PHOSPHONATES UTILIZATION IN MARINE AND FRESHWATER

PICOCYANOBACTERIA

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ABSTRACT

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A PCR-based assay is described, designed to detect expression of the phosphonate assimilation gene phnD from picocyanobacteria. The phnD gene encodes the phosphonate binding protein of the ABC-type phosphonate transporter, present in many of the picocyanobacterial genome sequences. Detection of phnD expression can indicate a capacity of picoplankton to utilize phosphonates, a refractory form of phosphorus that can represent 25% of the high molecular weight dissolved organic phosphorus pool in marine systems. Primer sets were designed to specifically amplify phnD sequences from marine and freshwater Synechococcus spp., Prochlorococcus spp. and environmental samples from the ocean and Laurentian Great Lakes. Marine strain Synechococcus WH8102 and some freshwater Synechococcus strains are able to grow on phosphonates as a sole P source; particularly, freshwater Synechococcus ARC-21 is able to utilize synthetic phosphonate herbicide (glyphosate). Quantitative RT-PCR from cultured marine Synechococcus sp. strain WH8102 and freshwater Synechococcus sp. ARC-21 demonstrated induction of phnD expression in P-deficient media, suggesting that phn genes are regulated coordinately with genes under phoRB control. However, pho box was not found in the putative promoters of phosphonate utilization genes in picocyanobacteria. Last, RT-PCR of environmental RNA samples from the Sargasso Sea, Pacific Ocean, and the Baltic Sea detected phnD expression from the endemic picocyanobacterial population. Synechococcus spp. phnD expression yielded a depth-dependent pattern following gradients of P bioavailability, and addition of phosphate to natural sample resulted in deactivation of Synechococcus phnD expression. By contrast, the Prochlorococcus spp. primers
revealed that in all samples tested, \textit{phnD} expression was constitutive. In overall, this study demonstrated the significance of phosphonates as a phosphorus source in the DOP pool for picocyanobacteria in P-depleted environments. The method described herein will allow future studies aimed at understanding the utilization of naturally-occurring phosphonates in the ocean as well as monitoring the acquisition of synthetic phosphonate herbicides (e.g. glyphosate) by picocyanobacteria in fresh waters.
Dedicated to my parents,
Shilova Rimma Mihailovna and Shilov Nickolai Nickolaevich,
and to my grandmother, Podolskaya Phekla Philippovna.
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1  CHAPTER I. INTRODUCTION

1. 1  Key primary producers in oceans and freshwaters

Marine *Synechococcus* spp. and *Prochlorococcus* spp. (*Synechococcus* and *Prochlorococcus* henceforth) are dominant photoautotrophic organisms in the open oceans, with a population often reaching $10^5$ cells ml$^{-1}$ (Olson et al., 1990a; Olson et al., 1990b; Campbell and Vaulot, 1993; Veldhuis and Kraay, 1993). In some oceanic regions, *Synechococcus* and *Prochlorococcus* are responsible for near 50% of carbon fixation (Li, 1994; Li et al., 1997; Veldhuis et al., 1997; DuRand et al., 2001). Similarly, freshwater unicellular *Synechococcus* comprises a major part of freshwater picoplankton (Weisse, 1993; Padisak et al., 1997; Wilhelm et al., 2006). It is now well acknowledged that freshwater *Synechococcus* and marine *Synechococcus* and *Prochlorococcus* contribute significantly to global primary production and represent the key position at the base of the freshwater and marine food web (Waterbury et al., 1986; Fahnenstiel and Carrick, 1992; Nagata et al., 1994; Nagata et al., 1996; Stockner and Shortshead, 1994; Partensky et al., 1999).

Both *Synechococcus* and *Prochlorococcus* are Gram-negative bacteria and belong to the phylum *Cyanobacteria*. This dissertation is concerned with utilization of phosphorus compounds by these ubiquitous cyanobacteria, and below is provided a more detailed discussion on these two taxa.

1.1.1 Genus *Synechococcus*

*Synechococcus* were first detected by virtue of intense orange phycoerythrin (PE) fluorescence in 1979 (Paerl, 1977; Waterbury et al., 1979). Members of this genus have cell size 0.6-0.8 X 0.6-1.6 µm (Waterbury et al., 1986). The name *Synechococcus* was given to a generic
group of small unicellular coccoid cyanobacteria lacking sheaths and dividing by binary fusion in a single plane (Waterbury and Rippka, 1989). The name is limited to strains that have monovinyl chlorophyll $a$ and light-harvesting phycobilisomes (Urbach et al., 1998).

The phylogeny of genus *Synechococcus* is rather complex. Based on G+C content, habitat, photosynthetic pigment content, and growth requirements, genus *Synechococcus* was divided into six strain clades: *Cyanobacterium*, *Synechococcus*, *Cyanobium*, 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 clade outside of all other cyanobacteria (Figure 1.1) (Urbach et al., 1998; Crosbie et al., 2003; Ernst et al., 2003; Fuller et al., 2003). The picophytoplankton clade was confirmed by sequence analysis of the *psbB*, a chlorophyll $a$ binding antennae protein CP47, and *petB/D*, the b-type cytochrome and subunit IV polypeptides of the photosynthetic b6/f complex respectively (Urbach et al., 1998).

In the present research, freshwater unicellular *Synechococcus*, marine *Synechococcus* (clades A and B), and *Prochlorococcus* strains were of main interest, and the name “picocyanobacteria” will refer to these clades hereafter.
Figure 1.1 Neighbor joining analysis of 16S-rRNA gene sequences (from Urbach et al., 1998).
Figure 1.2 Phylogenetic analysis of 16S-23S rDNA spacer sequences from *Synechococcus* and *Prochlorococcus* (from Rocap et al., 2002).
1.1.2 Diversity of marine *Synechococcus*

Marine *Synechococcus* is abundant in coastal and open oceans; the population sizes often range from $1 \times 10^3$ to $1 \times 10^4$ cells mL$^{-1}$ (Ahlgren and Rocap, 2006). It contributes to one fourth of the primary production in many oceanic regions (Li, 1994; DuRand et al., 2001). The two marine clusters A and B were combined into two subclusters within the *Synechococcus* Marine cluster and named 5.1 and 5.2 respectively (Herdman et al., 2001). These two clusters differ in the presence or absence of PE, habitat, and G+C content. Cluster 5.1 (WH7803,WH8102) consists of *Synechococcus* strains that possess PE, have high salt requirement for growth, with G+C contents of 55-62%, and are dominant in both open and coastal ocean (Partensky et al., 1999). Cluster 5.2 (WH 5701) consists of halotolerant *Synechococcus* strains isolated from coastal regions that lack PE, but have phycocyanin (PC), and with a G+C content of 63-66% (Scanlan and West, 2002).

Subcluster 5.1 is further divided into 10 lineages (Figure 1.2) based on the sequence analysis of the 16S-rRNA gene, their motility, a ratio of the pigments phycourobilin (PUB) to phycoerythrobilin (PEB), and ability for chromatic adaptation (Ferris and Palenik, 1998; Rocap et al., 2003; Fuller et al., 2003). For example, clade III (WH8102) is motile (Toledo et al., 1999; Waterbury et al., 1985). Clades I and III are capable of chromatic adaptation (Palenik, 2001). Clade VI lacks PUB, while clade VIII lacks both PUB and PEB (Waterbury et al., 1986).

Five more potential lineages have been described based on the sequence analysis of the *ntcA* gene encoding a nitrogen regulatory protein (Penno et al., 2006) and the 16S-23S rRNA internal transcribed sequence (ITS) (Ahlgren and Rocap, 2006); moreover, there is additional evidence for even more novel lineages (Muhling et al., 2006; Zwirglmaier et al., 2007).
1.1.3 Uniqueness and diversity of Prochlorococcus

Since the discovery of Prochlorococcus by a sensitive flow cytometry by virtue of its dim red fluorescence (Chisholm et al., 1988; Goericke and Rippka, 1993), it has received much attention.

It is the smallest photosynthetic organism (Morel et al., 1993) with the size 0.4-0.6 µm X 0.5-0.8 µm (Lichtlé et al., 1995). Prochlorococcus has unique photosynthetic pigment content of divinyl derivatives of chlorophyll a and b, chl $a_2$ and chl $b_2$ respectively (Goericke and Repeta, 1993). Both chl $a_2$ and chl $b_2$ have absorption and excitation maxima in the blue part of the visible spectra red shifted by 8-10 nm compare to their monovinyl counterparts (Moore et al., 1995; Morel et al., 1993). Some have chl $b_1$ and/or small amount of a particular type of PE (Partensky et al., 1993; Morel et al., 1993; Hess et al., 1996).

Due to its unique pigments and small size, Prochlorococcus has an advantage over Synechococcus for growth at depth. The absorption of Prochlorococcus is maximal in 430-490 nm, and thus Prochlorococcus is best suited for absorbing blue light, that can penetrate deeper in the water (Partensky et al., 1993; Moore et al., 1995). It has high photosynthetic yield at all wavelengths (Shimada et al., 1996) and higher probability of absorbing than scattering incident photons (Morel et al., 1993).

The phylogeny of Prochlorococcus is less complicated than Synechococcus. Six genotypes have been proposed so far based on the ratio of chl $b_2$ to chl $a_2$ content and analysis of 16S-rRNA gene sequences. Prochlorococcus with low chl $b_2$/chl $a_2$ ratio, ranging from 0.1 to 0.6, grows better at high irradiance, and is called high-light (HL) adapted ecotype. Prochlorococcus with high chl $b_2$/chl ratio grows better at lower irradiance, and is referred as low-light (LL) adapted (Moore et al., 1955, 1998; Urbach et al., 1998; Urbach and Chisholm, 1996).
1998; Moore and Chisholm, 1999; Moore et al., 2002). Later, two ecotypes were distinguished among HL strains, and 4 ecotypes were distinguished among LL strains based on 16S-23S-rDNA-ITS sequence analysis (Rocap et al., 2002) (Figure 2).

**1.1.4 Abundance and distribution of marine picocyanobacteria**

High diversity of both *Synechococcus* and *Prochlorococcus* allows them to dominate in oligotrophic systems by accommodating different niches.

Distribution of *Prochlorococcus* is restricted to the oligotrophic regions located between latitudes 40º N and 40º S, while *Synechococcus* is ubiquitous in all oceanic regions. In general, where they co-occur, *Synechococcus* and *Prochlorococcus* have a seasonal pattern of abundance with the former being more abundant in spring after winter mixing and the latter being more abundant in summer and early fall during period of stratification (Campbell et al., 1997; DuRand et al., 2001; Ahlgren et al., 2006). In the Sargasso Sea, *Synechococcus* populations reached a maximum of $5.6 \times 10^4$ cells ml$^{-1}$ in spring, and *Prochlorococcus* populations maximum was $2.6 \times 10^5$ cells ml$^{-1}$ in summer and fall during 1989-1994 years (DuRand et al., 2001).

With the increasing volume of available 16S-rRNA gene sequences for marine picocyanobacteria, design of molecular probe specific for different lineages of both *Synechococcus* and *Prochlorococcus* has become possible. Using dot-blot hybridization in parallel with clone library construction and sequencing, Zwirglmaier et al. (2008) analyzed the distribution pattern of *Synechococcus* and *Prochlorococcus* lineages in Pacific, Indian, and Arctic Oceans and together with the data from previous studies (Fuller et al., 2005; Bouman et al., 2006; Johnson et al., 2006; Garczarek et al., 2007; Zwirglmaier et al., 2007), in a global scale.
Four major ocean domains has been identified by Longhurst (1995, 2007): the polar domain, coastal boundary domain, westerly winds domain (temperate latitudes), and trade winds domain (subtropical and tropical latitudes) (Figure 1.3). The four domains characterized by different nutrient concentration, temperature range, degree of seasonal mixing, amount of light, and primary production.

*Synechococcus* clades I (e.g. CC9311) and IV (e.g. CC9902) dominate in the polar domain; other lineages of *Synechococcus* are present in low abundance, while *Prochlorococcus* is absent. *Synechococcus* clade II (e.g. RS9911, CC9605) and sometimes clade I and IV predominate in the coastal domain. In both trade winds and westerly winds domains, *Synechococcus* are present in low to moderate abundance and the diversity is similar with presence of clades II, III, V/VI/VII (Zwirglmaier et al., 2008).

In contrast, *Prochlorococcus* dominate in these two domains with HLII found in the trade winds domain, HLI found in the westerly winds domain (Zwirglmaier et al., 2008), and LL ecotypes are relatively evenly distributed. Therefore, temperature is the major factor determining distribution for *Prochlorococcus* ecotypes spatially. Indeed, temperature preferences were observed in cultures. Members of HLII ecotype prefer temperature in a range 23-30°C, while HLI and LL ecotypes grow in a temperature range 14-25°C (Johnson et al., 2006; Zwirglmaier et al., 2008).

Vertical separation of *Prochlorococcus* ecotypes has been observed since 1999, with HL ecotypes dominant in upper water column and LL ecotypes found at deeper depths (Partensky et al., 1999; Johnson et al., 2006; Garczarek et al., 2007; Zwirglmaier et al., 2007; Zwirglmaier et al., 2008). HLII ecotype might be dominant up to 120 m depth below the deep chlorophyll
maximum in the Sargasso Sea (Ahlgren et al., 2006), and a similar pattern was observed in the Red Sea (West et al., 2001).

Alternatively to *Prochlorococcus*, *Synechococcus* does not exhibit vertical separation (Fuller et al., 2006; Zwirglmaier et al., 2008). The major factor determining a distribution pattern of *Synechococcus* might be the concentration of nutrients (Zwirglmaier et al., 2008). A member of the clade I (CC9311) has limited P regulatory and scavenging systems (Ostrowski and Scanlan, unpublished) and might not survive in P limited environments; therefore, this clade is absent in open ocean regions.

![Figure 1.3](image_url) Four major ocean domains as identified by Longhurst (1995, 2007) (from Zwirglmaier et al., 2008).
1.1.5 Freshwater picocyanobacteria

Compared to marine, there is less information available on freshwater picocyanobacterial biodiversity, and the factors controlling the growth, distribution, and productivity (Stockner and Antia, 1986; Callieri and Stockner, 2002). Similar to marine, freshwater *Synechococcus* show high diversity with specific niche adaptation, and the abundance of freshwater *Synechococcus* follows a seasonal pattern with a peak in spring after winter mixing and a peak late summer (Weisse, 1993; Stockner et al., 2000; Callieri and Stockner 2002).

Both PE-rich and PC-rich *Synechococcus* exist in freshwater ecosystems and dominate in deep oligotrophic or in shallow eutrophic lakes respectively (Postius and Ernst, 1999; Ernst et al., 2003). For example, PE-rich *Synechococcus* yield a major form of biomass in oligotrophic lakes Huron and Michigan (Fahnenstiel and Carrick, 1992; Nagata et al., 1996), and also in Lake Superior (Fahnenstiel et al., 1986; Ivanikova et al., 2007), and the eastern and central basins of Lake Erie (Pick, 1991; Wilhelm et al., 2006; Ivanikova et al., 2008). In the Lake Erie western basin, which is eutrophic, PC-rich *Synechococcus* constitute more than a half of picocyanobacterial community (Pick, 1991).

Based on phylogenetic analysis of 16S-rRNA sequences and *cpcBA*-IGS, a phycocyanin intergenic sequence, freshwater *Synechococcus* was divided into six clusters within the picocyanobacterial lineage that includes marine *Synechococcus* and *Prochlorococcus*. Some lineages are cosmopolitan and some are specific for certain geographical regions (e.g. Group E) or ecological niche (cluster of Antarctic strains) (Ernst et al., 2003; Crosbie et al., 2003). For example, two unique clusters (LSI and LSII) were found to dominate in Lake Superior, while members of other cosmopolitan clusters were also present in nearshore samples (Ivanikova et al., 2007). In the Lake Erie central basin, PE-rich *Synechococcus* strains belonging to a cosmopolitan
cluster MH301 were predominant in July, and PE-rich strains from clusters Group E and MH305 were present in August 2004 (Wilhelm et al., 2006; Ivanikova et al., 2008; Cupp and Bullerjahn, unpublished data).

1.1.6 Nutrient assimilation in picocyanobacteria

One of the main adaptations of picocyanobacteria to growth in oligotrophic environments is their small cell size which results in high surface to volume ratio, and thus, rapid nutrient uptake rates (Chisholm, 1992; Friebele et al., 1978; Lehman and Sandgren, 1982; Suttle and Harrison, 1982). In general, *Synechococcus* can assimilate wider range of different nutrients compared to *Prochlorococcus*, which is reasoned by the distribution of these two clades. However, in both genera, the clades have adapted to utilize certain nutrients that are most available in their habitat.

*Synechococcus* can utilize both organic (amino acids and urea) and inorganic (ammonium, nitrate, nitrite) sources of nitrogen (Waterbury et al., 1986; Moore et al., 2002; Palenik et al., 2003). However, some strains grow better on ammonium than on nitrate or nitrite (Ahlgren and Rocap, 2006). Only one strain *Synechococcus* MIT S9220, isolated from equatorial Pacific, cannot utilize nitrate (Moore et al., 2002). Nitrate assimilation in *Synechococcus* is carried through nitrate (*narB*) and nitrite (*nirA*) reductases (Flores et al., 2005), but uptake of nitrate and nitrite is through NapA/NrtP, whereas in many freshwater cyanobacteria, it is through *nrtABCD*, an ABC type of transporter (Sakamoto et al., 1999).

By contrast, all tested *Prochlorococcus* strains grow on ammonium, but are not able to grow on nitrate (Dufesne et al., 2003; Palenik et al., 2003; Rocap et al., 2003). Indeed, the *narB* gene is absent in all known *Prochlorococcus* genomes. Moreover, only some *Prochlorococcus*
strains, belonging to LL ecotypes, were able to grow on nitrite, e.g. MIT9313 (Moore et al., 2002). *Prochlorococcus* PCC9511 failed to grow on nitrite as a sole N source (Rippka et al., 2000; El Alaoui et al., 2001). *Prochlorococcus* MED4 does not have the *nirA* gene (Scanlan and West, 2002). Heterogeneity of *Prochlorococcus* in N assimilation is related to niche partitioning. Both PCC9511 and MED4 are the HL strains and are abundant in the upper water column, where nitrite concentration is usually extremely low; by contrast, LL *Prochlorococcus* MIT9313 inhabits deeper water column where nitrite concentration is higher.

Phosphorus metabolism in picocyanobacteria has not been as well studied yet, but likely that both genera have relatively low phosphorus requirements (Bertilsson et al., 2003). For planktonic cells in the open ocean, two major reservoirs for P are nucleic acids and phospholipids (Van Mooy et al., 2006). Picocyanobacteria, especially *Prochlorococcus*, have evolved to have small genome and thus decreased P requirements (Dyhrman et al., 2007). Moreover, one of the major adaptations to low-P environment in *Synechococcus* and *Prochlorococcus* is synthesis of sulfolipids instead of phospholipids (Van Mooy et al., 2006; Dyhrman et al., 2007).

Phosphorus assimilation and its regulation are described in details in sections 1.2 and 1.3, where aspects of P nutrition and P-dependent gene expression are described both in general and in light of the research in this dissertation.

### 1.1.7 Picocyanobacteria: conclusion

In summary, picocyanobacteria have evolved adaptations for occupying specific niches. Such adaptations include but not limited by: high diversity inside the genera (more than 6 freshwater and more than 10 marine *Synechococcus, 6 Prochlorococcus*), specific photosynthetic
pigment composition (PE with different PUB/PEB ration or PC-rich *Synechococcus*, HL or LL adapted *Prochlorococcus*), ability to utilize a variety of nutrients depending on clade, their small size, small genome, and low P requirements. The heterogeneity of picocyanobacterial clades in nutrient metabolism should be considered when assessing their physiology and contribution into biogeochemical cycles. However, nutrient metabolism, especially phosphorus physiology, has not been comprehensively addressed yet in picocyanobacteria.

