

USING MOLECULAR PROBES TO DETECT CYANOBACTERIAL COMMUNITIES AND
PHOSPHORUS UTILIZATION IN THE GREAT LAKES

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ABSTRACT

Dr. George S. Bullerjahn, Advisor

We examined the genetic potential of picocyanobacteria to recruit different sources of organic phosphorus, as well as their capacity to switch from phospholipids to sulfolipids in both Lake Erie and Lake Superior. The pelagic regions of Lake Superior and eastern Lake Erie are typically P-limited environments, and picocyanobacteria of the genus *Synechococcus* are the dominant primary producers during the summer. Specifically, the ability of endemic microbes to assimilate organic phosphates and phosphonates have been examined. As a proxy for their utilization of these substrates, the expression of two genes, *phnD* and *phoX* have been monitored. The *phnD* gene encodes the phosphonate binding protein of the ABC-type phosphonate transporter, whereas the *phoX* gene encodes a calcium-dependent alkaline phosphatase. To assess the ability of freshwater *Synechococcus* spp. to substitute sulfolipids for phospholipids, *sqdX* gene expression was monitored. The *sqdX* gene is a gene essential for sulfolipid biosynthesis and encodes the cyanobacterial sulfolipid synthase. We have developed PCR primers to detect the presence of all three genes in the endemic picocyanobacteria, and RT-PCR is being used to examine the patterns of expression that serve to assess the degree of P-stress experienced in the phytoplankton. To date, we show that the *phnD* gene is constitutively expressed, suggesting that freshwater picocyanobacteria are metabolizing exogenous phosphonate compounds in the severely P-limited environments. In contrast, *phoX* is regulated by P bioavailability in Great

Lakes picocyanobacteria. We also provide evidence that *sqaX* is expressed during increased growth rates in phosphorus-replete conditions.

**Посвящается моим родителям, Кутовому Анатолию Степановичу и
Кутовой Наталье Вячеславовне, а также моей сестре, Кутовой Анне
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CHAPTER 1. INTRODUCTION

1.1 Characteristics of freshwater picocyanobacteria

Freshwater unicellular *Synechococcus* are the dominant autotrophic prokaryotic picoplankton (APP) that comprise a major part of freshwater picoplankton (Weisse 1993, Padisak et al. 1997, Wilhelm et al. 2006). It has been recognized that freshwater *Synechococcus* and marine *Synechococcus* and *Prochlorococcus* dominate the photosynthetic picoplankton and contribute significantly to chlorophyll (Chl) biomass and primary production. (Waterbury et al. 1986, Fahnenstiel and Carrick 1992, Nagata et al. 1994, Nagata et al. 1996, Stockner and Shortsheed 1994, Partensky et al. 1999). Indeed, picoplankton from oligotrophic lakes is responsible for up to 70% of the fixed carbon annually (Caron et al. 1985, Munawar & Fahnenstiel 1982). Total chlorophyll in the pelagic Laurentian Great Lakes contributed by picoplankton, mainly by *Synechococcus* spp., is typically 30-50% (Fahnenstiel and Carrick 1992, McKay et al. 2005, Ivanikova et al. 2007b). In general, temporal and spatial variations in freshwater cyanobacterial abundance and composition have been observed and associated with pH (Stockner and Shortreed 1991), light penetration (Pick 1991), nutrients (Wehr 1989), and food web interactions (Weisse et al. 1990). However, compared with marine picoplankton, there is not much information available on the ecology and molecular biology of freshwater APP (Stockner and Antia 1986, Callieri and Stockner 2002). Due to the lack of information on the freshwater APP, particularly the absence of sequenced genomes, and their close phylogenetic relatedness to their marine counterparts (Fahnenstiel et al. 1991[a, b], Ivanikova et al. 2007b, 2008), marine picocyanobacteria will be employed in this dissertation as a model system.

The main goal of this study is to expand our knowledge of how picocyanobacteria utilize P in the largely oligotrophic waters of the Laurentian Great Lakes.

1.1.1 Genus *Synechococcus*

Due to high diversity of *Synechococcus*, they represent a dominant organism in the oligotrophic environments and are able to accommodate diverse niches. *Synechococcus* were discovered because of their intense orange phycoerythrin (PE) fluorescence in 1979 (Waterbury et al. 1979). *Synechococcus* are Gram-negative bacteria belonging to the phylum *Cyanobacteria*. Their typical cell size is 0.6-0.8 X 0.6-1.6 μm (Waterbury et al. 1986). Genome size ranges from 2.2 to 2.86 Mb (Scanlan et al. 2009).

The phylogeny of genus *Synechococcus* is complex and based on G+C content, habitat, photosynthetic pigment content, and growth requirements. Genus *Synechococcus* has been divided into six strain clades: *Cyanobacterium*, *Synechococcus*, *Cyanobium*, and Marine clusters A, B, and C (Waterbury and Rippka 1989). Further phylogenetic analysis of 16S-rRNA gene sequences revealed a polyphyletic nature of the clades (Urbach et al. 1998, Honda et al. 1999). Based on the phylogenetic analysis of 16S-rRNA gene sequences, clades *Cyanobium*, Marine A and B, freshwater unicellular *Synechococcus* together with *Prochlorococcus* form a coherent phylogenetic group representing a single picophytoplankton lineage distinct from other cyanobacteria (Figure 1.1) (Urbach et al. 1998, Crosbie et al. 2003; Ernst et al. 2003, Fuller et al. 2003, Ivanikova 2007b). This research was focused on freshwater unicellular *Synechococcus*.

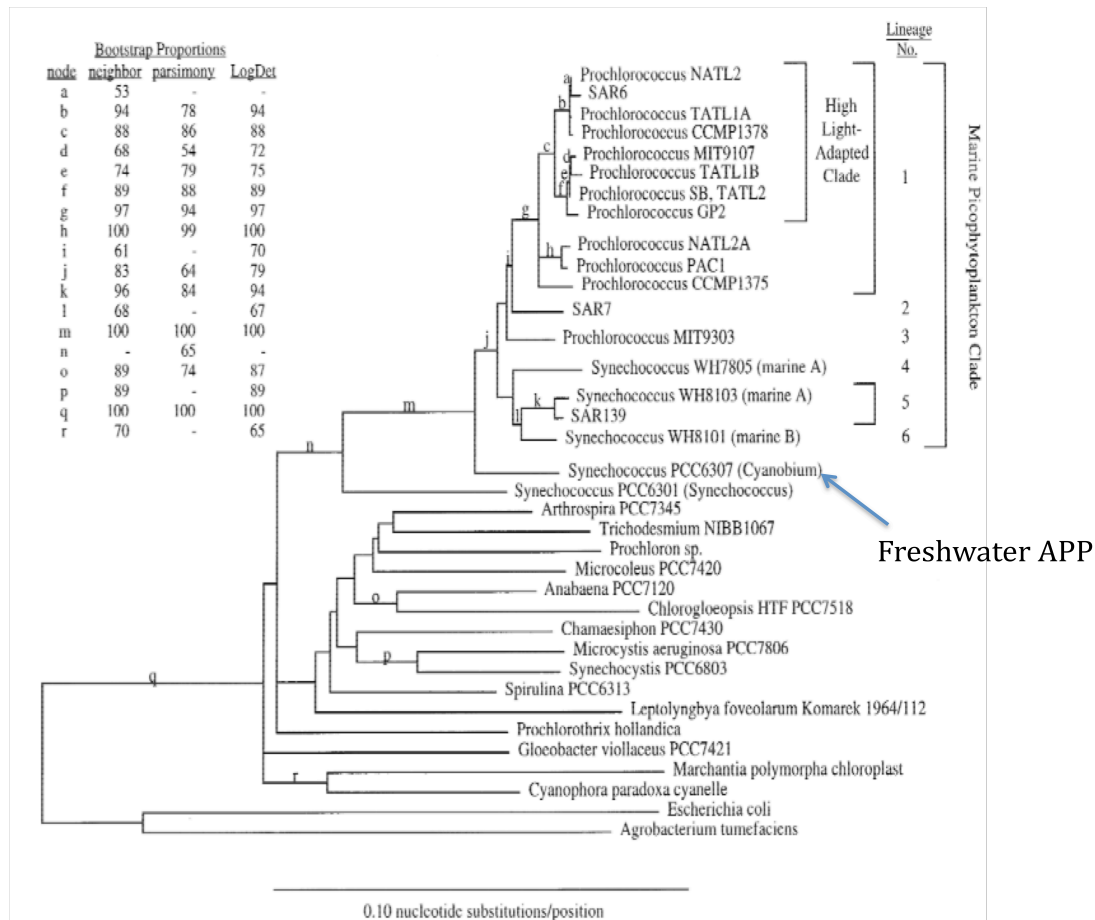


Figure 1.1. Neighbor joining analysis of 16S-rRNA gene sequences (from Urbach et al. 1998).

Based on 16S-rRNA sequences and *cpcBA*-IGS, an intergenic sequence between two phycocyanin apoprotein genes, freshwater *Synechococcus* were divided into six clusters within the picocyanobacterial lineage. The lineages are either cosmopolitan or unique to the particular geographical region and/or ecological niche (Ernst et al. 2003, Crosbie et al. 2003). For instance, it has been demonstrated that there are two dominant unique clusters (LSI and LSII) in Lake Superior, whereas cosmopolitan lineages were found in nearshore samples (Ivanikova et al. 2007b). Similarly, in the central basin of Lake Erie, PE-rich cosmopolitan cluster MH301 dominated in July, whereas PE-rich strains from clusters

Group E and MH305, described in Crosbie et al. (2003), were present in August 2004 (Wilhelm et al. 2006, Ivanikova et al. 2008, Cupp and Bullerjahn, unpublished data).

Synechococcus strains are broadly classified into two cell-types: the first with orange autofluorescing phycoerythrin (PE-rich cells) and an absorption peak at ~560 nm, and the second with red autofluorescing phycocyanin (PC-rich cells) (Wood et al. 1985, Ernst 1991) and an absorption peak at ~625 nm (Callieri et al. 1996, Haverkamp et al. 2008, Callieri 2010). PE-rich and PC-rich *Synechococcus* tend to dominate in deep oligotrophic and shallow eutrophic lakes respectively (Postius and Ernst 1999, Ernst et al. 2003). For instance, PE-rich *Synechococcus* are dominant in oligotrophic lakes Huron, Michigan (Fahnenstiel and Carrick 1992, Nagata et al. 1996), Superior (Fahnenstiel et al. 1987, Ivanikova et al. 2007), and in the central and eastern basins of Lake Erie (Pick 1991, Wilhelm et al. 2006, Ivanikova et al. 2008). Contrary, in the eutrophic western basin of Lake Erie, PC-rich *Synechococcus* represent more than half of the picocyanobacterial community (Pick and Agbeti 1991). Differences in ecological niches were also observed in Lake Superior (Sterner et al 2007, 2010). It has been illustrated for both Lake Erie and Lake Superior that there is a shift in the picocyanobacterial community from PE- and PC-rich *Synechococcus* in spring to PE-rich forms in late summer (Ivanikova et al. 2007, Benjamin Beall, personal communication). Epilimnetic picocyanobacteria abundance increases in late summer, during summer stratification, after temperatures reach 20 °C (Pick and Agbeti, 1991). In addition, Pick and Agbeti (1991) showed correlation between the average light extinction coefficient and the proportion of picoplankton biomass.

Marine *Synechococcus* strains have evolved specific adaptations to deal with horizontal gradients of nutrients and light quality (Fuller et al. 2006, Zwirgmaier et al.

2008). Nutrient concentration is perhaps the main reason explaining a distribution pattern of *Synechococcus* (Zwirgmaier et al. 2008). Similarly, freshwater *Synechococcus* appear to demonstrate high diversity and specific niche adaptation, with the peak abundance in late summer and decrease in abundance in spring, after winter mixing, suggesting a seasonal pattern dependent on nutrient availability, temperature, and light (Weisse 1993, Stockner et al. 2000, Callieri and Stockner 2002).

1.1.2 Nutrient acquisition in picocyanobacteria

The relatively small size of *Synechococcus* spp., and consequently high surface to volume ratios, provide an advantage for nutrient acquisition, thus representing one of the adaptations to oligotrophic environments (Friebele et al. 1978, Suttle and Harrison 1982, Lehman and Sandgren 1985). Moreover, picocyanobacteria have evolved small genome sizes to reduce their nutrient requirements (Dyhrman et al. 2007).

It has been documented that APP abundance declines in waters of higher nutrient content (Stockner and Antia 1976, Callieri and Stockner 2002). Many APP species have very rapid nutrient uptake rates and are capable of maintaining high uptake independent of cell quotas (Suttle and Harrison, 1986).

The success of *Synechococcus* spp. in oligotrophic systems can also be explained by their adaptations to low light and nutrient availability, as well as their ability to assimilate a wide range of nutrients.

1.1.3 Assimilation of nitrogen

APP, as photoautotrophs, require light and necessary elemental building blocks

including carbon, nitrogen, phosphorus, and a number of micronutrients. *Synechococcus* can utilize both: organic nitrogen compounds like amino acids and urea, and the inorganic nitrogen compounds ammonium, nitrate, and nitrite. (Waterbury et al. 1986, Moore et al. 2002, Palenik et al. 2003). Nitrate assimilation in *Synechococcus* is conducted through nitrate (*narB*) and nitrite (*nirA*) reductases (Flores et al. 2005), whereas an uptake of nitrate and nitrite is carried out by NapA/NrtP. However, in many freshwater picocyanobacteria, an uptake of nitrate and nitrite occurs through *nrtABCD*, an ABC transporter (Sakamoto et al. 1999). Nitrate, and nitrite, assimilation require energy to reduce N to ammonia, so most picocyanobacterial strains prefer ammonium as a nitrogen source over the oxidized inorganic forms and organic nitrogen compounds. Nitrogen often limits primary productivity in oceans due to loss of nitrogen through marine denitrification (Krom et al. 1991, Falkowski 1997, Moore et al. 2002, Gruber 2008).

1.1.4 Nutrient stoichiometry (N:P ratios) and APP growth

It has been hypothesized that nutrient acquisition in freshwater *Synechococcus* is likely restricted by different environmental limitations. Levels of inorganic and dissolved organic nitrogen are often much higher in freshwater than in marine environments (Kamennaya et al. 2008). Nitrogen stress responses, acquisition, and metabolism are thought to be distinctly different in freshwater APP and involve additional regulatory and transport components (Su et al. 2003). In fact, dissolved nitrate:phosphate ratios in freshwaters can approach 8,000 (as seen in Lake Superior), which significantly differs from the Redfield ratio 16:1 (Sterner et al. 2007). In particular interest is the potential of freshwater picocyanobacteria to thrive and dominate in ultraoligotrophic conditions with

extremely low P concentrations. Comparison of P-stress regulation in freshwater picocyanobacteria with marine *Synechococcus* spp. genomes can shed light on their adaptation to extreme P-limited habitats and one is of the main goals of this dissertation.

The success of picocyanobacteria in the oligotrophic environments is perhaps due to the number of adaptations they have evolved to low P concentrations and which are described in detail in section 1.3. In general, it is thought that picocyanobacteria have low P requirements compared to bacteria (Bertilsson et al. 2003), although recent work has refuted this (Scott et al. 2010). The major demand for P in planktonic cells are typically nucleic acids and phospholipids (Van Mooy et al. 2006).

Lastly, as photosynthetic organisms, light is also a limiting factor. The ability of APP to survive under low light conditions is tightly coupled with competition for limiting nutrients. It has been shown that picocyanobacterial production in some extremely oligotrophic lakes is related to photosynthetic active radiation but not to P (Callieri 2010). This observation suggests that low P concentration might be not the only limiting factor driving picoplankton production. Similarly, Lavallée & Pick (2002) have shown a lack of relationship between picophytoplankton growth rates and any form of dissolved phosphorus.

1.1.5 Picocyanobacteria: conclusion

To summarize, picocyanobacteria have developed a number of adaptations which are observed as high diversity within the genera (more than 6 freshwater clusters and more than 10 marine clusters of *Synechococcus*, and 6 *Prochlorococcus* clusters) for thriving in oligotrophic environments, including specific photosynthetic pigment composition (PE

or PC-rich *Synechococcus*), capacity to utilize a variety of nutrients, low P requirements, small size, and small genome. The diversity within the APP is observed as significant differences between picocyanobacteria strains in their specific adaptations to oligotrophic environments. Therefore, the picocyanobacterial heterogeneity is critical while assessing their physiology and contribution to biogeochemistry. However, nutrient metabolism, particularly the phosphorus physiology of freshwater APP, has not been clearly addressed.

1.2 Freshwater ecosystems: Lake Erie and Lake Superior

1.2.1 The Lake Erie ecosystem

Lake Erie is the smallest, the warmest, and the most productive of the Great Lakes (Munawar and Weisse, 1989). It is divided into three basins: the eutrophic western basin and its associated embayments, a mesotrophic central basin, and an oligotrophic eastern basin. Given its location, the lake is greatly influenced by humans. Because of the anthropogenic impact on the lake, nutrient cycling has received a great deal of attention. During the 1960s-1970s, Lake Erie experienced high loadings of anthropogenic phosphorus, which caused toxic phytoplankton blooms, decreased water quality, and resulted in hypoxia in some parts of the lake. After the Great Lakes Water Quality Agreement 1972, loads of phosphorus decreased to ca. 11,000 tons per year (Dolan 1993), resulting in a reduction in the productivity of the lake.

Lake Erie shows strong environmental gradients that likely support distinct microbial populations. Typical depth-dependent changes in temperature, dissolved oxygen, and phytoplankton abundance are detected throughout the year with the thermal

stratification and hypolimnetic hypoxia that typically begins in June (Charlton et al. 1993, Wilhelm et al. 2006). The APP consortium of the lake is mostly represented by cyanobacteria, specifically single-celled, phycoerythrin and phycocyanin – rich *Synechococcus*. The abundance of the APP for Lake Erie is on average on the order of 10^5 cell mL⁻¹ (Ivanikova et al. 2008). This value is comparable to the numbers reported for Lake Superior (Pick and Caron 1987, Rinta-Kanto et al. 2005, Ivanikova et al. 2007b). Additionally, photosynthetic measurements in Lake Erie revealed that, depending on the season, 30-100% of offshore photosynthetic activity is attributed to phytoplankton fraction (Ivanikova et al. 2007). Thus, cyanobacteria play an important role in the food web of the Great Lakes.

Atmospheric deposition, agricultural run-off, food chain recycling, and diazotrophs contribute to the total nitrogen pool in the lake. Phosphorus under natural conditions (without anthropogenic inputs) is usually scarce in water. Soil and rocks are the primary natural sources of phosphorus, usually in the form of phosphate. P bioavailability mostly depends on the recycling rate, as well as agricultural and industrial inputs. In general, the overall dynamics of nutrient availability in aquatic systems is influenced by many factors, such as upwelling (Wu et al. 2000), mixing of water masses (Gargett 1991), recycling by consumers (Landry et al. 1997), biological and chemical equilibria regulated by organism uptake (Maldonado et al. 2001), sorption to surfaces of particles (Ciffoy et al. 2001), and binding by biogenic and abiotic ligands of differing affinity and concentrations (Rue and Bruland 1997, Sterner 2004).

The N to P ratio in Lake Erie is often significantly higher than the 16:1 N:P Redfield ratio. Mean annual particulate value of (molar) N:P ranged from 12:1 to 29:1 (Millie et al.

2009) in 2003-2005. Geider and La Roche (2002) showed that the ratio of N:P in APP to be as high as 50:1. In addition, in phosphorus amendments experiment in which phosphate was added to whole lake water, an increase in phytoplankton production was observed indicating a limitation of primary production by phosphorus (Schindler 1977, Wetzel 2001, Heath et al. 2003 DeBruyn et al. 2004).

1.2.2 *Synechococcus* sp. KD3a and ARC-21

Dr. Bullerjahn's lab maintains culture collection of Great Lakes cyanobacteria from both ultraoligotrophic Lake Superior and the meso/oligotrophic Lake Erie central basin. Two major members of the Lake Erie endemic APP, *Synechococcus* sp. KD3a and ARC-21, are the strains selected for the nutrient amendments experiments in this dissertation. Both *Synechococcus* sp. KD3a and ARC-21 are phycoerythrin-rich members of the picocyanobacterial clade cultured from the central basin of Lake Erie, but are members of distinctly different clusters within the freshwater picocyanobacteria (Urbach et al. 1998, Laloui et al. 2002). By 16S rDNA sequences, KD3a groups with *Synechococcus* sp. MH301, a PE-rich strain cultured from Mondsee, Austria (Crosbie et al. 2003). By contrast, ARC-21 is a member of Crosbie Group B, whose members include cosmopolitan PE-rich strains from largely oligotrophic environments (Fig 1.2) (Crosbie et al. 2003, Ivanikova et al. 2008). Also, it has been shown that strain ARC-21 is the only strain currently in culture that can be lysed by cyanophages isolated from Lake Erie (Ivanikova et al. 2008, A.R. Matteson and S.W. Wilhelm, unpublished). KD3a is a member of MH301 cluster and thought to be the most abundant organism in Lake Erie during early summer (Ivanikova et al. 2008). It has been demonstrated that the most common sequences in the 16S rRNA libraries group in

the MH301 cluster in July (Wilhelm et al. 2006).

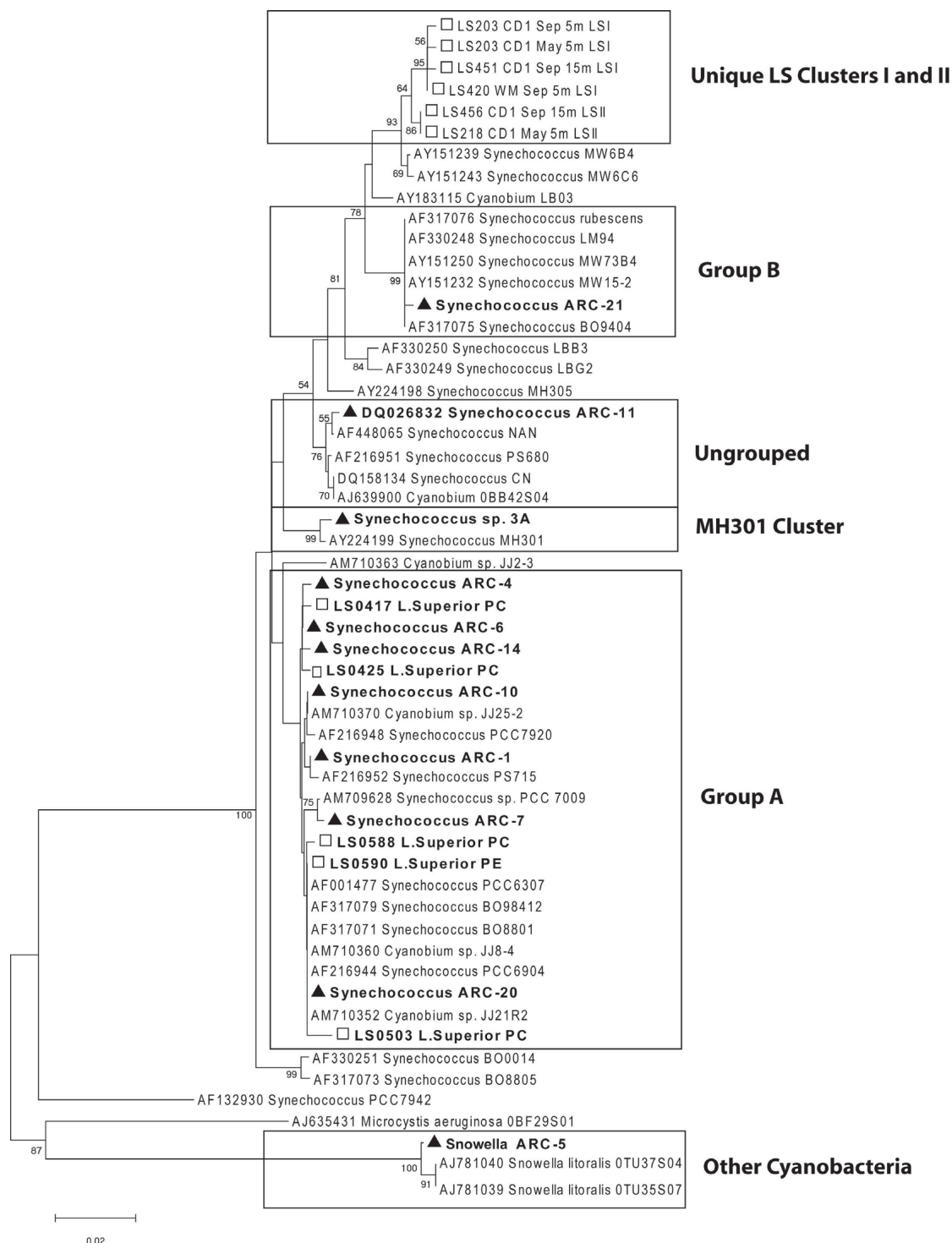


Fig. 1.2 Neighbor-joining tree of 16S rDNA amplicons from Lake Superior (open squares) and Lake Erie (triangles). Sequences from cultured cyanobacteria are shown in bold (reproduced from Ivanikova et al. 2008).

