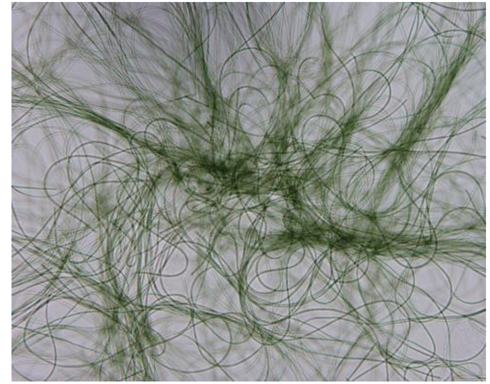

Accessing *Planktothrix* species diversity and associated toxins using quantitative real-time PCR in natural waters

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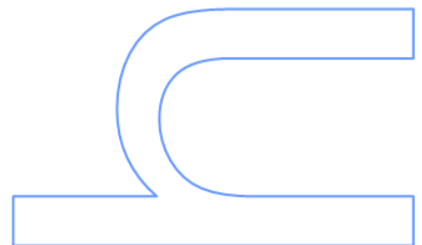

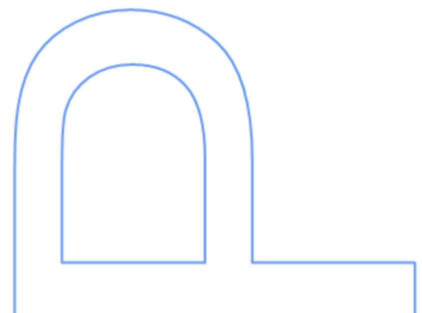

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RESUMO

Em Portugal, as cianotoxinas mais frequentes em águas doces são as microcistinas e a sua ocorrência tem sido sobretudo associada a cianobactérias do género *Microcystis*. No entanto, e mais recentemente, tem vindo a ser descrita a produção destas toxinas por espécies do género *Planktothrix*, sugerindo que este género é, também, um produtor importante de microcistinas nas águas doces superficiais portuguesas. Porém, e contrariamente às espécies de *Microcystis*, o conhecimento acerca da ocorrência, distribuição e toxigénese de *Planktothrix* é ainda limitado.
As espécies de *Planktothrix* apresentam algumas particularidades que dificultam a sua amostragem e identificação/quantificação em amostras naturais - **Capítulo 2**. Em particular, a morfologia das colónias de *Planktothrix* não permite distinguir facilmente as células individualizadas, o que torna a sua identificação e quantificação particularmente difíceis por microscopia óptica, o método geralmente usado na monitorização de cianobactérias no ambiente. Por outro lado, os métodos usados na detecção de microcistinas (ELISA, HPLC) não identificam as espécies produtoras. Estas estirpes podem ser identificadas por PCR convencional, mas este método não permite quantificar a sua densidade. Em suma, não está ainda disponível um método que, simultaneamente, identifique e quantifique as estirpes produtoras de microcistinas. Neste trabalho pretendeu-se desenvolver um método de PCR em tempo real aplicado à monitorização de espécies tóxicas de *Planktothrix* em reservatórios de água doce superficial destinados ao consumo humano e actividades recreativas.
O trabalho experimental desenvolveu-se de acordo com as fases que seguidamente se enumeram.
Numa primeira fase realizaram-se estudos de campo de forma a avaliar a ocorrência e distribuição de *Planktothrix* em diversas albufeiras - **Capítulo 3**. Concluiu-se que o *Planktothrix* tem uma distribuição abrangente e que a espécie *Planktothrix agardhii* é a mais comum. Foi identificada a produção de microcistinas em isolados desta espécie.
Numa segunda fase, foi desenvolvido um método de detecção e quantificação de *Planktothrix agardhii*, baseado na metodologia de PCR em tempo real - **Capítulo 4**. A metodologia de PCR em tempo real é bastante promissora para a investigação em cianobactérias, bem como para a sua monitorização no ambiente. A principal vantagem desta técnica, relativamente ao PCR convencional, é a possibilidade de quantificar o número de cópias do gene-alvo numa amostra. Assim, para além de identificar estirpes de *Planktothrix*, o PCR em tempo real é simultaneamente um método de quantificação,

o que constitui uma vantagem relativamente aos procedimentos de rotina utilizados na monitorização de cianobactérias. De salientar que a determinação da densidade celular é fundamental na avaliação de risco de ocorrência de cianobactérias tóxicas, visto que os valores guia para as cianotoxinas se baseiam nas concentrações cianobacterianas e na quota de cianotoxina por célula.

Outro aspecto importante na monitorização de cianobactérias é a utilização de amostras preservadas. A preservação é utilizada para manter as características morfológicas para posterior identificação e quantificação celulares e, também, para evitar a degradação do material biológico durante o transporte da amostra. Neste trabalho foi também avaliada a aplicabilidade do método de PCR em tempo real previamente desenvolvido, na amplificação de ADN de amostras preservadas - **Capítulo 5**. Os resultados indicam que o PCR em tempo real é uma técnica robusta e aplicável àquele tipo de amostras mas que os métodos mais comuns de preservação (Solução de Lugol, Formaldeído e Gluteraldeído) reduzem a quantidade/qualidade/disponibilidade de ADN. Como o ADN se degrada rapidamente após fixação das amostras, foi avaliada a aplicação do PCR em tempo real a amostras preservadas por outro método de preservação - **Capítulo 6**. A preservação em metanol 100% a -20ºC permitiu manter a integridade das amostras quer para análises morfológicas quer para análises moleculares até dois anos de preservação.

O último capítulo desta tese reporta o resultado de dois anos de monitorização de uma albufeira que apresenta uma florescência persistente de *P. agardhii* tóxico (**Capítulo 2**, **Anexo A**: Pág. 187) – **Capítulo 7**. Nesta albufeira, densidades celulares elevadas nem sempre correspondiam a quantidades elevadas de toxina e vice-versa. Utilizando a técnica de PCR em tempo real foi possível verificar que estão presentes estirpes tóxicas e não tóxicas e que podem florescer em alturas diferentes. Foi também detectado uma segunda espécie não pertencente ao género *Planktothrix* que poderá estar também a contribuir para a concentração de microcistinas. Durante a monitorização foi observado um parasita quitrídeo que infectava os filamentos de *Planktothrix* (**Anexo A**: Pág. 191). A densidade deste parasita foi também quantificada através de PCR em tempo real e verificou-se que o seu aparecimento e desenvolvimento coincidia com o aumento de estirpes tóxicas e concentração de microcistinas.

Palavras-chave

Cianobactérias, florescências, qPCR em tempo real, quitrídeos, *mcy*A, microcistinas, monitorização, *Planktothrix*, preservação, *Rhizophydium megarrhizum*, *rpo*C1.

ABSTRACT

The most common cyanotoxins in Portuguese freshwaters are microcystins and their occurrence has been mainly attributed to cyanobacteria from the *Microcystis* genus. However, most recently, it has been described the production of these toxins by species of *Planktothrix*, suggesting that this genus is also a major producer of microcystin in Portuguese surface waters. Nevertheless, and conversely to the *Microcystis* species, the knowledge on the occurrence, distribution and toxigenesis of *Planktothrix* is still limited.
Planktothrix species exhibits some particularities that difficult their sampling, identification, quantification and toxigenic characterization in natural samples - **Chapter 2**. Particularly, the morphology of *Planktothrix* colonies does not allow distinguishing easily the individual cells. This makes their identification and quantification by optical microscopy a very difficult task, although this is the method generally used in cyanobacteria monitoring. Moreover, the most common methods for the detection of microcystins (ELISA, HPLC) do not identify the producer strains. These strains can be identified by conventional PCR, but this method doesn't enable to quantify them. In resume, there is not yet available a method that allow simultaneously identify and quantify microcystin-producing strains.
This work aimed to develop a method based on Real-Time PCR applied to the monitoring of toxic species of *Planktothrix* in surface freshwater reservoirs used as drinking water supply and for recreational activities.
The experimental work was developed according to several phases that are listed and explained below.
In a first approach, field surveys were conducted to access the occurrence and distribution of *Planktothrix* – **Chapter 3**. It was observed that *Planktothrix* has a wide distribution in Portuguese lakes and that *Planktothrix agardhii* is the most commonly found species. Furthermore microcystin production was detected in isolates from this species.
In a second stage, it was developed a method based on real-time PCR to detect and quantify *Planktothrix agardhii* - **Chapter 4**. The real-time PCR is a promising technique for cyanobacteria research and monitoring. The main advantage of real-time PCR over conventional PCR is the ability to quantify the target gene copy numbers on a sample. Thus, in addition to identifying *Planktothrix* strains, the real-time PCR also enables to quantify those strains, which constitutes an advantage over the procedures used in the routine monitoring of cyanobacteria. It should be noted that the determination of the cell

density is critical in the risk assessment of toxic cyanobacteria, as the guideline values for cyanotoxins are based on cyanobacterial concentrations as well as on toxin cell quota.

Another important aspect in cyanobacteria monitoring is the use of preserved samples. Preservation is used to maintain the morphologic features of cyanobacterial cells to further be used in their identification/quantification and also to avoid sample degradation during transport. In this work, the applicability of the real-time PCR technique in the amplification of DNA from preserved samples was evaluated, by using the method previously developed for cell quantification – **Chapter 5**. The results indicate that real-time PCR is a robust technique applicable to those types of samples but that the most common preservation methods (Lugol's solution, formaldehyde, glutaraldehyde) reduce the DNA quantity and quality. Since DNA degrades fast in those samples, the applicability of real-time PCR on preserved samples was tested using other preservation procedure – **Chapter 6**. The preservation in methanol 100% at -20ºC allowed maintaining the integrity of the samples both for morphologic and molecular analysis up to two years after preservation.

The last chapter of this thesis reports the result of two years of monitoring a water reservoir having a persistent bloom of toxic *P. agardhii* (**Chapter 2**, **Appendix A**: Pág. 187) - **Chapter 7**. In this reservoir, high cell densities did not always correspond to high amounts of toxin and *vice versa*. Using the real-time PCR it was demonstrated that both toxic and non-toxic strains are present within the reservoir and that they can flourish at different times. It was also detected a specie from another genus that also contributes to the production of microcystins. During the monitoring it was observed a chytrid parasite that infected the filaments of *Planktothrix* (**Appendix A**: Pág. 191). The density of this parasite was also quantified by real-time PCR and the results showed that its development coincides with the increase of toxic *Planktotrhix* strains and of microcystin levels in the reservoir.

Key-Words

Cyanobacteria, blooms, chytrids, *mcy*A, microcystins, monitoring, *Planktothrix*, preservation, Real-time qPCR, *Rhizophydium megarrhizum, rpo*C1.

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ABREVIATIONS LIST

Ct – Threshold Cycle

CCALA – Acronym of cyanobacteria cultures from Culture Collection of Autotrophic Organisms

CREM - Centro de Recursos Microbiológicos

CIIMAR – Centro Interdisciplinar de Investigação Marinha e Ambiental. Interdisciplinary Centre of Marine and Environmental Research

DNA – Deoxyribonucleic acid

EDTA - Ethylenediamine tetraacetic acid

ELISA – Enzyme-linked immunosorbent assay

EN - European Standard

FCT – Fundação para a Ciência e Tecnologia/Foundation for Science and Technology.

GV - Guideline Values

HPLC-DAD – High Performance Liquid Chromatography with Diode Array Detector

ISO – International Organization for Standardization

LBE - Laboratory of Biology and Ecotoxicology

LC-MS/MS – Liquid Chromatography–Mass Spectrometry

LD50% - Lethal Dose, the dose needed to kill 50%.

LEGE – Laboratório Ecotoxicologia Genomica e Evolução. Acronym of cyanobacteria cultures from Cianoteca LEGE culture collection

LM - Light Microscopy

LMECYA – Laboratório Microbiologia Experimental Cyanobacteria. Acronym of cyanobacteria cultures from the Estela Sousa e Silva algal culture collection

LOAEL – Lowest Observed Adverse Effect Level

LPS – Lipopolysaccharide

MC – Microcystin

MCMC – Markov Chain Monte Carlo

NIH – National Institute of Health

NOAEL – No Observed Adverse Effect Level

NRPS – Non-Ribosomal Peptide Synthesis, involving peptide synthetases

NRPS/PKS – Non-Ribosomal Peptide Synthesis, involving peptide synthetases and polyketide synthases

NTC – No Template Control

OD – Optical Density

PCR – Polymerase chain reaction

PVPP - Polyvinypyrrolidone

Qpcr – Quantitative PCR

RBA – Receptor Binding Assays

RNA – Ribonucleic Acid

RT-qPCR – Reverse transcription quantitative polymerase chain reaction

RuBisCO – ribulose 1,5-bisphosphate carboxylase-oxygenase

SDS - sodium dodecyl sulfate

SEM – Scnaing Electron Microscopy

STX - Saxitoxin

TBE - Tris-borate EDTA

TNA - Taq Nuclease Assay

TDI - Tolerable Daily Intake

TE – Tris EDTA

Tris – (hydroxymethyl)aminomethane

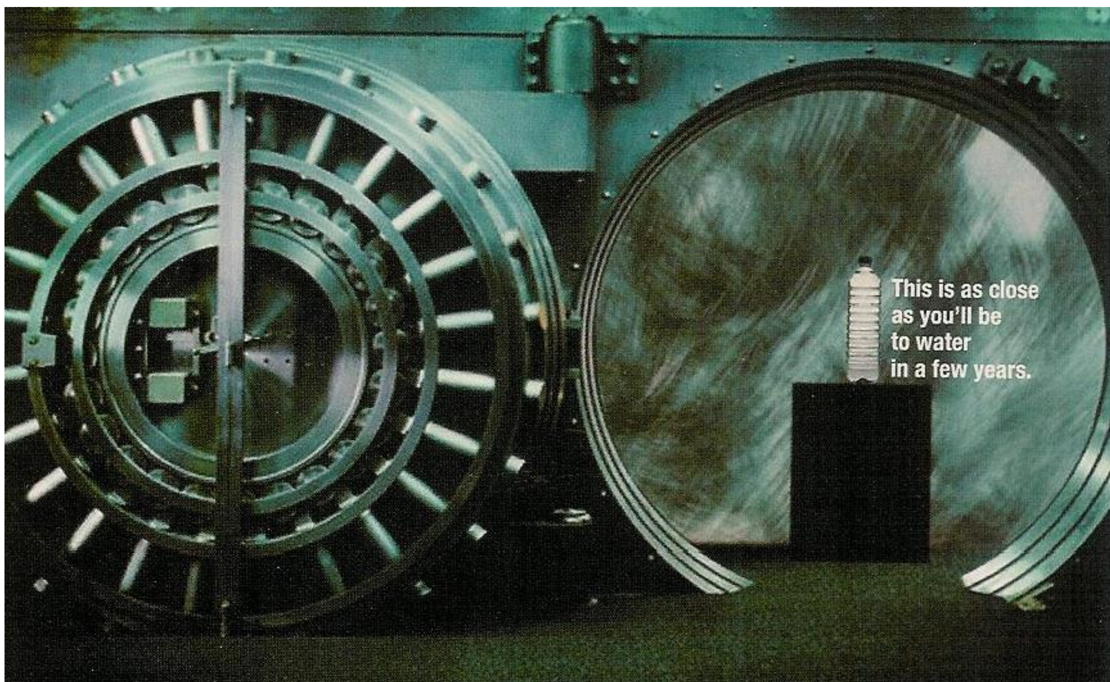
WHO – World Health Organization

WWTP – Waste Water Treatment Plant

WATER

A transparent and odorless liquid formed simply by two atoms of hydrogen and one of oxygen is essential to sustain life. The increased thirst for water influenced human migration since the beginning of times and shaped human distribution and agglomeration. Water necessity as changed landscapes – see the case of the Aral Sea a 68,000 km^2 that dried in 20 years. From the water that the earth contains approximately 96% is on the oceans and only 3% is fresh water[1a]. From these 3% about two-thirds is in glaciers and permanent ice cover[1a]. Believed to fall from the sky, water was taken for granted and one could not forecast the problems related with the need for water. Approximately 2300 people die per day from water related diseases; one child every minute and one in nine people lack access to safe water[1b]. With the increasing human population, anthropogenic and climate changes the water is becoming scarce and is no longer a common good.