### 1.2 Phosphorus (P) in aquatic ecosystems

Phosphorus is an essential macronutrient for all living organisms; life is truly built around phosphorus (deDuve, 1991). Phosphorus accounts for 2-4% of the dry weight of most cells (Karl, 2000); it is incorporated into a broad spectrum of vital compounds including nucleic acids, ATP, phosphoproteins, and phospholipids. Being discovered in 1669 by a German alchemist Hennig Brand as a “light bearing” solid substance, phosphorus was recognized as an essential participant in metabolism after almost 300 years (Bridger and Henderson, 1983; Lehninger et al., 1993). The averaged elemental composition of plankton estimated by Fleming in 1940 yielded a ratio of C:N:P equal to 106:16:1. Cooper and Richards found that inorganic C:N:P ratio varied in proportions of 105:15:1. Based on these two data set, Alfred Redfield (1958) concluded that 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 was named “Redfield ratio”, and it is used for calculations of export production in oceanography and limnology. The N:P ratio of 16:1 is used for differentiating between N-limitation and P-limitation (Geider and La Roche, 2002).
1.2.1 P-limitation in oceans and lakes

Inorganic phosphate ($P_i$) is the preferred form of phosphorus for microbial growth, and despite of its importance, in many aquatic environments $P_i$ is often near or below the detection limits (30 nmolar) by standard analytical methods (Karl, 2000). Indeed, primary production in diverse marine ecosystems is frequently P-restricted. This is true for the Mediterranean Sea where concentration of $P_i$ was less than 2 nM (Krom et al., 1991; Thingstad et al., 2005) and the Sargasso Sea with reported concentrations of 10 nm or less (Cotner et al., 1997). P-limitation has been observed in the Pacific and Atlantic Gyres (Björkman et al., 2000; Sañudo-Wilhelmy et al., 2001). There is often a seasonal N:P stoichiometric imbalance in coastal regions due to inputs of nitrogen-rich freshwater (Fisher et al., 1992, 1999; Dyhrman et al., 2007). In the North Pacific Subtropical Gyre, a shift from N-limited to P-limited has occurred recently likely due to increased temperature, and $P_i$ concentration was about 40 nM (Karl et al., 2001).

In freshwater environments, the limitation of primary production by P availability is a central tenet of modern day limnology (e.g. Schindler, 1977). Hudson et al. (2000) assessed concentration of $P_i$ in 56 lakes of North America with a steady state radiobioassay. The concentrations were orders of magnitude lower than estimates made by standard chromatic and radiochemical methods, with a range of 27 to 885 pM. Thus, the already low concentrations of $P_i$ reported in other oligotrophic lakes could be reestimated to be two orders of magnitude lower (Hudson et al., 2000).

1.2.2 Lake Erie as a P-limited environment

Lake Erie is another example of a P-limited freshwater system. It is the smallest, the warmest, and the most productive of the Great Lakes (Munawar and Weisse, 1989). The lake is
influenced significantly by anthropogenic impact from high-density population, industry, and agriculture. During 1960s-1970s, Lake Erie was extremely eutrophic due to high loadings of anthropogenic phosphorus, which caused proliferation of toxic phytoplankton, decreased water clarity, and anoxic conditions in some portions of the lake. After the Great Lakes Water Quality Agreement was implemented to reduce the amount of phosphorus loadings into the lake, loads of phosphorus decreased to ca. 11,000 tons per year or 240 nM if evenly distributed throughout the lake (Dolan, 1993). At present, the western basin of Lake Erie experiences seasonal eutrophication, and the central and eastern basins are oligotrophic (Makarewicz and Bertram, 1991). In July 2004, concentration of $P_i$ was reported ca. 0.093 and 0.056 µM for western and both central and eastern basins respectively. The N to P ratio was significantly higher than the Redfield ratio, and an addition of $P_i$ to whole water resulted in increase in phytoplankton biomass indicating a limitation of primary production by phosphorus (DeBruyn et al., 2004).

### 1.2.4 Standard method to assess P concentration

The most commonly used assay for measuring P concentration is the phosphomolybdate assay (Benitez-Nelson, 2000; Paytan and McLaughlin, 2007), which estimates what is defined as the soluble reactive phosphorus pool (SRP). SRP comprises a dissolved P fraction that reacts with molybdate ions in an acid solution resulting in phosphomolybdate complex, which is further reduced by ascorbic acid into blue molybdenum complex. Hydrophosphate anions form a majority of SRP (87%) compared to phosphate anions. Total dissolved phosphorus (TDP) consists of SRP and soluble non-reactive phosphorus (SNP). Exposing a water sample to high temperature and/or high pressure in the presence of a strong oxidizing agent converts TDP to SRP, which is then assessed by the phosphomolybdate method (McKelvie et al., 1995). Finally,
the SNP pool is calculated as a difference between TDP and SRP (Paytan and McLaughlin, 2007).

Several restrictions exist in applying this method. Not only it has detection limit of 30 nM (Karl, 2000; Benitez-Nelson, 2000), but also it does not estimate the bioavailable phosphorus (BAP) pool. Some components of SRP may not be bioavailable such as polyphosphates, and alternatively, some bioavailable compounds from SNP pool may not be readily hydrolyzed to SRP. Indeed, using \(^{32}\)P-labelling technique (Karl and Bossard, 1985), it was found that BAP pool exceeded the SRP pool at station ALOHA (Pacific oligotrophic gyre) indicating utilization of SNP (Björkman and Karl, 2003).

1.2.4 SNP composition

Despite the fact that SNP comprises 75% of TDP in surface waters of open oceans (Karl and Yanagi, 1997; Karl and Björkman, 2002) and up to 50% of TDP in coastal regions (Romakevich, 1984), little is known about SNP chemical composition and bioavailability. Low molecular weight dissolved organic phosphorus (LMW DOP) contribute from 50 to 80% to SNP pool (Benitez-Nelson, 2000), but composition of LMW DOP cannot yet be assessed (Dyhrman, 2007). High molecular weight (HMW) DOP comprises about a quarter of SNP in the upper ocean (Clark et al., 1998; Kolowith et al., 2001), and consists of two classes of P bonds, ester (both mono- and diester) and phosphonate (C-P) bonds (Benitez-Nelson, 2000; Karl and Björkman, 2002). Phosphonates, their origin and bacterial degradation are described below.
1.2.5 Phosphonates in marine ecosystems

Naturally-occurring phosphonates may compose up to 25% of HMW DOP in marine environment (Clark et al., 1998; Kolowith et al., 2001), but are generally thought to be more refractory to assimilation than organic monoesters (Benitez-Nelson, 2000). This may not be the case because phosphonates may be utilized at equivalent rates with phosphoesters from SNP. This suggestion is based on the observation that despite decreasing concentration of SNP with depth, the proportional composition of SNP remained the same (Clark et al., 1998). Moreover, phosphonates were selectively removed relative to phosphoesters from sinking particulate organic phosphorus in anoxic conditions (Benitez-Nelson et al., 2004). Thus, phosphonates may be an unrecognized source of bioavailable phosphorus in oligotrophic oceans. In addition, phosphonates are even implicated in the accumulation of greenhouse gases; recent studies have indicated that microbial utilization of methyl phosphonate in marine environments can yield the aerobic generation of atmospheric methane (Karl et al., 2008).

Being synthesized since 1944, phosphonates were discovered in living forms only in 1959 (Horigushi 1959). Naturally-occurring phosphonates in the DOP pool are derived from the degradation of glycolipids, glycoproteins, antibiotics and phosphonolipids (Clark et al., 1998; Kolowith et al., 2001; White and Metcalf, 2007). The most abundant naturally-occurring phosphonates are 2-aminoethylphosphonic acid (2-AEP), phosphonoformic acid and methylphosphonic acid (Horigushi 1984; Schowanek 1990; Karl and Björkman, 2002).

1.2.6 Phosphonates in freshwater environments (Glyphosate)

Whereas phosphonates in the marine environments are naturally-occurring, a possible significant source of phosphonates in freshwater environments is of anthropogenic origin. Since
the first synthesized phosphonate compound aminomethylphosphonic acid in 1944 (Hilderbrand & Henderson, 1983), anthropogenic phosphonates have been increasingly used in agriculture, pharmacy, and households due to their chemical stability and structural similarities to analogous phosphate esters, which make them potent enzyme inhibitors (Kolpin et al., 2006). For example, about 18.1 million kg of phosphonates are used annually in USA (Nowack and Stone, 1999), among them is the most widely used herbicide in the world, glyphosate (Roundup) (Woodburn, 2000). Dramatic increases in the agricultural use of glyphosate occurred in 1997 with the introduction of glyphosate-resistant crops (corn, soybeans, cotton) (Kolpin et al., 2006). Based on the documented use of glyphosate statewide, and that ~20% of the USA agricultural land in Ohio lies within the Erie watershed, the estimated deposition of phosphonates onto fields in the watershed may exceed one thousand metric tons per year (NSF Center for Integrated Pest Management, 2004). Whereas the formulation of glyphosate in commercially available Roundup promotes attachment to soil particles, microbial action could yield phosphonate degradation products becoming mobile within the watershed, yielding loadings of P into Lake Erie heretofore undescribed. Notably, a recent survey of streams in the Midwest documented the detection of glyphosate in 55 of 154 water samples, and aminomethylphosphonic acid (AMPA), a major glyphosate degradation product, was detected in 106 of the same samples (Scribner et al., 2003); similar distribution of glyphosate and AMPA detection was observed by Kolpin et al. (2006).

Many bacteria possess the ability for glyphosate degradation (Dick and Quinn, 1995), and since water quality legislation regulates phosphate sources, not phosphonates, loadings of anthropogenic phosphonates may contribute to the growth of bacterial planktonic community in P-stress conditions bringing new P into the food web at large. For instance, glyphosate, AMPA, as well as 2-AEP was shown to be used as a sole P source by bacterial community from different
environmental samples including one from an oligotrophic lake (Schowanek et al., 1990). Cyanobacteria are resistant to mM concentrations of glyphosate (Powell et al., 1991), and addition of glyphosate to water samples stimulates growth of picocyanobacteria over eukaryotic algae (Perez et al., 2007). However, it is not known if picocyanobacteria directly benefit from addition of glyphosate or AMPA or indirectly through receiving Pi from glyphosate and AMPA degradation by community bacteria.

Phosphonate assimilation by bacteria is described in section 1.4.

1.2.7 Phosphorus as a limiting nutrient: conclusion

Despite of being essential, phosphorus is often a limiting nutrient in marine and freshwater systems (e.g. Schindler, 1977; Karl et al., 2001). Understanding BAP concentration and composition is complicated due to the restrictions of applied standard methods, but increasing evidence suggests that phosphonates, previously thought to be recalcitrant, contribute significantly to the BAP pool (Clark et al., 1998; Kolowith et al., 2001; Dyhrman et al., 2006; Karl et al., 2008). Moreover, loadings of anthropogenic phosphonate may represent a new unrecognized P source in lakes.

1.3 P metabolism and pho regulon

In order to survive in P limited environments, bacterial have evolved a specific P stress response in addition to general reduction of metabolic processes. Genes that encode proteins participating in P-stress response are coordinately regulated as the pho regulon.
1.3.1 P metabolism and *pho* regulon in bacteria

The *pho* regulon of *Escherichia coli* has been the best studied and consists of at least 47 genes (Wanner, 1996; Han et al., 1994; Suziedeliene et al., 1999; Harris et al., 2004). Additional genes belonging to the *pho* regulon are likely to exist as well (Yuan et al., 2006).

The *pho* genes are controlled by a two-component regulatory system PhoR/PhoB. First evidence of P genes activation was described by Wanner in 1980, and PhoB participation was observed by Makino in 1986, who introduced the name “*pho* box”. PhoR is an inner membrane histidine kinase protein, which senses periplasmic $P_i$ concentration through interaction with the Pst transport system. PhoB is a response regulator that acts as a DNA binding protein to activate or inhibit transcription (Makino, 1986; Smith and Payne, 1992; Wanner, 1996, Harris et al., 2001). When P is limiting, PhoR is autophosphorylated on a histidine residue and then phosphorylates aspartate residue of the PhoB. Phosphorylated PhoB binds to a specific DNA sequences (*pho* box) in a promoter and activates transcription (Figure 1.4). Although it can bind to DNA in inactive state, the binding affinity is stronger when PhoB is phosphorylated. Interactions between PhoB and $\sigma_{70}$ facilitate strong binding of RNA-Polymerase which otherwise has a weak binding to the promoters of *pho* regulon due to *pho* promoters having a -35 sequence distinct from consensus sequence of bacterial promoters (Makino, 1993). When $P_i$ is in excess, Pst- PhoU system forms a repression complex with PhoR, which dephosphorylates PhoB (Muda, 1992; Steed and Wanner ,1993; Wanner, 1997). In some bacteria, PhoB can be activated by a different histidine kinase in the absence of PhoR; for example, CreC from the CreC/CreB two component regulatory system, involved in carbon metabolism, can induce PhoB activity in *E.coli* (Wanner and Wilmes-Riesenberg, 1992). In addition, acetyl phosphate may phosphorylate PhoB (Wanner and Wilmes-Riesenberg, 1992; Hiratsu et al.,1995).
In P-replete conditions, $P_i$ is transported into the periplasm either through porins or as a result of hydrolysis of some organic phosphates by phosphatases, which are constitutively expressed (Wanner, 1996). Then $P_i$ is translocated into the cytoplasm by low affinity and high velocity Pit transport system (Willsky and Malamy, 1980).

Bacteria have evolved two specific responses for P-stress: scavenging any available $P_i$ from surroundings and utilizing alternative P sources. Thus, when P is limiting, PhoB activates genes encoding for high affinity transport $pstABCS$, an outer membrane porin $phoE$, a special transporter for glycerol-3-phosphate $ugp$, alkaline phosphatase $phoA$ and other phosphatases, and the phosphonate assimilation genes $phn$ (Argast and Boos, 1980; Overbeeke and Lugtenberg 1980, Magota, 1984; Coleman, 1992; Wanner, 1994; Wanner, 1996). Additionally, PhoB activates expression of PhoBR operon (Makino, 1986).
Figure 1.4 Control of the *pho* regulon (from Lamarche et al., 2008)
1.3.2 P metabolism and *pho* regulon in cyanobacteria

In cyanobacteria, the *pho* regulon is relatively well studied in the model organisms *Synechocystis* PCC6803 and *Synechococcus* PCC7942. Mechanisms of P regulation are similar to that of *E.coli* with a two-component regulatory system that senses P\(_i\) concentration and controls expression of genes for high affinity P\(_i\) transport and assimilating alternative P sources. Orthologues to *E.coli* PhoR/PhoB, named SphS/SphR, have been shown to regulate expression of *pho* genes in *Synechococcus* PCC7942 (Aiba et al., 1993; Nagaya et al., 1994) and in *Synechocystis* PCC6801 (Hirani et al., 2001; Suzuki et al., 2004). Similarly, phosphorylated SphR binds to 2 or 3 *pho* boxes in the promoter of *pho* genes, but the *pho* consensus sequence is different: CTTAACCT in *Synechocystis* PCC6803 (Nagaya et al., 1994; Suzuki et al., 2004). The main difference of *pho* regulation in cyanobacteria compared with *E.coli* is that SphS (PhoR in *E.coli*) is a soluble protein, and thus it detects intracellular P\(_i\) concentration (Nagaya et al., 1994). How cyanobacteria detect extracellular concentrations of P\(_i\), if they do, is not yet known.

The number of *pho* genes in cyanobacteria is lower than in *E.coli*. Microarray analysis revealed 12 genes upregulated in *Synechocystis* PCC6803 when P is limiting. These genes are included in operons of *sphX-pstS1-C1-A1-B1-B1'* and *pstS2-C2-A2-B2*, both encoding high affinity P\(_i\) transport, the operon *phoA-nucA*, encoding alkaline phosphatase and extracellular nuclease (Suzuki et al., 2004). One gene, *urtA*, that codes for a urease transporter subunit, was repressed.

Other differences from the *E.coli* *pho* regulon include an absence of genes for low affinity transport (Wanner, 1996; Moore et al., 2005; Su et al., 2003). A negative regulator, PhoU, is present in many freshwater cyanobacteria (Juntarajumnong et al., 2007), but not in marine cyanobacteria (Moore et al., 2005). An exception is *Crocosphaera watsonii* WH8501, an
important marine unicellular diazotroph, which has both the Pit system and PhoU protein (Dyhrman and Halley, 2006).

Homologues to the SphS/SphR have been located in the sequenced genomes of many other cyanobacteria including the picocyanobacteria *Synechococcus* WH8102, CC9605, WH7803 and *Prochlorococcus* MIT9312, and MED4. However, the absence of SphR in *Trichodesmium* IMS101 or inactivity of SphR in *Prochlorococcus* MIT9313 does not exclude P responsive regulation. Thus, P limitation resulted in activation of the *ptsS* and phosphonate utilization genes in *Trichodesmium* IMS101 (Dyhrman et al., 2006). Similarly, *Prochlorococcus* MIT9313 upregulates expression of the *pho* genes under P stress, including *pstS*, *sphS*, *phoE* (Martiny et al., 2006). Therefore, other regulatory proteins should be involved regulating the *pho* genes instead of SphR.

**1.3.3 P metabolism and *pho* regulon in picocyanobacteria**

In picocyanobacteria, regulation of P genes has not been extensively studied, but available data suggest that P genes and their regulation vary among genera and among different isolates. This difference is related to the ecological niche from which isolates have been isolated (Martiny et al., 2006). Thus, *Synechococcus* and some *Prochlorococcus* (e.g. PCC9511) strains are able to grow on variety of organic phosphoesters (Scanlan et al., 1997; Rippka et al., 2000; Moore et al., 2005), and a *pho* box has been predicted in the *Synechococcus* WH8102 *phoA* promoter (Su et al., 2003; Su et al., 2007). By contrast, genome of *Prochlorococcus* MIT9313 lacks the *phoA* gene (Scanlan and West, 2002). As a member of a LL ecotype, MIT9313 inhabits below the nutricline in the water column (Ahlgren et al., 2006) where concentration of inorganic
phosphorus is higher, and concentration of DOP is lower than in the surface waters; therefore, the presence of phoA gene is not essential for this isolate.

In addition, expression of P genes differ between Prochlorococcus HL strain MED4 and LL strain MIT9313. While they both showed activation of sphS, pstABCS, and phoE gene expression upon P deprivation, overall, a more P-specific response was observed in MED4, and a more general stress response was observed in MIT9313 (Martiny et al., 2006).

Due to its existence in a relatively constant oligotrophic environment, Prochlorococcus have lost some of the P metabolism genes that are present in Synechococcus. For instance, the sphX gene encoding a phosphate binding protein of the ABC transporter and thought to be involved in P-stress regulation (Falkner et al., 1998), is absent in all Prochlorococcus genomes while present in Synechococcus WH8102 (Scanlan and West, 2002).

Despite the differences, picocyanobacteria share several core P genes. Thus, in addition to sphS, phoE, pstS, pstABC, genes for polyphosphate assimilation are present in genomes of all picocyanobacteria, and at least some strains are capable of growing on polyphosphate as a sole P source (Moore et al., 2005). However, the polyphosphate utilization genes ppK and ppX were not activated by P limitation in both Prochlorococcus MED4 and MIT9313 in the short term (Martiny et al., 2006).

Further, picocyanobacteria possess multiply copies or at least one copy of the pstS gene indicating importance of Pi scavenging in picocyanobacterial habitats. Expression of the pstS gene has been observed in both Prochlorococcus MED4 and MIT9313 under P limitation (Martiny et al., 2006). Synechococcus WH8102 has three pstS genes, and in promoter region of these genes, pho boxes equivalent to the pho box in E.coli were computationally predicted (Su et al., 2003); however, later the same researchers did not confirm their presence (Su et al., 2007).
Notably, all picocyanobacteria harbor \textit{phnCDE} genes for phosphonate transport and genes for potential phosphonate degradation \textit{phnX} and \textit{phnW} (Palenik et al., 2003; Su et al., 2003, Moore et al., 2005; Su et al., 2007). Considering that phosphonate may be a significant part of DOP pool in oligotrophic oceanic regions, the ability to utilize phosphonate can be highly beneficial for picocyanobacteria and support their abundance and dominance in P-limited environments.

1.3.4 P metabolism and \textit{pho} regulon: conclusion

To survive in P limited environment, bacteria and cyanobacteria including picocyanobacteria activate mechanisms for scavenging any remains of P and utilizing alternative P sources. These mechanisms are regulated by the two component regulatory system PhoR/PhoB in bacteria and SphS/SphR in cyanobacteria, and presence of PhoB or SphR is required for activation. Interestingly, the absence of SphR in some cyanobacteria and picocyanobacteria does not indicate the lack of a P stress response (Dyhrman et al., 2006; Martiny et al., 2006). Thus, additional regulators are to be discovered. While it is relatively well understood what genes for P metabolism are present in picocyanobacteria, functionality and regulation of these genes remains largely obscure, and an example are the phosphonate assimilation genes, which is the topic of my dissertation.

