LEBANESE AMERICAN UNIVERSITY

DEVELOPMENT OF AN *HCP*-BASED ASSAY FOR THE QUANTIFICATION OF PICOCYANOBACTERIA BY REAL-TIME PCR

By

STEPHANIE J. MEHANNA

A thesis
Submitted in partial fulfillment of the requirements
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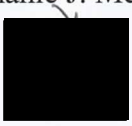
Signature: 

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To the memory of my beloved mother, Lena, I dedicate this thesis.

Development of an *hcp*-based Assay for the Quantification of Picocyanobacteria by Real-time PCR

Stephanie J. Mehanna

Abstract

Picocyanobacteria form most of the autotrophic picoplankton (APP) and are the major contributors to primary production in both freshwater ecosystems and oligotrophic oceans. Two related APP genera forming the picocyanobacterial clade are *Synechococcus* sp. and *Prochlorococcus* sp. Several methods have been used for the classification and quantification of picocyanobacteria including flow cytometry and analysis of photosynthetic pigments. However, more recently, quantitative real-time PCR (qPCR) has become the method of choice for quantification purposes as well as the assessment of biodiversity in picoplanktonic environments, including studies on cyanobacterial prevalence. Lately, both marine and freshwater picocyanobacteria were found to harbor a gene, *hcp*, 100% conserved among all the strains isolated and therefore representing a valuable target for quantification of picocyanobacteria by qPCR, which is the aim of the present study. The approach used is summarized by the creation of a plasmid that harbors the gene (based on one strain) to construct a standard curve for qPCR, followed by qPCR of environmental samples. We have carried out sequence alignment of *hcp* for *Synechococcus* sp. strains LS 0504, KD3a, ARC-11 and ARC-21. Based on the sequence alignment, we have designed primers for part of the *hcp* gene (186 bp). PCR reactions on both freshwater and marine DNA samples from strains LS 0504, KD3a, ARC-11, ARC-21 and WH 8102 verified the successful primer design. *hcp*, amplified from LS 0504 strain was then cloned and transformed into plasmid DNA and absolute quantification of samples collected from the Sargasso Sea, USA and from an artificial lake at Annaya, Lebanon was performed by qPCR using an

external standard curve based on serial dilutions of the plasmid. The numbers of picocyanobacteria from marine samples were in the range of 10^3 cells/mL and numbers from freshwater samples were in the range of 10^5 cells/mL. We have thus successfully designed an *hcp*-based assay that can be applied to quantify picocyanobacteria in local freshwater and marine environments.

Keywords: *Hcp*, QPCR, Standard curve, Plasmid, Picocyanobacteria.

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GLOSSARY

Amp: Ampicilline

ANN: Annaya

APP: Autotrophic Picoplankton

bp: base-pair

Chl: Chlorophyll

Conc.: Concentration

Ct: Cycle Threshold

Cq: Quantification Cycle

DNQ: Determined, Not Quantifiable

dsDNA: double-stranded DNA

Fe: Iron

GFP: Green Fluorescent Protein

LB: Luria-Bertani

LOD: Limit of Detection

LOQ: Limit of Quantification

$N_{2:}$ Nitrogen

P: Phosphorous

PC: Phycocyanin

PE: Phycoerythrin

PS: Photosystem

qPCR: Quantitative Real-time PCR

SD: Standard Deviation

SRG: Sargasso Sea

UD: Undetermined

CHAPTER ONE

INTRODUCTION

1.1 Cyanobacteria, an overview

Cyanobacteria, also known as cyanophyta or blue-green algae have a dual nomenclature system; they are named under both the botanical and the bacteriological codes which evokes great confusion; in fact, it is only in 1978 that two papers dealing with cyanobacteria were published in the International Journal of Systematic Bacteriology (IJSB) following the recognition that these organisms are prokaryotes (Oren, 2011).

Cyanobacteria are considered the most successful phototrophic organisms; being the first group capable of oxygenic photosynthesis (oxidizing water to produce oxygen), they are the key players in the transition of the Earth's atmosphere from an anaerobic to an aerobic state (Tomitani, Knoll, Cavanaugh, & Ohno, 2006). Oxygenic photosynthesis occurs in the thylakoid membranes; as shown in Figure 1, photosystem II (PSII) oxidizes water into oxygen (using the sun's energy), thus liberating electrons which are transferred to photosystem I (PSI) and ultimately used for storing compounds (respired for energy, in the dark) and fixing CO_2 into biosynthetic intermediates (by the Calvin cycle). H_2 is produced via hydrogenase or nitrogenase (McKinlay & Harwood, 2010).

Photosynthetic pigments are situated in the thylakoids; the green color of chlorophyll *a* is generally masked by accessory pigments called phycobiliproteins (phycocyanin and phycoerythrin) located in phycobilisomes, as well as carotenoids (beta-caroten). Chlorophyll and phycocyanin are present in all cyanobacteria and cell colors range from blue-green to violet-red (Chorus & Bartram, 1999).

Interestingly, the oldest fossil records on Earth (around 3,460 million years old) belong to the cyanobacterial phylum (Brasier, Green, Lindsay, & Steele, 2004). Moreover, some diazotrophic cyanobacteria (Nitrogen-fixing bacteria) are major contributors to the global nitrogen supply, hence the importance of these primary producers in the oxygen, nitrogen and carbon cycles (Oren, 2004; Tomitani et al., 2006).

Molecular phylogenetic studies conducted on rRNA and cytochrome c sequences have observed a relationship between cyanobacteria and chloroplasts, thus validating the prokaryotic origin of chloroplasts (Giovannoni et al., 1988).

Cyanobacteria are ubiquitously found in both aquatic and terrestrial milieus including extreme habitats such as deserts, hot springs and polar regions. They are very morphologically diverse monophyletic organisms and thus, classical phylogenetic analysis relies on morphological distinctions for their classification; five main groups are recognized: Group I (Chroococcales) is composed of unicellular cyanobacteria which cells can divide by binary fission, e.g. *Synechococcus*, Group II (Pleurocapsales) not only reproduce by binary fission, they are also capable of multiple fission to form small, dispersed cells called baeocytes. Group III (Oscillatoriales) consists of filamentous, non-differentiating bacteria, while filaments of Group IV (Nostocales) can differentiate into heterocysts and akinetes. Group V (Stigonematales) is also capable of cell differentiation but has more complex multicellular patterns of organization (Figure 2). Molecular classification conforms to all but some of these groupings; analysis of the 16S rRNA sequence supports the monophyly of taxa that are capable of multiple fission or cell differentiation (subsections II, IV and V), however analysis of the *hetR* and *rbcL* gene sequences (genes coding for nitrogenase enzyme which is responsible for fixing nitrogen) indicates that subsections II and V form paraphyletic grouping (E. Flores & F.G. Flores, 2008; Tomitani et al., 2006).
Figure 3 reveals the phylogenetic relationships among cyanobacteria based on the molecular analyses cited above.

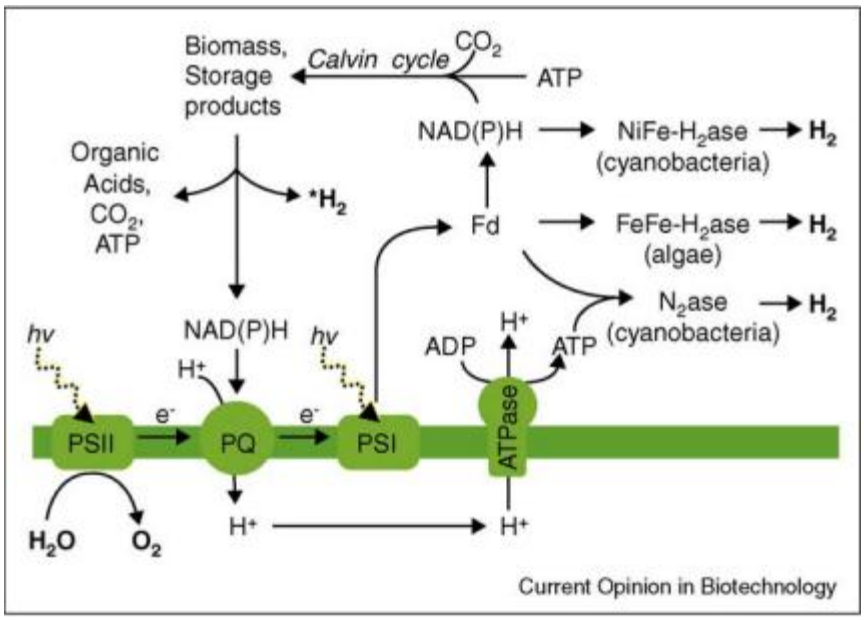

Figure 1: Oxygenic photosynthesis by cyanobacteria and algae (McKinlay & Harwood, 2010).

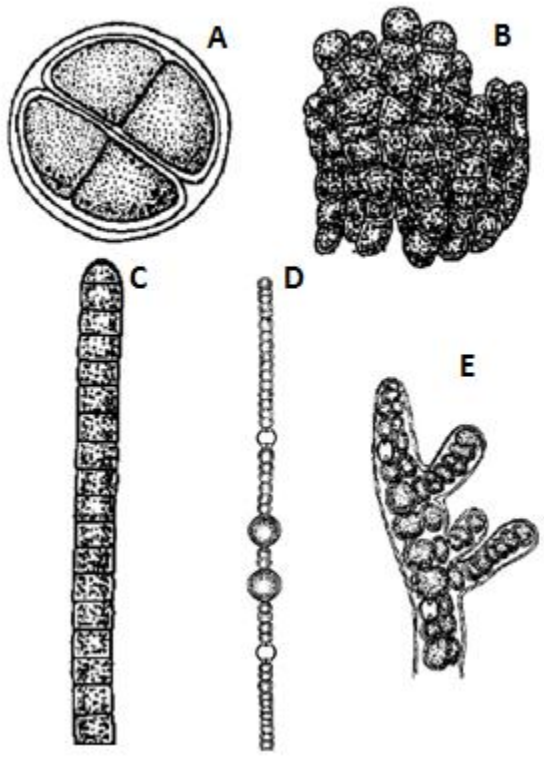

Figure 2: Morphologies of cyanobacteria. A: Chroococcales; B: Pleurocapsales; C: Oscillatoriales; D: Nostocales; E: Stigonematales (Chorus & Bartram, 1999).

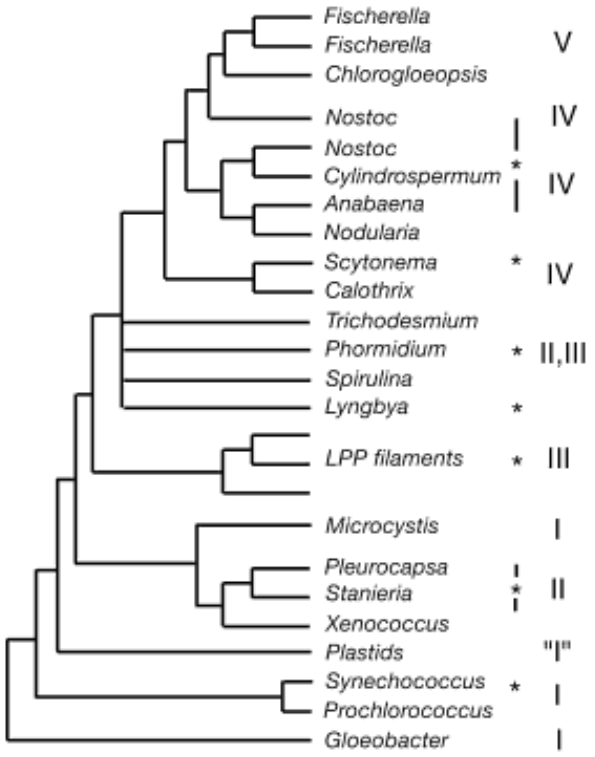

Figure 3: Phylogenetic relationships among cyanobacteria as inferred from molecular analyses on 16S rRNA, *hetR* and *rbcL* gene sequences. Roman numerals refer to cyanobacterial subsections I-V. Asteriscs mark morphotypes derived from 2000-1500 Ma fossil records (E. Flores & F.G. Flores, 2008).

During their course of evolution, cyanobacteria have acquired various mechanisms of adaptation; they are able to cope with alterations in nutrients' availability; for instance, under nitrogen-limiting conditions, they are able to utilize atmospheric nitrogen gas (N_2) as a source of nitrogen via the process of nitrogen fixation (converting N_2 into ammonia by nitrogenase enzyme). In addition, they are capable of storing the phosphorous (P) in excess for subsequent utilization when the latter in scarce (Paerl, Fulton, Moisander, & Dyble, 2001). To survive in oligotrophic ecosystems with low nutrient availability, cyanobacteria have also developed specialized nutrient uptake mechanisms; for example, when nitrogen is scarce, *Synechococcus* sp. utilize nitrate for their growth. When phosphorous is scarce, they are able to utilize phosphonates (Ilikchyan, McKay, Kutovaya, Condon, & Bullerjahn, 2010; Scanlan & West, 2002) Moreover, because of the high sensitivity of nitrogenase to molecular oxygen, cyanobacteria adopted certain

measures to protect nitrogenase from being inactivated by oxygen; filamentous bacteria have used spatial separation of the processes of nitrogen fixation and oxygenic photosynthesis by being capable of differentiating into heterocysts (non-dividing cells containing nitrogenase and incapable of oxygenic photosynthesis). Non-heterocystous filaments as well as nitrogen-fixing unicellar cyanobacteria in which photosynthesis happens in the same cell, have developed temporal separation of the processes of nitrogen fixation and oxygenic photosynthesis; under consecutive light and dark growth conditions, nitrogenase functions only in the dark period (Stal & Krumbein, 1987). Moreover, to regulate buoyancy at moderate pressures, cyanobacteria possess gas vesicles that enable them to adjust their location in the vertical column in order to sustain efficient mechanisms for capturing light (Steinberg & Hartmann, 1988; Walsby, Hayes, & Boje, 1995). Another mechanism of adaptation is chromatic adaptation which requires the regulation of the production of the main cellular chromophores phycobilins; phycoerythrin (PE) and phycocyanin (PC) by green and red light (Erokhina, Spirina, Shatilovich, & Gilichinskiĭ, 2000).

