How does nutrient limitation affect expression of assimilatory genes for a photosynthetic microbial mat community in Yellowstone National Park?

by

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ABSTRACT

Microbial mat communities inhabiting hot springs in Yellowstone National Park have been studied for their biodiversity, energetics, and evolutionary history, yet little is known about how these communities cope with nutrient limitation. In the present study, changes in assimilatory gene expression levels for acquisition of nitrogen (*nrgA*), phosphorus (*phoA*), and iron (*yusV*) were measured in a nutrient enrichment experiment that manipulated N, P, and Fe availability alone and in various combinations. While results for *nrgA* and *phoA* were inconclusive, results for *yusV* showed an increase in expression with the addition of N and Fe. These are the first data showing the impact of nutrients on siderophore uptake regulation in hot spring microbes.

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INTRODUCTION

Nitrogen (N) and phosphorus (P) are necessary building blocks for life as they are required for cells to synthesize key biomolecules such as proteins and nucleic acids. Iron (Fe) and other metals (Ni, Mo, W, etc.) are also important nutrients, as some metals serve as cofactors for enzymes that catalyze many chemical reactions within the cell (Andrew *et al.* 2003). Low availability of these nutrients can often limit the growth of organisms, initiating resource competition and thus affecting community composition (Rhee 1978, Tilman *et al.* 1982, Klausmeier *et al*. 2004). One means of assessing nutrient limitation in various ecosystems is by experimental enrichment assays in which nutrients are added to the biota and the chemical and biological responses are measured. Such approaches have shown that photoautotrophs in diverse ecosystems (freshwater, marine, and terrestrial) have consistent positive responses of growth rate to N or P enrichment while combined N and P enrichments produce a variety of strong synergistic responses (Harpole *et al.* 2011), revealing widespread prevalence of N and P limitation in most ecosystems on Earth (Elser *et al.* 2007).

To cope with nutrient limitation, organisms have evolved various ways to increase resource acquisition. Various studies have shown that, when limited by N, P, or Fe, model microorganisms (e.g. *E. coli* and *Bacillus subtilis*) will de-repress (up-regulate) their respective assimilatory genes. For example, under nitrogen limitation, some bacterial species de-

repress transcription of high affinity ammonium transport genes. *B. subtilis* uses the *nrgA* gene (Detsch *et al.* 2003), while *E. coli* uses a homologue *amtB* gene to encode for high affinity ammonium protein transporters (Hua *et al.* 2004). Under phosphorus limitation, *B. subtilis* and *E. coli* both use *phoA* in the production of alkaline phosphatases during P limitation (Hulett *et al.* 1994, Inouye *et al.* 1981). Alkaline phosphatases increase P availability by cleaving PO_4^{2-} groups from molecules located in the periplasmic space, increasing P supply to the cell for uptake and use during P limitation. Finally, when bacteria are iron-limited they release siderophores (Fe-chelating compounds) into their environment and use siderophore transporters (ATP binding components) to bring siderophore-bound Fe into the cell. *yusV* is an example of one such protein used by *B. subtilis* (Ollinger *et al.* 2006). Overall, these studies show that, when nutrient-limited, various microbes increase the activation of specific assimilatory genes in order to cope with that limitation.

However, most studies on such nutrient-driven changes in gene expression have been performed with model microbes in laboratory settings; experiments assessing gene expression changes (transcriptional or translational responses) to nutrient availability *in situ* are relatively few. In one example involving nitrogen enrichment experiments targeting the cyanobacterium *Cylindrospermopsis* sp. in the St. Johns River estuary in Florida, Moisander *et al.* (2008) observed that transcription of the *nifH* (nitrogenase encoding) gene increased in microcosms that did not receive