1.4 Phosphonates and their utilization by bacteria

Phosphonates are organic molecules containing a covalent bond between atoms of phosphorus and carbon (Figure 1.5); the recalcitrance of the bond is due to higher activation energy for hydrolysis compared to the phosphate monoester linkage (Black, 1991). Reactive
phosphonic acids are the only organic P compounds documented in meteorites and have been proposed as the first prebiotic organic P during the early stages of Earth’s evolution (Degraaf et al., 1997; Quinn, 2000).

Bacteria, some lower eukaryotes, and plants are known to synthesize phosphonates (Horigushi, 1984; Kugler et al., 1990; Shoji et al., 1986); however, only prokaryotes and some fungi are capable of assimilating phosphonates as a source of P, N, or C (Kononova and Nesmeyanova, 2002).

![Structures of orthophosphate and some phosphonates](from Kononova and Nesmeyanova, 2002)

**Figure 1.5** Structures of orthophosphate and some phosphonates (from Kononova and Nesmeyanova, 2002)

### 1.4.1 C-P lyase

Four pathways have been described to date for phosphonate assimilation. The C-P lyase pathway is a broad specificity multienzyme system, which cleaves the C-P bond by a yet unknown mechanism resulting in a hydrocarbon and P$_i$ (Wackett et al., 1987; Wanner, 1994). In *E. coli*, this system is encoded by a 10.9 kb operon of 14 genes including the ABC transporter
system *phnCDE* (Yakovleva et al., 1998). The C-P lyase pathway has been found in many bacteria, mostly in proteobacteria (Quinn et al., 2007), but also in cyanobacteria. Thus, *Trichodesmium* IMS101 expresses the lyase-associated genes in cultures under P limitation. Expression was observed in environmental samples as well (Dyhrman et al., 2006). For a thorough review on C-P lyase see Ternan et al., 1998; Kononova and Nesmeyanova, 2002; and Huang et al., 2005.

### 1.4.2 Phosphonatases

In contrast to C-P lyase, three other pathways appear to be substrate specific, as well as their transport system (Ternan et al., 1998). Degradation of 2-AEP includes two reactions; first is transamination from 2-AEP to pyruvate resulting in phosphonoacetaldehyde and alanine, and second reaction is hydrolysis of phosphonoacetaldehyde to acetaldehyde and P\(_i\) (Figure 1.6).

Two enzymes responsible for 2-AEP degradation, 2-AEP:pyruvate aminotransferase and phosphonoacetaldehyde hydrolase (phosphonatase), were first discovered in *Bacillus cereus* (LeNauze et al., 1977), later in *Pseudomonas aeruginosa* (Dumora et al., 1989), *Enterobacter aerogenes*, *Salmonella typhimurium* (Jiang et al., 1995), and *Pseudomonas putida* (Ternan and Quinn, 1998). These two enzymes are encoded by *phnW* and *phnX* respectively.

Assimilation of phosphonoacetate, the natural existence of which is not known, is performed by phosphonoacetate hydrolase in *Pseudomonas fluorescens* (McMullan and Quinn, 1994; McGraph et al., 1995). Finally, *Burcholderia* spp. possess genes responsible for hydrolysis of 2-amino-3-phosphonopropionate, a component of phosphonolipids (Ternan and Quinn, 1998b). Similarly, to 2-AEP, degradation of 2-amino-3-phosphonopropionate includes two
reactions, transamination and hydrolysis. Both pathways exist in many other bacteria (review by Quinn et al., 2007).

In addition to these described above, novel pathways likely to be discovered. Thus, growth of *Campylobacter* spp. has been observed on a variety of phosphonates as a sole P source, but genes for none of the described pathways have been found in the genomes of these species (Mendz et al., 2005).

![Phosphonatase pathway](image)

**Figure 1.6** Phosphonatase pathway (from White and Metcalf, 2007)

1.4.3 Regulation of phosphonate assimilation

Genes for phosphonate assimilation by C-P lyase are induced by P limitation (Chen et al., 1990; Metcalf and Wanner, 1993). However, genes for phosphonatase pathways may be part of the *pho* regulon (Lee et al., 1992; Baker et al., 1998; Ternan and Quinn, 1998), as well as P independent and substrate inducible. For example, expression of phosphonatase is activated
under P limitation in *S. typhimurium*, *E. aerogenes*, and *Pseudomonas* spp., except *P. putida* (Jiang et al., 1995). By contrast, in *P. putida* and *P. fluorescens*, genes are induced by the presence of 2-AEP and phosphonoacetate respectively in P-replete conditions (Quinn, 2002; Kulakova et al., 2001). The general tendency is that bacteria with *pho* regulation of the pathway are able to utilize phosphonate as a P source only, and bacteria with a substrate inducible pathway assimilate phosphonate as a source of P and/or N and/or C.

1.4.4 Picocyanobacteria and phosphonates

In picocyanobacteria, potential phosphonate utilization pathways are represented by a hybrid system. The *phnCDE* operon encodes an ABC transporter for a wide variety of phosphonates, and this transporter is usually associated with the C-P lyase pathway. However, the presence of *phnX* and *phnW* genes (Palenik et al., 2003; Su et al., 2003; Moore et al., 2005), that encode the phosphonatase pathway, suggests the capability of picocyanobacteria to utilize 2-AEP only. Thus, ability of *Synechococcus* WH8102 to grow on 2-AEP and ethylphosphonate (Palenik et al., 2003; Su et al., 2003) indicates that either phosphonatase is capable of hydrolyzing other phosphonates or there are additional yet unknown enzymes present. The attempts to grow *Prochlorococcus* on phosphonate as a sole P source have failed so far, and activation of the *phn* gene expression has not been observed under P limitation in a short term in MED4 and MIT 9313 (Martiny et al., 2006), but assimilation of phosphonates in picocyanobacteria has not been comprehensively addressed yet. Moreover, no research has been conducted on utilization of phosphonates in freshwater picocyanobacteria. Since no genome of freshwater *Synechococcus* has been sequenced, it is not known if they have a genetic potential to assimilate phosphonate as both marine *Synechococcus* and *Prochlorococcus* do.
1.4.5 Phosphonates assimilation: conclusion

Four general pathways for phosphonate utilization have been described for bacteria; picocyanobacteria possess a hybrid system consisting of a C-P lyase transporter and 2-AEP phosphonatase genes (Palenik et al., 2003; Su et al., 2003). Largely unknown is whether or not all picocyanobacteria can assimilate phosphonates, and if so, the phosphonates substrate range and the genetic regulation of phosphonate utilization.

1.5 Rationale for this dissertation

To summarize, phosphorus is an essential element, but it is often limiting in many marine and freshwater ecosystems (e.g. Schindler, 1977; Björkman et al., 2000; Karl et al., 2001). Cyanobacteria have evolved adaptations to P limitation by producing proteins for high affinity P scavenging and utilization of alternative P sources, including the phosphonates. Naturally occurring phosphonates may contribute significantly to the bioavailable phosphorus pool in P-limited marine regions (Clark et al., 1998; Kolowith et al., 2001; Dyhrman et al., 2007; Karl et al., 2008). In lakes, the presence of naturally derived phosphonates has not been reported or studied; nevertheless, bacterial degradation of the anthropogenically-derived phosphonates (e.g. Glyphosate) may introduce a new unrecognized P; into the food web at large (Dick and Quinn, 1995; this dissertation). Picocyanobacteria are abundant and dominant in oligotrophic aquatic systems (e.g. Olson et al., 1990a; Campbell and Vaulot, 1993; Weisse, 1993). The high diversity of Synechococcus and Prochlorococcus results in niche partitioning that ensures their abundance. Despite the differences in P metabolism among picocyanobacteria (Moore et al., 2005; Martiny et al., 2006), the ability for phosphonate utilization may be a common feature. If so, picocyanobacteria collectively would benefit in surviving and thriving in P limited environments.
Considering that picocyanobacteria are major contributors to the primary production in oligotrophic waters (e.g. Waterbury et al., 1986; Nagata et al., 1994, Partensky et al., 1999; DuRand et al., 2001), understanding the P physiology of picocyanobacteria is important in terms of global biogeochemical cycles.

1.6 Objectives

In the present research, phosphonate utilization by marine and freshwater picocyanobacteria genera *Synechococcus* and *Prochlorococcus* has been assessed. Specific objectives that were addressed are following:

- to determine the genetic potential that freshwater *Synechococcus* spp. possess to assimilate phosphonates via a PCR based assay designed for the phosphonate transporter gene *phnD*;
- to assess expression of the *phnD* gene in cultures and environmental RNA by applying an RT-PCR assay;
- to analyze regulation of the phosphonate assimilation in marine *Synechococcus* WH8102 and freshwater *Synechococcus* ARC-21 by using quantitative RT-PCR;
- to assess the ability to utilize phosphonate as a sole P source in marine and freshwater *Synechococcus* spp. by performing amendment experiments; particularly, utilization of the glyphosate by freshwater *Synechococcus*. 
2 CHAPTER II. DETECTION AND EXPRESSION OF THE PHOSPHONATE TRANSPORTER GENE phnD IN MARINE AND FRESHWATER PICOCYANOBACTERIA

In press in *Environmental microbiology*.

Supplementary material is included as Appendix I.

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2.1 Abstract

We describe a PCR-based assay designed to detect expression of the phosphonate assimilation gene *phnD* from picocyanobacteria. The *phnD* gene encodes the phosphonate binding protein of the ABC-type phosphonate transporter, present in many of the picocyanobacterial genome sequences. Detection of *phnD* expression can indicate a capacity of picoplankton to utilize phosphonates, a refractory form of phosphorus that can represent 25% of the high molecular weight dissolved organic phosphorus pool in marine systems. Primer sets were designed to specifically amplify *phnD* sequences from marine and freshwater
*Synechococcus* spp., *Prochlorococcus* spp. and environmental samples from the ocean and Laurentian Great Lakes. Quantitative RT-PCR from cultured marine *Synechococcus* sp. strain WH8102 and freshwater *Synechococcus* sp. ARC-21 demonstrated induction of *phnD* expression in P-deficient media, suggesting that *phn* genes are regulated coordinately with genes under *phoRB* control. Last, RT-PCR of environmental RNA samples from the Sargasso Sea and Pacific Ocean detected *phnD* expression from the endemic picocyanobacterial population. *Synechococcus* spp. *phnD* expression yielded a depth-dependent pattern following gradients of P bioavailability. By contrast, the *Prochlorococcus* spp. primers revealed that in all samples tested, *phnD* expression was constitutive. The method described herein will allow future studies aimed at understanding the utilization of naturally-occurring phosphonates in the ocean as well as monitoring the acquisition of synthetic phosphonate herbicides (e.g. glyphosate) by picocyanobacteria in fresh waters.

2.2 **Introduction**

Phosphorus has been described as the “staff of life – the most essential of nutrients” owing to its importance to biota and the low ambient concentrations at which it is present in many surface waters (Karl, 2000). Phosphorus is an essential component of DNA, ATP and phospholipids and accounts for about 2–4% of the dry weight of most cells (Karl, 2000). Yet in many aquatic environments, dissolved inorganic phosphate (DIP) is near detection limits using standard analytical methods (Karl and Tien, 1992; Anagnostou and Sherrell, 2008). Indeed, the limitation of primary production by P availability is a central tenet of modern day limnology.
(Schindler, 1977), and P limitation is also a common feature in diverse marine environments (Krom et al., 1991; Wu et al., 2000; Karl et al., 2001; Sañudo-Wilhelmy et al., 2001; Dyhrman et al., 2007; Paytan and McLaughlin, 2007).

Complicating our understanding of P bioavailability is that the presumed dissolved P fraction may contain some non-reactive inorganic species such as polyphosphates (Diaz et al., 2008). Additionally, some components of the dissolved organic phosphorus (DOP) fraction may be less readily converted to total dissolved phosphorus (TDP) by conventional assay methods (Monaghan and Ruttenberg, 1999; Benitez-Nelson, 2000). Included in this category are naturally-occurring phosphonates that can comprise up to 25% of the high molecular weight DOP pool in the open ocean (Clark et al., 1998; Kolowith et al., 2001), and are generally thought to represent a P source more refractory to assimilation than organic phosphomonoesters (Benitez-Nelson, 2000; Dyhrman et al., 2007).

Phosphonates are organic molecules containing a covalent bond between carbon and phosphorus, thus differing from organic phosphates that contain a more easily hydrolyzed monophosphate ester linkage. The sources of phosphonates in the DOP pool are often unclear, but are likely derived from the degradation of glycolipids, glycoproteins, antibiotics and phosphonolipids (Clark et al., 1998; Kolowith et al., 2001; Dyhrman et al., 2006; White and Metcalf, 2007). Phosphonates can also be derived from anthropogenic activity. Indeed, agricultural runoff can contain phosphonates derived from glyphosate, a common herbicide originally produced under patent to Monsanto as Roundup® (Scribner et al., 2003; Byer et al. 2008).
Both *Trichodesmium* spp. and marine picocyanobacteria (*Synechococcus* spp. and *Prochlorococcus* spp.) have genes necessary for phosphonate utilization (Palenik et al., 2003; Su et al., 2003; Dyhrman et al., 2006). Whereas all the groups appear to harbor the *phnCDE* genes related to transport, phosphonate metabolism is mediated by a C-P lyase pathway in *Trichodesmium* sp. (Dyhrman et al., 2006) and likely by a proposed phosphonatase pathway encoded by *phnX* (also labeled *cbbY*) and *phnW* in the picocyanobacteria (Su et al., 2003). *Trichodesmium* sp. *phn* genes (including *phnD*) are inducible under P limitation (Dyhrman et al., 2006). In *Prochlorococcus* MIT9313 and MED4, induction of the *phnCDE* genes was not observed under short term (48 h) P limitation (Martiny et al., 2006), but the expression and regulation of the *phn* genes have not been comprehensively addressed across the multiple *Synechococcus* spp. and *Prochlorococcus* spp. ecotypes or in varying conditions of phosphorus bioavailability.

In freshwater ecosystems, it is well accepted that microorganisms (i.e. bacteria and cyanobacteria) comprise the dominant taxa capable of utilizing phosphonates (Huang et al., 2005). Assimilation of anthropogenically-derived phosphonates such as the herbicide glyphosate by microbes thus affords a mechanism for introducing new P, as microbially-derived phosphate, into the food web at large. Despite the possible significance of phosphonate compounds in P metabolism and biogeochemistry in freshwater systems, the presence and expression of this capacity has not been examined in detail in freshwater cyanobacteria. In this paper we describe the development and proof-of-concept of an assay to detect the presence and regulated
expression of the phosphonate transporter gene \textit{phnD} by picocyanobacteria in natural samples from a variety of both freshwater and marine environments.

2.3 Results

Oligonucleotide primers were developed for both \textit{Synechococcus} spp. and \textit{Prochlorococcus} spp. \textit{phnD} based on the PhnD primary structures deduced from the available genomic sequences. Primer design took into account the amino acid sequence dissimilarities between \textit{Prochlorococcus} spp. and \textit{Synechococcus} spp. \textit{phnD} at sites conserved within each genus. Forward and reverse primers employed the conserved amino acid sequence from \textit{Synechococcus} spp. PhnD residues 39-48, and 244-253, respectively (numbered using PhnD from WH8102). The \textit{Synechococcus} spp. specific primers were designed recognizing the variability in amino acid sequence in open ocean and coastal strains at positions 249 and 251, yielding two different reverse primers that in turn generated PCR products of either 616 or 618 basepairs (bp). Primers for \textit{Prochlorococcus} spp. \textit{phnD} were designed from the conserved PhnD sequences beginning at residues 103 and 184, yielding a PCR product of 242 bp. The specificity of the \textit{Synechococcus} spp. primers was assessed following PCR of DNA from \textit{Prochlorococcus} spp. Testing all representative strains from all \textit{Prochlorococcus} spp. clades (MIT9312, MED4, MIT9313, NATL2A, and CCMP1375) no PCR product was observed (Fig. S1A, Supplementary material). Similarly, the \textit{Prochlorococcus} spp. primers failed to amplify \textit{Synechococcus} spp. \textit{phnD} from marine strains WH5701, WH7803 and WH8102 as well as freshwater strains (Fig. S1B, Supplementary material, also see Fig. 1C).
2.3.1 PCR of *phnD* from *Synechococcus* and *Prochlorococcus* spp.

PCR with the *Synechococcus* spp. primer set successfully amplified *phnD* sequences from most environmental DNA and cultured picocyanobacteria tested from freshwater (Fig. 2.1A) and marine (Fig. 2.1B) habitats. Freshwater environmental DNA included four sites in Lake Erie (LE samples, Fig. 2.1A) and eleven cultured *Synechococcus* sp. isolates from Lake Erie (Maumee, KD3a, ARC-11) and Lake Superior (LS prefix, Fig. 2.1A). *Prochlorococcus* spp. *phnD* were not amplified using this primer set, and no amplicon was observed in PCR of *Synechococcus elongatus* sp. strain PCC 7942 DNA, a strain from outside the picocyanobacterial lineage that lacks *phnD* (GenBank accession number NC007604). This demonstrated the specificity of the PCR for *Synechococcus* spp. of the picoplankton clade *sensu* Urbach et al. (1998). Similarly, PCR yielded the ca. 620 bp *phnD* product from all cultured marine *Synechococcus* spp., as well as from most environmental DNA samples from the Sargasso Sea, South Pacific, and Monterey Bay (Fig. 2.1B). Only one sample from the deep chlorophyll maximum (95 m) at the Bermuda Atlantic Time-Series (BATS) station (Fig. 2.1B) did not amplify. Last, the *Prochlorococcus* spp. primer set specifically amplified *phnD* sequences from all oceanic environmental samples tested, but did not amplify *phnD* from *Synechococcus* sp. WH8102 (Fig. 2.1C). Thus, the PCR assay exhibits the specificity necessary to differentiate *phn* gene expression in mixed picocyanobacterial populations.

All cloned amplicons obtained were verified as *Synechococcus* sp. or *Prochlorococcus* sp. *phnD* sequences by searching the GenBank nonredundant database (summarized in Table S1, Supplementary Material). Neighbor-joining phylogenetic analysis of representative PhnD
sequences from cultured picocyanobacteria (open symbols) and environmental DNA (closed symbols) revealed that the freshwater *Synechococcus* spp. sequences yielded a cluster distinct from the marine sequences, with the exception of the euryhaline *Synechococcus* sp. WH5701 (Fig. 2.2). *Prochlorococcus* spp. sequences yielded an additional cluster, and all three picocyanobacterial clusters were phylogenetically distant from PhnD sequences obtained from both filamentous cyanobacterial genomes and *Synechococcus* sp. strain PCC 7002, a strain excluded from the picoplankton clade (Fig. 2.2).

**Figure 2.1** PCR of *phnD* from environmental DNA and cultured picocyanobacteria.  
*Panel A:* PCR of *phnD* from freshwater environmental DNA and cultured freshwater *Synechococcus* spp. Environmental DNAs are from Lake Erie stations 961, Sandusky Bay (SB), MB20 and from the Maumee River that flows into Lake Erie’s western basin. Cultured cyanobacteria of the picoplankton clade include KD3a and ARC-11 from Lake Erie, and isolates from Lake Superior (LS prefix). Negative controls include *Prochlorococcus* sp. MIT9312 and *Synechococcus* sp. strain PCC 7042.  
*Panel B:* PCR of *phnD* from cultured marine *Synechococcus* (WH5701, WH8102 and WH7803) and marine environmental DNA from the Bermuda Atlantic Time Series (BATS) station in the Sargasso Sea, two South Pacific stations (SP), and seasonal samples from Monterey Bay, CA station M1 (MB).  
*Panel C:* PCR of *Prochlorococcus* spp. *phnD* from environmental samples from BATS, two South Pacific stations (SP), and *Prochlorococcus* sp. strains NATL2A, MIT9211, MED4, MIT9313, MIT9312, and CCMP1375. NTC, no template control.
Figure 2.1 PCR of *phnD* from environmental DNA and cultured picocyanobacteria.
Figure 2.2 Phylogenetic tree of PhnD sequences deduced from DNA sequences obtained from freshwater *Synechococcus* spp., marine *Synechococcus* spp., *Prochlorococcus* spp., filamentous cyanobacteria, and amplicons from environmental DNA. Accession numbers are included where appropriate. Open symbols, cultured picocyanobacteria; closed symbols, environmental samples; squares and circles represent freshwater and marine environments, respectively.
2.3.2 *phnD* gene expression in *Synechococcus* sp. WH8102 and ARC-21 under P-limitation

It has been reported that *Synechococcus* WH8102 is capable of growing on 2-aminoethylphosphonate (2-AEP) as a sole P source (Palenik et al. 2003; Su et al. 2003); similarly, we were able to grow both marine (WH8102) and a freshwater *Synechococcus* (ARC-21) (Ivanikova et al. 2008) on 2-AEP (Figs. S2A and S2B, Supplementary material). Freshwater *Synechococcus* ARC-21 was able to utilize glyphosate as a sole P source as well. Based on these observations, we next assessed the *phnD* expression pattern during P limitation and growth on phosphonate in these two strains by quantitative RT-PCR.