1.2 Uses of cyanobacteria

1.2.1. *Cyanobacterial bioreporters*

Given that cyanobacteria are the major primary producers in aquatic environments, they have become ideal targets for the identification and the monitoring of factors that may restrict productivity in such environmental settings; for this purpose, genetically engineered cyanobacterial biosensors or bioreporters have been designed and have become widely used. Typically, the promoter of a gene known to be regulated by the factor studied is fused to reporter genes encoding proteins with detectable activities such as luciferase, the green-fluorescent protein (GFP) and β-galactosidase. Therefore, bioreporters allow the study of the bioavailability of a particular analyte to the living cell, thus complementing the parallel measurements for physical and chemical factors in the ecosystem studied (T. Bachmann, 2003; Bullerjahn, Boyanapalli, Rozmarynowycz, & McKay, 2010). In a study by Schreiter et. al (2001) *Synechococcus* PCC 7942 strain was used to monitor the bioavailability of

phosphate by fusing the *phoA* promoter (*PphoA)* to luciferase gene *(luxAB)* from *Vibrio harveyi*. Moreover, Mbeunkui et. al (2002) assessed the bioavailibilty of nitrate by using the cyanobacterial *Synechocystis* PCC 6803 strain harboring a fusion of *luxAB-kmr* with *nblA1*; the latter is up-regulated when nitrate is deficient. In fact, *nblA1* codes for the nblA1 polypeptide that is responsible for phycobilisome degradation and consequently, the cessation of phycobiliproteins' production as well as the degradation of pre-existing ones. Also, Boyanapalli et. al (2007) monitored the bioavailability of Iron (Fe) by fusing the promoter of the Fe-responsive gene *isiAB* to *luxAB* and integrating the fusion into *Synechococcus* sp. Strain PCC 7002. Actually, not only have cyanobacteria been used for monitoring nutrient bioavailability, but they also allowed to detect environmental pollutants including herbicides and other toxins; for this purpose, Shao, Howe, Porter and Glover (2002) designed a biosensor in which he simply integrated the luciferase gene *luc* into the chromosome of *Synechocystis* sp. Strain PCC6803; sensitivity to the doses of pollutants studied such as herbicides (Atrazine, Propazine, Simazine and Diuron), heavy metals and volatile organic pollutants is detected by decreased bioluminescence. Results showed that the *Synechocystis* sp. Strain PCC6803 biosensor was much more sensitive for the detection of these compounds than the previously used *E. coli* HB101 biosensor.

1.2.2. *Generation of bioenergy*

Another use of cyanobacteria is in the generation of energy that can substitute the finite and toxic fossil fuels; as mentioned earlier, cyanobacteria are highly efficient photosynthetic organisms that produce their main storage compounds (lipids, proteins and carbohydrates) by using CO_2, H_2O and the sun's energy (Figure 1). These synthesized components can be potentially used as feedstock to be converted into biofuels, with lipids having the highest load of energy. In fact, bioenergy has recently started to gain interest with the use of first and second generation products such as wheat, sunflower, peanuts, soybean, rapeseed, sesame seeds and switch-grass. However, the major drawback of using such kinds of biofuels is their competition with food sources for water and farmland. Therefore, cyanobacteria are today considered as third-generation biofuels that

can overcome this constraint; they possess some unique properties which make them the most promising source for generating bioenergy:

- They can store large amounts of lipids in their thylakoids membranes
- They are the most efficient photosynthetic organisms
- They require basic conditions of growth and can be cultured to very large quantities
- Cultivation is thus easy and inexpensive

Cyanobacterial biomass allows the generation of a wide range of biofuels including hydrogen, ethanol, photanol, diesel, methane and electric energy (Quintana, Van der Kooy, Van de Rhee, Voshol, & Verpoorte, 2011). Recently, the American University of Beirut (AUB) in partnership with researchers from five selected Mediterranean countries (Egypt, Cyprus, Malta, Italy and Greece), is studying the potential of using microalgae to generate biofuels. According to Yusuf Abou Jawdah, the principal researcher from AUB, the project will represent the first step towards creating a biotechnology unit, with the expertise and the research needed for future development of biofuel markets in Lebanon. He added that this would help in the achievement of self sufficiency as regards energy and sustainable development ("AUB - 2012 - AUB joins Mediterranean consortium in EU-funded research study on producing biodiesel from algae," n.d.).

Not only do cyanobacteria generate bioenergy, but they can also be used for the generation of high-value products such as isoprene (to produce synthetic rubber) and sugars. More importantly, cyanobcteria were found to encode for various natural compounds of medical and industrial use including potential pharmaceuticals, anti-fouling agents, insecticides and mycosporines which are UV-absorbing products capable of protecting biological samples or coating surfaces (Ducat, Way, & Silver, 2011).

To compete with existing sources of energy, several strategies should be designed to optimize the yield of cyanobacterial products. Currently, the major concern is the development of cost-efficient photosynthetic bioreactors; while

enclosed bioreactors are expensive; “open-pond” models are more sensitive to contaminations by foreign organisms as well as weather variation. Another concern is the necessity to enhance the efficiency of photosynthesis. One possible strategy would be through genetic engineering of cyanobacterial strains that are close to achieving maximal photosynthetic efficiency (Angermayr, Hellingwerf, Lindblad, & de Mattos, 2009; Ducat et al., 2011).

1.3 Picocyanobacteria

The autotrophobic picoplankton (APP) (0.2-2 μm) is composed predominantly of picocyanobateria which are the major contributors to primary production in both freshwater ecosystems and oligotrophic oceans (Callieri & Stockner, 2002; Ivanikova, Popels, McKay, & Bullerjahn, 2007).

Two related APP genera forming the picocyanobacterial clade are *Synechococcus* sp. and *Prochlorococcus* sp. (Sánchez-Baracaldo, Handley, & Hayes, 2008). Their genome consists of a single circular chromosome and lacks plasmid DNA. *Synechococcus* has a genome size ranging from 2.2 to around 2.86 Mb and the genome of *Prochlorococcus* ranges between 1.64 and 2.7 Mb. Compared to other cyanobacteria and average-sized bacteria, picocyanobacterial species are considered to have small genomes (Noordewier et al., 2005; Scanlan et al., 2009). Thus, they have a limited set of genes; *Synechococcus* harbors only 2,358 to 3,129 genes and *Prochlorococcus,* 1,716 to 3,022 genes with a small number of paralogous genes. Despite their very few protein-coding genes, Prochlorococci can still maintain their vital biosynthetic pathways and hence, their autotrophic life would be unaffected (Johnson et al., 2007; Scanlan et al., 2009).

Although Synechococci and Prochlorococci co-occur in the environment, their patterns of abundance differ both seasonally and spatially; Synechococci usually favor nutrient-rich, irradiated waters and become more abundant in mixed water columns during spring, whereas Prochlorococci prefer oligotrophic waters and bloom in stratified waters during summer and fall; they are known to inhabit deeper layers than synechococci (Mella-Flores et al., 2011).

To collect photosynthetic light, picocyanobateria, like cyanobacteria use the accessory pigments, phycobilisomes, which determine the composition of the picoplankton; while phycoerythrin (PE)-rich and phycocyanin (PC)-rich Synechococci predominate freshwater ecosystems, phycoerythrin (PE)-rich *Synechococcus* strains and chlorophyll (Chl) *b*-containing *Prochlorococcus* cohabit in oceanic environments (Baoli, Cong-Qiang, Fushun, Yuanxiu, & Yanyiu, 2009; Ivanikova et al., 2007).

Whereas most of the research involving picocyanobacteria was conducted on marine species, only few studies focused on freshwater bacterial strains. Several methods have been used for the classification and quantification of picocyanobacteria based on the analysis of their photosynthetic pigments (red or green) as well as their size. These methods include, flow cytometry, chromatographic assays, electron and epifluorescence microscopy observations (Figure 4) in addition to immunofluorescence techniques (Callieri & Stockner, 2002). While quantification by imaging have shown to be time-consuming, labor-intensive and prone to counting errors, especially with large samples, flow cytometry has proven to offer outstanding enumeration statistics for picocyanobacteria since it is rapid, accurate and possesses a high sorting capacity. However, the use of flow cytometry is accompanied by many limitations including high cost, requirement of highly skilled operators and proper choice of fluorochromes (Vives-Rego, Lebaron, & Nebe-von Caron, 2000; Wang, Hammes, De Roy, Verstraete, & Boon, 2010). Therefore, quantitative real-time PCR (qPCR) has become the method of choice for quantification purposes as well as the assessment of biodiversity in picoplanktonic environments (Malinen, Kassinen, Rinttilä, & Palva, 2003).

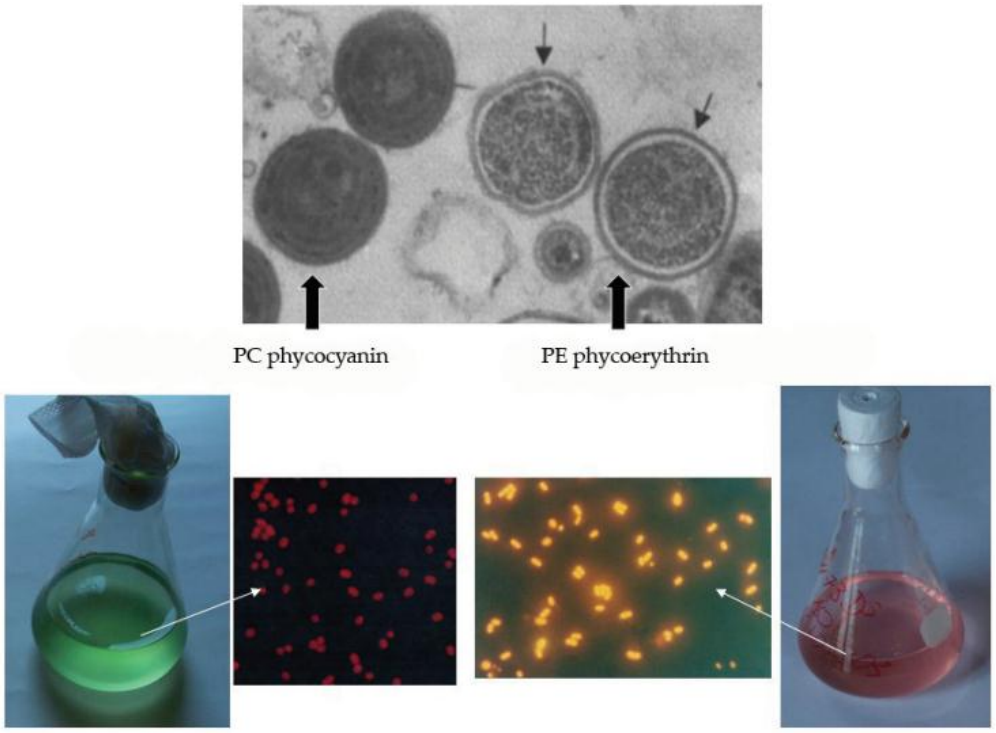

Figure 4: Imaging of *Synechococcus sp*. Lower panel: Cultures of *Synechococcus sp.*: PE-rich (right), PC-rich, lacking phyoerythrin (left). The microscopic pictures are taken under blue excitation at 1250x with Zeiss AXIOPLAN epifluorescence microscope. Upper panel: Transmission electron microscopy field showing the internal structure of the two types of cells (Callieri, 2007).

1.4 qPCR

qPCR is a modification of the classical PCR method for gene amplification. It uses fluorescent dyes or probes to monitor the increase in the amount of amplicons during the course of the experiment. In other words, fluorescence varies proportionally to the amount of DNA amplified and this variation is recorded as a graph that allows quantitative analysis. Qualitative analysis is also possible using end-point and melting-curve measurements (Strömbom et al., 2006); the melting-curve is generated to validate the specificity of the product amplified without the need for sequencing or gel electrophoresis (Andree et al., 2011).

Today, researchers are provided with more than one type or variation of the qPCR method, each with its advantages; the most commonly used are: the TaqMan PCR, the molecular beacon and the SYBR green technique (Tse & Capeau, 2003).