1.2.3 The Lake Superior ecosystem

Lake Superior has the largest surface area (82,100 km²) of any freshwater lake worldwide, and is the deepest (maximum depth, 406 m) and coldest (average temperature, 4°C) of the Laurentian Great Lakes (Hassler et al. 2009). Even though Lake Superior is described as “ultraoligotrophic” lake based on chlorophyll concentration, the mean water column production is ca. 300 mg C m⁻² d⁻¹ (Sturner 2011) which is according to Wetzel (2001) is a “mesotrophic” type of a lake. A potential 12-month growing season in Lake Superior contributes to this perhaps higher than expected level of annual production (Sturner 2010). Primary production is thought to be the major source of organic carbon in Lake Superior and APP contributes into the primary production at large. It has been estimated that approximately 30- 50% of autotrophic production of Lake Superior was attributed to the APP (Fahnenstiel et al.1986, McKay et al. 2005, Ivanikova et al. 2007). Ivanikova et al. (2007) showed that Lake Superior harbors unique picocyanobacteria (Fig. 2). In addition, the authors demonstrated that the majority of sequences correspond to two new clusters of *Synechococcus*, which have not been previously described in any other environments (Ivanikova et al. 2007, Ivanikova et al. 2008). Contrary, picocyanobacteria from a near-shore area in Lake Superior were related to the cosmopolitan *Synechococcus* found worldwide (Ivanikova et al. 2007).

Sturner et al. (2004) suggested that Lake Superior represents the closest freshwater analog of the open ocean for of a number of reasons. First, direct atmospheric inputs are very important to the lake’s biogeochemistry due to the large surface area and relatively small ratio of watershed. Second, due to the limited watershed input, there is not much continental influence on the lake. However, there has been 6-fold increase in Lake Superior

nitrate levels over the past 100 years. Currently, nitrate concentration is between 20 and 30 $\mu\text{mol L}^{-1}$ (Weiler 1978, Bennett 1986, McManus et al. 2003, Sterner et al. 2007). The reasons behind the accumulation of nitrate in Lake Superior are still unknown, but atmospheric and terrestrial inputs have been ruled out (Sterner et al. 2007).

Third, Lake Superior's APP is mainly P and Fe limited (Sterner et al. 2004). Dissolved phosphorus is below the detection limit using the common soluble reactive phosphorus method (Weiler 1978, Baehr and McManus 2003). Total dissolved phosphorus is typically in a range from 4 to 30 nmol L^{-1} (Field and Sherrell 2003, Sterner et al. 2007). It has been acknowledged that APP growth in the lake is mainly limited by phosphorus. Sterner et al. (2004) showed growth increase in response to the phosphorus treatment in the range of 0.05 to 0.15 d^{-1} . Also, it has been documented that DIN:DIP ratios in Lake Superior often exceed 8,000, whereas for marine phytoplankton the ratio is typically closer to 16:1 (Sterner et al. 2007).

1.2.4 Freshwater ecosystems: conclusion

In summary, Lake Erie and Lake Superior are unique freshwater ecosystems with APP as the essential contributors into primary productivity. Both Erie and Superior harbor a large phycoerythrin-rich population. However, Lake Superior represents low nutrient environment in which both phosphorus and trace metals are present in very low concentration (Sterner et al. 2004, Ivanikova et al. 2007a). In contrast, Lake Erie is more eutrophic, with lower a N:P ratio, and great anthropogenic influence. The absence of Superior phylotypes in Erie suggests the unique Superior population that is likely outcompeted by endemic Erie picoplankton (Ivanikova et al. 2008). Conversely, the

nutrient poor, cold, and light-limited Lake Superior appears to have selected for unique *Synechococcus* ecotypes (Ivanikova et al. 2007b). Owing the uniqueness of both Lake Erie and Lake Superior in terms of nutrient availability and contribution of the APP in biogeochemical cycles, this work was focused on assessing of the physiology of the Superior and Erie APP. Particularly, the phosphorus assimilation was of main interest due to it has not been comprehensively addressed in freshwater picocyanobacteria, and freshwaters are often P-limited.

1.3 Phosphorus (P) in aquatic ecosystems

Phosphorus (P) is a fundamental element in a number of metabolic pathways, as well as an essential component of phospholipids, nucleic acids, and ATP in a cell. Most P in living systems is in the reduction state of phosphate (White and Metcalf 2007). Dissolved inorganic phosphate (DIP) is thought to be directly available to microbes but often is limited in aquatic environments. According to Alfred Redfield (1958) the elemental composition of plankton was uniform, and synthesis or decomposition of organic matter were responsible for the changes in C, N, and P concentrations in marine waters. The ratio of 106:16:1 C:N:P is known as “Redfield ratio”, and it is widely used in oceanography and limnology. The N:P ratio is employed for assessing N- and P-limitations in aquatic systems (Geider and La Roche 2002).

1.3.1 P-limitation in lakes

Inorganic phosphate (Pi) is the preferred form of phosphorus for microbial growth, but in many aquatic environments Pi is present in low concentration. It has been estimated

that Pi concentrations are often near or below the detection limit (30 nmolar) by standard analytical methods (Karl, 2000). In fact, primary production in freshwater systems is frequently P-restricted (e.g. Schindler, 1977). For example, it has been shown by a steady state radiobioassay that the concentration of Pi in 56 lakes of North America were orders of magnitude lower than estimates made by standard colorimetric and radiochemical methods (Hudson et al. 2000). Therefore, the already low concentrations of Pi reported in other oligotrophic lakes could be reestimated to be two orders of magnitude lower (Hudson et al. 2000). Similarly, the pelagic regions of Lake Superior and eastern Lake Erie are typically P-limited environments, and picocyanobacteria of the genus *Synechococcus* are the dominant primary producers during the summer. Indeed, on an annual basis, the typical median surface concentration of total phosphorus (TP) in Lake Superior ranges from ~0.10 to 0.15 μM (McManus et al. 2003) and 0.03–0.9 μM (average 0.17 μM) in Lake Erie (Elsbury et al. 2009).

In order to adapt to a changing P environment, marine picophytoplankton have evolved two primary adaptations to the variable P: first, P conservation through a switch from phospholipids to sulfolipids; second, P-limitation responses that recruit alternative P sources such as phosphonates and P esters. It has been previously shown that these strategies are used widely by picophytoplankton in regions of the oligotrophic ocean where phosphate is scarce (Dyhrman et al. 2007, Martiny et al. 2006, Van Mooy et al. 2009). Unlike marine planktonic microbes, information on the freshwater picocyanobacterial P cycle is limited.

1.3.2 P-conservation: Non-phosphorus lipids in response to phosphorus scarcity

As one of the adaptations to a changing P environment, eukaryotic phytoplankton and cyanobacteria have evolved mechanisms that allow them to reduce their P demands. Van Mooy et al. (2009) showed that phytoplankton are able to economize their biochemical P requirements and still maintain growth by substituting non-phosphorus membrane lipids for phospholipids. For instance, in the surface waters of the North Pacific Subtropical Gyre (NPSG) 18 to 28% of the P is used by the total planktonic community for phospholipid synthesis (Van Mooy et al. 2006, 2008b; Bjorkman 2003, Zubkov et al. 2007). However, phytoplankton have the ability to reduce the amount of phosphate uptake used for phospholipid synthesis from 17% in a P replete environment, such as the South Pacific Subtropical Gyre, to 1.3% in oligotrophic Sargasso Sea (Van Mooy et al. 2008a). Given that nucleic acids and phospholipids are the major pools of P in planktonic cells, this biochemical mechanism could provide a significant advantage for phytoplankton over other marine microbes to dominate low-P environments (Dyhrman et al. 2007, Moore 2005).

As suggested above, Cyanobacteria of the genera *Synechococcus*, *Prochlorococcus*, *Crocospaera*, and *Trichodesmium* substitute the non-phosphorus membrane lipid sulphoquinovosyldiacylglycerol (SQDG) for the phospholipid phosphatidylglycerol in P-limited environments (Weissenmayer et al. 2002, Van Mooy et al. 2009). There are four genes—*sqdA*, *sqdB*, *sqdC*, and *sqdD*—encoding enzymes of the sulfolipid biosynthetic pathway, which were first described in the purple bacterium *Rhodobacter sphaeroides*.

Among these genes, only a putative *sqdB* ortholog was present in the available cyanobacterial genomes (Guler et al. 2000, Benning 2004).

It was also shown that *Synechococcus* sp. strain PCC7942 possesses a new open reading frame (ORF) directly downstream of *sqdB*. This ORF encodes a putative protein of 377 amino acids with no sequence similarity to any of the described *sqd* gene products of *R. sphaeroides* (Guler et al. 1996). The novel ORF was named *sqdX*. As it has been established later, *sqdX* is a gene essential for sulfolipid biosynthesis in the cyanobacterium *Synechococcus* sp. strain PCC7942 and encodes the cyanobacterial sulfolipid synthase. Furthermore, it was illustrated that inactivation of *sqdX* in *Synechococcus* caused complete sulfolipid deficiency which could be repaired by introducing the *sqdX* genes in *trans* (Guler et al. 2000, Weissenmayer et al. 2000). In conclusion, the *sqdX* gene product is crucial for cyanobacterial sulfolipid biosynthesis in *Synechococcus* and could serve as a genetic marker for assessing P status of aquatic environments.

1.4 P-limitation responses

1.4.1 *pho* regulon

The P-limitation response of picocyanobacteria is one of the phytoplankton's adaptations to deal with the scarce P in an aquatic environment (Dyhrman et al. 2007). Phosphorus stress genes are repressed or activated by the phosphorylation state of the transcription factor PhoB, and together comprise the so-called Pho regulon (Wanner et al. 1994). There are two main P-limitation responses known to date. The first response involves scavenging for trace Pi by the Pst system, a high affinity and low velocity transport system (Willsky and Malamy 1980). Pst is an ABC type transporter with PstB as an ATP-

binding protein and PstS as a Pi-binding protein that scavenges scarce phosphate (Magota et al. 1984). The Pst system is also coregulated with other proteins, such as PhoE, an outer membrane porin which is responsible for passage of phosphates into the periplasm (Overbeeke and Lugtenberg 1980).

Alkaline phosphatases are another class of enzymes with considerably increased activity in Pi-limiting conditions (Wanner et al. 1996). Their function is to supply Pi for the Pst transport system by hydrolyzing P esters (Coleman et al. 1992). Another P-specific response is activation of genes responsible for transport and utilization of alternative sources of P, such as phosphonates, which comprise a fraction of the dissolved organic P (DOP) pool.

1.4.2 DOP

Despite the fact that DOP represents a much greater fraction of total P than Pi in the surface waters of the open ocean, little is known about the chemical composition or bioavailability of DOP (Karl and Björkman 2002). DOP comprises both low molecular weight (LMW) DOP, which is greater part of the DOP, and high molecular weight (HMW) DOP, which is about 25% of DOP in the upper ocean (Clark et al. 1998; Kolowitz et al. 2001). HMW DOP in turn, provides two classes of P bonds: ester (both mono- and diester) and phosphonate (C-P) linkages (Karl and Björkman 2002), of which phosphonates represent 25% of the total. Therefore, P esters represent a larger potential P source than phosphonates. Furthermore, phosphonates are thought to be more refractory to assimilation than organic P esters (Karl and Björkman 2002).

1.4.3 P esters

Many marine organisms possess alkaline phosphatase (APase) enzymes to meet their P requirement by hydrolyzing ester bonds between phosphates and dissolved organic molecules and releasing P for cellular assimilation (Luo et al. 2009; Martinez et al. 1996). Measurements of APase activity have been extensively used as a proxy to assess the degree of P-starvation in microbial communities (Cotner et al. 1997, Van Wambeke et al. 2002). Particularly, phytoplankton associated alkaline phosphatase activity (APA) can serve as an indicator of P deficiency because the enzyme is induced when P is low (Pettersson et al. 1985) and inhibited when P becomes available (Perry et al. 1972, Elser & Kimmell 1986). However, it has been proved that the assay has some limitations (Rose and Axler 1998, White et al. 2009). For example, Rose and Axler (1998) demonstrated that in the event of colimitation or secondary N limitation, APA might show P deficiency even after additions of P. It has also been shown that the expression of AP activity in different species from the same environment and at the same external P concentration varies significantly (Rengefors et al. 2003, Litchman and Nguyen 2008). In fact, Litchman and Nguyen (2008) suggested that this variability is due to a species-specific dependence of AP expression on the internal P concentration. Furthermore, the authors have demonstrated that two tested freshwater phytoplankton species varied in AP expression “under the same external P concentrations, temperature, and light conditions” which corresponded with the previous observations by Rengefors et al. (2003), Dyhrman (2006), and Ruttenberg (2006c). In conclusion, the internal P pool differs among species, even when external P is the same (Shuter 1978), and the relationship between internal P and synthesis of AP can vary for different species

(Litchman and Nguyen 2008). This might limit the assay utility and suggests usage of more direct measures of P stress, such as evaluating the expression of P-stress enzymes.

To date, at least 3 prokaryotic APase gene families have been characterized, encoding the products PhoA, PhoD, and PhoX (White et al. 2009).

Originally it was thought that alkaline phosphatases in bacteria are in the periplasm, where they hydrolyze phosphoesters passing through the cell envelope without damaging intracellular phosphorylations (Wanner et al. 1996). However, Luo et al. (2009), using the metagenomic database of the Global Ocean Survey and a consensus classification algorithm, predicted three major modes of DOP metabolism by marine bacteria. First, small P-esters can be transported across the cell membrane and hydrolyzed in the cytoplasm by APases, predominantly by PhoA and PhoD. Second, there are APase activities associated with periplasmic, membrane-attached proteins. Third, enzymes released from the cell (mainly PhoX proteins) cause release of extracellular Pi, followed by the assimilation of Pi into the cell (Luo et al. 2009, White et al. 2009).

Regarding the transport systems, different pathways responsible for transferring the proteins. PhoA is transferred to the periplasm by the Sec pathway, while the twin-arginine translocation pathway is used to export PhoX (Zaheer et al. 2009). In addition, these two enzymes have different metal requirements: PhoA is activated by zinc and magnesium ions and PhoX by calcium ions (Roy et al. 1982, Coleman et al. 1992). Indeed, it has been shown that because the total zinc concentration is low in some P limiting regions (Jakuba et al. 2008), it may be a restrictive factor for alkaline phosphatase activity and DOP assimilation (Shaked et al. 2006). In other words, cells containing a Ca-dependent PhoX may have an advantage over a zinc-dependent PhoA (Kathuria et al. 2010).

It has also been shown that *phoA* gene families from *Escherichia coli* and *Synechocystis* PCC6803 are under the control of a histidine-kinase transcriptional regulator PhoB (Vershinina and Znamenskaia 2002, Su et al. 2003), which binds to the Pho box in the promoter region of each target gene or gene cluster (Orchard et al. 2009).

PhoA is a major member of Pho regulon in many bacteria. It has been extensively studied in *E.coli* as a part of the P-starvation response and proved to hydrolyze P esters, and it is induced several hundred fold when P is deficient (Wanner et al. 1996, Sebastian and Ammerman 2009). In contrast, PhoX is more widely distributed than PhoA within the cyanobacteria. Even though PhoX is an alkaline phosphatase and under *phoRB* control (Von Kruger et al. 2006), it lacks any homology to PhoA. Metagenomic analysis revealed that PhoX is more widespread than PhoA in oligotrophic marine environments (Sebastian and Ammerman 2009). Because PhoX is induced upon P-starvation, facilitating organic P acquisition, it was suggested that the gene could be used as a molecular marker to estimate P-status in marine environments (Sebastian and Ammerman 2009).

PhoD, a secreted phosphodiesterase/alkaline phosphatase, was first purified from a culture of *Bacillus subtilis*. It has been shown that the gene is induced under P-starvation, thus it is a part of Pho regulon. As well as PhoX, PhoD requires calcium for its activity (Luo 2009). It has been demonstrated that the amino acid sequence of PhoD has no similarity to any protein in the databases available and is different from other APases (Eder et al. 1995). According to the metagenomic database of GOS, PhoD is the most abundant APase in marine cyanobacteria (White et al. 2009).

1.4.4 Phosphonates

Phosphonates (Pns) are organic compounds containing a covalent bond between phosphorus and carbon atoms. The C-P bond is more difficult to hydrolyze than phosphate esters because of their high activation energy for hydrolysis (Black et al. 1991). Known Pns include biogenic and xenobiotic compounds (Horiguchi 1984, Ternan et al. 1998a). The most abundant biogenic phosphonates, such as 2-aminoethylphosphonic acid (2-AEP), phosphonoformic acid and methylphosphonic acid, are derived from the degradation of glycolipids, glycoproteins, antibiotics, and phosphonolipids (Karl and Björkman 2002, Clark et al. 1998, Kolowith et al. 2001, Dyhrman et al. 2006, White and Metcalf 2007). Specifically, 2-AEP is found in phosphonoglycolipids and it is believed to be the most abundant Pn in the oceans (Horiguchi 1984, Martinez et al. 2010).

Since 1944, when the first phosphonate was synthesized (Hilderbrand & Henderson 1983), synthetic phosphonates have been extensively used in agriculture, pharmacy, and households because of the stability of the C-P bond that makes them resistant to chemical and enzymatic hydrolysis (Kolpin et al. 2006). Some of the most common synthetic Pns include the antiviral compound phosphonoacetate, the herbicide glyphosate, and a number of detergents. It has been estimated that more than 2×10^4 tons of organophosphonates are discharged into the environment each year (Egli et al. 1988). Furthermore, it is thought that reactive phosphonic acids were the first prebiotic organic P on Earth (Degraaf et al. 1997), which all suggest that phosphonates may be a potential source of organic P for bacteria. In fact, it has been known for some time that phosphonates are readily assimilated by many bacteria (Wackett et al. 1987, Quin et al. 1989, Schowanek 1990, Liu et al. 1991, Huang et al. 2005), including cyanobacteria (Dyhrman et al. 2002, Palenik et al. 2003, Su et

al. 2003, Moore et al. 2005, Dyhrman et al. 2005, Ilikchyan et al. 2009). In order to better understand how microorganisms assimilate, transform, and turn over elemental resources in the ocean, genetic and biochemical analyses have been applied. Many studies in this area have provided detailed information on the Pn utilization pathways (Ternan et al. 1998, Quinn et al. 2007, White and Metcalf 2007).

1.4.5 Assimilation and degradation of phosphonates

Known C-P hydrolases include the C-P lyase and the phosphonatase pathway. The C-P lyase is a complex membrane-bound system encoded by 14 genes - *phnC-P*, which together encode a broad substrate specificity enzymatic pathway belonging to the Pho regulon (Metcalf and Wanner 1991, 1993[a, b], Yakovleva et al. 1998). The first three genes in the operon *phnCDE* encode a Pns-specific ABC transporter, whereas *phnG-phnM* are used for catalytic activity. The C-P lyase operon is found in distantly related bacteria indicating horizontal gene transfer (Huang et al. 2005).

The second mechanism of phosphonate degradation is a hydrolysis by different phosphonatases. In contrast to the C-P lyase, phosphonatases are substrate specific, and the genes may be under Pho regulation or be substrate-inducible (LeNauze et al. 1977, Dumora et al. 1989, Baker et al. 1998, Ternan et al. 1998b, Kim et al. 2002, Quinn et al. 2007). The phosphonatase pathway is encoded by two genes: pyruvate transaminase (*phnW*) and phosphonoacetaldehyde hydrolase or phosphonatase (*phnX*) (Martinez et al. 2010).

It has been demonstrated by Dyhrman et al. (2006) that the marine N-fixing filamentous cyanobacterium *Trichodesmium erythraeum* harbors the complete C-P lyase

operon. It has been expressed in P-depleted cultures, as well as in the oligotrophic Sargasso Sea, suggesting that members of this genus can use Pns as an alternative source of P (Dyhrman et al. 2006).

It has been proved that marine *Synechococcus* sp. WH8102, which harbors the *phnWX* phosphonatase pathway, is able to grow on the Pns 2-AEP and ethylphosphonate as a P source (Palenik et al. 2003, Su et al. 2003). Additionally, the transporter protein PhnD has been shown to be one of the most abundant polypeptides in a metaproteomic analysis of the Sargasso Sea (Sowell et al. 2009). All these findings suggest that Pns may be an important P source for marine microbes.

In conclusion, one of the adaptations of the marine and freshwater bacterial community to low P environments is to evolve mechanisms for recruiting alternative P sources, such as P esters and Pns. Whereas it has been proved for many marine cyanobacteria to possess assimilation mechanisms for P esters and Pns utilization, little is known about phosphonate utilization in the freshwater bacterial community.

1.4.6 Apases and Pns in freshwater environments

Unlike carbon and nitrogen, Pi cannot be replenished from the atmospheric sources, thus, the C:N:P ratios, which are reflective of the demands for nutrients, are often not maintained at the optimal level for bacterial growth (Schindler 1977, Hecky and Kilham 1988). Indeed, in freshwater, Pi often becomes a limiting factor for phytoplankton production (Elser et al. 1990, Huisman and Weissing 2001). One of the factors contributing to the P limitation is the low solubility of phosphates in the presence of Ca^{2+} and Mg^{2+} , which can be abundant in some freshwaters (Falkowski and Knoll 2007).

There are a number of phosphohydrolases, such as phosphomonoesterases, phosphodiesterases, and triphosphoric monoester hydrolases which hydrolyze phosphate ester bonds facilitating a release of P_i . The potential substrates for phosphatases are phosphomonoesters, nucleic acids, nucleotide monomers, inorganic pyrophosphate, polyP, and short chain metapolyP (Siuda et al. 1984). There are two types of phosphatases: alkaline and acid phosphatases, which have extracellular and intracellular functions respectively (Jansson et al. 1988, Bentzen 1992). As mentioned above, APases are induced upon P stress conditions and the product of their activity, P_i , is readily assimilated by phytoplankton (Jansson et al. 1988). Furthermore, it has been hypothesized that organic P is the prevalent source of P for phytoplankton in P-limited freshwater environments (Currie and Kalf 1984, Huisman 2005). All these results support the idea that phosphatases are a significant source of P_i for phytoplankton and have a critical function in the P dynamics of lakes (Jansson et al. 1988, Huisman 2005).

Whereas in marine systems, biogenic Pns may contribute up to 25% of DOP, there is no information on freshwater Pns available to date. Nevertheless, the amount of anthropogenic Pns, such as phosphonate herbicides, loaded in freshwater systems have been drastically increased and shown to be utilized by microbial communities under P-limited conditions (Ilikchyan et al. 2009).

1.5 Objectives

Here, I propose to assess different mechanisms to adapt to a low P environment in both Lake Erie and Lake Superior. Specifically, I examined the ability of endemic microbes

to utilize organic phosphates and phosphonates, as well as monitored the distribution and abundance of *sqdX* and *pstB* genes.

This research is significant because of the following reasons:

1. An understanding of mechanisms for acquisition of P compounds other than Pi will give a better insight into P physiology of freshwater picocyanobacteria. Specifically, an assessment of the microbes' capacity to assimilate organic phosphates and phosphonates are of primary concern.
2. Due to the pelagic regions of Lake Superior and eastern and central basins of Lake Erie which are typically P-limited environments, and picocyanobacteria of the genus *Synechococcus* are the dominant primary producers, it is important to understand how endemic microbes thrive in such low P environments. For instance, it has been documented that DIN:DIP ratios in Lake Superior often exceed 8,000, whereas for marine phytoplankton the ratio is typically 16:1, an ideal stoichiometry known as the Redfield ratio (Sterner et al. 2007). The *pstB* gene, which is upregulated to cope with low phosphorus availability, was employed to assess the P-status of the environment.
3. Whereas the utilization of phosphonates has been proved for marine environments, it is still unclear for freshwater environments. Therefore, testing oligotrophic freshwater environments for the presence and utilization of naturally-occurring phosphonates by picoplankton is essential for the understanding of P-stress induced gene mechanisms (Benitez-Nelson et al. 2000).
4. Pelagic Lake Superior was used as an example of an extremely oligotrophic system to better understand the physiological ecology of the endemic phytoplankton population, which is thought to be unique to the Lake and adapted to the vanishingly

low concentrations of total phosphorus (Ivanikova et al. 2007).

5. The expression of *sqdX* gene, the cyanobacterial sulfolipid synthase (*sqdX*) essential for sulfolipid biosynthesis, was monitored as one of the cyanobacterial adaptations to economize their P quota.

CHAPTER 2. MATERIALS AND METHODS

2.1. Environmental samples

Information on sampling and station locations can be found in Table 2.1 and Figures 2.1 and 2.2. Seston was collected onto 0.22 μ m Stervex cartridges (Millipore) as described below.