In both low phosphate (LowP) and 2-aminoethylphosphonate (2-AEP) treatments, expression of WH8102 *phnD* and *phnX* as well as the *pstS* genes was activated in five days, and the expression of *phnD* and *phnX* paralleled the activation of the *phoB* gene (Fig. 2.3A). From three replicates, normalized fold expression was equal for both *phnD* and *phnX* genes in all samples, suggesting similar regulation for the phosphonate transport and phosphonatase operons. The decline in expression in LowP media after nine days was due to the loss of cell viability as suggested by bleaching of the cultures.

Expression of *pstS* in WH8102 was activated in 24 hours in 2-AEP but not in the LowP samples, where concentration of external inorganic P was higher at this time (Fig. 2.3B). All genes, except *pstS*, yielded greater fold expression in LowP than in 2-AEP after 5 days, which may be explained by an additional P availability after hydrolysis of 2-AEP by phosphonatase activity.
The expression pattern of \textit{phnD} in the Lake Erie freshwater \textit{Synechococcus} sp. strain ARC-21 revealed that the gene is inducible in LowP and P-free BG-11 media containing 2-AEP, although the induction is modest by comparison to WH8102 (Fig. 2.3C). Routinely, mRNA levels increased only 3-4 fold over P-replete growth conditions. Under these culture conditions, ARC-21 grows utilizing both 2-AEP and glyphosate as P sources (Fig. S2B, Supplemental material). A full understanding of the regulation of P-responsive genes in this strain awaits further investigation, when a genome sequence will allow development of appropriate PCR primers for \textit{phoB}, \textit{pstS} and \textit{phnX}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2_3.png}
\caption{Quantitative RT-PCR. Panels \textit{A} and \textit{B}: Quantitative RT-PCR of genes involved in phosphorus acquisition in WH8102 cultures following transfer to LowP medium (Panel \textit{A}) and P-free medium containing 10 mM 2-aminoethylphosphonate (Panel \textit{B}).}
\end{figure}
Figure 2.3 Quantitative RT-PCR (cont.) Expression of *pstS, phoB, phnD* and *phnX* were normalized to *rnpB* expression and corresponding P-replete samples. Panel C: Quantitative RT-PCR of *phnD* in ARC-21 cultures following transfer to LowP BG-11 or P-free BG-11 containing 10 mM 2-AEP.
2.3.3 RT-PCR of *phnD* sequences from environmental RNAs

To test the proof-of-concept that *phnD* expression in environmental samples can be monitored by both primer sets, the *Synechococcus* and *Prochlorococcus* spp. *phnD* primers were employed in RT-PCR assays with RNA extracted from marine seston. Samples from surface waters of Monterey Bay, the South Pacific and the Sargasso Sea each yielded a *phnD* RT-PCR product (Figs. 2.4A and 2.4B). One sample (95 m from the BATS station in the Sargasso Sea) yielded *Prochlorococcus* spp. *phnD* amplicons, but no PCR or RT-PCR products were detected with the *Synechococcus* spp. primer set (Figs. 2.1B and 2.4A).

During October 2007, at Sargasso Sea stations BATS (Sta. 2), 4, 6 and 9, expression of the *Synechococcus* spp. *phnD* was observed in all surface mixed layer samples. At depth, whereas *phnD* was detected by PCR in seston collected from stations 4, 6 and 9, RT-PCR demonstrated that the gene was not expressed (Fig. 2.5A). This expression profile largely coincided with the concentration of DIP in the samples (Table 2.1) showing depletion of DIP at the surface and its increase at depth. In general, expression of *phnD* was observed at DIP concentrations below 1 nM. The sensitivity of the PCR assay allowed the detection of *Synechococcus* spp. *phnD* at cell density as low as 130 cells mL$^{-1}$ (Table 2.1). Alternatively, expression of the *Prochlorococcus* spp. *phnD* was observed in all Sargasso Sea samples at all depths (Fig. 2.5B).

Sequencing of the cloned PCR and RT-PCR products revealed that the *Synechococcus* *phnD* primer set amplified diverse *Synechococcus* spp. strains from open ocean, neritic, and freshwater environments (Table S1). The Monterey Bay samples yielded *phnD* sequences with
the closest nucleotide identity to coastal *Synechococcus* strains (BL107, CC9902), and the Sargasso Sea samples yielded sequences with the highest identity to an open ocean strain (WH8102). The *Prochlorococcus* *phnD* primer set amplified *phnD* sequences from *Prochlorococcus* spp. AS9601, MIT9301, MIT9312, and MIT9215 from both South Pacific and Sargasso Sea samples taken at the surface and the deep chlorophyll maximum depth, reflecting the dominance of the HL II ecotype (Moore et al. 1998, Rocap et al. 1999) in these regions (Ahlgren et al. 2006, Zinser et al. 2006, Zinser et al. 2007).
Figure 2.4 RT-PCR of \textit{phnD} from environmental samples. \textit{Panel A}: RT-PCR of \textit{phnD} from seston RNA from the Bermuda Atlantic Time Series (BATS) station in the Sargasso Sea and Monterey Bay station MB1 (MB); \textit{Synechococcus} sp. WH8102 RNA was used as a positive control. \textit{Panel B}: RT-PCR of \textit{Prochlorococcus} spp. \textit{phnD} sequences from environmental RNA from BATS and two South Pacific Ocean stations (SP).
Figure 2.5 RT-PCR of *phnD* from Sargasso Sea samples taken in October 2007. Panel A: *Synechococcus* spp. *phnD*. Panel B: *Prochlorococcus* spp. *phnD*. *Synechococcus* spp. *phnD* expression in samples from the surface (5 m) and the lack of expression at deep chlorophyll maximum (95 – 120 m) suggest the availability of P with depth (summarized in Table 2.1).

<table>
<thead>
<tr>
<th>STATION</th>
<th>DEPTH (m)</th>
<th>DIP (nM)</th>
<th>SYN cells ml⁻¹</th>
<th>PRO cells ml⁻¹</th>
<th>SYN PHND DNA</th>
<th>RNA</th>
<th>PRO PHND DNA</th>
<th>RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>BATS</td>
<td>4</td>
<td>0.5</td>
<td>6424</td>
<td>NA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
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<td></td>
<td>95</td>
<td>12.89</td>
<td>2688</td>
<td>133597</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sta. 4</td>
<td>5</td>
<td>1.1</td>
<td>NA</td>
<td>NA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>119</td>
<td>1.3</td>
<td>130</td>
<td>45612</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sta. 6</td>
<td>5</td>
<td>1.2</td>
<td>NA</td>
<td>NA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>4.6</td>
<td>246</td>
<td>55673</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sta. 9</td>
<td>4</td>
<td>0.1</td>
<td>NA</td>
<td>NA</td>
<td>+</td>
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<td>NA</td>
<td>NA</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 2.1 Phosphate (DIP) concentration, picocyanobacterial cell number, and *phnD* amplification at Sargasso Sea stations, October 2007
2.4 Discussion

2.4.1 Detection of *phnD* in cultured picocyanobacteria and environmental samples

In this paper, we describe a PCR-based assay to detect the presence and expression of *phnD*, and thus the potential assimilation of phosphonates by picocyanobacteria in diverse aquatic environments. Indeed, an initial survey of environmental samples from the Laurentian Great Lakes, Atlantic and Pacific Oceans indicate that *Synechococcus* spp. and *Prochlorococcus* spp. *phnD* is detectable with our primer sets and assay conditions. The one exception was the lack of amplification of *Synechococcus* spp. *phnD* from a deep chlorophyll maximum sample from the Sargasso Sea, a location where the *Synechococcus* spp. abundance was relatively high (2700 cells mL\(^{-1}\); M. Lomas, personal communication). The failure to detect PCR amplicons in this instance was most likely due to errors in extracting DNA from this sample.

Clustering of *phnD* sequences in the phylogenetic analysis of the freshwater and marine members of the picocyanobacterial lineage demonstrates that the phosphonate transporter is likely an ancient feature of the picocyanobacterial core genome (Fig. 2.2). By contrast, *phnD* sequences from filamentous cyanobacteria are phylogenetically distinct from the picocyanobacterial orthologs, exhibit greater diversity and commonly form multigene families that may have arisen by gene duplication.
2.4.2 Regulation of *phnD* in cultured picocyanobacteria

Overall, the expression patterns in LowP media for *phnD*, *phnX*, and *pstS* (SYNW1018) genes in *Synechococcus* sp. WH8102 are consistent with *pho*-dependent regulation. Variations in the pattern of *pstS* expression (Fig. 2.3) also suggest that *pstS* may be under control by additional mechanisms independent of *phoRB*. PhoRB-dependent regulation for phosphonate utilization genes has been documented previously for many bacteria (Jiang et al. 1995, Kononova and Nesmeyanova 2002, Dyhrman et al. 2006). Su et al. predicted the PhoB box in the *Synechococcus* WH8102 promoters of phosphonate transporter (e.g. *phnD*) and phosphonatase (e.g. *phnX*) operons, and in all *pstS* genes (Su et al. 2003), consistent with many of the regulation patterns observed herein. However, the same research group later did not report the presence of a PhoB box in all above-mentioned genes (Su et al., 2007). Therefore, further investigation is needed to demonstrate whether phosphonate utilization genes are directly regulated by PhoBR or indirectly by some yet unknown mechanism.

The lack of *phnD* expression prior to 5 days in LowP SN medium may be a response to changes in the internal, not external, Pi concentration, so that gene expression occurs following depletion of the internal Pi quota. This suggestion is consistent with the findings of a computational analysis suggesting that PhoR in *Synechococcus* sp. WH8102 is a soluble protein (Su et al. 2003) in contrast to a cytoplasmic membrane protein in *E.coli* (Scholten and Tommassen, 1993).

Since *phnD* is typically cotranscribed with the *phnC* and *phnE* genes that together encode the complete phosphonate ABC transporter (e.g. Huang et al., 2006), we suggest that RT-PCR of
*phnD* can serve as a proxy for picocyanobacterial phosphonate acquisition. The ARC-21 strain of freshwater *Synechococcus* sp. tested here also expresses *phnD* as a consequence of P limitation. As such, the *phnD* RT-PCR assay may provide information regarding the P status of picocyanobacteria in both marine and freshwaters. In particular, we are interested in determining the threshold (if any) at which picocyanobacteria specifically activate *phn* expression as phosphate pools become depleted. These detailed expression studies are critical given the potential heterogeneity of expression patterns and P acquisition strategies present among strains of picocyanobacteria (Martiny et al., 2006; Moore et al., 2005).

### 2.4.3 Regulation of *phnD* in field samples

Analysis of environmental samples demonstrated that expression of *Synechococcus* spp. *phnD* was detectable in surface samples from sites exhibiting low P availability. In agreement with the expression data from the WH8102 cultures, expression at depth was repressed with increasing DIP. However, a few nearshore samples analyzed suggested that *pho*-independent mechanisms might regulate *phn* expression in at least some *Synechococcus* spp. strains. Sequences from a cloned RT-PCR product from Monterey Bay resulted in the closest nucleotide identity (ca. 95%) to *Synechococcus* sp. CC9902 (Table S1). Since this strain lacks the *phoB* gene (Su et al. 2007), *phnD* might be under alternative control or expressed constitutively if assuming *pho* regulation for the phosphonate transporter genes. A future widespread depth-dependent survey of *phn* expression from nearshore and pelagic ecosystems will help clarify whether multiple mechanisms exist for *phn* transcriptional regulation.
By contrast, \textit{phnD} expression in \textit{Prochlorococcus} spp. was uniformly constitutive in all environmental samples tested thus far (Figs. 2.4C, 2.5B and Table 2.1), suggesting a lack of regulation of the \textit{phnCDE} operon in HLII clade. Whether the uniform presence of the \textit{phnD} RT-PCR amplicon is truly due to constitutive expression awaits further investigation, because the samples employed typically contained 50-60 fold more \textit{Prochlorococcus} spp. vs. \textit{Synechococcus} spp. cells in the DCM (Table 2.1). Indeed, Martiny et al. (2006) demonstrated that \textit{Prochlorococcus} spp. \textit{phn} genes were not activated during a 48 h adaptation to P deficiency.

Analysis of a larger set of environmental samples providing detailed spatial and depth resolution across the nutricline will help determine the threshold DIP concentration yielding \textit{phnD} transcription among the picocyanobacteria.

\section*{2.5 Concluding remarks}

Phosphonates represent a potentially important reservoir of P in aquatic environments depleted of DIP. Naturally-occurring phosphonate compounds can account for 25\% of the high molecular weight DOP pool in the open ocean (Clark et al., 1998; Kolowith et al., 2001). Anthropogenic phosphonates, such as the herbicide glyphosate, contribute to the phosphonate pool in lakes and their watersheds (Byer et al. 2008) and possibly to the ocean margins. Phosphonates are even implicated in the accumulation of greenhouse gases; recent studies have indicated that microbial utilization of methyl phosphonate in marine environments can yield the aerobic generation of atmospheric methane (Karl et al. 2008).
The RT-PCR results herein demonstrate expression of \textit{phnD} in a wide variety of environments in both the Pacific and Atlantic, and spanning both open ocean and coastal ecosystems. These results underscore the utility of the method, and suggest that phosphonate acquisition could be broadly important to the physiological ecology of marine picocyanobacteria. The development and application of quantitative RT-PCR assays for \textit{phnD} in these groups, in combination with additional studies on the regulation of \textit{phnD}, would be a valuable mechanism for examining the phosphorus physiology of the picocyanobacteria in more detail from both marine and freshwater environments.

2.6 Experimental procedures

2.6.1 Environmental samples and picocyanobacterial strains

Table 2.2 provides details of sampling and sources of picocyanobacterial cultures. Seston from the environmental samples were harvested by filtering onto 0.22 $\mu$m polycarbonate filters (Millipore) that were transferred into 2 mL cryo tubes and preserved with RNALater (QIAGEN), or alternatively, seston was collected onto 0.22 mm Sterivex cartridge filters (Millipore) and immediately frozen in liquid N$_2$. The filters and cryo tubes were stored at -80°C in the lab prior to DNA or RNA extraction.

2.6.2 Cell culturing methods

Freshwater \textit{Synechococcus} spp. cultures were maintained at 23°C in BG-11 medium (Allen, 1968; as described at http://www-cyanosite.bio.purdue.edu) under continuous
illumination of 5 - 10 µmol quanta m\(^{-2}\) s\(^{-1}\). ARC-21 is a phycoerythrin-rich freshwater strain isolated from pelagic Lake Erie (Ivanikova et al., 2008). *Synechococcus* sp. WH 8102 was grown at 25°C in SN medium (Waterbury et al., 1986) under continuous illumination of 25 µmol quanta m\(^{-2}\) s\(^{-1}\). Under these illumination conditions, growth rates of newly inoculated cultures were typically ca. 0.6 d\(^{-1}\). K\(_2\)HPO\(_4\) was omitted in P-deplete BG-11, added at concentration of 8.6 µM in LowP SN, or substituted by 10 µM 2-aminoethylphosphonate (Sigma) in 2-AEP BG-11 and 2-AEP SN. For quantitative RT-PCR, *Synechococcus* sp. ARC-21 and WH8102 were cultured to mid exponential phase in BG-11 and SN media, respectively. Cells were harvested by filtration, washed twice with a P-free medium, and inoculated into BG-11 or SN with the different P amendments described above. Three replicates were performed. Samples for RNA extraction were harvested by centrifugation and preserved with RNAlater (QIAGEN).

2.6.3 Preparation of DNA and RNA from samples and cultures

DNA was isolated from culture material using either phenol-chloroform extraction or the DNeasy Tissue Kit (QIAGEN) according to the manufacturer’s procedures. In the phenol-chloroform method, 5 mL of late exponential phase culture was used. Cells were harvested by centrifugation (10 min, 3,000 x g), washed with 1.5 mL of TE buffer (10 mM Tris-HCl, 1 mM Na\(_2\) EDTA [pH 8.0]) and resuspended in 300 µL of STET buffer (50 mM Tris-HCl, 50 mM sodium EDTA, 5% Triton X-100, 8% sucrose [pH 8.0]), containing lysozyme (10 mg mL\(^{-1}\)). The tubes were incubated at 37°C for 30 min followed by addition of 0.3 volume of 10% (wt/vol) sodium dodecyl sulfate (SDS) and incubation at 65°C for 40 min. Further, 0.3 volume of 5 M
NaCl was added, and tubes were incubated at 65°C for additional 20 min. After incubation, the phenol-chloroform-isoamyl alcohol (25:24:1) extraction was performed 2 times followed by a chloroform extraction. For the DNeasy Tissue Kit, 1.5 mL of culture material was used, and the manufacturer’s lysis buffer was replaced with the STET buffer. For environmental samples filtered onto 0.22 mm polycarbonate membranes (Millipore), 2 mL of TE buffer was added to a 15 mL Falcon tube containing a filter, and the tube shaken to resuspend the filtered seston. Following centrifugation, DNA was extracted from the pellet as described above or with the DNeasy Tissue Kit. For extraction of DNA from a Sterivex filter, 2 ml of the 0.4 x STET buffer containing lysozyme (10 mg mL⁻¹) were injected into the Sterivex filter, and the filter was incubated at room temperature for 1 h. The solution was removed with a syringe and placed into a 15 ml Falcon tube. DNA extraction was continued by the phenol-chloroform method as described above.

RNA was extracted from the culture material and environmental samples using the RNeasy Mini Kit and RNAProtect Bacteria reagent (QIAGEN) with minor modifications from the manufacturer’s instructions. The protocol for enzymatic lysis of bacteria was used with TE buffer containing 15 mg mL⁻¹ lysozyme. For extraction of RNA from a Sterivex filter, 2 mL of RNAProtect Bacteria reagent (QIAGEN) were passed through the filter. Next, 400 µl of the TE/lysozyme buffer were injected into the filter. After incubation for 10 min at room temperature with vortexing every 2 min, 1.4 mL of the RNA Lysis buffer were added, and the filters were incubated for 1 h at room temperature. The solution was removed through an inlet with a syringe and decanted into a 15 ml Falcon tube, and RNA extraction was continued
according to the manufacturer’s instructions. The Sterivex filter could be reused for DNA extraction. On-column DNase digestion was performed on RNA samples using RNase-free DNase (QIAGEN). RNA samples were stored at -80°C.

2.6.4 PCR and RT-PCR amplification of phnD from *Synechococcus* and *Prochlorococcus* spp.

To amplify the phnD sequence from *Synechococcus* spp., following primers were designed: *phnD* _syn119F: 5’-TCGGNGCMATYCCSGATCAGAACCCSG-3’; *phnD* _syn734R1: 5’-TTGGGCTGSGCGASCCAGTGGTARTC-3’; *phnD* _syn731R2: 5’-GGNCGNGCCACCCAGTGGTARTC-3’. Both reverse primers were used in a single reaction. For amplification of the phnD sequence from *Prochlorococcus* spp., following primers were designed: *phnD* _pro307F: 5’-GTNATWGCTCAAAGAGATATWGAT-3’; *phnD* _pro551R: 5’-GTTGCATCATGACTNCCRCTATANCC-3’. The phnD sequences were amplified using a PTC-100 Programmable Thermal Controller (MJ Research, Inc). Each PCR reaction (25 µL) contained 1 x PCR buffer (Promega), 0.2 mM of each deoxynucleotide (Promega), 0.5 µM of each primer, and 1.0 unit of GoTaq DNA polymerase (Promega), and ca 10 ng of the template DNA. For *Synechococcus* spp. phnD amplification, the temperature profile was 95°C for 5 min, 40 cycles of 95°C for 1 min, an initial annealing temperature of 65°C for 1 min decreasing by 0.5°C each cycle until 55°C was reached, 72°C for 1 min, followed by extension at 72°C for 20 min. For *Prochlorococcus* spp. phnD primers, the annealing temperature was 57°C decreasing by 0.5°C each cycle until 50°C was reached, and the remaining PCR profile was the same as for
Synechococcus spp. *phnD*. RT-PCR was performed using a OneStep-RT-PCR kit (QIAGEN) according to the manufacturer’s instructions with a PCR profile as stated above. Amounts of RNA used per reaction were ca. 10 ng. Additionally, each reaction was performed without RT to ensure the absence of genomic DNA in the RNA samples. PCR and RT-PCR products were resolved on 2% agarose gels. All PCR and RT-PCR amplifications were repeated at least three times. Selected bands were analyzed after cloning into TOPO plasmid vectors (Invitrogen), and amplicons were sequenced at the University of Chicago Cancer Research Center using the T7 primer.

