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# Detection and expression of the phosphonate transporter gene *phnD* in marine and freshwater picocyanobacteria

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## Summary

**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 phoshonates in the ocean as well as monitoring the acquisition of synthetic phosphonate herbicides (e.g. glyphosate) by picocyanobacteria in freshwaters.**

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## 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 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 hydrolysed 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 harbour 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 labelled *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 have 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 *phnD* by picocyanobacteria in natural samples from a variety of both freshwater and marine environments.

## Results

Oligonucleotide primers were developed for both *Synechococcus* spp. and *Prochlorococcus* spp. *phnD* based on the PhnD primary structures deduced from the available genomic sequences. Primer design took into account the amino acid sequence dissimilarities between *Prochlorococcus* spp. and *Synechococcus* spp. *phnD* at sites conserved within each genus. Forward and reverse primers employed the conserved amino acid sequence from *Synechococcus* spp. PhnD residues 39–48 and 244–253 respectively (numbered using PhnD from WH8102). The *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 base pairs (bp). Primers for *Prochlorococcus* spp. *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 *Synechococcus* spp. primers was assessed following PCR of DNA from *Prochlorococcus* spp. Testing all representative strains from all *Prochlorococcus* spp. clades (MIT9312, MED4, MIT9313, NATL2A and CCMP1375) no PCR product was observed (Fig. S1A). Similarly, the *Prochlorococcus* spp. primers failed to amplify *Synechococcus* spp. *phnD* from marine strains WH5701, WH7803 and WH8102 as well as freshwater strains (Fig. S1B, also see Fig. 1C).

### *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. 1A) and marine (Fig. 1B) habitats. Freshwater environmental DNA included four sites in Lake Erie (LE samples, Fig. 1A) and 11 cultured *Synechococcus* sp. isolates from Lake Erie (Maumee, KD3a, ARC-11) and Lake Superior (LS prefix, Fig. 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 and colleagues (1998). Similarly, PCR yielded the *c*. 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. 1B). Only one sample from the deep chlorophyll maximum (95 m) at the Bermuda Atlantic Time-Series (BATS) station (Fig. 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. 1C). Thus, the PCR assay exhibits the specificity necessary to differentiate *phn* gene expression in mixed picocyanobacterial populations.

 

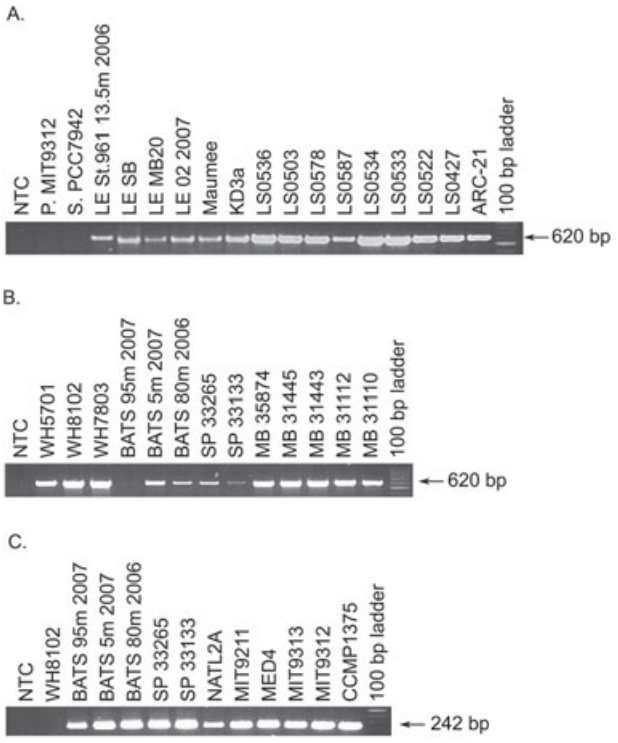


**Fig. 1.** A. PCR of *phnD* from freshwater environmental DNA and cultured freshwater *Synechococcus* spp. Environmental DNAs are from Lake Erie stations 961, Sandusky Bay (SB), MB20 and from the Maumee River that flows into Lake Erie's western basin. Cultured cyanobacteria of the picoplankton clade 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 7942.
B. PCR of *phnD* from cultured marine *Synechococcus* (WH5701, WH8102 and WH7803) and marine environmental DNA from the BATS station in the Sargasso Sea, two South Pacific stations (SP) and seasonal samples from Monterey Bay, CA station M1 (MB).
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.