Lake/Station	Date	Depth (m)	Volume (L)	Lat/long
LE/957	08/05/2009	1	1	41 40.59N 81 44.33W
LE/84	08/05/2009	1	0.95	41 56.08N 81 39.14W
LE/946	08/05/2009	1	1	42 10.00N 80 38.31W
LE/23	08/05/2009	1	1	42 11.32N 80 02.12W
LE/452	08/05/2009	1	1	42 34.52N 79 55.17W
LE/CCB (Cleveland Central Bay)	04/24/2009	Surface	1.5	41 44.07N 81 41.54W
LE/7M	06/23/2009	Surface	1.1	41 43.97N 83 17.78W
LE/MB20	06/24/2009	Surface	0.8	41 42.90N 83 27.32W
Maumee River/Napoleon	05/01/2009	Surface	0.3	41 22.55N 84 08.198W
LS/WM	06/10/2009	5	2	47 20.00N 89 48.00W
LS/WM	08/23/2009	5	3	~
LS/WM	08/23/2009	127	3	~

LS/WM	05/15/2010	2	3	~
LS/WM	05/15/2010	155	4	~
LS/CD1	05/14/2010	5	4	47 03.90N 91 25.90W
LS/CD1	05/14/2010	235	5	~
LS/SteC	08/20/2010	5	8	46 48.11N 91 51.50W
LS/WM	08/18/2010	5	10	~
LS/CD1	10/05/2010	10	4	~
LS/CD1	10/05/2010	245	4	~
LS/SteI	10/06/2007	25	0.96	46 99.80N 90 72.50W

Table 2.1. Summary of samples collected from Lake Erie and Lake Superior

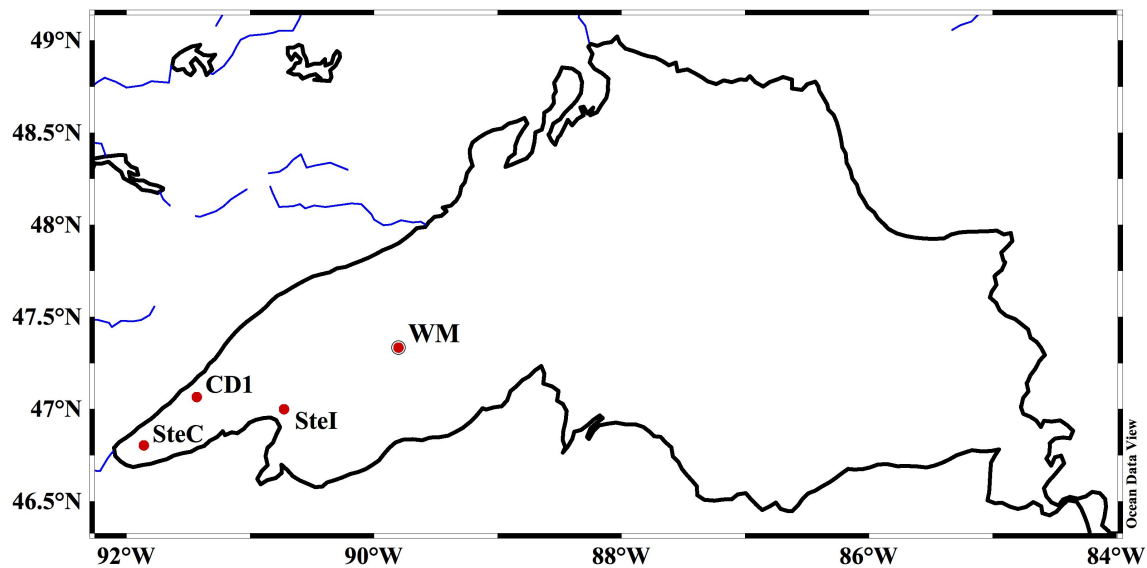


Figure 2.1. Map of Lake Superior, with the locations of hydrographic stations indicated. Stations CD1 and WM are pelagic stations. CD1 is close to shore by distance (~ 10 km) but due to its depth ($z_{\max}=249$ m) and exclusion from the nearshore current exhibits the features of an offshore station

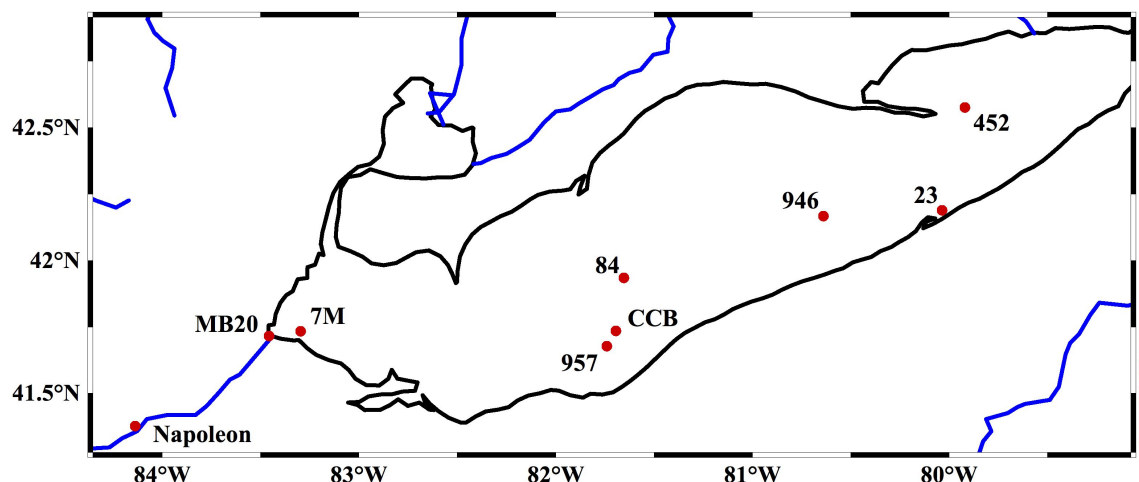


Figure 2.2. Sampling locations within Lake Erie

2.2 Cell culturing conditions

Two Lake Erie strains, *Synechococcus* spp. ARC-11 and KD3a (Cupp et al. 2007, Ivanikova et al. 2008), were maintained in BG11 medium (Allen et al. 1968) at 23 °C on a shaker table under continuous illumination of 5 - 10 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$. Both of the strains are phycoerythrin-rich strains isolated from pelagic Lake Erie (Ivanikova et al. 2008). Under these conditions, the growth rates of newly inoculated cultures were ca. 0.7 d⁻¹. In the first series of experiments with *Synechococcus* KD3a, the culture was tested for the ability to induce the phosphonate transporter gene in presence of 2-AEP. There were two individual experiments. In the first set, cultures were grown in regular BG-11 medium with the following addition of 100uM 2-AEP. Control flasks had regular BG-11 medium. In the second set, P-free BG-11 medium with the following 2-AEP addition was used. The control samples had P-free BG-11 medium. All of the experiments were set in triplicate. The growth was monitored by chlorophyll *in vivo* fluorescence and cell abundances measured by flow

cytometry. The alkaline phosphatase activity and RT-PCR were conducted to assess the P status of the cultures.

The second series of growth experiments, the re-feed experiments, were performed with *Synechococcus* ARC-21 and were aimed to identify the ability to induce both the phosphonate transporter gene and alternative alkaline phosphatases. In the re-feed experiments cells were starved for P and then P was added back. The source of P added to P-starved cells was either 2-AEP or inorganic P (K_2HPO_3). Positive control was regular BG-11 medium whereas negative control was a culture with no P added. All of the experiments were set in triplicate. Similarly, the growth of the cultures was monitored by *in vivo* fluorescence and flow cytometry; the alkaline phosphatase activity and RT-PCR of *phnD* and *phoX* was performed to evaluate the P status of the cultures. Both cultures, *Synechococcus* sp. KD3a and ARC-21, were cultured to mid exponential phase in BG-11. Cells were harvested by filtration, washed three times with P-free medium, and inoculated into BG-11 with the different P amendments described above. Samples for RNA extraction were collected by centrifugation and preserved with RNeasy (QIAGEN) (Ilikchyan 2009).

Another growth experiment was set *in situ* using Lake Superior water, on the R/V *Blue Heron*, in October 2010. The experiment was conducted in acid-washed 4 L cubitainers with water collected by CTD (Conductivity, Temperature, Depth recorder) rosette at 10 m depth. The +P treatments were spiked with 20 μ M (final concentration) of Pi (sodium phosphate salt) and placed in a flowing water incubator for 2 days (~47 hours, 12:50h on 5 Oct through 12:05h on 7 Oct). Control sample was with no Pi added. Treatments were performed in duplicates. The experiments were terminated by filtration through Sterivex cartridge filters.

2.3 Nucleic acid extraction

DNA was isolated from culture material using either phenol-chloroform extraction (Chomczynski & Sacchi 1987) or DNeasy Tissue Kit (QIAGEN) according to the manufacturer's procedures. For DNA extraction from environmental samples and from cultures the phenol-chloroform method and the DNeasy Tissue Kit (QIAGEN) were used respectively. Seston from environmental samples was collected onto 0.22 μm Sterivex cartridge filters and frozen immediately in liquid nitrogen prior to DNA extraction. For extraction of DNA from a Sterivex filter, TE buffer (10 mM Tris-HCl, pH 8; 1mM EDTA, pH 8) containing 15 mg mL⁻¹ lysozyme was injected into the Sterivex filter, and the filter was incubated at room temperature for 1 h. After incubation the solution was removed with a syringe and placed into a 15 ml Falcon tube. The solution was extracted twice with equal volumes of phenol-chloroform (24:1) followed by a chloroform extraction. The supernatant was collected, and DNA was precipitated by addition of one volume of isopropanol followed by centrifugation for 10 min at 13,000 $\times g$ at room temperature. The precipitated DNA was washed with 70% (v/v) ethanol, dried, and resuspended in TE buffer (Hisbergues et al. 2003).

For the DNeasy Tissue Kit, 2 mL of culture material was used and the manufacturer's lysis buffer was replaced with the STET buffer (50 mM Tris-HCl, 50 mM sodium EDTA, 5% Triton X-100, 8% sucrose [pH 8,0]).

RNA was extracted from environmental samples following collection of seston onto 0.22 μm Sterivex cartridge filters. Samples for RNA extraction from the growth experiments were harvested by centrifugation and preserved with RNeasy Protect Bacteria reagent (QIAGEN) at -80°C. RNA extraction from both environmental and growth

experiment samples was performed using the RNeasy Mini Kit with slight modifications from the manufacturer's instructions. Extraction of RNA from a Sterivex filter was performed as previously described in Ilikchyan et al., 2009. The RNA was then treated with RNase-free DNase (QIAGEN) to remove any trace amounts of genomic DNA. RNA concentrations were measured with a NanoDrop ND-1000 spectrophotometer. All RNA samples were screened for genomic contamination by PCR.

2.4 PCR and RT-PCR conditions

Table 2 provides details on the primers have been used for PCR and RT-PCR amplifications with *Synechococcus* spp. All the sequences were amplified using a PTC-1148 MJ Mini Gradient Thermal Cycler (Bio-Rad, Inc). PCR amplification was done with 1 ml of normalized DNA template (ca 10 ng) in a final reaction mixture (25 ml) containing 1 x PCR buffer (Promega), 0.2 mM of each deoxynucleotide (Promega), 0.5 μ M of each primer, and 1.0 unit of GoTaq DNA polymerase (Promega). The PCR conditions for *phnD*, *sqdX*, and *pstB* were 95°C for 5 min, 30 cycles of 95°C for 1 min, an annealing temperature varied depending on the primer sets (see Table 2) for 1 min, 72°C for 1 min, followed by extension at 72°C for 10 min. For *phoX* and *rnpB* primers the conditions were the following: 95°C for 5 min, 30 cycles of 95°C for 30 sec, an annealing temperature varied depending on the primer sets (Table 2) for 30 sec, 72°C for 30 sec, followed by extension at 72°C for 10 min.

RNA was reverse-transcribed to single-stranded complementary DNA using an iScript cDNA Synthesis kit (Bio-Rad). To normalize the cDNA, the volume of RNA template added to the reaction mix was adjusted to yield a final concentration of 50 ng/ μ L.

Additional reactions were done without reverse transcriptase (RT) to ensure the absence

of genomic DNA in all cDNA samples (Dyhrman et al. 2006). PCR amplification was done in the 25 ul reaction mixture with the cDNA template normalized to ca. 10ng. PCR and RT-PCR products were resolved on a 2% agarose gel. Selected gene products were cloned into TOPO plasmid vectors (Invitrogen) and sequenced at the University of Chicago Cancer Research Center using specific gene primers. Obtained sequences were analyzed manually and aligned using ClustalX – 2.0.12 software (Thompson et al. 1997). Phylogenetic analysis was performed using Mega 5.0 software (Kumar et al. 2007) and Phylogeny.fr (Dereeper et al. 2008).

Gene	Primer code	Primer sequence	Annealing temperature (°C)	Product size (bp)	Target region (from/to)
phnD*	SynFW280_F	5'ACCGGNGATCTSGAYCTGGTNTGGTTCGG	65	320	280/600
	SynFW600_R	5'ATGGTGGYGTCRTGGCTGCCRCTGAANCC			
phoX_1	phoX120_F	5'CTGGCSGGCAACAARTACGGCTGG	65	180	120/300
	phoX300_R	5'GTAGAGGTGGNCGCCGCGGCGATCGC			
phoX_2	phoX774_F	5'TGGGGCACGGTGCTGAGCGCCGAG	65	296	774/1070
	phoX_1070	5'GTAGAGGTGGCCGCCGCGGCGATCG			
phoX_3	phoX915_F	5'GTGCTSAGYGCBGARGAGAA	60	160	915/1075
	phoX1075_R	5'CCACCATCCARCCGTAYTTGTT			
sqdX	sqdX228_F	5'CCRTCSTRATGATGTCVGG	61	582	228/810
	sqdX810_R	5'GAGCACTACGGYWTGGGVATGC			
pstB	pstB73_F	ACGYCACSATCAGYTACGGC	55	407	73/480
	pstB480_R	CCRCTYTCTTSAGCTTGTC			
rnpB*	rnpB_F	5'CCGTGAGGAGAGTGCCACAG	65	114	280/600
	rnpB_R	5'CAGCACCTCTCGATGCTGCTGG			

Table 2.2. *Synechococcus* spp. PCR and RT-PCR primers

2.5 Alkaline phosphatase activity

Alkaline phosphatase activity of the samples was measured fluorometrically using 4-methylumbelliferyl phosphate (MUBP; Sigma) as a substrate (Sinsabaugh et al. 1997, DeBruyn et al. 2004, Sterner et al. 2004). Samples from the nutrient amendments experiment were collected every 24 hours. To ensure the absence of microbial contamination in substrate mixes, sodium bicarbonate (5 mM) was substituted for the samples in substrate controls while quench samples were prepared using the samples and 4-methylumbelliferone. The assay was performed in black 96-well microtiter plates at ambient room temperature (23°C). Each well received 200 μ L of the sample and 50 μ L of sterile substrate solution. Fluorescence readings were recorded at 0.1 – 1- h intervals, depending on the amount of activity present, with a Dynatech MicroFLUOR platereader with a 365-nm broadband excitation filter and a 450-nm narrowband emission filter. Activities were expressed as the rate of accumulation of methylumbelliferone equivalents (μ mol liter⁻¹ h⁻¹) by using an emission coefficient of 4,100 nmol⁻¹ (calculated by regression from 0.25-3.5 μ M MUF standards in 5 mM bicarbonate).

2.6 Flow cytometric analysis

Three 1.5 ml aliquots of each sample were fixed for 20 min at room temperature in the dark with 0.1% (final concentration) glutaraldehyde grade II (Sigma). Samples were flash-frozen in liquid nitrogen and then stored at -80°C until analysis. Samples were analyzed on a Becton-Dickinson FACSCalibur (BD Biosciences, San José, California) using standard flow cytometry protocols (Marie et al. 2005). Picocyanobacterial cells were identified by orange (FL2) fluorescence from phycoerythrin and light scatter (SSC) and

enumerated using the CellQuest software package (BD Biosciences, San José, California).

Volumetric cell abundances were calculated from flow cytometric cell counts (cell acquisition⁻¹), acquisition duration (min acquisition⁻¹), and independently calibrated flow rates (volume min⁻¹).

CHAPTER 3. RESULTS

3.1 Flow cytometric analysis

Flow cytometric enumeration of picocyanobacteria in the transfer experiment with *Synechococcus* KD3a and the re-feed experiment with *Synechococcus* ARC-21 was conducted. Growth of *Synechococcus* sp. KD3a in P-replete and P-depleted media measured by *in vivo* chlorophyll *a* (ug per L) and by flow cytometry (cells per mL) (Fig. 3.1). In general, growth rate in -P cultures was lower than in +P. Additionally, -P cultures reached stationary phase on day 18, while +P cells continued growing, increasing in abundance and chlorophyll concentration. Chlorophyll and cell abundance values were not significantly different during lag and exponential phases; however, they started showing slight dissimilarities by the end of the experiment. Cell size was also evaluated by flow cytometry and yielded an increase in the cell size of P-stressed cells compared to P-replete cultures (Fig. 3.2).

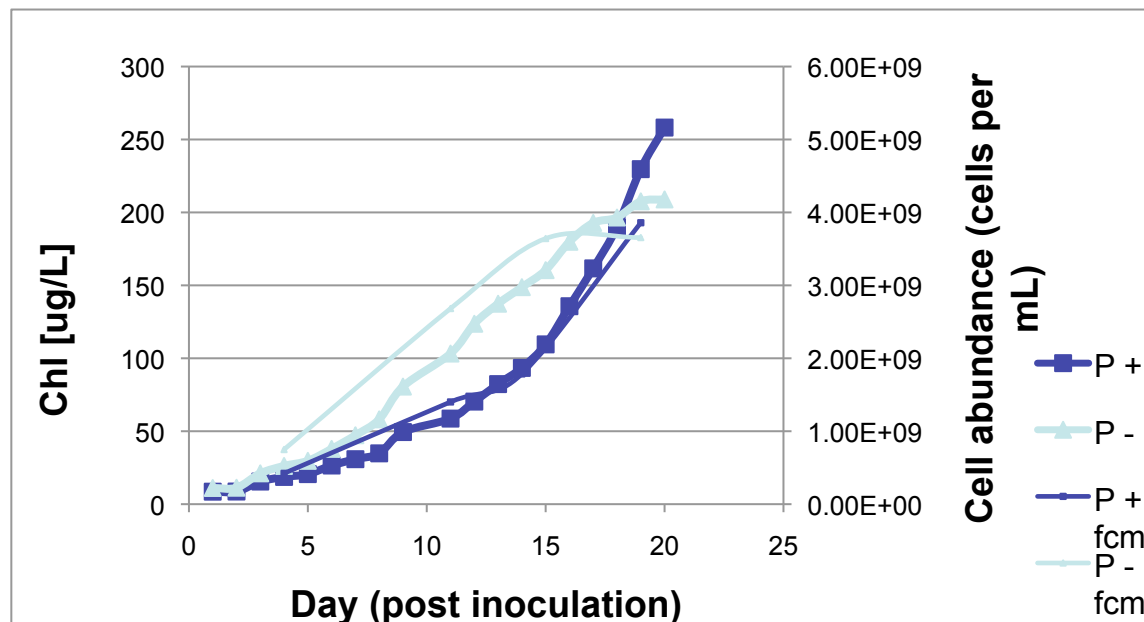


Figure 3.1. *Synechococcus* KD3a chlorophyll and cell abundance measurements. Dark blue and light blue lines represent P-replete and P depleted cultures, respectively. Thick and thin lines show cell abundance and chlorophyll measurements, respectively.

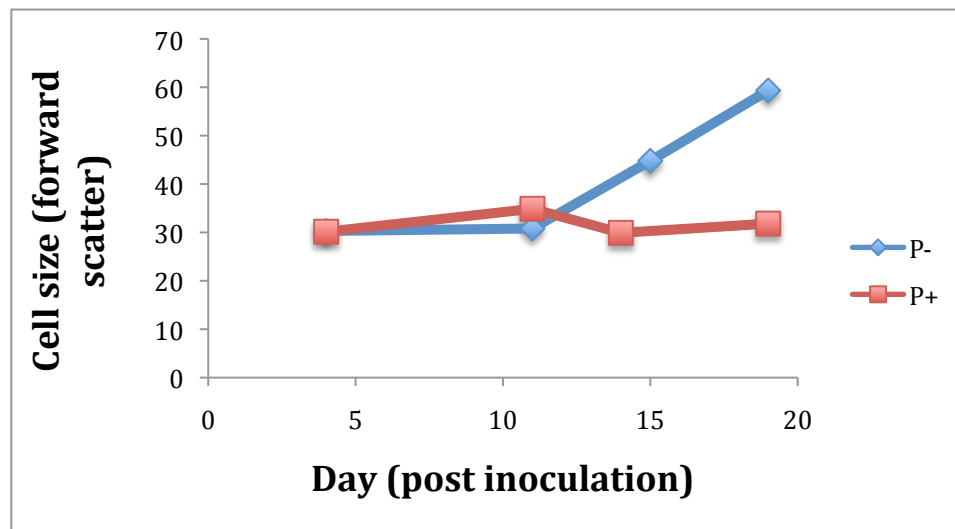


Figure 3.2. Cell size of *Synechococcus* KD3a measured by flow cytometry. Red and blue lines are P-replete and P depleted cultures, respectively.

In the re-feed experiment with *Synechococcus* ARC-21 cell abundance and cell size were measured by flow cytometry. Cell abundance in BG-11 cultures (positive control) was the highest compared to other cultures. Cultures where either 2-AEP or K_2HPO_4 was added, and negative control (no P added) showed no significant difference in their cell abundance (Fig. 3.3). As for cell size, after the addition of P sources the largest cell size was observed in cultures treated with phosphate (Fig. 3.4). Cultures with 2-AEP added and cultures with no P source (negative control) were comparable. Cell size in BG-11 cultures (positive control) remained stable throughout the experiment and was the lowest among all the treatments.

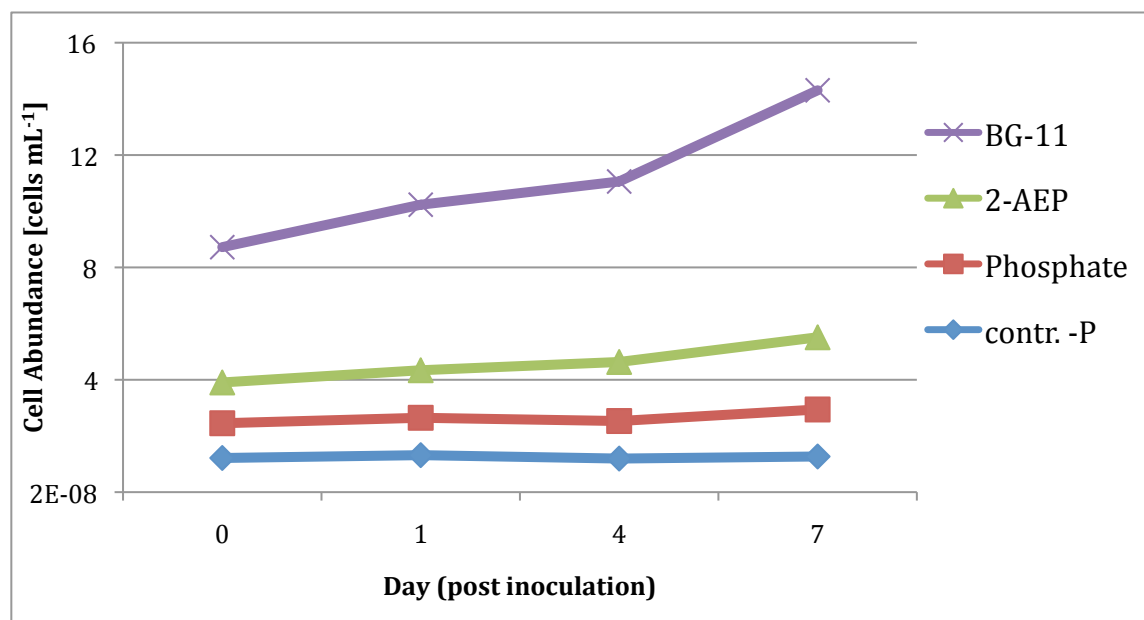


Figure 3.3. *Synechococcus* ARC-21 cell abundance in the re-feed experiment measured by flow cytometry. Measurements are taken during 7 days after inoculation, including time 0 – cultures before adding the amendments. **2-AEP** – after the addition of phosphonate 2-AEP; **Phosphate** – after the addition of phosphate source; **BG-11** – positive control, regular BG-11 medium; **contr.-P** – negative control, with no P added.