2.6.5 Real-time PCR

RNA extracted from the *Synechococcus* sp. WH8102 and ARC-21 P amendment experiments was reverse-transcribed by using iScript™ cDNA Synthesis kit (BIO-RAD) following the manufacturer’s instructions. Real-Time PCR was performed with the MyiQ™ Real-Time PCR Detection System (BIO-RAD) using IQ™ SYBR® Green Supermix (BIO-RAD) with 0.25 μM of each primer per reaction. Primers for the *rnpB*, *hcp*, *phnD*, *phoB*, *pstS*, and *phnX* genes were designed to amplify ca 89-150 nucleotides, and their efficiencies were estimated by dilution series over 2 orders of magnitude on cDNA derived from WH8102 or ARC-21 RNA from P-replete samples (Table 2.3). All real-time PCR amplifications were performed in triplicate and no-template controls were run in parallel. No-RT controls were performed for each reaction. After reverse transcription, cDNA was diluted three fold, and 5 μl of the diluted cDNA were used per reaction. For the *rnpB* gene, the cDNA was diluted 50-fold
additionally. The protocol for real-time PCR was as follows: 95°C for 3 min, 40 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 15 sec with fluorescence data collection, followed by a standard melting curve cycle. Normalized expression was calculated relative to a reference gene (Martiny et al. 2006) and P-replete condition using MyiQ Optical System software (ΔΔCₜ method). As a reference, the rnpB gene was chosen for *Synechococcus* sp. WH8102 and ARC-21 (Martiny et al. 2006). Fortuitously, the rnpB primers developed for marine picocyanobacteria amplified freshwater ARC-21 sequence with identical efficiency (Table 2.3).

### 2.6.6 Bioinformatics tools

Primers for *phnD* were designed based on the alignment of the *phnD* genes from available genomic sequences of *Synechococcus* spp. (NC 008319.1; NC 007516.1; NC 007513.1; NC 009481.1; NC 005070.1; NZ AATZ00000000; NZ AANP00000000; NZ AANO00000000; NZ AAOK00000000) and *Prochlorococcus* spp. (NC 008816.1; NC 009976.1; NC 008820.1; NC 007577.1; NC 005071.1; NC 008819.1; NC 007335.2; NC 005042.1; NC 005072.1) by using ClustalX – 1.83 software (Thompson et al., 1997). All sequence data obtained from PCR or RT-PCR amplification and subsequent cloning were analyzed manually by using FinchTV 1.4 (http://www.geospiza.com/finchtv/index.htm), ClustalX, BLASTX and BLASTN (http://www.ncbi.nlm.nih.gov/BLAST/). The phylogenetic tree was constructed with Mega 4.0 software (Kumar et al., 1994; http://www.megasoftware.net) using the neighbor-joining method and 1000 bootstrap replicates. The homologous PhnD
sequence of 206 amino acids deduced from the *Synechococcus* spp. *phnD* amplicon was used for phylogenetic analysis.

2.6.7 Accession numbers

*phnD* sequences obtained in this study were deposited in GenBank under accession numbers EU362636 - EU362729 and FJ172179 - FJ172204 (Table S1).

2.7 Acknowledgements

The authors thank Erik Zinser for providing *Prochlorococcus* spp. DNAs to all available ecotypes and Steven Wilhelm and Tom Bridgeman for collecting environmental DNA samples from Lake Erie. Emeka Anyawu assisted in the culturing of strain ARC-21. The work described in this paper was supported by awards OCE-0727644 from the National Science Foundation and R/ER-73 and R/ER-71 from the Ohio Sea Grant College Program (to G.S.B. and R.M.L.M.). The authors thank Michael Lomas (Bermuda Institute of Ocean Sciences) for sharing nutrient and cell count data from the Sargasso Sea.
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Table 2.2A  Environmental samples (A) and cultures (B) employed in this study
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Table 2.3 *Synechococcus* sp. WH8102 and ARC-21 specific primers for real-time PCR (E – efficiency)
References


3 CHAPTER III. EXPRESSION OF THE PICOCYANOBACTERIAL PHOSPHONATE TRANSPORTER GENE IN DIVERSE ENVIRONMENTS

3.1 Introduction

Phosphonates, previously thought to be recalcitrant, are utilized as a phosphorus source by microbial communities in aquatic environments deprived of inorganic phosphates (Clark et al., 1998; Benitez-Nelson et al., 2004; Dyhrman et al., 2006; Karl et al., 2008). Earlier, we have demonstrated that picocyanobacteria possess and express phnD encoding the phosphonate binding protein in a variety of environments. Moreover, expression of Synechococcus spp. phnD reflected the bioavailability of DIP, whereas constitutive expression was observed for Prochlorococcus spp. phnD (Ilikchyan et al., In press). Here, we extended our survey of environmental samples that ranged in space vertically and horizontally, as well as in time. The results of this study supported the importance of phosphonate acquisition to the physiology of marine picocyanobacteria and the utility of the RT-PCR assay with Synechococcus spp. phnD primers in assessing the P status in this picocyanobacterium. Three distinct aquatic ecosystems characterized by P limitation were considered: the Sargasso Sea, the Baltic Sea, and one of the Laurentian Great Lakes, Lake Erie.

The seasonal physical, chemical, and biological characteristics of the Sargasso Sea are well studied (Schroeder and Stommel, 1969). In winter, deep mixing results in bringing the cold, nutrient rich waters to the surface, whereas throughout summer and fall, the surface waters in the Sargasso Sea are stratified with a shallow (50 m), nutrient poor mixed layer. The physical and chemical changes of the surface waters are reflected in the abundance and productivity of phytoplankton (e.g. Menzel and Ryther, 1960, 1961). Picocyanobacteria constitute the majority
of photoautotrophic organisms in the Sargasso Sea. *Synechococcus* spp. reach maximum abundance in April-May shortly after winter mixing, and *Prochlorococcus* spp. maximum is in summer and fall (DuRand et al., 2001). The Sargasso Sea is an oceanic phosphorus depleted region with DIP concentrations less than 10 nM (e.g. Cotner et al., 1997; Karl, 2002). Under these conditions, utilization of phosphonates from the DOP pool can be an important survival strategy for picocyanobacteria. Indeed, the expression of phosphonate transporter genes were observed by endemic picocyanobacteria in the Sargasso Sea in October (Ilikchyan et al., In press). In this study, we have assessed picocyanobacterial phnD expression at different depths in May and October.

The Baltic Sea is a brackish environment connected to the North Sea through the Skagerrak and the Kattegat straits surrounding Denmark and Sweden. Cyanobacteria form extensive blooms in the Baltic Sea during stratification each summer (Sellner et al., 1997; Kononen et al., 1996). The blooms consist of filamentous nitrogen-fixing cyanobacteria and PE and PC-rich picocyanobacteria (*Synechococcus* spp.) (Stal et al., 2003; Haverkamp et al., 2008). Picocyanobacteria account for an average 80\% of total cyanobacterial biomass and contribute up to 50\% to the primary production of the cyanobacterial blooms (Stal et al., 2003). Although picocyanobacteria are in general nitrogen limited in the Baltic Sea (Stal et al., 2003), P limitation has been detected in the central Baltic Sea in July based on POP to biomass ratio, phosphate turnover time, and alkaline phosphatase activity (Nausch et al., 2004). The limitation of primary production by P in the Baltic Sea was deduced from DIP measurements earlier (Paasche and Erga, 1988). However, in contrast to extensive studies conducted on nitrogen limitation in the Baltic Sea (e.g. Ohlendieck et al., 2000; Stal and Walsby, 2000; Wasmund et al., 2001), P limitation has not received much attention. An increased occurrence and abundance of blooms
has been documented recently (Larsson et al., 2001) leading to higher amounts of fixed nitrogen in the ecosystem that could promote a shift in the Redfield N:P ratio. Here, we have attempted to assess possible P limitation and phosphonate utilization in the picocyanobacteria in the Baltic Sea, Skagerrak, and Kattegat with the RT-PCR method developed previously (Ilikchyan et al., In press).

In freshwater environments, the limitation of primary production by phosphorus is well known (e.g. Schindler, 1977). Lake Erie is the smallest yet the most productive of the Laurentian Great Lakes (Munawar and Weisse, 1989). While the western basin of Lake Erie is shallow and mesotrophic, the central and eastern basins are deeper and considered oligotrophic (Makarewicz and Bertram, 1991). Picocyanobacteria yield a significant biomass fraction of the Lake Erie phytoplankton (Fahnenstiel et al., 1986), and the distribution of diverse Synechococcus spp. follows limnological distinctions of the basins. PC-rich Synechococcus spp. are dominant in the western basin, and PE-rich Synechococcus spp. are dominant in the central and eastern basins (Pick, 1991; Wilhelm et al., 2006; Ivanikova et al., 2008; Cupp and Bullerjahn, unpublished). Previously, we have shown that primers for Synechococcus spp. phnD yielded a PCR amplicon from all available cultured isolates from Lake Erie and from several natural surface water samples, thereby establishing the genetic potential for phosphonate utilization. In the present study, we have analyzed a time series of environmental samples from Lake Erie for picocyanobacterial phnD expression.
3.2 Results

3.2.1 Picocyanobacterial phnD expression in the Sargasso Sea in May and October 2008.

The presence and expression of Synechococcus spp. and Prochlorococcus spp. phnD was assessed in samples collected in the Sargasso Sea cruises in May and October 2008. Samples were analyzed from three depths (surface, 40 m, and 100 m) in May and from four depths (surface, 40 m, DCM, and 200 m) in October. Additionally, a nutrient amendment incubation experiment was conducted on board during the October cruise.

DNA extracted from samples taken at three different depths in the Sargasso Sea in May 2008 all yielded a PCR amplicons for both Synechococcus spp. phnD and Prochlorococcus spp. phnD (Fig. 3.1). Expression of Prochlorococcus spp. phnD was observed in almost all 24 samples (Figs. 3.2). The following RNA samples: BATS at 40 m, Station 3 at 100 m, and Station 4 at 40 m did not amplify picocyanobacterial rnpB (Fig. 3.3) and Prochlorococcus spp. phnD. Low RNA content in samples BATS at 40 m, Station 3 at 100 m, and Station 4 at 40 m due to errors in RNA extraction might be a reason for the failure to detect a Prochlorococcus spp. RT-PCR phnD amplicon. By contrast, the samples from all stations at all depths from the Sargasso Sea May 2008 cruise did not show any expression of the Synechococcus spp. phnD (data not shown), despite the fact that all corresponding DNA samples yielded a PCR amplicon with Synechococcus spp. phnD primers (Fig. 3.1A).

Samples from two stations, Station 1 and BATS, were processed from the October 2008 cruise. All DNA samples yielded Prochlorococcus spp. phnD amplicon; though a weak band was observed in the sample BATS at 200 m (Fig. 3.4B). Similarly, Synechococcus spp. phnD was present in all DNA samples with low abundance in the sample BATS 200 m (Fig. 3.4A).
*Synechococcus* spp. *phnD* expression was detected in the samples taken at the surface and 40 m depth, but not at the DCM or 200 m (Fig. 3.5A). *Prochlorococcus* spp. *phnD* was expressed in all samples except BATS 200 m, a sample found to be negative for picocyanobacterial RNA (Figs. 3.5B and 3.5C).

Despite the fact that PCR, as performed here, is not a quantitative assay, the intensity of bands corresponding to *phnD* and *rnpB* reflects the abundance of picocyanobacteria at depth with highest number of *Prochlorococcus* spp. cells at the DCM and *Synechococcus* spp. cells at the surface (DuRand et al., 2001).

A nutrient amendment incubation experiment was performed with water collected from BATS at the surface in October 2008. The RT-PCR assay revealed that an addition of 0.5 µM phosphate quenched expression of the *Synechococcus* spp. *phnD* in 48 h while not affecting expression of the *Prochlorococcus* spp. *phnD* (Figs. 3.6A and 3.6B). The amendments with 0.5 µM 2-AEP yielded both *Synechococcus* spp. and *Prochlorococcus* spp. *phnD* amplicons. The control sample failed to amplify *Synechococcus* spp. *phnD* and yielded weak bands for *Prochlorococcus* spp. *phnD* and *rnpB* (Fig. 3.6). This suggests the overall low picocyanobacterial cell abundance in the control at 48 h. At this time, we await cell counts by flow cytometry to confirm the low population size in the control sample. Overall, these data confirm that *phnD* in *Synechococcus* spp. is regulated by phosphate availability. The activity of alkaline phosphatase was measured before and 48 h after amendments with ration ranging from 0.7 to 1.31 pmol h⁻¹ ml⁻¹, and there was no significant difference between the samples.
Figure 3.1 PCR from the Sargasso Sea samples taken in May 2008, with *Synechococcus phnD* primers (Panel A) and *Prochlorococcus phnD* primers (Panel B). RNA extracted from *Synechococcus* WH8102 was used as a positive control for *Synechococcus* spp. *phnD* primers.
Figure 3.2 Expression of the *Prochlorococcus phnD* in the Sargasso Sea samples taken in May 2008 assayed by RT-PCR. NTC – no template control. To help affirm the negative result, PCR from samples BATS 40m, St.3 100m, St.4 1m, St.4 40m, St.5 1m, and St.5 40m were performed additionally with the double volume of the RNA; no-RT control was negative.
Figure 3.3 RT-PCR from the Sargasso Sea samples taken in May 2008 with picocyanobacterial *rnpB* primers.

Figure 3.4 PCR from the Sargasso Sea samples taken in October 2008 with *Synechococcus phnD* primers (Panel A) and *Prochlorococcus phnD* primers (Panel B). DNA extracted from *Synechococcus* WH8102 (Panel A) and *Prochlorococcus* MED4 (Panel B) were used as positive controls.
Figure 3.5 RT-PCR from the Sargasso Sea samples taken in October 2008. Expression of the *Synechococcus* phnD (Panel A), *Prochlorococcus* phnD (Panel B), and *rnpB* (Panel C) was assayed with RT-PCR.

Figure 3.6 RT-PCR from the growout experiment RNA extracted in 48 h after the addition of nutrients. Control – no amendments, Phosphate - addition of 0.5 μM of phosphate, and 2-AEP – addition of 0.5 μM of 2-aminoethylphosphonate. Panel A - *Synechococcus* phnD expression, Panel B - *Prochlorococcus* phnD expression, and Panel C – *rnpB* expression.
3.2.2 *Synechococcus* spp. *phnD* expression in the Baltic Sea

The PCR and RT-PCR were employed with the Baltic Sea samples collected in July 2008 from the surface water mixed layer (9 m) at eight hydrographic stations (Fig. 3.7). Limited depth profiles were conducted at two additional stations; the Landsort Deep process station MM08-3056 (St. 57) at the surface and 50 m, and the Skagerrak process station MM08-3101 (St. 76) at the surface and 22 m and 27 m, representing both peaks of a deep chlorophyll maximum (DCM). The PCR amplicon for *Synechococcus phnD* was observed in all samples although with a band of weak intensity at 50 m, located in anoxic waters at Landsort Deep (St. 57) (Fig. 3.8). The RT-PCR showed expression of *Synechococcus phnD* in most samples (Fig. 3.9). Dissolved inorganic phosphorus (DIP) concentrations, where known, were low in all surface mixed layer and DCM samples (Fig. 3.9). The highest surface mixed layer DIP concentrations were 0.18 and 0.15 µM from St. 62 and St. 65, respectively.

Two samples did not yield RT-PCR amplicons for *Synechococcus phnD*; namely the sample from 50 m at Landsort Deep and the surface mixed layer sample from the Skagerrak process station (the amplicon corresponding to St. 54 is still under investigation as we have obtained conflicting results with this sample). While the lack of a *phnD* RT-PCR amplicon from the anoxic zone at Landsort Deep may be related to the elevated DIP concentration measured at this depth (3.37 µM), this sample also yielded a weak signal for *rnpB* thus suggesting low cell abundance as a possible reason for the lack of an amplicon (Fig. 3.10). By contrast, the surface mixed layer sample from the Skagerrak yielded a distinct *rnpB* amplicon indicating sufficient cyanobacterial abundance at this site (Fig. 3.10). Yet, with surface water DIP below the limit of detection at this station, the absence of a *phnD* RT-PCR amplicon is difficult to reconcile.
Prochlorococcus phnD primers did not amplify any of the DNA or RNA samples from the Baltic Sea transect (data not shown) thus reflecting the absence of Prochlorococcus spp. in nutrient rich marine waters and at higher latitudes (Campbell et al., 1997; DuRand et al., 2001; Ahlgren et al., 2006).

Sequencing of the RT-PCR amplicons from the Landsort Deep (St. 57), Skagerrak (St. 76) and Kattegat (MM08-3074; St. 74) process stations as well as from St. 65 (MM08-3062) confirmed the specificity of the Synechococcus phnD amplification. Phylogenetic analysis of the obtained sequences reflected some of the diversity of the Synechococcus spp. previously described in the Baltic Sea (Haverkamp et al., 2008). Most of the sequences were closely related to Synechococcus spp. BL107 and CC9902, members of Clade IV, while some sequences belonged to Clade III (WH8102) (Fig. 3.11).
Figure 3.7 The Baltic Sea, Kattegat, and Skagerrak transect.
Figure 3.8 PCR from the Baltic Sea samples with the *Synechococcus* *phnD* primers. DNA extracted from *Synechococcus* sp. WH8102 was used as a positive control. Unless indicated otherwise, samples were taken at the 9 m depth.

Figure 3.9 RT-PCR from the Baltic Sea samples with the *Synechococcus* *phnD* primers. *Synechococcus* sp. WH8102 RNA was used as a positive control. The concentration of DIP (µM) is shown where available.
Figure 3.10 RT-PCR of the Baltic Sea samples with the rnpB primers.

Figure 3.11 Phylogenetic tree of phnD sequences obtained from the amplicons from the Sargasso Sea, Baltic Sea (green triangles), Lake Erie (diamonds) environmental samples and from Synechococcus spp. genomes (circles). The phylogenetic analysis was performed by the neighbor-joining method with 1,000 bootstrap replicates. phnD of euryhaline Synechococcus WH5701 was used as an outgroup. Sequences obtained in a previous study were also included: from Monterey Bay and the Sargasso Sea in October 2007.
3.2.3 Analysis of samples from Lake Erie in 2008

Samples from Lake Erie were taken from the western, eastern, and central basins in April (ER samples) and in the western basin from May to July (St. 7M and MB20). A PCR amplicon for *Synechococcus phnD* was identified in all DNA samples (Table 3.1). RT-PCR assay revealed no expression of *Synechococcus phnD* in any samples (data not shown), but expression of the picocyanobacterial *rnpB* (Fig. 3.12) and cyanobacterial 16S-rRNA genes (Table 3.1) expression were detected.

Sequencing of the *Synechococcus phnD* PCR amplicons from samples ER59, ER78M (April), and 7M from May resulted in sequences from the marine Clade III *Synechococcus* that includes pelagic marine strain WH8102 (Fig. 3.11). *Prochlorococcus phnD* primers amplified a product of a correct size in all RNA samples from Lake Erie (Fig. 3.13), as well as in DNA samples (data not shown). The amplicon was verified as *Prochlorococcus phnD* by sequencing, and the highest nucleotide identity was to the *Prochlorococcus* HLII clade (94-97 %). To ensure that there was no contamination in RT-PCR and PCR assays, a negative control was successfully performed with water used for RNA and DNA extraction and found to be negative.

An increase in chlorophyll *a* for pico, nano, and microplankton was observed in May 29th and later in July 10th at 7M and MB20 stations (Figs. 3.14A and 3.14B). Station MB20 was characterized by 3-5 times higher chlorophyll content than Station 7M in general. Alkaline phosphatase activity was detected in samples taken from May 29th, June 24th, and July 10th at St. 7M and St. MB20 (Figs. 3.15A and 3.15B). The highest APase activity was in June 24th for St. 7M and May 29th and June 24th for St. MB20 consistent with a P limitation in the plankton community. Similar to chlorophyll, St. MB20 exhibited higher APase activity than St. 7M.
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**Table 3.1** PCR and RT-PCR amplification of Lake Erie water samples with the *Synechococcus* spp. *phnD* and cyanobacterial 16S-rRNA primers.