N but remained low in N-enriched treatments. Similarly, in phosphorus enrichment experiments involving the marine cyanobacterium *Synechococcus* sp., unamended cultures showed increased translation of the phosphate-binding protein (PstS) in picoplankton (Scanlan *et al.* 1997). Although this was measured as an increase in PstS protein abundance, it more than likely reflects an increase in the expression of the *pstS* gene. While a metatranscriptomics study of effects of iron enrichment has been completed on a marine phytoplankton community, its focus was on the physiological response of iron-binding proteins to Fe limitation and not on observing transcriptional or translational changes of iron metabolism (Marchetti *et al.* 2012). Notably, each of the studies just described focused on how enrichment of a single nutrient affected transcription and translation; none of them considered how additions of nutrients in combination affected the response of those genes. Furthermore, these studies were all focused on either estuarine or oceanic phytoplankton; none of them assessed responses of microbial mat communities nor have extreme environments been considered.

Microbial mats are highly structured benthic communities found in a wide variety of ecosystems. Microbial mat communities that inhabit hot springs of Yellowstone National Park (YNP) have been extensively studied for their biodiversity (Ward *et al.* 1998, Meyer-Dombard *et al.* 2005), energetics (Amend *et al.* 2001), and evolutionary history (Nisbet *et al.* 1999). While little is known about nutrient cycling in these mats nor about

how their microbial communities cope with nutrient limitation, some responses of high temperature microbial mat communities to N and P limitation have recently been investigated (Steunou *et al.* 2008). These researchers observed a correlation between the up-regulation of N assimilatory genes (the N-fixation genes *nifH* and *nifD*) and the down-regulation of an N metabolism gene (*glnA*- glutamine synthetase) in *Synechococcus* ecotypes found within a microbial mat in Mushroom Spring at Yellowstone National Park (USA). Since down-regulation of *glnA* is typically induced by N limitation (Gussin *et al.* 1986), the presence and expression of *nif* genes may indicate that these microbial communities use N_2 fixation to increase the supply of reduced nitrogen under N limitation. Interestingly, the *nif* gene has been shown to be distributed in hot springs encompassing a wide range of pH and temperatures at Yellowstone National Park (Hamilton *et al.* 2011) as well as expressed in other hot springs (Loiacono *et al.* 2012). Phosphorus enrichment studies conducted in the laboratory on *Synechococcus* sp. isolated from Octopus Spring at Yellowstone National Park (USA) found that transcription levels of *phoA* genes increase with P limitation (Adams *et al.* 2008). Together, these data indicate the microbes that make up hot spring microbial communities also possess mechanisms to cope with N and P limitation and that they use them when N and P are limited under *in situ* conditions.

While these experiments help inform our understanding of how microbial mats in hot springs might deal with nutrient limitation, they are

limited because they focus solely on single genes and single microbial species from the community. To move towards a more complete assessment, in this study we use RT-qPCR to assess the changes in gene expression for N, P, and Fe assimilatory genes (respectively: *nrgA, phoA,* and *yusV*) after nutrient enrichment for a complex microbial mat community inhabiting Bison Pool, a hydrothermal spring at Yellowstone National Park, USA.

METHODS

Study Site:

The enrichment experiment was performed from August 15-16 2010 at the Bison Pool hot spring, which is located in Sentinel Meadow of the Lower Geyser Basin in Yellowstone National Park (44° 34' 10.9'' N, 110° 51' 54.7'' E). The main source pool overflows to forms two outflow channels, resulting in downstream gradients in temperature and water chemistry. For more details about environmental conditions in Bison Pool see Meyer-Dombard *et al.* (2005, 2011). This study focuses on the microbial mats that inhabit the lower temperature (<75° C) photosynthetic zone of the outflow channel.

Physical-chemical conditions:

Temperature and pH were measured *in situ* at the start of the experiment using a YSI^{TM} Model 30 meter. Source water was filtered using a 60-ml syringe and 0.8 membrane filter (Pall Corporation, Port Washington, NY)

followed by a 0.2 µm membrane filter (Pall Corporation, Port Washington, NY). Two 120-ml polyethylene (PET) amber bottles were filled with 30 ml of this filtered water for analysis of initial concentrations of total dissolved phosphorus (TDP) and total dissolved nitrogen (TDN). A further 30 ml was collected into a HDPE bottle and preserved with 0.4 ml ultrapure nitric acid for later analysis of dissolved iron concentrations. Ammonium concentrations were determined using the fluorometric method of Holmes *et al.* (1994). Total phosphorus levels were measured using the standard 4500-P G method by colorimetric flow injection analysis (Clesceri *et al.* 1998). Iron concentrations were determined using inductively coupled plasma mass spectrometry (ICP-MS).