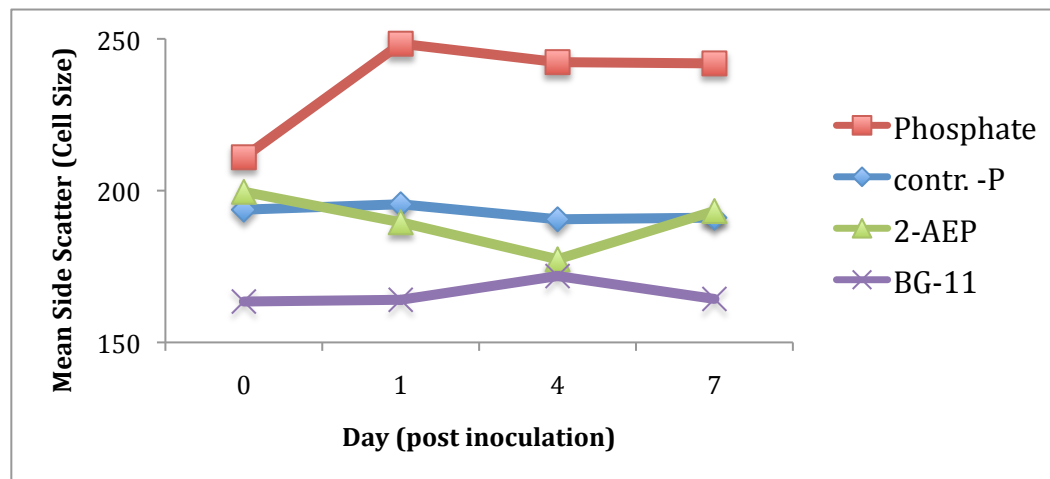


Figure 3.4. *Synechococcus* ARC-21 cell size in the re-feed experiment measured by flow cytometry.

3.2 Alkaline phosphatase assay

The activity of alkaline phosphatase was assessed in both the transfer experiment with *Synechococcus* KD3a and the re-feed experiment with *Synechococcus* ARC-21 (as described earlier). In the transfer experiment exponentially growing cells were transferred to either replete (+P) or depleted (-P) media whereas in the re-feed experiment cells were starved for P and then P was added back. In the transfer experiment, APA increased in the -P culture to a final rate of $10.8 \text{ nmol P h}^{-1} \text{ mg Chl } a^{-1}$, while in +P cultures APA was never above $0.06 \text{ nmol P h}^{-1} \text{ mg Chl } a^{-1}$ (Fig. 3.5). In the re-feed experiment, APA was measured before and after the amendments with activities ranging from 1.13 to $10.75 \text{ nmol P h}^{-1} \text{ mg Chl } a^{-1}$, this rate declined upon P addition, and was 0.97, 2.45, and $6.34 \text{ nmol P h}^{-1} \text{ mg Chl } a^{-1}$ in +P, +2AEP, and Ctrl. (no P) treatments, respectively (Fig. 3.6A and 3.6B). APA in P replete cultures (positive control) never exceeded $0.04 \text{ nmol P h}^{-1} \text{ mg Chl } a^{-1}$.

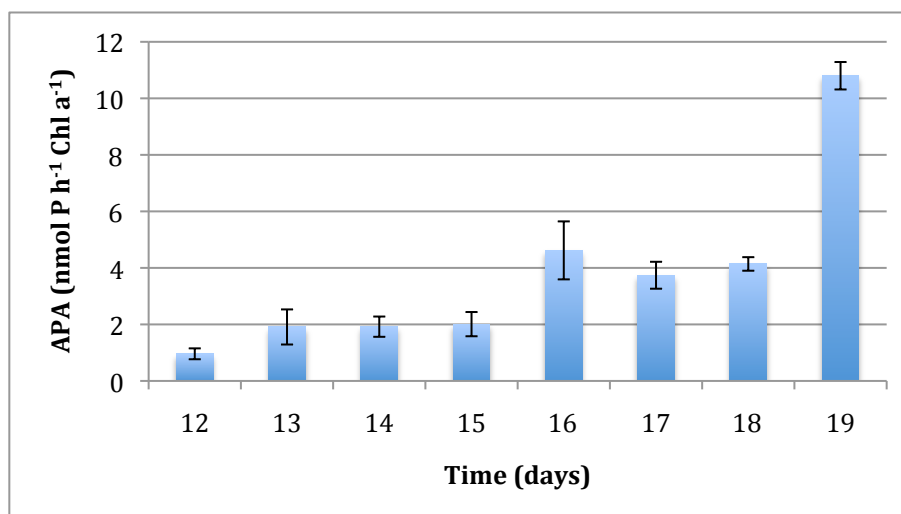
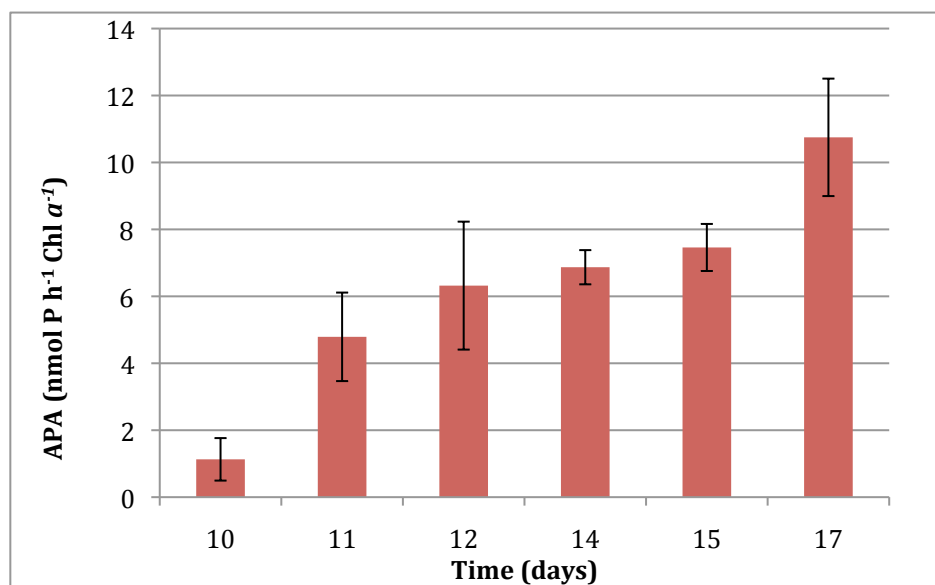


Figure 3.5. Alkaline phosphatase activity for the transfer experiment with *Synechococcus* KD3a in P depleted cultures. APA was measured during 19 days after inoculation.

A.



B.

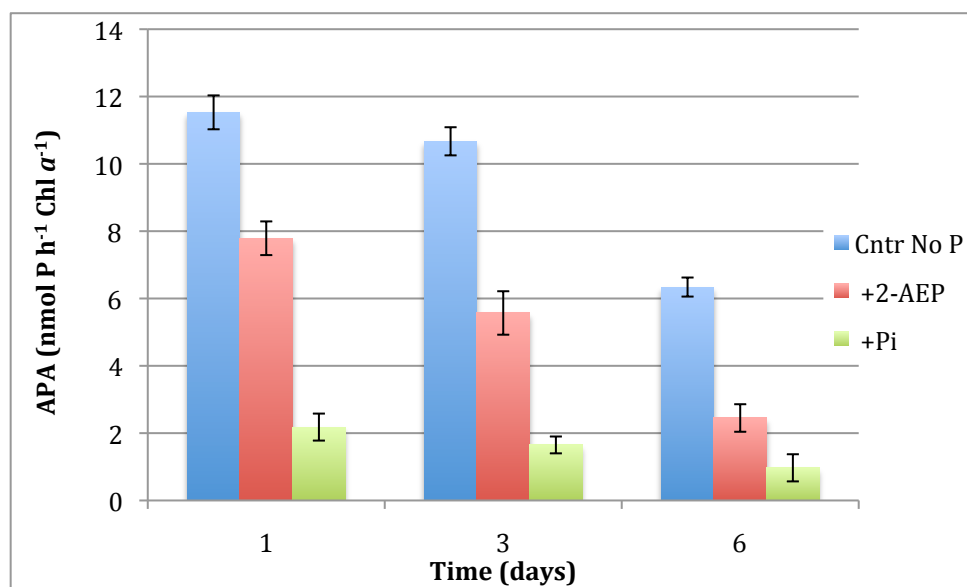


Figure 3.6. Plots of APA for the re-feed experiment with *Synechococcus* ARC-21. **Plot A:** APA in cultures before the addition of amendments; **Plot B:** APA after the addition of P (green bar), 2-AEP (red), and control samples with no P source added (blue). APA was measured during 17 days before the addition of P source and 6 days afterwards.

3.3 PCR of *phnD*, *phoX*, *sqdX*, and *pstB* from *Synechococcus*

To detect the presence of the *phnD*, *phoX*, *sqdX*, and *pstB* genes, a PCR-based assay was designed as a proxy for the genetic potential of freshwater *Synechococcus* spp. to adapt to low P and assimilate alternative P sources. The assay for *sqdX*, and *pstB* was developed based on the gene sequences for marine *Synechococcus* spp. The *Synechococcus* spp. *phoX* specific primers were designed recognizing the variability in amino acid sequence in open ocean and coastal strains. The *phnD* primers were adapted from Ilikchyan et al. (2009). Consequently, specific oligonucleotide primers of low-degeneracy were designed (Table 2.2). The primers for *phnD*, *sqdX*, and *pstB* amplify 620, 580 and 407 basepairs fragments, respectively. The *pstB* gene was initially designed from the misannotated *pstS* gene in *Synechococcus* WH8102. There were three sets of *phoX* primers designed: *phoX_1*, *phoX_2*, and *phoX_3*, each of them giving 180, 296, and 160 basepairs fragments, respectively. All four genes were detected in cultured freshwater *Synechococcus* spp. from Lake Erie and Superior and environmental freshwater samples (Figures 3.7-3.10). Sequencing of the amplicons confirmed the PCR results.

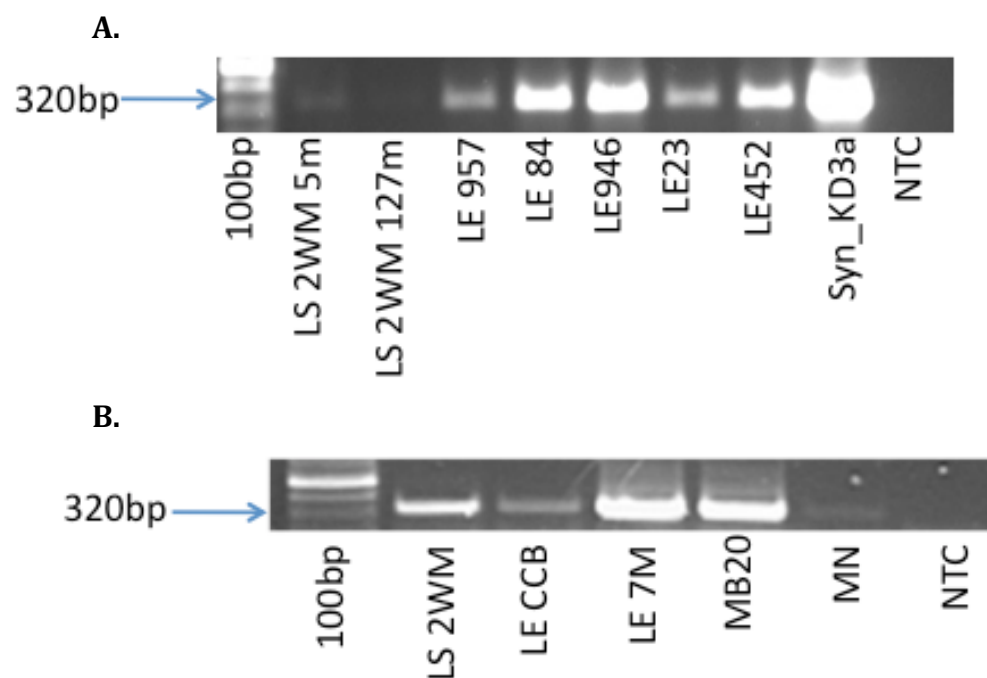
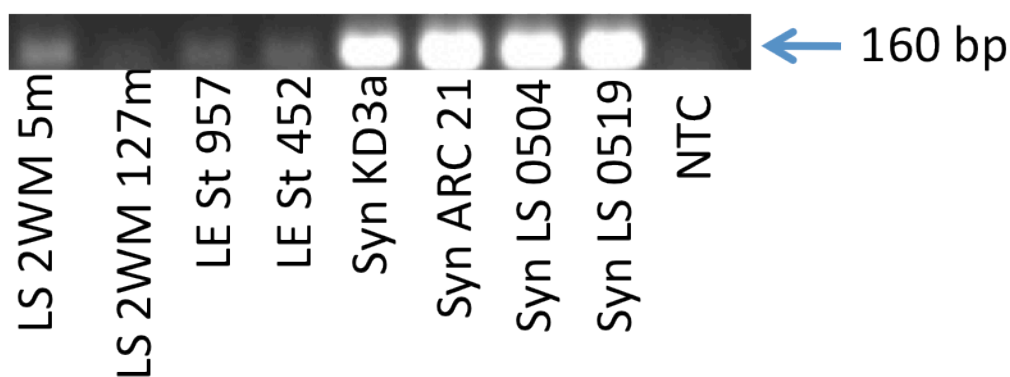


Figure 3.7. PCR from cultured *Synechococcus* KD3a (Lake Erie strain), Lake Superior (LS), and Lake Erie (LE) environmental samples with *Synechococcus phnD* primers. **Panel A:** samples from August 2009; **Panel B:** samples from April (LE CCB and MN, Napoleon site in the Maumee River) and June (LS 2WM, LE 7M, and LE MB20) 2009; samples from all of the stations are surface samples; NTC, no template control.

A.



B.

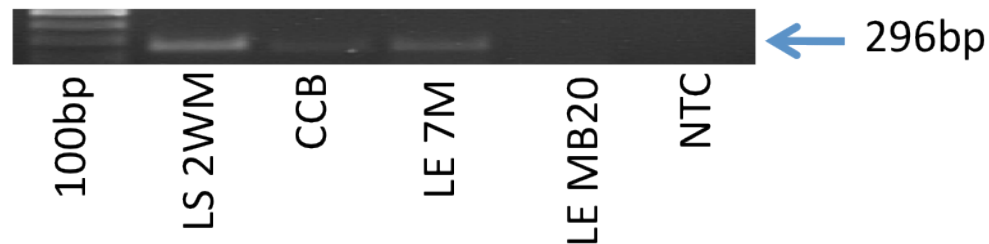


Figure 3.8. PCR from cultured *Synechococcus* spp. (strains KD3a, ARC-21 from Lake Erie and LS0504 and 0519 from Lake Superior), Lake Superior (LS), and Lake Erie (LE) environmental samples with *Synechococcus* primers *phoX_3* (A) and *phoX_2* (B). **Panel A:** samples from August 2009; **Panel B:** samples from April (LE CCB) and June (LS 2WM, LE 7M, and LE MB20) 2009; NTC, no template control.

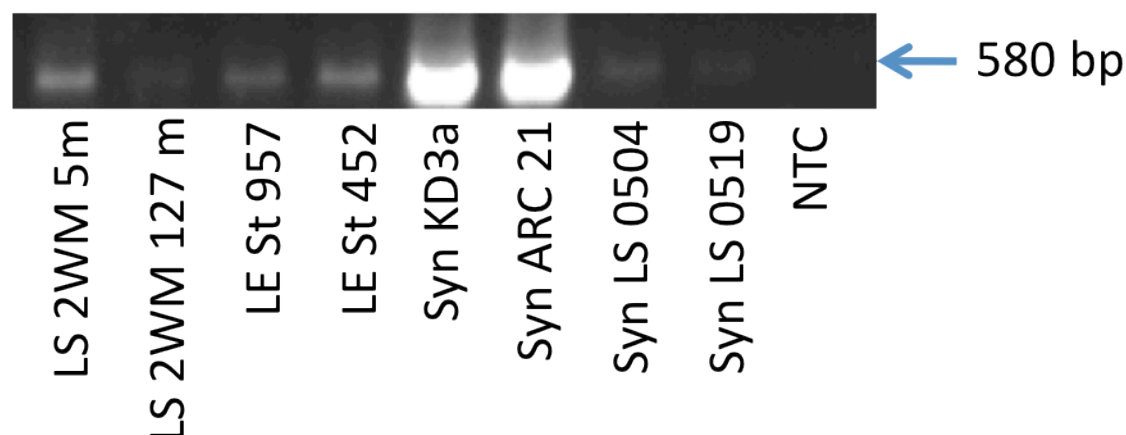


Figure 3.9. PCR from cultured *Synechococcus* spp., Lake Superior (LS), and Lake Erie (LE) samples with *Synechococcus sqdX* primers. Samples were taken in August 2009. **LS2WM 5m** – surface sample from Lake Superior station 2WM; **LS2WM 127m** – depth sample from Lake Superior 2WM; **LE St957** – surface sample from central basin of Lake Erie; **LE St452** – surface sample from eastern basin of Lake Erie; **Syn KD3a and ARC-21** – cultured *Synechococcus* sp. from Lake Erie; **Syn LS0504 and LS0519** - cultured *Synechococcus* sp. from Lake Superior; NTC, no template control.

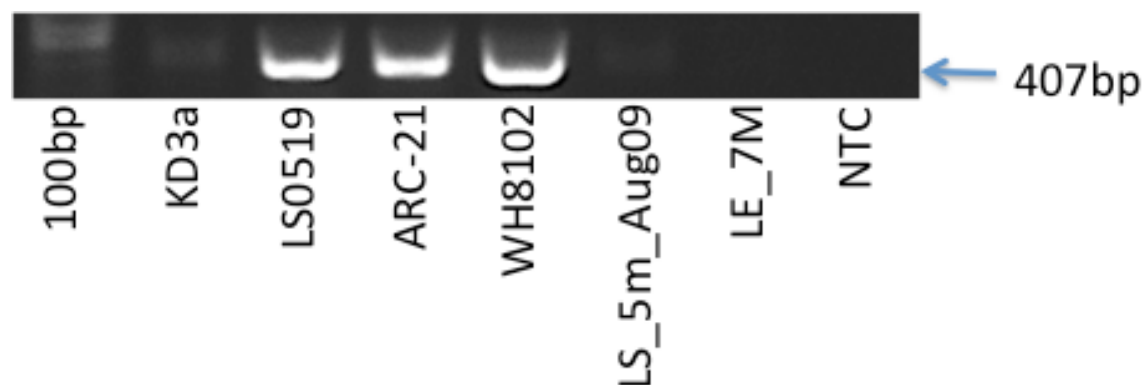


Figure 3.10. PCR from cultured *Synechococcus* spp. (strains KD3a, ARC-21 from Lake Erie; LS0519 from Lake Superior; WH8102 marine *Synechococcus* strain), Lake Superior (LS), and Lake Erie (LE) environmental samples with *Synechococcus pstB* primers.

LS_5m_Aug09 – Lake Superior surface sample taken at 2WM in August 2009; **LE7M** – Lake Erie surface sample from June 2009; NTC, no template control.

3.4 *phnD*, *phoX*, *sqdX*, and *pstB* gene expression in *Synechococcus* sp. KD3a and ARC-21 under P-limitation

The regulation of *phnD*, *phoX*, *sqdX*, and *pstB* expression in freshwater *Synechococcus* spp. was examined by RT-PCR. The analysis of *phnD* gene expression from the experiment with *Synechococcus* KD3a revealed that it is expressed constitutively at a low level (Fig. 3.11), in all the treatments. However, after the addition of 2-AEP to P-starved cultures that have bleached due to P stress, a clear difference was observed visually. Indeed, the color of the cultures became more intense indicating phycoerythrin production, and by fluorometry, amount of chlorophyll increased significantly (data not shown).

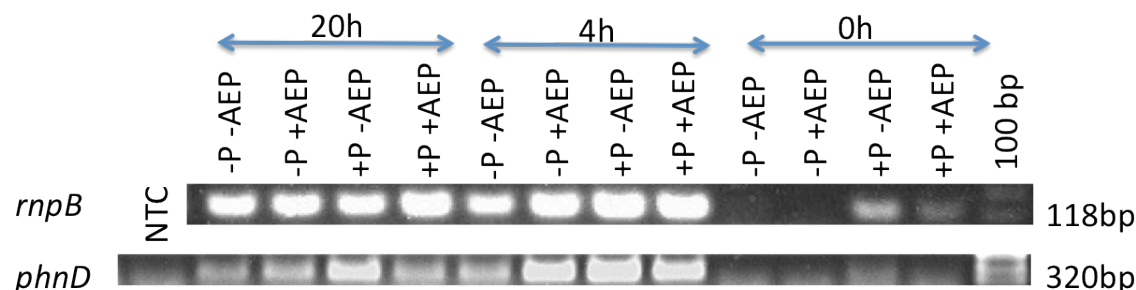


Figure 3.11. Reverse transcriptase PCR from a nutrient amendment growth experiment. RNA extracted in 0, 24, and 48 h after the addition of nutrients. Top row, RT-PCR of picocyanobacterial *rnpB* transcripts as a positive control for picocyanobacterial RNA. Bottom row, *Synechococcus* KD3a *phnD* expression. **+P+AEP**: BG-11 medium with 100uM 2-AEP added; **+P-AEP**: control, regular BG-11 medium; **-P+AEP**: P-free BG-11 medium with 2-AEP added; **-P-AEP**: control, P-free BG-11 medium; NTC, no template control.

Based on the results from the transfer experiment with *Synechococcus* KD3a, we next assessed the *phnD*, *phoX*, *sqdX*, and *pstB* expression patterns during P limitation in *Synechococcus* ARC-21. In the re-feed experiment, where cells were starved for P and then P was amended post-starvation, the *phnD* gene was constitutively expressed in the amendments with 2-AEP and K_2HPO_3 , in the positive control with P-replete BG-11, and the negative control, with no P added (Fig. 3.12). These data are similar to our observation of the *phnD* expression pattern in *Synechococcus* KD3a. In contrast, the *phoX* gene was regulated depending on P bioavailability. *phoX* gene expression was repressed in 24 hours after the addition of K_2HPO_3 (Fig. 3.12).

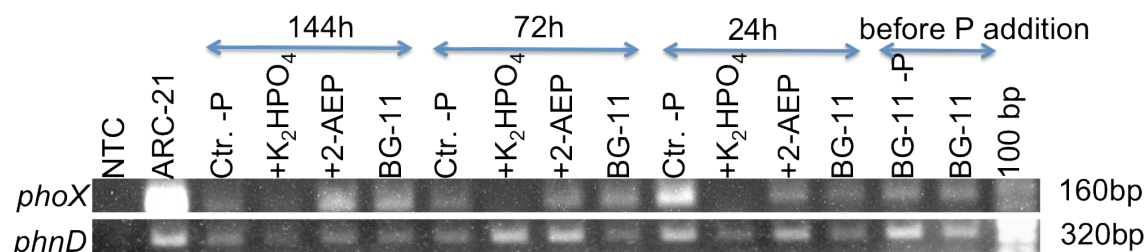


Figure 3.12. Reverse transcriptase PCR from a nutrient amendment growth experiment. RNA extracted before the addition of P source, in 24, 72, and 144 hours after the addition of nutrients. Top row, *Synechococcus* ARC-21 *phoX* expression, bottom row, *phnD* expression. **BG-11**: regular BG-11 medium; **BG-11 -P**: P-free BG-11 medium; **+2-AEP**: P-free BG-11 medium with 2-AEP added; **+K₂HPO₄**: P-free BG-11 medium with phosphate added; **Ctrl. -P**: control, no phosphorus source added; **NTC**, no template control.

sqdX, the gene essential for sulfolipid biosynthesis, was induced after the addition of P to P-starved cultures. The gene was activated in 24 hours after the addition of K₂HPO₄ and 72 hours after the addition of 2-AEP to P-starved cultures (Fig. 3.13). *sqdX* showed little or no expression in the negative control, cultures with no P source added, nor positive control, cultures with regular BG-11 medium. *sqdX* from Lake Superior environmental samples from August and October was also not induced.

pstB gene showed very moderate expression in both *Synechococcus* cultures and Lake Superior environmental samples (Fig. 3.13).

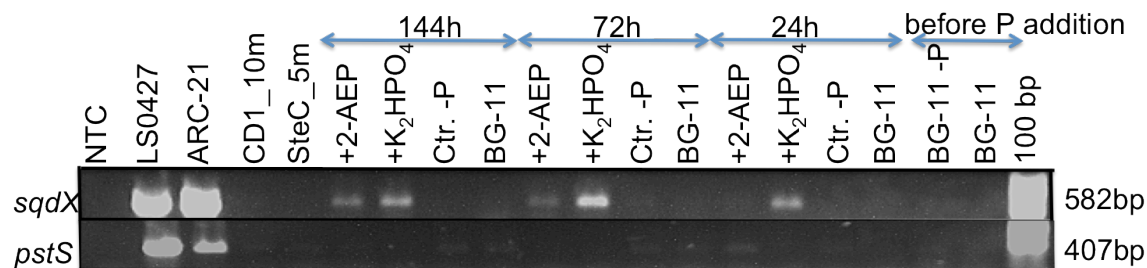


Figure 3.13. Reverse transcriptase PCR from a nutrient amendment growth experiment. RNA extracted before the addition of P source, in 24, 72, and 144 hours after the addition of nutrients. **BG-11**: regular BG-11 medium; **BG-11 -P**: P-free BG-11 medium; **+2-AEP**: P-free BG-11 medium with 2-AEP added; **+K₂HPO₄**: P-free BG-11 medium with phosphate added; **Ctrl. -P**: control, no phosphorus source added; **SteC_5m**: Lake Superior station SteC from August 2010; **CD1**: Lake Superior station CD1 from October 2010; **ARC-21**: Lake Erie isolate *Synechococcus* ARC-21; **LS0427**: Lake Superior isolate *Synechococcus* LS 0427; **NTC**: neagative template control.