All cloned amplicons obtained were verified as *Synechococcus* sp. or *Prochlorococcus* sp. *phnD* sequences by searching the GenBank non-redundant database (summarized in Table S1). 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). *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).

### 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). 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-AEP treatments, expression of WH8102 *phnD* and *phnX* as well as the *pstS* genes was activated in 5 days, and the expression of *phnD* and *phnX* paralleled the activation of the *phoB* gene (Fig. 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 9 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 h in 2-AEP but not in the LowP samples, where concentration of external inorganic P was higher at this time (Fig. 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 *phnD* in the Lake Erie freshwater *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 with WH8102 (Fig. 3C). Routinely, mRNA levels increased only three to fourfold over P-replete growth conditions. Under these culture conditions, ARC-21 grows utilizing both 2-AEP and glyphosate as P sources (Fig. S2B). 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 *phoB, pstS* and *phnX.*

### *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

 

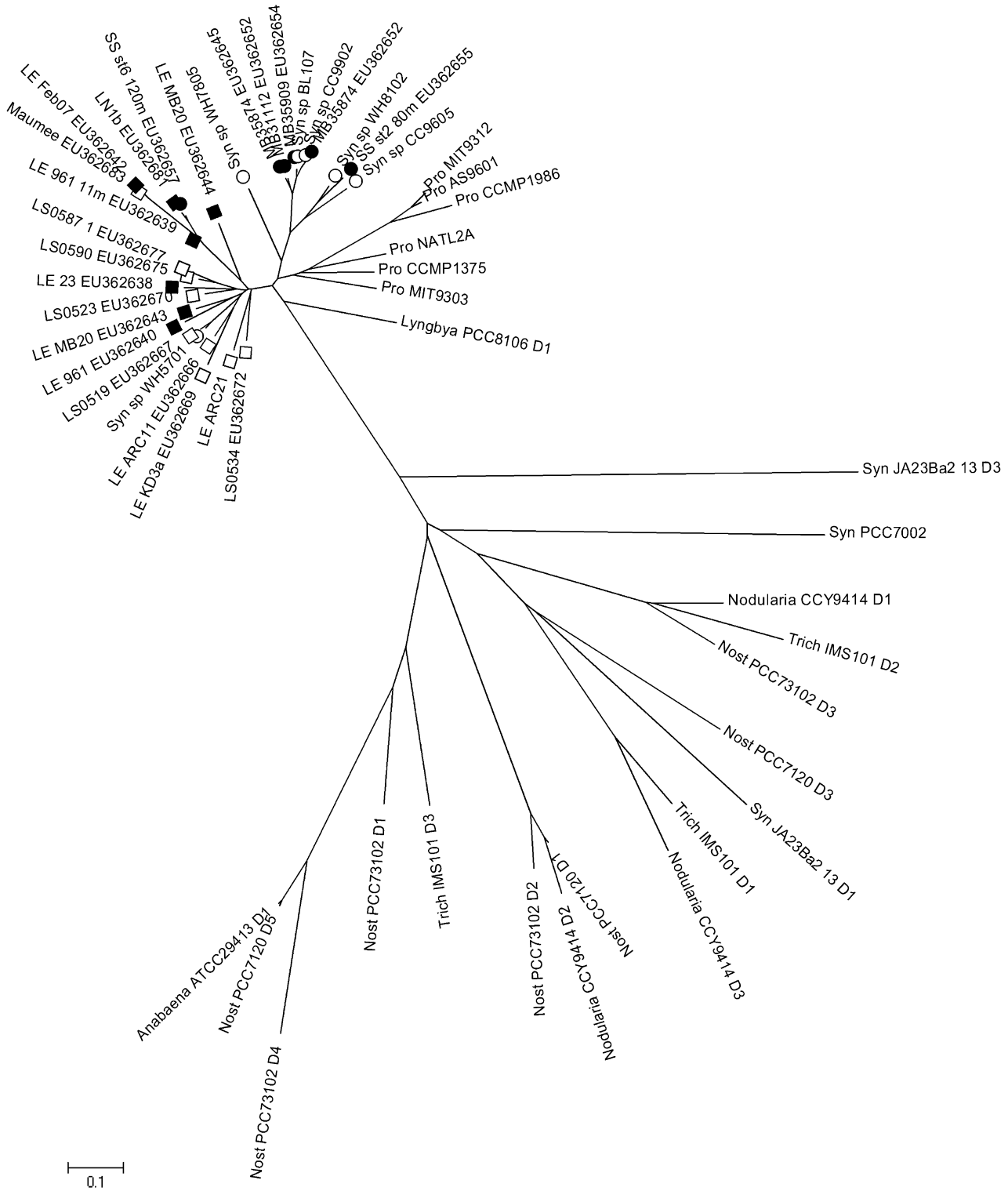


**Fig. 2.** Phylogenetic tree of PhnD sequences deduced from DNA sequences obtained from freshwater *Synechococcus* spp., marine *Synechococcus* spp., *Prochlorococcus* spp., filamentous cyanobacteria and amplicons from environmental DNA. Accession numbers are included where appropriate. Open symbols, cultured picocyanobacteria; closed symbols, environmental samples; squares and circles, freshwater and marine environments respectively.

 

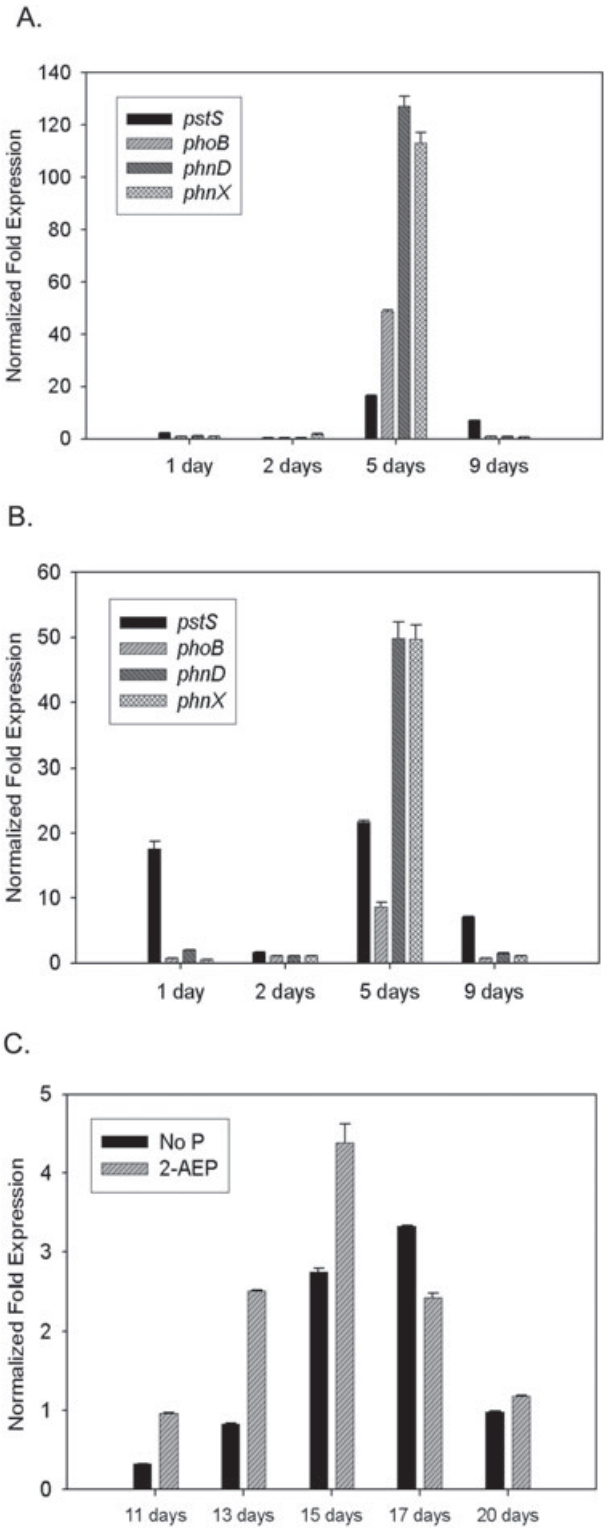


**Fig. 3.** A and B. Quantitative RT-PCR of genes involved in phosphorus acquisition in WH8102 cultures following transfer to LowP medium (A) and P-free medium containing 10 μM 2-AEP (B). Expressions of *pstS*, *phoB*, *phnD* and *phnX* were normalized to *rnpB* expression and corresponding P-replete samples.
C. Quantitative RT-PCR of *phnD* in ARC-21 cultures following transfer to LowP BG-11 or P-free BG-11 containing 10 μM 2-AEP.

