Lenes *et al.* 2001; Mills *et al.* 2004; Hanington, Rose and Johnstone 2016), but little is known about stream ecosystems, where iron is generally insoluble (Vuori 1995). Considering that many streams are nitrogen (Vitousek and Howarth 1991; Elser *et al.* 2007) and/or iron limited (Passy 2010; Larson, Liu and Passy 2015), and that phosphorus may stimulate stream nitrogen fixation (Marcarelli and Wurtsbaugh 2006; Marcarelli and Wurtsbaugh 2007), it is plausible that phosphorus-iron colimitation may govern the composition of stream diazotrophs in nitrogen-poor systems. The paucity of studies testing the influence of phosphorus-iron colimitation on stream diazotrophs in the presence of increasing anthropogenic nutrient inputs speaks to the need for more investigation into the impacts of phosphorus-iron enrichment in streams.

Recently, experiments in aquatic microcosms have demonstrated significantly greater taxa richness and biomass of N_2-fixing organisms under treatments of phosphorus-iron addition compared to treatments with only phosphorus addition (Larson, Liu and Passy 2015). Increased richness of non-N_2-fixing organisms were also correlated with greater abundance of nitrogen fixers, highlighting the potential indirect impacts that nitrogen fixation may have on the rest of the stream benthos (Larson, Liu and Passy 2015). Nevertheless, compositional shifts in N_2-fixing organisms were not elucidated and are the focus of this report. Nitrogen fixation can be an important source of nitrogen in nutrient limited streams (Peterson and Grimm 1992; Henry and Fisher 2003); however, under optimal conditions (e.g. prolonged nitrogen limitation and increased water temperatures), N_2-fixing cyanobacteria can also reach nuisance levels (Lopez *et al.* 2008; Pearl, Hall and Calandrino 2011). With increasing occurrences of CyanoHABS (Lopez *et al.* 2008), it is imperative to determine the relative importance of the various factors contributing to the growth and proliferation of cyanobacteria, including those with the capacity for nitrogen fixation and bloom formation. Gaining a greater understanding of the factors influencing the growth of cyanobacteria in mixed species assemblages will help us devise ways to control their growth and prevent them from reaching nuisance levels (Pearl, Hall and Calandrino 2011; Pace *et al.* 2016).

It is likely that many of the factors influencing the abundance of phototrophic N_2-fixing cyanobacteria, including nutrient availability, are also important to N_2-fixing non-photosynthetic bacteria (Caron 1994; Kohler *et al.* 2016). As with N_2-fixing cyanobacteria, little is known about how phosphorus-iron colimitation influences N_2-fixing stream heterotrophs. Thick biofilms have a complex three-dimensional structure, leading to heterogeneous nutrient and oxygen conditions within the biofilm matrix. It is conceivable that even in nutrient replete conditions, non-photosynthetic bacteria having the capacity for nitrogen fixation could persist within the lowest layers of the biofilm mat, where nutrient limitation and anaerobic conditions develop due to separation from oxygenic photosynthesis within the top layer of the biofilm (Berrenero *et al.* 2016). In fact, high nitrogenase activity has been observed in the lowest layers of a cyanobacterial mat, separated from oxygenic photosynthesis in the top layer of the mat (Stal, Grossberger and Krumbein 1984).

Phosphorus concentrations are increasing in streams and lakes due to land use for agriculture and increasing atmospheric deposition (Foley *et al.* 2005; Stoddard *et al.* 2016). To better understand the impacts of iron and phosphorus colimitation on stream primary producers, most notably N_2-fixing cyanobacteria and bacteria, we initiated a set of experiments in artificial stream microcosms. We manipulated nitrogen, phosphorus and iron, and quantified the nutrient effects on the composition of N_2-fixing organisms in the benthos by high-throughput DNA sequencing techniques. We focused on the nitrogenase reductase (*nifH*) gene, which is responsible for encoding part the of nitrogenase enzyme complex (Zehr, Mellon and Zani 1998; Zehr *et al.* 2003; Berrendero *et al.* 2016). As molecular studies are increasingly being used to investigate microbial communities, while most of the stream cyanobacterial research has relied on microscopic identifications, we were also interested in comparisons between molecular and microscopic results. We had the following objectives: (i) to examine whether iron limitation and iron-phosphorus colimitation generated distinct responses in nitrogen fixer composition (photosynthetic and non-photosynthetic organisms) under nitrogen limited versus nitrogen replete conditions, and (ii) to assess the discriminative power of conventional microscopy techniques versus molecular approaches and whether they provided complementary or overlapping information.