"All people, whatever their stage of development and their social and economic conditions, have the right to have access to an adequate supply of safe drinking water."[1c]



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[1] Poster: Awareness campaign "Stop Global Warming", Postal Free, Young & Rubicam Redcell, Corbis, PubliCards, ACT.

[a] http://water.usgs.gov/edu/earthwherewater.html [b] http://water.org [c] World Health Organization, Cyanobacterial toxins: Microcystin-LR in Drinking-water, Guidelines for drinking-water quality 2nd ed. Health criteria and other supporting information, Geneva, 1998.

"Water is the driving force of all nature"

Leonardo da Vinci

"No water, no life. No blue, no green"

Sylvia Earle

"Ocean, a body of water occupying about two-thirds of a world made for man — who has no gills."

Ambrose Bierce

"Thousands have lived without love, not one without water"

W. H. Auden

"We never know the worth of water till the well is dry."

Thomas Fuller

"It is life, I think, to watch the water.

A man can learn so many things."

Nicholas Sparks

"We forget that the water cycle and the life cycle are one."

Jacques Cousteau

Chapter

01_

INTRODUCTION

The Organism_

Cyanobacterial Blooms

Cyanobacteria are an integral part of phytoplankton and primary production in aquatic systems. They are ubiquitous, well-adapted, microscopic oxygenic prokaryotic phototrophs. The adaptation ability that cyanobacteria possess dictates their success in the water column. These organisms require low light intensities and exhibit optimal growth rates at relatively high temperatures in comparison with other microalgae (Reynolds 1984, Paerl et al. 2011, Paerl & Paul 2012). They also have a higher ability to uptake nutrients, phosphorous and nitrogen, than for many other photosynthetic organisms (Paerl et al. 2011, Paerl & Paul 2012). They possess storage capacity for phosphorous and are capable of nitrogen fixation. This means that they can compete with other phytoplankton under conditions of nutrient limitation (Mur et al. 1999, Briand et al. 2003, Sunda et al. 2006). Their success is also due to the presence of gas vacuoles that provide buoyancy regulation in stratified waters. They can migrate in the water column and regulate their vertical position, accessing light in the surface layers and nutrients near the sediment (Mur et al. 1999, Paerl et al. 2011, Paerl & Paul 2012). They uptake CO_2 efficiently and have high tolerance to UV radiation (Paerl & Paul 2012). Furthermore cyanobacteria also produce active substances that inhibit the growth algae competitors and grazers (Mur et al. 1999, Briand et al. 2003, Sunda et al. 2006, Apeldoorn et al. 2007). This robustness of cyanobacteria allows them to develop, dominate and persist in freshwater lakes. The development of cyanobacteria is, most of the times, excessive resulting in nuisance blooms. Cyanobacterial blooms have a negative impact in water ecosystems: i) they accumulate in the surface and restrict light penetration in the water, which can impair the growth of other phytoplankton, benthic microalgae and aquatic macrophytes: ii) they induce oxygen depletion and clogging of the fish gills, causing massive fish killing; iii) they produce secondary metabolites that are toxic for other organisms. In sum, cyanobacteria blooms decreases aquatic biodiversity and can disrupt the entire water ecosystem.

From a public health perspective, cyanobacteria are a health risk because they produce secondary metabolites that are harmful for humans that can be exposed

through drinking/recreational waters and through the food web due to toxin accumulation in aquatic animals.

Cyanobacterial Toxins

"A thick scum like green oil paint, some two to six inches thick unwholesome for cattle and other animals to drink at the surface, bringing on a rapid and sometimes terrible death" from Francis (1878) in Chorus & Salas (1997).

Cyanobacteria produce multiple toxic compounds that are grouped according to their primary target organ - hepatotoxins, neurotoxins, cytotoxins and lipopolysaccharide endotoxins. Detailed information about cyanobacteria toxins, such as, the types of toxins, the producer species, the effects and lethal doses can be found in Chapter 2. Microcystin is one of the most common cyanotoxins found in freshwaters. It is a hepatotoxin and it has over 100 variants that differ in structure and toxicity (Tonk et al. 2005, Welker & Döhren 2006, del Campo & Quahid 2010, Bortoli & Volmer 2014; Qi et al. 2015). Microcystins are produced by several bloom forming cyanobacteria such as: *Microcystis*, *Planktothrix*, *Anabaena, Hapalosiphon, Phormidium* and *Nostoc* (Haider et al. 2003, Oksanen et al. 2004, Codd et al. 2005, Quiblier et al. 2013). The most severe case of human intoxication by microcystins took place in Caruaru, Brazil, which resulted in 60 deaths in patients from a hemodialysis unit (Pouria et al. 1998, Jochimsen et al. 1998). In China (Ueno et al. 1996) and recently in Serbia (Damjana et al. 2011) the high incidence of primary liver cancer has also been attributed to microcystins-contaminated drinking water. More recently, Ohio, in USA, was declared a state of emergency as a result of elevated levels of microcystins in tap water (Jetoo et al. 2015). An advisory “do not drink” was imposed for 3 days, to the population, which became known as the “Toledo water crisis” (Jetoo et al. 2015).

Therefore, cyanobacterial blooms and their toxins represent a serious threat to human health and to the quality and sustainability of freshwater resources through contamination of water supplies.

Cyanobacteria in Portugal

Cyanobacterial blooms are frequent worldwide and Portuguese inland waters are no exception, with toxic blooms being a recurrent phenomenon (Vasconcelos 1999) (Fig.1). Blooms have been reported throughout the country (Vasconcelos 1999, Caetano et al. 2001, Saker et al. 2004, Pereira et al. 2004, Teles et al. 2006, Galvão et al. 2008, Figueiredo et al. 2010, Vasconcelos et al. 2011, Bellém et al. 2013). The

species accounting for most of the blooms and toxic events in Portugal are *Dolichospermum* (former *Anabaena*), *Aphanizomenon*, *Cylindrospermopsis*, *Microcystis* and *Planktothrix* (Saker et al. 2004, Figueiredo et al. 2006, Pereira et al. 2004, Vasconcelos 1999, Churro et al. 2012 – Chapter 2). Furthermore, most cyanobacterial toxins have also been detected in those blooms or in cultured strains isolated from natural samples, such as microcystins (Vasconcelos et al. 1995; 1996), saxitoxins (Pereira et al. 2000; 2004, Ferreira et al. 2001) and anatoxin (Osswald et al. 2009).

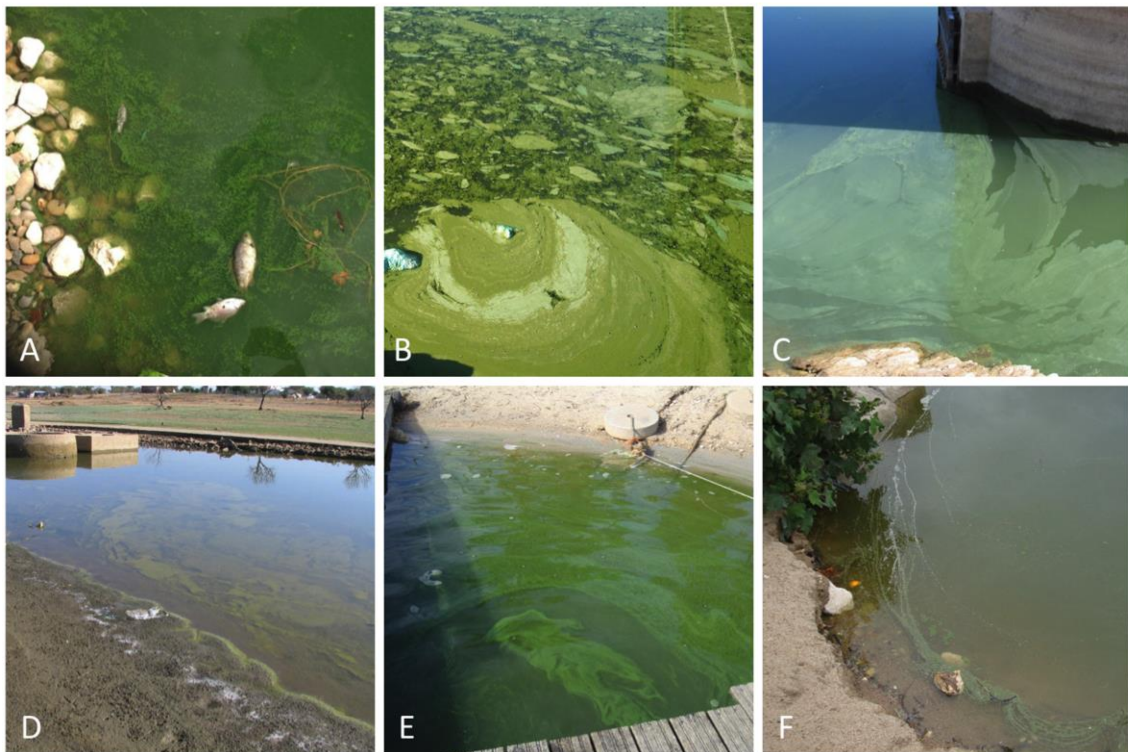

Fig. 1 - Cyanobacteria blooms in Portuguese lakes. A – Fish mortality in Patudos lake undergoing a cyanobacterial bloom (source: Publico newspaper by Marisa Soares, September 9th, 2014); B – *Aphanizomenon* bloom in central Portugal, May 2015; C – *Planktothrix* bloom in Roxo Lake, September 2005; D – *Aphanizomenon* and *Microcystis* bloom in Alvito Lake, September 2005; E – *Microcystis* bloom in Magos Lake, October 2012; F – *Planktothrix* bloom in Patudos Lake, October 2012.

Planktothrix

Planktothrix is one of the most widespread bloom forming cyanobacteria genera in Europe (Fig. 2). From the fourteen species described, four - *Planktothrix agardhii*, *P. rubescens*, *P. prolifica* and *P. suspensa* - have been reported to cause toxic blooms. *P. agardhii* and *P. rubescens* are the most commonly reported species and are both microcystin procucers (Tonk et al. 2005, Keil et al. 2002).

P. agardhii is frequently found in shallow eutrophic to hypereutrophic lakes all across middle Europe. In France is one of the most frequently found species (Catherine et al. 2008) and was also found as a dominant species in 40% of Czech fishponds (Poulícková et al. 2004). Toxic blooms are also common in Poland, Hungary and Germany (Budzyńska et al. 2009, Pawlik-Skowroµska et al. 2008, Farkas et al. 2014). This cyanobacterium tolerates a wide range of temperatures and light intensities so it can prevail all year around. It is generally mixed in the water but it can also form metalimnetic blooms, survive under ice-covered lakes and tolerate shade from other phytoplankton under eutrophic conditions (Rücker et al. 1997, Hašler and Poulícková 2003, Halstvedt et al. 2007, Pawlik-Skowroµska et al. 2008, Bonilla et al. 2012).

P. rubescens is found in deep meso to oligotrophic lakes, all across the north and midle European lakes especially in alpine Lakes (Legnani et al. 2005, Halstvedt et al. 2007, Bogialli et al. 2012). This species has the ability to grow at low irradiances, tolerates lower temperatures and have stronger gas vacuoles when compared to other cyanobacteria, so this species usually forms perennial and metalimnetic blooms (Walsby et al. 1998, Walsby and Schanz 2002, Akçaalan et al. 2014). However, *P. rubescens* blooms can also occur in eutrophic lakes since concentration of phytoplankton in the upper water layer increases the light attenuation (Walsby and Schanz 2002). Furthermore, a shallow lake dominated by *P. rubescens* was reported in Hungary (Vasas et al. 2013).

P. agardhii and *P. rubescens* occupy different ecological niches and experiments have showed that some strains segregate each other (Oberhaus et al. 2008). Nevertheless, although rare, there are some reports on the co-occurrence of these species (Davis and Walsby 2002, Halstvedt et al. 2007, Oberhaus et al. 2008). In Lake Steinsfjorden, southeastern Norway *P. rubescens* and *P. agardhii*, had similar depth distributions and both formed metalimnetic blooms (Halstvedt et al. 2007). In addition, Fastner et al. (1999) reported that *Planktothrix* blooms produce the highest level of toxins per biomass when compared to *Microcystis* and *Anabaena* and that *P. rubescens* is more toxic then *P. agardhii* (Fastner et al., 1999, Akçaalan et al. 2006).

In Portugal, *P. agardhii* and *P. rubescens* have been pointed out to cause blooms associated with microcystin production (Churro et al. 2012 – Chapter 2, Paulino et al. 2009). However little is known about the occurrence, distribution and toxin production of these harmful bloom-forming cyanbacteria in southwest European lakes and in particular, in Portugal (Fig. 2).