“+” and “–” are the presence and absence of the amplification, respectively; “w” – a band of weak intensity.
Figure 3.12 RT-PCR from Lake Erie samples with the *rnpB* primers showing the presence of picocyanobacterial RNAs in all samples.

Figure 3.13 Expression of the *Prochlorococcus phnD* in Lake Erie samples. Samples taken in April: ER15M – Eastern basin, ER59, ER60, and ER61 – Western basin, ER78M – Central basin. Samples taken from May to July from the Western basin: St. 7M and MB20.
Figure 3.14 Chlorophyll $a$ concentrations for pico, nano, and microplankton at St. 7M (Panel A) and St. MB20 (Panel B) in Lake Erie, $\mu$g L$^{-1}$.

Figure 3.15 Alkaline phosphatase activity in the samples from St. 7M (Panel A) and MB20 (Panel B) in Lake Erie.
3.3 Discussion

In this study, we have assessed expression of the *phnD* in a broad range of environmental samples, including the Sargasso Sea samples taken from eight stations in May and from two stations in October 2008, Baltic Sea transect samples from ten stations in July 2008, and Lake Erie samples from seven stations in 2008.

3.3.1 Expression of the picocyanobacterial *phnD* in the Sargasso Sea

*Prochlorococcus* spp. *phnD* expression was observed in all samples from the Sargasso Sea drawn in May and October 2008, except BATS at 40 m, St. 3 at 100 m, and St. 4 at 40 m in May and BATS 200 m in October. These negative samples likely contained prohibitively low total picocyanobacterial RNA. The constitutive presence of the *Prochlorococcus* spp. *phnD* RNA is consistent with the data obtained earlier for the Sargasso Sea samples taken in October 2007 (Ilikchyan et al., In press). In contrast, while a PCR amplicon was detected for *Synechococcus* spp. *phnD* in all samples at all depths, expression of this gene was not observed in May when the concentration of phosphate is generally higher throughout the water column due to recent winter mixing (DuRand et al., 2001). In October, *Synechococcus* spp. expressed *phnD* in the mixed water layer but not at the DCM (ca. 100 m) or at 200 m. This is consistent with low DIP levels typically observed in the surface layer, which is only 50 m in depth in the fall. The DNA samples from 200 m depth yielded *Synechococcus* spp. *phnD* amplicons, but there are likely fewer living *Synechococcus* spp. cells at this depth. In addition to the elevated DIP concentration at 200 m, this leads to an absence of *phnD* expression. At the DCM depth, *Synechococcus* spp. are present (DuRand et al., 2001; Ahldren et al., 2005) though in lower abundance than in the surface waters, and the lack of phosphonate gene expression may be
explained by phosphate availability below the mixed layer depth. We await core nutrient measurements from these 2008 cruises to assess the DIP concentration that may repress phnD expression.

Indeed, addition of phosphate in the nutrient amendments experiment yielded no Synechococcus spp. phnD expression at 48 h while the biomass has increased as suggested by RT-PCR with the rnpB primers. An addition of 2-AEP resulted in increased picocyanobacterial biomass as well, although Synechococcus spp. phnD expression was evident in this sample. Since only 2 L of water were available for RNA extraction and biomass in the control growout sample did not significantly increase in 48 h, concentration of the obtained RNA was insufficient to assess Synechococcus spp. phnD expression. Prochlorococcus spp. phnD and rnpB expression was barely detectable in the control as well. Synechococcus spp. expressed phnD at time zero because the water for the experiment was taken from the surface at the BATS station where in an identical sample, expression of the Synechococcus spp. phnD was detected in the samples taken from the surface and 40 m (Fig. 3.5A).

Alkaline phosphatase activity was not significantly different in all samples of the nutrient amendments experiment at time zero and in 48 h. Since eubacteria likely contribute the largest component of total alkaline phosphatase activity in environmental samples, this assay was not an accurate indication of picocyanobacterial P status. An assessment of the P cell quota would help to determine the P status in picocyanobacteria.

Information on DIP concentration and cell counts for May and October 2008 Sargasso Sea cruises will help to explain the phnD expression results. However, P limitation in the Sargasso Sea surface waters is well documented (Cotner et al., 1997; Karl, 2000; Wu et al., 2000; Dyhrman et al., 2007), and a significant depletion in P occurs during late summer and
early fall when the Sargasso Sea is stably stratified (DuRand et al., 2001; Ahlgren et al., 2006). Our data suggest that *Synechococcus* spp. regulate expression of the phosphonate transporter gene in response to phosphate availability. *Prochlorococcus* spp., at least members of HLII clade, express *phnD* independently from P limitation. This corresponds with a reduced genome size of *Prochlorococcus* HL spp. and loss of some of the functional and regulatory genes in comparison to the LL clade and to *Synechococcus* spp. (Scanlan and West, 2002; Moore et al., 2002; Dufresne et al., 2003; Palenik et al., 2003; Rocap et al., 2003; Moore et al., 2005). To confirm that *Prochlorococcus* spp. express the phosphonate uptake genes independently of P limitation, an RT-PCR assay could be designed with degenerate primers specific for a *Prochlorococcus* spp. gene known to belong to the *pho* regulon, such as *pstS* or *phoE* genes. Although the *sphS* (*phoR*) gene is absent in some *Prochlorococcus* spp., genes responsible for P stress response are activated (Martiny et al., 2006).

### 3.3.2 Expression of *Synechococcus* spp. *phnD* in the Baltic Sea

The expression of *phnD* in most samples collected from the surface mixed layer of the Baltic Sea demonstrated that the endemic *Synechococcus* spp. are utilizing phosphonates. These data coincided with relatively low phosphate concentrations compared to samples collected at depths below the mixed layer at the process stations studied. In all surface samples, the ratio of DIN:DIP was above the Redfield ratio of 16 suggesting phosphorus limitation. Thus, expression of *phnD* by *Synechococcus* spp. could be attributed to the P limitation response. Although nitrogen and iron rather than phosphorus limitation are general features of the waters in the Baltic Sea (Stal et al., 2003), P limitation may occur during or after a bloom of diazotrophs in stratified conditions (Paasche & Erga 1988, Granéli et al. 1990, Kononen et al. 1993). Indeed, P
limitation was observed in the Baltic Sea during a bloom of heterocystous cyanobacteria in July 2001 (Nausch et al., 2004).

The lack of phnD expression in the surface mixed layer at the Skagerrak site is an enigma since DIP was below detection limits, and both picocyanobacterial rnpB and a PCR amplicon for phnD were detected in this sample. A possible explanation may be rapid P cycling, which cannot be assessed by synoptic chemical measurements (Dyhrman et al., 2006). In addition, changes in Synechococcus spp. pho regulon expression is likely a response to the internal, not external, P quota (Su et al., 2003). Thus, a transient low DIP concentration might not influence phnD expression. A lack of expression in the deep water sample from Landsort Deep was likely due to very low cell abundance of Synechococcus spp. combined with the elevated DIP concentration at this depth.

Sequences obtained from RT-PCR amplicons from several stations were identified as phnD from marine Synechococcus spp., from both open ocean (Clade III) and coastal region clades (Clade IV). No members of freshwater clades were found in contrast to reported diversity of Synechococcus spp. in the Baltic Sea composed of members from marine as well as from freshwater clades (Haverkamp et al., 2008). Whether this was due to the limitation or selectivity of the primer set remains to be investigated.

3.3.3 The absence of Synechococcus spp. phnD expression in Lake Erie

The absence of Synechococcus spp. phnD expression in Lake Erie in April coincided with the replete phosphorus conditions during the spring mixing event. Samples taken from May to July were from the mesotrophic western basin (Makarewicz and Bertram, 1991). This may explain the lack of Synechococcus spp. phnD expression at St. 7M and St. MB20. The observed
activity of alkaline phosphatase in May, June, and July was attributed to the whole microbial community response that includes picocyanobacteria as a minor fraction; thus, the P status of *Synechococcus* spp. was not known.

Since *Synechococcus* spp. *phnD* expression was not detected, phosphonates that may be present in Lake Erie were not assimilated by these picocyanobacteria. The occurrence of natural phosphonates in freshwater environments is not yet known. However, anthropogenic phosphonate in the form of glyphosate was detectable by ELISA in samples from St. 7M and St. MB20 taken in May 2008 (George S. Bullerjahn, personal communication). In July 2007, $^{31}$P-NMR analysis of lyophilized water samples from Lake Erie at Sandusky Bay station showed the presence of abundant total phosphonates (Claudia Benitez-Nelson, personal communication). Thus, the utilization of glyphosate by heterotrophic bacteria and other cyanobacteria should be assessed at these sites to estimate the impact of anthropogenic phosphonates loadings into the lake.

The presence of marine *Synechococcus* spp. revealed by sequencing of a DNA amplicon from several samples, as well as the presence and expression of *Prochlorococcus* spp. *phnD* in Lake Erie samples might be due to amplification of *phnD* sequences from marine picocyanobacteria released from ballast water discharge (George Bullerjahn, personal communication). The occurrence of other common marine bacteria sequences in Lake Erie environmental samples, e.g. from SAR11, will help to clarify this. The fact that *Prochlorococcus* spp. express genes in freshwater environment indicates their survival at least for a short period of time. However, the cells of *Prochlorococcus* spp. were not detected by flow cytometry thus far (George Bullerjahn, personal communication) and by amplification of the cyanobacterial 16S-rRNA gene from Lake Erie water samples (Ivanikova et al., 2008; Cupp and Bullerjahn,
unpublished). A future investigation may include the detection and expression of other Prochlorococcus spp. specific genes and flow cytometry of concentrated water samples due to the extremely low abundance of Prochlorococcus spp., if present.

Freshwater Synechococcus spp. phnD sequences were not observed by sequencing in contrast to our early survey of Lake Erie environmental samples. Whether it was due to primers selectivity or the representation of picocyanobacterial communities at the time of sampling remains to be determined. Given the reproducible observations on the endemic Lake Erie picocyanobacteria (Wilhelm et al., 2006; Ivanikova et al., 2008), the former explanation is more likely.

### 3.4 Concluding remarks

The fact that picocyanobacteria are utilizing phosphonates in marine environments underscores the significance of phosphonates in the bioavailable DOP pool. Synechococcus spp. expressed phnD gene under P-limited conditions in samples from the Sargasso Sea and Baltic Sea transects, and HLII Prochlorococcus spp. phnD expression was constitutive in the Sargasso Sea. The same pattern of phnD expression was observed previously (Ilikchyan et al., In press). Development of clade specific molecular probes for phosphonate utilization genes in picocyanobacteria will bring insight into differences in regulation of phosphonate assimilation among the clades and improve our overall understanding of P physiology in picocyanobacteria. In addition, more detailed analysis of phnD expression in cultured picocyanobacteria will help to determine the mechanism of phnD activation under P limitation.

For utilization of phosphonates by picocyanobacteria in Lake Erie, samples from oligotrophic eastern and central basins have to be analyzed for the presence of phnD expression.
Also, sequencing the genome of a dominant member of Synechococcus spp. in Lake Erie will facilitate the design of primers with higher specificity for the phosphonate utilization genes in freshwater picocyanobacteria.

In overall, the pattern of Synechococcus spp. \textit{phnD} expression observed in natural water samples herein and earlier (Ilikchyan et al., In press) together with the results of nutrient amendment experiment conducted in the field suggest applicability of the developed RT-PCR method in assessing the P status of marine picocyanobacteria, particularly \textit{Synechococcus} spp.

\section*{3.5 Experimental procedures}

\subsection*{3.5.1 Environmental samples}

Table 3.2 provides details of sampling. The samples for RNA and DNA extraction were processed as described in Ilikchyan et al., In press. Briefly, seston from environmental samples was collected onto 0.22 $\mu$m Sterivex cartridge filters and frozen immediately in liquid nitrogen. The cartridges were stored at -80°C prior to RNA and DNA extraction. For chlorophyll \textit{a} extraction, seston from environmental samples was harvested onto 20 $\mu$m, 2 $\mu$m, and 0.2 $\mu$m polycarbonate filters (Millipore), transferred into cryo tubes and stored at -20°C until processing. Chlorophyll \textit{a} concentration was measured fluorometrically after extraction with 90\% acetone.

\subsection*{3.5.2 DNA and RNA extraction}

RNA and DNA were extracted from Sterivex filters with RNeasy kit (Qiagen) and phenol-chloroform method respectively; the methods were described earlier (Ilikchyan et al., In press). The volume of RNase free water for elution of RNA was adjusted based on the volume of filtered water sample.
3.5.3 PCR and RT-PCR

All PCR and RT-PCR amplifications with *Synechococcus* and *Prochlorococcus* spp. *phnD* primers were done as described earlier (Ilikchyan et al., In press). *rnpB* is a single copy gene and encodes for RNase P RNA. The *rnpB* primers were designed to amplify a 118 bp region specific for picocyanobacteria, RNPB-F: CCGTGAGGAGAGTGCCACAG; RNPB-R: CAGCACCTCTCGATGCTGCTGG. Specificity of the primers was confirmed by the absence of amplification from DNA extracted from *Synechococcus* sp. PCC7942, *Synechocystis* sp. PCC6803, *Microcystis* sp. M300. All available marine and freshwater picocyanobacterial DNA yielded an amplicon of the correct size. The PCR conditions for amplification with the *rnpB* primers were following: 95°C for 5 min, 30 cycles of 95°C for 30 s, 65°C for 30 s, and 72°C for 30 s, followed by 72°C for 10 min.

3.5.4 Nutrient amendment incubation experiment

Twenty-four L of surface water from the Sargasso Sea BATS were gravity filtered through 5 µm membrane, and 2 L were distributed into each of nine polycarbonate bottles for control and amendments with 0.5 µM K₂HPO₄ and with 0.5 µM 2-aminoethylphosphonate in triplicate. The bottles were incubated at 70 umol quanta m⁻² s⁻¹ in a deck incubator for 48 h. After incubation, seston from 2 L of each treatment and control was filtered into a 0.22 µm Sterivex cartridge filter and frozen in liquid nitrogen immediately. Additionally, 500 ml from each amendments and control were filtered onto a 0.2 µm polycarbonate filters (Millipore) for chlorophyll *a* extraction, and 500 ml were filtered onto precombusted GF/F filters to assay for POP, placed into Petri dishes and stored at -20°C until processing. Samples for chlorophyll *a* and POP assays were collected in duplicate at time zero and after 48 h incubation.
3.5.5 Alkaline phosphatase activity

Alkaline phosphatase activity (APA) was measured fluorometrically with methylumbelliferyl phosphate (Sigma) as described elsewhere (Hoppe, 1993; Sterner et al., 2004). Fluorescence was measured with Turner TD-700 fluorometer at 364 nm excitation and 445 nm emission. For the nutrient amendments experiment with water collected in the Sargasso Sea, fluorescence was measured at 3 hour intervals over 26 hours. For Lake Erie water samples, the measurements were done at 30 min interval over 2 hours. To ensure the absence of contaminating phosphatase activity in the solutions, a negative control was included with 50 µM of methylumbelliferyl phosphate added to 5 mM sodium bicarbonate solution and incubated in parallel with the water samples. To correct for endogenous fluorescence quenching by the sampled water, a quench standard was included with 1 µM of methylumbelliferone product added to the water sample. The data were calibrated with three standard concentrations of methylumbelliferone: 0.1, 0.5 and 1 µM. The assay was done in triplicates and at the same day when the water samples were collected.
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Table 3.2 Environmental samples employed in this study (cont.)
CHAPTER IV. PHOSPHONATE UTILIZATION AND REGULATION OF THE PHOSPHONATE GENES IN PICOCYANOBACTERIA: GROWTH ASSAYS AND phn PROMOTERS ANALYSIS

4.1 Introduction

Phosphonates have been receiving an increasing attention as an important alternative phosphorus (P) source in P-limited aquatic environments. Indeed, the nitrogen-fixing cyanobacterium *Trichodesmium* ISM101, possessing the C-P lyase genes for phosphonate degradation is able to grow on phosphonates as a sole P source, and the expression of the phosphonate utilization genes is activated under P limitation in cultures as well as in environmental samples (Dyhrman et al., 2006). Gilbert et al. (2008) reported that *Vibrionaceae* grow on phosphonoacetate as a sole P and C source, and the expression of *phnA* gene for phosphoacetate hydrolysis was observed in water samples from the Western English Channel. Under P starvation, thermophilic *Synechococcus* sp OS-B’ utilizes methylphosphonate as a P source (Adams et al., 2008). Moreover, utilization of phosphonates contribute to the accumulation of greenhouse gases; methylphosphonate is utilized aerobically as a P source by the bacterial community in P stressed marine waters yielding the production of methane (Karl et al., 2008).

Picocyanobacteria of the genera *Synechococcus* and *Prochlorococcus* contribute significantly to global primary production and represent the key position at the base of the freshwater and marine food web (e.g. Waterbury et al., 1986; Nagata et al., 1994; Stockner and Shortsheed, 1994). Both genera harbor genes for phosphonate transport and utilization, and expression of the *phnD* gene encoding the phosphonate binding protein of an ABC type transporter was observed in marine environmental samples under P-limited conditions for
Synecococcus spp. and constitutively for Prochlorococcus spp. HLII (Ilikchyan et al., In press and 3). Though attempts to grow Prochlorococcus spp. on phosphonates as a sole P source have been unsuccessful to date (Moore et al., 2005), marine Synecococcus WH8102 is capable of growing on 2-aminoethylphosphonate (Palenik et al., 2003; Su et al., 2003; Ilikchyan et al., In press) and ethylphosphonate (Palenik et al., 2003; Su et al., 2003). Similarly to marine, freshwater Synecococcus ARC-21, isolated from Lake Erie (Ivanikova et al., 2008), demonstrated growth on 2-AEP and additionally on glyphosate, the phosphonate herbicide known as Roundup® (Ilikchyan et al., In press). Glyphosate was detected by ELISA in samples from western basin of Lake Erie taken in May (Chapter 3), and thus a new P source enters the food chain in the lake as a result of glyphosate assimilation by Synecococcus spp. This is relevant, given the fact that Synecococcus spp. constitute a significant fraction of Lake Erie picoplankton (Wilhelm et al., 2006). Whether or not other freshwater Synecococcus spp. can utilize phosphonates was addressed in this study.

In addition, we were interested in determining the range of phosphonate compounds that picocyanobacteria can use as a P source. While many filamentous cyanobacteria and some Synecococcus spp. of non-picocyanobacterial clade possess C-P lyase genes (Dyhrman et al., 2006; Gilbert et al., 2008) and are capable of utilizing a variety of phosphonates, picocyanobacteria have genes for the phosphonatase pathway, that is considered to be specific for 2-AEP hydrolysis. However, the presence of a non-specific phosphonate transport system and the ability to grow on glyphosate (Ilikchyan et al., In press) and ethylphosphonate (Palenik et al., 2003; Su et al., 2003) suggest that other phosphonates besides 2-AEP can be assimilated by phosphonatase in picocyanobacteria.
Last, possible regulation of phosphonate utilization genes expression was suggested based on in-situ analysis of promoters and operons encoding the phosphonate transporter and phosphonatase in picocyanobacteria. Our previous results demonstrated a pho–like regulation of the phnD in *Synechococcus* WH8102, but a pho box was not identified in promoters of phosphonate utilization genes in this strain and other picocyanobacteria (Su et al., 2007). The phosphonatase pathway is known to be either part of the pho regulon or regulated by a LysR family transcription factor (Lee et al., 1992; Jiang et al, 1995; Baker et al., 1998; Ternan and Quinn, 1998; Quinn, 2002). Here, I speculate that neither one directly regulates phosphonate utilization in picocyanobacteria. Examination of phosphonate utilization operons revealed the presence of conserved transcription factors, specifically, an alternative type III sigma factor may be responsible for the transcriptional activation of the phosphonate transporter operon, and a two-component regulatory system may regulate phosphonatase gene expression. Also, the promoter structure and presence of transcription factor genes suggest that the pattern of gene regulation differs for *Synechococcus* and *Prochlorococcus* spp., consistent with the field observations (Ilikchyan et al., In press; Chapter 3).

