CONSTRUCTION AND CHARACTERIZATION OF CYANOBACTERIAL BIOREPORTERS TO ASSESS PHOSPHORUS BIOAVAILABILITY IN MARINE ENVIRONMENTS

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ABSTRACT

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Deficiency of essential nutrients for phytoplankton such as iron and phosphorus is well documented in the world oceans. As a relatively novel approach for identification of bioavailable P in marine systems, this research is focused on the construction and initial characterization of P-responsive cyanobacterial bioreporters for assessing the bioavailability of P in marine environments. Three bioreporters were constructed by transforming the fusion of P-responsive promoter sequences with *luxAB* into *Synechococcus* sp. PCC 7002. All obtained bioreporters were shown to produce luminescence after addition of aldehyde; however, the intensity of bioluminescence did not always correlate to the concentration of inorganic phosphate in medium. The analysis of the promoter sequences revealed a similar 15 bp motif that can be a potential consensus sequence conserved in P-responsive promoters of *Synechococcus* sp. PCC 7002 and some other genetically similar microorganisms.

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INTRODUCTION

Representing the foundation of the marine food chain, phytoplankton play the essential role in primary production of the world's oceans and contributes to the nutrient cycling in aquatic environments. Thus in the carbon cycle, due to the photosynthetic ability of many organisms belonging to this ecological niche, phytoplankton are responsible for the conversion of carbon dioxide into organic materials which are further available for higher organisms in the ocean and on land. Carbon dioxide is liberated as the result of their respiration and several other factors, and the cycle starts over. In turn, the limitation of phytoplankton growth and production can disturb global carbon cycling and indirectly contribute to climate changes. Therefore, one of the fundamental questions of marine ecology is what factors and, particularly, nutrients can limit phytoplankton growth?

This problem has been intensively studied during last several decades. Although many regions of the ocean have been documented to be limited by nitrogen (Smith, 1984; Howarth and Marino, 2006) or iron (Mills et al. 2004; Boyanapalli et al. 2007), some marine systems were suggested to be limited by phosphorus. Phosphate depletion in marine diazotrophic cyanobacteria has been reported in the western regions (Wu et al. 2000; Moore et al. 2008) and the eastern tropics (Mills et al. 2004) of the North Atlantic Ocean, Mediterranean Sea (Thingstad et al. 2005) and in different coastal ecosystems (Sundareshwar et al. 2003; Rejmankova and Komarkova, 2000).

The possibility of phosphate depletion in marine systems can be explained if we consider the phosphorus cycle. It starts from weathering rock formations which contain phosphate salts, yielding phosphates dissolving in soil waters and rivers. Riverine flux

provides phosphates to the world oceans. However, due to low solubility of phosphate salts in water and their high capability to bind to soil particles, only a small fraction of total phosphate is able to reach the oceans.

While phosphorus deficiency is a limiting factor for growth in all groups of phytoplankton, phosphate requirements differ from group to group and even from one bacterial strain to another. Basically, they depend on two major factors – the size of a cell and intracellular phosphate transport mechanism. In oligo- and mesotrophic areas of oceans, the majority of the phytoplankton community is represented by photosynthetic picocyanobacteria *Prochlorococcus* and *Synechococcus* (Partensky et al. 1996; Jacquet et al. 1998). Both of these clades are characterized by slow rates of metabolism and growth, the features which are necessary to survive in oligotrophic environment. Permanent life in nutrient's depleted waters has resulted in cyanobacteria having evolved to have extremely sophisticated, regulated nutrient uptake systems. The next section will describe how phosphate deficiency influences their metabolism and the mechanisms controlling P-uptake and transport in cyanobacteria.

Phosphorus deficiency of cyanobacteria

Phosphorus is an essential component of all living cells. It comprises the backbone of DNA and RNA molecules which are responsible for such important functions in organisms as hereditary information storage and protein synthesis. Adenosinetriphosphate (ATP) molecules perform energy transfer functions in almost all known forms of life also are P-rich molecules. Phospholipids serve as key components of cellular membranes.

For photosynthetic organisms, phosphorus and other nutrient limitations cause a reduction in growth and photosynthesis (Scanlan and Wilson, 1999; Collier and Grossman, 1992). Living under the conditions when phosphorus availability is variable, cyanobacteria possess a highly developed P-uptake regulating system that can allow them to survive even under P depletion. Although genetic responses on P-limitation in cyanobacteria are not completely understood, the responses of *Escherichia coli* have been studied in great detail. Considering the fact that some P-responsive genes in cyanobacteria have been shown to be homologous (Whitton and Potts, 2000) to *Escherichia coli* genes, an idea on P regulation in cyanobacteria can be obtained.

The *pho* regulon in *Escherichia coli* includes at least 31 genes (Wanner, 1993) performing different functions related to phosphorus uptake, metabolism and transport. Under P_i (inorganic phosphate) starvation the PhoR protein (sensor) autophosphorylates by using an ATP molecule and then transfers the phosphate group to the PhoB protein. Phosphorylated PhoB serves as a transcriptional activator that is able to specifically recognize promoters of the *pho* regulon. At this point, transcription and translation of the genes belonging to the *pho* regulon begins, and, as a result, cells obtain a set of proteins which allow them to aquire P at high affinity. These genes include *phoA,* encoding alkaline phosphatase, an enzyme that can cleave P_i from organic phosphates (Kikuchi et al. 1981), *phoH* encoding an ATP-binding protein (Kim et al. 1993), and the *phn* operon that includes fourteen genes responsible for utilization and transportation of organic phosphonates. It should be noticed that *phn* genes have been shown to be active only in a subset of *Escherichia coli* strains; in other words, not every *Escherichia coli* strain is able to use phosphonates as a sole source of P (Wanner and Boline, 1990).