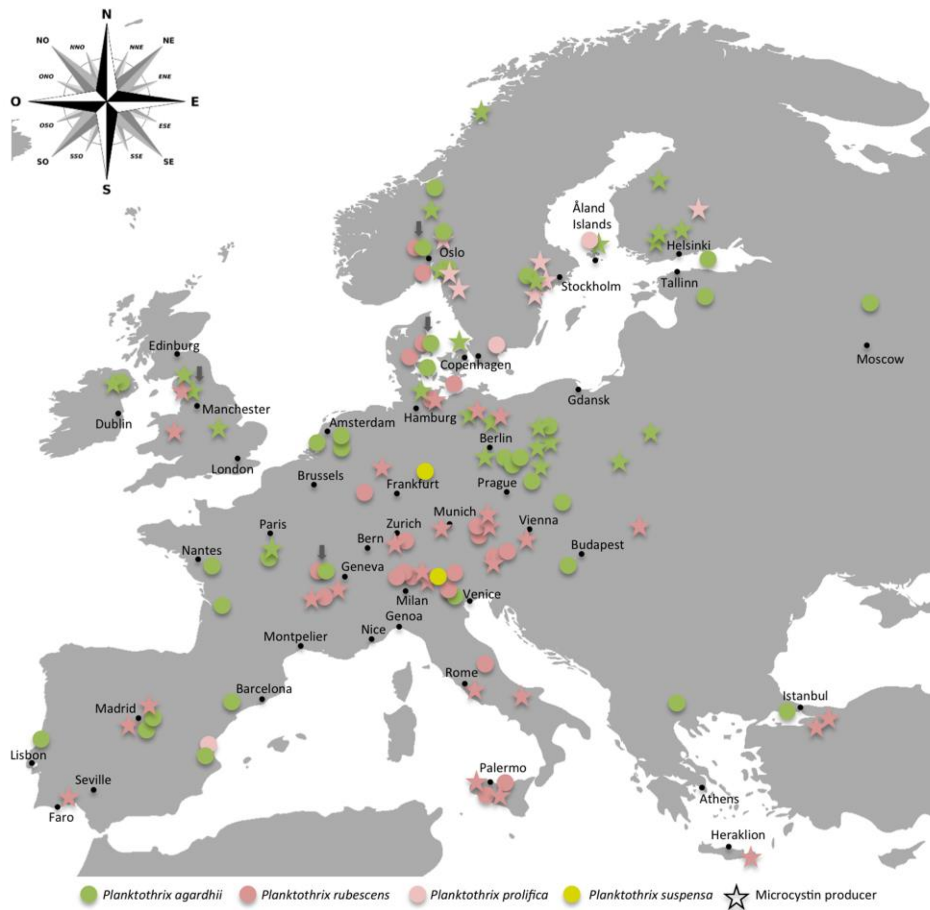

Fig. 2 - Occurrence and distribution of *Planktothrix* in European Lakes. The arrow indicates the co-occurrence of *P. agardhii* and *P. rubescens*. (Rojo and Cobelas 1994; Montealegre et al. 1995; Rücker et al. 1997; Rudi et al 1998; Lindholm et al. 1999; Bettinetti et al. 2000; Salmaso 2000; Bright & Walsby 2000; Davis & Walsby 2002; Buzzi 2002, Morabito et al. 2002, Viaggiu et al. 2003,Davis et al. 2003; PoulíČková et al. 2004; Almodóvar et al. 2004; Barco et al. 2004; Kurmayer et al. 2004, Hoyos et al 2004; Viaggiu et al. 2004; Briand et al. 2005; Nõges & Kangro 2005; Kangro et al. 2005, Briand et al. 2005, Jacquet et al. 2005, Rudi et al. 2005, Mbedi et al 2005; Legnani et al. 2005; Stefaniak et al. 2005, Messineo et al. 2006; Akcaalan et al. 2006; Kurmayer & Gumpenberger 2006; Yéprémian et al. 2007, Halstved et al. 2007; Honti et al. 2007; Naselli-Flores et al. 2007; Catherine et al. 2008; Baumann and Juttner 2008; Vareli et al. 2009; Ernst et al. 2009; Budzyńska et al. 2009; Paulino et al. 2009; Manganelli et al. 2010; Salmaso 2010; Kormas et al. 2011; Al-Tebrineh et al. 2011; D'Alelio et al. 2011; D'Alelio and Salmaso 2011; Mankiewicz-Boczek et al. 2011; Bogialli et al. 2012; Messyasz et al. 2012; Vasas et al. 2013; Akçaalan et al. 2014; Grabowska and Mazur-Marzec 2014; Larson et al. 2014; Kurmayer et al. 2015, NIVA Culture Collection of Algae Catalog).

Cyanobacteria and cyanotoxins monitoring

After the Caruaru incident most countries became aware of the risks posed by the contamination of drinking water with toxic cyanobacteria. Consequently, many implemented regulatory programs and guidelines for the monitoring of cyanobacteria and cyanotoxins, particularly microcystins (see Chapter 2). Cyanobacteria are identified by optical microscopy, using classical taxonomy based on morphological features. The cell quantification is determined by counting individual cells in

sedimentation chambers using the Utermöhl method (Lund et al. 1958). This process is highly dependent on the operator's extensive knowledge of taxonomy, which ensures a correct identification of the species. Furthermore, some cyanobacterial species are difficult to distinguish, even for an experienced taxonomist, and sometimes it is also difficult to distinguish the limits between cells, which compromise the correct determination of cell densities. This is an important aspect because the cyanobacterial concentration is a parameter used to define the guideline levels.

The detection and quantification of many cyanotoxins (microcystin, cylindrospermopsin, anatoxin and saxitoxin) can be done using enzyme-linked immunosorbent assay (ELISA) (An & Carmichael 1994), receptor binding assays (RBA) (Rubio et al. 2014) and protein phosphatase inhibition assay (PPIA) (An & Carmichael 1994, Metacalf et al. 2001). These assays are based in the recognition and binding of the toxins to specific antibodies or receptors. These methods are fast and sensitive but nonspecific reactions may occur leading to an overestimation of the amount of toxin (Merel et al. 2013). The high-performance liquid chromatography (HPLC) and liquid chromatography–mass spectrometry (LC-MS/MS) enables in-depth study of toxins and its variants (Lawton et al. 1994, Draper et al. 2013, Guzmán-Guillén et al. 2012, Sanchez et al. 2014). However these methods require extensive processing of the sample and standards for each specific toxin and/or variants (Merel et al. 2013, Metcalf and Codd 2014).

Genetic base of microcystin production

Microcystins are cyclic peptides that are assembled non-ribosomically by peptide synthetases (NRPS) and polyketide synthases (NRPS/PKS) coded by nine to eleven genes organized in a cluster named *mcy* (Welker and Döhren 2006) (Fig. 3). The *mcy* gene clusters are large in size, have approximately 48 to 57 kb, and were already sequenced in the cyanobacteria *Microcystis* (Tillett et al. 2000), *Planktothrix* (Christiansen et al. 2003), *Anabaena* (Rouhiainen et al. 2004) and *Nostoc* (Fewer et al. 2013). The *Planktothrix mcy* gene cluster is the one that possesses major differences in its organization when compared to the other three genera, lacking the genes coding for an aspartate racemase and a dehydrogenase (*mcy*I and F) and having the *mcy*T that encodes a putative thioesterase which is only found in this genus (Christiansen et al. 2003, Kurmayer and Christiansen, 2009) (Fig.3). The organization of *mcy* genes also differs between genera. In *Microcystis*, *Anabaena* and *Nostoc* the genes are transcribed bidirectional from a central promoter region, while almost all the genes in

the genus *Planktothrix* are unidirectional transcribed from a promoter located upstream of the gene *mcy*D (Christiansen et al. 2003, Kurmayer and Christiansen 2009).

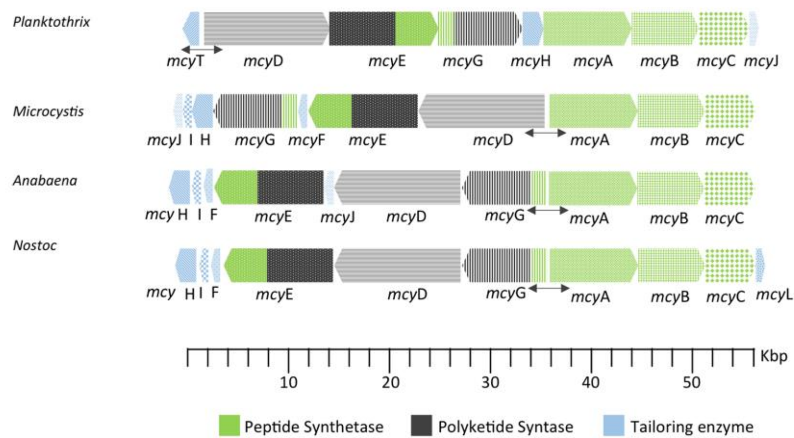

Fig. 3 - Representation of the microcystin synthetase gene cluster (*mcy*) in *Planktothrix* (Christiansen et al. 2003), *Microcystis* (Tillett et al. 2000), *Anabaena* (Rouhiainen et al. 2004) and *Nostoc* (Fewer et al. 2013). Adapted from Kurmayer and Christiansen (2009) and Fewer et al. (2013). The arrows indicate the transcription direction.

The Method_

Real-time PCR

Real-time Polymerase Chain Reaction (Real-time PCR) is similar to a conventional PCR with the exception that the progress of the reaction is monitored as it occurs, meaning, in “real-time” (Heid et al. 1996). The data is collected, through fluorescence, during the DNA fragment amplification at each cycle, rather than at the end of the reaction when the amount of fragments accumulates. This means that by monitoring the PCR reaction it is possible to quantify the initial number of copies of the target fragment since the amplification is exponential. Furthermore, the technique is highly sensitive and can be tailored according to the desired specificity that is determined by primer and probe target sequence. These primers and probes can be designed either for specific DNA sequences or for conserved regions. Since the reaction is performed in a closed vessel reaction and all the PCR reagents can be bought together in a mastermix, and since, there is no need of post handling of PCR products, this technique is less prone to contaminations (Espy et al. 2006).

There are several fluorophores that can be used in real-time PCR, the most common are Intercalating fluorescent dyes – SYBR® green - and fluorescent probes - Taqman®. The SYBR® green dye emits fluorescence when intercalates with dsDNA

(Fig. 4). The Taqman® probes are DNA sequences complementary of the target fragment, labeled with a fluorescent reporter dye at one end and a quencher dye at the other. When the Taq polymerase cleaves the 5' end of the probe releases the reporter dye that emits fluorescence (Fig. 4).

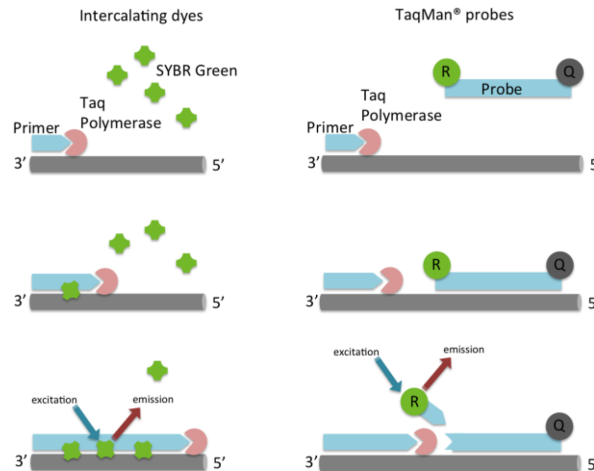

Fig. 4 - Mode of action of the fluorophores used in real-time PCR, SYBR® Green and TaqMan® assays. R – reporter, Q – quencher.

For the past two decades the real-time PCR technique has been used across multiple fields of biological research and is nowadays broadly applied in routine monitoring and diagnostics. It is commonly used in the quantification of viral and bacterial loads (Mackay et al. 2002, Maurin 2012), food authentication (Mafra et al. 2008), detection of genetically modified organisms (Lin and Pan *in press*), food (Postollec et al. 2011) and water (Ramírez-Castillo et al. 2015) borne pathogens.

Likewise, in cyanobacteria research the real-time qPCR has proved useful in studies involving cyanobacteria population dynamics and distribution permitting gene quantification in environmental communities (Kurmayer and Kutzenberger 2003, Rantala et al. 2006, Hoto et al. 2008, Davis et al. 2009, Tai and Palenik 2009, Orr et al. 2010). Tailored real-time qPCR reactions have been developed for the detection and quantification of genotypes related to several toxins, such as, microcystins (Furukawa et al. 2006, Al-Tebrineh et al. 2011, Baxa et al. 2010, Hautala et al. 2013), nodularins (Koskenniemi et al. 2007, Al-Tebrineh et al. 2011), cylindrospermopsin (Rasmussen et al. 2008, Marbun et al. 2012, Orr et al. 2010), saxitoxin (Al-Tebrineh et al. 2010) and multiplex real-time PCR targeting genes involved in the production of the previously mentioned toxins (Al-Tebrineh et al. 2012). Protocols have also been developed for the

detection and quantification of potentially toxic cyanobacteria species, such as, *Microcystis* (Baker et al. 2002, Baxa et al. 2010), *Anabena* (Baker et al. 2002, Rueckert et al. 2007, Al-Tebrineh et al. 2010), *Cylindrospermopsis* (Rasmussen et al. 2008, Orr et al. 2010) and *Planktothrix* (Schober & Kurmayer 2006, Ostermaier and Kurmayer 2009). Furthermore, studies using real-time PCR are applied in quantification of nucleotide variations responsible for microcystin variants (Ostermaier and Kurmayer 2010), non-toxic mutants caring microcystin related genes (Ostermaier and Kurmayer 2009) and detection of cyanobacteria genes from sediments (Savichtcheva et al. 2011, 2015, Kyle et al. 2015). The aforementioned quantitative real-time PCR assays are robust, reliable and sensitive, with low gene copy number detection limits. These studies showed promising results in the simplification of cyanobacteria identification and enumeration for routine monitoring programs (Pearson and Neilan 2008, Humbert et al. 2010, Martins and Vasconcelos 2011). The characteristics of the real-time PCR make it an appealing tool for cyanobacteria research and monitoring, although, is not yet applied in monitoring.

The Aims_

The main objective of this thesis was to develop a qPCR technique applied to cyanobacteria monitoring, focused in the *Planktothrix* species. The specific goals of this study were:

1. To develop a protocol for the specific detection and quantification of the commonly found *Planktothrix agardhii*, thus contributing for the simplification of common cell counting procedures;

2. To test the applicability of the real-time PCR in the amplification of target DNA from preserved samples, which can be useful in retrospective analyses of samples;

3. To monitor through real-time PCR the *Planktothrix agardhii* in environmental samples;

4. To increase the knowledge on the occurrence, toxin production and bloom dynamics of *Planktothrix* in Portuguese inland waters.

Chapter

02_

4

Risk Assessment of Cyanobacteria and Cyanotoxins, the Particularities and Challenges of *Planktothrix* spp. Monitoring

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1. Introduction

Cyanobacteria are a diverse well adapted group of organisms that presents amazing morphological diversity. Cyanobacteria can be unicellular or colonial (filamentous, spherical or amorphous) (Fig. 1). Since cyanobacteria have cells larger than normal bacterial cells and behavior more similar to algae, they were classified under the microalgae for a long time and acquire the name of blue-green algae or Cyanophyta (Whitton & Potts 2000). Cyanobacteria is a phylum of bacteria that obtain their energy through photosynthesis. The name "cyanobacteria" comes from their coloration (cyano = blue). The vegetative cell wall is of Gram-negative type and in some species the peptidoglycan layer is considerably thicker than in other bacteria. Many unicellular and filamentous cyanobacteria possess an "envelope" outside the lipopolysaccharide (LPS) "outer membrane", which is called: sheath, glycocalyx, or capsule, and depending on the consistency, gel, mucilage or slime. The sheaths of cyanobacteria are predominantly polysaccharide, but a part of its weight may be polypeptides, and depending on the species, some types of sugar residues may be involved (Castenholz, 2001).

Fig. 1. Optical microscopy photographs of cyanobacteria presenting diffe
The arrow indicates the heterocyst cell in *Anabena circinalis*.