Experimental set-up and sampling:

A semi-intact section of microbial mat was transferred into a 2-l beakers with a sterile spatula, homogenized, and then divided between 24 100-ml Pyrex^{TM} bottles each containing 80 mL of source water. Eight different treatments were imposed in triplicate; Control, +N, +P, +Fe, +N&P, +N&Fe, +Fe&P, and +N,P,&Fe. Nutrients were added to the following final concentrations: +125 uM for nitrogen (as NH_4NO_3), +7.8 uM for phosphorus (as $NaHPO_4$), and +78 nM iron (as $FeC_6H_6O_7$). The bottles were capped and incubated at random positions near each other in the outflow channel near the initial sampling site. The outflow channel temperature at this site was ~ 57° C. After 24 hours, water from each of

the three replicates of each treatment was filtered as described above and pooled into a 1-L Whirlpak™ bag and held on ice until being frozen (-20° C) for later chemical analysis. Biomass (approximately 2 grams) from each bottle was divided and placed into separate 2-mL cryovials and flash-frozen in liquid nitrogen. Vials were stored at -80° C until DNA and RNA extraction for gene expression analysis.

Nucleic acid extraction, cloning and DNA sequencing:

DNA was extracted from 0.5 g of wet microbial mat using the FastDNA SPIN kit for soil (Bio101, Inc., Carlsbad, CA). Samples were then stored at -20° C.

Clones were created by purifying PCR products using a QIAquick kit (QIAGEN, Valencia, CA, USA), ligation was completed using the pGEM-T easy Vector System (Promega, Madison, WI, USA), and transformation was completed using JM109 *E.coli* competent cells (Promega, Madison, WI, USA). Plasmid DNA was extracted using Wizard *Plus SV* minipreps DNA purification System (Promega, Madison, WI, USA). PCR bands were purified using Wizard SV Gel and PCR Clean-Up system (Promega, Madison, WI, USA) and PCR products were sequenced by the ASU DNA Sequencing Facility (Arizona State University, Arizona, USA) using standard M13 primers. Five clones from each of the primers were sequenced.

Reverse transcription and qPCR:

RNA was extracted from mat samples using the RNA PowerSoil Total RNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA) according to the manufacturer's instructions. DNA was removed using the TURBO DNA-free DNase Kit (Ambion, Austin, TX) following the manufacturer's instructions. RNA was purified via chloroform extraction and precipitated with isopropanol at -20° C overnight and re-suspended in 25 uL of nuclease free water. RNA quality was checked on a gel and quantified via Nanodrop (Thermo Fisher Scientific, Watham, MA). 10 uL of this DNase-treated RNA was reverse transcribed with random nonamers as primers using 200 units of reverse transcriptase (RT) (Superscript III, Invitrogen^{TM}) and the other 10 uL was saved and diluted to a final volume of 50 uL with nuclease-free water to serve as a non-reverse transcribed control in qPCR.

qPCR-Target Genes:

Specific primers were designed to target *nrgA, phoA,* and *yusV* genes (Table 1) based on sequence information from the Bison Pool Metagenome database (Swingley et al., 2012). cDNA, DNase-treated RNA, and plasmids to construct standard curves were subjected to qPCR to construct standard curves, targeting genes listed in Table 1.