MATERIALS AND METHODS

Microcosm study

In September–October 2011, we performed a nutrient manipulation experiment in a facility with 24 stream microcosms located at the University of Texas at Arlington. Briefly, 30.5 cm diameter round glass dishes containing 4.5 L of nutrient media (*see below*) were lined with 36 natural stone tiles (3.1 cm × 1.5 cm × 0.7 cm) for algal colonization. Inoculation of all microcosms took place on day 1 of the experiment by seeding with algae scraped from several streams of varying nitrate, phosphate and iron levels in the Dallas-Fort Worth area and pooled together. The microcosms were illuminated by 250 W metal halide lamps for 14 h daily. A constant current velocity of 8 cm·sec^{-1} was maintained in each microcosm by an IKA RW-20 digital overhead stirrer (IKA®

Works, Inc., Wilmington, North Carolina, U.S.A.). A more thorough description of these microcosms can be found in Larson, Liu and Passy (2015).

The microcosms were filled with modified COMBO medium, prepared with carbon-filtered water and supplemented with $NaNO_3$ (14.0 mg·L^{-1} N), K_2HPO_4 (1.55 mg·L^{-1} P), EDTA + $FeCl_3 \cdot H_2O$ (0.21 mg·L^{-1} Fe), and all their combinations or left as control (none of the aforementioned nutrients added). Other than the manipulated nutrients, modified COMBO medium included all constituents (major stocks and algal trace elements) in their normal concentrations (Kilham *et al.* 1998). Employing a full factorial design resulted in eight different nutrient treatments with three replicates each, hereafter referred to as NPFe, PFe, NFe, NP, N, P, Fe and Control. However, we focus here only on the NP, NPFe, P, and PFe treatments since biofilms in all other treatments consisted almost entirely of diatoms and did not accumulate sufficient biomass to successfully extract enough DNA (<10 ng/μL) for 16S rRNA and *nifH* genes molecular analyses (Fig. S1, Supporting Information). Every three days, one third of the medium in each microcosm was replaced with fresh medium of the respective nutrient combination and the nutrient levels were checked for consistency with the COMBO levels with an Auto-Analyzer III (SEAL Analytical Inc., Mequon, Wisconsin, U.S.A.). On day 60, algal samples from three tiles were randomly chosen from each microcosm and scraped with a toothbrush until visibly cleaned.

Algal processing and microscopic identification

The collected algae were fixed with 4% formaldehyde. Soft algae units (unit = a cell for unicellular algae, a colony, or 25 μm of a filament) were counted in 30 random fields in a Palmer–Maloney cell. Samples were acid digested and mounted with Naphrax® for diatom identification. At least 400 diatom frustules were enumerated per sample.

Molecular analysis of microbial communities

DNA extraction and PCR amplification

Total genomic DNA was extracted in triplicate from each treatment using PowerLyzer PowerSoil Isolation Kit (MO BIO, Carlsbad, CA). All extractions steps were performed according to manufacturer's instructions. Extracted DNA samples were stored at −20°C until further analyzed.

16S rRNA gene amplification and sequencing

The 16S rRNA gene from different DNA samples was amplified with sequencing primers F515 (5′-GTGCCAGCMGCCGCGG-3′) and R907 (5′-CCGTCAATTCMTTTRAGTTT-3′). These primers were attached with a unique identifier and DNA sequencing adapter. The details of the 16S rRNA gene amplification were the same as described previously in Mirza *et al.* (2014). In short, 50 μL PCR amplification reactions contained a 1X buffer, 0.2 μM of each primer, 1.8 mM $MgCl_2$, 200 μM deoxynucleoside triphosphates (dNTPs), 20 ng of template and 1 μL FastStart high-fidelity PCR system enzyme (Roche Applied Sciences). The PCR conditions were as follows: 3 min at 96°C, followed by 30 cycles of denaturation at 94°C for 45 s, primer annealing at 56°C for 45 s, extension at 72°C for 45 s and final extension for 7 min. Amplified PCR products were purified with Agencourt AMPure beads (Beckman Coulter, Brea, CA). Purified PCR products from different samples were pooled in equimolar concentrations and pyrosequencing was performed on the mixture with the 454 GS FLX sequencer (454 LifeSciences) at the Utah State University Center for Integrated Biosystems (CIB), USA.