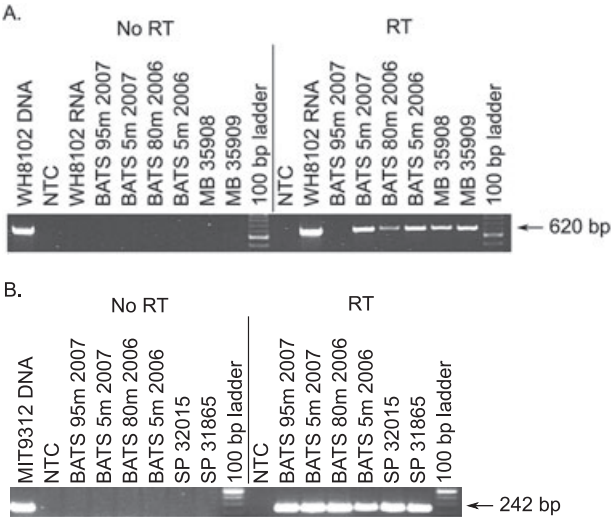


**Fig. 4.** A. RT-PCR of *phnD* from seston RNA from the BATS station in the Sargasso Sea and Monterey Bay station MB1 (MB); *Synechococcus* sp. WH8102 RNA was used as a positive control.
B. RT-PCR of *Prochlorococcus* spp. *phnD* sequences from environmental RNA from BATS and two South Pacific Ocean stations (SP).

(Fig. 4A and B). 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 1B and 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. 5A). This expression profile largely coincided with the concentration of DIP in the samples (Table 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

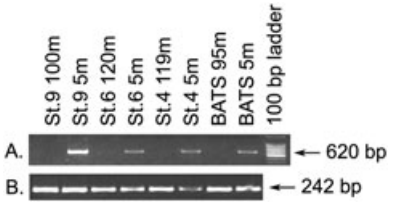


**Fig. 5.** RT-PCR of *phnD* from Sargasso Sea samples taken in October 2007.
A. *Synechococcus* spp. *phnD*.
B. *Prochlorococcus* spp. *phnD*. *Synechococcus* spp. *phnD* expression in samples from the surface (5 m) and the lack of expression at deep chlorophyll maximum (95–120 m) suggest the availability of P with depth (summarized in Table 1).

 

**Table 1.** Phosphate (DIP) concentration, picocyanobacterial cell number and *phnD* amplification at Sargasso Sea stations, October 2007.

| Station | Depth (m) | DIP (nM) | Syn (cells ml$^{-1}$) | Pro (cells ml$^{-1}$) | Syn *phnD* DNA | Syn *phnD* RNA | Pro *phnD* DNA | Pro *phnD* RNA |
|---|---|---|---|---|---|---|---|---|
| BATS | 4 | 0.5 | 6424 | NA | + | + | + | + |
| | 95 | 12.89 | 2688 | 133597 | – | – | + | + |
| Sta. 4 | 5 | 1.1 | NA | NA | + | + | + | + |
| | 119 | 1.3 | 130 | 45612 | + | – | + | + |
| Sta. 6 | 5 | 1.2 | NA | NA | + | + | + | + |
| | 120 | 4.6 | 246 | 55673 | + | – | + | + |
| Sta. 9 | 4 | 0.1 | NA | NA | + | + | + | + |
| | 100 | 4 | NA | NA | + | – | + | + |

NA, data not available; +/– indicates the presence/absence of a *phnD* amplicon.

as low as 130 cells ml$^{-1}$ (Table 1). Alternatively, expression of the *Prochlorococcus* spp. *phnD* was observed in all Sargasso Sea samples at all depths (Fig. 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; 2007).

## Discussion

### *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 indicates that *Synechococcus* spp. and *Prochlorococcus* spp. *phnD* are 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, pers. comm.). 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). By contrast, *phnD* sequences from filamentous cyanobacteria are phylogenetically distinct from the picocyanobacterial orthologues, exhibit greater diversity and commonly form multigene families that may have arisen by gene duplication.

### *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. 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 and colleagues (2003) 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, 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 abovementioned 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 *Escherichia coli* (Scholten and Tommassen, 1993).

As *phnD* is typically co-transcribed with the *phnC* and *phnE* genes that together encode the complete phosphonate ABC transporter (e.g. Huang *et al*., 2005), 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 (Moore *et al*., 2005; Martiny *et al*., 2006).

### *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 near-shore samples analysed 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 (*c*. 95%) to *Synechococcus* sp. CC9902 (Table S1). As 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 near-shore and pelagic ecosystems will help clarify whether multiple mechanisms exist for *phn* transcriptional regulation.