Although the genome of cyanobacteria is not characterized in such details, some evidence exists on the existence of a system that resembles the *pho* regulon in *Escherichia coli* can be found in the literature. Thus, J. Eaton-Rye has shown that *Synechocystis* sp. PCC 6803 has a two-component signal trunsduction system similar to PhoR-PhoB in *Escherichia coli* (Hirani et al. 2001). Computational analysis of 19 sequenced cyanobacterial genomes has demonstrated that "*pho* regulons" in cyanobacteria are highly diversified, and the genes responsible for phosphorus assimilation are not always under control of regulator SphR which is homologous to PhoB in *Escherichia coli* (Su et al. 2007). In spite of this diversity in P-regulation system, many genes which are functionally similar to *Escherichia coli* genes can be found in genomic databases. Such information offers the great opportunities to study phosphorus regulation mechanisms in cyanobacteria and provides us the basis for the construction of cyanobacterial bioreporters which are able to report the data on bioavailable phosphorus in aquatic environments.

Cyanobacterial bioreporters and bioavailability

Bioreporters are powerful tool for investigation of a microorganism's perception of an environment. A whole-cell bioreporter is an organism engineered with a fusion of a responsive promoter and a reporter gene and, therefore, able to provide us the information on its immediate surrounding through the changes in metabolic and transcriptional behavior (Liveau and Lindow, 2002). Up to date, a wide range of biorepoters sensitive to different kinds of physical, chemical or biological stimuli have been developed. Among them are temperature sensors (ex. Ullrich et al. 2000), antibiotics

detectors (ex. Hansen et al. 2001), and nutrients or metals status reporting systems (ex. Miller et al. 2001; Mioni et al. 2003; Ivanikova et al. 2005).

All bioreporters can be divided on two categories (Fig. 1) (Belkin, 2003). So-called "lights off" bioreporters decrease their output signal in the presence of an analyte due to inhibition of some step in the process leading to the emission of the signal. The most part of cyanobacterial bioreporters including Fe-responsive (Durham et al. 2002, Boyanapalli et al. 2007) and P-responsive (Gillor et al. 2002) belong to this category. In contrast, in "lights on" bioreporters the analyte acts as the activator for transcription or translation of the reporter gene giving an increase in output signal. A good example of "lights on" bioreporters is the nitrate sensitive Synechocystis sp. Strain PCC 6803 reporter developed by Ivanikova et al. (2005).

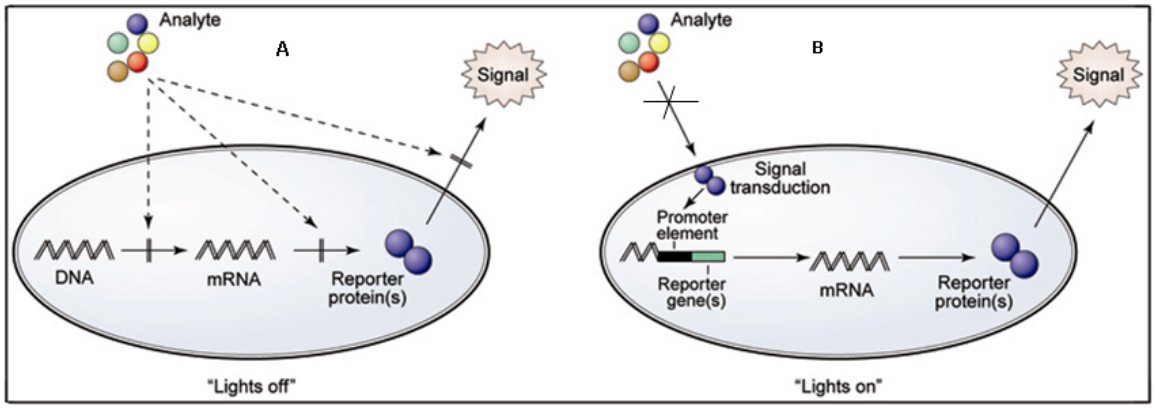


Figure 1. The difference between two classes of bioreporters: "lights off" bioreporters decrease or eliminate output signal in the presence of an analyte; "lights on" bioreporters increase or produce output signal in the presence of an analyte. (from Belkin, 2003)

It should be stressed that unlike other analytical methods, bioreporters provide the information on bioavailability of a nutrient such as phosphorus but not on its total

concentration. Bioavailability is defined as an amount of a nutrient that can freely penetrate through the membrane of an organism at a given time (Semple et al. 2004). It means that not all amount of a nutrient physically presented in a medium is actually available for biota. Bioavailability is a property which depends on organism qualities (physiology, membrane properties), nutrient qualities (specification, solubility, etc.) and surrounding conditions (temperature, pH, etc.). All these factors have to be considered during construction of a bioreporter.

For example, if we want to construct cyanobacterial bioreporter to access phosphate bioavailability in an oligotrophic oceanic region, it is better to choose the most relevant strain for this region. However, due to genetic complications, it is not always possible. To overcome this difficulty, other oceanic strains can be used but there is no guarantee that they will sense the same range of bioavailable phosphate. Generally, all bioreporters are restricted by their ecological niche.

There were several attempts to construct P-sensitive bioreporters. Applicable to monitoring P-deficiency in fresh water systems, a luminiscent cyanobacterial bioreporter has been created by Gillor et al. (2002) by the integration of *phoA:luxAB* fusion into the chromosome of the freshwater cyanobacterium *Synechococcus* sp. PCC 7942 where *phoA* is P-sensitive promoter and *luxAB* are the genes encoding bacterial luciferase, an enzyme that catalyzes a light-emitting reaction. As a result, luminescent bioreporter that can assess phosphorus bioavailability in the range of 2 nM to 2 μM has been constructed and successfully implemented. Two other P-sensitive bioreporters were designed by Dollard and Billard (2003) who introduced a *phoA:luxCDABE* construct into the heterotrophic bacteria *E. coli* MG1655 and *Pseudomonas fluorescence* DF57 chromosomes. The

obtained constructions were dose-responsive when phosphate concentrations fell below 60 and 40 μM, respectively.