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RISK ASSESSMENT OF CYANOBACTERIA AND CYANOTOXINS, THE PARTICULARITIES AND CHALLENGES OF *PLANKTOTHRIX* SPP. MONITORING

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Introduction

Cyanobacteria are a diverse well-adapted group of organisms that presents amazing morphological diversity. Cyanobacteria can be unicellular or colonial (filamentous, spherical or amorphous) (Fig. 1). Since cyanobacteria have cells larger than normal bacterial cells and behavior more similar to algae; they were classified under the microalgae for a long time and acquire the name of blue-green algae or Cyanophyta (Whitton & Potts 2000). Cyanobacteria are a phylum of bacteria that obtain their energy through photosynthesis. The name "cyanobacteria" comes from their coloration (cyano = blue). The vegetative cell wall is of Gram-negative type and in some species the peptidoglycan layer is considerably thicker than in other bacteria. Many unicellular and filamentous cyanobacteria possess an “envelope” outside the lipopolysaccharide (LPS) “outer membrane”, which is called: sheath, glycocalyx, or capsule, and depending on the consistency, gel, mucilage or slime. The sheaths of cyanobacteria are predominantly polysaccharide, but a part of its weight may be polypeptides, and depending on the species, some types of sugar residues may be involved (Castenholz, 2001). Cyanobacteria are autotrophs and possess all the photosynthetic pigment (chlorophyll a, carotenoids, allophycocyanin, phycobilins, phycoeritrins) except chlorophyll b (Castenholz, 2001). Prochlorophytes are also cyanobacteria that contain chlorophyll a and b, but, opposing to other cyanobacteria, lack phycobiliproteins (Castenholz, 2001). Cyanobacteria have the ability to use low light intensities effectively, since they are able to produce the accessory pigments needed to adsorb light most efficiently in the habitat in which they are present, providing them a great advantage for the colonization of a wide range of ecological niches (van den Hoek et al., 1995; WHO, 1999).

Fig. 1 - Optical microscopy photographs of cyanobacteria presenting different morphologies. The arrow indicates the heterocyst cell in *Anabena circinalis*.

Phycobiliprotein synthesis is particularly susceptible to environmental influences, especially light quality. The chromatic adaptation is largely attributable to a change in the ratio between phycocyanin and phycoerythrin in the phycobilisomes. The photosynthetic pigments are located in thylakoids that are free in the cytoplasm near the cell periphery (Fig. 2). Cell colors vary from blue-green to violet-red due to the chlorophyll a masking by the carotenoids and accessory pigments. The pigments are involved in phycobilisomes, which are found in rows on the outer surface of the thylakoids (Fig. 2) (WHO, 1999).

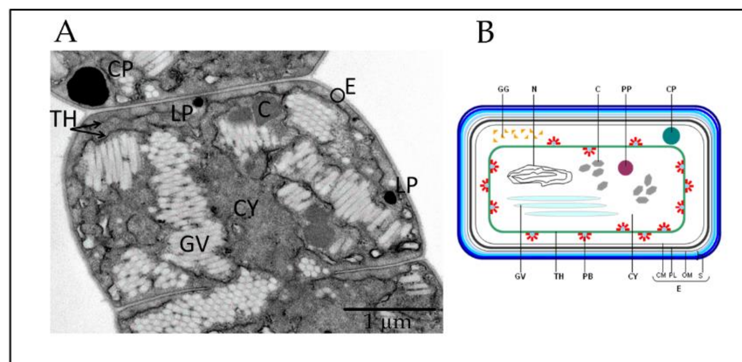

Fig. 2 - Cyanobacteria cell structure. (A)Transmission electron micrographs showing the ultrastructure of an *Anabena circinalis* vegetative cell; (B) Schematic diagram of a cyanobacterial vegetative cell. S: external 4-layered cell wall; OM: outer membrane; PL: peptidoglycan layer; CM: cytoplasmic membrane; CW: cell wall; E: cell envelope; TH: thylakoid; PB: phycobilisome; CY: cytoplasm; GV: gas vesicle; GG: glycogen granules; N: nucleoplasmic region; C: carboxysome; PP: polyphosphate granule; CP: cyanophycin granule; LP: lipid droplets (adapted from van den Hoek et al., 1995; Castenholz, 2001).

Cyanobacteria are also able of storing essential nutrients and metabolites within their cytoplasm. Prominent cytoplasmic inclusions such as glycogen and cyanophycin granules (polymers of the amino acids arginine and asparagine), polyphosphate bodies, carboxysomes (containing the primary enzyme for photosynthetic CO2 fixation, ribulose 1,5-bisphosphate carboxylase-oxygenase: RuBisCO) and gas vacuoles (Fig. 2) can be observed by electron microscopy. The occurrence of fimbriae (pili) is abundant in many cyanobacteria with varying patterns. Some filamentous forms are also able of gliding (sliding) (van den Hoek et al., 1995; WHO, 1999; Castenholz,

2001). Cyanobacteria can be found in the most diverse environments like hot springs, salt marshes, soils, fresh, brackish, and marine waters (Sze, 1986). In sum, cyanobacteria are ubiquitous oxygenic photosynthetic prokaryotes.

Why the surveillance on Cyanobacteria?

Cyanobacteria are common constituents of the phytoplankton in aquatic environments. In optimal conditions these phytoplankton's can develop massively and form blooms, becoming the dominant organism in the water column and creating serious problems in water quality (Cood, 2000; Vasconcelos, 2006). The water quality deterioration produced by cyanobacterial blooms includes foul odors and tastes, deoxygenation of bottom waters (hypoxia and anoxia), fish kills, food web alterations and toxicity. Other threatening characteristic of these organisms is their ability to produce toxins that affects other living organisms and humans (Carmichael, 2001). The capacity of mass development together with the ability to produce potent toxins enlightens the importance of implementing regular monitoring programs for cyanobacteria and cyanotoxins in freshwater environments, in order to minimize potential health risks to animal and human populations that results from exposure through drinking and recreational activities. The implementation of surveillance programs on cyanobacteria involves understanding the ecophysiology of cyanobacteria, bloom dynamics, conditions that promote blooms, production of toxins and their impact in human and animal health (McPhail & Jarema, 2005). Cyanobacteria possess some ecostrategies that allows them to overcome other organism and become dominant. In general there are four constraints on cyanobacteria growth as pre-requisites for bloom enhancement: light, nutrients, temperature and stability of the water column. Cyanobacteria require low light intensities for growth, compared with algae, which provides competitive advantages in lakes which are turbid due to growth of other phytoplankton. They also have a higher affinity for uptake phosphorous and nitrogen than many other photosynthetic organisms and they have a substantial storage capacity for phosphorous (Mur et al., 1999). Some genera like *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Nodularia* and *Nostoc* have specialized cells (heterocysts) (Fig. 1) for nitrogen fixation and blooms of these genera can often be related with periodic nitrogen limitation. This means that they can compete other phytoplankton under conditions of phosphorous and nitrogen limitation (Briand et al., 2003; Sunda et al., 2006). The success of some cyanobacteria is also due to the presence of gas vacuoles that provide buoyancy regulation. During water stratification conditions cyanobacteria can migrate in the water column, accessing light in the surface layers and nutrients

near the sediment. During photosynthesis, carbohydrates are accumulated which makes them heavy and sinking away from light and when the carbohydrates are respired, buoyancy is restored. As large colonies sink faster than small ones or single cells, genera like *Microcystis*, *Anabaena*, *Aphanizomenon* and *Nodularia* have scum-forming strategies (Vance, 1965; Mur et al., 1999). Cyanobacteria also produces active substances that inhibits the growth of competing algae and grazers that feed upon them, this can also promote cyanobacteria proliferation (Briand et al., 2003; Granéli & Hansen, 2006; Sunda et al., 2006; van Apeldoorn et al., 2007; Figueredo et al. 2007). As a consequence of the characteristics mentioned above the cyanobacterial cells numbers in water bodies vary seasonally. In temperate regions, seasonal successions of organisms belonging to different phytoplankton taxa are often observed. Whereas at the beginning of the summer a great variety of microalgae and cyanobacteria usually co-exist in the same water body, towards the end of summer this diversity may drop drastically as the result of the mass development of the cyanobacterial communities (blooms) (Sze, 1986). These blooms may be formed by a consortium of cyanobacteria producing different amounts of toxins at different rates, with the same bloom-forming species having both toxigenic and non-toxigenic strains, indistinguishable by morphological examination. Cyanobacterial blooms are complex and can develop in a rather sudden and unpredictable way.

Cyanotoxins

Cyanobacteria are able to produce secondary metabolites that present a vast diversity of structures and variants. Most of cyanobacterial secondary metabolites are alkaloids, or possess peptidic substructures synthesized by NRPS (non-ribosomal peptide synthesis, involving peptide synthetases) or NRPS/PKS (involving peptide synthetases and polyketide synthases) hybrid pathways (Valério et al., 2010). Cyanotoxins are usually classified according to their target in mammals, being divided in hepatotoxins (liver damaging), neurotoxins (nerve damaging), cytotoxins (cell damaging) and toxins responsible for allergenic reactions (dermatotoxins), presenting several kinds of mechanisms of action. A considerable number of these different types of toxins have been isolated from cyanobacteria, belonging to different taxa, as summarized in Table 1.

Toxin	Taxon	LD_{50} * (i.p., mouse) of pure toxin	Primary target in mammals	Mechanism of action
Hepatotoxins				
Microcystins (~80 variants) (Cyclic heptapeptides)	*Microcystis, Planktothrix, Oscillatoria, Nostoc, Anabaena, Anabaenopsis, Hapalosiphon, Snowella, Woronichinia, Aphanocapsa*	25 to ~1000μg/kg bw	Liver	Multi-pathway process. MCs inhibit the serine/threonine protein phosphatases type 1 and type 2A (PP1/PP2A) and induces oxidative stress leading to a cascade of events responsible for the MC cytotoxic and genotoxic effects in animal cells
Nodularin (9 variants) (Cyclic pentapeptides)	*Nodularia spumigena*	30–50 μg/kg bw	Liver	Similar to MCs
Cytotoxin				
Cylindrospermopsin (3 variants) (Guanidine alkaloid)	*C. raciborskii, Umezakia natans, Aph. Ovalisporum, Rapidiopsis curvata, Anabaena bergii, Aphanizomenon, Lyngbya*	200 -2100 μg/kg bw/d 200 μg/kg bw/5–6 d	Liver, kidneys, lungs, heart	Inhibition of glutathione (GSH) and protein synthesis, as well as the inhibition of cytochrome P450
Dermatotoxins				
Aplysiatoxin (phenolic bislactone)	*Lyngbia, Planktothrix, Schizothrix*	107-117 μg/kg	Skin	Inflammatory agent, protein kinase C activator
Debromoaplysiatoxin (phenolic bislactone)	*Lyngbia*	107-117 μg/kg	Skin	Inflammatory agent, protein kinase C activator
Lyngbiatoxin-A (Alkaloid)	*Lyngbia*	250 μg/kg (?LD100)	Skin	Inflammatory agent, protein kinase C activator
Neurotoxins				
Anatoxin-a (5 variants) (Tropane-related alkaloids)	*Anabaena* spp., *Oscillatoria, Cylindrospermum, Microcystis, Aphanizomenon, Planktothrix*	250 μg/kg bw	Post-synaptic neuromuscular junction	Agonism of the muscular and neuronal nicotinic acetylcholine receptor
Homoanatoxin-a (alkaloid)	*Planktothrix, Oscillatoria, Anabaena, Raphidiopsis, Phormidium*	250 μg/kg bw	Post-synaptic neuromuscular junction	Same as Anatoxin-a
Anatoxin-a(s) (Guanidine methyl phosphate ester)	*Anabaena, Aphanizomenon*	40 μg/kg bw	Post-synaptic neuromuscular junction	Irreversible inhibition of acetylcholinesterase
Saxitoxin (20 variants) (Carbamate alkaloids)	*Anabaena, Aphanizomenon, C. raciborskii, Lyngbya, Planktothrix*	10-30 μg/kg bw	Axons	Blockage of the sodium or calcium channels of the nerve axon membranes

Table 1 - Cyanotoxins detected and correspondent taxa from which have been isolated as well as their primary target in mammals. Based on the information from (Chorus et al., 2000; Charmichael, 2001; Codd et al., 2005; Stewart et al., 2006; van Apeldoom et al., 2007; Bláha et al., 2009; Valério et al., 2010; Mihali et al., 2009). * - the dose needed to kill 50% of mice.

Cyanobacteria/cyanotoxins risk assessment

Risk assessment consists in the identification and determination of quantitative or qualitative value of risk related to the exposure to a given hazard, taking into account possible harmful effects on individuals or populations exposed to that hazard and all the possible routes of exposure. The risk assessment process includes four steps: the hazard identification, hazard characterization, exposure assessment, establishment of dose–effect and dose–response relationships in likely target individuals and populations (Duffus et al., 2007). A schematic representation of the steps involved in risk assessment of cyanotoxins is depicted in Fig. 3.

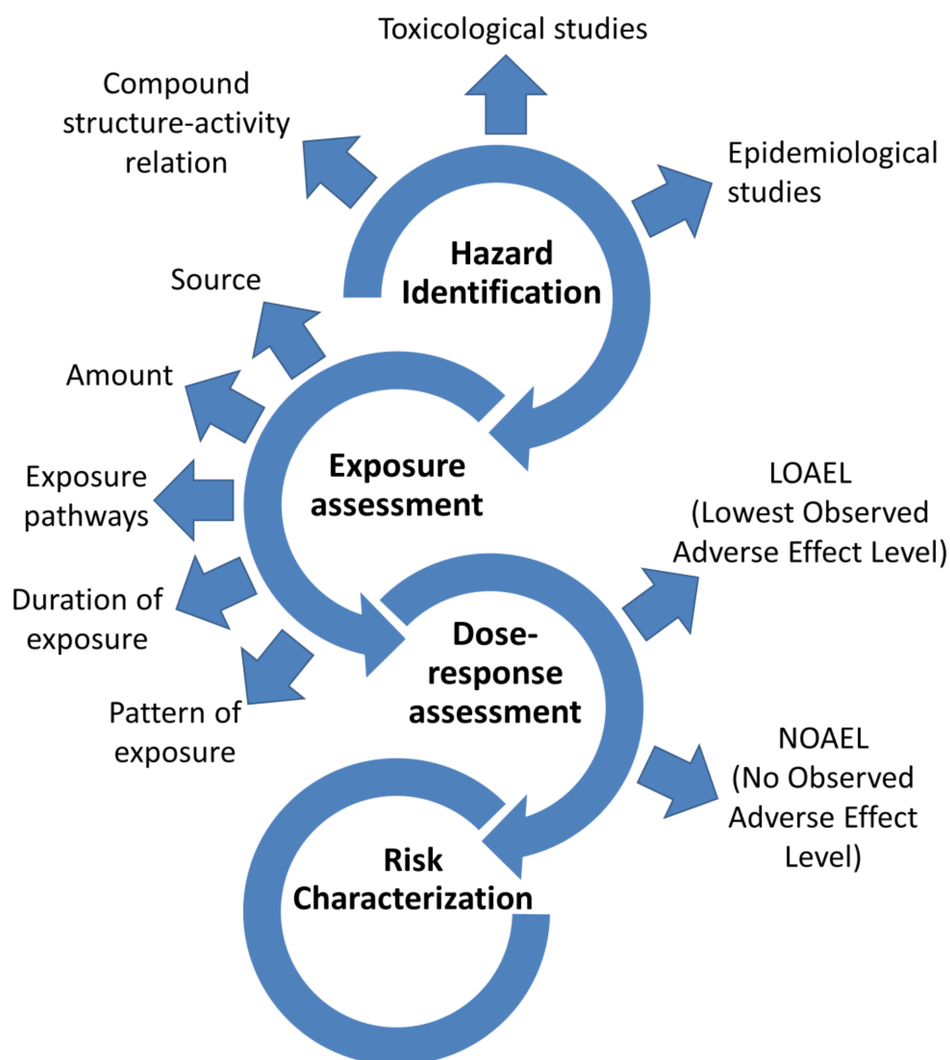

Fig. 3 - Organizational chart of the steps involved in risk assessment (adapted from Dolah et al. 2001).