3.5 RT-PCR assay for *phnD* and *phoX* expression in environmental samples

To prove our finding about the *phnD* and *phoX* expression patterns and assess the P status of the cyanobacterial picoplankton in oligotrophic environments, reverse transcriptase PCR was used with RNA extracted from Lake Superior and Lake Erie. Lake Superior samples were collected in May, August, and October, at different sites and depths. Lake Superior was chosen because it represents an extreme case of a P-limited environment. The samples were obtained during Spring, Summer, and Fall to reveal whether there is any seasonal variation of *phnD* and *phoX* expression. Different sampling locations and depths were chosen to identify the correlation between these parameters and gene expression. Lake Erie samples for RT-PCR were obtained at a depth of 1 m from offshore stations in August 2009. The samples were collected from the central and eastern basins as representatives of mesotrophic and oligotrophic environments, respectively. Lake Superior samples were of main interest due to the oligotrophic nature of the lake. Lake Erie

samples were used for comparison with Lake Superior, since both of these ecosystems are unique and host phylogenetically distinct consortia of APP.

The *Synechococcus* spp. *phnD* and *phoX* primers were used in RT-PCR with RNA extracted from both Lake Erie and Lake Superior. Lake Superior surface samples of 2WM, CD1, and SteC from May and August each yielded a *phnD* and *phoX* RT-PCR product (Figs. 3.14A and 3.14B). Samples from depth, 155 m from 2WM and 235 m from CD1, yielded no *Synechococcus* spp. *phnD* or *phoX* RT-PCR amplicons (Figs. 3.14A and 3.14B), which was likely due to low abundance of *Synechococcus* spp. in deep waters.

During October 2010, at Lake Superior station CD1, expression of the *Synechococcus* spp. *phnD* was detected, but no RT-PCR products were observed with the *Synechococcus* spp. *phoX* primer set (Figs. 3.15B and 3.15C). The absence of amplicons at depth (245 m) can be explained by the lack of *Synechococcus* spp. in deep waters.

The RT-PCR assay was also conducted with RNA extracted from the Lake Superior amendment samples where the +P treatments were spiked with 20 μ M (final concentration) of Pi and placed in a flowing water incubator for 2 days. Control samples were with no Pi added. The presence of *rnpB* RT-PCR products confirmed that RNA was successfully extracted and can be further used for detection of *Synechococcus* spp. *phnD* and *phoX* in the samples. Expression of the *phnD* gene was observed in both +P and control samples, whereas the *phoX* gene showed no response to the addition of P to the samples (Figs. 3.15B and 3.15C). In contrast to the Lake Superior expression pattern, expression of the *Synechococcus* spp. *phnD* and *phoX* was observed in all Lake Erie samples (Figs. 3.16A and 3.16B).

sqdX and *pstB* were not expressed in the environmental samples from Lake Superior (Fig. 3.13).

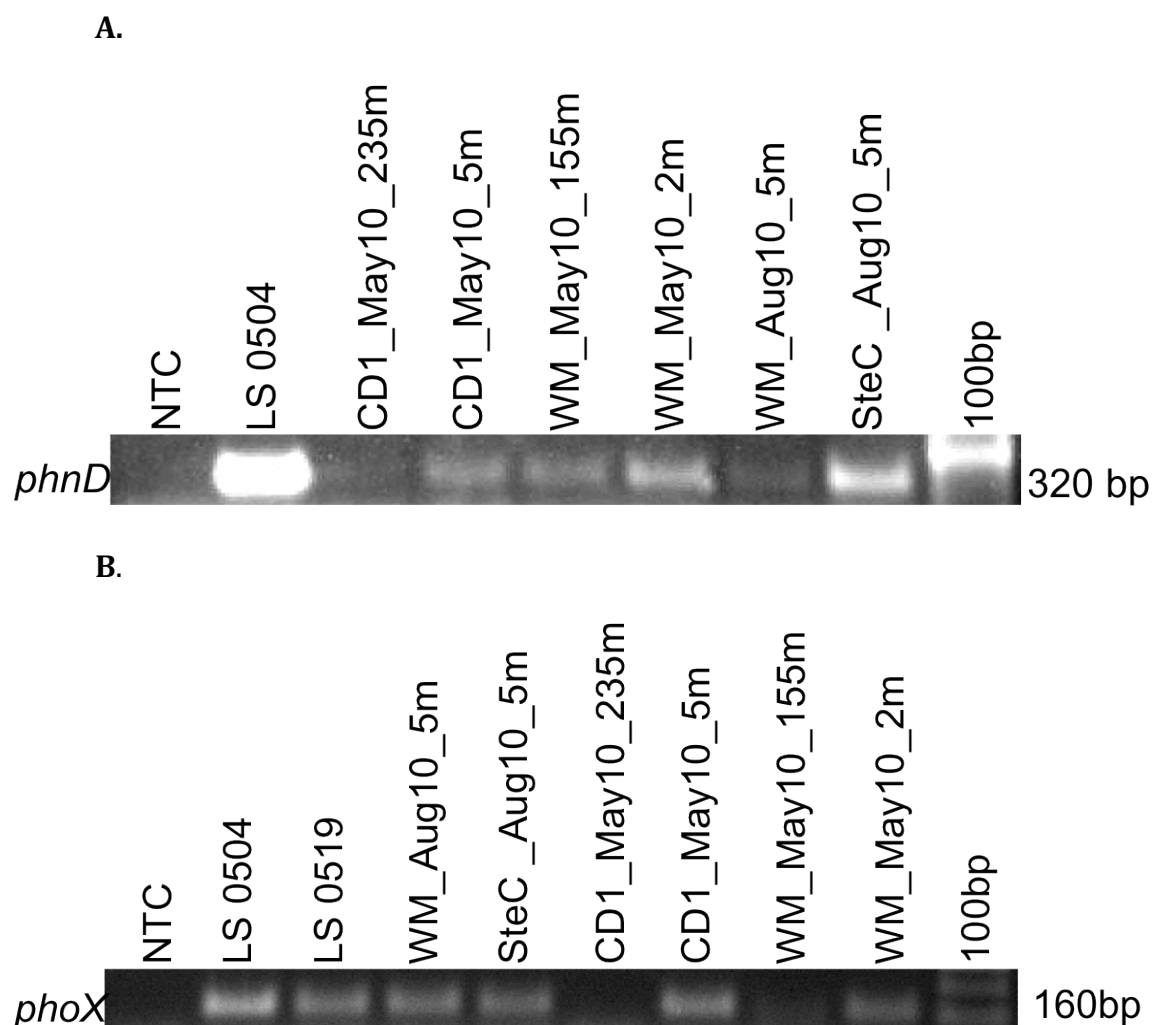


Figure 3.14. RT-PCR of *phnD* and *phoX* from Lake Superior. The surface layer was either 2 or 5 m, depth samples were either from 155 or 235 m. **Panel A:** RT-PCR of *phnD* from seston RNA from Lake Superior stations 2WM, CD1, and SteC; the samples were taken in May and August 2010; *Synechococcus* sp. LS0504 and LS0519 RNA were used as a positive control. **Panel B:** RT-PCR of *Synechococcus* spp. *phoX* sequences from environmental RNA from Lake Superior stations 2WM, CD1, and SteC; NTC, no template control.

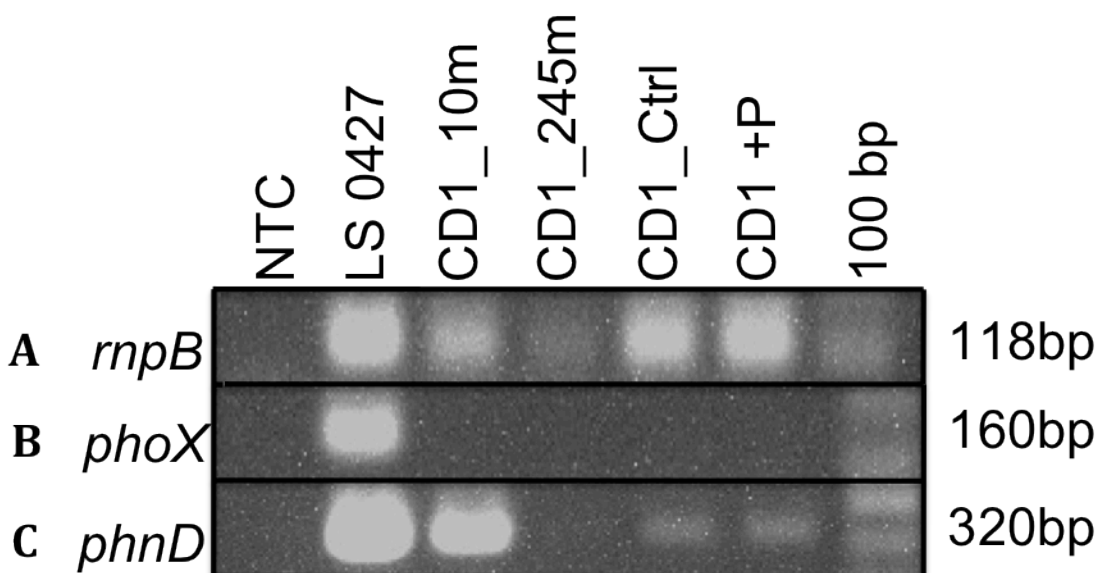


Figure 3.15. RT-PCR of *phnD*, *phoX*, and *rnpB* from Lake Superior CD1 and the amendment experiment in October 2010. RNA extracted in 48 h after the addition of P. **Panel A:** RT-PCR of picocyanobacterial *rnpB* transcripts as a positive control for picocyanobacteria; **Panel B:** RT-PCR of *Synechococcus* spp. *phoX*; **Panel C:** RT-PCR of *Synechococcus* spp. *phnD*. **CD1+P** – addition of 20 μ M of phosphate to water collected at CD1; **CD1_Ctrl.** – water from CD1 with no phosphate added; **CD1_245m** – sample from the depth of 245 m collected at CD1; **CD1_10m** – sample from the surface (10 m) collected at CD1; LS0427 RNA was used as a positive control; NTC, no template control.

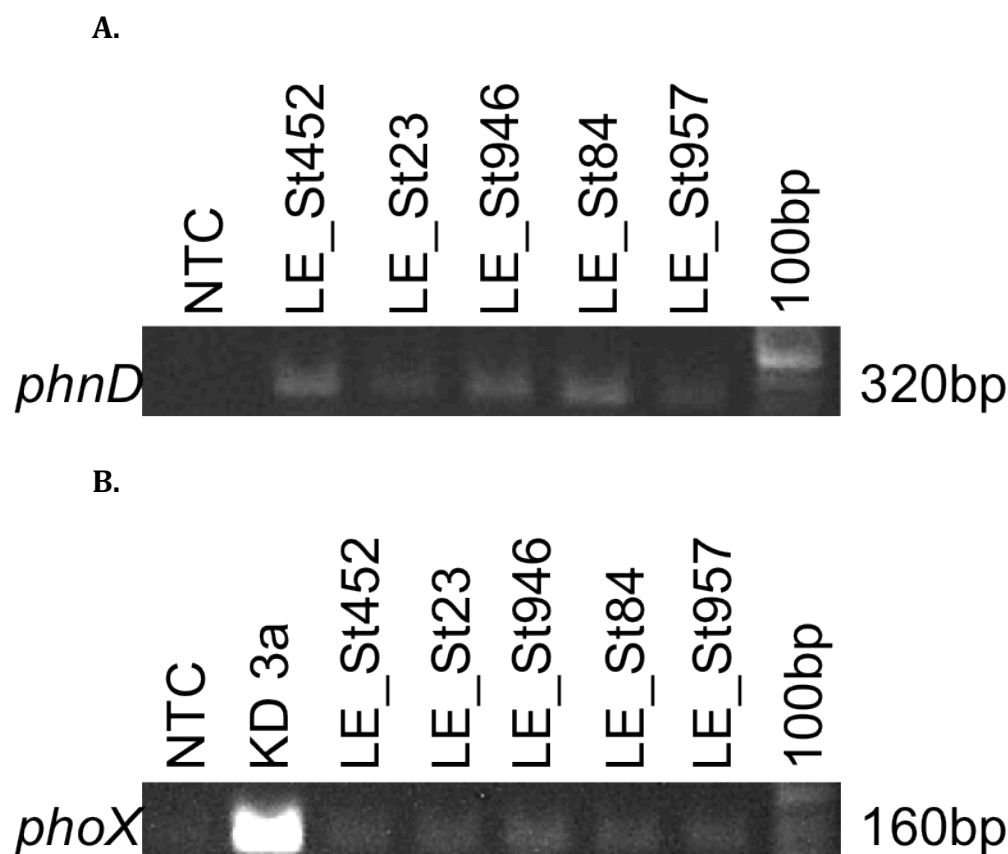


Figure 3.16. RT-PCR of *phnD* and *phoX* from Lake Erie samples taken in August 2009. **Panel A:** *Synechococcus* spp. *phnD*; **Panel B:** *Synechococcus* spp. *phoX*; *Synechococcus* spp. KD3a used as positive control; Lake Erie stations 957, 84, and 946 represent central basin of Lake Erie; stations 23 and 452 are from eastern basin of Lake Erie; NTC, no template control.

3.6 Phylogenetic analysis of picocyanobacterial *phnD*, *phoX*, and *pstB* from Lake Erie and Lake Superior samples

Sequencing and phylogenetic analysis of the cloned PCR products indicates that Lake Superior *Synechococcus phnD* sequences from several dates and stations form a separate cluster, distinct from both marine *Synechococcus* cultures and cosmopolitan cultured Lake Superior isolates (Fig. 3.17). This finding is consistent with the previous work by Ivanikova et al. (2007b, 2008). Sequences obtained from three different stations in

Lake Superior (stations 2WM, CD1, and SteC) do not branch according to their location.

Comparison of Lake Erie *phoX* sequences from central and eastern stations revealed two distinct clusters that diverge from Lake Superior and marine *Synechococcus* cultures (Fig. 3.18). Lake Erie central basin stations 946 and 957, Lake Erie eastern station 452, marine *Synechococcus* cultures, and Lake Superior isolates, each of them forms an individual group.

Analysis of Lake Erie and Lake Superior *phoX* sequences revealed that they are phylogenetically distinct and show individual clustering (Fig. 3.19). Samples from Lake Erie eastern basin station 452 from August 2009, Lake Superior samples from the stations 2WM and SteC from different dates, and *Synechococcus* cultures were compared.

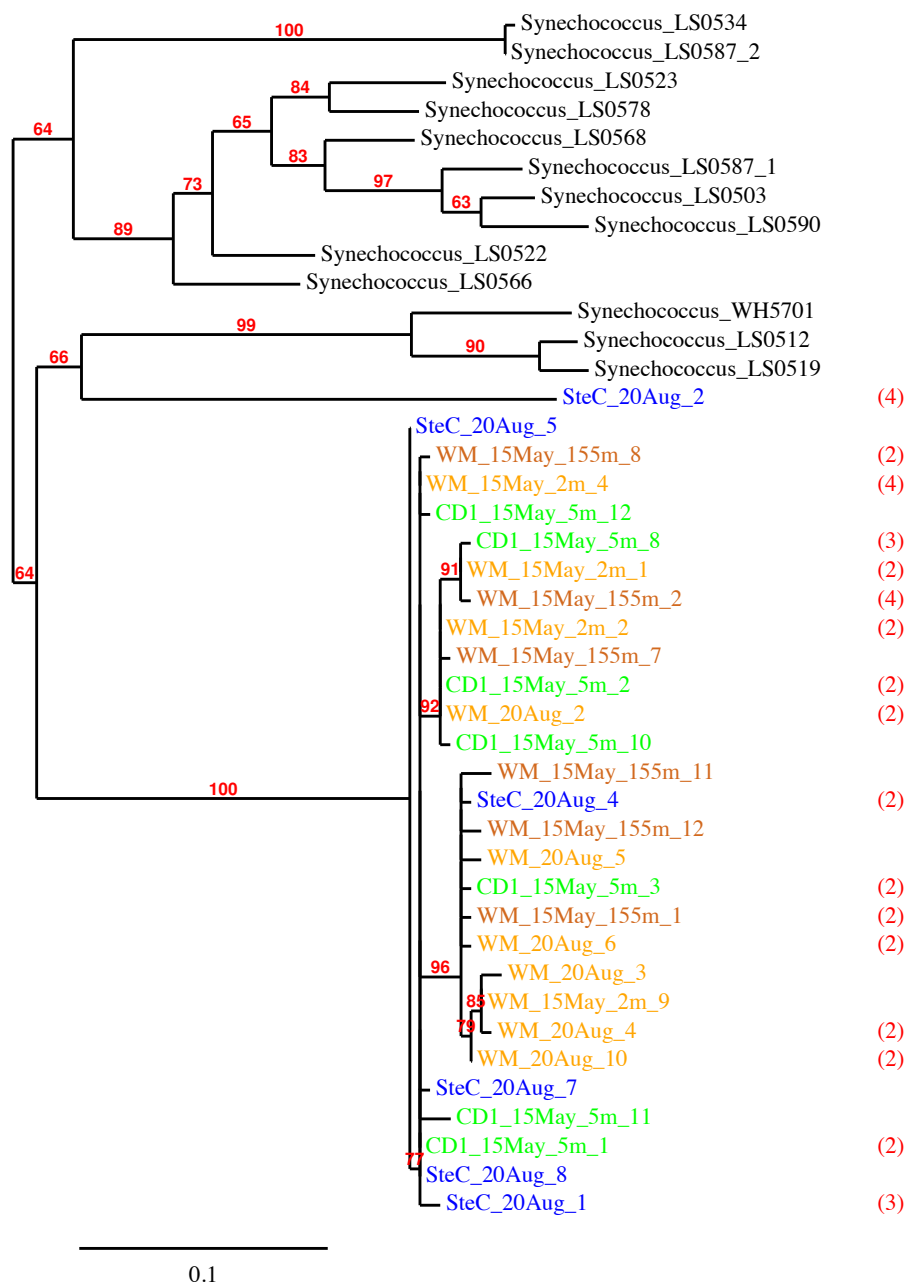


Figure 3.17. Phylogenetic tree showing relationship between *phnD* amplicon sequences from Lake Superior and *Synechococcus* cultures. Lake Superior surface samples from the stations 2WM (orange), CD1 (green), and SteC (blue) from different dates are shown. *Synechococcus* cultures are in black. The number of clones is indicated in the brackets.

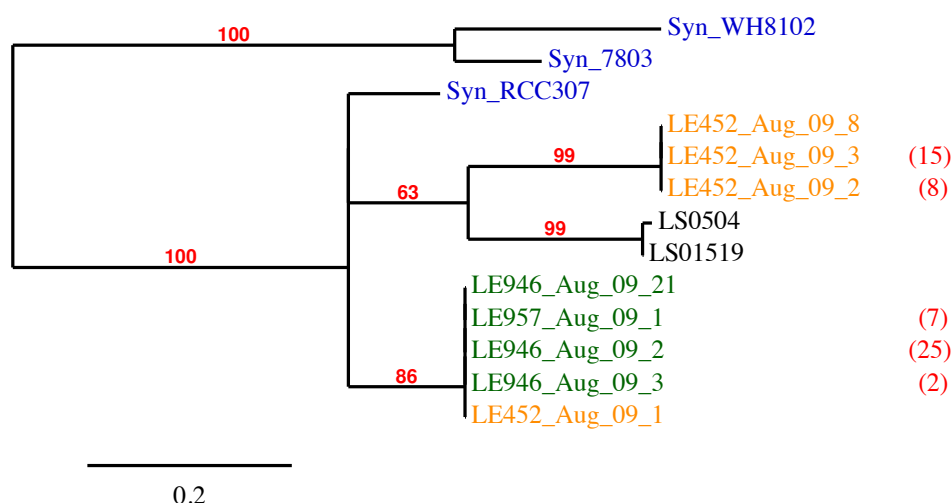


Figure 3.18. Phylogenetic tree of *Synechococcus phoX* sequences deduced from Lake Erie DNA sequences and *Synechococcus* cultures. **LE452 (orange)** – surface samples from eastern basin obtained in August 2009; **LE946 (green) and LE957** - surface samples from central basin obtained in August 2009. Marine *Synechococcus* strains are indicated in blue. Two Lake Superior cultures, LS0504 and LS0519 are shown in black. The number of clones is in brackets.

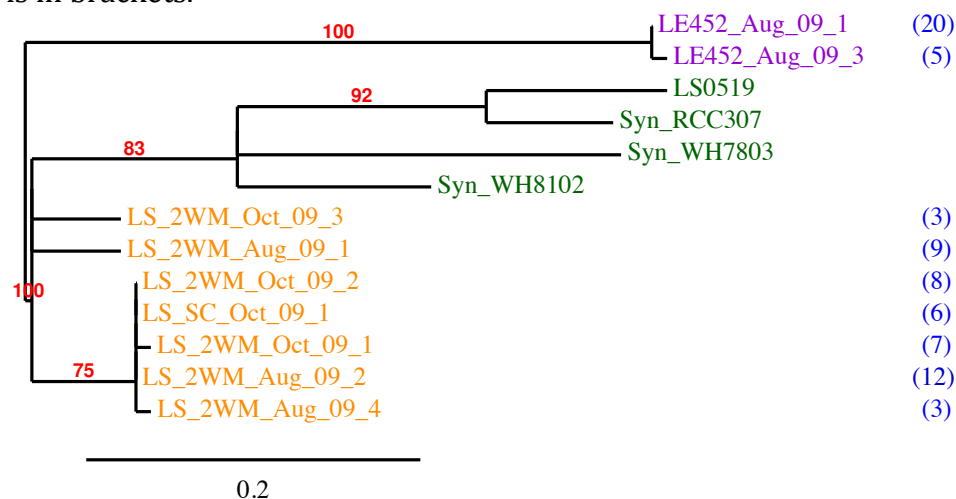


Figure 3.19. Phylogenetic tree showing relationship between *phoX* amplicon sequences from Lake Superior and Lake Erie cultures. Lake Superior surface samples from the stations 2WM (LS_2WM) and SteC (LS_SC) from different dates are shown in orange; surface samples from Lake Erie eastern basin station 452 from August 2009 are shown in purple; *Synechococcus* cultures are in green; the number of clones is indicated in the brackets.

Synechococcus pstB sequences from Lake Superior stations 2WM, SteC, and SteJ, Lake Erie central (St. 84) and eastern basins (St. 23), and *Synechococcus* cultures illustrate three distinct clusters (Fig. 3.20). Lake Superior sequences form one cluster with sub-cluster of Lake Erie eastern basin sequences within this group. The sequences from Lake Erie central basin form a separate cluster. Finally, sequences from *Synechococcus* cultures reveal another discrete cluster. The samples from Lake Superior stations 2WM, SteC, and SteJ were taken in fall 2009 and 2007, while samples from two Lake Erie stations, eastern basin station 23 and central basin station 84 were obtained August 2009.

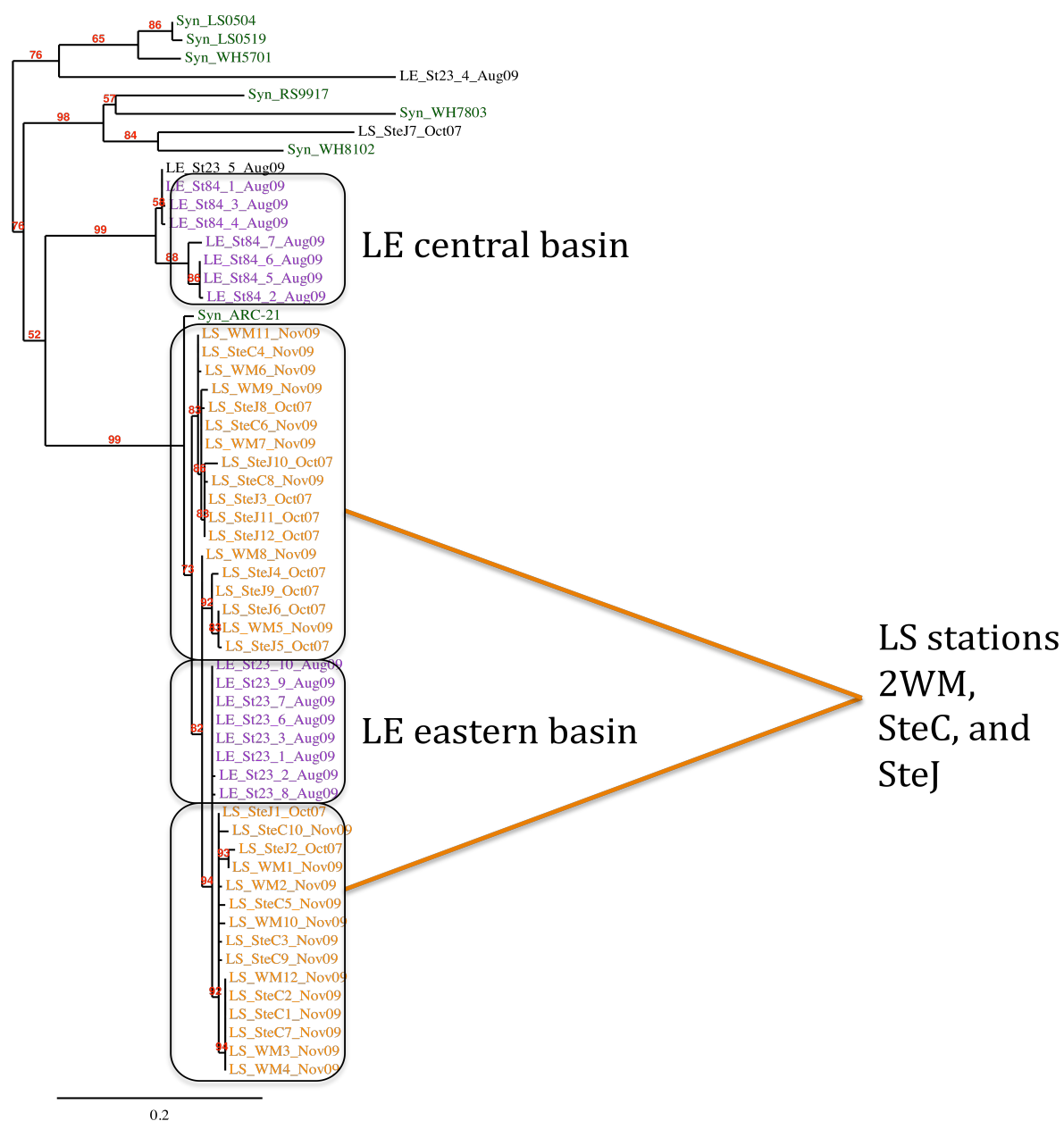


Figure 3.20. Phylogenetic tree showing relationship between *pstB* amplicon sequences from Lake Superior and Lake Erie cultures. Lake Superior surface samples from the stations 2WM (LS_WM), SteC, and SteJ from fall 2009 and 2007 are shown in orange; Lake Erie surface samples from eastern basin station 23 and central basin station 84 from August 2009 are shown in purple; *Synechococcus* cultures are in green.