NifH gene amplification and sequencing

The *nifH* gene was amplified using PolF (TGCGAYCCSAARGC BGACTC) and PolR (ATSGCCATCATYTCRCCGGA) primers Poly, Monrozier and Bally (2001). The primers were attached with the unique identifier and DNA sequencing adapter. All reagents for *nifH* gene amplification, except the PCR primers, were the same as described above. The PCR conditions used for *nifH* gene amplification were 2 min of denaturation at 95°C, followed by 35 rounds of temperature cycling (95°C for 30 s, 59°C for 30 s and 72°C for 45 s), and a final extension at 72°C for 7 min. The *nifH* amplicons were purified with Agencourt AMPure beads and sequenced at CIB as described above for the 16S rRNA gene.

Sequence analyses

Prokaryotic 16S rRNA genes obtained from different treatments were filtered for initial quality control by removing DNA sequences containing >1 inexact match with the unique barcode identifier, read length <370 bp, containing >8 homopolymer bases and sequences with unidentified bases (N). After initial screening, chimeric sequences were identified and removed as described previously Mirza *et al.* (2014). Sequences that passed quality control were submitted to the Ribosomal Database Project (RDP; http://rdp.cme.msu.edu), Naive Bayesian Classifier 2.5 (Wang *et al.* 2007). All DNA sequences were also aligned and clustered into operational taxonomic units (OTUs) at 97% DNA identity. This OTU-based abundance data were used to assess the changes in overall community structure in response to different nutrient treatments. Representative sequences from each OTU containing more than 10 sequences were phylogenetically analyzed using MEGA version 5.2 (Tamura *et al.* 2007).

The *nifH* gene sequences were processed for initial quality control parameters (as described above) using the functional gene pipeline of the RDP II Fish *et al.* (2013) (http://rdp.cme.msu.edu). Chimeric sequences were identified and removed using the USEARCH 6 chimera check. Frame-shift errors were adjusted by running FrameBot, and protein sequences were aligned through Hidden Markov Models (HMMER3 aligner). Aligned DNA sequences were clustered into OTUs at 97% DNA similarity by complete linkage clustering using RDP's mcClust. The OTUs were then used in multivariate analyses. Identification of the *nifH* gene sequences was carried out by running an NCBI Standalone Blast Setup and also compared against a *nifH* gene reference database of about 1100 sequences obtained from Wang *et al.* (2013). To evaluate the relative distribution of the most abundant phylotypes/clusters, we selected the representative sequences from the OTUs containing more than 100 sequences. These representative DNA sequences were used to create a Maximum-likelihood based phylogenetic tree to indicate the relative distribution of these most abundant phylotypes across different treatments and also their phylogenetic affiliations in relation with other microorganisms.

Nucleotide sequence accession numbers

Both 16S rRNA and *nifH* gene sequences were submitted to NCBI Sequence Read Archive (Sequence submission ID = SUB3205505 and Bioproject ID = PRJNA417492).

Statistical analysis

All statistical analyses were carried out using R (version 3.2.3, R Core Team 2015). Using the vegan package (Oksanen *et al.*