nrgA and *yusV* underwent qPCR using the 2x Brilliant SYBR green QPCR kit (Agilent, Santa Clara, CA). Briefly, for the *yusV* and *nrgA*

genes, the 25 uL reaction mixture contained 5 uL of sample (10ng/ul) or 1 uL of 10-fold serially diluted standards, 12.5 uL of 2x SYBR Green, 2 uL (7.5 uM) of the appropriate forward and reverse primers (IDT), and 0.375 uL of 1:500 diluted reference dye. Samples were run on the MX3000Pro thermocycler with the following cycling conditions: *yusV*- denaturation step at 95 °C (10 min), followed by 40 cycles of 95°C (30 s), 67°C (1:00), 72°C (1:30), 85°C (15 s), with a final extension of 95°C (10 min); *nrgA*- 95 °C (10 min), followed by 40 cycles of 95 °C (30 s), 55 °C (1.5 min), 72°C (1 min), with a final extension of 95 °C (10 min).

phoA underwent qPCR using the Environmental Taqman kit (Invitrogen, Carlsbad, CA). Briefly, each 25 uL reaction contained 2 uL of sample or 1 uL of 10-fold serially diluted standards, 1 X Taqman Environmental PCR master mix, 0.2 nM of Taqman Probe (Applied Biosystems, Foster City, CA), 1 nM of the forward and reverse primer for *phoA* (IDT). Samples were run on an MX3000Pro themocycler with the following cycling conditions: 95 °C (10 min), followed by 45 cycles of 95 °C (30 s), 53° C (1.5 min), with a final extension of 95°C (10 min). All samples and standards were analyzed in triplicate for each experimental bottle.

qPCR- 16S rRNA Bacteria:

Primer sequences for the 16S rRNA bacterial gene can be found in Table 1. The 25 uL reaction mixture contained 1 uL of sample (14.6 ng/uL) or 1 ul of 10-fold serially diluted standards, 12.5 uL of Fast SYBR Green, 0.6

uM of the appropriate forward and reverse primers, and 0.375 uL of 1:500 diluted reference dye. Samples were run on the MX3000 Pro thermocycler with the following cycling conditions: denaturation step at 95 °C (3 min), followed by 40 cycles of 95 °C (20 s), 67°C (20 s), 72°C (20 s), 85 °C (15 s), with a final extension of 95 °C (10 min).

Data Analysis:

Gene expression data for *nrgA*, *phoA* and *yusV* were normalized by the 16S rRNA gene expression data to account for bacterial total RNA input variability between samples. These samples were then normalized to the input RNA concentration. RNA concentrations were determined using methods described in Kyle *et al. (*2003). One-way ANOVA and power statistics were used on log-transformed data to assess statistical significance between treatments and to evaluate the probability of type II errors for the analysis. DNA sequences from cloned plasmids were analyzed using BLASTX to estimate phylum-level taxonomic coverage of the primers.

Table 1: qPCR primer sequences and size (# of base pairs)

Gene	Gene function	Sequence	Bp size
nrgA	Ammonium transporter	for (5'- AAGCCGCGATCAATTCCA 3') rev (5'-GCCGAGCCAGAGGATGAA-3')	63
yusV	Siderophore transporter	for (5'-SGGCAAGACSACSCTGYTGCGC-3') rev (5'-GCTGGCGYTSSCCGCCGC-3')	315
phoA	Alkaline phosphatase	for (5'-GGTSCTGGGCTGTTCT-3') rev (5'-GAYSCGCCCSCCYTC-3'). Probe (6FAM-AAGGCNCTGGAGATTCTMGBNFQ).	168
16S rRNA bacteria		for (5'-ACTCCTACGGGAGGCAGCAG-3') rev (5'-ATTACCGCGGCTGCTGG-3')	180

RESULTS

Nutrient uptake

Measurements of dissolved nutrient concentrations in the incubation bottles indicated strong uptake after fertilization in most cases (Table 2). In the +P microcosms, added P was completely depleted back to initial concentrations after the 24-h incubation period. A similar depletion of added NH_4^+ was seen in all amended nitrogen treatments, as NH_4^+ declined from 64 uM to below 1.79 uM. A reduction of 50% of the NO_3^- concentration was observed in two N-enriched treatments (+N, +NP&Fe). However, an increase in NO_3^- was observed in the other two N-enriched treatments (+N&P, +N&Fe). Most of post-incubation Fe concentrations were below the limit of detection, also showing a depletion

of the added nutrient. The substantial reductions of amended treatments suggest a strong microbial demand for the added nutrients under the conditions of the incubation.