The SYBR green method uses the SYBR green dye, a very sensitive intercalating agent that binds only to double-stranded DNA (ds). After each cycle of annealing and elongation, SYBR green intercalates between base-pairs and emits fluorescence when excited by ultraviolet light (Figure 5). The signal is thus proportional to the amount of dsDNA produced and is recorded by a computer (Ririe, Rasmussen, & Wittwer, 1997).

As shown in Figure 6, the amplification reaction is translated as a sigmoid curve that represents the cycle fluorescence signal as a function of cycle number; this curve can be divided into three major phases: phase I corresponds to the lag phase where fluorescence cannot be detected due to background noise. Phase II represents the exponential phase where fluorescence just starts to be detected and phase III is the last stage of amplification where fluorescence is no more exponential and reaches a plateau. Ideally the amount of DNA should double after each amplification cycle, however this is not the case in experimental settings where efficiency can range between 1 (in the absence of amplification) and 2 (when the amount of DNA doubles) (Karlen, McNair, Perseguers, Mazza, & Mermod, 2007).

Data processing can be achieved using different methods; the choice of qPCR calculations is between “absolute” and “relative”. Absolute quantification depends on a standard curve to determine the exact copy number of the target sequence. This curve is created based on known copy numbers of commercially available DNA, target sequences amplified by PCR or plasmids housing the gene of interest. Relative quantification, on the other side, is commonly used for clinical studies and is based on the comparison between the expression of a target gene and a reference gene (Peirson, Butler, & Foster, 2003; Rutledge & Côté, 2003; Yuan, Reed, Chen, & Stewart, 2006). Using current available softwares, data analysis is performed through the “cycle-threshold” method which determines the gene copy number as a function of the cycle threshold (Ct); the point at which fluorescence just starts to be detectable but is still within the exponential phase of amplification. This reduces the limitations of interfering factors linked to the late stages of PCR and thus increases

the precision of quantification. Two methods may be used for determining the Ct value: the fit point and the second derivative. In the fit point method, the Ct value is the value at which a line parallel to the x-axis and the fluorescence curve intersect. In the second derivative method, the Ct or so-called Cq (Quantification cycle) is the second derivative maximum of the fluorescence curve. The "cycle-threshold" approach is only valid if Cq values are measured during phase II or the exponential phase and assumes that efficiency is the same between all PCR reactions (Guescini, Sisti, Rocchi, Stocchi, & Stocchi, 2008; Rutledge & Côté, 2003).

qPCR has been used lately for the enumeration of cyanobacteria and picocyanobacteria. In a study conducted by Furukawa et al. (2006), real-time PCR was used for the quantification of microcystin-producing cyanobacteria in a fresh water environment based on Microcystin Synthetase A (mcyA) gene. Similarly, marine hepatotoxin nodularin-producing Nodularia were quantified by qPCR using primers specific to ndaF, the subunit F of the nodularin synthetase gene (Koskenniemi, Lyra, Rajaniemi-Wacklin, Jokela, & Sivonen, 2007). Both studies designed qPCR standard curves as a basis for the assay of gene copy number per volume of sample used. Another study by Sánchez-Baracaldo, Handley and Hayes (2008) assessed the abundance of picocyanobacteria from seven freshwater lakes by qPCR based on small subunit (ssu) rDNA sequences.

1.5Cyanobacteria as indicators of water quality

It is of great importance to monitor the abundance of cyanobacteria and picocyanobacteria since they adapt to both nutrient-enriched and nutrient-deficient environments and thus can represent major indicators of water quality.

1.5.1. *Significance of cyanobacterial blooms*

When water becomes enriched with nutrients, especially nitrogen and phosphorous, this will lead to a condition known as eutrophication, which is the excessive growth of algae and microalgae, thus altering the structure and the stability of the ecosystem as well as water quality compared to reference conditions

(Andersen, 2006). Eutrophication occurs mainly due to anthropogenic disturbances of the natural system such like domestic and agricultural run-off. Under these conditions and upon high exposure to light and temperature, surface and subsurface blooms of cyanobacteria develop. Such blooms appear as a yellowish scum and result in a disagreeable smell and taste (Figure 7) (Chorus & Bartram, 1999; Mbeunkui, Richaud, Etienne, Schmid, & Bachmann, 2002). Elliot, Jones and Thackeray (2006) showed, for instance, that the abundance of cyanobacteria was maximal: 62.4% upon exposure to the highest temperature and nutrient loads, a value that is 46.9% higher than that obtained under reference conditions. This supports the idea that an increase in the biomass of bloom-forming cyanobacteria might be an indication of water pollution. Moreover, Douterelo, Perona and Mateo (2004) observed an increase in the abundance of cyanobacteria belonging to the Oscillatoriales order at stations with elevated nutrient levels in running waters of Madrid (Spain). In fact, massive loads of cyanobacteria in water systems are of particular concern for both human and animal health; such blooms interfere with potable water due to the process of deoxygenation, as well as the unpleasant smell and taste that form. In addition, some strains of these bacteria such as Anabaena, Nodularia, Oscillatoria, Nostoc and Microcystis even produce toxins that deteriorate water quality, aquaculture, fishing and most importantly, cause serious health problems for both humans and animals; these problems include liver diseases, cancers and ultimately, death (Paerl et al., 2001; Schreiter et al., 2001). Moreover, cyanobacterial blooms are directly correlated with the formation of hypoxic and anoxic regions leading to mortalities among fish and fauna in underlying waters (Paerl et al., 2001); Wilhelm et al. (2006) observed an increase in cyanobacterial loads (above 10^5 mL^{-1}) in hypoxic regions (dead zone) of Lake Erie. Because of all the problems related to massive growth of cyanobacteria in water, especially drinking water, the World Health Organization (WHO) suggested a series of guidelines and protocols required to deal with the situation; today, many countries have put these protocols in practice to avoid the consequences of such blooms (Leboulanger et al., 2002).

In what concerns Lebanon, whereas no data of cyanobacterial counts from freshwater samples were available (as mentioned above), the Lebanese littoral and its microbial populations were the focus of several research conducted by the National Council for Scientific Research; Abboud-Abi Saab, Fakhry, Kassab and Mattar (2004) performed a microscopic counting of phytoplanktonic organisms to assess water quality in the Southern littoral; results have shown high cell abundances at three stations: Saida, Sarafand and Sour during the month of October (in the range of 10^4 and 10^5 cells/ L). These numbers correlated with the enrichment of these stations with nutritive salts (especially N and P) and thus concluding that enumerating phytoplanktonic organisms is an important tool to estimate the degree of eutrophication in coastal regions. Another study by Abboud-Abi Saab, Fakhri, Sadek and Matar (2008) also aiming at studying eutrophication, assessed the phytoplankton biomass along the Lebanese littoral by measuring the levels of chlorophyll-*a* pigments; mean values of chl-*a* did not exceed 1 mg/m^3 for all stations except for the regions of Antelias, Saida and Ramlet El Bayda where chl-*a* levels reached values between 5 and 20 mg/m^3, indicating that these stations are eutrophied; untreated waste water as well as urbanization are behind this large biomass of phytoplankton in these regions.

1.5.2. *Significance of picocyanobacteria*

Not only do cyanobacteria account for polluted water (nutrient-enriched water), but some strains are also indicators of nutrient-deficient environments. Actually, because of the specialized nutrient-uptake mechanisms they have developed, picocyanobacteria are highly abundant in oligotrophic ecosystems where competition with other phytoplankters becomes hindered by low concentrations of nutrients (Callieri, 2007). Ernst, Deicher, Herman and Wollenzien (2005) showed, for instance, that freshwater ecosystems are dominated by PE-rich *Synechococcus* sp. when nutrient availability is low.

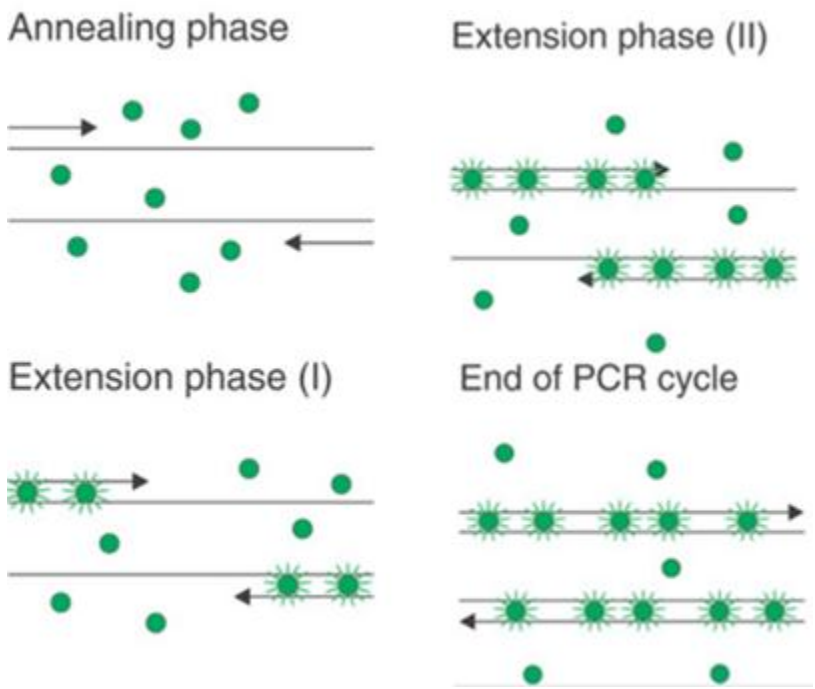

Figure 5: Principle of qPCR by the SYBR green method (van der Velden et al., 2003).

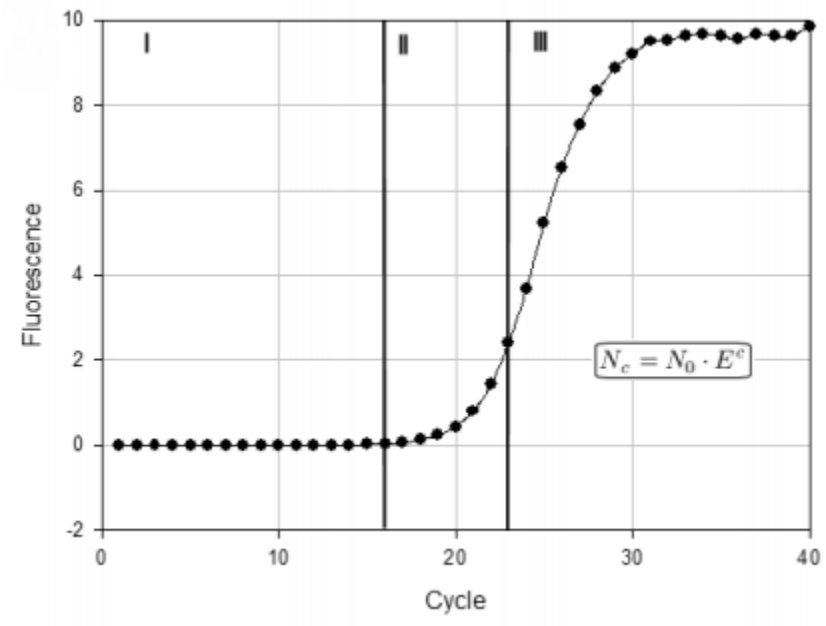

Figure 6: The three phases of the amplification reaction in qPCR (Karlen et al., 2007).

Figure 7: : Examples of cyanobacterial blooms: Left panel: blooms of *Anabaena* and *Microcystis aeroginosa* spp. in the lower St. Johns river (Florida); upper right: bloom of *Nodularia* spp. in the Baltic Sea off the Southern coast of Finland; lower rightbloom of *Anabaena* spp. in lake Okaro, New Zealand (North island) (Paerl et al., 2001).

1.6 *hcp* gene

Recently, both marine and freshwater picocyanobacteria were found to harbor a gene, *hcp*, 100% conserved among all the strains isolated. The gene encodes a 62 amino acid, hyperconserved polypeptide and is exclusive to picocyanobacterial species (Kutovaya, McKay, & Bullerjahn, 2010). Furthermore, *hcp* is flanked by highly conserved tRNA genes that are in turn located between rpl19, coding for large ribosomal protein 19 and gltX that encodes a glutamyl tRNA synthetase. Thus, the syntheny of the *hcp* gene is also conserved. Studying the resulting protein deduced a possible role of *hcp* in binding DNA or RNA as well as a probable involvement in the translation process (Zhaxybayeva, Gogarten, & Doolittle, 2007). Being highly conserved, *hcp* region is a very good target for quantification of picocyanobacteria by qPCR.

1.7 Aim of the study

The aim is of this study is to develop an *hcp*-based assay for the quantification of picocyanobacteria in marine and freshwater samples by qPCR. The approach used is summarized by the creation of a plasmid that harbors the gene (amplified from DNA of the LS0504 strain), the construction of a standard curve for qPCR, and the *hcp*-based qPCR on DNA extracted from samples of the Sargasso Sea, USA and an artificial lake in Annaya, Lebanon.

CHAPTER TWO

MATERIALS AND METHODS

2.1 DNA samples

DNA samples from freshwater *Synechoccus* sp. strains (collected from Laurentian Great Lakes) LS 0504, KD3a, ARC-11, ARC-21 and marine strain WH 8102 were provided by Dr. George Bullerjahn from the Department of Biological Sciences, Bowling Green State University.