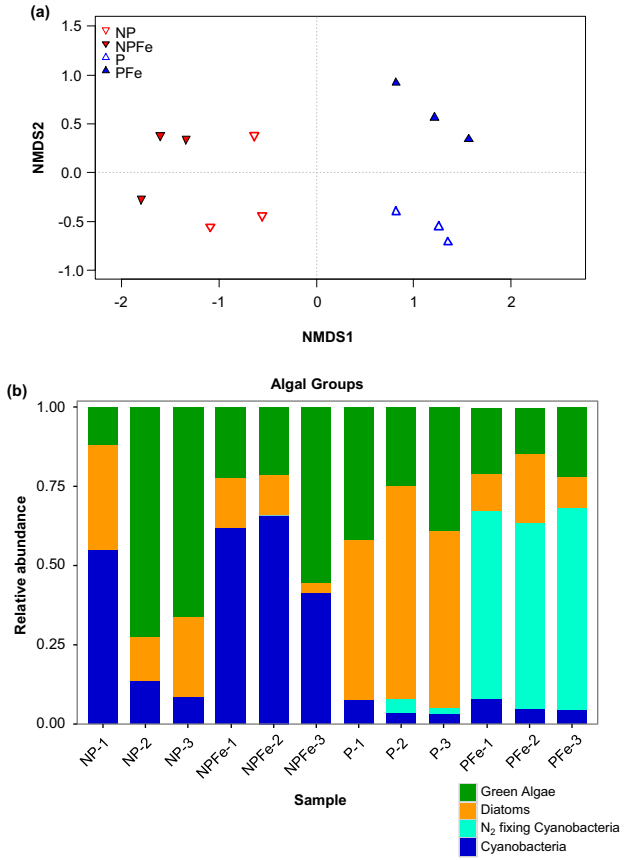

Figure 1. Non-metric multidimensional scaling of periphyton communities from nutrient addition microcosm experiments using microscopic count data (**a**). Relative abundance of the different algal groups in each replicate and each nutrient treatment (**b**).

2016), we performed separate non-metric multidimensional scaling (NMDS) using Bray–Curtis dissimilarities to identify compositional patterns across treatments using data from microscopic counts, 16S rRNA and *nifH* gene sequence analyses. PERMANOVAs were used to test for differences in species composition across treatments using 999 permutations. Additionally, Mantel tests were performed to test for correlations between dissimilarity matrices obtained from microscopic counts, 16S rRNA and *nifH* molecular analyses using 999 permutations.

RESULTS

Microscopic counts

NMDS of microscopic counts of the four studied treatments on day 60 of the experimental run revealed distinct groupings: first, the nitrogen treatments (NP and NPFe) separated from the nitrogen-free treatments (P and PFe) on the first axis, and the Fe treatments PFe and NPFe) separated from the iron-free treatments (P and NP) on the second axis but the latter separation was less clear (Fig. 1a). PERMANOVA revealed significant differences in composition between the four treatments (Pseudo-$F_{(3,11)} = 7.42$, $P \leq 0.001$, Fig. 1a). Abundance of green algae was the highest in two replicates of the NP treatment, with diatoms most abundant in the P treatment (Fig. 1b). Cyanobacteria were generally most abundant in the treatments with iron, with putative N_2-fixing cyanobacteria having the highest abundance in the PFe

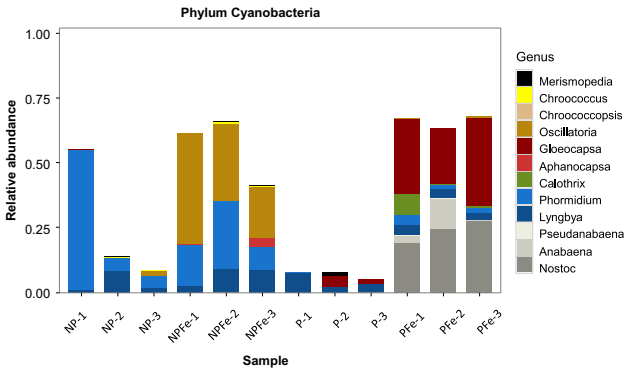

Figure 2. Relative abundance of cyanobacteria genera for each replicate of the various nutrient treatments. Results based on microscopic count data.