While several P-sensitive bioreporters for measuring phosphate in fresh water have been developed, there are no data on bioreporters which are able to assess bioavilable P in marine systems. Taking into account potential depletion by P of some oceanic regions, such sensors may serve as an effective tool in the recognition of these regions and estimation of bioavailable. This research project is a part of more global investigation, the objective of which is to yield an array of complementary phosphorus-responsive bioreporters capable of sensing a broad range of bioavailable P in natural marine systems.

Overview of the research

Phosphorus is well documented to be a limited nutrient in some regions of the ocean. Up to date, plenty of chemical and biological approaches for measuring the amount and speciation of P have been developed. However, these methods do not provide any information on how much phosphorus is actually available for oceanic phytoplankton. A bioreporter approach can be helpful in answering this question.

At least three things are required to construct cyanobacterial bioreporter that will allow to measure bioavailable P in marine systems: a P-sensitive promoter, a reporter gene and a relevant cyanobacterial strain. We decided to use promoters of the genes *phoH* that encodes a protein catalyzing the removal of phosphate residues from ATP molecules and *phnD* that encodes a phosphonates transporter. Both genes are actively transcribed under phosphate starvation. However, the concentration of P under which transcriptional activation takes place, is not necessarily the same for these promoters;

therefore, potentially two bioreborters with different ranges of measured P can be obtained. Moreover, working with the *phnD* promoter may give us the information on bioavailable phosphonates, as it is currently unclear what precise conditions yield transcription of phosphonate utilization genes (ex. Ilikchyan et al. 2009).

As reporter genes we have used *luxAB,* which encode bacterial luciferase. This enzyme catalyzes the oxidation of reduced riboflavin phosphate ($FMNH_2$) and a long-chain fatty aldehyde by oxygen with emission of light (Fig. 2). If transcription and translation of *luxAB* genes takes place in a cell, after delivering an amount of aldehyde we should have bioluminescence as an output signal.

$$FMNH_2 + R\text{-}CHO + O_2 \rightarrow FMN + H_2O + RCOOH + h\nu\ (490\ nm)$$

Figure 2. Reaction catalyzed by bacterial luciferace

Finally, in the capacity of bacterial strain we have chosen *Synechococcus* sp. PCC 7002. While this strain lies outside the the picocyanobacterial clade (Urbach et al. 1998), it possesses high rate of growth compare to other cyanobacteria, can survive under wide range of salinities (Engelbrecht et al. 1999), and is naturally transformable (Stevens et al. 1980).

To sum up, the purpose of this project is to make two plasmid vectors containing *phoH:luxAB* and *phnD:luxAB* fusions, correspondingly, and deliver them into *Synechococcus* sp. PCC 7002. I tested the obtained mutants on their capability to give a bioluminescent signal depending on the availability of phosphate in the medium. Once tested in the laboratory, the bioreporters can be used further to provide the information on phosphorus bioavailability in natural marine systems.

MATERIALS AND METHODS

Media and growth conditions

Synechococcus PCC 7002 was cultured in modified ASN-III medium containing vitamin B_{12} (Appendix) (Wurster et al. 2003) at 25° C with continuous illumination of 45 µmol quanta $m^{-2}s^{-1}$. The P-bioreporter mutants were grown in ASN-III medium containing 20 µg/mL spectinomycin to maintain the integrity of the chromosomal insert. *Escherichia coli* strains Dh5α, used for maintenance of TOPO-vector, and TOP10, used for maintenance of the pMBB vector and plasmid construct, were grown in LB medium containing 100 µg mL^{-1} spectinomycin at 37 °C (Sambrook et al. 1989). All media were prepared using Milli-Q water (Millipore Corp.).

To prevent contamination by phosphate, all glassware and polycarbonate bottles used to work with bioreporters have been kept in 10% hydrochloric acid overnight and rinsed 5 times with Milli-Q water. Bacterial growth was monitored by measuring *in vivo* chlorophyll (chl)-*a* fluorescence (model TD-700 fluorometer; Turner Designs) or by measuring optical density of cultures at 730 nm (Spectronic® 20 Genesys™ spectrophotometer; Thermo scientific).

Construction of plasmids

The promoter sequences of the P-stress induced genes *phoH* (Genbank accession number AF035751) and *phnD* (GeneID 6054969) were recovered by PCR. Since we do not know the exact sequence of *phnD* promoter, two different alternatives have been used – *phnD* and *phnD*_short. PCR amplification of the sequences was performed with

*Synechococcu*s 7002 total DNA and Taq polymerase (Promega), according to the manufacturers instructions, for 30 cycles of the following temperatures: 94 °C for 1 min, annealing at 55 °C *(phnD* and *phnD*_short promoters) or 60 °C (*phoH* promoter) for 2 min, 72 °C for 3 min, with a final extension at 72 °C for 15 min. Prior to PCR, the reaction mixture was preheated for five minutes at 95 °C. Three sets of primers have been used (Table 1).

Table 1. Primers used to amplify promoters sequences. 5' → 3'

Name	Primer sequence	Amplify
phoH_For	GCGGCCGCAATTGCCTAAATAC	130 bp *phoH*
phoH_Rev	GGATCCAGAGATCTGCCAGG	
phnD_For	GGCCCGCGGCCGCGGGAGGCTTTTTATCACTCTA GCGAAGATCCC	396 bp *phnD*
phnD_Rev	CCGGGGGATCCGGCGGTTCTCGTTGGGCGTTGGG	
phnD_For	GGCCCGCGGCCGCGGGAGGCTTTTTATCACTCTA GCGAAGATCCC	186 bp *phnD*_short
phnD_short_ Rev	CCGGGGGATCCGCCTAAACCTTGATTTAGACCCA GCTACG	
pMBB_For.	CTAAGCTGATCCGGTGGATGACC	217 bp fragment in pMBB
pMBB_Rev.	AGCGACGTTCATTCACAGTCGC	

The insertional plasmid vector pMBB containing the *luxAB* genes, *Not* I *and Bam* HI restriction sites and a spectinomycin resistance gene has been obtained before

(Boyanapalli et al. 2007) (Fig. 3). In this vector, the *desB* gene encoding omega-3 fatty acid desaturase, serves as a recombination site. Disruption of *desB* does not influence the bacterial phenotype at standard growth temperature, but yields mutants not able to survive at temperatures below 15 °C. At this point, inadvertent release of the engineered strains into environment cannot cause any consequences because they would not survive most marine habitats.