2.2 PCR amplification of *hcp*

PCR was used to amplify specific DNA sequences of the *hcp* gene in a 50 µL reaction.

2.2.1. *Primer design for hcp*

Primers were designed based on alignments of DNA sequences of the *hcp* gene from species LS 0504, KD3a, ARC-11 and ARC-21 (GenBank) and other surrounding DNA regions using ClustalW software (www.genome.jp/tools/clustalw). NetPrimer software (www.premierbiosoft.com/netprimer/index.html) was used for primer analysis and design.

Forward primer:

hcp23F 5’ ATg gAg TTg gAT TTA CAA CCC gg 3’

Reverse primer:

hcp134R 5’ AAT TCA Cgg CCC Tgg TCg CT 3’

2.2.2. *PCR conditions and reagents*

The reaction mix (50 µL) included 0.2 mM of dNTPs, 1X of Taq buffer, 2.5 mM $MgCl_2$, 0.4 µM of each primer, 50 ng of template DNA and 0.1 U/µL of AmpliTaq Gold (Roche).

The reaction was initially held at 95 °C for 2 min followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 57 °C for 30 s, extension at 72 °C for 15 s and final extension at 72 °C for 10 min. Samples were left in a final hold for infinity at 4 °C.

The PCR products were visualized on a 1% agarose gel via electrophoresis at 100 V.

2.3 Cloning procedure

2.3.1. *PCR*

hcp was amplified from LS 0504 strain by PCR in a 50 µL reaction using the protocol described in section 2.2.2 with primers hcp23F and hcp134R. Products were visualized on a 1% agarose gel via electrophoresis at 100 V.

2.3.2. *DNA purification from PCR product*

DNA was purified directly from the PCR product using the NucleoSpin® Extract II Kit (MACHEREY-NAGEL GmbH & Co. KG) according to manufacturer's instructions.

2.3.3. *Cloning reactions*

Cloning reactions and transformation procedures were performed as described by the TOPO TA Cloning Kit (Invitrogen) using TOP10 competent cells.

2.3.4. *Screening for transformants*

From each transformation reaction, cells (10-50 µL) were spread on a prewarmed ampicilline (Amp) selective Luria-Bertani (LB) plate and incubated overnight at 37 °C. The protocol for the preparation of LB plates is described in TOPO TA Cloning Kit (Invitrogen). Two to three white colonies were sub-cultured several times. Cryobanks were also created and stored in quadruplets at – 80 °C.

2.3.5. *Extraction of pCR2.1-TOPO-hcp plasmid*

Plasmid DNA was extracted using Zyppy™ Plasmid Miniprep Kit according to manufacturer's instructions.

2.3.6. *Amplification of the cloned PCR insert*

PCR using the protocol described in section 2.2.2 with primers hcp23F and hcp134R was performed on pCR2.1-TOPO-*hcp* plasmid to check for successful cloning and transformation and PCR products were visualized on a 1% agarose gel via electrophoresis at 100 V.

2.4 Environmental samples

2.4.1. *Marine DNA samples*

Marine samples were collected from the Sargasso Sea which is located in the middle of the North Atlantic Ocean. The northwest region of the sea, at the Bermuda Atlantic Time-series Study site (BATS) is the most studied and characterized (Venter et al., 2004). DNA samples were provided by Dr. George Bullerjahn from the Department of Biological Sciences, Bowling Green State University; details on each sample are represented in Table 1.

Table 1: Details on marine samples collected from the Sargasso Sea.

DNA sample	Station	Depth of collection site (m)	Date of collection (day/month /year)	Volume of extracted DNA (µL)	Volume of water filtered (L)
SRG 1	2 (BATS)	5	22/10/07	150	6
SRG 2	2 (BATS)	95	22/10/07	150	6
SRG 3	4	5	23/10/07	150	6
SRG 4	4	119	23/10/07	150	6
SRG 5	9	5	25/10/07	150	6
SRG 6	9	100	25/10/07	150	6

DNA extraction was performed as described by Ilikchyan, McKay, Zehr, Dyhrman and Bullerjahn (2009) and stations are as shown in Figure 8.

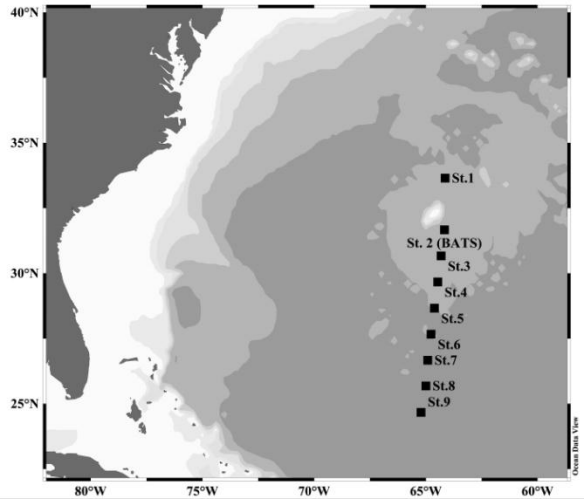

Figure 8: Map of Sargasso Sea stations from which water was sampled (Ilikchyan et al., 2010).

2.4.2. *Freshwater samples*

Freshwater water samples were collected from an artificial lake

Sample collection

- Cleaning of collection containers

1 L glass bottles were cleaned by autoclaving and 19 L polycarbonate containers were cleaned by acid wash (1:20 HCl).

- Collection site

Superficial water was collected from an artificial lake in Annaya (Jbeil, Mount Lebanon) on November 12, 2011 by immersing glass bottles and dumping the contents into the large polycarbonate container.

- Water filtration

Water (500 mL) was filtered using 0.2 μm filters; five filters were flash frozen in a microfuge tube by immersion in liquid nitrogen followed by immediate storage at -80 °C and the other five were incubated on BG-11 agar plates prepared as descrribed by Stanier, Kunisawa, Mandel and Cohen-Bazire (1971).

- DNA extraction

DNA extraction from both filters and colonies was performed according to the protocol described by Mella-Flores et. al (2011) and sample name for DNA extracted from the filter is ANN.

- PCR

PCR using the protocol described in section 2.2.2 with primers hcp23F and hcp134R was performed on DNA extracted from colonies (grown on BG-11 media) to check for the presence of picocyanobacteria. PCR products were visualized on a 1% agarose gel via electrophoresis at 100 V.

2.5 qPCR

The hcp23F and hcp134R primers were used to create the external standard curve for the absolute quantification of environmental samples. qPCR protocol was conducted using Roche LightCycler 1.0 instrument with Roche LightCycler software version 3.5:

The reaction mix (20µL) included 3 mM $MgCl_2$, 2 µL of Reaction Mix (LightCycler FastStart DNA Master SYBR Green I; Roche), and 0.35 µM of each primer.

Thermal cycling consisted of the following steps: preheating at 95 °C for 10 min, with a heating rate of 20 °C s^{-1}, quantification, including 45 cycles (95 °C for 0 s, 57 °C for 5 s, and 72 °C for 8 s), fluorescence measurement at the end of each cycle at 72 °C through channel F1 (530 nm), and a heating rate of 20 °C s^{-1}, melting curve analysis through heating from 77 °C to 97 °C at a rate of 0.1 °C s^{-1} and fluorescence measurement continuously through channel F1 (530 nm).

The standard curve was designed as a correlation between known gene copy numbers and Cq values. Therefore, a serial dilution of plasmid pCR2.1-TOPO-*hcp* was performed including two 5-fold dilutions (at low concentrations): $3.56x10^2$ and $1.78x10^3$ gene copies/20 µL reaction and four 10-fold dilutions (at higher concentrations): $8.89x10^3$-$8.89x10^6$ gene copies/20 µL reaction. Gene copy numbers were derived from measured concentrations (by the NanoDrop spectrometer, thermo scientific, software version 3.7.1) according to the formula:

[DNA concentration (g μL^{-1}) x Avogadro constant (6.022×10^{23} copies mol^{-1}) x sample volume (µL)] [Vector molecular weight Mw (g mol^{-1})]$^{-1}$ = Gene copy numbers.

MW = vector size (bp) x 660 Da/bp = Da (or g mol^{-1}) (Fu et al., 2009)

The vector size pCR 2.1-TOPO (Invitrogen) was used to calculate the vector molecular weight (vector size 3900 Da + PCR product 186 Da) x 660 = 2.7×10^6 Da.

To minimize error, triplicates of each standard were used.

The second derivative maximum was the analysis method since it is a robust and a reliable method; by doing an automatic data calculation, it excludes the user influence upon threshold selection and excludes background noise (Larionov, Krause, & Miller, 2005).

qPCR on environmental DNA samples (section 2.4) was performed using the same conditions described above with an increased annealing temperature (68 °C) to reduce mispriming. Triplicates from each DNA sample were also used and one of the standards (8.89×10^5 Gene copy numbers/20 µL) was included in the run (a step necessary for absolute quantification using an external standard curve).

Absolute quantification was performed by importing the standard curve constructed into the run. All calculations (including statistical analysis) were performed by LightCycler software version 3.5 (Roche).

CHAPTER THREE

RESULTS

3.1 Sequence alignment

hcp sequences of picocyanobacterial strains LS 0504, KD3a, ARC-11 and ARC-21 were downloaded from GenBank and aligned using ClustalW software. Results are shown in Figure 9.

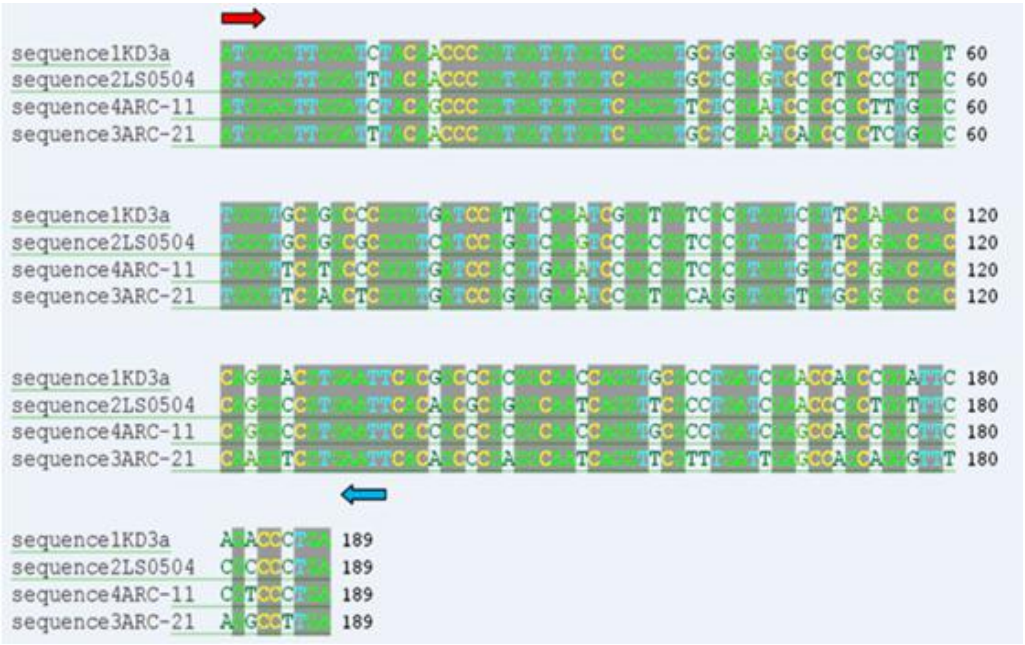

Figure 9: Sequence alignment of picocyanobacterial strains using ClustalW software. Grey highlights homologous sequences. Red arrow: forward primer, blue arrow: reverse primer.

3.2 Primer design

Primers were developed based on the most homologous sequences at or near the 5' end and the 3' end (Figure 9):

Forward primer:

hcp23F 5' ATg gAg TTg gAT TTA CAA CCC gg 3'

Reverse primer:

hcp134R 5’ AAT TCA Cgg CCC Tgg TCg CT 3’

3.3 Successful PCR after optimization

hcp from all picocyanobacterial strains LS 0504, KD3a, ARC-11, ARC-21 and WH8102 was amplified using the primers hcp23F and hcp134R. Results of the amplicons visualized on a 1% agarose gel are shown in Figure 10. Lanes 3 to 7 show a band at around 180 bp corresponding to the size of the *hcp* gene and demonstrate successful primer design.

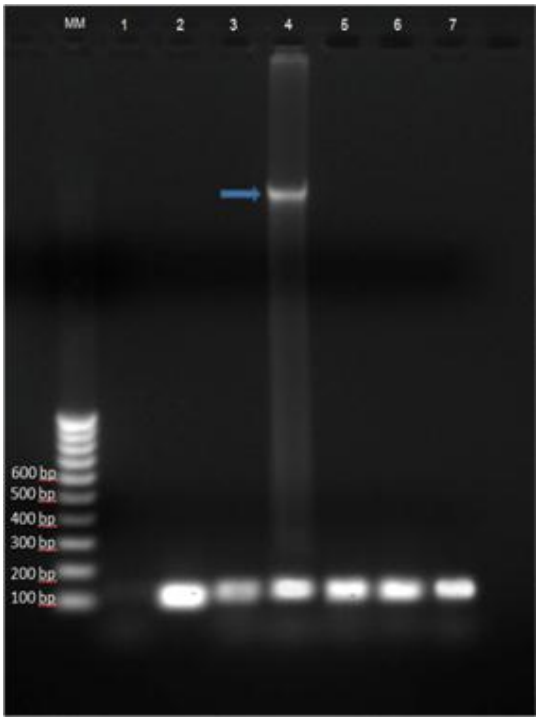

Figure 10: PCR amplification of *hcp* from all picocyanobacterial strains visualized on a 1% agarose gel stained with ethidium bromide. Lane MM: 100 bp molecular marker, lane 1: negative control, lane 2: positive control, lane 3: ARC-11 strain, lane 4: ARC-21 strain, lane 5: LS0504 strain, lane 6: KD3a strain, lane 7: WH8102 strain (marine). The band indicated by the blue arrow corresponds to genomic DNA.