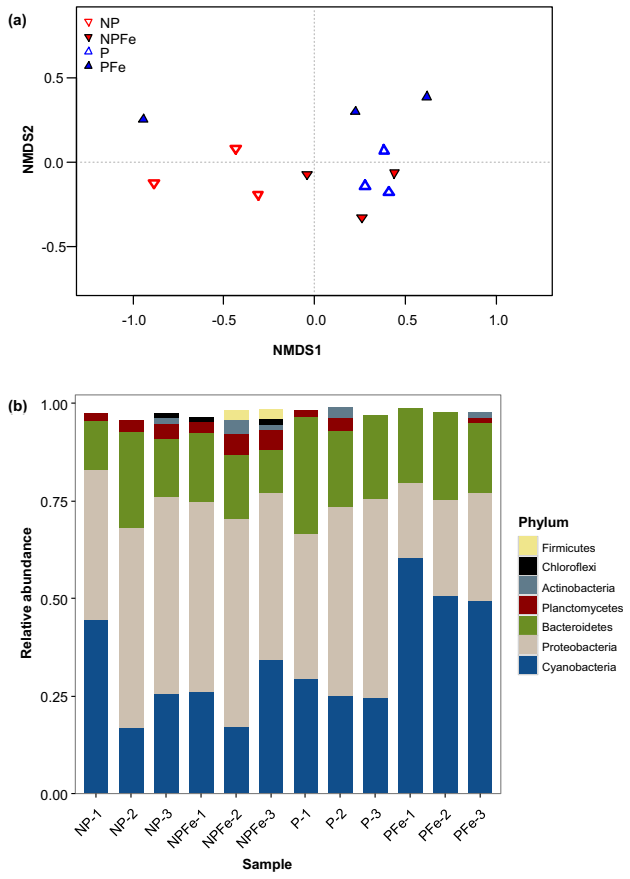

Figure 3. Non-metric multidimensional scaling of microcosm periphyton communities based on 16S rRNA sequence data (**a**). Relative abundance of the different phyla from phototrophic and heterotrophic microorganisms in each replicate from the various nutrient treatments based on 16S rRNA sequence data (**b**).

treatment (Fig. 1b). In the treatments with nitrogen, cyanobacteria consisted mainly of species from the genera *Phormidium* and *Oscillatoria* (Fig. 2). In the treatments lacking nitrogen, species of cyanobacteria consisted of multiple N_2-fixing genera, most notably *Nostoc*, *Anabaena* and *Gloeocapsa* (Fig. 2).

Molecular analyses

Phylum level analysis of 16S rRNA gene data revealed less distinct groupings of the four treatments (i.e. P, PFe, NP and

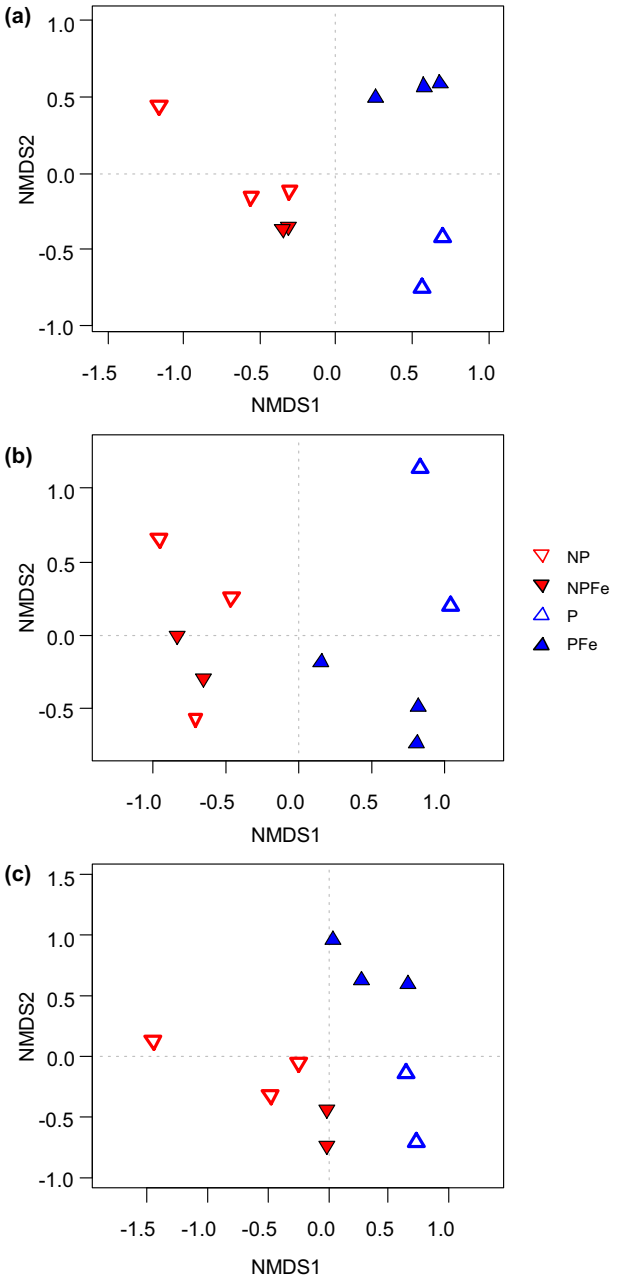

Figure 4. Non-metric multidimensional scaling of microcosm periphyton communities based on *nifH* sequence data for all organisms (**a**), photosynthetic cyanobacteria (**b**) and non-photosynthetic bacteria (**c**).