CHAPTER 4. DISCUSSION

4.1 Detection of *phnD*, *phoX*, *sqdX*, and *pstB* in cultured picocyanobacteria and environmental samples

The main goal of this study was to assess different mechanisms to adapt to a low P environment in both Lake Erie and Lake Superior. Specifically, we examined the ability of endemic microbes to utilize organic phosphates and phosphonates, as well as monitoring the distribution and abundance of *sqdX* and *pstB* genes. A PCR-based assay was employed to detect the presence and expression of *phnD* and *phoX*, and presence and abundance of *sqdX* and *pstB* in freshwater cyanobacterial cultures and environmental samples. We have assessed presence and expression of these genes from 9 Lake Erie and 12 Lake Superior stations taken on different dates and seasons to ensure reliability of the results. Indeed, we have demonstrated by PCR primer sets that all four genes are detectable in both *Synechococcus* cultures and environmental samples from Lake Erie and Lake Superior. The presence and expression of the phosphorus stress response genes in all tested samples suggests that freshwater picocyanobacteria have evolved diverse mechanisms to deal with changing P-limited environments. In fact, P limitation in Lake Erie and Lake Superior has been acknowledged (Sternner et al. 2004, Schelske et al. 2006, North et al. 2007). Because phosphorus limits APP growth rates, this may lead to the selective advantages and disadvantages among species and consequently control primary production and community composition (Tilman 1976, North et al. 2007). To better understand how APP respond to P starvation we chose to examine four genes involved in P acquisition and conservation: *phnD*, *phoX*, *sqdX*, and *pstB*. Phylogenetic analysis of the *phnD*, *phoX*, and *pstB*

freshwater and marine members of the picocyanobacterial sequences revealed that freshwater APP form separate clusters distinct from their marine counterparts (Fig. 3.17-3.20). Similarly, Ivanikova et al. (2007b, 2008) demonstrated the presence of distinct picocyanobacterial populations in two Laurentian Great Lakes: Lake Erie and Lake Superior.

To summarize, our results have shown that *phnD*, *phoX*, *sqdX* and *pstB* genes are present in both cultured freshwater *Synechococcus* spp. from Lake Erie and Superior and environmental freshwater samples. The wide distribution of the genes suggests their potential importance in freshwater systems; therefore, the assessment of the gene expression pattern might help identify the P status of *Synechococcus* spp. in the lakes. Whereas genomes of some marine *Synechococcus* spp. are available, thus facilitating genomic and metagenomic studies, there are no genomes sequenced for freshwater *Synechococcus* spp. to date.

4.2 Regulation of *phnD*, *phoX*, and *sqdX* in cultured picocyanobacteria

To evaluate the regulation of the *phnD*, *phoX*, and *sqdX* expression in freshwater *Synechococcus* spp. RT-PCR assay was employed. It has been shown that both marine (WH8102) (Palenik et al. 2003; Su et al. 2003) and a freshwater *Synechococcus* (ARC-21) (Ilikchyan et al. 2009) are capable of growing on 2-aminoethylphosphonate (2-AEP) as a sole P source. Similarly, we were able to grow *Synechococcus* KD3a on 2-AEP. Our results indicate that freshwater *Synechococcus* sp. KD3a expresses the *phnD* gene constitutively at a low level (Fig. 3.11), which is contrary with the data for P-dependent regulation in *Trichodesmium* (Sanudo-Wilhelmy et al. 2001, Dyhrman et al. 2006) and for many other

bacteria (Jiang et al. 1995; Kononova et al. 2002). In the re-feed experiment with *Synechococcus* ARC-21, the *phnD* gene also showed constitutive expression in all the amendments (fig. 3.12). Therefore, our results suggest that the *phnD* gene in freshwater picocyanobacteria is a constitutive housekeeping gene regulated by mechanisms independent from *phoRB*. In addition, this finding indicates variability within different ecotypes of *Synechococcus* spp. in their P-responses, such as uptake-, storage-, and conversion-capacities in relation to the availability of P (Huisman 2005). Such variability was also suggested by Gomez-Garcia and colleagues (2010) studying *Synechococcus* isolates from Yellowstone National Park, in which two different strains possess different mechanism for *phn* gene expression.

In contrast, the *phoX* gene was regulated in response to P bioavailability in the environment. The gene was repressed within 24 hours after the addition of K_2HPO_3 (Fig. 3.12) and remained repressed over the time course in -P relative to +P conditions. Indeed, culture studies have demonstrated that gene expression and activity of alkaline phosphatase are commonly regulated by P availability via the two-component regulatory system *phoBR* (e.g. Torriani-Gorini 1987, Wanner 1996, Moore et al. 2005, Martiny et al. 2006, Sebastian and Ammerman 2009, Tetu et al. 2009, Zaheer et al. 2009, Kathuria and Martiny 2010). Moderate expression of *phoX* gene in 144h can be explained by the consumption of P source by P-starved cells and consequently depletion of P in the medium. The expression pattern of the *phoX* in the re-feed experiment with *Synechococcus* sp. ARC-21 is consistent with the previous studies showing that cultures and field populations of cyanobacteria are capable of hydrolyzing P esters (Stihl et al. 2001, Dyhrman et al. 2002, Orchard et al. 2009). Also, it has been demonstrated that the *phoA* and *phoX* genes are both

upregulated by P starvation, with *phoX* showing a much stronger response (Orchard et al. 2009). Many families of phosphatases involved in P assimilation have been described, but the key enzymes in marine bacteria for utilizing dissolved organic phosphate are the *phoA*- and *phoX*-types (Kathuria and Martiny 2010). It has been demonstrated that *phoX* and *phoA* have different requirements, including different substrate specificity and cofactor requirements. For example, *phoX*, an alternative APase, has broad substrate specificity and unlike *PhoA* family it does not require Zinc as a co-factor. Considering that many aquatic systems are P- and Zn-limited, possessing an enzyme with broad substrate specificity and which does not require Zn as a co-factor, may provide a significant growth advantage. In fact, Zn is among the trace metals that are often below the detection limit. For example, it has been shown that concentration of zinc in Lake Superior is as low as 2 µg/L, whereas Ca, which is one of the most abundant ions in Lake Superior, is ca. 14 mg/L (Matheson and Munavar 1978). In addition, the analysis of metagenomic sequence databases from the Global Ocean Sampling revealed the high abundance of cytoplasmic APases, thus the transport and intracellular hydrolysis of small organophosphate molecules is an important mechanism for bacterial acquisition of P in oligotrophic aquatic systems (Luo et al. 2009). Because APases play a crucial ecological role in organic P processing, understanding of mechanisms regulating gene function in freshwater picocyanobacteria was of primary importance in this study.

sqdX gene expression pattern indicated that the gene is activated in P-starved cells after the addition of a P source. In the re-feed experiment the gene was induced upon P addition in 24 hours and remained activated throughout the time the samples have been collected (six days after the addition of the amendments) (Fig.3.13). After the addition of

phosphonate source (2-AEP) to P-starved cultures, *sqdX* showed expression in 72 hours. In positive controls, with regular BG-11 medium, and negative controls, with no P source, *sqdX* was downregulated. Total duration of the re-feed experiment including the time before the addition of the amendments and afterwards was 24 days. *sqdX* expression was not detected in Lake Superior environmental samples collected in August and October 2010 from stations SteC and CD1 (surface water samples). By contrast, Van Mooy et al. (2009) demonstrated that phytoplankton, in regions of oligotrophic ocean where phosphate is scarce, reduce their cellular phosphorus requirements by substituting non-phosphorus, sulfo – and betaine membrane lipids for phospholipids. Similarly, *Rhizobium (Sinorhizobium) meliloti*, under phosphate stress, produces phosphorus-free lipids. It has been demonstrated that reduced transport of phosphorus sources to the cytoplasm in *Rhizobium (Sinorhizobium) meliloti* causes diacylglycerol trimethylhomoserine synthesis and that this synthesis is controlled by the *phoB* regulatory gene (Geiger et al. 1999). Overall, it has been acknowledged that marine picophytoplankton and other bacteria use phospholipid substitutions as a strategy to deal with phosphorus limitation (Benning et al. 1993, Geiger et al. 1999, Guler et al. 2000, Van Mooy et al. 2006, Zubkov et al. 2007). Based on gene expression studies our results suggest that in freshwater picocyanobacteria the *sqdX* gene is not involved in a P conservation mechanism. We hypothesize that *sqdX* is activated in response to cell requirement for membrane biosynthesis under conditions of active nutrient-replete growth. The possibility remains that betaine lipids replace phospholipids in P-limited Lake Superior, as suggested by a preliminary study elsewhere (James Cotner, personal communication). Since Lake Superior has high levels of bioavailable nitrogen, nitrogen-containing betaine lipids might serve as an alternative to

phospholipids.

4.3 Flow cytometric analysis

Flow cytometric analysis revealed increase in cell size of P-stressed cells (3.2). P deficiency is associated with the inability to replicate DNA, thus blocking cell division (Latasa and Berdalet 1993). Presumably, synthesis of other compounds that does not require P (such as storage polymers) would be unaffected, resulting in increased cell size. Overall, flow cytometric analysis of the samples from both the growth experiment with *Synechococcus* KD3a and the re-feed experiment with *Synechococcus* ARC-21 has shown an effect of P-starvation on cell and confirmed the results of the genetic analysis.

4.4 Alkaline phosphatase assay

APase assay was employed with both, the transfer and the re-feed experiments as a physiological indicator of P limitation of the cultures. In both experiments phosphate limitation results in the induction of alkaline phosphatase activity of *Synechococcus* KD3a (Fig. 3.5) and ARC-21 (3.6A). In the transfer experiment P-starved cultures started showing increase in APA only after day 11, whereas the marine filamentous diazotroph *Trichodesmium* shows APA within 24 hours (Orchard et al. 2009). So slow response of freshwater APP to P starvation might be due to their lower growth rate and the differences in P-demands of a smaller picocyanobacterial cell (Sebastian and Ammerman 2010).

In the re-feed experiment, where P was added back to the P-starved cultures, APA decreased dramatically in cultures with K_2HPO_4 added and there was a slower decrease in APA observed in the cultures with 2-AEP added (3.6B). This response of the APA to the

addition of a P source can be explained by the differences in availability of inorganic phosphate and phosphonates to APP. Indeed, it has been acknowledged that DIP is directly available to cyanobacteria (Sanudo-Wilhelmy et al. 2004, Dyhrman et al. 2006), whereas phosphonates, as a part of DOP pool, are more refractory, thus energetically more “expensive” to a cell (Palenik et al. 2003, Moore et al. 2005). The decline in APA in control sample, with no P source added, after twenty days of the experiment was due to the loss of cell viability as suggested by bleaching of the cultures.

Indeed, it has been indicated that the regulation of phosphatases of phytoplankton is mediated by the external P_i concentration (Sakshaug et al. 1984, Chróst & Overbeck 1987, Paasche & Erga 1988, Uchida 1992) or, additionally, by the internal N:P ratio (Mykkestad & Sakshaug, 1983) (Fig. 4.1). Additionally, Paasche and Erga (1988) demonstrated that phosphatase activity might also depend on the P-demand of the organisms and on the season of observation (Huang and Hong, 1998, Hoppe 2003).

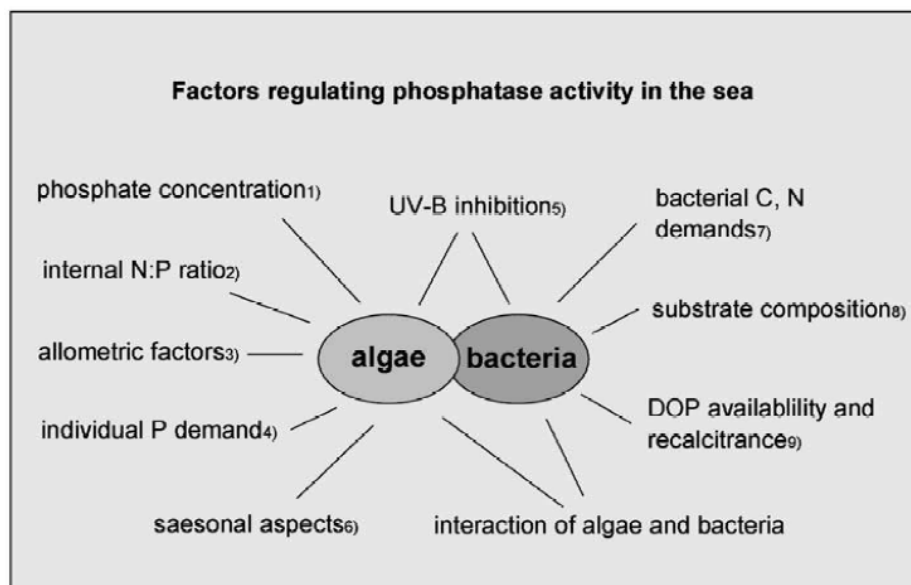


Figure 4.1. Important factors regulating phosphatase activity in the sea. Figure adopted from Hoppe (2003) and references therein.

4.5 Regulation of *phnD* and *phoX* in field samples

4.5.1 *phnD* expression profile

Analysis of Lake Erie and Lake Superior samples demonstrated uniformly constitutive expression of *phnD* gene in *Synechococcus* spp. (Figs. 3.14A, 3.15C and 3.16A), suggesting lack of regulation of the *phnCDE* operon. In contrast, in marine cyanobacteria *Trichodesmium* and *Synechococcus* the *phnD* gene is expressed in phosphate-deficient cultures and is not detected in phosphate-replete cultures (Dyhrman et al. 2006, Ilikchyan et al. 2009). Similar phosphate regulation of *phn* genes was observed in other microbes (Kononova & Nesmeyanova 2002). Lack of *phnD* gene regulation in the freshwater samples is perhaps due to similar loss of regulatory functions as seen in marine *Prochlorococcus*. Indeed, *Prochlorococcus phnD* expression is constitutive (Ilikchyan et al. 2009, 2010) and likely reflects the loss of transcriptional regulators through genomic reduction.

Therefore, our data suggest that constitutive expression of a gene involved in phosphonate transport is due to phosphonate compounds serving as a phosphorus source for freshwater picocyanobacteria in this low DIP system (Dyhrman et al. 2002 and 2006).

Whereas it has been shown that phosphonates are readily assimilated by freshwater, marine, and other bacteria (Kononova & Nesmeyanova, 2002, Dyhrman et al. 2006, Ilikchyan et al. 2009), the availability of phosphonates to marine bacteria has been recognized just recently (Dyhrman et al. 2006, Karl et al. 2008, Ilikchyan et al. 2009, 2010). Additionally, Dyhrman et al. (2009) demonstrated by using ³¹P NMR spectroscopy that *Trichodesmium* sp. produce phosphonates (possibly as a storage compound) and thus might be a significant source of these compounds in the ocean, particularly in nutrient poor regions. In turn, we screened our freshwater picocyanobacteria cultured strains for the

presence of phosphonates using solid-state nuclear magnetic resonance spectroscopy (Claudia R. Benitez-Nelson). The results indicate that all the tested strains, *Synechococcus* ARC-21, *Synechococcus* LS 0427, *Synechococcus* LS 0503, and *Synechococcus* LS 0519, lack intracellular phosphonates. Therefore, since phosphonate transporter gene is constitutively expressed in freshwater environmental samples, but phosphonates were not detected in the cultures, the potential source of phosphonates is in the environment. Indeed, phosphonates have been detected in freshwater systems before (Nanny and Minear 1997). The authors showed that phosphonates were present in pelagic lake water samples from Crystal Lake, a man-made mesotrophic lake in Champaign County, Illinois. It was also indicated that the only water sources are a 200 ft (61 m)-deep well and surface runoff from surrounding area, which is wooded with deciduous trees. The authors report no storm sewers flow into the lake, therefore, an anthropogenic origin of phosphonates in the lake is unlikely. Thus far, analysis of water from a single station did not reveal phosphonates in Lake Superior water. However, the rapid turnover of P in the system could make total P of all forms vanishingly low (Stets and Cotner 2008, Brown and Sherrell 2004). Additional samples will be tested in the future to determine whether phosphonates are routinely detectable in Lake Superior DOP.

4.5.2 *phoX* expression profile

It has been acknowledged that *phoX*, an alternative alkaline phosphatase, is predominantly responsible for extracellular phosphatase activity during P limitation and is part of the Pho regulon (Monds et al. 2006, Jin-Ru et al. 2007, Sebastian & Ammerman 2009). The *phoX* gene was expressed in all tested Lake Erie samples (3.16B), however, we

have not tested any samples after fall mixing events. Analysis of the Lake Erie and Lake Superior samples indicates that *phoX* gene expression is regulated by P availability. *phoX* is induced in Lake Superior environmental samples from May and August, and repressed in the samples from October (3.14B, 3.15B, 3.16B). The *phoX* expression pattern is consistent with nutrient availability that would increase following autumn mixing of the lake.

Similarly, PhoX was found to be induced solely by P-starvation and accounted for 90% of the activity in the model marine bacterium, *Silicibacter pomeroyi* (Sebastian & Ammerman 2009). Also, Kathuria and Martiny (2010) observed that both *phoA* and *phoX* genes associated with *Prochlorococcus* and SAR11 were primarily found in cells from low P environments. Overall, these data suggest that *phoX* expression in freshwater *Synechococcus* spp. was regulated by phosphorus availability, and that *phoX* may be used as a molecular marker to assess the P status of individual populations of bacteria or the microbial community at large.

4.6 Phylogenetic analysis of picocyanobacterial *phnD*, *phoX*, and *pstB* from Lake Erie and Lake Superior samples

Sequencing of Lake Superior *Synechococcus* spp. *phnD* from environmental samples revealed that the sequences cluster separately from marine *Synechococcus* cultures and freshwater isolates, thus Lake Superior *Synechococcus* spp. represent a taxonomically unique group (Fig. 3.17). Sequences obtained from Lake Superior stations 2WM, CD1, and SteC clustered together regardless of their location and date they were sampled. The fact that Lake Superior environmental sequences are distinct from Lake Superior isolates (from Dr. Bullerjahn's culture collection) suggests that the culture collection from Superior does

not reflect the endemic diversity present in the lake, a finding consistent with other environmental sequencing and culturing efforts (Crosbi et al. 2003a, Ivanikova et al. 2008). Overall, our results agree with previous work by Ivanikova et al. (2007b, 2008) who demonstrated by analyzing 16S rDNA the presence of two unique Lake Superior clusters termed LSI and LSII.

Analysis of Lake Erie *phoX* sequences revealed that they form two distinct clusters: central basin (stations 946 and 957) and eastern basin (station 452) clusters (Fig. 3.18). Marine and Lake Superior *Synechococcus* branched independently from each other and Lake Erie samples. This grouping pattern of Lake Erie samples suggests that microbial population of central basin differs from eastern basin and represents two diverse micro-environments: the mesotrophic, relatively warm and shallow central basin and oligotrophic, cold and deeper eastern basin. We have not analyzed samples from western basin due to its eutrophic nature and lower abundance of picocyanobacteria.

Comparison of sequences from Lake Erie eastern station 452, August 2009, and Lake Superior sequences from the stations 2WM and SteC, August and October 2009, indicated that they form two separate, phylogenetically distinct branches (Fig. 3.19). Three marine *Synechococcus* strains, *Synechococcus* RCC307, *Synechococcus* 7803, and *Synechococcus* WH8102, and one freshwater strain from Lake Superior *Synechococcus* LS 0519 form an individual cluster as well. Overall, our data correspond with the finding by Ivanikova et al. (2008) who demonstrated that Lake Superior APP is unique to the lake, whereas Lake Erie APP is largely populated cosmopolitan strains found in freshwater lakes throughout the world. The authors demonstrated that even though Lake Erie is downstream of Lake Superior within the Laurentian Great Lake system, the Lake Superior

16S rDNA sequences do not persist in Lake Erie.

In contrast to the phylogenetic pattern of *phnD* and *phoX* genes in both Lake Erie and Lake Superior, analysis of *pstB* sequences revealed that the Lake Erie eastern basin sequences group with Lake Superior sequences (Fig. 3.20). Moreover, the central basin's Lake Erie *Synechococcus* spp. were clustered into an independent phylogenetic group. The *pstB* distribution pattern suggests that the gene that is a part of an ABC transporter may be crucial in freshwater picocyanobacteria for coping with low phosphate concentrations. Because the P-limited eastern basin and Lake Superior share a phylogenetically distinct PstB ortholog we propose its best adapted to a low P environment. In fact, PstB was found to be specifically expressed when the availability of inorganic phosphate was low (Tetu et al. 2009). Reflecting the importance of PstB proteins in P assimilation, the ABC transporter component gene *pstB* appear to be present in all marine picocyanobacterial genomes analyzed so far (Ostrowski and Scanlan 2006). In the *Crocospaera* WH8501(marine diazotroph) *pstB* gene is in a cluster with *pstS* and has been shown to be upregulated by P deficiency in culture studies (Dyhrman and Haley 2006). Clearly, *pstB* is the part of an essential mechanism in cyanobacteria to thrive in the environments with extremely low P concentrations.

CHAPTER 5. CONCLUDING REMARKS

Cyanobacteria play a significant role in the food web of the Great Lakes, however, despite their importance, very little is known about ecology and physiology of freshwater picoplankton. The purpose of this study was to expand our knowledge on freshwater picocyanobacteria P physiology, specifically, how picocyanobacteria utilize P in the largely oligotrophic waters of the Laurentian Great Lakes.

This study revealed the following results:

1. *phnD*, *phoX*, *sqdX*, and *pstB* genes are present in both cultured freshwater *Synechococcus* spp. and environmental samples from Lake Erie and Superior. The wide distribution of the genes suggests that the assessment of the gene expression pattern might help identify the P status of *Synechococcus* spp. in the lakes.
2. *phnD* conclusions:
 - a. *phnD* gene is expressed constitutively at a low level in freshwater *Synechococcus* sp. cultures and environmental samples from Lake Erie and Lake Superior
 - b. Expression of the gene is not controlled through a general stress response, unlike in many marine cyanobacteria (Dyhrman et al. 2006) which suggests multiple strategies for phosphonate utilization among cyanobacteria
 - c. Constitutive expression of the *phnD* gene suggests significance of Pns as P source for freshwater picocyanobacteria
3. *phoX* conclusions

- a. *phoX* was repressed in P-starved cultures after the addition of Pi in the re-feed experiment and in Lake Superior environmental samples from October (repression following fall turnover)
 - b. *phoX* expression pattern in freshwater *Synechococcus* spp. is consistent with phosphorus bioavailability
 - c. *phoX* may be used as a molecular marker to assess P status of freshwater picocyanobacteria
4. *sqdX* conclusions
- a. *sqdX* is activated in P-starved cells upon the addition of Pi or phosphonate (2-AEP)
 - b. Expression of *sqdX* is not consistent with it serving a role in P conservation
5. *pstB* conclusions
- a. phylogenetic analysis of *pstB* suggests that the gene may be crucial in freshwater picocyanobacteria for coping with low phosphate concentrations
 - b. Because the P-limited eastern basin and Lake Superior share a phylogenetically distinct PstB ortholog we propose its best adapted to a low P environment
 - c. The distribution pattern of *pstB* sequences in freshwater picocyanobacteria may implicate horizontal gene transfer of *pstB* as an adaptation to P-limited environments

Overall, this study demonstrated that freshwater picocyanobacteria have evolved different adaptations to cope with P-limited environments, such as the ability to utilize DOP and substitute sulfolipids for phospholipids. This study also indicates that P metabolism in freshwater *Synechococcus* spp. differ from P responses in their marine

counterparts, therefore, representing a unique ecotype. In regards to future work, development of specific molecular probes for P-stress genes to assess P status of freshwater picocyanobacteria might be one of the future approaches. In addition, quantitative RT-PCR can be applied to assess P stress gene regulation pattern in freshwater picocyanobacteria. We argue that the genome sequences will be instrumental in determining the above-mentioned approaches and provide better insight into how these ecologically relevant *Synechococcus* strains contribute to the food webs of oligotrophic lakes in general.