The *phoH* and *phnD* promoters were ligated into the *Not* I *and Bam* HI sites of the pMBB vector. The resulting constructs were transformed into *Escherichia coli* TOP10, grown, then extracted and purified in the amount of 100-500 μg/mL using Qiagen® Plasmid Maxi Kit. The obtained solutions of the plasmid vectors were used to perform the genetic transformation of *Synechococcus* PCC 7002 (Stevens et al. 1980).

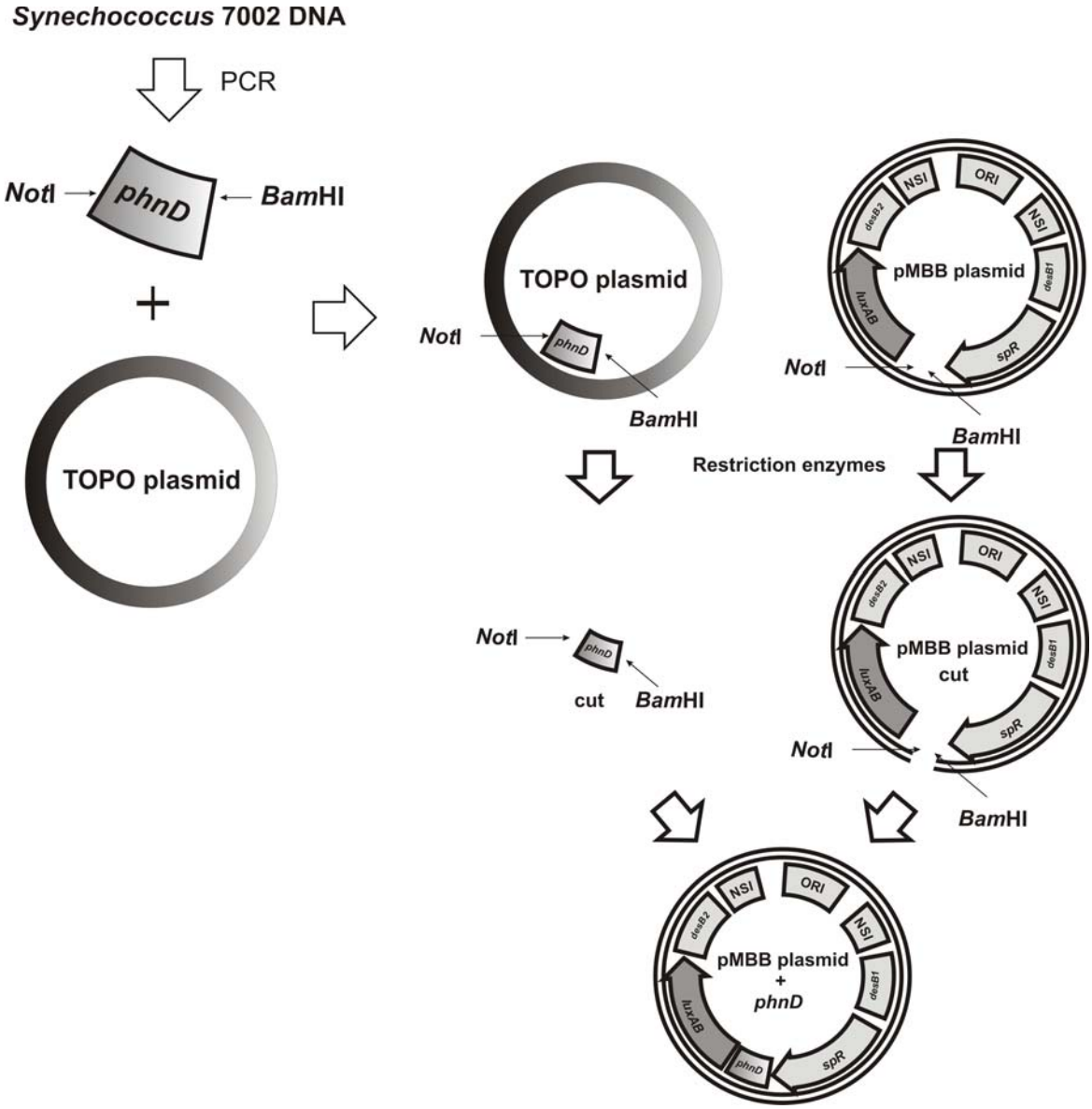


Figure 3. Design of the plasmid containing the *phnD:luxAB* fusion. *luxAB*: promoterless luciferase gene, *spR*: spectinomycin resistance gene, *desB1* & *desB2*: consecutive *desB* gene fragments, ORI: *E. coli* origin of replication initiation, NSI: neutral sites for *Synechococcus* PCC 7942, *phnD:* P-responsive promoter. The same scheme was applied for *phoH*- and *phnD*_short-based constructs.

Transformation procedure

Two mL of *Synechococcus* PCC 7002 culture were collected by centrifugation at 3000 g and then resuspended in 300 µL of ASN-III medium. Five µL of a plasmid

solution were mixed with 30 μL of 0.15 M NaCl/0.015 M Na_3citrate; 33.5 μL of the obtained mixture were transferred to the recipient cells. After 3 hours exposure at 39 °C, 300 μL of the mixture were placed on a freshly prepared medium ASN-III agar plate. To aid in the recovery of transformants, put nitrocellulose filter paper on the surface of the plate before inoculation of the cells. The plate was incubated at 30-32° C with continuous illumination of 50 μmol quanta $m^{-2}s^{-1}$ for 40 hours. Then we transferred the nitrocellulose filter (containing the cultured cells) onto the surface of freshly prepared medium ASN-III agar plate containing 20 μg/mL of spectinomycin. The plate was incubated in the same conditions. Transformants began to appear in about 4-5 days. The number of positive colonies varied from 1 to 15.