By contrast, *phnD* expression in *Prochlorococcus* spp. was uniformly constitutive in all environmental samples tested thus far (Figs 4C and 5B and Table 1), suggesting a lack of regulation of the *phnCDE* operon in HLII clade. Whether the uniform presence of the *phnD* RT-PCR amplicon is truly due to constitutive expression awaits further investigation, because the samples employed typically contained 50- to 60-fold more *Prochlorococcus* spp. versus *Synechococcus* spp. cells in the DCM (Table 1). Indeed, Martiny and colleagues (2006) demonstrated that *Prochlorococcus* spp. *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 *phnD* transcription among the picocyanobacteria.

### *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 *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 *phnD* in these groups, in combination with additional studies on the regulation of *phnD*, would be a valuable mechanism for examining the phosphorus physiology of the picocyanobacteria in more detail from both marine and freshwater environments.

## Experimental procedures

### *Environmental samples and picocyanobacterial strains*

Table 2 provides details of sampling and sources of picocyanobacterial cultures. Seston from the environmental samples was harvested by filtering onto 0.22 μm polycarbonate filters (Millipore) that were transferred into 2 ml cryo tubes and preserved with RNALater (QIAGEN), or alternatively, seston was collected onto 0.22 μm 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.

### *Cell culturing methods*

Freshwater *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

**Table 2A.** Environmental samples employed in this study.

| Location | Sample ID | Date | Latitude | Longitude | Depth (m) | Volume filtered (ml) |
|---|---|---|---|---|---|---|
| *Marine environmental samples:* | | | | | | |
| *Atlantic Ocean:* | | | | | | |
| Sargasso Sea | BATS 5m 2006 | 10/2006 | 31°39′N | 64°09′W | 5 | 4000 |
| | BATS 80m 2006 | | | | 80 | 4000 |
| | BATS 5m 2007 | 10/2007 | | | 5 | 4000 |
| | BATS 95m 2007 | | | | 95 | 4000 |
| | St.4 5m | 10/2007 | 29°40′N | 64°28′W | 5 | 4000 |
| | St.4 119m | | | | 119 | 4000 |
| | St.6 5m | 10/2007 | 27°40′N | 64°46′W | 5 | 4000 |
| | St.6 120m | | | | 120 | 4000 |
| | St.9 5m | 10/2007 | 24°40′N | 65°13′W | 5 | 4000 |
| | St.9 100m | | | | 100 | 4000 |
| *Pacific Ocean:* | | | | | | |
| Monterey Bay | MB31110 | 09/27/2006 | 36°75′N | 122°03′W | 5 | 1000 |
| St. MB1 | MB31112 | | | | 10 | 1000 |
| | MB31443 | 11/08/2006 | | | 5 | 700 |
| | MB31445 | | | | 10 | 1000 |
| | MB35874 | 02/15/2007 | | | 10 | 750 |
| | MB35908 | | | | 5 | 1000 |
| | MB35909 | | | | 10 | 1000 |
| *South Pacific Ocean:* | | | | | | |
| St. KM0703.004.01.24 | SP33133 | 03/18/2007 | 14°50′S | 155°W | 5 | 4000 |
| | SP31865 | | | | 5 | 3900 |
| St. KM0703.007.05.24 | SP33265 | 03/21/2007 | 22°30′S | 157°W | 5 | 3000 |
| | SP32015 | | | | 5 | 3000 |
| *Freshwater environmental samples:* | | | | | | |
| *Lake Erie:* | | | | | | |
| St. 23 | LE23 | 08/2007 | 42°30′N | 79°53′W | 5 | 100 |
| St. 961 | LE961_11 | 08/2007 | 41°55′N | 82°11′W | 11 | 100 |
| | LE961_13.5 | 08/2007 | | | 13.5 | 100 |
| | Feb07 | 02/2007 | | | 5 | 100 |
| Maumee Bay | MB20 | 06/2007 | 41°43′N | 83°27′W | 1 | 100 |
| Sandusky Bay | SB | 08/2007 | 41°28′N | 82°43′W | 1 | 50 |
| *Nettle Lake, Ohio* | LN | 06/2007 | 41°41′N | 84°44′W | 1 | 50 |