3.4 Amplification of *hcp* from pCR2.1-TOPO-*hcp* plasmid

PCR amplification with primers hcp23F and hcp134R was performed on the extracted plasmid DNA to check for successful cloning. PCR products were visualized on a 1% agarose gel and results are shown in Figure 11. Lane 3 shows a

band at around 180 bp which corresponds to the size of the *hcp* gene and hence demonstrates successful cloning and transformation.

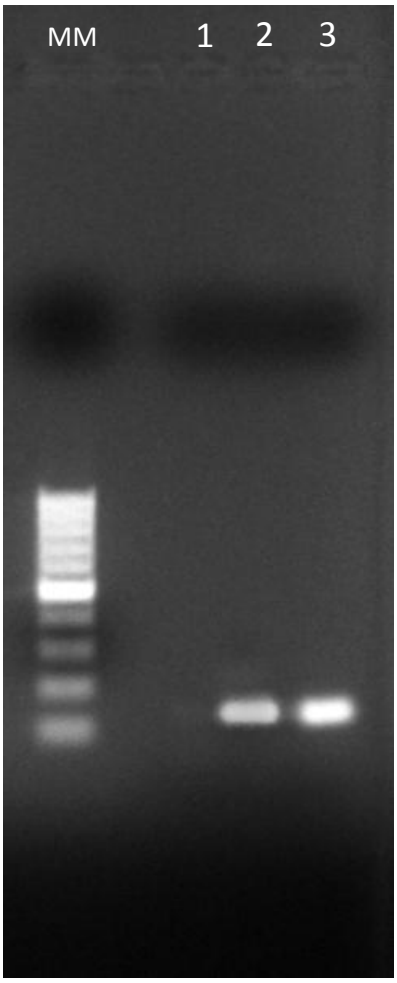

Figure 11: PCR amplification of *hcp* from pCR2.1-TOPO-*hcp* plasmid visualized on a 1% agarose gel stained with ethidium bromide. Lane MM: 100 bp molecular marker, lane 1: negative control, lane 2: positive control, lane 3: plasmid DNA.

3.5 Amplification of *hcp* from colonies grown on BG-11 plates

PCR amplification with primers hcp23F and hcp134R was performed on DNA extracted from colonies (grown on BG-11 plates). PCR products were visualized on a 1% agarose gel and results are shown in Figure 12. Lane 3 shows a band at around 180 bp which corresponds to the size of the *hcp* gene and hence demonstrates the presence of picocyanobacteria in freshwater samples (Annaya).

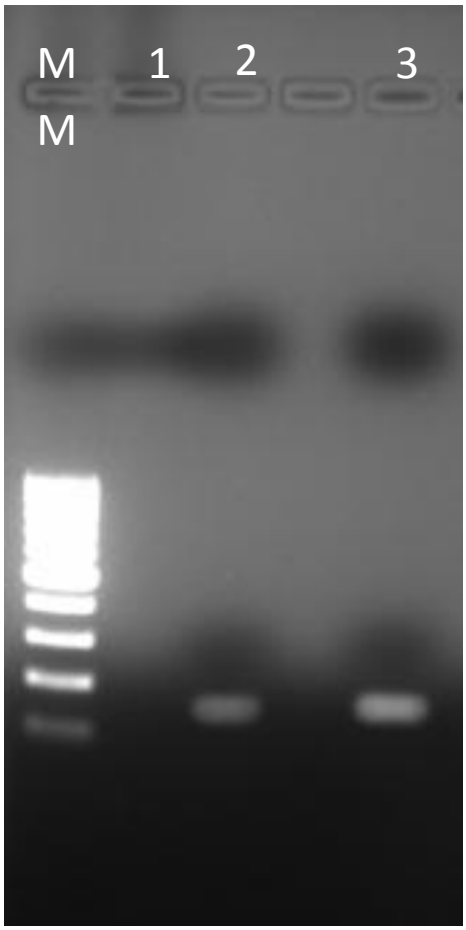

Figure 12: PCR amplification of *hcp* from colonies grown on BG-11 plates (Annaya) visualized on a 1% agarose gel stained with ethidium bromide. Lane MM: 100 bp molecular marker, lane 1: negative control, lane 2: positive control, lane 3: extracted DNA.

3.6 qPCR results

3.6.1. *Standard curve*

qPCR on triplicates of the six standards (two 5-fold-dilutions of pCR2.1-TOPO-*hcp* : $3.56x10^2$ and $1.78x10^3$ gene copies/20 µL reaction and four 10-fold dilutions of pCR2.1-TOPO-*hcp*: $8.89x10^3$-$8.89x10^6$ gene copies/20 µL reaction) was performed using hcp23F and hcp134R primers.

- Fluorescence curves

Fluorescence was measured through channel F1 (530 nm) and Figure 13 shows the fluorescence curves obtained.

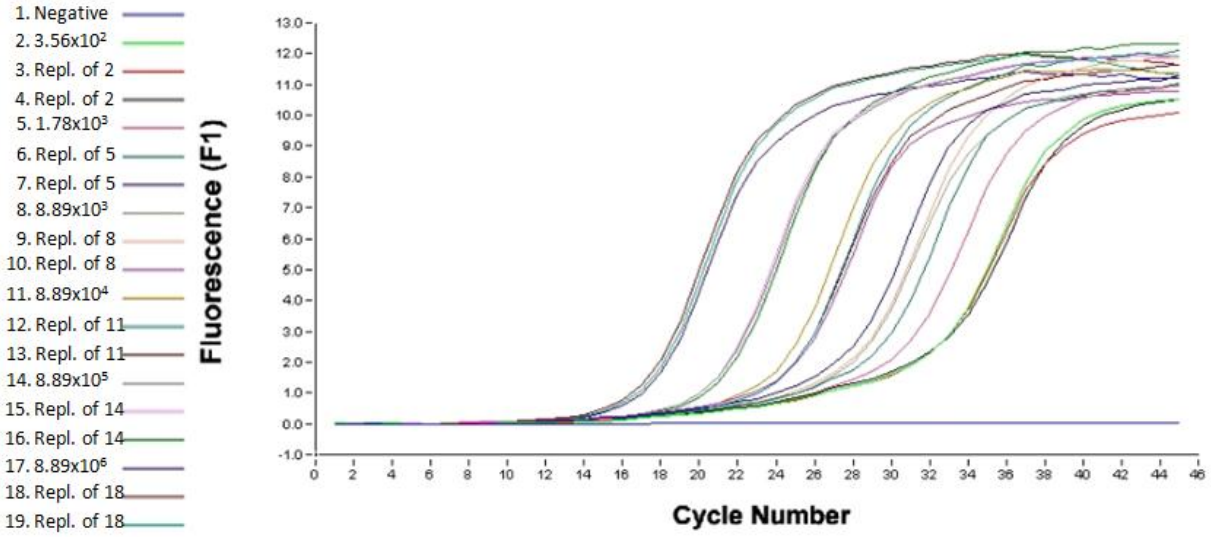

Figure 13: Fluorescence curves obtained by qPCR on standards used to create the standard curve (two 5-fold-dilutions of pCR2.1-TOPO-*hcp* : $3.56x10^2$ and $1.78x10^3$ gene copies/20 µL reaction and four 10-fold dilutions of pCR2.1-TOPO-*hcp*: $8.89x10^3$-$8.89x10^6$ gene copies/20 µL reaction). Curves represent the variation in fluorescence as a function of the cycle number.

- Melting curves

Melting curve analysis was performed by heating the samples from 77 °C to 97 °C and fluorescence was measured through channel F1 (530 nm). Results show that all standards have the same melting temperature (around 90.5 °C) (Figure 14), thus proving the specificity of the products obtained; in other words, primers were specific to the *hcp* gene and therefore, quantification would be indeed based on *hcp.*

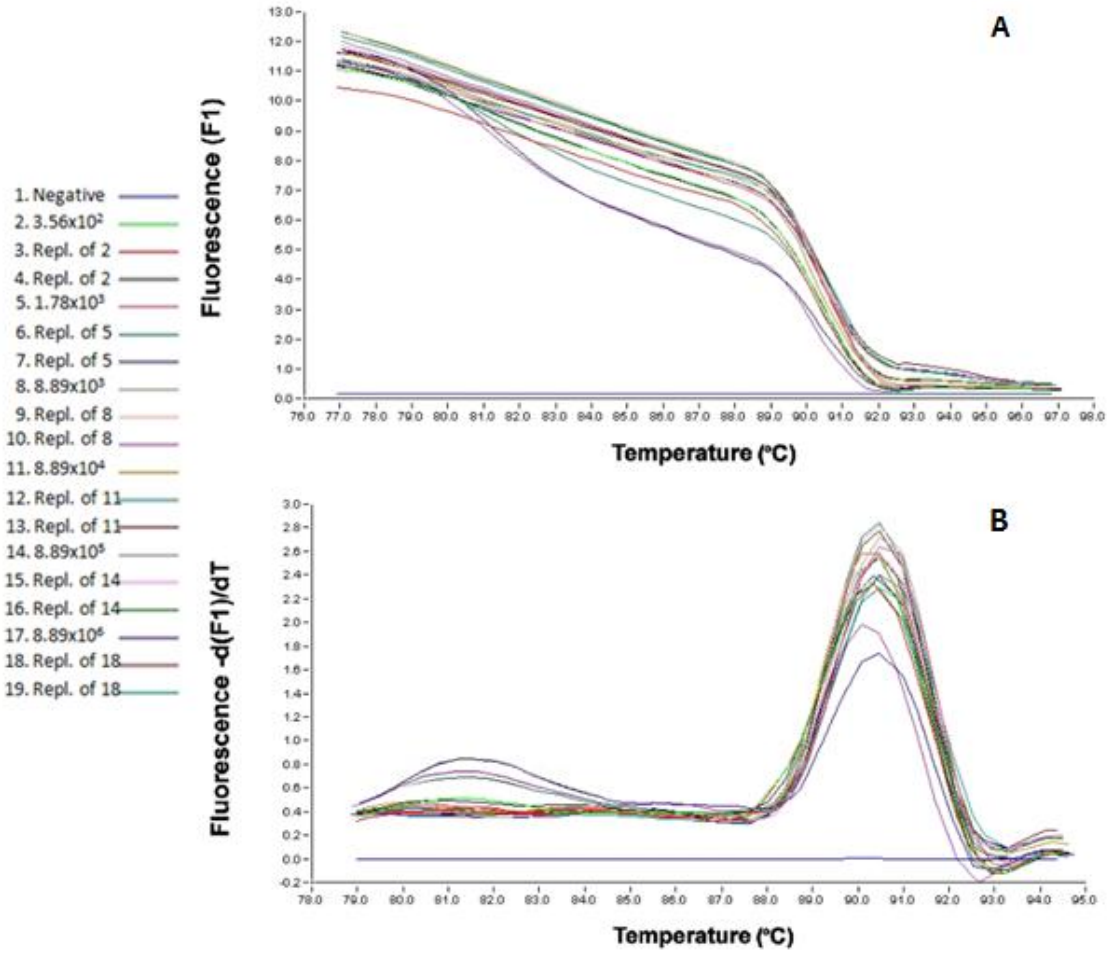

Figure 14: Melting curve data of standards (measured in triplicates) used to create the standard curve (two 5-fold-dilutions of pCR2.1-TOPO-*hcp* : $3.56x10^2$ and $1.78x10^3$ gene copies/20 µL reaction and four 10-fold dilutions of pCR2.1-TOPO-*hcp*: $8.89x10^3$-$8.89x10^6$ gene copies/20 µL reaction). A: Melting curves showing the variation in fluorescence as a function of temperature; B: Melting curve analysis data.

- Standard curve

The standard curve was designed as a correlation between Gene copy numbers of the standards and their corresponding Cq values (Figure 15). The efficiency obtained (E = 1.983) is at the higher extreme of the usual range (between 1 and 2, as mentioned earlier). Thus, amplification is highly efficient.