Bioreporter response to P-deficiency

Prior to measuring bioluminescence response of bioreporters, the cultures were grown in low phosphate water from North Atlantic Ocean amended with $NaNO_3$, trace metals and 55 μM K_2HPO_4. Exponential phase cells were collected by centrifugation at 5000 g for 10 min, washed twice with low P oceanic water, and resuspended in triplicate polycarbonate bottles containing the oceanic water amended with $NaNO_3$, trace metals and defined phosphate concentration. Luminescence of the cultures was measured with a Wallac 1420 multilabel counter with setting of 0 s delay and 10 s measurements. Two hundred μL of culture were delivered to a plate's well followed by the addition of 10 μL of 5.3 nM decanal solution. The measurements started 5 min. after addition of decanal and continued about one hour. The experiment yielded about 40 numbers for each measured sample since 6-7 samples were taken simultaneously. The last 20 numbers

were averaged. A negative control (no decanal) provided us with the background level of luminescence. The background luminescence was substructed from the luminescence of the working sample, divided by (chl)-*a* fluorescence and multiplied by 100. The values finally obtained by this method were used for data analysis. The procedure was repeated every 3-4 hours over a period of 3 days.

RESULTS AND DISCUSSION

Construction of bioreporters

To analyze the obtained plasmids for the presence of a promoter insert we have used pMBB_For. and pMBB_Rev. primers (Table 1) which amplify a 186 bp fragment covering the *Not*I and *Bam*HI restriction sites. Figure 4 (*Panel A*) shows that 130 bp *phoH*, 396 bp *phnD* and 186 bp *phnD*_short sequences were successfully ligated into pMBB. The same set of primers has been used for testing the chromosomal DNA of the obtained bioreporters. Electrophoresis revealed that all sequences were successfully transformed into *Synechococcus* sp. PCC 7002 chromosomal DNA (Fig. 4, *Panel B*).

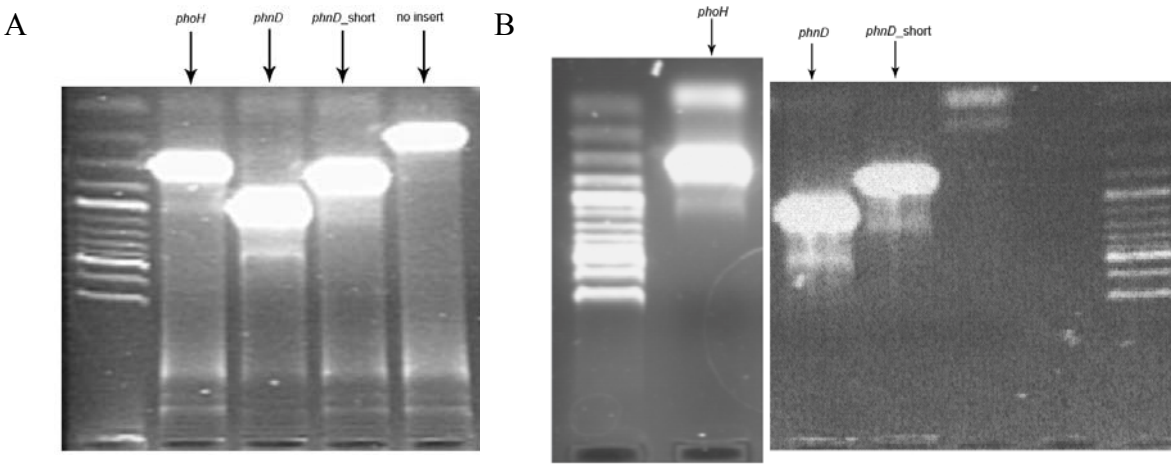


Figure 4. *Panel A.* Electrophoresis diagram of the sequences amplified by pMBB_For. and pMBB_Rev. primers from the pMBB vector with *phoH*, *phnD* and *phnD*_short inserts. *Panel B*. Electrophoresis diagram of the sequences amplified by pMBB_For. and pMBB_Rev. primers from *Synechococcus* 7002 chromosomal DNA after transformation.

Growth of wild type strain vs. bioreporters

There is no considerable difference in the growth of wild *Synechococcus* sp. PCC 7002 strain and the constructed bioreporters. The measured rates of growth in ASNIII medium at room temperature with illumination of 45 μmol quanta $m^{-2}s^{-1}$ were in the range of 0.09-0.12 h^{-1} for all organisms. It means that the performed genetic manipulations did not cause any major changes in *Synechococcus* sp. PCC 7002 phenotype under laboratory conditions.

Bioreporters response to P-deficiency

All constructed bioreporters have been demonstrated to emit light after the addition of decanal. However, correlation between the intensity of bioluminescence and concentration of phosphate in the medium is not clear.

In the case of the *phoH* bioreporter the output signal does not seem to be dependent on the phosphate concentration. An example the of *phoH* bioreporter response to P-deficiency is represented in Figure 5 where we can see the dependence of the luminescence intensity over time for cultures maintained under different concentrations of P. While all curves have approximately the same trend and reach maxima in 25-35 hours after beginning the experiment, the intensities of bioluminescence measured at the same period of time are indistinguishable.