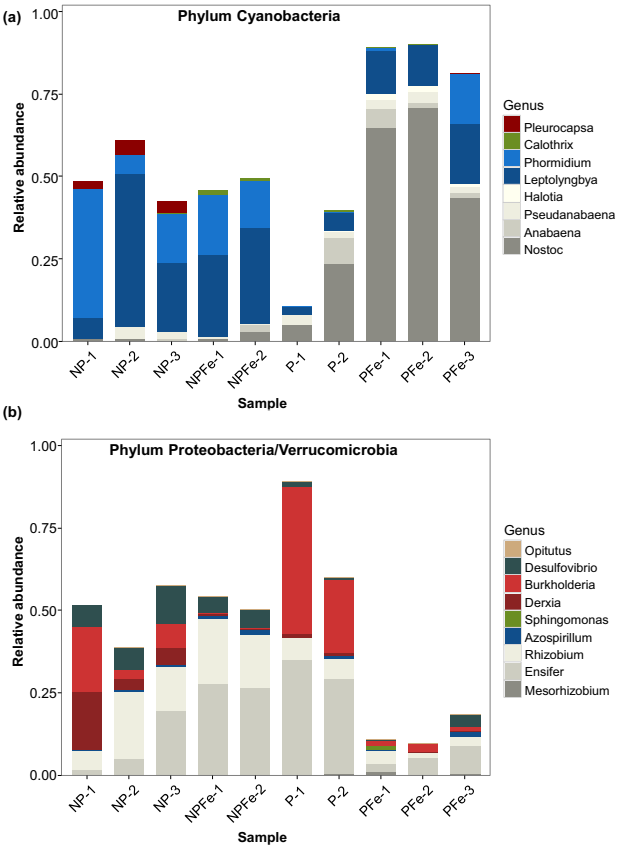

Figure 5. Relative abundance of nitrogen-fixing cyanobacteria genera (**a**) and nitrogen-fixing bacteria (**b**) for each replicate of the various nutrient treatments based on *nifH* sequence data.

NPFe) with greater variability of the PFe treatment compared to microscopic data (Fig. 3a). There was also considerable overlap between the P and NPFe treatments. Despite this, PERMANOVA results for composition revealed marginally significant treatment effects (Pseudo-$F_{(3,11)} = 2.76$, $P \leq 0.049$). Compared to the other treatments, the PFe treatments showed the highest relative abundance of cyanobacteria at the expense of *Proteobacteria* (Fig. 3b). Accordingly, in all but the PFe treatments, most of the 16S rRNA sequences came from non-photosynthetic bacteria.

Results obtained from the analysis of the *nifH* gene showed more distinct multivariate groupings than those observed with the 16S rRNA data (Fig. 4a–c; Fig. S2, Supporting Information). Distinct groupings across all treatments were observed for all *nifH* gene-containing organisms (Fig. 4a) and bacteria (Fig. 4c), yet there was overlap between the NP and NPFe treatments for cyanobacteria (Fig. 4b). Despite losing two samples to insufficient DNA material for *nifH* analysis (one P and one NPFe treatment replicate), PERMANOVA of composition demonstrated significant treatment effects for all *nifH* gene-containing organisms, including photosynthetic and non-photosynthetic ones (all *nifH*: pseudo-$F_{(3,9)} = 4.68$, $P \leq 0.001$, Fig. 4a, cyanobacteria: pseudo-$F_{(3,9)} = 3.87$, $P \leq 0.002$, Fig. 4b, bacteria: pseudo-$F_{(3,9)} = 4.60$, $P \leq 0.001$, Fig. 4c). There was a greater relative abundance of phototrophic *nifH* organisms (i.e. cyanobacteria) in the PFe treatments relative to the other treatments (Fig. 5a). The majority of cyanobacteria in the PFe treatment was represented by *Nostoc*, but other heterocyst forming taxa from the family *Nostocaceae*, i.e, *Anabeana*, *Pseudanabaena* and *Halotia*, were also found (Fig. 5b). Composition of *nifH*-containing bacteria consisted mostly of the genera *Ensifer*, *Rhizobium*, *Burkholderia* and *Derxia*.