The scientific knowledge on cyanotoxins still does not enable to correctly assess the risk of human exposure to toxic cyanobacteria. Many toxicological aspects remain to clarify, epidemiological data are insufficient and the exposure assessment is a very complex task. The human exposure to cyanobacterial cells and/or its toxins may occur through water swallowing or inhalation during recreational activities, such as swimming, canoeing, sailboarding and paddling, through the intake of contaminated drinking water and through hemodialysis treatment. Most episodes of human illness related with cyanobacetria/cyanotoxins resulted from an acute intoxication though the exposure routes mentioned above (for review see Chorus et al., 2000; Duy et al., 2000; van Apeldoom et al., 2007), such as the following examples:

Example 1_ Symptoms after exposure through recreational activity: nausea, abdominal pain, fever, dyspnea, respiratory distress, atypical pneumonia and hepatotoxicosis with a significant increase of hepatic damage biomarkers (Giannuzzi et al., 2011);

Example 2_ Symptoms after exposure during heamodialysis treatment: weakness, muscular pain, nauseas, vomiting, neurologic symptoms (head pain, vertigo, deafness, blindness and seizures), increase of hepatic damage biomarkers, hepatomegaly, hepatic failure and death (reviewed in Pouria et al, 1998).

Besides the acute effects mentioned above, few papers reports the association between the ingestion of water contaminated with microcystins and the increase of hepatocarcinoma (Yu, 1995; Ueno et al., 1996) and colorectal cancer (Zhou et al, 2002) in human populations supplied with untreated- or ineffective-treated water. Laboratorial studies have demonstrated that, in fact, microcystins, nodularins and cylindrospermopsin are genotoxic (reviewed in Zĕgura et al., 2011) and the carcinogenic potential of these toxins have been postulated (Gehringer, 2004; Kinnear, 2010). However, there are still many uncertainties that difficult an unequivocal conclusion about this issue. The problem of chronic effects are particularly relevant in the case of continuous exposure to low levels of cyanotoxins, even at residual levels, that are not detected by the conventional methods employed in the monitoring procedures. Moreover, the scientific and analytical limitations hinder the complete determination of the toxicological properties of cyanotoxins, and the correct assessment of human exposure to cyanotoxins, as well as lack to provide epidemiological evidence that could confirm the chronic effects of cyanotoxins on human health. Therefore, although the surveillance programs can somehow protect against the cyanotoxins acute effects, risk assessment procedures should be

developed and implemented, particularly in what concerns to chronic exposure to cyanotoxins. During the last decade, the WHO has been regularly reviewing the public health significance of cyanobacteria occurrence in freshwater and developed guidelines for drinking and recreational water environments (WHO, 1998, 2003). This organization recommends that the approach to developing guidelines for cyanobacteria in freshwater should consider:

_the occurrence of cyanobacteria in general (in addition to their toxins) as part of the hazard, because it is not clear that all known toxic components have been identified and irritation symptoms reported may be caused by these unknown substances;

_the particular hazard caused by the well-known cyanotoxins; and

_the hazard associated with the potential for scums formation, which increase the local hazard concentration.

WHO (2003) has divided the health effects into two categories:

_Symptoms associated with skin irritation and allergic reactions resultants from dermal exposure to unknown cyanobacterial substances, and

_Potentially more severe effects due to the exposure to high concentrations of already known cyanotoxins, particularly microcystins (the most commonly found and more studied cyanotoxins).

Given the two types of severity of the symptoms, the WHO considered that the establishment of a single guideline value was not appropriate and, therefore, it has defined several guideline values associated with increasing severity and probability of impact in health at three levels (Table 2).

WHO guideline levels	Cyanobacterial cells and chlorophyll levels	Health risks	Recommended action
Low	< 20,000 of total cyanobacterial cells mL^{-1} OR < 10 μg L^{-1} chlorophyll-a with dominance of cyanobacteria OR < 2.5 mm^3 L^{-1} cyanobacterial biomass	Short term adverse health outcomes unlikely	Cyanobacteria either absent or present at low levels - continue monitoring
Moderate	20,000 - 100,000 of total cyanobacterial cells mL^{-1} OR 10 - 50 μg L^{-1} chlorophyll-a with dominance of cyanobacteria OR 2.5 - 12.5 mm^3 L^{-1} cyanobacterial biomass	Short term adverse health outcomes, e.g. skin irritations, gastrointestinal illness, probably at low frequency	Add signs to indicate MODERATE alert level - increased health risk for swimming and other water contact activities
High	Cyanobacterial scum formation in contact recreation areas OR > 100,000 of total cyanobacterial cells mL^{-1} OR > 50 μg L^{-1} chlorophyll-a with dominance of cyanobacteria OR > 12.5 mm^3 L^{-1} cyanobacterial biomass	Short term adverse health outcomes such as skin irritations or gastrointestinal illness following contact or accidental ingestion Severe acute poisoning is possible in worst ingestion cases	Immediate action to prevent contact with scums Add signs to indicate HIGH alert level - warning of danger for swimming and other water contact activities

Table 2 - WHO guideline values for safe practice in managing bathing waters that may contain cyanobacterial cells, according to the level of probability of adverse health effects (WHO, 2003).

Cyanotoxin analysis will generally be required in one of the following circumstances (WHO, 1999):

1_Action Level 1 status (i.e. > 2000 cells mL^{-1}) predominated by *Microcystis aeruginosa*, or when concentrations of other potentially toxic taxa (see Table 1) exceed 15 000 cells mL^{-1}.

2_Action Level 2 status where numbers of a cyanobacterial taxa not previously recorded as toxic exceed 100,000 cells mL^{-1} (recommended toxicity analysis by mouse bioassay or comparative method).

A brief summary of the steps that must be taken into account, when performing cyanobacteria monitoring, are presented in Fig. 4.

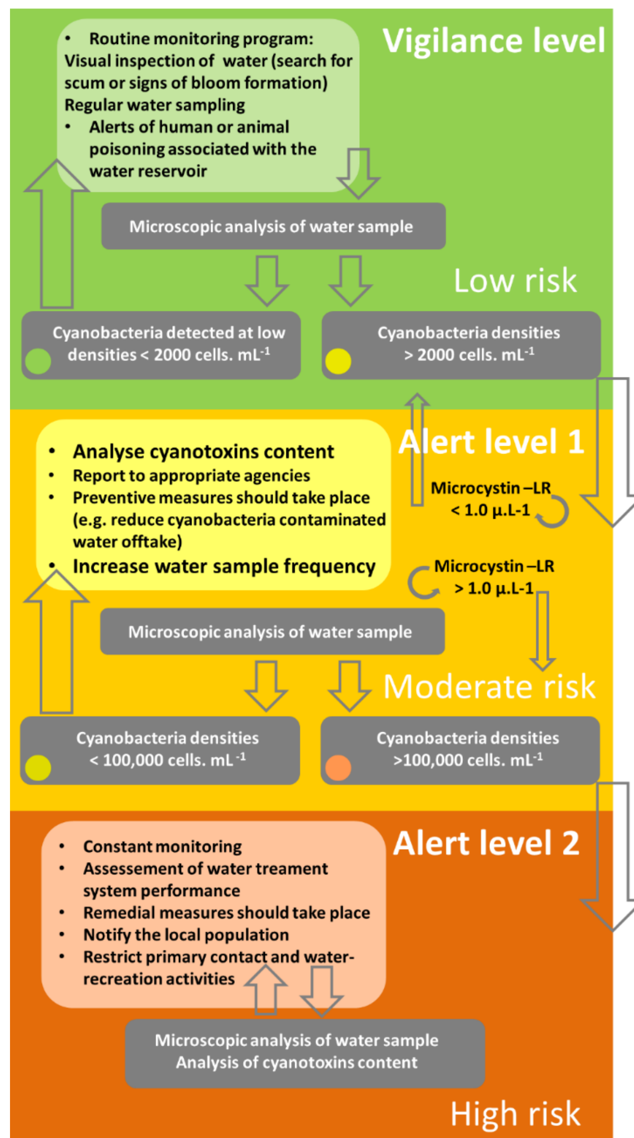

Fig. 4 - Organizational chart of the steps involved in cyanobacteria risk management (adapted from Bartram et al. 1999).

Derivation of guideline values

Characterization of human hazards usually relies mainly on animal studies, or incidents from which quantitative estimates of the hazards to humans must be extrapolated. Few studies in rodents and pigs enabled to estimate the tolerable daily intake (**TDI**) of some cyanobacterial toxins (Duy et al., 2000; Falconer et al., 1999; Humpage and Falconer, 2003). Usually, studies with different quantitative animal dosing data, with follow-up over extended periods (preferably over the lifetime of the animal being tested) are necessary to estimate a no-observed-adverse-effect level (**NOAEL**), or at least a lowest-observed-adverse-effect-level (**LOAEL**).

For drinking water, the TDI for cyanotoxins can be estimated as:

$$\text{TDI}=\frac{\text{NOAEL or LOAEL}}{\text{UF}} \qquad (1)$$

Where, TDI units are mg/kg body wt/day, or μg/kg body wt/day, and UF is the product of uncertainty factors, e.g

$$\text{UF}=1000\begin{Bmatrix}\text{10 (intra-specific variations)}\\ \text{10(inter-specific variations)}\\ \text{10 (less-than-lifetime study)}\end{Bmatrix} \qquad (2)$$

Additionally, it may be also necessary consider a UF of 5 if the LOAEL is used and a UF of 3, if tumor promotion is considered (Codd et al., 2005).

The guideline value (**GV**; μg/l water) can be calculated as:

$$\text{GV}=\frac{(\text{TDI })\times\text{body wt }\times\text{AF}}{\text{C}} \qquad (3)$$

Where body weight is usually assumed to be 60 kg for a human adult and AF is the allocation factor, which is the proportion of daily exposure arising from drinking water ingestion. Because some oral exposure may occur via food or dietary supplements or other route, therefore, an AF of 0.8 (80% of total intake) is assumed for drinking water. Finally, C is the volume of drinking water consumption per day, assumed to be 2 L for an adult (Codd et al., 2005; van Apeldoorn et al., 2007).

Guidelines for Microcystins

The drinking water guideline for microcystins was determined from a sub-cronic study (Fawell et al., 1994) with mice orally administered with microcystin-LR (since it is one of the most toxic and frequent microcystin variant and for which more information is available). In this study a NOAEL of 40 μg/kg bw was derived and a TDI of 0.040 was calculated using an uncertainly factor of 1000 (10 for intra-specific variations, 10 for inter-specific variations and 10 for limitations in the database). The resulting guideline value, using an allocation factor of 0.80 for total microcystin-LR (free plus cell bound), was aprox. 1 μg/L in drinking water. A similar TDI for microcystins was obtained (0.067 *vs.* 0.040) from a study with pigs using freeze-thawed *Microcystis* cells containing quantified microcystins (Falconer et al., 1994). These resulted in similar GVs: 1 μg/L for mice *vs.* 1.61 μg/L for pigs. For safety reasons, the World Health Organization (WHO) has adopted the lowest value (1 μg/L) as the GV for microcystin in drinking water for adults (WHO, 1998). However, if tumour-promoting actions of microcystins are also

considered, then an additional UF of 3 for this hazard must be used, thus originating a GV of about 0.3 µg/L (Codd et al., 2005). The Australian guideline is 1.3 µg/L for total microcystin. This slightly differs from the WHO provisional guideline of 1 µg/L microcystin-LR due to the use of a different average body weight for an adult (70 kg *vs.* 60 kg) and different Alocation Factor (0.9 vs. 0.8).

Guidelines for Nodularin

No NOAEL can be derived for nodularin(s) due to the absence of suitable toxicological data. However, since nodularin(s) and microcystin-LR have identical mechanisms of action, the guideline value determined for MC-LR (1 µg/L) can also be used for nodularin(s).

Guidelines for Anatoxin-a

A NOAEL of 98 µg/kg has been derived from a 28-day gavage study using mice (Fawell et al., 1999). If a uncertainly factor (UF) of 1000 (10 for intra-specific variations, 10 for inter-specific variations and 10 for limitations in the database) is used, a TDI of 0.1 µg/kg bw can be reached. Svrcek & Smith (2004) have suggested a guideline limit of 3.0 µg/L.

Guidelines for Anatoxin-a(S)

There are no sufficient data to derive an NOAEL or LOAEL and, consequently, insufficient data to determine a TDI for anatoxin-a(S). However, in the Guidelines for Drinking-Water Quality Management for New Zealand 2005, a Maximum Acceptable Values (MAVs) for anatoxin-a(S) of 1.0 µg/L is suggested (Chorus, 2005).

Guidelines for Cylindrospermopsin

According to the 90-day study of Shaw et al. (2000) using drinking water in mice a NOAEL of 150 µg/kg bw was obtained. A second study with mice administered by gavage with cylindrospermopsin for 11-weeks from Humpage and Falconer (2003) resulted on a NOAEL of 30 µg/kg bw. If a uncertainly factor (UF) of 1000 (10 for intra-specific variations, 10 for inter-specific variations and 10 for limitations in the database) is used, a TDI of 0.03 µg/kg bw can be calculated. Considering the “standard” adult body wt of 60 kg and a 0.9 AF, a GV of 0.81 is obtained , leading the authors to propose a Guideline Value of 1 µg/L (Humpage and Falconer, 2003).

Guidelines for Saxitoxin

There are no attempts to determine a NOAEL or LOAEL and thus calculate a TDI for saxitoxin, because the range of lowest concentration where adverse effects were observed varies greatly. Given the different susceptibilities of person, it has been difficult to decide which uncertainty factor should be also used (van Appeldoorn et al., 2007). Although there are no official guidelines, Australia considers a GV of 3 μg STX eq/L of drinking water, which was based on the data from marine shellfish toxicity (van Appeldoorn et al., 2007).

Guidelines for Aplysiatoxin and Lyngbyatoxins

There are no sufficient data to derive an NOAEL or LOAEL and thus calculate a TDI for these toxins. The members of the population presenting greatest risk when exposed to cyanotoxins are children because of their water intake: body weight ratio, which is higher than that of adults (Falconer, 1999). Also the people having already certain pathologies may be more susceptible to the intake of the toxins (Falconer, 1999). Ideally, the guidelines values established should protect against acute and chronic effects derived from the contact with cyanobacteria and their toxins, although, such it was stated above, the knowledge on the chronic effects of cyanotoxins still presents many gaps. The guideline values determined/suggested for each known cyanotoxin are summarized in Table 3.