**Table 2B.** Cultures employed in this study.

| Source | Name | Reference |
|---|---|---|
| *Cultures:* | | |
| Lake Superior | LS 0427 | Ivanikova *et al.* (2007) |
| | LS 0503 | Ivanikova *et al.* (2008) |
| | LS 0522 | |
| | LS 0533 | |
| | LS 0534 | |
| | LS 0536 | |
| | LS 0578 | |
| | LS 0587 | |
| Lake Erie | LE ARC-11 | Ivanikova *et al.* (2007) |
| | LE KD3a | Ivanikova *et al.* (2008) |
| | LE ARC-21 | |
| Maumee river | Maumee | R.M.L. McKay |
| Axenic cultures | WH 8102 | CCMP |
| | WH 5701 | R.M.L. McKay |
| | WH 7803 | R.M.L. McKay |
| | PCC 7942 | Pasteur Culture Collection |
| *Genomic DNA:* | | |
| Prochlorococcus | MIT 9211 | Eric Zinser |
| | MIT 9312 | |
| | MIT 9313 | |
| | MED4 | |
| | NATL2A | |
| | CCMP 1375 | |

these illumination conditions, growth rates of newly inoculated cultures were typically *c.* 0.6 $day^{-1}$. $K_2HPO_4$ was omitted in P-deplete BG-11, added at concentration of 8.6 μM in LowP SN, or substituted by 10 μM 2-AEP (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).

### *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, 3000 *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 vol. of 10% (wt/vol) sodium dodecyl sulfate and incubation at 65°C for 40 min. Further, 0.3 vol. 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 two 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 μm 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× STET buffer containing lysozyme (10 mg ml$^{-1}$) was 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) was passed through the filter. Next, 400 μl of the TE/lysozyme buffer was 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 was 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.

### *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′-TCGG NGCMATYCCSGATCAGAACCCSG-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). Each PCR reaction (25 μl) contained 1× 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 *c.* 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 *c.* 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 analysed after cloning into TOPO plasmid vectors (Invitrogen), and amplicons were sequenced at the University of Chicago Cancer Research Center using the T7 primer.

### *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 *c.* 89–150 nucleotides, and their efficiencies were estimated by dilution series over two orders of magnitude on cDNA derived from WH8102 or ARC-21 RNA from P-replete samples (Table 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 threefold, and 5 μl of the diluted cDNA was 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 s, 55°C for 30 s and 72°C for 15 s 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 ($\Delta\Delta C_T$ 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 3).

### *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

 

**Table 3.** *Synechococcus* sp. WH8102 and ARC-21 specific primers for real-time PCR.

| Name (gene-strain) | Sequence (5′-3′) | Efficiency (%) | Amplicon size (bp) |
|---|---|---|---|
| CBBY-WH-F | GACCATGGTCTGAAGGAGCG | 80 | 89 |
| CBBY-WH-R | CCGCTGAACTGTGGTGCAGC | | |
| PHND-WH-F | ACCACCCTCCGCAGATCAGGTTC | 87 | 115 |
| PHND-WH-R | CCACATTGAGGGAATCACTCAG | | |
| PHOB-WH-F | ATCTACAGCCACGCCAACC | 100 | 103 |
| PHOB-WH-R | AGGTCGAGGATCTTGTACTC | | |
| PSTS-WH-F | GCACCATCGCCTTCGGCTACAAC | 94 | 114 |
| PSTS-WH-R | GCTGGCAACCGAGGTCTTGC | | |
| RNPB-F | CCGTGAGGAGAGTGCCACAG | 100 | 114 |
| RNPB-R | CAGCACCTCTCGATGCTGCTGG | | |
| PHND-A21-F | ATCTGGTCTGGTTTGGCGGTC | 97 | 148 |
| PHND-A21-R | TGGTTCTGGATCGGTTTGATG | | |

sequence data obtained from PCR or RT-PCR amplification and subsequent cloning were analysed 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.

### *Accession numbers*

*phnD* sequences obtained in this study were deposited in GenBank under Accession Numbers EU362636–EU362729 and FJ172179–FJ172204 (Table S1).

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## Supporting information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Specificity of *Synechococcus* spp. *phnD* (A) and *Prochlorococcus* spp. *phnD* (B) primers. Lanes are labelled with the specific strain DNAs tested. NTC, no template control.
**Fig. S2.** Growth of *Synechococcus* sp. WH8102 (A) and ARC-21 (B) on phosphonates measured by extracted chlorophyll *a* (mg $l^{-1}$).
**Table S1.** Amplicon sequences from environmental samples generated by PCR and RT-PCR.

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