Table 2: Water chemistry data for the experimental enrichment treatments. "I" is the initial concentration of nutrients in water from the outflow channel. "Calculated treatment" indicates the estimated concentration for that nutrient after experimental enrichment; "C" is the control treatment. The values given reflect only one observation for each parameter after the 24-h incubation period, as water from the three different microcosms was pooled together in order to have enough water for the chemical analyses. BD = below detection limit.

Treatment	P (umol L^{-1})	NH_4 (umol L^{-1})	NO_3 (umol L^{-1})	Fe (nmol L^{-1})
I	0.15	1.5	1.51	BD
Calculated treatment	7.95	64	64	78
C	0.09	0.79	21.6	26.9
+P	**0.12**	1.43	4.08	40.1
+Fe	0.07	1.93	36.3	**BD**
+N	0.15	**1.5**	**32.8**	BD
+P&Fe	**0.05**	1.36	13.6	**38.5**
+N&P	**0.12**	**1.79**	**100**	BD
+N&Fe	0.07	**1.71**	**85.7**	**BD**
+NP&Fe	**0.34**	**1.79**	**35**	**BD**

Gene expression

The *phoA, nrgA,* and *yusV* genes were all successfully reverse-transcribed and amplified from the microbial mat. However, considerable variability among treatment replicates was observed (Figure 1). Based on the RT-qPCR results, a significant effect of nutrient amendment was seen for *yusV* expression (Figure 1-C; P=0.0008); specifically, expression was notably high with the combined addition of N and Fe. For *phoA* (Figure 1-A), expression appeared somewhat elevated in treatments receiving combined nutrient amendments (+N&P, +N&Fe, +NP&Fe) and low in +N and +P&Fe treatments but the overall ANOVA was not statistically significant. Finally, as shown in Figure 1-B, *nrgA* gene expression levels appeared to be higher in treatments receiving N in combination with P or Fe (+N&P, +N&Fe, +NPFe) but this pattern was not statistically significant due to high variation within treatments.

To evaluate the extent to which the lack of effect of enrichment on gene expression reflected a combination of sample variability and small sample size, we calculated power statistics. Power statistics on log-transformed data for *phoA* and *nrgA* did not yield high probability of achieving a significance level of 0.05 with the number of samples used in this study (27% and 35%, respectively). Thus, there is high probability of type II error for the outcome for these two genes and thus we cannot confidently conclude that nutrient amendment does not affect their expression.