### 4.2 Results

#### 4.2.1 Growth of picocyanobacteria on phosphonates as a sole P source

Table 4.1 summarizes the results of picocyanobacterial growth on different phosphonates as a source of P, measured by extracted chlorophyll *a*. Marine *Synechococcus* WH8102 and freshwater *Synechococcus* ARC-21 demonstrated the most abundant growth on 2-aminoethylphosphonate (2-AEP), and ARC-21 was capable of utilizing glyphosate as well (also Figs. 4.1A and Ilikchyan et al., In press). The extracted chlorophyll *a* of both *Synechococcus*
WH8102 cultures grown on 2-AEP and *Synechococcus* ARC-21 grown on 2-AEP and glyphosate as a sole P source were similar to chlorophyll values from P-replete cultures. *Synechococcus* ARC-11, an isolate from Lake Erie, and *Synechococcus* LS0512, isolated from Lake Superior, showed moderate growth on both 2-AEP and glyphosate (Figs. 4.1B and 4.1C). *Synechococcus* KD3a isolated from Lake Erie was not able to utilize either phosphonates as a source of P; the same was true for Lake Superior isolate LS0504 despite the fact that both strains possess the *phnD* gene (Ilikchyan et al., In press). None of tested *Synechococcus* spp. cultures (six) were able to utilize aminomethylphosphonate (AMPA), a product of glyphosate microbial degradation, and both WH8102 and ARC-21 did not assimilate methylphosphonate (MP) (also Fig. 4.1A).

Ethylphosphonate (EP) and phosphonoacetic acid (PA) were also tested as a source of alternative P, and growth was observed for *Synechococcus* ARC-11, ARC-21, and WH8102; however, *Synechococcus* KD3a ceased to grow on PA (data not shown). Due to the presence of phosphate contamination in commercially available EP (0.2%) and PA solutions (0.055%, *which may not be significant during a long term growth experiment*) measured by the molybdate assay, work with these two phosphonates was discontinued. Glyphosate and 2-AEP solutions did not contain any detectable phosphate.

Further, to show an ability of picocyanobacteria to utilize phosphonates, particulate organic phosphorus (POP) was assessed in *Synechococcus* ARC-21 after 17 days of growth at different P conditions, P-replete (86 µM), P free, low P (8.6 µM), and P free with an addition of 10 µM of 2-AEP. The POP per cell in 2-AEP cultures was significantly (p=0.047) higher from values obtained from No P cultures (Fig. 4.2), indicating assimilation of 2-AEP as a P source.
Table 4.1 Growth of picocyanobacteria on phosphonates as a sole P source. Cultures tested: marine *Synechococcus* WH8102; Lake Erie isolates: *Synechococcus* ARC-21, ARC-11, and KD3a; Lake Superior isolates: *Synechococcus* LS0512 and LS0504. Phosphonates abbreviations are given in Table 2.

<table>
<thead>
<tr>
<th></th>
<th>2-AEP</th>
<th>Glyphosate</th>
<th>AMPA</th>
<th>Methyl-Pn</th>
</tr>
</thead>
<tbody>
<tr>
<td>WH8102</td>
<td>++</td>
<td>NA</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ARC-11</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>NA</td>
</tr>
<tr>
<td>ARC-21</td>
<td>++</td>
<td>++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>KD3a</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>NA</td>
</tr>
<tr>
<td>LS0512</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>NA</td>
</tr>
<tr>
<td>LS0504</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>NA</td>
</tr>
</tbody>
</table>

++ Growth at the same abundance as in P-replete media;
+ Growth at lower abundance than in P-replete, but significantly different than in NoP media (with $\alpha=0.05$);
- No growth was observed;
NA Not tested.

**Figure 4.1** Growth of picocyanobacteria on phosphonates. *Panel A. Synechococcus* WH8102 grown on 2-aminoethylphosphonate (2-AEP) and methylphosphonate (MP) as a sole P source. The growth was measured by chlorophyll *a* fluorescence. NoP – P-free medium.
Figure 4.1 Growth of picocyanobacteria on phosphonates. (cont.)
Panel B. *Synechococcus* ARC-11 growth on 2-AEP and glyphosate; the growth measured by extracted chlorophyll.
Panel C. *Synechococcus* LS0512 grown on 2-AEP, aminoethylphosphonate, and glyphosate as a sole P source measured by extracted chlorophyll on 20th day. The chlorophyll *a* concentration was significantly (p=0.042) higher in 2-AEP compared to NoP cultures, and no significant difference was observed for cultures grown on glyphosate (p=0.586).
Figure 4.2 Particulate organic phosphorus (POP) per cell in *Synechococcus* ARC-21 grown on 2-AEP as a sole P source on 17th day after inoculation. Cultures grown in 2-AEP showed significantly (p=0.048) higher POP per cell than in NoP media.

4.2.2 Phosphonate operons in picocyanobacteria

The structure of operons for phosphonate transport and phosphonatase was analyzed from those picocyanobacteria, whose genome have been sequenced. In all analyzed *Synechococcus* (7 genomes) and *Prochlorococcus* spp. (10 genomes), the phosphonate transporter operon consists of genes in the following order: *aspC* encoding an aminotransferase (presumably *phnW*), *phnD* encoding for the phosphonate binding protein, *phnC* encoding for an ATPase, and *phnE* encoding for a permease protein (Fig. 4.3). Except *Synechococcus* RCC307, picocyanobacteria have the *tesA* gene, that codes for lysophospholipase L1, located just downstream of *phnE* gene in the same operon. *Synechococcus* spp. WH8102, RCC307, WH9605, WH9902, CC9311, WH5701, and *Prochlorococcus* MIT9313 possess a gene for a putative sigma factor type III located downstream of the *phn* genes (Fig. 4.3). Alignment of the amino acid sequence of the
sigma factor gene with BLASTp against nr database in GenBank revealed 35% identity to SigF of *Synechocystis* PCC 6803, 40% to the *Synechococcus elongatus* PCC 7942 SigF. In addition, a gene for a small hypothetical protein is also present within or downstream of the operon in all *Synechococcus* spp. except RCC307. The *phnE* genes in *Prochlorococcus* MIT9301, MIT9215, and AS9601 have a frameshift encoding the 125th amino acid residue which may indicate that PhnE protein is nonfunctional in these strains.

Several strains have acquired a second copy for some of the phosphonate transporter genes. Thus, *Synechococcus* CC9605 have a *phnCD* operon (Syncc9605_1097 and Syncc9605_1096) similar to the *phnCDE* operon of *Trichodesmium erythraeum* IMS101 (61% aa identity). The *Synechococcus* RCC307 has a second *phnD* (SynRCC307_2410) that is similar to *Synechococcus* RSS9916 *phnD* (33% aa) and is contrtrascribed together with a two-component sensor histidine kinase with a response regulator gene located upstream (SynRCC307_2412). In *Prochlorococcus* MIT9301, *phnD* (P9301_12511) from a second operon for the phosphonate transporter *phnCDE* shares 52% amino acid sequence identity with *Trichodesmium erythraeum* IMS101 *phnD*. The *phnCDE* operon is located near the *phoBR* operon in the *pho* region (Martiny et al., 2006) of MIT9301 genome. The primers designed earlier (Ilikchyan et al., In press) for *Synechococcus* and *Prochlorococcus* spp. *phnD* are not able to amplify the second *phnD* gene since it is phylogenetically distinct from the picocyanobacterial *phnD*.

The phosphonatase operon is composed of the *phnX* gene encoding phosphonatase and a gene for conserved hypothetical protein which has transmembrane domains as suggested by topology prediction with TMHMM (CBS, Denmark) (Fig. 4.4). *Prochlorococcus* MIT9313 and all *Synechococcus* spp. except RCC307 possess a third gene encoding for a phosphoribulokinase/uridine kinase family protein.
Figure 4.3 Phosphonate transporter operons structure in picocyanobacteria, where *aspC* encodes aminotransferase, *phnD* encodes phosphonate binding protein, *phnC* encodes ATPase, *phnE* encodes a permease, *tesA* encodes the lysophospholipase, and *hp* encodes a hypothetical protein. Note: missannotation in some genomes with *phnC* and *phnE* has been corrected during this analysis.
Figure 4.4 Phosphonatase operons structure in picocyanobacteria, where *phnX* encodes phosphonatase (*cbbY* in genome annotation), *chp* encodes a conserved hypothetical protein with transmembrane domains, *chp-kinase* encodes a putative phosphoribulokinase/uridine kinase family protein.
4.2.3 Analysis of 5′-untranslated regions of the phosphonate utilization and \textit{pho} genes in picocyanobacteria

Putative promoters were selected for phosphonate utilization operons and for genes and operons known or assumed to be under \textit{pho} regulation in picocyanobacteria. Motif search with MEME software (Timothy et al., 1994; Timothy et al., 2006) identified a motif with a sequence highly similar to the \textit{pho} box for cyanobacteria, but in complementary reverse orientation (Figs. 4.5, 4.6 and 4.7). The \textit{pho} box in cyanobacteria consist of two tandem repeats CTAAACCT separated by three nucleotides (Su et al., 2007). The same distance between tandems is in the motif identified with MEME; thus, we considered this motif to be the \textit{pho} box. In general, promoters of \textit{phoBR}, \textit{pstS}, \textit{pstCAB}, \textit{phoE}, and \textit{phoA} genes, as was expected, contain the \textit{pho} box (Figs. 4.5, 4.6, and 4.7A and B). The \textit{pho} box was found in the promoter of the second copy of the \textit{phnCDE} operon of \textit{Prochlorococcus} MIT9301, which as mentioned above is similar to \textit{Trichodesmium erythraeum} IMS101, where this operon is likely to be under \textit{pho} regulation (Dyhrman et al., 2006).

The \textit{pho} box was not found in the promoters of phosphonate utilization operons in both \textit{Synechococcus} and \textit{Prochlorococcus} spp. Alternatively, phosphonate promoters contain motifs distinct from the \textit{pho} promoters. In all \textit{Synechococcus} spp. except RCC307 and in \textit{Prochlorococcus} MIT9313, putative promoters of the phosphonate transporter operons include three motifs; SPT1 (stands for \textit{Synechococcus} phosphonate transporter), SPT2, and SPT3 (Fig. 4.8). The SPT1 motif is also present in two out of seven phosphonatase promoters (\textit{cbbY} as in genome annotations) and in several other promoters (Fig. 4.9). The phosphonatase promoter contains also a SPA1 motif (stands for \textit{Synechococcus} phosphonatase) in six out of seven \textit{Synechococcus} spp. strains, but not in \textit{Prochlorococcus} MIT9313.
In *Prochlorococcus* spp. members of HL clades, promoters of *phnDCE* and phosphonatase share more similarity between each other than promoters in *Synechococcus* spp. Both PPT2 (for *Prochlorococcus* phosphonate transporter) and PPT3 motifs occur in all *phnDCE* and nearly all phosphonatase promoters (Figs.4.10 and 4.11). The PPT1 motif is unique to *phnDCE* promoters, and both PPA1 (for *Prochlorococcus* phosphonatase) and PPA2 are unique to the phosphonatase promoter.

From the four analyzed LL *Prochlorococcus* genomes, only promoters of MIT9313 contained motifs that were similar to *Synechococcus*; no motifs were identified in phosphonate promoters of *Prochlorococcus* CCMP1375, MIT9211, and NATL1A.

No similarity in motifs of phosphonate promoters were found between *Synechococcus* and *Prochlorococcus* spp, except *Prochlorococcus* MIT9313.
Figure 4.5 Picocyanobacterial promoters containing a consensus *pho* box identified with MEME. Note: not all possible P genes were analyzed. The promoters where a *pho* box was found by Su et al., 2007, are marked with a red circle. Plus and minus signs indicate location of the box on plus and minus DNA strands. The zero on the scale represents a translational start site.
Figure 4.6 The *pho* box consensus sequence. Panel A. The *pho* box identified with MEME in both *Synechococcus* and *Prochlorococcus* spp. promoters analyzed together. Panel B: The *pho* box consensus sequence identified by Su et al, (2007). The reverse complemented sequence of the *pho* box in Panel A is similar to the one in Panel B.
Figure 4.7 The *pho* box identified with MEME in *Synechococcus* (Panel A) and *Prochlorococcus* spp. (Panel B) promoters analyzed separately.
Figure 4.8 Motifs and their arrangement in promoters of the phosphonate transporter operons in *Synechococcus* spp. Panel A. Arrangement of motifs found in promoters of phosphonate transporter operons in *Synechococcus* spp. and *Prochlorococcus* MIT9313. SPT – *Synechococcus* phosphonate transporter.
Figure 4.8 Motifs and their arrangement in promoters of the phosphonate transporter operons in *Synechococcus* spp. (cont.) Panel B. Motifs found in promoters of the phosphonate transporter operons in *Synechococcus* spp. and *Prochlorococcus* MIT9313.
Figure 4.9 Motifs and their arrangement in promoters of the phosphonatase operons in *Synechococcus* spp. *Panel A.* Arrangement of motifs identified in phosphonatase operon promoters in *Synechococcus* spp. SPA – *Synechococcus* phosphonatase. *Panel B.* Motifs identified in phosphonatase operon promoters in *Synechococcus* spp.
Figure 4.10 Motifs and their arrangement in promoters of the phosphonate transporter operons in *Prochlorococcus* spp. Panel A. Arrangement of motifs identified in promoters of the phosphonate transporter operons in *Prochlorococcus* spp. PPT – *Prochlorococcus* phosphonate transporter.
Figure 4.10 Motifs and their arrangement in promoters of the phosphonate transporter operons in Prochlorococcus spp. (cont.) Panel B. Motifs identified in promoters of the phosphonate transporter operons in Prochlorococcus spp.
Figure 4.11 Motifs and their arrangement in promoters of the phosphonatase operons in *Prochlorococcus* spp. Panel A. Arrangement of motifs identified in phosphonatase operon promoters in *Prochlorococcus* spp. PPA – *Prochlorococcus* phosphonatase. Panel B. Motifs identified in phosphonatase operon promoters in *Prochlorococcus* spp.
4.3 Discussion

4.3.1 Utilization of phosphonates by picocyanobacteria.

The capability of *Synechococcus* WH8102 to grow on 2-AEP and EP as a sole P source was documented previously (Palenik et al., 2003; Su et al., 2003). Here, we confirmed that this marine strain can utilize 2-AEP, and the rate of growth was the same as during P-replete conditions. However, growth on EP (Sigma) could not be established since this compound was contaminated with phosphate, thus it remains unclear whether or not EP can be used as a sole P source by *Synechococcus* spp.

Similar to the marine strain, freshwater *Synechococcus* ARC-21 yielded abundant growth on 2-AEP. Determination of POP per cell confirmed the ability of ARC-21 to assimilate 2-AEP. The higher ratio of POP per cell in 2-AEP cultures in comparison to NoP cultures on the 17th day corresponded with the activation of *phnD* gene expression on 15th and 17th days measured by quantitative RT-PCR (Ilikchyan et al., In press). In addition, ARC-21 utilized glyphosate as a P source, suggesting that phosphonate herbicides are utilized by endemic cyanobacteria of Lake Erie. Indeed, glyphosate was detected in Lake Erie by ELISA in samples taken in July 2007 and in May 2008 (George Bullerjahn and Claudia Benitez-Nelson, personal communication).

Despite the presence of a broad specificity phosphonate transporter system, *Synechococcus* spp. possess a more substrate selective mechanism of phosphonate utilization. Indeed, among all tested phosphonate compounds, 2-AEP and glyphosate (and maybe PA) were assimilated by *Synechococcus* spp. as a source of P. These phosphonates have a polarized C-P bond in contrast to AMPA, EP, and MP, and probably polarization of C-P bond is a requirement for hydrolysis by the phosphonatase pathway.
4.3.2 A possible mechanism for phosphonate utilization

The conservation of the operon structure for both phosphonate transporter and phosphonatase genes indicates an ancient trait for phosphonate utilization in picocyanobacteria. This was also suggested by phylogenetic analysis of PhnD sequences showing that picocyanobacterial PhnD form a cluster distinct from PhnD sequences obtained from filamentous cyanobacteria and other bacteria (Ilikchyan et al., In press).

The phosphonatase pathway is performed by two enzymes, aminotransferase (*phnW*) and phosphonatase (*phnX*) (Ternan et al., 1998) (for phosphonatase pathway, see Fig. 1.6 in Chapter 1). Genes for both proteins were found in picocyanobacteria (Su et al., 2003), though located in different operons with *phnW* likely being the *aspC* gene in the phosphonate transport operon and *phnX* (named *cbbY*-like in annotation of genomes) in a separate operon together with a conserved hypothetical protein. This arrangement is unusual for phosphonatase pathway found in *Pseudomonas aeruginosa*, *Salmonella typhimurium* and other heterotrophic bacteria (Dumore et al., 1998; Jiang et al., 1995; Ternan et al., 1998), which also have a specific 2-AEP transport system (Ternan et al., 1998). It is likely that phosphonatase is not restricted to 2-AEP degradation, but can hydrolyze a variety of phosphonates that have an electron-withdrawing substituent at beta carbon polarizing the C-P bond. Since these cyanobacteria have a non-specific transport system, some of them may exploit the capacity of a phosphonatase utilizing 2-AEP, glyphosate, and PA as observed by the growth assays described herein.

A possible loss of function of PhnE protein in *Prochlorococcus* MIT9301 may be compensated by the PhnE encoded in a second *phnCDE* operon that has been likely acquired by a horizontal gene transfer. In *Prochlorococcus* MIT9215 and AS9601, the second operon was not found, thus these strains may have lost their ability for phosphonate uptake. To demonstrate
the diversity of picocyanobacteria that are capable of utilizing different phosphonate compounds, more extensive growth studies have to be performed on other cultured *Synechococcus* and *Prochlorococcus* spp.

4.3.3 A possible regulatory mechanism for phosphonate utilization gene expression in picocyanobacteria.

The *pho* box identified in this study resembles the cyanobacterial *pho* box described by Su et al. (2007). The presence and location of the *pho* box in the putative promoter regions of the *phoA* gene in *Synechococcus* WH8102 and *Prochlorococcus* NATL2A and the *pstS* and *phoE* genes in *Prochlorococcus* MED4 are consistent with the prediction by Su et al. (2007). In addition, *Prochlorococcus* MED4 *pstC, phoE, pstS, phoB* and *phoR* genes were upregulated in 48h under P limitation as determined by a microarray (Palenik et al., 2006), and a *pho* box was found in promoters of these genes in the present study.

In our previous survey, quantitative RT-PCR revealed that *Synechococcus* WH8102 activated expression of the *phnD* and *phnX* in parallel to *phoB* and *pstS* under P limitation (Ilikchyan et al., In press). Expression of *phnD* was activated in *Synechococcus* ARC-21 as well. However, a *pho* box was not found in the promoters of both *phnDCE* and phosphonatase in all analyzed picocyanobacteria (this study and Su et al., 2007). The exception was a second *phnCDE* operon copy, phylogenetically distinct from picocyanobacterial *phnDCE*, found in *Prochlorococcus* MIT9301. Indeed, *phnDCE* genes were not activated in *Prochlorococcus* MED4 and MIT9313 in 48h under P limitation when the *pho* genes were upregulated (Martiny et al., 2006). All together this indicates that phosphonate utilization genes in picocyanobacteria are
either indirectly regulated by \textit{phoBR}, or they are regulated in parallel by a mechanism independent of \textit{phoBR}.

The presence of a gene encoding a type III sigma factor (\textit{sigF}) within or downstream of the \textit{phnDCE} operon in all \textit{Synechococcus} spp. except WH7803 and in \textit{Prochlorococcus} MIT9313 suggests a possible regulation mechanism. SigF regulates transcription of genes in response to cell surface associated processes such as pilus formation and motility in \textit{Synechocystis} sp PCC 6803 (Asayama and Imamura, 2008). A type III sigma factor is usually contranscribed with a membrane-spanning anti-sigma factor and also activates expression of itself (Sakamoto et al., 2007; Asayama and Imamura, 2008). A gene for putative anti-sigma factor was not evident in \textit{Synechococcus} spp. and \textit{Prochlorococcus} MIT9313 \textit{phnDCE} operons. However, the putative promoters of both \textit{phnDCE} and phosphonatase are distinct from the promoters of \textit{pho} genes, and in \textit{Synechococcus} spp. and \textit{Prochlorococcus} MIT9313, the \textit{phnDCE} promoters are GA-rich, which is one of the main features of SigF recognizable promoters (Asayama and Imamura, 2008).