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INTRODUCTION TO THE APPENDICES

There are two appendices to the dissertation: “Analysis of toxic cyanobacterial populations in the Maumee River and Lake Erie western basin” and “Expression of *hcp* in freshwater *Synechococcus* spp., a gene encoding a hyperconserved protein in picocyanobacteria.” Even though the topics of the appendices are not closely related to the body of the dissertation, the availability of lake and river samples obtained by the lab, along with the resource of Dr. Bullerjahn’s freshwater *Synechococcus* strain collection, enabled some additional studies outside of the main focus of the dissertation. Both of the studies were on the endemic cyanobacteria of the Great Lakes, therefore, the rationale for doing these studies is appropriate to lend some cohesion to the entire dissertation.

**APPENDIX 1. Analysis of toxic cyanobacterial populations
in the Maumee River and Lake Erie western basin**

Chapter A1. INTRODUCTION

The aim of this study was to use genetic tools to investigate the potential relationship between the spatial distribution of toxic cyanobacteria in the Maumee River and western basin of Lake Erie. Indeed, Rinta-Kanto et al. (2005) suggested that the Maumee River and Maumee Bay may serve as a source for *Microcystis* spp. to western and central basins of Lake Erie. *Microcystis mcyA*, gene encoding a subunit of the microcystin synthase complex, was employed as a proxy for identification of toxic cyanobacteria in both the lake and river samples. The *Microcystis* 16S -23S ITS, the internal transcribed spacer of the ribosomal RNA operon, was used to provide an overall picture of the cyanobacterial population in the Maumee River and western basin of Lake Erie. Phylogenetic analysis followed by statistical tests assessed the relationships between the Maumee River and Lake Erie cyanobacterial populations.

Chapter A2. MATERIAL AND METHODS

Table A1 and Figure A1 provide details on sampling and station locations. The samples were processed, extracted and analyzed as described in Rinta-Kanto and Wilhelm (2006). All PCR amplifications with *Microcystis* spp. *mcyA* (Hisbergues et al. 2003) and ITS (Janse et al. 2003) primers were performed as described earlier (Rinta-Kanto et al. 2006). Sequencing was performed at the University of Chicago Cancer Research Center using specific gene primers. Obtained sequences were analyzed manually and aligned using ClustalX – 2.0.12 software (Thompson et al. 1997). Phylogenetic analysis was performed using Mega 5.0 software (Kumar et al. 2007) and Phylogeny.fr (Dereeper et al. 2008).

UniFrac software was used for comparing microbial community diversity as described in Lozupone and Knight (2005).

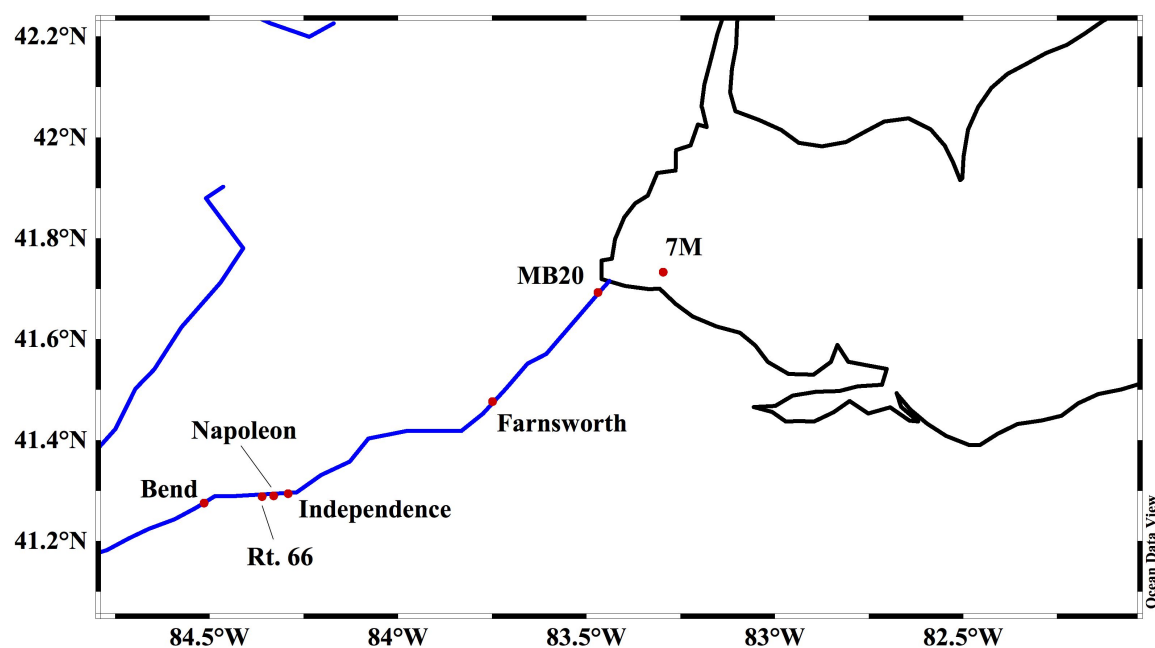


Figure A1. Sampling locations visited in this study. The Maumee River sites are The Bend, Rt. 66 Bridge, Napoleon, Independence Dam, and Farnsworth. Maumee Bay sites are MB20 and 7M.

Site	Date	Volume (L)	Lat/long
882	07/12/05	0.3	41 44 02N 83 23 08W
558	07/12/05	0.2	41 41 56N 83 27 39W
973	07/12/05	0.3	41 47 30N 83 20 20W
MR/Napoleon	05/07/2009	0.25	41 22 55N 84 8 19.8W
MR/Napoleon	05/12/2009	0.13	~
MB/MB20	06/23/2009	0.8	41 41 58N 83 28 12W
MB/MB20	07/02/2009	0.35	~
LE/7M	06/23/2009	1.5	41 43 97N 83 17 78 W
LE/7M	07/02/2009	2	~
LE/7M	07/13/2009	2	~
MR/Independence Dam	03/20/2010	0.18	41 17 40.5N 84 17 29.2W
MR/Waterville (Farnsworth)	03/20/2010	0.12	41 28 36.2N 83 44 55.4W
MR/Bend	04/20/2010	0.4	41 16 32.1N 84 30 53W
MR/Independence Dam	04/20/2010	0.3	~
MR/Rt66 Bridge	04/20/2010	0.375	41 17 18.8N 84 21 39.2W

Table A1. Information on sampling sites. MR – Maumee River, MB- Maumee Bay, LE – Lake Erie, 882 – mouth of the river, 558 – the river sample, 973 – western basin of Lake Erie. All samples were drawn from surface water (<1 m depth).

Chapter A3. RESULTS AND DISCUSSION

A3.1 *mcyA* phylogeny

Phylogenetic analysis of *McyA* sequences from the Maumee River revealed that majority of the sequences group with *Planktothrix* spp. (Fig. A2). In fact, among forty one *McyA* clones thirty eight clones were clustered with *Planktothrix* spp. while only three Maumee River clones grouped with *Microcystis* spp. These results suggest that toxic cyanobacteria in Maumee River were mostly represented by *Planktothrix* spp.

Comparison of *McyA* amplicon sequences from the Maumee River, Sandusky Bay, and western basin of Lake Erie demonstrated two distinct clusters (Fig. A3). The Sandusky Bay sequences cluster with the Maumee River sequences and together represent *Planktothrix* cluster, whereas Lake Erie sequences group separately and appear as *Microcystis* spp. cluster. Therefore, the presence of two distinct clusters Maumee River-Sandusky Bay and Lake Erie suggests that they exist independently from each other, thus the Maumee River and Maumee Bay do not serve as a source for *Microcystis* spp. to western basin of Lake Erie.

Phylogenetic analysis of *McyA* sequences taken the same day in the summer revealed that the majority of the sequences from both the Maumee River and the western basin of Lake Erie are *Microcystis* sequences whereas only two river sequences grouped with *Plankthotrix* spp. (Fig. A4). This result suggests a shift between *Microcystis* and *Plankthotrix*.

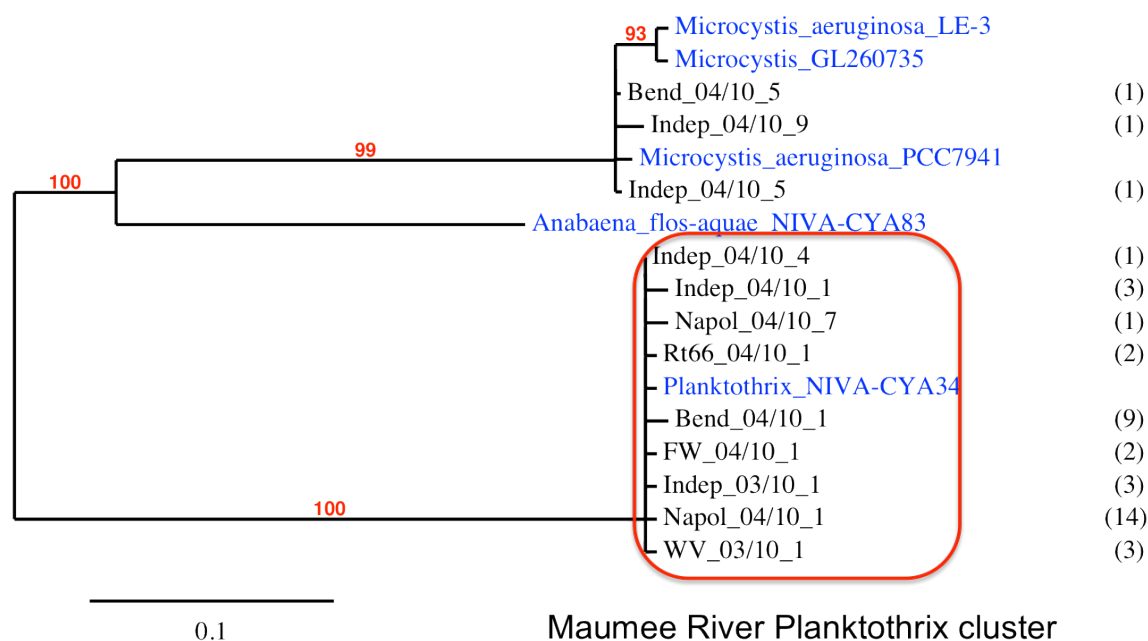


Figure A2. Phylogenetic tree showing relationship between McyA sequences from the Maumee River and potentially toxic genotypes of cyanobacteria. WV_03/10 and Indep_03/10 are from March 2010. The rest of the sequences are from April 2010. Bend, Indep - Independence Dam, Napol - Napoleon, Rt66 - Rt66 Bridge, FW - Farnsworth, and WV - Waterville are sites in the Maumee River. The numbers in parentheses indicate the number of identical sequences represented these sequences.

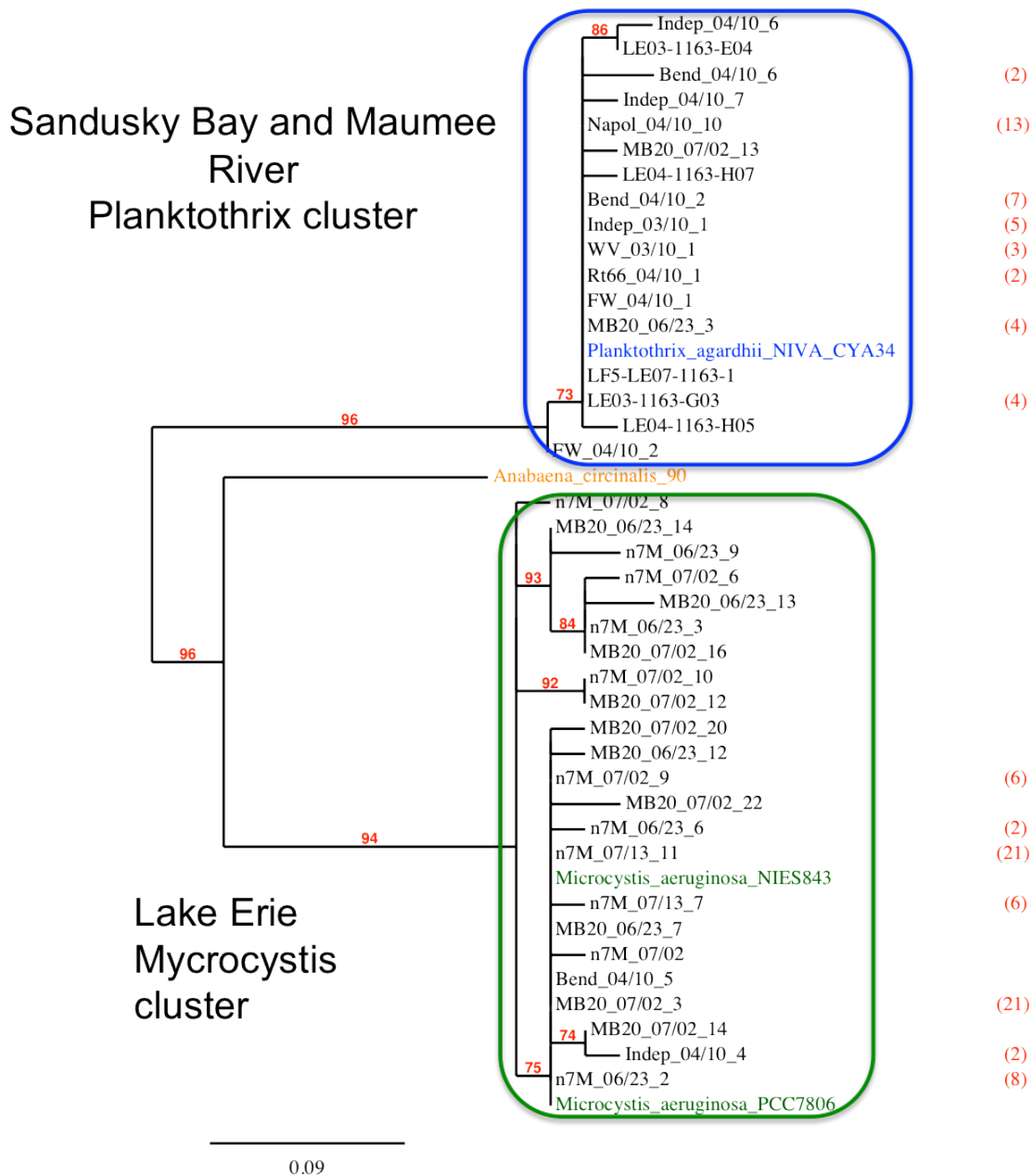


Figure A3. Phylogenetic tree showing relationship between McyA amplicon sequences from the Maumee River, Sandusky Bay, and western basin of Lake Erie. The coding system for the clones is as follows. Maumee River: Bend, Indep -Independence Dam, Napol - Napoleon, Rt66 - Rt66 Bridge, FW - Farnsworth, and WV - Waterville are sites in the Maumee River. The Maumee River sequences are from April 2010. Lake Erie samples from 23 June (7M and MB20), 1 July (7M and MB20), and 13 July (7M and MB20) 2009 are shown. Sandusky Bay sequences (labeled LEO-1163) were obtained from GenBank (Rinta-Kanto 2006). The numbers in parentheses indicate the number of identical sequences represented these sequences.

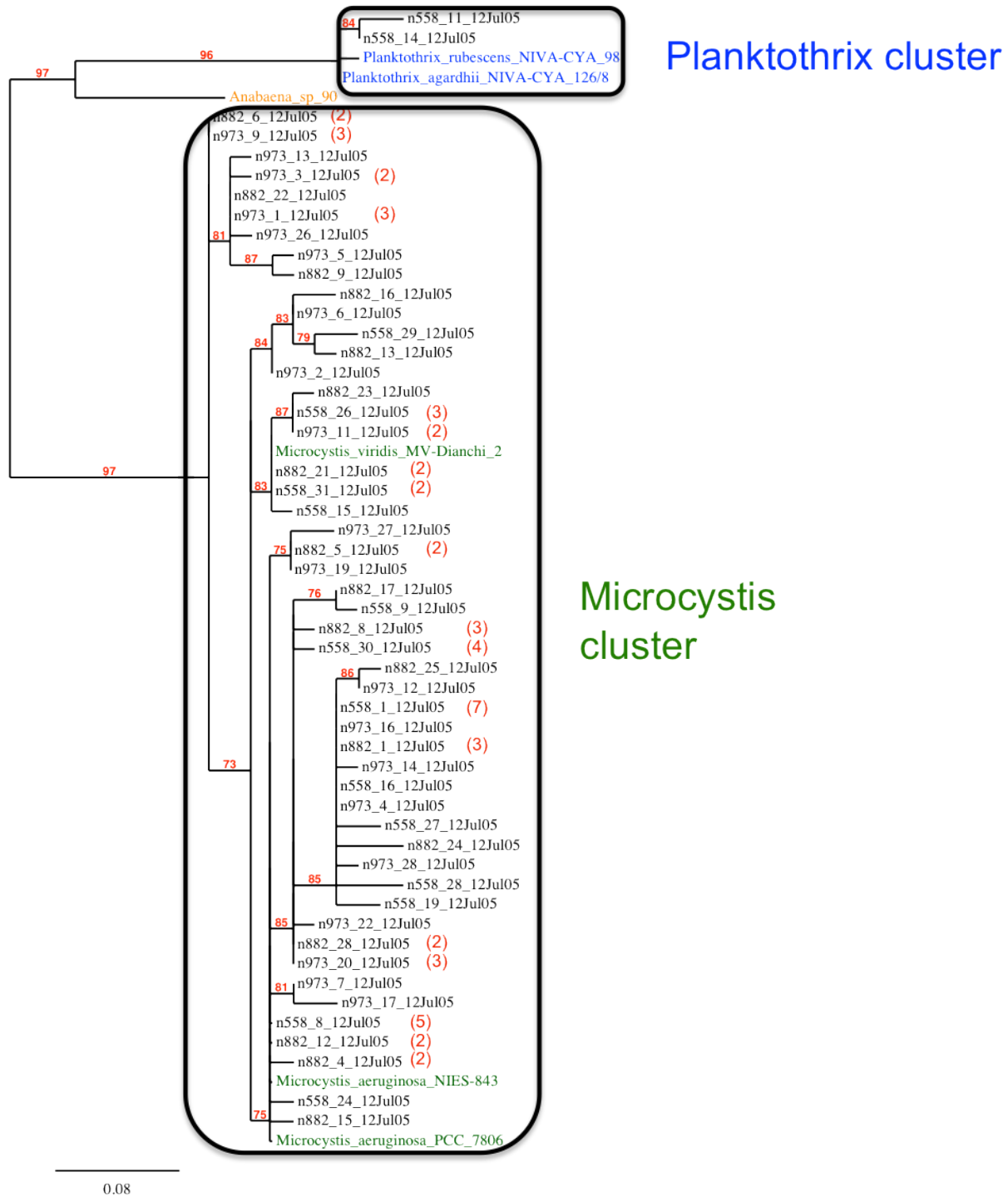


Figure A4. Phylogenetic tree showing relationship between McyA amplicon sequences from the Maumee River and western basin of Lake Erie taken on the same day. **882** – mouth of the river, **558** – the river sample, **973** – western basin of Lake Erie.

A3.2 ITS phylogeny

Since non-toxigenic strains are usually present in the population and *mcyA* does not assess an overall picture of the cyanobacterial population, the 16S - 23S ITS locus was employed to better understand relationships between the community composition in the Maumee River and Lake Erie, especially with respect to toxic vs nontoxic genotypes.

Phylogenetic analysis of ITS sequences from the Maumee River and Lake Erie did not show distinct clusters (Fig. A5). Thirty one Lake Erie sequences and twenty seven Maumee River sequences grouped with *Microcystis* spp., whereas sixty six Lake Erie sequences and fifteen river sequences clustered with *Planktothrix* spp. The results of ITS phylogeny indicate that toxic and non-toxic representatives of both *Planktothrix* spp. and *Microcystis* spp. were present in both the river and lake samples. However, from our results, it was not clear whether there is a relationship between the community composition of the Maumee River and Lake Erie samples.

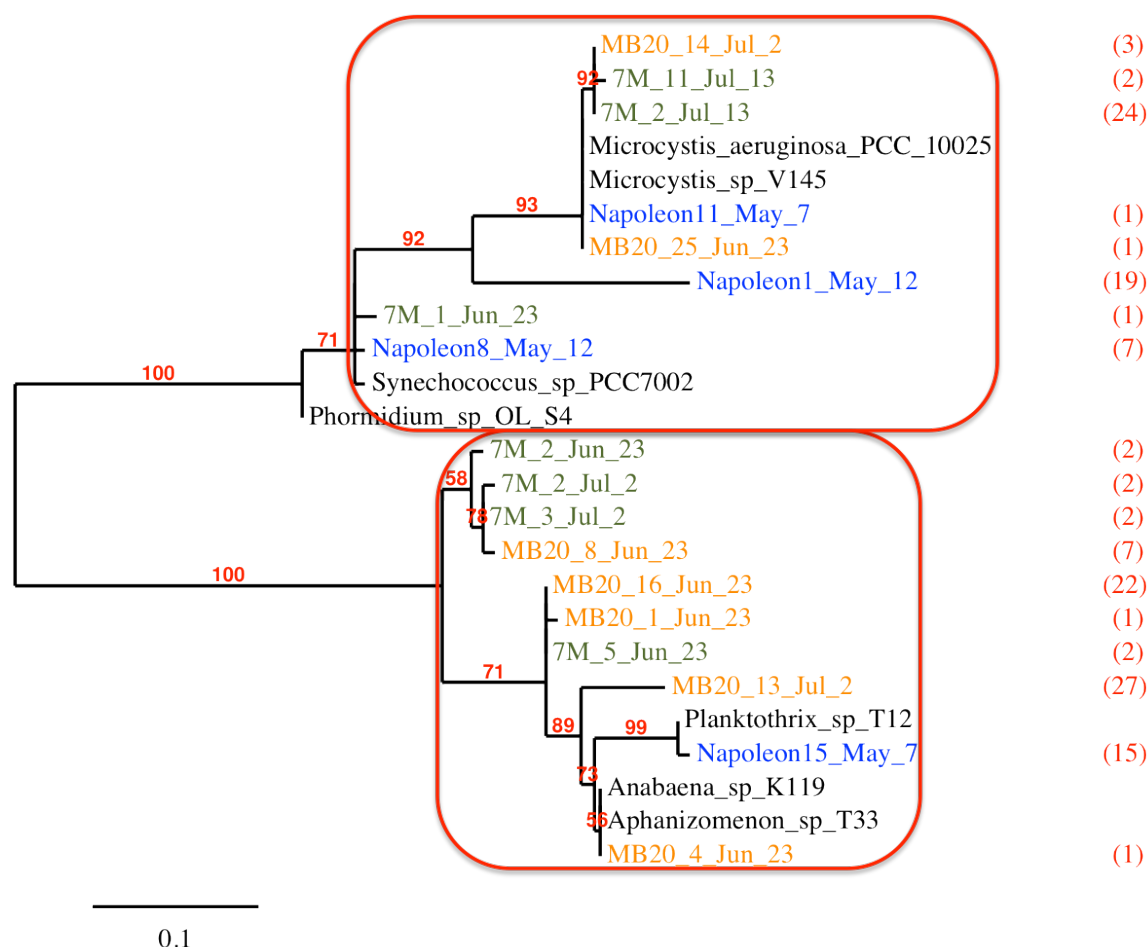


Figure A5. Phylogenetic tree displaying relationship between ITS amplicon sequences from the Maumee River site Napoleon and western basin of Lake Erie, MB20 and 7M. Napoleon samples are from different dates in May 2009. Lake Erie samples are from 23 June (7M and MB20), 1 July (7M and MB20), and 13 July (7M and MB20) 2009. The numbers in parentheses indicate the number of identical sequences represented these sequences.

A3.3 UniFrac analysis of ITS sequences

UniFrac was applied to the ITS sequences from the Maumee River and western basin of Lake Erie to examine the similarities in the entire cyanobacterial community between the different locations. UniFrac tests for differences between locations based on the frequency of sampled sequences. In order to assess whether cyanobacterial community

from the river and the lake were significantly different, a series of UniFrac analyses were applied.