Mantel tests revealed significant correlation between the dissimilarity matrices for microscopic counts (reduced matrix with P, PFe, NP, and NPFe treatments only) and the *nifH* gene results (Mantel $r = 0.5038$, $P = 0.0024$). There was no significant correlation between the dissimilarity matrices for 16S rRNA and microscopic counts (Mantel $r = 0.0347$, $P = 0.3423$) as well as for the 16S rRNA gene and *nifH* gene results (Mantel $r = 0.0944$, $P = 0.2633$).

DISCUSSION

Results from our microcosm study demonstrate the potential for iron limitation to influence the composition and relative abundance of stream putative N_2-fixing organisms. This result is consistent with those observed for lacustrine and marine phytoplankton (Wurtsbaugh and Horne 1983; Lenes *et al.* 2001; Mills *et al.* 2004; Hanington, Rose and Johnstone 2016). To our knowledge, this is the first study of stream diazotrophs to use both molecular and cell count approaches to report a negative impact of iron-phosphorus colimitation within the phytobenthos, including reduced abundance of heterocyst forming putative N_2-fixing cyanobacteria (e.g. *Nostoc* and *Anabaena*). We also found lower abundance of cyanobacteria generally in our treatments without iron compared to those with it using microscopic count data. Even in the treatments with nitrogen (i.e. NP and NPFe), there was lower relative abundance of non-heterocyst forming cyanobacteria (i.e. *Oscillatoria*, *Phormidium* and *Lyngbya*) in the NP treatment compared to the NPFe treatment. Considering that *Nostoc*, *Anabaena*, *Oscillatoria*, *Phormidium* and *Lyngbya* all have the ability to form blooms, the present findings may have important implications for the study of CyanoHABs, where the influence of iron limitation has been studied far less than the impacts of nitrogen and phosphorus.

Numerous studies in marine and lacustrine systems have explored diazotrophs in phytoplankton using the *nifH* gene as a proxy for their diversity, yet almost no studies to date have done so in stream benthic communities. We are aware of only one other investigation that examined *nifH* in the stream benthos (Berrendero *et al.* 2016), reporting greater nitrogen fixation in a mat dominated by the filamentous non-heterocystous cyanobacterium *Schizothrix*, under conditions of low nitrogen. Unlike Berrendero *et al.* (2016), we observed primarily heterocystous putative N_2-fixing cyanobacteria under conditions of low nitrogen, but non-heterocystous species, most notably *Leptolyngbya* and *Phormidium*, under high nitrogen. We did not measure nitrogenase activity directly, but owing to the high N availability provided, it is unlikely that non-heterocystous taxa were actively fixing N. As stated previously, production of the nitrogenase enzyme is energetically expensive and likely to be expressed only under conditions of prolonged low nitrogen availability. Yet, to our knowledge, our study is the first to measure a major shift from non-heterocyst to heterocyst-forming cyanobacteria in the benthos, most notably in the treatment with adequate phosphorus and iron.

Despite growing recognition that iron limitation can influence the abundance of diazotrophic cyanobacteria, particularly in pelagic environments, such as oceans and lakes (Wurtsbaugh and Horne 1983; Lenes *et al.* 2001; Mills *et al.* 2004; Hanington, Rose and Johnstone 2016), iron has generally not been thought to be at limiting concentrations in streams. This oversight may partially explain why many of the previous studies of nitrogen fixation in benthic stream producer communities focused largely on phosphorus additions, while omitting iron. It may be that failure to measure iron in addition to nitrogen and phosphorus in previous experiments may have hindered our ability to understand the response or lack thereof of phosphorus additions on nitrogen fixation in streams. For example, one other observational study, we are aware of that investigated the impacts of iron addition on stream nitrogen fixers, did not detect a significant effect, yet they were unable to determine whether iron was limiting in the streams they tested (Kunza and Hall 2013). In our study, putative N_2-fixing cyanobacteria were present, although not particularly abundant in treatments with adequate phosphorus, yet limiting iron concentrations (i.e. P-only treatments). As results from our study suggest, the greatest response in heterocyst forming putative N_2-fixing cyanobacteria was observed in treatments with non-limiting concentrations of both iron and phosphorus. Greater understanding of phosphorus-iron colimitation may help to reconcile some of the inconsistencies observed in previous studies between nitrogen fixation and phosphorus additions. Given that several studies have suggested that iron concentrations in a considerable proportion of streams within the United States could be at levels that may be limiting to the benthos (Passy 2010; Larson, Liu and Passy 2015), future studies of stream nitrogen fixation should evaluate iron concentrations in addition to nitrogen and phosphorus.