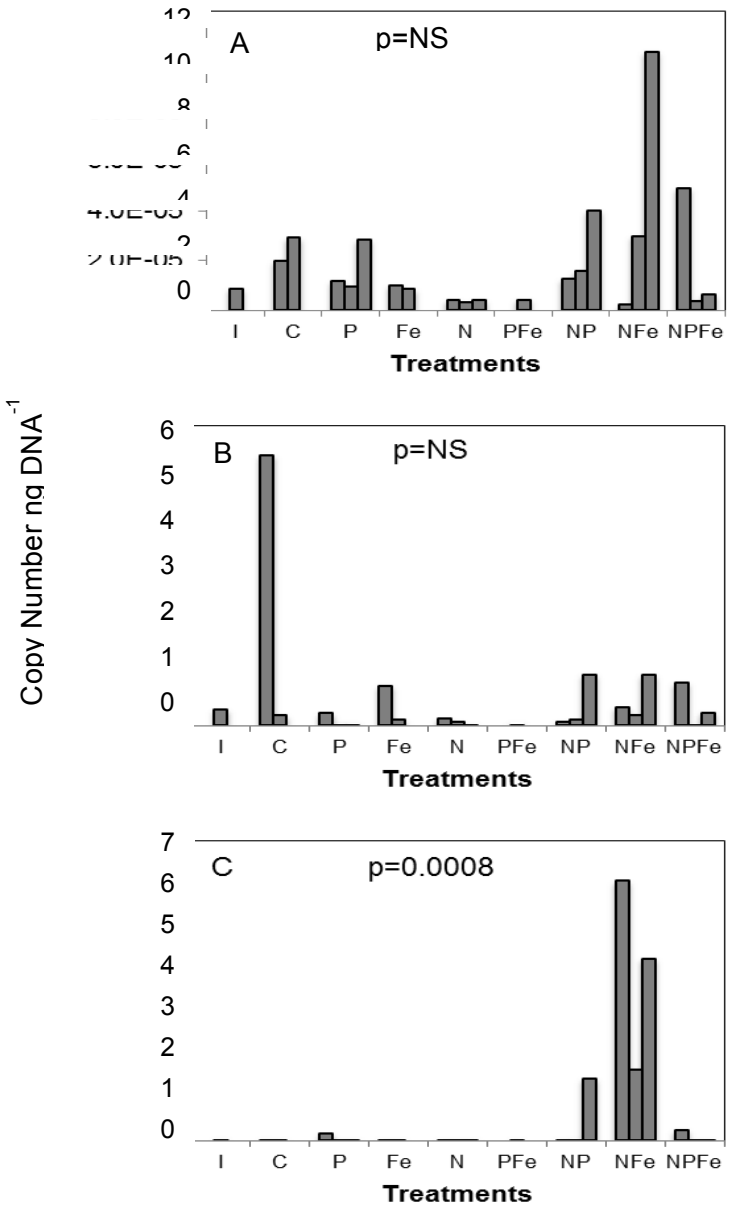

Figure 1. Effect of various nutrient enrichment treatments (n=3) on expression of (A) *phoA*/16S bacteria, (B) *nrgA*/16S bacteria, and (C) *yusV*/16S bacteria determined by RT-qPCR. *P* values reflect the result of the one-way ANOVA for the overall effect of experimental treatment on log-transformed data. "NS" = non-significant ($p > 0.05$).

Relative taxonomic coverage of primers

To assess the coverage of the primers used in this study, DNA sequences from cloned plasmids were analyzed using BLASTX (Fig. 2). The results show considerable differences in the breadth (number of different taxa) and depth (relative abundances within taxa) covered by each primer. The *phoA* primers targeted 14 bacterial and two archaeal taxonomic phyla. A majority of the sequences targeted were identified as Proteobacterial, indicating considerable depth for this one taxon but very low breadth for the community as a whole. The *yusV* primer had even less breadth, targeting only four bacterial taxonomic phyla and a single archaeal taxon. However, it did provide considerable depth within the Proteobacteria. *nrgA* primers encompassed greater breadth of the community, capturing eleven bacterial and two archaeal phlya, while also having more depth within each taxon compared to the *yusV* and *phoA* primers.