The Environment Counts analysis shows detailed information on how many Operational Taxonomic Units (OTUs) from each environment were used in the study (Fig. A6.A). OTUs were set at 97% sequence identity. To evaluate how the three environments related to one another, we used the Environment Distance Matrix analysis (Fig. A6.B). Lower values represent communities that were more similar. The analysis demonstrated that Maumee Bay sequences MB20 and 7M were more similar to each other (UniFrac value = 0.5717) than to the Maumee River site Napoleon (UniFrac values = 0.7238 and 0.7597) (Fig. A6.B). For better visualization the overall patterns of variation we next used the Principal Coordinates Analysis (PCA). A scatter-plot of the first two principal coordinates was created and coded the points by environment (Maumee Bay and Maumee River) (Fig. A7). PC1 had twice as much amount of the variation in the data than PC2 (66.00 and 34.00% of the variation respectively). PC1 separated MB20 and Napoleon communities from 7M (Fig. A7). PC2 separated the environments from MB20 and 7M from Napoleon. Overall, the analysis indicated that cyanobacterial lineages in the Maumee River are different from the Maumee Bay counterparts.

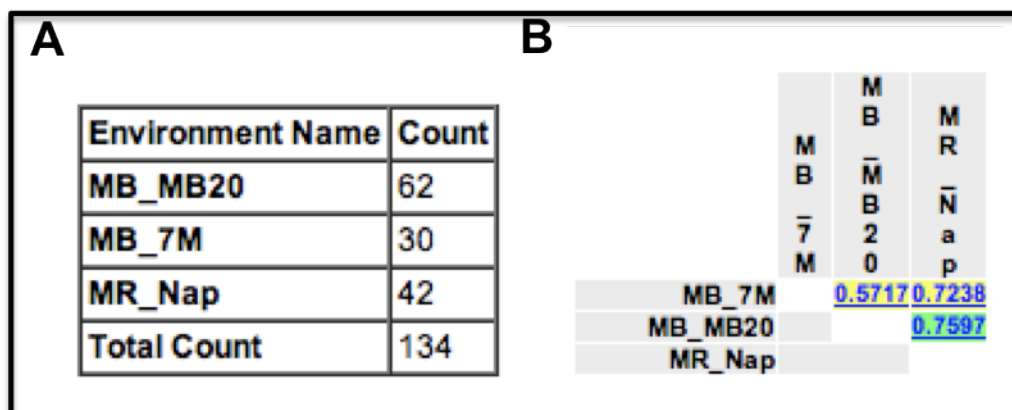


Figure A6. Screenshots of analysis results. For the environment names, the letter before the underscore indicates whether the sequences were from Maumee Bay (MB), or the Maumee River (MR). The letters after the underscore indicate whether the sequences were from the site 7M (7M), site MB20 (MB20), or from the Napoleon site in the Maumee River (Nap). **A.** Result of running the **Environment Counts Analysis** option with *Use abundance weights* set to *No*, so that the counts represent the number of OTU rather than the total number of clones evaluated. **B.** Result of running **Environment Distance Matrix**. The raw UniFrac values for all pairs of environments are colored by quartile; values in the 25–50% range are yellow, 50– 75% are green.

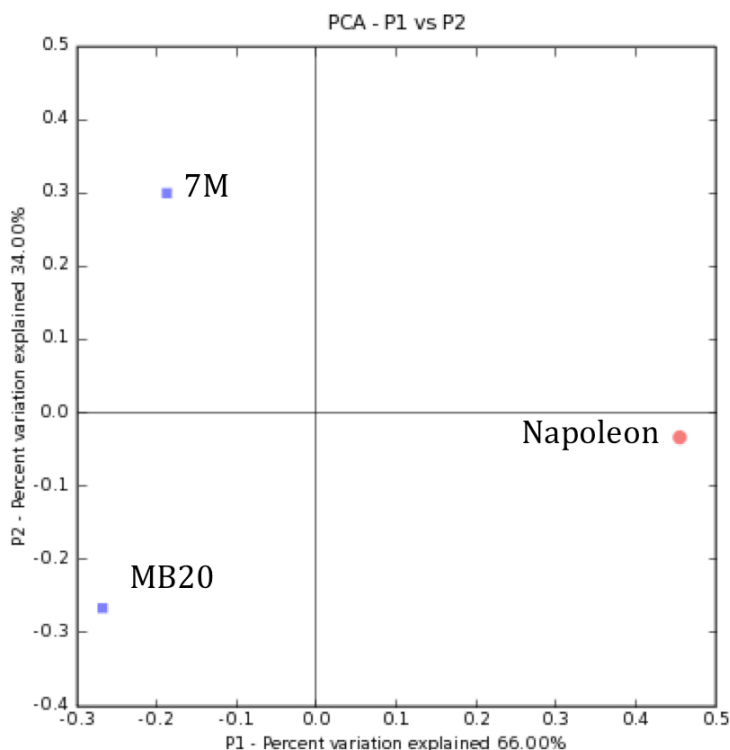


Figure A7. Result of running **PCA** and choosing to output a *ScatterPlot* and the *Bin envs by:first letter* option. Blue squares represent Maumee Bay (sites MB20 and 7M) and red circles represent the Maumee River (site Napoleon). Two points on the left side of the x-axis represent Maumee Bay samples. A point on the right side of the x-axis is the Maumee River site Napoleon. The axes are labeled with the percent of the variation explained by each principal component.

The Jackknife Environment Clusters analysis was performed to estimate the robustness of the results to sampling effort and evenness. The *Number of sequences* was set to 30 and *Number of Permutations* to 100. The Number of sequences was set to 30 due to this was the number of sequences in the environment represented by the fewest OTUs (MB_7M) (Fig. A6.A). Similarly to PCA results, the Jackknife Environment Clusters results indicated that Maumee Bay sequences (MB20 and 7M) were more similar to each other than they were to sequences from the river (Fig. A8). MB20 and 7M clustered together and the node that grouped them was supported > 99.9% of the time (Fig. A8).



Figure A8. Jackknife Environment Clusters results with the *Number of sequences* to set at 30 and *Number of Permutations* set to 100. The environment abbreviations are the same as described for Fig. A5. Each node is colored by the fraction of times it was recovered in the jackknife replicates. Nodes recovered >99.9% of the time are shown in red. The fraction can also be viewed in the interface by moving the pointer over the colored bar.

P Test Significance and UniFrac Significance analysis were next applied to estimate whether the communities in the different samples were significantly different from each other. P Test Significance analysis was performed using the *Each pair of Environments* option with *Number of Permutations* set to 100. The results of the analysis suggest that all three environments were statistically significantly different from each other (Fig. A9.A). The P-value of < 0.03 indicates that a lower number of parsimony changes was never observed in the 100 random permutations that were performed. UniFrac Significance test was performed using the *Each Environment Individually* option with the *Number of Permutations* set to 100. Of the three environments, Maumee River sequences were significantly different (MR_Nap P = 0.01) from Maumee Bay sequences (MB_MB20 P = 0.43

and MB_7M $P = 0.51$) (Fig. A9.B). This result is consistent with the results from other analysis and indicates that samples from the Maumee River and Maumee Bay were statistically different from each other.

A				B	
	M B 7 M	M B 2 0	M R N a p	Environmental Distance P Value(s)	
				Environment	P Value
MB_7M		≤ 0.03	≤ 0.03	MB_MB20	0.43
MB_MB20			≤ 0.03	MB_7M	0.51
MR_Nap				MR_Nap	≤ 0.01

Figure A9. A. Result of running **P-Test Significance** with the *Each pair of environments* option. The P-values have been colored by significance. P-values in a range from 0.01 to 0.05 are shown green. **B.** Result of running **UniFrac Significance** on *Each environment individually* with *Number of Permutations* set to 100. Environment abbreviations are the same as described for Fig. 5.

Chapter A4. CONCLUSIONS

Phylogenetic analysis of *mcyA* sequences from the Maumee River and western basin of Lake Erie revealed that the lake sequences are primarily *Microcystis* sequences while the river sequences form a separate cluster that is homologous to *Planktothrix* spp. This result suggests that the Maumee River does not serve as a source of toxic *Microcystis* spp. to western Lake Erie.

Parallel analysis of ITS sequences using UniFrac further supports our data indicating distinct clusters of cyanobacterial communities exist in the Maumee Bay/western basin and the river.

APPENDIX 2. Expression of *hcp* in freshwater *Synechococcus* spp., a gene encoding a hyperconserved protein in picocyanobacteria

Research Paper

Expression of *hcp* in freshwater *Synechococcus* spp., a gene encoding a hyperconserved protein in picocyanobacteria

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Marine picoplankton of the genus *Synechococcus* and *Prochlorococcus* spp. are widely studied members of the picocyanobacterial clade, composed of unicellular cyanobacteria that dominate pelagic regions of the ocean. Less studied are the related freshwater *Synechococcus* spp. that similarly dominate the euphotic zone of oligotrophic lakes. Previous work has shown that marine picocyanobacteria harbor a small gene, *hcp*, that encodes a 62 amino acid protein 100% conserved among all strains examined. The gene is restricted exclusively to the picocyanobacterial lineage. The current study reveals that *hcp* is also 100% conserved in four freshwater *Synechococcus* spp. strains isolated from the Laurentian Great Lakes, and that the gene constitutively expressed with genes encoding a ribosomal protein and two tRNA genes. The synteny of the *hcp* region is also conserved between the marine and freshwater strains. Last, the *hcp* gene and the organization of the surrounding genetic region has been retained in the reduced genome of a picocyanobacterial endosymbiont of the amoeba *Paulinella* sp.

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Introduction

The autotrophic picoplankton are major primary producers in both the oligotrophic ocean and in large lakes. Such biomass in both marine and freshwater ecosystems is composed predominantly of cyanobacteria <3 µm in size, including the genera *Synechococcus* spp. (in marine and freshwaters) and *Prochlorococcus* spp. (in oligotrophic tropical waters). Whereas a great body of research has been focused on the genetic and physiological biodiversity between different picocyanobacterial ecotypes in the ocean [1, review], the freshwater picocyanobacteria have received far less attention [2]. Indeed, the ecophysiology and evolution of marine picocyanobacteria has been aided by the vast amount of genomic data currently available for *Synechococcus* spp.

and *Prochlorococcus* spp., and from these studies, a core picocyanobacterial genome of 1,228 orthologs has been established [3]. Despite their close relatedness to the marine forms, no such comparative genomics is available for the strains inhabiting exclusively freshwater environments.

Of the picocyanobacterial core genome, Zhaxybayeva and colleagues identified a conserved gene present in all marine picocyanobacteria, but lacking in the genomes of other cyanobacterial clades [4]. Surprisingly, the gene, termed *hcp* (for hyperconserved protein), encodes an invariant polypeptide of 62 amino acid residues. Among these marine taxa, the synteny of the *hcp* region is highly conserved, in that *hcp* is located between two tRNA genes that are in turn flanked by *rpl19*, encoding large ribosomal subunit protein 19 and *gltX*, encoding a glutamyl tRNA synthetase. Analysis of the deduced amino acid sequence suggested that Hcp is a DNA or RNA binding protein, and its functional partitioning with genes involved in translation suggest a role in this process.

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In this paper, we extend the analysis of the *hcp* genetic region to the less widely studied freshwater picocyanobacteria of the genus *Synechococcus* spp. These organisms dominate oligotrophic lacustrine environments both in numbers [5–7] and in overall primary productivity [8]. Freshwater members of the genus yield several phylogenetic clusters [9], some of which are cosmopolitan, while other clusters appear to exhibit endemism, dominating in a limited number of aquatic habitats [8, 10, 11]. The *hcp* gene is present in all isolates tested and encodes the identical 62 amino acid protein described in marine *Synechococcus* spp. and *Prochlorococcus* spp. As has been suggested for the marine forms [4], *hcp* may be useful as a proxy for quantifying *Synechococcus* spp. in lakes by quantitative PCR. Finally, we note the *hcp* gene has been retained in the *Paulinella chromatophora* picocyanobacterial endosymbiont genome, suggesting that *hcp* remains under selection following the transition to the intracellular organellar environment.

Materials and methods

Strains and growth conditions

Synechococcus spp. strains LS 0504, KD3a, ARC-11 and ARC-21 were analyzed from our culture collection from the Laurentian Great Lakes, and have been described previously [8, 10]. Phycoerythrin (PE)-rich LS 0504 was isolated from pelagic waters of Lake Superior, whereas KD3a, ARC-11 and ARC-21 were isolated from the central basin of Lake Erie. *Synechococcus* spp. KD3a and ARC-21 are PE-rich, whereas ARC-11 is phycocyanin (PC)-rich. Each isolate is a member of a different cluster among the freshwater picocyanobacteria [9, 10]. All strains were grown in BG-11 medium [12] at 20 °C and an irradiance of 10 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$. Irradiances were manipulated as required to examine *hcp* expression in different light conditions. For these experiments, irradiances employed were 100 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$, 10 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ and darkness.

PCR primers and conditions

DNA and RNA were prepared from cultured picocyanobacteria as described previously [13]. Table 1 presents sequences of the PCR primers and the genes targeted for amplification. PCR amplicons were cloned into TOPO plasmid vectors (Invitrogen) prior to automated sequencing by the chain termination method. Reverse transcriptase PCR (RT-PCR) was performed with 10 ng total RNA using the Go-Green kit (Promega) with select primer pairs from Table 1. Samples from no-RT controls

were electrophoresed in parallel with RT-PCR products to confirm that no contaminating DNA was present.

GenBank accession numbers

DNA sequences of the *hcp* region from *Synechococcus* spp. LS 0504, KD3a, ARC-11 and ARC-21 were deposited in GenBank under accession numbers GQ243719, GQ243720, GQ243721 and GQ243722.

Results and discussion

PCR amplification of the *hcp* region of freshwater *Synechococcus* spp.

PCR primers (Table 1) were designed based on alignments of *hcp* and surrounding genes from marine picocyanobacterial genomic sequences. No freshwater *Synechococcus* spp. sequences were available for comparison. Owing to the remarkable conservation of sequences among marine picocyanobacteria and freshwater *Synechococcus* spp. *rpl19*, tRNA-Trp, tRNA-Asp, *hcp* and *gltX*, universal primer sets were developed that allowed the amplification and sequencing of the *hcp* and surrounding genes from freshwater *Synechococcus* spp. ARC-11, ARC-21, LS 0504 and KD3a (Table 1). Assembling overlapping sequences of amplicons, the *hcp* region of the four freshwater strains were compared (Fig. 1). As is the case with the marine strains, the gene order is conserved, in that *hcp* is located between *rpl19* and *gltX*, and immediately flanked by tRNA-Trp and tRNA-Asp genes. The deduced Hcp amino acid sequence is 100% conserved, indicating a polypeptide of 62 amino acids:

${}^2\text{HN-MELDLQPGDVVKVLESAALGWVRARVIRVKSGGRV}$
 $\text{VVQSDQGREFTARGNQVRLIEPAGFRP-COOH}$

It should be noted that the marine picocyanobacterial genomes available in GenBank indicate the *hcp* gene encodes a polypeptide of 95 amino acids, as the *hcp* coding region was annotated to begin at an in-frame methionine codon 66 nucleotides 5' to the start codon used in this paper. This revised annotation is in agreement with the analyses by Zhaxybayeva and colleagues [4]. It is more likely that the *hcp* gene encodes the smaller 62 residue polypeptide, because the upstream alternative start codon is located in the middle of the tRNA-Trp gene.

Comparing the four sequences from the freshwater strains, the only notable difference in how the genetic region is organized in these strains is seen in strain ARC-11, in which the intergenic region between *hcp* and tRNA-Asp is over 400 nucleotides, whereas in the other

Table 1. PCR oligonucleotide primers employed in this study.

Primer	Sequence	Region targeted (ARC-11 sequence)
HCP_1F	5'-ATGGARTTGGATCTWCAACCYGG	Nucleotides 1-23, <i>hcp</i> (forward)
HCP_140R1	5'-GCNGTGAATTCNCGRCCCTGRTC	Nucleotides 137-114, <i>hcp</i> (reverse)
HCP_140R2	5'-GCNGTGAATTCNCGRCCTTGRTC	Nucleotides 137-114, <i>hcp</i> (reverse)
HCP_190R1	5'-TCAGGGACGGAANCCAGCNGGYTC	Nucleotides 189-165, <i>hcp</i> (reverse)
HCP_190R2	5'-TCAGGGACGGAANCCNCGCNGG	Nucleotides 189-167, <i>hcp</i> (reverse)
L19_F	5'-AAGGYCACCGCGTGAARCAGCGC	33 nucleotides upstream from stop codon (forward)
Asp-tRNA_R	5'-CCGCCGTGACAGGGCGGTGCTC	Nucleotides 44-21, Asp-tRNA (reverse)
Trp-tRNA_F	5'-AGGTCTCCAAAACCTGATGTCGG	Nucleotides 28-51, Trp-tRNA (forward)
GltX_R	5'-GCTTCNGCGGTGTANCCCAT	Nucleotides 795-784 <i>gltX</i> (reverse)

strains it is much shorter (Fig. 1). Analysis of this 407 bp sequence revealed an open reading frame of 312 nucleotides whose deduced amino acid sequence yielded no similarity to proteins in the GenBank database.

Despite the 100% conservation of the polypeptide sequence, the nucleotide sequence of *hcp* genes from marine and freshwater strains reflects changes in codon usage and GC content since the divergence of the picocyanobacterial lineage [4]. The freshwater *hcp* genes described here range from 80–82% identical to *Synechococcus* sp. WH8102 at the nucleotide sequence level, and average 73% identity to *hcp* from *Prochlorococcus* sp. MED4. Overall, the complete conservation of the protein among the picocyanobacteria demonstrates that the gene has been under stabilizing selection prior to the divergence of the freshwater picocyanobacteria from the marine forms.

Expression of *hcp* in *Synechococcus* sp. ARC-11

To test whether environmental changes influence the expression of the *hcp* gene, we grew *Synechococcus* sp.

ARC-11 under a variety of growth conditions, detecting the presence of *hcp* transcripts by RT-PCR. Examining the *hcp* expression under varying irradiances, RT-PCR amplicons were detected in high light, low light and in total darkness (Fig. 2), indicating constitutive transcription of *hcp*. Additional experiments monitoring *hcp* transcripts under conditions of nitrogen and phosphorus limitation, and during nutrient-replete growth to stationary phase yielded no differences in expression level (data not shown).

Additional PCR experiments examined the cotranscription of genes in the *hcp* region. RNA from *Synechococcus* spp. WH8102 and ARC-11 yield RT-PCR amplicons if primer sets extend from *rpl19* through tRNA-Asp, but no amplicons are detectable that include *gltX*, suggesting that *rpl19* and *hcp* are cotranscribed independently of *gltX* (Fig. 3).

A variant Hcp in retained in the *Paulinella chromatophora* endosymbiont

Whereas *hcp* has not been identified in any organism outside of the picocyanobacterial clade, we examined

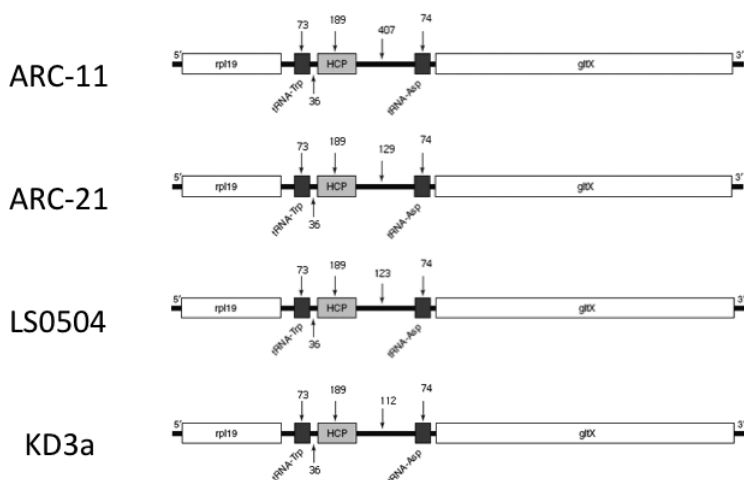


Figure 1. Genetic organization of the *hcp* region in the four freshwater strains (Diagram modified from Zhaxybayeva *et al.* [4]). The number of nucleotides comprising tRNA-Trp, tRNA-Asp, *hcp* and the intergenic regions are indicated. The ARC-11 intergenic region between *hcp* and tRNA-Asp contains a small ORF (see Results and Discussion for details).

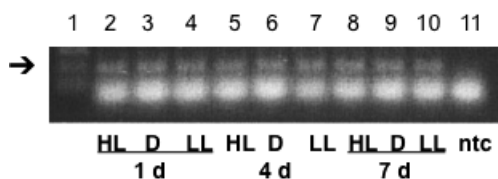


Figure 2. Expression of the *hcp* gene in freshwater *Synechococcus* sp. ARC-11. RNA extracted from cultures exposed to different light regimes was amplified by RT-PCR, employing the *hcp*-specific primer set (HCP_F1 and HCP_140R1). Arrow indicates the presence of the 140 basepair *hcp* amplicon. Products were electrophoresed on 1% agarose gels. Lane 1, 100 bp ladder; lanes 2–4, *hcp* expression from 24 h incubations in high light (HL; $100 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$, dark and low light (LL; $10 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$), respectively; lanes 5–7, same cultures following 4 d incubation; lanes 8–10, same cultures following 7 d incubation. Lane 11, no template control.

whether the hyperconserved protein could be present in a photosynthetic endosymbiont sharing common ancestry with *Synechococcus* spp. The amoeba *Paulinella chromatophora* contains an organelle arising from a primary endosymbiosis with a picocyanobacterium [14, 15], and the endosymbiont genome (termed a chromatophore) is significantly reduced compared to free-living cyanobacteria. The complete genome sequence is approximately 1 Mb, encoding 867 protein coding genes, compared to the ca. 3 Mb genomes of over 3,000 genes found in free-living *Synechococcus* spp. [16]. Despite the fact that over 2/3 of the picocyanobacterial genome have proven to be dispensable in the chromatophore, the synteny of the *hcp* region from *rpl19* to *gltX* remains intact, suggesting that *hcp* remains under positive selection. Notably, the deduced protein sequence differs at 7 out of 62 positions from Hcp from free-living picocyanobacteria (Fig. 4), suggesting that the endosymbiotic environment may in turn have selected for structural and functionally important variability in the polypeptide.

Concluding remarks

Despite the evolutionary distance between freshwater *Synechococcus*, marine *Synechococcus* and *Prochlorococcus*, the *hcp* gene and its surrounding genetic region remain intact, and the Hcp polypeptide is 100% conserved. Thus, Hcp is an ancient trait maintained in the picocyanobacterial lineage long before the divergence of freshwater strains from the marine forms. Given that the picocyanobacteria represent one of the deepest

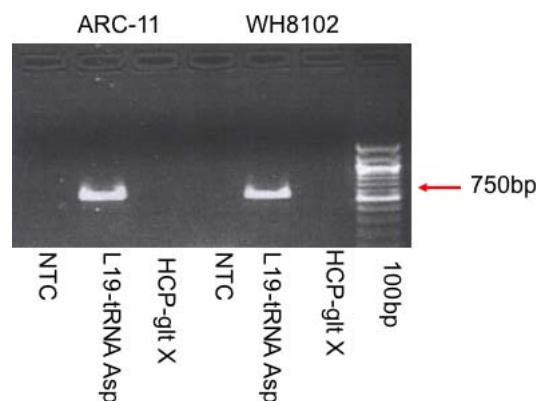


Figure 3. Cotranscription of *hcp* with *rpl19* but not *gltX*. RNA from *Synechococcus* sp. ARC-11 and marine *Synechococcus* sp. WH8102 was extracted and amplified by RT-PCR with primers specific for *rpl19* and tRNA-Asp (L19_F and GltX_R) and *hcp* and *gltX* (HCP_F1 and GltX_R). In both species, the ca. 750 basepair amplicon corresponding to the *hcp* transcript contains *rpl19* and tRNA-Asp, but no amplicons are detected that include *gltX*. NTC, no template control.

branches of the Cyanobacteria, branching just after *Gleobacter* [17], *hcp* may have been selectively lost from other lineages over time. The retention of Hcp in the picocyanobacterial endosymbiont of *Paulinella*, in which the majority of the genome has been lost, further suggests that Hcp plays a critical role in picocyanobacterial physiology. Given the coexpression of *hcp* with genes involved in protein synthesis, and the characteristics of the protein suggesting a nucleic acid binding function [4], we speculate that Hcp could be a novel ribosomal protein specific to this group of cyanobacteria. Precedence for the detection of novel ribosomal proteins has been documented for chloroplasts [18, 19], and we raise the possibility that the picocyanobacterial ribosome may also contain distinct and unique components.

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Pico MELDLQPGDVVKVLESAALGWVRARVIRVKS~~G~~GRVVVQSDQGREFTARGNQVRLIEPAGFRP
Paul MELNLQPGDMVKVLESAALGWVRARVIRVKS~~S~~GRIVVQSDQGREFTARRNQIRLIEPSGFRP

Figure 4. Sequence comparison between Hcp from free-living picocyanobacteria (Pico) and the *Paulinella* chromatophore symbiont (Paul).

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