Toxin	Drinking water guideline values	Countries using the GV	References
MC-LR	1.0 µg/L (most generally accepted)	Brazil, Czech Republic, Denmark, France, Great Britain, Greece, Italy, New Zealand, Poland, Portugal, South Africa, Spain, U.S.A.	Chorus, 2005; Codd et al., 2005; Van Apeldoorn et al., 2007
MC-LR	1.3 µg/L	Australia Canada	Chorus, 2005; Van Apeldoorn et al., 2007
Nodularin	No guideline, however, hazard assessment can be guided by that for microcystins 1.0 µg/L	New Zealand	Fitzgerald et al., 1999; Chorus, 2005; Van Apeldoorn et al., 2007
Anatoxin-a	3.0 µg/L (no official guideline) 6.0 µg/L	New Zealand	Codd et al., 2005; Svrcek & Smith, 2004; Chorus, 2005
Homoanatoxin-a	2.0 µg/L	New Zealand	Chorus, 2005
Anatoxin-a(S)	Nd 1.0 µg/L	New Zealand	Chorus, 2005
Cylindrospermopsin	1.0 µg/L (suggested) 15.0 µg/L	Canada, New Zealand Brazil	Humpage &Falconer, 2003; Svrcek & Smith, 2004; Chorus, 2005
STX	3.0 µg STX eq/L	Australia Brazil New Zealand	Svrcek & Smith, 2004; Chorus, 2005; Codd et al., 2005
Aplysiatoxins	nd		
Lyngbyatoxins	nd		

Table 3 - Guideline values (GV) estimated for cyanobacterial toxins in drinking water. Nd – not determined.

Challenges and gaps in *Planktothrix* spp. risk assessment and management

Usually, *Microcystis* is the genus that occurs more frequently and is usually considered the main responsible for the production of microcystins. However, there is another emergent genus that has also the ability to produce microcystins, which is *Planktothrix*. The cyanobacteria of the genus *Planktothrix* have a planktonic life style, occur in solitary filaments and lack sheaths, heterocytes and akinetes. Formerly classified into to the genus *Oscillatoria*, *Planktothrix* now represents a well-defined independent genus based in phylogenetic and morphologic characteristics and comprises 13 species (Komárek & Komárkova 2004). Similar to other cyanobacteria, *Planktothrix* can achieve high cellular densities in water forming blooms that unbalance the ecosystem and it can also produce several types of cyanotoxins, namely microcystins, homoanatoxin-a, anatoxin-a, aplysiatoxins, saxitoxins, anabaenopeptins, (Luukkainen et al., 1993; Erhard et al., 1999; Kouzminov, 2001; Viaggiu et al., 2004; Wood, 2005; Kosol et al., 2009), thus threatening humans and animals. From the 13 species described *Planktothrix rusbescens* and *Planktothrix agardhii* are the most studied and common species reported to cause water related problems. A summary of *Planktothrix* occurrence in European lakes where they form recurrent blooms and the associated toxicity found is presented in Table 4. Unlike other cyanobacteria, *P. agardhii* and *P. rubescens* are well adapted to low light intensities and this characteristic provides to them several advantages. For *P. agardhii* it allows them to grow in waters with high turbidity, in which it can be homogeneously dispersed throughout the epilimnion in eutrophic waters having an competitive advantage upon other phytoplankton specis For *P. rubescens* the low light intensity requirements together with the high content of the red pigment phycoeritrin enables it to growth in the metalimnetic layer in thermally stratified waters away from the phototic surface zone (Mur et al., 1999; Bright & Walsby, 2000). Furthermore, these two species have different irradiance tolerances; *P. agardhii* is more tolerant to high irradiance than *P. rubescens*, what is related with their occurrence in different ecological niches in the water column and inhabit in different types of water systems (Oberhaus et al., 2007). Therefore *P. agardhii* grows well in the upper part of the water column of shallow eutrophic lakes, however it can also growth at several depths along the water column (Halstvedt et al., 2007). On the other hand, *P. rubescens* is well adapted in forming metalimnic populations of deep stratified lakes in spring and summer and when the lake loses its thermal stratification in the winter, it can be disperse through the entire water column (Bright & Walsby, 2000; Briand et al., 2005). *Planktothrix* also has different water temperature tolerances when compared to

other cyanobateria, making them organisms that can be easily found in subalpine lakes or in temperate regions during winter, so *Planktothrix* blooms may persist all year around and not only during summer or spring where temperatures and light irradiance are higher.*P. agardhii* has been found viable under ice covers (Sivonen & Jones 1999; Oberhaus et al., 2007). Since both species occupies different water niches they can coexist in the same water body forming surface and deep layer blooms, althougth this coexistence is rare it has been reported (Davis & Walsby, 2002; Halstvedt et al., 2007). Regarding the risk management measures that are usually followed to overcome the presence of cyanobacteria and cyanotoxins in the freshwater, *Planktothrix* has some particularities that need to be taken into account. One of them is *Planktothrix*´s ability to establish populations at several depts. in the water column that allows them to access nutrients located near the bottom and still have enough light for photosynthesis, making them able to form blooms away from the surface. This unique characteristic of *Planktothrix* may possess a problem for the water monitoring authorities, since their bloom may be overlook by surface monitoring inspection (Sivonen & Jones 1999). Furthermore, *Planktothrix* blooms may co-occur with other cyanobacterial surface blooms what can also be misleading in water monitoring. Generally cyanobacteria blooms are expected to occur in highly nutrient rich waters during summer or spring months (Chorus et al., 2000). The responsible agencies for the reservoirs monitoring often restricts or increases to normal level the water inspection and water sampling frequency. *Planktothrix* species such as *P. rubescens* occurs in low nutrient oligotrophic waters forming perennially blooms that can prevail for many years. Furthermore since nutrients are not a limiting factor for *P. rubescens* it has been reported the lodging and development of population of this species after restoration lake activities and decrease in nutrient input since it improves trophic level and increases water transparency (Jacquet et al., 2005; Legnani et al., 2005; Ernst et al., 2009). So, in lakes were *Planktothrix* species occur the surveillance must be during all year (Utkilen et al., 1999; Naselli-Flores et al., 2007). Other important feature is that *Planktothrix* may contain higher microcystins content per cell, when compared with other microcystins producers; and that the proportion of toxic strains is higher in *Planktothrix* blooms than for example *Microcystis* blooms, this may result in the occurrence of high toxin concentrations in water without scum formation (Falconer et al., 1999; Briand et al., 2008; Ernst et al., 2009).

Country	Lake	Maximum depth (m)	Trophic status	Species	Toxins detected	References
Austria	Mondsee	68	Mesotrophic	*P. rubescens*	---	Kurmayer et al. 2004; Kurmayer & Gumpenberger 2006
	Irrsee	32	Mesotrophic	*P. rubescens*	---	Kurmayer et al. 2004; Kurmayer & Gumpenberger 2006
	Afritzer	2.5	Mesotrophic	*P. rubescens*	---	Kurmayer & Gumpenberger 2006
	Wörthersee	85.2	Mesotrophic	*P. rubescens*	---	Kurmayer & Gumpenberger 2006
Czech Republic	Bílá Lhota	1	Hypertrophic	*P. agardhii*	---	PoulíČková et al. 2004
England	Blelham Tarn	14.5	Eutrophic	*P. rubescens, P. agardhii*	---	Davis & Walsby 2002; Davis et al. 2003
	Elmhirst	Shallow	---	*P. agardhii*	3.2 $\mu.L^{-1}$ MC-LR [a,c]	Akcaalan et al. 2006
	Bassenthwaite	21	Mesotrophic	*P. agardhii*	27- 41 $pg.filament^{-1}$ MC-LR [b,c]	Akcaalan et al. 2006
Estonia	Verevi	11	Hypertrophic	*P. agardhii*	---	Nõges & Kangro 2005; Kangro et al. 2005
Finland	Vargsundet	32	Oligotrophic	*P. agardhii*	11.1 $\mu g.L^{-1}$ MC-RR [a,d]	Lindholm et al. 1999
France	Bourget	145	Mesotrophic	*P. rubescens*	Max. 6.7 $\mu.L^{-1}$ MC-LR [a,d]	Briand et al. 2005, Jacquet et al. 2005
	Viry-Châtillon	5.3	Hypertrophic	*P. agardhii*	Máx. 7.4 $\mu g.L^{-1}$ MC-LR [a,e], Máx. 34.5 $\mu g.L^{-1}$ MC-LR [a,d]	Catherine et al. 2008, Yéprémian et al. 2007
Germany	Ammersee	81.1	Mesotrophic	*P. rubescens*	Mean 0.43 ± 0.06 $\mu g.L^{-1}$ MC-LR [a,c]	Ernst et al. 2009
	Stolpsee	Shallow	Eutrophic	*P. agardhii*	1.81 $\mu g.L^{-1}$ MC-LR [a,d]	Mbedi et al 2005
	Breiter Luzin	58.3	Mesotrophic	*P. rubescens*	2.31 $\mu.L^{-1}$ MC-LR [a,d]	Mbedi et al 2005
	Langer See	3.8	---	*P. agardhii*	----	Rücker et al. 1997

	Wolziger See	13	---	*P. agardhii*	----	Rücker et al. 1997
	Lebbiner See	4	---	*P. agardhii*	----	Rücker et al. 1997
Greece	Ziros	56	Oligotrophic	*P. rubescens*	Máx. 199 μ.L^{-1} MC-LR [a,c]	Vareli et al. 2009
Hungary	Balaton	3.2[f]	Eutrophic	*P. agardhii*	---	Honti et al. 2007
Italy	Pusiano	24.3	Eutrophic	*P. rubescens*	---	Legnani et al. 2005
	Como	410	Mesotrophic	*P. rubescens*	---	Bettinetti et al. 2000; Buzzi 2002
	Maggiore	370	Oligo-mesotrophic	*P. rubescens*	---	Morabito et al. 2002
	Fiastrone			*P. rubescens*	---	Viaggiu et al. 2003
	Spino	8		*P. rubescens*	12.13 ng.mg Anatoxin-a [a,d]	Viaggiu et al. 2003, Viaggiu et al. 2004
	Gerosa	50	Oligotrophic	*P. rubescens*	Máx. 1.94 μg.L^{-1} MC-LR [a,c]	Manganelli et al. 2010
	Pozzillo	50	Meso-eutrophic	*P. rubescens*	34 mg.L^{-1} MC-LR [a,d]	Naselli-Flores et al. 2007
	Prizzi	44	Meso-eutrophic	*P. rubescens*	7 μg L^{-1} MC-LR [a,d]	Naselli-Flores et al. 2007
	Nicoletti	36	Mesotrophic	*P. rubescens*	---	Naselli-Flores et al. 2007
	Garcia	43	Meso-eutrophic	*P. rubescens*	---	Naselli-Flores et al. 2007
	Garda	350	Oligo-mesotrophy	*Planktothrix* sp., *P. rubescens*	---	Salmaso 2000; Salmaso 2010
Netherlands	Vechten	11.9	---	*P. agardhii*	---	Montealegre et al. 1995
Norway	Steinsfjorden	24	Mesotrophic	*P. rubescens, P. agardhii*	---	Halstved et al. 2007; Rudi et al. 2005
Poland	Bytynskie	3.5[f]	Eutrophic	*P. agardhii*	15.8 μ.L^{-1} MC-LR [a,e]	Mankiewicz-Boczek et al. 2009

	Lubosinskie	2.6[f]	Eutrophic	*P. agardhii*	21.9 µ.L^{-1} MC-LR [a,e]	Mankiewicz-Boczek et al. 2009
	Laskownickie	7.4	Hypertrophic	*P. agardhii*	---	Stefaniak et al. 2005
	Grylewskie	6.5	Hypertrophic	*P. agardhii*	---	Stefaniak et al. 2005
Portugal	Beliche	52	Eutrophic	*P. rubescens*	1.1 µg.L^{-1} MC-LR [a,c]	Paulino et al. 2009a
Spain	El Atazar	100	Oligo-mesotrophic	*P. rubescens*	---	Almodóvar et al. 2004
Switzerland	Zürich	143	Mesotrophic	*P. rubescens*	---	Bright & Walsby 2000; Kurmayer & Gumpenberger 2006
	Hallwilersee	48	Mesotrophic	*P. rubescens*	---	Kurmayer & Gumpenberger 2006
Turkey	Spanca	55	Oligo-Mesotrophic	*P. rubescens*	18.4-66.1 pg.filament^{-1} MC-LR [b,c]	Akcaalan et al. 2006
	Iznik	70	Mesotrophic	*P. rubescens*	29.2-114 pg.filament^{-1} MC-LR [b,c]	Akcaalan et al. 2006
	Küçükçekmece	20	---	*P. agardhii*	Nontoxic [a]	Al-Tebrineh et al. 2011
	Sapanca	52	---	*P. rubecens*	Mean 8.4 ± 0.5µL^{-1} MC-LR [a,d]	Al-Tebrineh et al. 2011

Table 4 - Lakes were *Planktothrix* spp. has been reported to form recurrent blooms. **(a)** Bloom Sample/Environmental sample, **(b)** Filaments isolated from bloom samples, **(c)** anti-Adda ELISA Kit, **(d)** HPLC, **(e)** protein phosphatase 2A inhibition assay (PP2A), **(f)** Mean depth., (---) Information not available.

Planktothrix spp. occurrence in Portugal

For a long time, the presence of microcystins in Portuguese freshwater reservoirs has always been attributed to *Microcystis aeruginosa*. However, more recent studies have shown that their occurrence is also associated with other cyanobacteria. The occurrence of *Planktothrix* spp. in freshwater reservoirs in the centre and south of Portugal has been increasing in the last years. This last section is dedicated to the particular case of a freshwater reservoir that is being monitored over the last years, where a continuous *Planktothrix* bloom is present. *Planktothrix* species can be commonly found in Portuguese freshwater reservoirs. Some of the species reported are *P. mougeotii/P. isothrix* from a wastewater treatment plant in the north of Portugal (Vasconcelos & Pereira 2001, Martins et al. 2010), *P. rubescens* from Beliche reservoir in the South of Portugal (Paulino et al. 2009a) and *P. agardhii* and *P. pseudoagardhii* isolated from several reservoirs in the center and south of Portugal that are maintained in laboratory cultures (Paulino et al. 2009b). However, their occurrence is more pronounced in the center and south of Portugal where it has been increasing and causing problems in some water reservoirs over the last years, such as the deep layer *P. rubescens* bloom with associated microcystin production reported by Paulino et al. 2009. This last section is dedicated to the particular case of a drinking water reservoir located in the center of Portugal that has been monitored over the last eight years and where a continuous *Planktothrix* spp. bloom persists since 2006 (Fig. 5).