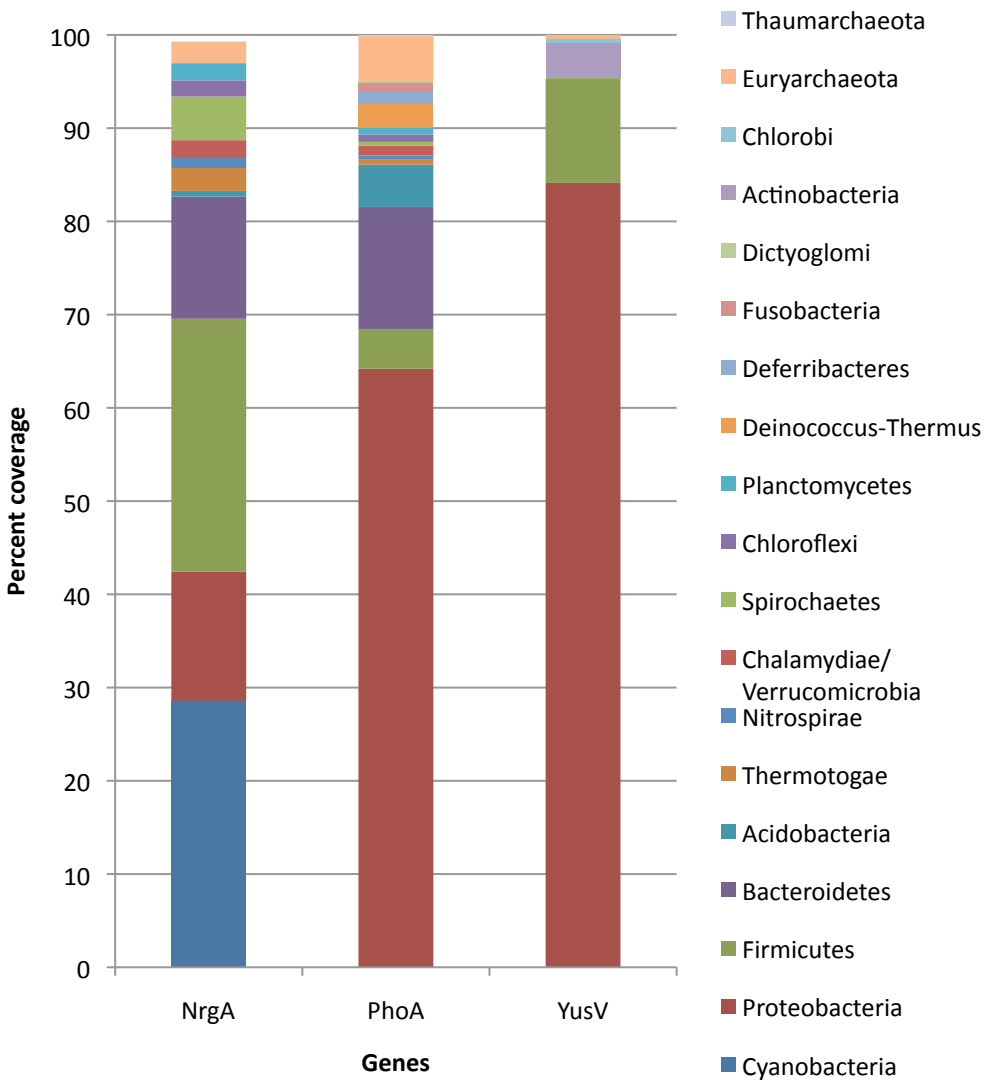

Figure 2. Relative abundance of phylum-level taxonomic coverage as calculated from BLAST (BLASTX) based on phylum-level taxonomic evaluations.

Expected primer coverage of the microbial mat community

To assess primer coverage of the microbial mat community, the meta-genomic BPEG ("Bison Pool Environmental Genome)" data from site 5 at Bison Pool (Swingley *et al.* 2012) was compared to the phlya covered by the primers designed for this study. Table 3 summarizes the outcome for the four most dominant members of the microbial mat community (which accounts for 55.8% of the total): Chlorofexi, Cyanobacteria, Proteobacteria, and Planctomycetes. The primer coverage for the targeted genes are as follows: *nrgA* (55.8%), *phoA* (44.4%) and *yusV* (11%).

Table 3: Primer coverage of the microbial mat community.

Phlya	% total sequence at site	Phlya covered by primers		
		NrgA	PhoA	YusV
Chloroflexi	27.8%	✔	✔	
Cyanobacteria	11.4%	✔		
Proteobacteria	11%	✔	✔	✔
Planctomycetes	5.60%	✔	✔	
Expected primer coverage of microbial mat community		55.8%	44.4%	11.0%

DISCUSSION

This study was designed to assess how nutrient amendments alter expression patterns for nutrient assimilatory genes in a hot spring microbial mat. The observed drawdown of nutrients to their initial levels within the 24-hour incubation showed high demand for those added nutrients (Table 2). Given that primary production in most ecosystems is N- and/or P-limited (Elser *et al.* 2007) and that iron can also frequently act as a primary or as a co-limiting nutrient (Mills *et al.* 2004), we expected that the added N, P, and Fe would be actively assimilated by the microbial community. While the NO_3^- increase in the +N&P and +N&Fe treatments was an exception to this expected drawdown of amended nutrients, the overall depletion of added nutrients is consistent with high nutrient demand, and possible nutrient limitation, by biota in the Bison Spring mat.