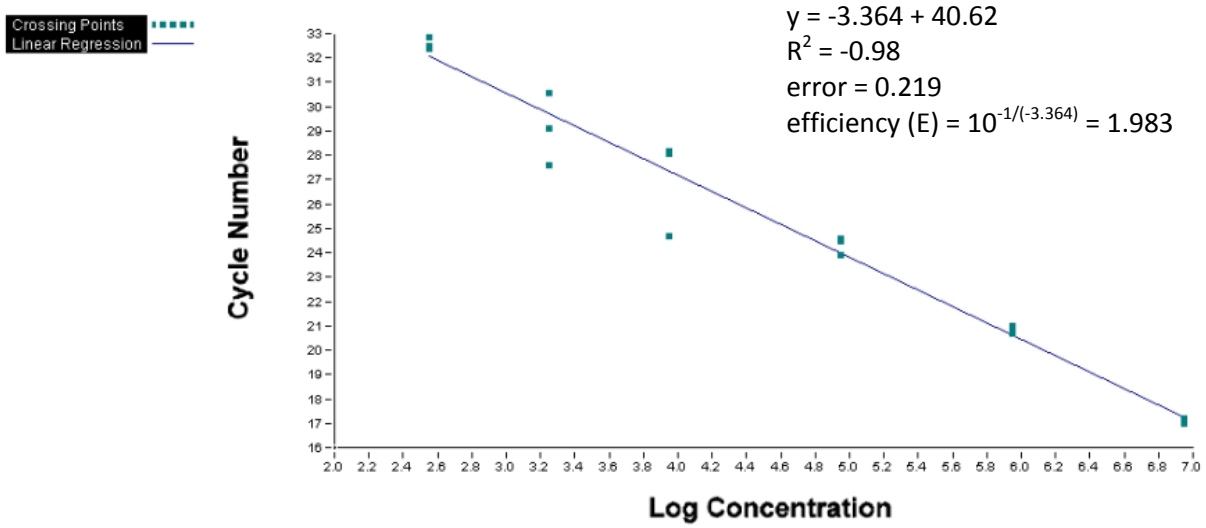

Figure 15: Standard curve developed as a correlation between Log Concentration of the standards (two 5-fold-dilutions of pCR2.1-TOPO-*hcp*: $3.56x10^2$ and $1.78x10^3$ gene copies/20µL reaction and four 10-fold dilutions of pCR2.1-TOPO-*hcp*: $8.89x10^3$-$8.89x10^6$ gene copies/20µL reaction) and the quantification cycle. Measurements were done on triplicates of each standard. Slope: -3.364; regression coefficient (R^2): - 0.98; error: 0.219 and efficiency: 1.983.

- Quantification of standards and statistical analysis

Calculations and statistical analysis related to the construction of the standard curve were performed by LightCycler software version 3.5 (Roche) and are summarized in Table 2. According to Karlen et. al (2007), qPCR does not allow the determination of the statistical significance of data obtained. However, in his study, he suggested a statistical analysis that would lead to better DNA quantification estimates; as regards the standard deviation, he concluded that a value larger than 0.4 decreases the reproducibility of the

results. As shown in Table 2, SD>0.4 for low-concentrated standards (1.477 and 1.988 for DNA concentrations of 1.78 x 10^3 and 8.89 x 10^3 gene copies/ 20 µL reaction, respectively), which was also observed by Karlen et. al (2007) for samples with a Cq higher than 30 (corresponding to low concentrations). Therefore, standard deviation normally increases with decreasing DNA concentrations.

Table 2: Calculations and statistical analysis related to the construction of the standard curve as performed by LightCycler software version 3.5 (Roche).

Sample	Measured conc. (Gene copies/ 20 µL reaction)	Cq	Grouped mean of Cq	Grouped SD of Cq	Calculated conc. (Gene copies/ 20 µL reaction)	Grouped mean of calculated conc. (Gene copies/ 20 µL reaction)
Negative						
3.56 x 10^2	3.56 x 10^2	32.49	32.58	0.238	2.62 x 10^2	2.48 x 10^2
Replicate of 3.56 x 10^2	3.56 x 10^2	32.40			2.79 x 10^2	
Replicate of 3.56 x 10^2	3.56 x 10^2	32.85			2.05 x 10^2	
1.78 x 10^3	1.78 x 10^3	30.56	29.09	1.477	9.78 x 10^2	3.67 x 10^3
Replicate of 1.78 x 10^3	1.78 x 10^3	29.11			2.64 x 10^3	
Replicate of 1.78 x 10^3	1.78 x 10^3	27.61			7.40 x 10^3	
8.89 x 10^3	8.89 x 10^3	28.08	26.99	1.988	5.37 x 10^3	2.16 x 10^4
Replicate of 8.89 x 10^3	8.89 x 10^3	29.19			4.97 x 10^3	
Replicate of 8.89 x 10^3	8.89 x 10^3	24.69			5.45 x 10^4	
8.89 x 10^4	8.89 x 10^4	23.90	24.32	0.361	9.35 x 10^4	7.19 x 10^4
Replicate of 8.89 x 10^4	8.89 x 10^4	24.56			5.97 x 10^4	
Replicate of 8.89 x 10^4	8.89 x 10^4	24.49			6.24 x 10^4	
8.89 x 10^5	8.89 x 10^5	20.78	20.82	0.145	7.93 x 10^5	7.73 x 10^5
Replicate of 8.89 x 10^5	8.89 x 10^5	20.70			8.36 x 10^5	
Replicate of 8.89 x 10^5	8.89 x 10^5	20.98			6.90 x 10^5	
8.89 x 10^6	8.89 x 10^6	17.20	17.11	0.124	9.21 x 10^6	9.81 x 10^6
Replicate of 8.89 x 10^6	8.89 x 10^6	16.97			1.08 x 10^7	
Replicate of 8.89 x 10^6	8.89 x 10^6	17.16			9.45 x 10^6	

Conc.: concentration; Cq: Quantification cycle; SD: standard deviation.

3.6.2. *DNA quantification from environmental samples*

qPCR on triplicates of each of the environmental samples (sections 2.4.1 and 2.4.2) was performed using hcp23F and hcp134R primers and one of the standards (8.89 x 10^5 gene copies/ 20 µL) was included in the run.

- Fluorescence curves

Fluorescence was measured through channel F1 (530 nm) and Figure 16 shows the fluorescence curves obtained.

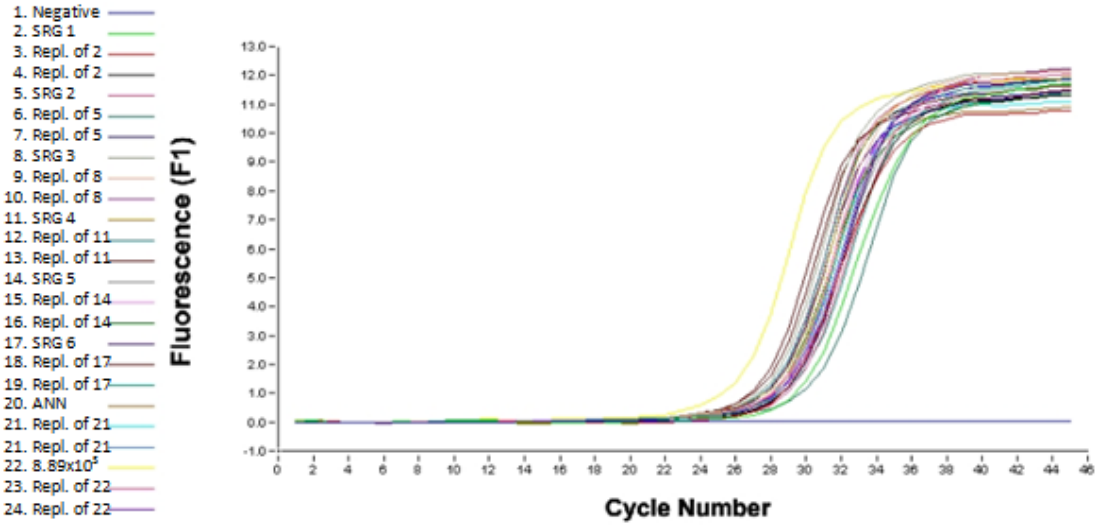

Figure 16: Fluorescence curves obtained by qPCR on triplicates of each DNA sample extracted from environmental water samples and triplicates of one of the standards (8.89 x 10^5 gene copies/ 20 µL reaction). SRG: DNA samples extracted from water sampled at the Sargasso Sea, USA; ANN: DNA samples extracted from water sampled at Annaya, Lebanon. Curves represent the variation in fluorescence as a function of the cycle number.

- Melting curves

Melting curve analysis was performed by heating the samples from 77 °C to 97 °C and fluorescence was measured through channel F1 (530 nm). Results show that all samples have almost the same melting temperature (around 90.5 °C) (Figure 17), thus proving the specificity of the products obtained; quantification is indeed based on *hcp.*

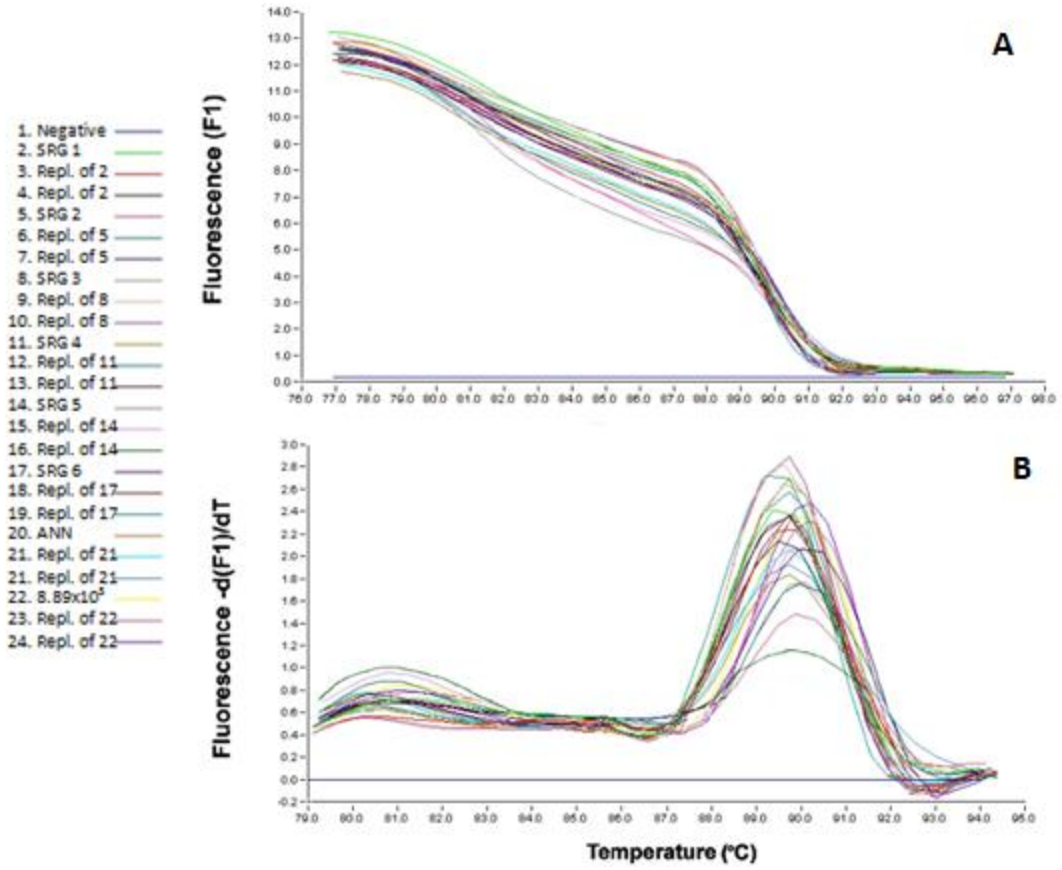

Figure 17: Melting curve data of environmental DNA samples (measured in triplicates) and one of the standards: 8.89 x 10^5 gene copies/ 20 µL reaction. A: Melting curves showing the variation in fluorescence as a function of temperature; B: Melting curve analysis data.

- Quantification of environmental DNA samples and statistical analysis

Absolute quantification of environmental DNA samples was performed by importing the standard curve constructed in section 3.5.1 into the run. All calculations (including statistical analysis) were performed by LightCycler software version 3.5 (Roche) and are summarized in Table 3. DNA concentrations for environmental samples of both Sargasso Sea, USA and Annaya, Lebanon were quantifiable since they exceeded the limit of quantification (LOQ); numbers obtained for the Sargasso Sea were in the range of 10^3 gene copies/1 mL of water filtered and those obtained for freshwater samples (Annaya) were in the range of 10^5 gene copies/ 1 mL. Standard deviations, however, are higher than 0.4 for most samples which can be explained by the high corresponding Cq values (Karlen et al., 2007).

Table 3: Calculations and statistical analysis related to the quantification of DNA from environmental samples.