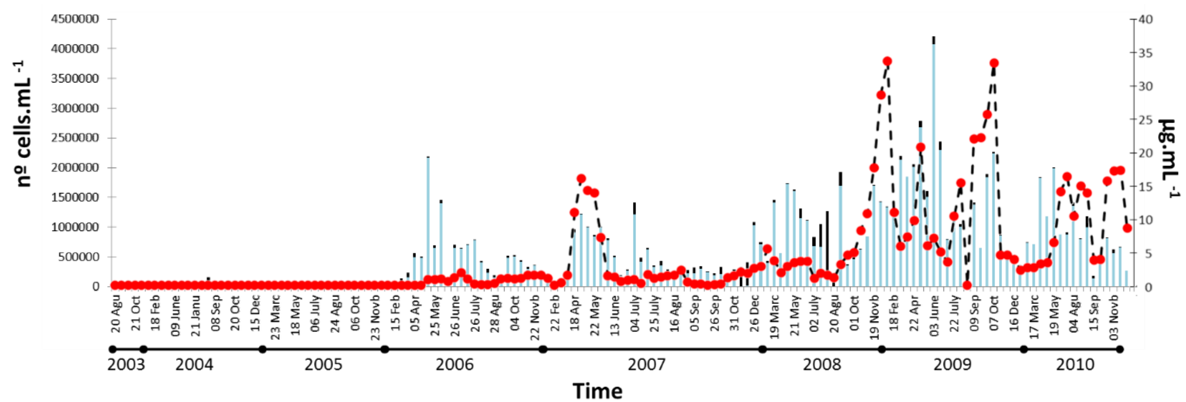

Fig. 5 - *Planktothrix* occurrence in a freshwater reservoir located in the center of Portugal and microcystin-LR concentration in raw water over the same sampling period (--•-- microcystin concentration in µg.mL $^{-1}$, light blue bars represent *Planktothrix* spp. cell concentration in nºcells.mL^{-1}, black bars represent total phytoplankton cell concentration in nºcells.mL^{-1}).

As it can be depicted from Fig. 5 high *Planktothrix* cell concentrations started to appear in the reservoir in 2006 and microcystin concentration increased significantly since 2007. Furthermore, the microcystin concentrations in raw water does not correlate with

Planktothrix cell numbers, since a high cell concentration does not indicate the presence of high microcystin concentrations and high concentrations of microcystins are not directly associated with high cell densities. This is probably because distinct strains/species of this genus with distinct ability to produce microcystins may occur together. In fact, a natural cyanobacterial population is usually a consortium of toxic and nontoxic strains, and this is believed to be the reason why the population toxicity can vary over time and between samples (WHO, 1999). As it can be seen by this monitoring data, *Planktothrix* can suddenly reach high cell densities and dominate the phytoplankton community presenting cell densities values close to total phytoplankton concentration. The figure also shows that *Planktothrix* can form perennial blooms but during this time no visible scum formation was observed within the reservoir. It is still unknown why this bloom of toxic *Planktothrix* persists for 5 years in this reservoir and the answer to this issue will be certainly an important contribution to the knowledge of cyanobacteria ecotoxicology. Since the begining of this P*lanktothrix* bloom this reservoir has been under strict vigilance: monitoring sampling is regular, cellular composition/densities and microcystin content in the samples are always screened and the water treatment plant efficiency analysed to avoid any possible harmfull effect on the population. Nevertheless, due to the persistence of high cell densities and high toxin contents occasionally observed, the reservoir represents a potential risk for human and wild life. Therefore, studies must be performed in order to understand the factors underlying the bloom appearance, persistence and toxicity and to access the risk that this reservoir represents to human health, in order to apply measures to prevent and manage the risk of *Planktothrix* occurrence in the reservoir and to restore the quality of this water-supply.

Conclusion

The risk of human exposure to toxic cyanobacteria is very difficult to assess because many scientific issues remain to be clarified, such as the toxicological properties of cyanotoxins and their real impact on human health. Nevertheless, the establishment of several guidelines for the most common toxins and the establishment of surveillance programs have contributed to minimize the human exposure to toxic cyanobacteria. However, particular attention should be taken for those species, such as *Planktothrix,* that develop particular strategies to adapt, survive and proliferate in freshwater environments. Therefore, the monitoring programs in water reservoirs where *Planktothrix* species occur must have into account that samples should be taken at several depths, microcystin concentration should be accessed constantly and the water

system should be monitored regularly throughout the years since perennial persistence of *Planktothrix* may occur. In water capture for potable water treatment plants the selection of water off-take depth is important and the infrastructures must be equipped with multiple off-takes. In water reservoirs where *Planktothrix* species occurs, certain particularities must be taken into account (Fig. 6) in order to implement the most adequate risk assessment procedures, monitoring programs and preventive measures to protect public health from cyanotoxin occurrence in freshwater supplies.

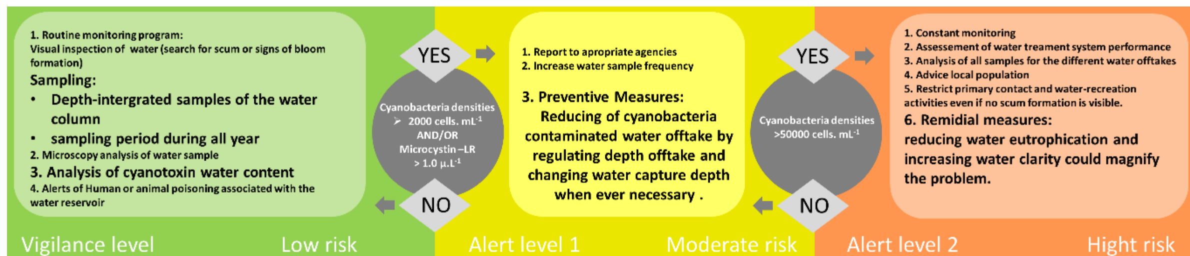

Fig. 6 - Schematic scheme of the steps involved in *Planktothrix* risk management.

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Chapter

03_

Profiling the cyanobacteria *Planktothrix* from Portuguese freshwaters: Identification, occurrence, distribution, and phylogeny

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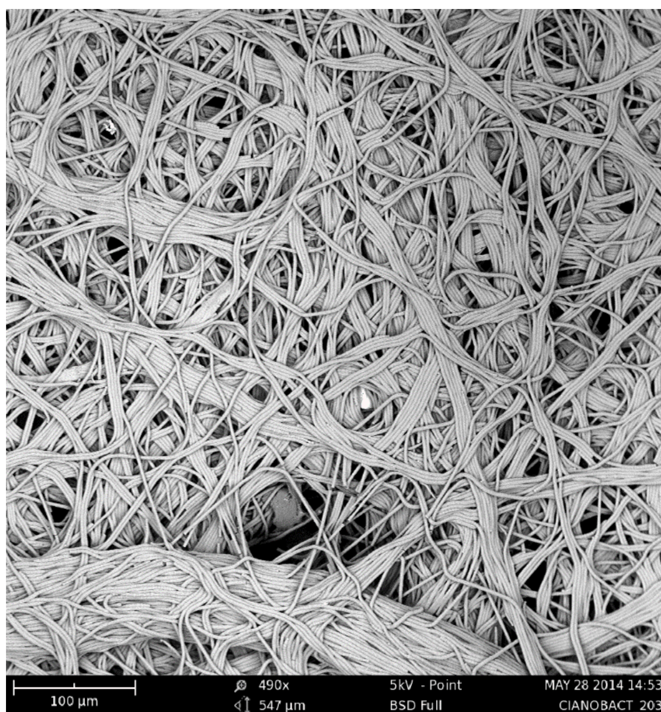

PROFILING THE CYANOBACTERIA *PLANKTOTHRIX* FROM PORTUGUESE FRESHWATERS: IDENTIFICATION, OCCURRENCE, DISTRIBUTION AND PHYLOGENY

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Abstract

Planktothrix is one of the most prevalent and widespread bloom-forming cyanobacteria and has been the cause of increasing public health concern due to the production of the hepatotoxins microcystins associated with the blooming. Although empirically recognized as a common constituent of the phytoplankton of Portuguese lakes the available information has never been gathered and little is known on the occurrence, distribution and species composition of *Planktothrix* in southwest European lakes.

In this study several *Planktothrix* monocultures isolated from Portuguese lakes were characterized according to their morphology, microcystin production and *rpo*C1 gene phylogeny. Additionally, based on the origin of the obtained cultures, observations/presence in environmental samples, monitoring and bibliographic records the occurrence of *Planktothrix* in Portuguese Lakes was mapped.

The Portuguese cultures isolated and analyzed in this study were identified morphologically as *P. agardhii*, *P. rubescens* and *P. mougeotii*. However, based on the *rpo*C1 phylogeny the isolates were clustered into three clades: *P.agardhii*/*P.rubescens*, *P. pseudoagardhii* and *P. mougeotii*. Microcystins were detected in 7 *P. agardhii* strains.

Gathering the results from the field samples with those from previous reports we conclude that *Planktothrix* is widely distributed in Portuguese freshwaters from north to south and that *P. agardhii* is the most commonly found and reported species with microcystin producers among them.

Keywords: Cyanobacteria, bloom-forming, microcystins, *Planktothrix*, *rpo*C1, systematics.

Introduction

Planktothrix Anagnostidis *et* Komárek 1988 is one of the most widespread bloom forming and microcystin producer cyanobacteria genera in freshwaters. These organisms form blooms that can prevail all year around (Pawlik-Skowroµska et al. 2008, Ernst et al. 2009, Kokocinski et al. 2011) and accumulate in the metalimnion, away from the surface of stratified lakes (Walsby and Schanz 2002), which may be overlooked by monitoring programs. An additional matter of concern is that, metalimnetic blooms of this genus can produce high levels of microcystins per biomass (Fastner et al. 1999). In Finland and Sweden, acute human illness was reported after microcystin contamination of drinking water collected from reservoirs undergoing *Planktothrix* blooms (Annadotter et al. 2001).

This genus is well delimited by morphologic characteristics and also according to 16S rRNA gene sequences similarity (Suda et al. 2002, Komárek and Komárková 2004) and is placed in the family Microcoleaceae within the order Oscillatoriales according to recent revisions by Komárek (2015) and Komárek et al. (2014). Nevertheless species delineation and identification within this genus is difficult and its intrageneric diversity and systematics is yet to be defined (D'Alelio and Gandolfi 2012, D'Alelio and Salmaso 2011, Komárek and Komárková 2004). There are currently fourteen *Planktothrix* species described plus three recently proposed species (*P. paucivesiculata*, *P. tepida*, *P. serta*) – see Table 1 (Gaget *et al.* 2015). Since most phenotypic characteristics of the several species overlap, the genetic characteristics are fundamental to define species boundaries.

Table 1 - Currently described *Planktothrix* species based on Suda *et al.* 2002, Komárek and Komárková 2004, Komárek and Anagnostidis 2005, Liu *et al.* 2013 and Gaget *et al.* 2015.

Species	Color	Cell width (µm)	Trichome end Apical cells	Calyptra	Constrictions at Cross-Walls	Observations
P. agardhii	Blue-green	2.3 - 9.8	Gradually attenuated Narrowed	+	-	
P. suspensa	Yellow-green Olive-green	2 - 4	Slightly attenuated Narrowed or conical	+	-	
P. prolifica	Reddish	2 - 5.8	Gradually attenuated Narrowed	+	-	
P. rubescens	Reddish	3.9 - 9.4	Gradually attenuated Narrowed	+	-	
P. paucivesiculata	blue-green	4.4 - 5.5	Not attenuated Rounded	-	-	
P.tepida	Blue-green	4.3 – 4.9	Not attenuated Rounded	-	-	
P. serta	Blackish-green	5.5 – 6.0	Not attenuated Rounded	-	-	
P. spiroides	Blue-green Olive-green	3.7 – 6.4	Gradually attenuated Rounded	-	-	Screw-like coiled filaments
P. arnoldii	Blue-green	9 - 10	Gradually attenuated Rounded	+	+	Freshwater; Asia
P. clathrata	Blue-green	5 - 8	Not attenuated Rounded	-	+	Freshwater; Oceania
P. compressa	Blue-green	5 - 9. 5	Not attenuated Rounded	-	+	
P. geitleri	Blue-green	5 - 11	Gradually attenuated widely rounded-obtuse	-	+	Marine; Asia
P. mougeotii	Dark blue-green to olive-green	5.9 – 7.8	Not attenuated Rounded	-	-	
P. planctonica	Blue-green	7.5 - 11.5	Not attenuated Rounded	-	+	screw-like coiled
P. isothrix	Blue-green	5.5 - 10	Slightly attenuated Rounded	+	-	
P. pseudagardhii	Pale blue-green, blue-green, yellow-green	3 - 6.4	Slightly attenuated Rounded	+	-	
P. zahidii	Olive-blue-green	2.5 - 3.5	Not attenuated Rounded	-	-	Freshwater; Asia

The first polyphasic approach to study this species was made by Suda et al. (2002), which resulted in the description of a new species – *P. pseudoagardhii* – and defined three distinct clusters with 16S rRNA phylogenies (*P. agardhii*, *P.pseudoagardhii* and *P. mougeotii*). Since then the genus received some attention and, based on molecular analysis, new species have been added – *P. spiroides* – (Liu et al. 2013) and withdraw - *P. cryptovaginata* is now *Limnoraphis cryptovaginata* – (Komárek et al. 2013).

The resolution of the intrageneric diversity within *Planktothrix* and the knowledge on its distribution and species diversity is important for identification and management in routine monitoring programs. In Portugal, *Planktothrix* has been pointed out to cause

blooms associated with microcystin production (Churro et al. 2012, Paulino et al. 2009). However little is known on the occurrence, distribution, species composition and toxin production of these harmful bloom-forming cyanbacteria in southwest European lakes particularly in Portugal.

In this study, we examined the morphology; phylogeny and microcystin production of *Planktothrix* cultures isolated from different Lakes and also gathered bibliographic and monitoring information on *Planktothrix* occurrences in order to access the diversity and distribution of this genus in Portuguese freshwaters.

Methods

Bibliographic and monitoring records

Bibliographic records of *Planktothrix* occurrence and also monitoring data held at the laboratory of Biology and Ecotoxicology from the National Institute of Health Doutor Ricardo Jorge were consulted to narrow down the field surveys for *Planktothrix* sampling and map the occurrence of *Planktothrix* species in Portuguese inland waters.

Field surveys and sample collection

Between 2007 and 2013 a total of 23 lakes across Portugal were sampled. The samples were checked for the presence of *Planktothrix* and used for the isolation of filaments. The list of the water bodies sampled is presented in Table 2. The composed samples were taken at several depths with a Van Dorn bottle and transported in refrigerated conditions to the laboratory. In lakes were sampling in depth could not be performed only surface samples were collected.

Table 2 - Portuguese Lakes sampled during field surveys from 2007 to 2013.