Non-photosynthetic putative N_2-fixing bacteria were also observed in greater relative abundance in the NP, NPFe and P-only treatments in comparison to the PFe treatment. *Proteobacteria* have been observed to display a strong response to nutrient addition in other aquatic studies, most notably phosphorus additions (Newton and McMahon 2011; Staley *et al.* 2014; Kohler *et al.* 2016), so the high relative abundance of *Proteobacteria* in our P-only treatment is consistent with other studies. However, the high relative abundance of *Proteobacteria* in the NP and NPFe treatments was somewhat surprising, given the abundant nitrogen in these treatments. It is possible that the non-photosynthetic bacteria we observed were surviving in these conditions without carrying out N_2-fixation. Since biofilms can accumulate considerable biomass, it is also conceivable that nitrogen fixation could occur within thick biofilms, which may provide opportunities for heterogeneous nutrient and oxygen conditions to develop. While our study was not designed to measure the direct effect of biofilm thickness on nitrogen fixation, it is plausible that biofilm heterogeneity may have favored nitrogen fixation by non-photosynthetic organisms, especially towards the bottom of the biofilm mat, where nutrient and light limitation may have taken place. It is also likely that competition between phototrophic and heterotrophic bacteria could be occurring in a thick biofilm as the abundance of non-phototrophic bacteria decreased in our experiment with the increase in relative abundance of N_2-fixing cyanobacteria, most notably heterocyst forming taxa. While not plainly obvious with our molecular dataset, yet observed in the microscopic count data, the increase in photosynthetic cyanobacteria with nutrient addition suggests that certain species of cyanobacteria can be good indicators of high nutrient availability, with high abundance of heterocyst forming taxa also indicating low nitrogen levels.

The comparisons of microscopic count data with molecular data in our study also yielded interesting results. Analyses of molecular phylum data for the 16S rRNA gene and count data revealed that for all but the PFe treatments, non-photosynthetic bacteria were most abundant, while cyanobacteria were most abundant in the PFe treatments. Without the microscopic count data, we would have not known that cyanobacteria were actually quite abundant in the NPFe treatment. Conversely, we observed good correspondence between *nifH* and microscopic analyses with regard to dominant cyanobacteria taxa, other than *Gloeocapsa*, which was found in the microscopic counts but not with *nifH* sequencing. For example, using both techniques, *Nostoc* and *Anabaena* were detected in the PFe treatments, while *Phormidium*, in the NPFe treatments. Yet, results from molecular sequencing of *nifH* revealed more cyanobacteria taxa than the microscopy study, a result that was generally expected as molecular techniques can be more discriminatory than visual counts, particularly with regard to morphologically similar taxa.

The comparisons of both procedures in our experiments were informative in that the microscopic data gave context for the results from sequencing *nifH*. Specifically, microscopic results showed that cyanobacteria were generally more abundant in the treatments with iron, which was less obvious in the molecular data. Since molecular data tended to underestimate abundance, while count data underestimated diversity, both procedures provided complementary information. A significant Mantel test ($P = 0.0024$) also confirmed the strong association between count and *nifH* data in our study.

As the occurrence of cyanoHABs appears to be increasing, it becomes vital to gain greater understanding of the mechanisms behind these blooms. Many of the taxa contributing to cyanoHABs are nitrogen fixers, including *Nostoc* and *Anabaena*, which were shown here to be Fe and P-colimited. We also observed greater relative abundance of cyanobacteria in treatments with iron, suggesting the role of iron should be examined in greater detail in future studies of cyanobacteria in general, and cyanoHABs in particular. However, we also acknowledge that our experiments took place under highly controlled conditions, where we were able to limit grazing and flow, two processes known to also influence the dominance of cyanobacteria in streams (Scott and Marcarelli 2012). Yet despite this, the results of our study clearly demonstrate the potential for phosphorus-iron colimitation to influence the relative abundance of diazotrophic cyanobacteria within the benthos.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSEC* online.

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Conflicts of interest. None declared.

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