Sample	Measured conc. (Gene copy numbers/ 20 µL reaction)	Cq	Grouped mean of Cq	Grouped SD of Cq	LOQ (Gene copies/DNA extract)	LOD (Gene copies/DNA extract)	Calculated conc. (Gene copies/ 20 µL reaction)	Calculated conc. (Gene copies/ DNA extract)	UD, DNQ determination	Grouped mean of calculated conc. (Gene copies/ 20 µL reaction)	Volume of water filtered/ 2 µL DNA (mL)	Grouped mean of calculated conc. (Gene copies/ 1 mL volume filtered)
Negative												
SRG 1	Unknown	29.61	29.04	0.491	600	75	2.20×10^5	3.02×10^4	Ok	3.31×10^5	80	4.14×10^3
Replicate of SRG 1	Unknown	28.71			600	75	4.00×10^5	5.67×10^4	Ok		80	
Replicate of SRG 1	Unknown	28.81			600	75	3.74×10^5	5.28×10^4	Ok		80	
SRG 2	Unknown	29.21	29.51	0.660	600	75	2.87×10^5	4.00×10^4	Ok	2.50×10^5	80	3.13×10^3
Replicate of SRG 2	Unknown	30.27			600	75	1.42×10^5	1.91×10^4	Ok		80	
Replicate of SRG 2	Unknown	29.06			600	75	3.18×10^5	4.44×10^4	Ok		80	
SRG 3	Unknown	27.79	28.44	0.605	600	75	7.37×10^5	1.08×10^5	Ok	5.06×10^5	80	6.33×10^3
Replicate of SRG 3	Unknown	28.54			600	75	4.49×10^5	6.38×10^4	Ok		80	
Replicate of SRG 3	Unknown	28.99			600	75	3.32×10^5	4.66×10^4	Ok		80	
SRG 4	Unknown	28.23	27.90	0.361	600	75	5.50×10^5	7.92×10^4	Ok	6.98×10^5	80	8.73×10^3
Replicate of SRG 4	Unknown	27.96			600	75	6.60×10^5	9.56×10^4	Ok		80	
Replicate of SRG 4	Unknown	27.52			600	75	8.85×10^5	1.30×10^5	Ok		80	
SRG 5	Unknown	28.98	28.53	0.404	600	75	3.34×10^5	4.69×10^4	Ok	4.60×10^5	80	5.75×10^3
Replicate of SRG 5	Unknown	28.43			600	75	4.81×10^5	6.89×10^4	Ok		80	
Replicate of SRG 5	Unknown	28.19			600	75	5.65×10^5	8.14×10^4	Ok		80	
SRG 6	Unknown	28.00	28.16	1.208	600	75	6.43×10^5	9.30×10^4	Ok	7.01×10^5	80	8.76×10^3
Replicate of SRG 6	Unknown	27.04			600	75	1.21×10^6	1.82×10^5	Ok		80	
Replicate of SRG 6	Unknown	29.44			600	75	2.46×10^5	3.40×10^4	Ok		80	
ANN	Unknown	28.48	28.65	0.204	4000	500	4.66×10^5	4.44×10^5	Ok	4.19×10^5	1	4.19×10^5
Replicate of ANN	Unknown	28.59			4000	500	4.33×10^5	4.11×10^5	Ok		1	
Replicate of ANN	Unknown	28.88			4000	500	3.58×10^5	3.35×10^5	Ok		1	
8.89×10^5	8.89×10^5	25.91	27.52	1.430	-	-	2.58×10^5	-	-	1.21×10^5	-	-
Replicate of 8.89×10^5	8.89×10^5	27.99			-	-	6.45×10^5	-	-		-	
Replicate of 8.89×10^5	8.89×10^5	28.65			-	-	4.17×10^5	-	-		-	

SRG: DNA samples from Sargasso Sea, USA; ANN: DNA samples from Annaya, Lebanon; Cq: Quantification cycle; SD: standard deviation; conc.: concentration; LOQ: limit of quantification = 8 gene copies/reaction; LOD: limit of detection = 1 gene copy/reaction; UD: undetermined if calculated conc. (Gene copies/ DNA extract) is undetermined or < LOD (Gene copies/DNA extract); DNQ: determined, not quantifiable if conc. (Gene copies/ DNA extract) < LOD (Gene copies/DNA extract); Ok: if conc. (Gene copies/ DNA extract) > LOQ (Gene copies/DNA extract).

CHAPTER FOUR

DISCUSSION

4.1 qPCR results

In this study, we have developed an *hcp*-based assay that allowed the enumeration of picocyanobacteria at three different stations on the Sargasso Sea, USA as well as from freshwater sampled from an artificial lake in Annaya, Lebanon by qPCR. As such, this is the first record for picocyanobacteria isolated from a local lake.

Cell numbers obtained at station 2 (BATS) (Sargasso Sea) are in accordance with flow cytometric cell counts performed by Ilikchyan et. al (2010) in 2008 on water sampled at the same station, during the same period (October) (in the range of 10^3 cells/mL); these numbers reflect the physical and chemical alterations of surface waters of the Sargasso Sea. Generally, in winter, surface layers become cold and enriched with nutrients due to deep mixing of water (more than 80 m), favoring the growth of synechoccoci which peak in April-May, shortly after water mixing, whereas through summer and fall, surface water becomes stratified, with a nutrient-deficient mixed shallow layer, favoring the growth of prochlorococci (Ilikchyan et al., 2010). Therefore, the results obtained account more for *Prochlorococcus* sp. than for *Synechococcus* sp. since water samples were collected in October, during the fall season.

As mentioned earlier, freshwater ecosystems are dominated by synechococci and therefore, cell numbers obtained at the Annaya station probably account more for *Synechococcus* sp. No records of cyanobacterial or picocyanobacterial cell counts from Lebanese freshwater are available.

Previously in our laboratory, El Andari (2010) quantified picocyanobacteria in the western arm of Lake Superior using qPCR based on specific DNA sequences of the 16S rRNA gene. Numbers obtained in samples collected from CD1or Castle Danger 1 station on September, 2004 (2.7×10^3 cells/mL) correlated with flow cytometric counts performed by Ivanikova et al. (2007) on PC-rich and PE-rich picocyanobacteia collected from CD1 (August, 2006). These results further support the advantage of using qPCR for quantifying picocyanobacteria; relatively to flow-cytometric techniques which, as mentioned previously, proved to offer outstanding enumeration statistics for picocyanobacteria, qPCR is providing comparable results in addition to being more time-effective. The latter is of particular concern in quantification experiments since cyanobacteria have a slow growth-rate (colonies appear after one month of incubation on selective BG-11 plates) and thus, methods requiring cultivation of these organisms, particularly flow cytometry, become time-consuming.

In the study performed by Sánchez-Baracaldo et. al (2008) to assess the abundance of picocyanobacteria from seven freshwater lakes, qPCR was used based on small subunit (ssu) rDNA sequences. The specificity of the primers used to target sequences was assessed by adding serial dilutions of non-target sequences. However, multiple primer sets were required to amplify all sequences of the ssu rDNA. This was not the case in our study where universal primers were designed to amplify *hcp* sequences from all isolated picocyanobacterial strains.

It was mentioned that picocyanobacteria are indicators of nutrient-deficient environments and thus, the high abundance of picocyanobacteria obtained from freshwater samples (Annaya) (in the range of 10^5 gene copies/ mL) is indicative of an oligotrophic ecosystem. This might be due to the fact that such environments (notably lakes) are not subject to anthropogenic disturbances of the system such like domestic and agricultural run-off which lead to nutrient enrichment.

4.2 Drawbacks of qPCR method used

Since *hcp* gene is highly conserved among all picocyanobacterial strains, the assay we have developed can be reliably reproduced to quantify picocyanobacteria in both marine and freshwater environments.

However, as previously mentioned, quantification by qPCR has still not been fully evaluated yet and methods for both experimental and statistical validation of results have still not been developed (Karlen et al., 2007). Moreover, another drawback of the qPCR assay performed in this study may be the method used for data analysis. As cited earlier, the "cycle-threshold" approach assumes that efficiency is the same between all PCR reactions; according to Guescini, Sisti, Rocchi, L. Stocchi and V. Stocchi (2008), a slight decrease in PCR efficiency (4%) could produce an error of up to 400%. He therefore compared the Ct method with another method, Cy_0; results showed that under optimal PCR conditions, both methods are comparable as regards precision and accuracy. However, upon a decrease in efficiency, Cy_0 method resulted in significantly more precise and accurate results.

4.3 Future work

Therefore, in future work, other data analysis methods such as the Cy_0 method can be used to increase the precision and the accuracy of quantification results. In addition, since monitoring picocyanobacterial loads has great environmental and industrial importance, quantification studies should be performed on the Lebanese littoral as well as on freshwater ecosystems across Lebanon.

CHAPTER FIVE

CONCLUSION

Owing to the remarkable conservation of sequences among marine and freshwater picocyanobacterial strains, we have designed an *hcp*-based assay that allowed the quantification of these bacteria in aquatic environments. This was achieved by the development of universal primers for *hcp* followed by the creation of a plasmid that harbors the gene. Absolute quantification of samples collected from the Sargasso Sea, USA and Annaya, Lebanon was performed by qPCR using an external standard curve based on serial dilutions of the plasmid. The design of our study was applied to quantify picocyanobacteria in both marine and freshwater ecosystems.

REFERENCES

AUB - 2012 -AUB joins Mediterranean consortium in EU-funded research study on producing biodiesel from algae. (n.d.). *American University of Beirut.* Retrieved from http://www.aub.edu.lb/news/2012/Pages/biodiesel-algae.aspx

Abboud-Abi Saab, M., Fakhri, M., Sadek, E., & Matar, N. (2008). An estimate of the environmental status of Lebanese littoral. *Lebanese Science Journal*, *9*(1), 43-60.

Abboud-Abi Saab, M., Fakhry, M., Kassab, M.T., & Mattar, N. (2004). Les sels nutritifs et le phytoplancton sur la côte sud du Liban: Evaluation d'indices ecologiques [Nutritive salts and phytoplankton populations on the south coast of Lebanon: Evaluation of ecological factors]. *Lebanese Science Journal*, *6*(2), 27-44.

Andersen, J.H. (2006). Coastal eutrophication: Recent developments in definitions and implications for monitoring strategies. *Journal of Plankton Research*, *28*(7), 621-628. Doi: 10.1093/plankt/fbl001

Andree, K.B., Fernández-Tejedor, M., Elandaloussi, L.M., Quijano-Scheggia, S., Sampedro, N., Garcés, E., … Diogène, J. (2011). Quantitative PCR Coupled with Melt Curve Analysis for Detection of Selected Pseudo-nitzschia spp. (Bacillariophyceae) from the Northwestern Mediterranean Sea. *Applied and Environmental Microbiology*, *77*(5), 1651-1659. Doi: 10.1128/AEM.01978-10

Angermayr, S.A., Hellingwerf, K.J., Lindblad, P., & de Mattos, M.J.T. (2009). Energy biotechnology with cyanobacteria. *Current Opinion in Biotechnology*, *20*(3), 257-263. Doi: 10.1016/j.copbio.2009.05.011

Bachmann, T. (2003). Transforming cyanobacteria into bioreporters of biological relevance. *Trends in Biotechnology*, *21*(6), 247-249.

Baoli, W., Cong-Qiang, L., Fushun, W., Yuanxiu, Y., & Yanyiu, W. (2009). Flow cytometric observation of picophytoplankton community structure in the cascade reservoirs along the Wujiang River, SW China. *Journal of Limnology*, *1*(68), 53-63.

Boyanapalli, R., Bullerjahn, G.S., Pohl, C., Croot, P.L., Boyd, P.W., & McKay, R.M.L. (2007). Luminescent whole-cell cyanobacterial bioreporter for measuring Fe availability in diverse marine environments. *Applied and Environmental Microbiology*, *73*(3), 1019-1024. Doi: 10.1128/AEM.01670-06

Brasier, M., Green, O., Lindsay, J., & Steele, A. (2004). Earth's oldest (approximately 3.5 Ga) fossils and the "Early Eden hypothesis": Questioning the evidence. *Origins of Life and Evolution of the Biosphere: The Journal of the International Society for the Study of the Origin of Life*, *34*(1-2), 257-269.

Bullerjahn, G.S., Boyanapalli, R., Rozmarynowycz, M.J., & McKay, R.M.L. (2010). Cyanobacterial bioreporters as sensors of nutrient availability. *Advances in Biochemical Engineering/Biotechnology*, *118*, 165-188. Doi: 10.1007/10_2009_23

Callieri, C. (2007). Picophytoplankton in freshwater ecosystems: The importance of small-sized phototrophs. *Freshwater Review*, *1*(1), 1-28.

Callieri, C., & Stockner, J. (2002). Freshwater autotrophic picoplankton: A review. *Journal of Limnology*, *61*(1), 1-14.

Chorus, I., & Bartram, J. (1999). *Toxic cyanobacteria in water: A guide to their public health consequences, monitoring, and management*. London, England: Taylor & Francis.

Douterelo, I., Perona, E., & Mateo, P. (2004). Use of cyanobacteria to assess water quality in running waters. *Environmental Pollution (Barking, Essex: 1987)*, *127*(3), 377-384.

Ducat, D.C., Way, J.C., & Silver, P.A. (2011). Engineering cyanobacteria to generate high-value products. *Trends in Biotechnology*, *29*(2), 95-103. Doi: 10.1016/j.tibtech.2010.12.003

El Andari, J. (2010). *Enumeration of Novel Lake Superior Cluster I (LSI) as a Fraction of the Total Picocyanobacteria in the Autotrophic Picoplankton (APP) using Quantitative Real-Time PCR* (Unpublished master's thesis). Lebanese American University, Lebanon.