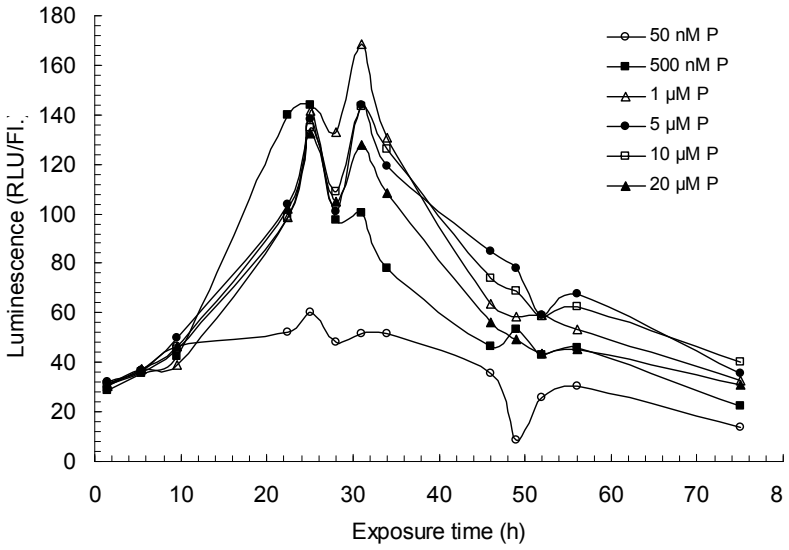


Figure 5. Bioluminescence of the *phoH Synechococcus* 7002 bioreporter. Log phase cells grown in ASNIII were transferred at the same time to ASNIII with various concentrations of K_2HPO_4. Luminescence was normalized against (chl)-*a* fluorescence.

By contrast, the data for *phnD* bioreporter bioluminescence were demonstrated to be in accordance with phosphate concentration. After the exposure of *phnD* bioreporter under different concentrations of P for 40-60 hours, the intensity of bioluminescence varies quiet strongly depending on the availability of P in the medium (Fig. 6). However, these data were obtained recently and require further confirmation. Furthermore, the graph shows that the range of phosphate which can be measured by the *phnD* bioreporter is from 1 to 10 µM. This is much higher concentration than phosphate concentration in natural marine systems.

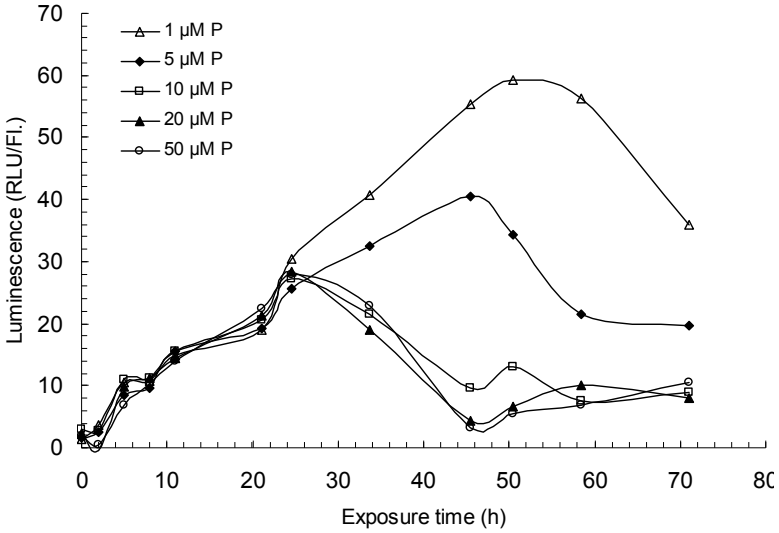


Figure 6. Bioluminescence of *phnD Synechococcus* 7002 bioreporter. Log phase cells grown in ASNIII were transferred at the same time to ASNIII with various concentrations of K_2HPO_4. Luminescence was normalized against (chl)-*a* fluorescence.

PhoH sequence analysis

Since the *phoH* bioreporter did not show P-dependent luminescence, the *phoH* sequence has been analyzed with bioinformatics tools for the presence of a *pho* box. The *pho* box is a consensus sequence which is found in the promoters in the P regulon of *E. coli* and several other microorganisms. The *pho* box is a DNA sequence recognized by the phosphorylated transcriptional activator PhoB. There is no strong evidence of the *pho* box existence in Synechococcus sp. PCC 7002; however, some research has indicated that a "*pho* box" is present in marine picocyanobacteria (Su et al. 2007; Ilikchyan 2009, Ph.D. dissertation).

While only one *phoH* sequence has been used for the construction of bioreporter, later we considered several other candidates for potential P-responsive promoters like

phoH (Fig. 7). All of them represent the upstream region of a P-responsive gene (or a potential P-responsive gene). Interestingly, *phoH*$_2$ and *phoH*$_3$ sequences are the part of the system which includes also the sequences encoding a two component response regulator, a PhoH-like protein and a sensory box protein/response regulator. Such an operon resembles the *E. coli* P regulon, and may represent *phoRB* regulated operons in *Synechococcus* sp. PCC 7002.

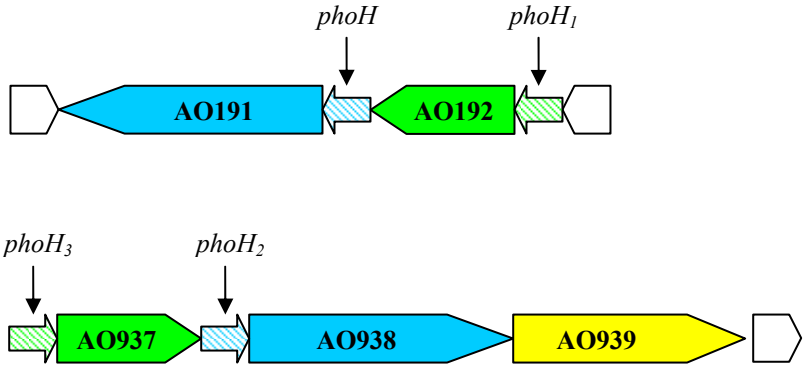


Figure 7. *Synechococcus* 7002 genomic sequences which can be potentially used as P-responsive promoters. *Panel A*. DNA region that includes *phoH*-like gene (AO191) and a gene encoding a hypothetical protein (AO192). *phoH* and *phoH*$_1$ sequences might contain P-responsive promoters. *Panel B*. DNA region that encodes a two component response regulator (AO937), a PhoH-like protein (AO938) and a sensory box protein (AO939). *phoH*$_1$ and *phoH*$_2$ sequences might contain P-responsive promoters. The bioreporter constructed in our lab has its promoter marked as *phoH*.