If transcription of the \textit{phnDCE} is regulated by an alternative sigma factor, transcription of the phosphonatase operon might be regulated by a two-component regulatory system based on the presence of a kinase encoding gene in the operon of a majority of analyzed \textit{Synechococcus} spp., as well as \textit{Prochlorococcus} strain MIT9313. A conserved hypothetical protein, encoded by a gene located downstream from \textit{phnX}, contains transmembrane domains, and therefore, might be a potential response regulator. Supporting a distinct regulation, the structure of \textit{phnDCE} and phosphonatase promoters is also different in all analyzed \textit{Synechococcus} spp. and \textit{Prochlorococcus} MIT9313 (Figs. 4.8 and 4.9); nevertheless, expression of \textit{phnD} and \textit{phnX} in WH8102 were similar under P limitation (Ilikchyan et al., In press).
**Synechococcus** RCC307, a strain isolated from Mediterranean Sea, lacks the *tesA* gene in the *phnDCE* operon and the chp-kinase gene in the phosphonatase operon. Motifs found in the *phnDCE* promoter of all other *Synechococcus* spp. were not identified in *Synechococcus* RCC307, suggesting a different mode of regulation in this strain.

*Prochlorococcus* spp. except MIT9313 lack the sigma factor and kinase encoding genes in phosphonate transporter and phosphonatase operons respectively. Therefore, with the exception of LL adapted clade IV, *Prochlorococcus* spp. possess conserved functional genes for phosphonate utilization, but have lost the regulatory genes. This is one of many examples (Scanlan and West, 2002; Moore et al., 2002; Dufresne et al., 2003; Palenik et al., 2003; Rocap et al., 2003; Moore et al., 2005) of genes encoding for regulatory mechanisms in picocyanobacteria that have been lost during evolution in *Prochlorococcus* spp. ecotypes. This is likely due to redundancy of possessing such genes in strains adapted for life in relatively constant marine environments. The absence of regulatory genes in HL *Prochlorococcus* spp. confirms and explains a constitutive expression of the *phnD* in this clade observed in our previous studies (Ilikchyan et al., In press).

Putative promoters of the *phnDCE* and *phnX* operons share some similarity in *Prochlorococcus* spp. belonging to HL clades, however, the significance of similarity is unclear due to an overall high AT genomic content. The motifs found in phosphonate utilization promoters of *Prochlorococcus* HL spp. are different from motifs in *Synechococcus* spp. suggesting distinct regulation mechanisms. Reflecting the genomic and physiological differences in HL and LL clades (Scanlan and West, 2002; Moore et al., 2005; Martiny et al., 2006), no shared motifs were found in *Prochlorococcus* MIT9211, CCMP1375, and NATL1a, all
belonging to LL clades, despite the fact that the structure of the operons is the same as in HL adapted *Prochlorococcus* spp.

### 4.4 Concluding remarks

Differences in P metabolism among clades of picocyanobacteria have been reported previously (Rippka et al., 2000; Scanlan and West, 2002; Moore et al., 2005; Martiny et al., 2006; Su et al., 2007), and attributed to the conditions at a specific occupied ecological niche (Martiny et al., 2006). Herein, the growth of freshwater *Synechococcus* spp. on phosphonates demonstrated that some, but not all are capable of utilizing phosphonates as a sole P source, despite the fact that functional genes for phosphonate utilization are conserved among all picocyanobacteria analyzed. For example, *Synechococcus* KD3a, a Lake Erie isolate, which is abundant in early summer (Wilhelm et al., 2006) failed to grow on either of phosphonate sources tested. This culture also showed poor alkaline phosphatase activity when transferred into P-free media (data not shown) suggesting that KD3a has adapted to more nutrient rich conditions and thus does not need an ability for phosphonate utilization. By contrast, marine *Synechococcus* WH8102 inhabits the open ocean where P limitation is a common feature in many regions, and phosphonates may contribute up to 25% of HMW DOP pool (Clark et al., 1998). Additional growth experiments will help to estimate the significance of phosphonates as an alternative P source for other marine and freshwater *Synechococcus* and *Prochlorococcus* spp.

Moreover, *in silico* analysis of promoter sequences and operon structure for *phnDCE* and phosphonatase revealed a potential regulation mechanism for phosphonate utilization not reported before for phosphonatase pathway. However, reflecting differences in P metabolism among *Synechococcus* and *Prochlorococcus* spp. clades, the potential regulatory genes are
missing in most *Prochlorococcus* spp. This supports constitutive expression of the *Prochlorococcus* spp. *phnD* observed in the field samples (Chapters 2 and 3). Thus, more work remains to be done to dissect expression pattern while considering picocyanobacterial clades diversity. Mutagenesis of the promoters for *phnDCE* and phosphonatase operons in cultured picocyanobacteria will help to elucidate the regulation of the phosphonate utilization in these genera.

### 4.5 Experimental procedures

#### 4.5.1 Cultures

*Synechococcus* spp. cultures used in this study included a marine strain WH8102 (CCMP), freshwater strains isolated from Lake Erie ARC-11, ARC-21, KD3a (Ivanikova et al., 2008), and strains isolated from Lake Superior LS0504 and LS0512 (Ivanikova et al., 2008).

#### 4.5.2 Growth conditions

*Synechococcus* WH8102 was maintained in SN medium (Waterbury et al., 1986) under continuous illumination of 25 μmol quanta m$^{-2}$ s$^{-1}$ at 25°C. Freshwater *Synechococcus* spp. were maintained in BG-11 medium (Allen, 1968; as described at [http://www.cyanosite.bio.purdue.edu](http://www.cyanosite.bio.purdue.edu)) under continuous illumination of 5-10 μmol quanta m$^{-2}$ s$^{-1}$ at 23°C. For phosphonate utilization experiments, all cultures were grown to mid exponential phase, cells were harvested by centrifugation, and washed twice with P-free medium, SN for WH8102 and BG-11 for all freshwater strains. After centrifugation, cells were resuspended in P-free medium, and aliquots were inoculated into media with different P amendments in triplicates, where P-replete was regular media, SN or BG11; NoP was phosphate free media, and
Phosphonates were added in concentration 10 µM to substitute phosphate in all other media. The abbreviations, source, and purity of phosphonates are given in Table 4.2. All glassware for growth experiments was acid-washed and rinsed with ddH₂O to eliminate any phosphate residue. Phosphate concentrations contaminating 10 mM phosphonate solutions was measured with the ascorbate-reduced molybdenum-blue assay.

4.5.3 Motif search

Putative promoter sequences of phosphonate utilization and P genes were selected from genomes of *Synechococcus* spp. WH8102 (NC_005070), WH7803 (NC_009481), WH9605 (NC_007516), WH9902 (NC_007513), CC9311 (NC_008319), WH5701 (NZ_AANO00000000), RCC307 (NC_009482) and *Prochlorococcus* spp. MIT9313 (NC_005071), MIT9211 (NC_009976), CCMP1375 (NC_005042), NATL1A (NC_008819), MIT9515 (NC_008817), MED4 (NC_005072), MIT9312 (NC_007577), MIT9301 (NC_009091), AS9601 (NC_008816), and MIT9215 (NC_009840). The length of the putative promoter was determined by the distance between translational start site and the closest upstream gene (but not less than 75 nucleotides). Motif searches were done with MEME software at [http://meme.sdsc.edu/meme/meme.html](http://meme.sdsc.edu/meme/meme.html) (Timothy et al., 1994; Timothy et al., 2006). The following parameters were chosen: any number of the occurrences of a single motif among the sequences, with minimum width of six nucleotides and maximum width of 24 nucleotides, and a maximum of ten motifs per search. The cutoff p-value for identified motifs was 1e-6. Three separate searches were performed, *Synechococcus* spp. promoters (including LL clade *Prochlorococcus* MIT9313), *Prochlorococcus* spp. promoters, and promoters of both *Synechococcus* and *Prochlorococcus* spp. together.
4.5.4 Other bioinformatics tools

The presence of phosphonate genes in picocyanobacterial genomes was identified with BLAST searches. Alignment of hypothetical protein sequences was done with BLASTp against the nr database at http://blast.ncbi.nlm.nih.gov/. The presence of transmembrane domains in proteins was analyzed with TMHMM at http://www.cbs.dtu.dk/ (CBS, Denmark).

4.5.5 POP analysis

*Synechococcus* ARC-21 was grown at different P conditions as described in Ilikchyan et al., In press. Forty mL of culture from each replicate was filtered onto a GF/F (Millipore) pre-combusted filter that was washed with 10% hydrochloric acid and rinsed with ddH₂O. Filters were stored at -20°C until processed. Blank filters with were prepared as well. Particulate organic phosphorus was measured by the ascorbate-reduced molybdenum-blue method after persulfate oxidation at 120°C for 30 min. The POP was normalized to the cell number. Cells were directly enumerated by autofluorescence as previously described (Caron et al., 1985). In brief, 1 mL of each culture was fixed with glutaraldehyde (2% v/v final) and stored at -4°C in dark until processing. Five mL of fixed cells diluted 150 folds were filtered onto 0.20 µm black polycarbonate filters, and filters were mounted on glass slides. Cells were counted using an epifluorescence microscope in at least 10 field views. Statistical analysis was done using ANOVA with α=0.05.
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Table 4.2 Phosphonates abbreviations, source, and purity (as stated by manufacturer).
CHAPTER IV. CONCLUSIONS AND FUTURE DIRECTIONS

Picocyanobacteria are abundant and dominant photoautotrophic organisms in diverse marine and freshwater environments, especially in oligotrophic ecosystems (Olson et al., 1990a; Olson et al., 1990b; Campbell and Vaulot, 1993; Veldhuis and Kraay, 1993; Weisse, 1993; Padisak et al., 1997; Ahlgren and Rocap, 2006; Wilhelm et al., 2006). Given their significant contribution to the primary production (Li, 1994; Li et al., 1997; Veldhuis et al., 1997; DuRand et al., 2001), understanding the picocyanobacterial physiology, such as phosphorus metabolism, is important in terms of global biogeochemical cycles. Phosphorus is often a limiting nutrient of primary productivity in the pelagic and coastal marine regions and in freshwaters (Schindler, 1977; Krom et al., 1991; Wu et al., 2000; Karl et al., 2001; Sañudo-Wilhelmy et al., 2001; Dyhrman et al., 2007; Paytan and McLaughlin, 2007). When inorganic phosphorus is depleted, the phosphorus demand in microorganisms is fulfilled from the dissolved organic phosphorus pool, that includes organic phosphate and phosphonate compounds. Recently, phosphonates have been considered as a substantial alternative source of phosphorus for microorganisms in marine waters under P limited conditions (Dyhrman et al., 2006; Adams et al., 2008; Gilbert et al., 2008; Karl et al., 2008). In lakes, where P limitation is a central feature (Schindler, 1977), naturally derived phosphonates have not yet been detected; however, agricultural runoff can contain anthropogenically derived phosphonates in the form of herbicide glyphosate (Scribner et al., 2003; Byer et al., 2008). Based on sequenced genomes, picocyanobacteria possess genes for the phosphonate uptake and hydrolysis (Moore et al., 2005; Su et al., 2003). The focus of this study was to assess the capability of picocyanobacteria to assimilate phosphonates in
diverse aquatic environments by applying molecular approaches, culturing methods, and analysis in silico.

A PCR-based method was designed to detect the presence and expression of *phnD* gene encoding the phosphonate binding protein of an ABC type transporter in picocyanobacteria. The degenerate primers for *Synechococcus* spp. and *Prochlorococcus* spp. specifically amplified a region of *phnD* gene from all cultured marine and freshwater picocyanobacteria examined. The presence of picocyanobacterial *phnD* was detected in diverse natural water samples taken from the Sargasso Sea, South Pacific Ocean, Monterey Bay, Baltic Sea, and Lake Erie (Ilikchyan et al., In press; Chapter 3). Further, an RT-PCR assay identified expression of the *Synechococcus* spp. and *Prochlorococcus* spp. *phnD* gene indicating utilization of phosphonates by endemic picocyanobacteria in these environments. The exception was Lake Erie, where *phnD* expression was not observed due to insufficient range of sampling (Chapter 3).

The functionality of the phosphonate genes in picocyanobacteria was confirmed by the observed growth of *Synechococcus* spp. on phosphonate compounds as a sole P source (Chapter 4). Although not all freshwater *Synechococcus* spp. tested were able to utilize phosphonates as a P source, a marine strain *Synechococcus* WH8102 and a freshwater strain *Synechococcus* ARC-21 showed an abundant growth on 2-aminoethylphosphonate (Ilikchyan et al., In press; Chapter 4). From the diversity of phosphonate compounds analyzed, *Synechococcus* spp. were able to assimilate phosphonates that have a polarized C-P bond. This probably reflects the specificity of the phosphonatase pathway present in picocyanobacteria.

To analyze the regulation of phosphonate utilization genes expression, quantitative RT-PCR was conducted from marine *Synechococcus* WH8102 and
freshwater *Synechococcus* ARC-21 cultures grown at various P conditions. The genes for phosphonate utilization were activated in P-deficient media, and in WH8102, the activation was parallel to induction of *phoBR* regulated genes (Ilikchyan et al., In press). Further, the detection of *Synechococcus* spp. *phnD* expression in environmental samples followed the bioavailability of inorganic phosphate at a particular site. In samples from the Sargasso Sea, the *Synechococcus* spp. *phnD* expression was activated in the mixed surface layer in October 2007 and 2008 (Ilikchyan et al., In press; Chapter 3) reflecting the low concentrations of dissolved inorganic phosphorus (DIP) usual for this time of year (e.g. Schroeder and Stommel, 1969; DuRand et al., 2001). Relatively high DIP concentrations in spring due to recent mixing event (e.g. Schroeder and Stommel, 1969; DuRand et al., 2001) accounted for the lack of the *Synechococcus* spp. *phnD* expression in samples taken in May 2008 (Chapter 3). In the Baltic Sea, the limitation of primary production by inorganic phosphorus, thought is not as common as nitrogen limitation, occurs at certain regions and times (Stal et al., 2003). The expression of *Synechococcus* spp. *phnD* was seen in a majority of samples taken from the mixed surface waters of the Skagerrak, Kattegat, and the Baltic Sea in July 2008 (Chapter 3). The absence of *Synechococcus* spp. *phnD* expression in nearly all samples taken from the Monterey Bay station coincided with usually high DIP concentrations in coastal marine regions and winter mixing (Ilikchyan et al., In press). Last, the expression of the *Synechococcus* spp. *phnD* was deactivated by an addition of phosphate to natural water samples in the nutrient amendment experiment conducted during the Sargasso Sea cruise in October 2008 (Chapter 3).

Despite the observed P dependent activation of the *phnD* gene in *Synechococcus* spp., *pho* box was not found in putative promoters of the phosphonate utilization operons
in these picocyanobacteria and in *Prochlorococcus* spp. (Chapter 4). The lack of *pho* box in these promoters is consistent with the data obtained by Su et al. (2007). An alternative regulatory mechanism in response to P deprivation was proposed with the participation of type III sigma factor and a two-component regulatory system (Chapter 4).

By contrast to *Synechococcus* spp., *Prochlorococcus* spp. members of HLII clade yielded constitutive expression of the *phnD* gene in all samples where *Prochlorococcus* spp. DNA was detected (Ilikchyan et al., In press; Chapter 3). The expression was not affected by an addition of phosphate in the nutrient amendment experiment (Chapter 3). Although it is not known if *Prochlorococcus* HLII spp. were P-limited at the time of sampling, the constitutive expression observed in this study might be explained by the lack of regulatory genes in the phosphonate utilization operons of these picocyanobacteria (Chapter 4).

In overall, this study demonstrated the significance of phosphonates as a phosphorus source in the DOP pool for picocyanobacteria in P-limited environments. The RT-PCR assay with *Synechococcus* spp. *phnD* primers can be applied to assess the P status of these picocyanobacteria in diverse aquatic ecosystems. Development of clade specific molecular probes for the phosphonate utilization genes in picocyanobacteria can be a future approach to study the expression in marine and freshwater environments in detail with regards to the observed heterogeneity of P physiology among *Synechococcus* spp. and *Prochlorococcus* spp. clades (Moore et al., 2005; Martiny et al., 2006; this study). In addition, to understand the regulatory mechanism, expression of the phosphonate genes in other picocyanobacterial cultures can be analyzed by quantitative RT-PCR. These approaches would complement the findings of the present research.
REFERENCES


Kononen, K., Lahdes, E.O., & Grönlund, L. (1993) Physiological and community responses of summer plankton to nutrient manipulation in the Gulf of Finland (Baltic Sea) with special references to phosphorus. *Sarsia* 78: 243–253


APPENDIX I. SUPPLEMENTARY MATERIAL FOR CHAPTER II

This section is included as supplementary material for the manuscript Ilikchyan et al. in press in

*Environmental Microbiology.*

**Table A1.** List of amplicon sequences generated from environmental samples obtained from the Pacific Ocean and the Atlantic Ocean. *Synechococcus* sequences were obtained with the *Synechococcus* spp. *phnD* primers, and *Prochlorococcus* sequences were obtained with the *Prochlorococcus* spp. *phnD* primers. Amplicon sequences were searched against nr database with BLAST. Percent sequence identities are shown to *phnD* sequences from cultured picocyanobacteria. The *Synechococcus* spp. and *Prochlorococcus* spp. *phnD* sequences were 565 and 193 nucleotides in length, respectively.
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**Table A1:** List of amplicon sequences generated from environmental samples obtained from the Pacific Ocean and the Atlantic Ocean.
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Table A1: List of amplicon sequences generated from environmental samples obtained from the Pacific Ocean and the Atlantic Ocean (cont.)
Figure A1. Specificity of the *Synechococcus* spp. *phnD* (Panel A) and *Prochlorococcus* spp. *phnD* (Panel B) primers. Lanes are labeled with the specific strain DNAs tested. NTC, no template control.
Figure A2. Growth of *Synechococcus* sp. WH8102 (Panel A) and ARC-21 (Panel B) on phosphonates measured by extracted chlorophyll a (mg L$^{-1}$).
APPENDIX II. PHYLOGENETIC ANALYSIS OF THE PICOCYANOBACTERIAL \textit{phnD} SEQUENCES OBTAINED IN THIS STUDY

Figure B1. Phylogenetic tree of the \textit{Synechococcus} spp. \textit{phnD}. The tree was constructed from the \textit{Synechococcus} spp. \textit{phnD} sequences obtained in this study from amplification from environmental samples (Lake Erie, the Sargasso Sea, Baltic Sea, and Monterey Bay), cultured freshwater strains (isolated from Lake Erie and Lake Superior), or retrieved from available \textit{Synechococcus} genomes (filled circle). The \textit{Prochlorococcus} CCMP1986 \textit{phnD} sequence was used as an outgroup. The phylogenetic analysis was performed by the neighbor-joining method with 1,000 bootstrap replicates.
Figure B2. Phylogenetic tree of *phnD* from the freshwater *Synechococcus* cluster. The tree was constructed from the *Synechococcus* spp. *phnD* sequences obtained in this study from amplification of environmental samples from Lake Erie and the Sargasso Sea and cultured freshwater strains (isolated from Lake Erie and Lake Superior), or retrieved from available *Synechococcus* genomes (diamond). The *Synechococcus* WH8102 *phnD* sequence was used as an outgroup. The phylogenetic analysis was performed by the neighbor-joining method with 1,000 bootstrap replicates.

Figure B3. Phylogenetic tree of *phnD* from *Prochlorococcus* spp. HL clade. The sequences were obtained from amplification of environmental samples (the Sargasso Sea and South Pacific) or retrieved from available *Prochlorococcus* genomes (filled circle). The *Prochlorococcus* NATL1A *phnD* sequence was used as an outgroup. The phylogenetic analysis was performed by the neighbor-joining method with 1,000 bootstrap replicates.
### APPENDIX III. ABBREVIATIONS

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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>2AEP</td>
<td>2-aminoethylphosphonate</td>
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<tr>
<td>AMPA</td>
<td>Aminomethylphosphonate</td>
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<td>APA</td>
<td>Alkaline phosphatase activity</td>
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<td>BAP</td>
<td>Bioavailable phosphorus</td>
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<td>BATS</td>
<td>Bermuda Atlantic Time Series</td>
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<tr>
<td>BS</td>
<td>Baltic Sea</td>
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<tr>
<td>DCM</td>
<td>Deep chlorophyll maximum</td>
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<td>DIP</td>
<td>Dissolved inorganic phosphorus</td>
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<tr>
<td>DOP</td>
<td>Dissolved organic phosphorus</td>
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<td>High-light adapted Prochlorococcus</td>
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<td>HMW</td>
<td>High molecular weight</td>
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<td>Phycocyanin</td>
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<td>Reverse-Transcription PCR</td>
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<td>TDP</td>
<td>Total dissolved phosphorus</td>
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