Elliott, J.A., Jones, I.D., & Thackeray, S.J. (2006). Testing the sensitivity of phytoplankton communities to changes in water temperature and nutrient load, in a temperate lake. *Hydrobiologia*, *559*(1), 401-411. Doi: 10.1007/s10750-005-1233-y

Ernst, A., Deicher, M., Herman, P.M.J., & Wollenzien, U.I.A. (2005). Nitrate and phosphate affect cultivability of cyanobacteria from environments with low nutrient levels. *Applied and Environmental Microbiology*, *71*(6), 3379-3383. Doi: 10.1128/AEM.71.6.3379-3383.2005

Erokhina, L.G., Spirina, E.V., Shatilovich, A.V., & Gilichinskiĭ, D.A. (2000). Chromatic adaptation of ancient viable cyanobacteria from arctic permafrost. *Mikrobiologiia*, *69*(6), 855-856.

Flores, E., & Flores, F.G. (2008). *The cyanobacteria: Molecular biology, genomics, and evolution*. Norfolk, UK: Horizon Scientific Press.

Fu, J., Li, D., Xia, S., Song, H., Dong, Z., Chen, F., … Tang, Z. (2009). Absolute quantification of plasmid DNA by real-time PCR with genomic DNA as external standard and its application to a biodistribution study of an HIV DNA vaccine. *Analytical Sciences: The International Journal of the Japan Society for Analytical Chemistry*, *25*(5), 675-680.

Giovannoni, S.J., Turner, S., Olsen, G.J., Barns, S., Lane, D.J., & Pace, N.R. (1988). Evolutionary relationships among cyanobacteria and green chloroplasts. *Journal of Bacteriology*, *170*(8), 3584 -3592.

Guescini, M., Sisti, D., Rocchi, M.B.L., Stocchi, L., & Stocchi, V. (2008). A new real-time PCR method to overcome significant quantitative inaccuracy due to slight amplification inhibition. *BMC Bioinformatics*, *9*, 326. Doi: 10.1186/1471-2105-9-326.

Ilikchyan, I.N., McKay, R.M.L., Kutovaya, O.A., Condon, R., & Bullerjahn, G.S. (2010). Seasonal expression of the picocyanobacterial phosphonate transporter gene phnd in the Sargasso Sea. *Frontiers in Microbiology*, *1*, 135. Doi: 10.3389/fmicb.2010.00135

Ilikchyan, I.N., McKay, R.M.L., Zehr, J.P., Dyhrman, S.T., & Bullerjahn, G.S. (2009). Detection and expression of the phosphonate transporter gene phnD in marine and freshwater picocyanobacteria. *Environmental Microbiology*, *11*(5), 1314-1324. Doi: 10.1111/j.1462-2920.2009.01869.x

Ivanikova, N.V., Popels, L.C., McKay, R.M.L., & Bullerjahn, G.S. (2007). Lake superior supports novel clusters of cyanobacterial picoplankton. *Applied and Environmental Microbiology*, *73*(12), 4055-4065. Doi: 10.1128/AEM.00214-07

Johnson, J., Steglich, C., Church, G.M., Richardson, P., Chisholm, S.W., Kettler G.C., … Chisholm, S.W. (2007). Patterns and implications of gene gain and loss in the evolution of Prochlorococcus. *PLoS Genetics*, *3*(12), e231. DOI:10.1371/journal.pgen.0030231

Karlen, Y., McNair, A., Perseguers, S., Mazza, C., & Mermod, N. (2007). Statistical significance of quantitative PCR. *BMC Bioinformatics*, *8*, 131. Doi: 10.1186/1471-2105-8-131

Koskenniemi, K., Lyra, C., Rajaniemi-Wacklin, P., Jokela, J., & Sivonen, K. (2007). Quantitative real-time PCR detection of toxic Nodularia cyanobacteria in the Baltic Sea. *Applied and Environmental Microbiology*, *73*(7), 2173-2179. Doi: 10.1128/AEM.02746-06

Kutovaya, O.A., McKay, R.M.L., & Bullerjahn, G.S. (2010). Expression of hcp in freshwater Synechococcus spp., a gene encoding a hyperconserved protein in picocyanobacteria. *Journal of Basic Microbiology*, *50*(3), 227-231. Doi: 10.1002/jobm.200900337

Larionov, A., Krause, A., & Miller, W. (2005). A standard curve based method for relative real time PCR data processing. *BMC Bioinformatics*, *6*, 62. Doi: 10.1186/1471-2105-6-62

Leboulanger, C., Dorigo, U., Jacquet, S., Berre, B.L., Paolini, G., & Humbert, J. (2002). Application of a submersible spectrofluorometer for rapid monitoring of

freshwater cyanobacterial blooms: A case study. *Aquatic Microbial Ecology*, *30*(1), 83-89. Doi: 10.3354/ame030083

Malinen, E., Kassinen, A., Rinttilä, T., & Palva, A. (2003). Comparison of real-time PCR with SYBR Green I or 5'-nuclease assays and dot-blot hybridization with rDNA-targeted oligonucleotide probes in quantification of selected faecal bacteria. *Microbiology (Reading, England)*, *149*(Pt 1), 269-277.

Mbeunkui, F., Richaud, C., Etienne, A.-L., Schmid, R.D., & Bachmann, T.T. (2002). Bioavailable nitrate detection in water by an immobilized luminescent cyanobacterial reporter strain. *Applied Microbiology and Biotechnology*, *60*(3), 306-312. Doi: 10.1007/s00253-002-1139-9

McKinlay, J.B., & Harwood, C.S. (2010). Photobiological production of hydrogen gas as a biofuel. *Current Opinion in Biotechnology*, *21*(3), 244-251. Doi: 10.1016/j.copbio.2010.02.012

Mella-Flores, D., Mazard, S., Humily, F., Partensky, F., Mahé, F., Bariat, L., … Garczarek, L. (2011). Is the distribution of Prochlorococcus and Synechococcus ecotypes in the Mediterranean Sea affected by global warming? *Biogeosciences Discussions*, *8*(3), 4281-4330. Doi: 10.5194/bgd-8-4281-2011

Noordewier, M., Rappe, M. S., Short, J. M., Carrington, J. C., Mathur, E. J., Giovannoni, S.J., … Tripp, J. (2005). Genome streamlining in a cosmopolitan oceanic bacterium. *Science*, *309*(5738), 1242-1245. Doi: 10.1126/science.1114057

Oren, A. (2004). A proposal for further integration of the cyanobacteria under the bacteriological code. *International Journal of Systematic and Evolutionary Microbiology*, *54*(5), 1895 -1902. Doi: 10.1099/ijs.0.03008-0

Paerl, H.W., Fulton, R.S., Moisander, P.H., & Dyble, J. (2001). Harmful freshwater algal blooms, with an emphasis on cyanobacteria. *The Scientific World Journal*, *1*, 76-113. Doi: 10.1100/tsw.2001.16

Peirson, S.N., Butler, J.N., & Foster, R.G. (2003). Experimental validation of novel and conventional approaches to quantitative real-time PCR data analysis. *Nucleic Acids Research*, *31*(14), e73. Doi: 10.1093/nar/gng073

Quintana, N., Van der Kooy, F., Van de Rhee, M.D., Voshol, G.P., & Verpoorte, R. (2011). Renewable energy from Cyanobacteria: Energy production optimization by metabolic pathway engineering. *Applied Microbiology and Biotechnology*, *91*(3), 471-490. Doi: 10.1007/s00253-011-3394-0

Ririe, K.M., Rasmussen, R.P., & Wittwer, C.T. (1997). Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. *Analytical Biochemistry*, *245*(2), 154-160. Doi: 10.1006/abio.1996.9916

Rutledge, R.G., & Côté, C. (2003). Mathematics of quantitative kinetic PCR and the application of standard curves. *Nucleic Acids Research*, *31*(16), e93.

Scanlan D.J., & West, N.J. (2002). Molecular ecology of the marine cyanobacterial genera Prochlorococcus and Synechococcus. *FEMS Microbiology Ecology*, *40*(1), 1-12. Doi: 10.1111/j.1574-6941.2002.tb00930.x

Scanlan D.J., Ostrowski, M., Mazard, S., Dufresne, A., Garczarek, L., Hess, W. R., … Partensky, F. (2009). Ecological genomics of marine picocyanobacteria. *Microbiology and Molecular Biology Reviews: MMBR*, *73*(2), 249-299. Doi: 10.1128/MMBR.00035-08

Schreiter, P.P., Gillor, O., Post, A., Belkin, S., Schmid, R.D., & Bachmann, T.T. (2001). Monitoring of phosphorus bioavailability in water by an immobilized luminescent cyanobacterial reporter strain. *Biosensors & Bioelectronics*, *16*(9-12), 811-818.

Shao, C. Y., Howe, C.J., Porter, A.J.R., & Glover, L.A. (2002). Novel cyanobacterial biosensor for detection of herbicides. *Applied and Environmental Microbiology*, *68*(10), 5026-5033. Doi: 10.1128/AEM.68.10.5026-5033.2002

Stal, L.J., & Krumbein, W.E. (1987). Temporal separation of nitrogen fixation and photosynthesis in the filamentous, non-heterocystous cyanobacterium Oscillatoria sp. *Archives of Microbiology*, *149*(1), 76-80. Doi: 10.1007/BF00423140

Stanier, R.Y., Kunisawa, R., Mandel, M., & Cohen-Bazire, G. (1971). Purification and properties of unicellular blue-green algae (order Chroococcales). *Bacteriological Reviews*, *35*(2), 171-205.

Steinberg, C.E.W., & Hartmann, H.M. (1988). Planktonic bloom-forming Cyanobacteria and the eutrophication of lakes and rivers. *Freshwater Biology*, *20*(2), 279-287. Doi: 10.1111/j.1365-2427.1988.tb00452.x

Strömbom, L., Ståhlberg, A., Zoric, N., Kubista, M., Andrade, J. M., Bengtsson, M., … Sjögreen, B. (2006). The real-time polymerase chain reaction. *Molecular Aspects of Medicine, 27*(2-3), 95-125. Doi: 10.1016/j.mam.2005.12.007

Sánchez-Baracaldo, P., Handley, B.A., & Hayes, P.K. (2008). Picocyanobacterial community structure of freshwater lakes and the Baltic Sea revealed by

phylogenetic analyses and clade-specific quantitative PCR. *Microbiology (Reading, England)*, *154*(Pt 11), 3347-3357. Doi: 10.1099/mic.0.2008/019836-0

Tomitani, A., Knoll, A. H., Cavanaugh, C.M., & Ohno, T. (2006). The evolutionary diversification of cyanobacteria: Molecular–phylogenetic and paleontological perspectives. *Proceedings of the National Academy of Sciences*, *103*(14), 5442 - 5447. Doi: 10.1073/pnas.0600999103

Tse, C., & Capeau, J. (2003). Real time PCR methodology for quantification of nucleic acids. *Annales De Biologie Clinique*, *61*(3), 279-293.

Venter, J.C., Remington, K., Heidelberg, J.F., Halpern, A.L., Rusch, D., Eisen, J.A., … Smith, H.O. (2004). Environmental genome shotgun sequencing of the Sargasso Sea. *Science (New York, N.Y.)*, *304*(5667), 66-74. Doi: 10.1126/science.1093857

Vives-Rego, J., Lebaron, P., & Nebe-von Caron, G. (2000). Current and future applications of flow cytometry in aquatic microbiology. *FEMS Microbiology Reviews*, *24*(4), 429-448.

Walsby, A.E., Hayes, P.K., & Boje, R. (1995). The gas vesicles, buoyancy and vertical distribution of cyanobacteria in the Baltic Sea. *European Journal of Phycology*, *30*(2), 87-94. Doi: 10.1080/09670269500650851

Wang, Y., Hammes, F., De Roy, K., Verstraete, W., & Boon, N. (2010). Past, present and future applications of flow cytometry in aquatic microbiology. *Trends in Biotechnology*, *28*(8), 416-424. Doi: 10.1016/j.tibtech.2010.04.006

Wilhelm, S.W., Bullerjahn, G.S., Eldridge, M.L., Rinta-Kanto, J.M., Poorvin, L., & Bourbonniere, R.A. (2006). Seasonal hypoxia and the genetic diversity of prokaryote populations in the central basin hypolimnion of lake Erie: Evidence

for abundant cyanobacteria and photosynthesis. *Journal of Great Lakes Research*, *32*(4), 657-671. Doi: 10.3394/0380-1330(2006)32[657:SHATGD]2.0.CO;2

Yuan, J.S., Reed, A., Chen, F., & Stewart, C.N. (2006). Statistical analysis of real-time PCR data. *BMC Bioinformatics*, *7*, 85. Doi: 10.1186/1471-2105-7-85

Zhaxybayeva, O., Gogarten, J.P., & Doolittle, W.F. (2007). A hyperconserved protein in Prochlorococcus and marine Synechococcus. *FEMS Microbiology Letters*, *274*(1), 30-34. Doi: 10.1111/j.1574-6968.2007.00806.x

van der Velden, V.H.J., Hochhaus, A., Cazzaniga, G., Szczepanski, T., Gabert, J., & van Dongen, J.J.M. (2003). Detection of minimal residual disease in hematologic malignancies by real-time quantitative PCR: Principles, approaches, and laboratory aspects. *Leukemia: Official Journal of the Leukemia Society of America, Leukemia Research Fund, U.K*, *17*(6), 1013-1034. Doi: 10.1038/sj.leu.2402922