Each sequence was compared to P-dependent promoters from other cyanobacteria containing "*pho* box" with MEME (Bailey and Elkan, 1994; http://meme.nbcr.net/meme4/cgi-bin/meme.cgi). Although all examined *Synechococcus* 7002 P-responsive promoters were demonstrated to share some motifs with other cyanobacteria, the 18 bp cyanobacterial "*pho* box" sequence described by Su et al. (2007) and Ilikchyan (2009, Ph.D. dissertation) was found only in the *phoH*$_3$ promoter (Fig. 8).

However, we should mention that *Synecococcus* sp. PCC 7002 strain is divergent from the *Synechococcus* and *Prochlorococcus*, and there is no strong homology between genomic DNA of *Synecococcus* sp. PCC 7002 and the *pho* box sequences of picocyanobacteria.

NAME	STRAND	START	P-VALUE	SITES
WH7803_pstCAB_PHO	+	77	1.70e-08	AGCAGCTTGC GTTAAAGCGTCCTTAGTG AATTCCATG
WH8102_pstCAB_PHO	+	48	1.93e-08	GGGGAATGTC GTTAAGCCGTTCTTAGTG ACTGAAACC
Rcc307_phoBR_PHO	-	72	9.75e-08	TAAACAAATT TTAAACGCGTTCTTAATG AGCCCAAAA
RCC307_pstS2_PHO	+	92	1.87e-07	ATACAAAAGA GATAACTGGTCCTTAAGG GATGCCTCG
WH8102_phoA_2391_PHO	+	90	3.63e-07	TTAAACTATT CCTAACTTGTTCTTTATT CAACGCTGC
RCC307_pstS1_PHO	+	72	3.63e-07	TCTTAGTGTC GCTAACCAGTCTTTAACT GCGACTTAA
MIT9301_phnCDE_from_Nostoc_PHO	+	50	4.59e-07	TTTTAATTCG CTTAACCTTTTATTAATT TTAAGTTAA
Syn7002_phoH1	+	33	2.27e-06	CCGATAGACA GTTAACGTTGCTTTGAAG CCAATATTA

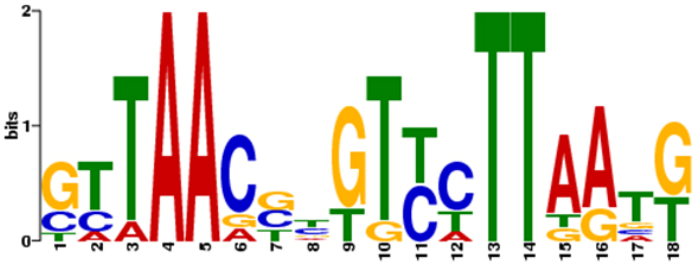


Figure 8. The "*pho* box" consensus sequence identified with MEME in some cyanobacterial P-responsive promoters and *Synechococcus* 7002 *phoH*$_1$. This sequence corresponds to the data obtained by Su et al. (2007) and Ilikchyan (2009). P-value for *phoH*$_1$ is relatively low compare to other cyanobacterial sequences indicating that the similarity could occur randomly.

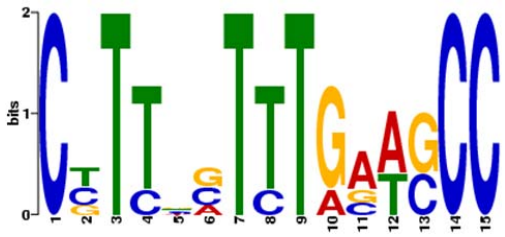
The suggested P-responsive promoter sequences were also compared against each other. It was demonstrated that they have a similar 15 bp motifs laying 35-45 bp upstream of the corresponding translational starting sites (Fig. 9). These motifs can probably represent a a "*pho* box" in *Synechococcus* sp. PCC 7002. The data represented in Figure 9 show that the statistical significance of the "*pho* box" occurrence is lower in *phoH*$_1$ and *phoH*$_3$ sequences which are situated upstream of the sequences encoding a hypothetical protein and a two component response regulator, respectively. In other words, the

probability that the "*pho* box" motif randomly occurred in these sequences is close to zero.

A

NAME	STRAND	START	P-VALUE	SITES		
Syn7002_phoH1	+	38	7.34e-09	AGACAGTTAA	CGTTGCTTTGAAGCC	AATATTAAGA
Syn7002_phoH3	+	81	7.14e-08	ATCAAACAAG	CTTTAGTTTGAAGCC	TTGATTTACT
Syn7002_phoH	+	40	1.08e-07	GTTCTATCGT	CCTTTGTTTGATCCC	GTCAGTGTTA
Syn7002_phoH2	+	81	7.17e-07	GGTCTTTTCT	CTTCCCTCTGCTGCC	TATAATTAAG
Syn7002_phnDCE	+	45	8.34e-07	AAAGCCTAAA	CCTTGATTTAGACCC	AGCTACGTTC

B



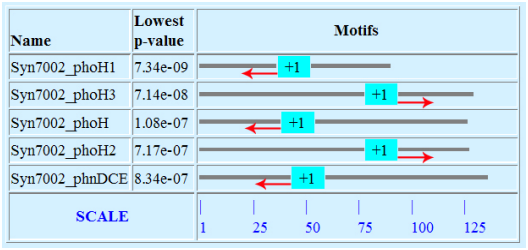


Figure 9. The possible "*pho* box" sequence identified with MEME in suggested *Synechococcus* 7002 P-responsive promoters. *Panel A*. The list of analyzed sequences which were found to share the same motif. *Panel B*. General representation of the "*pho* box" sequence and its position in the promoters. Arrows show the direction of transcription.