The unexpected increase in NO_3^- in the +N&P and +N&Fe treatments suggests that ammonium oxidizing bacteria (AOB) or archaea (AOA) were present in the microbial community and their nitrification activities were co-limited by N along with P or Fe. Usually an increase in NO_3^- production is seen when ammonia is added to AOB (Geets *et al.* 2006) or AOA (Tourna *et al.* 2011) communities. The increase measured in the +N&Fe treatment supports other observations that iron is required in order to achieve maximum conversion of nitrite to nitrate (Aleem *et al.* 1958). While some previous studies have not observed increases in nitrification (e.g. Robertson 1984) or in N-mineralization (e.g. Marrs *et al.*

1988) with amended phosphorus, these data show a potential increase in nitrification with the simultaneous addition of N and P. Considering that this effect was not seen when these N or P were added in isolation indicates potential co-limitation by N and Fe as well as by N and P on nitrification (Harpole *et al.* 2011).

The *yusV* gene expression data also suggest that N and Fe also interact in regulating Fe transport and siderophore processing in this microbial community. Siderophore uptake has been studied before in hot springs but these studies have generally focused on developing cultivation methods to increase the number of "uncultured" microbes from hot springs that can be grown in a laboratory setting (e.g. Lewis *et al.* 2010). Thus, little is known how siderophore transport is regulated within hot spring microbial communities. Planktonic cyanobacteria and microalgae in marine ecosystems have been shown to increase production and uptake of siderophores at high iron concentrations under N limitation (Kerry *et al.* 1988) and under N enrichment (Wang *et al.* 2001); our study is the first to show potential impacts of nutrients on siderophore uptake regulation in hot spring microbes.

Nutrient impacts on the expression of *yusV* were the only ones found to be statistically significant due to high variability within treatment replicates for the other two genes, *nrgA* and *phoA*. As a consequence of the resulting low statistical power, we can make no conclusions about the effect, or lack of effect, of addition of P and N on the P and N assimilatory

genes that we studied. The high variability we observed likely reflects difficulties with executing the experiment in a field environment with complex microbial mat communities; noise in the data appears to be less about the depth and breadth of primer coverage. Indeed, given that the YusV protein is found predominantly in the Proteobacteria phylum that constitutes just ~11% of the microbial mat community (Swigley *et al*. 2012), if narrow coverage was the main factor for sample variability we would have anticipated greater variability for those data than for the other two genes. Instead, the variability of these data most likely reflects effects of spatial heterogeneity amongst replicates, not only in the physical distribution of various microbial taxa (i.e. was that microorganism evenly distributed within replicates?) but also in the within-replicate distribution (i.e. was that microorganism located in the microbial mat in a manner that allowed it to experience the nutrient enrichment?). Future studies of this type should take measures to more completely homogenize the mat communities (though doing so would disrupt potentially important microstructure) or work with more microbial communities that are inherently more uniform. Alternatively, use of meta-transcriptomic technology would avoid issues related to primer coverage and specificity. If meta-transcriptomic approaches are not employed, then primers need to be designed to ensure consistency in the breadth and depth of the taxonomic groups assessed.

This is one of few studies that have attempted to assess *in situ* nutrient use and nutrient-driven expression changes for nutrient assimilatory genes in a complex microbial mat community in an extreme high temperature environment. Further studies relating nutrient limitation or co-limitation to global community expression response of assimilatory genes in such systems will increase our understanding of how these genes aid in maintaining homeostasis for complex microbial communities as they cope with the diversity of thermal and chemical conditions found in hot springs.

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