To sum up, although it is not completely clear why the *phoH* promoter used in this research project does not work properly, some suggestions on the design of another P-responsive promoter can be made. In this capacity I would choose the part of sequence *phoH*$_1$ upstream of the hypothetical protein *phoH*$_1$ (Fig. 7) because it includes the -35-45 *Synechococcus* sp. PCC 7002 "*pho* box" region and shares a consensus sequence with other cyanobacteria that was demonstrated in literature to be the possible cyanobacterial "*pho* box".

PhnD sequence analysis

Examining the *phnD* promoter, we used two sequences – *phnD* and *phnD*_short which are schematically represented in Figure 10. The *phnD* partially includes the

sequence of the *phnD* gene, while *phnD*_short is a deleted version of *phnD* positioned upstream of the *phnDEC* genes.

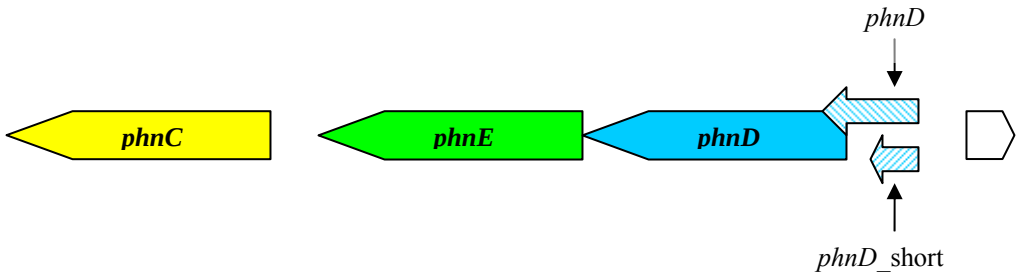


Figure 10. PhnDEC genes in Synechococcus 7002 and two P-responsive promoters (*phnD* and *phnD*_short) used for construction of the bioreporters.

We did not find any similar motifs between *Synechococcus* sp. PCC 7002 *phnD* and *phnDEC* promoters from other picocyanobacteria. However, comparison of *phnD* sequence to *phoH*, $phoH_1$, $phoH_2$ and $phoH_3$ revealed the presence of the 15 bp "*pho* box" region incidental to *Synechococcus* sp. PCC 7002 and described earlier (Fig. 7). In other words, we can propose that the *phnDEC* genes in *Synechococcus* sp. PCC 7002 are controlled by the same regulatory system as the *phoH* gene (and probably other P-responsive genes) which is unusual for cyanobacteria, although further confirmation is required. Indeed, evidence exists in marine picocyanobacteria (marine Synechococcus and Prochlorococcus) the *pho* genes and *phn* genes are regulated independently (Su et al. 2007; Ilikchyan et al. 2009; Ilikchyan 2009, Ph.D. dissertation).

CONCLUSION

Within the framework of this research project three cyanobacterial bioreporters maintaining the fusion of P-responsive promoters with *luxAB* genes were constructed and tested on their ability to glow under phosphate starvation. While all mutants have demonstrated the ability to emit light, the intensity of bioluminescence of the *phoH*-based bioreporter did not correlate to the phosphate concentration in the medium. At the same time, the *phnD*-based bioreporter has shown the maximum of luminescence after 50 hours exposure in the medium containing 1 μM of K_2HPO_4. A ten-fold increase of phosphate concentration has caused suppressed bioreporter luminescence. These data allow us to suggest that the *phnD*-based bioreporter can be used to measure phosphorus bioavailability in marine systems with high P content that is within the range of 1-10 μM; however, additional experiments are required to confirm this conclusion. No reliable data were obtained on the P-dependent bioluminescence of the *PhnD*_short-based bioreporter which has to be tested hereafter.

To make some predictions on a P-responsive promoter that can be used instead of *phoH* in future work on *Synechococcus* sp. PCC 7002 bioreporters, we compared the sequences of *phoH* to other potential promoter sequences from the same organism. It was demonstrated that there is a 15 bp motif 35-45 bp upstream of the corresponding genes in all analyzed sequences including *phoH*. Possibly, this consensus sequence is a homolog of the 18 bp *pho* box which is found in almost all P-responsive promoters of *E. coli*. While in *E. coli* the *pho* box sequence is the same for all genes, in *Synechococcus* sp. PCC 7002 we can find only some degree of similarity. As the analysis has shown, the

statistical significance of the 15 bp "*pho* box" occurrence in *phoH* is quite high, making this sequence not the optimal for the usage in the capacity of P-responsive promoter in cyanobacterial bioreporters. Another sequence has been suggested as a potential P-responsive promoter.

The phnD promoter has also been compared to the proposed promoter sequences. Intersting, we found that it also has the 15 bp "*pho* box" which is positioned 45 bp upstream of *phnDEC* genes. Therefore, we can propose that the *phnDEC* genes in *Synechococcus* sp. PCC 7002 are under control by the same regulatory system as the *phoH* gene which is unusual for cyanobacteria, although further confirmation is required.

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APPENDIX. The composition of modified medium ASNIII.

Ingredient	**g l^{-1}**	**mM**
NaCl	25.0	427
$MgCl_2.6H_2O$	2.0	9.8
KCl	0.5	6.7
$NaNO_3$	0.75	8.8
K_2HPO_4	0.02	0.55
$MgSO_4.7H_2O$	3.5	14.2
$CaCl_2.2H_2O$	0.5	3.4
Citric acid	0.003	0.015
Ferric ammonium citrate	0.003	0.015
EDTA (disodium magnesium)	0.0005	0.0015
Na_2CO_3	0.02	0.19
Trace metal mix	1 ml	
Vitamin B12		$1.5x10^{-5}$
MilliQ water	to 1 l	
pH after autoclaving and cooling: 7.5		

Trace minerals:

H_3BO_3	4.6×10^{-3}
$MnCl_2 \times 4H_2O$	9.1×10^{-4}
$ZnSO_4 \times 7H_2O$	7.7×10^{-5}
$Na_2MoO_4 \times 2H_2O$	1.6×10^{-4}
$CuSO_4 \times 5H_2O$	3.2×10^{-5}
$CoCl_2 \times 6H_2O$	1.7×10^{-7}