Lake	Coordinates	*Planktothrix* Presence
Agolada de Baixo	38°58'03.5"N 8°33'51.5"W	Yes
Alvito	38°16'56.2"N 7°54'48.1"W	Yes
Belver	39°28'57.4"N 8°00'01.5"W	Yes
Castelo de Bode	39°32'39.5"N 8°19'07.3"W	No
Cinco Reis	38°00'59.6"N 7°56'15.2"W	Yes
Fratel	39°32'35.4"N 7°48'06.2"W	Yes
Lucefecit	38°38'06.8"N 7°24'28.0"W	Yes
Magos	38°59'35.2"N 8°41'00.3"W	Yes
Marechal Carmona	39°58'11.6"N 7°11'42.1"W	No
Mte da Rocha	37°42'34.9"N 8°17'22.4"W	Yes
Mte Novo	38°31'47.3"N 7°43'16.7"W	Yes
Odivelas	38°11'23.1"N 8°05'40.9"W	Yes
Patudos	39°14'50.7"N 8°35'20.5"W	Yes
Pegões	38°40'01.6"N 8°31'38.6"W	No
Pego do Altar	38°25'10.5"N 8°23'25.3"W	Yes
Pracana	39°33'51.5"N 7°48'39.7"W	Yes
Rio de Mula	38°45'50.2"N 9°25'20.9"W	Yes
Roxo	37°56'45.0"N 8°03'24.8"W	Yes
S. Domingos	39°20'02.4"N 9°19'02.4"W	Yes
Sta Águeda	39°58'09.1"N 7°28'52.6"W	Yes
Vale do Cobrão	38°49'23"N 8°46'4"W	No
Vale do Gaio	38°15'58.7"N 8°15'55.7"W	Yes
Viriato	40°18'38.9"N 7°33'48.1"W	No

n.a – not applicable

Planktothrix isolation and culture conditions

Filaments from the genus *Planktothrix* were isolated from fresh samples with a micropipette under the inverted microscope Olympus® CK40. Cultures were established by transferring the isolated filaments into Z8 culture medium (Staub 1961). Successful cultures were maintained at 20 ± 1 °C with a light intensity of 15 $\mu mol.photons.m^{-2}.s^{-1}$ and a 16:8 h light:dark cycle. The list of *Planktothrix* cultures established and used in this study is displayed in Table 3.

Morphological analysis

Light microscopy

The isolates were studied using an Olympus® BX60 light microscope. Photographs were taken with an Olympus® DP11digital camera under 1000x magnification. The cell measurements were performed using ImageJ software (Schneider et al. 2012). The morphological characters evaluated were: cell dimensions (length and width), presence of sheath, cell wall constrictions, presence/absence of calyptra, shape of apical cell, presence/absence of necredia cells, filament color and shape. At least 50 measurements were done for calculating the size of the cells and 30 filaments of each isolate were characterized.

Scanning electron microscopy (SEM)

Samples of the isolates LMECYA203, LMECYA162, LEGE07229 and CHURRO10, representing the species *P. rubescens*, *P. pseudoagardhii*, *P. mougeotii* and *P. agardhii* respectively, were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer and dehydrated through a series of ethanol. The prepared material was examined with a Phenom® ProX desktop scanning electron microscope. The characteristics evaluated were: filament shape, structure of the apical cell, filament end and calyptra.

Phylogenetic analysis

DNA extraction

Cultures were grown in 4L bioreactors, containing Z8 medium, under continuous aeration to obtain biomass for DNA extraction. The biomass was harvested after 24h decantation and lyophilized in a freeze drier (Micromodul Y10, Savant®). The DNA was extracted from the lyophilized biomass using the Invisorb® Spin Plant Mini Kit. Total DNA concentration was quantified using a NanoDrop® ND-1000 (Thermo Fisher Scientific®) spectrophotometer by measuring UV absorbance at 260 nm.

Fragment amplification and sequencing

A 608 bp fragment within the *rpo*C1 gene was amplified with the primers RPOF (5'-TGGTCAAGTGGTTGGAGA-3') and RPOR (5'- GCCGTAAATCGGGAGGAA-3') (Lin et al., 2010). The amplifications were performed in a reaction mixture containing 1× PCR buffer (Invitrogen™), 0.05 mM dNTPs (Invitrogen™), 0.2 µM each primer, 2 mM MgCl2 (Invitrogen™), 2 µL of DNA extract and 1 U of Taq DNA polymerase (Invitrogen™) using a TGradient Thermocycler (Biometra) programed with a PCR cycle consisting of an initial denaturation step at 94 °C for 3 min, followed by 40 cycles of 20 s at 94 °C, 30 s at 55 °C and 20 s at 72 °C and a final extension step of 5 min at 72 °C. The amplified fragments were visualized under UV light after electrophoretic analysis performed in 0.8 % w/v agarose gel with GelRed™ DNA staining (Biotium®), at 80 V in 0.5× Tris-borate EDTA (TBE) buffer for 40 min. The amplified PCR products were purified using the PCR DNA and gel band purification kit ilustra™ GFX™ (GE Healthcare®). The DNA sequences from the amplified fragments were obtained by Sanger sequencing on a 3130xl Genetic Analyzer equipped with a 50 cm-capillary array (Applied Biosystems®) using BigDye® Terminator v1.1 solution (Life Technologies®).

Sequence analysis

A BioEdit (Hall et al. 1999) file was created with a total of 186 *rpo*C1 sequences including: 132 database sequences (NCBI), 54 sequences from the isolates in analysis in this study and the outgroup (*Prochlorococcus marinus* NATL1A). The nucleotide alignment was performed on MAFT using the accuracy-oriented model L-INS-I.

MrBayes v.3.2.2 (Ronquist et al., 2012) was used to implement a Bayesian inference (BI) consisting in a Bayesian MCMC program that creates phylogenetic inferences using all existing models of nucleotide, amino acid and codon substitutions (Yang and Rannala, 2012). The MCMC method guaranteed consistency (Yang and Rannala, 2012), assuring that the sampling of trees is proportional to their probability of occurrence with the chosen evolution model. The Bayesian analysis conditions were set in order that the likelihood scores of the trees reach stationary within the generation's time, with a total of 1.5x106 generations sampled every 100 generations from which the first 5% of the trees were discarded.

The MCMCMC sampling algorithm was used to estimate posterior probabilities (pp) of the phylogenetic trees. In this work we consider that phylogenetic relations are trustable and strong when presenting a pp above 0.7.

Microcystin production analysis

Microcystins were extracted from the lyophilized biomass with 70% methanol (10 mL/100 mg dry weight) for 2h under magnetic stirring. The extracts were further sonicated with an ultrasonic probe (Sonics Vibra-Cell CV33, Sonics & Materials Inc.), centrifuged and the pellets were re-extracted overnight by the same procedure. Supernatants of the two extractions were combined and subjected to rotary evaporation at 35ºC (Buchi-R, Flawil, Switzerland) to eliminate methanol. The resulting aqueous extracts were cleaned-up by solid phase extraction on Sep-Pak C18 cartridges (500 mg Waters, Massachusetts) previously activated with 20 mL of ethanol and equilibrated with 20 mL of distilled water. The microcystin containing fraction was eluted with methanol at 80% (v/v) and the methanolic fraction evaporated. The resulting solution was filtered through 0,45 μm syringe filters and analyzed by HPLC-DAD according to the ISO 20179:2005, using commercially available MC-LR, MC-RR and MC-YR standards (Alexis® Biochemicals).

Results

Identification of *Planktothrix* isolates and microcystin production

A total of 32 *Planktothrix* isolates from field surveys were cultured with success in samples from field surveys that originated from 5 different lakes (Table 2 and 3). All the isolates were morphologically examined and identified as belonging to *Planktothrix agardhii* group according to the descriptions in Suda et al. (2002) and Komárek and Anagnostidis (2005). The filaments were blue-green, straight, solitary and planktonic. Although they were buoyant and form mats in the surface of the liquid medium they also formed biofilms in the bottom and sides of the culture flasks. All the filaments from *P. agardhii* cultures had the ability to move with oscillation especially in recent established cultures. The cells were in average 3.64 ± 0.376 µm wide and 2.32 ± 0.490 µm long and no constrictions at the cross-walls were observed (Table 4). Two types of filament ends and apical cells were observed and typically belonging to the same filament (Fig. 1). In one end the filaments were gradually attenuated and the apical cells narrowed towards the end with a well-developed triangular hyaline calyptra whereas in the other end

Table 3 - *Planktothrix* cultures used in the study.

Species	Author Strain number*[1]	Other culture collections Strain numbers	Origin	Date of Collection	Toxin Production (ug/mg dry Weight)	Reference for toxin production
Planktothrix agardhii		LMECYA 153A	Enxoé	February 2003	Non toxic for MCYS	This study
P. agardhii		LMECYA 153B	Enxoé	February 2003	Non toxic for MCYS	This study
P. agardhii		LMECYA 153C	Enxoé	February 2003	Non toxic for MCYS	This study
P. agardhii		LMECYA 155	Enxoé	February 2003	Non toxic for MCYS	This study
P. agardhii	CHURRO2	LMECYA 229	S. Domingos	March 2007	Non toxic for MCYS	This study
P. agardhii	CHURRO3	LMECYA 229A	S. Domingos	March 2007	Non toxic for MCYS	
P. agardhii	CHURRO14	LMECYA 230	S. Domingos	March 2007	Non toxic for MCYS	This study
P. agardhii	CHURRO5	LMECYA 250	S. Domingos	March 2009	Non toxic for MCYS	This study
P. agardhii	CHURRO7	LMECYA 256	S. Domingos	June 2009	MCY-RR 0,92 MCY-LR 0,09	This study
P. agardhii	CHURRO6	LMECYA 257	S. Domingos	June 2009	Non toxic for MCYS	This study
P. agardhii	CHURRO12	LMECYA 258	S. Domingos	June 2009	MCY-RR 0,17	This study
P. agardhii	CHURRO15	LMECYA 259	S. Domingos	June 2009	MCY-RR 0,35	This study
P. agardhii	CHURRO10	LMECYA 260	S. Domingos	June 2009	Non toxic for MCYS	This study
P. agardhii	CHURRO11	LMECYA 269	Magos	July 2009	Non toxic for MCYS	This study
P. agardhii	CHURRO8	LMECYA 270	Magos	July 2009	Non toxic for MCYS	This study
P. agardhii	CHURRO13	LMECYA 275	Lucefecit	July 2009	Non toxic for MCYS	This study
P. agardhii	CHURRO8	LMECYA 277	Portalegre	August 2011	Non toxic for MCYS	This study
P. agardhii	CHURRO16	LMECYA 280	S. Domingos	August 2012	Non toxic for MCYS	This study
P. agardhii	CHURRO17	LMECYA 281	S. Domingos	August 2012	Non toxic for MCYS	This study
P. agardhii	CHURRO18	LMECYA 283	Patudos	October 2012	Non toxic for MCYS	This study
P. agardhii	CHURRO19	LMECYA 284	Patudos	October 2012	Non toxic for MCYS	This study
P. agardhii	CHURRO20	LMECYA 285	Patudos	October 2012	Non toxic for MCYS	This study
P. agardhii	CHURRO21	LMECYA 286	Patudos	October 2012	Non toxic for MCYS	This study
P. agardhii	CHURRO22	LMECYA292	S. Domingos	January 2013	Non toxic for MCYS	This study
P. agardhii	CHURRO23	LMECYA293	S. Domingos	January 2013	Non toxic for MCYS	This study
P. agardhii	CHURRO26	LMECYA294	S. Domingos	January 2013	Non toxic for MCYS	This study
P. agardhii	CHURRO25	LMECYA297	S. Domingos	May 2013	Non toxic for MCYS	This study
P. agardhii	CHURRO24	LMECYA298	S. Domingos	May 2013	MCY-RR 1,83 MCY-LR 0,11	This study
P. agardhii	CHURRO29	LMECYA301	S. Domingos	May 2013	Non toxic for MCYS	This study
P. agardhii	CHURRO30	LMECYA302	S. Domingos	June 2013	Non toxic for MCYS	This study
P. agardhii	CHURRO31	LMECYA303	S. Domingos	June 2013	Non toxic for MCYS	This study
P. agardhii	CHURRO38	LMECYA304	S. Domingos	June 2013	Non toxic for MCYS	This study
P. agardhii	CHURRO35	LMECYA305	S. Domingos	June 2013	MCY-RR 1,60 MCY-LR 0,12	This study
P. agardhii	CHURRO34	LMECYA306	S. Domingos	June 2013	MCY-RR 1,10 MCY-LR 0,09	This study
P. agardhii	CHURRO33	LMECYA307	S. Domingos	June 2013	MCY-RR 1,81 MCY-LR 0,10	This study
P. agardhii		CCALA159	Plon, Germany	1969	MCYS*[2]	This study
Planktotrix pseudoagardhii		LMECYA 162	Guadiana River, Mértola	July 2003	Non toxic for MCYS	This study
P. pseudoagardhii		LMECYA 224	Bufo	August 2005	Non toxic for MCYS	This study
P. pseudoagardhii	CHURRO4	LMECYA 276	Magos	May 2011	Non toxic for MCYS	This study
P. pseudoagardhii	CHURRO32	LMECYA 310	Magos	October 2010		
Planktothrix rubescens		LMECYA 203	Beliche	June 2005	MCY-RR 0,93 MCY-LR 0,07	This study, Paulino et al. 2009
Planktothrix mougeotii		LEGE 06224	Febros WWTP, Gaia	2006-2007	Non toxic for MCYS	This study, Martins et al. 2010
P. mougeotii		LEGE 06225	Febros WWTP, Gaia	2006-2007	Non toxic for MCYS	Martins et al. 2010
P. mougeotii		LEGE 06226	Febros WWTP, Gaia	2006-2007	Non toxic for MCYS	Martins et al. 2010
P. mougeotii		LEGE 06233	Febros WWTP, Gaia	2006-2007	Non toxic for MCYS	Martins et al. 2010
P. mougeotii		LEGE 07227	Febros WWTP, Gaia	2006-2007	Non toxic for MCYS	This study, Martins et al. 2010
P. mougeotii		LEGE 07229	Febros WWTP, Gaia	2006-2007	Non toxic for MCYS	This study, Martins et al. 2010
P. mougeotii		LEGE 07230	Febros WWTP, Gaia	2006-2007	Non toxic for MCYS	This study, Martins et al. 2010
P. mougeotii		LEGE 07231	Febros WWTP, Gaia	2006-2007	Non toxic for MCYS	This study, Martins et al. 2010

*[1] These strains were isolated by C.Churro during field surveys. *[2] Strain tested with ELISA Kit. WWTP - waste water Treatment Plant. MCY – Microcystin.

the filament was not attenuated and the apical cells were rounded without calyptra (Fig.1A-C). The cells had prominent gas vacuoles giving a golden sparkle color to the filaments. Two different morphotypes were detected: the isolate CHURRO5 and the

isolates CHURRO4 and CHURRO32. The cells from isolate CHURRO5 were wider (5.42 ± 0.371 µm) and longer (3.39 ± 0.660 µm) the filament ends were slightly attenuated with a smaller calyptra (Fig. 1F-G). The isolates CHURRO4 and CHURRO32 were paler in color with gas vacuoles scattered around the periphery of the filament, the apical cells were slightly attenuated with a small hyaline calyptra. All other characteristics were as described above; therefore these isolates were also identified as *P. agardhii*. The Portuguese isolates belonging to *P. rubescens* and *P. mougeotii* were previously identified by other authors (Paulino et al. 2009 and Martins et al. 2010, respectively) but were also characterized in this study (Table 4). *P. rubescens* LMECYA203 was morphologic identical to *P. agardhii* but exhibit red colored filaments (Fig. 1K-M). The filaments were markedly narrowed towards the ends with a characteristic bottle shape end and pronounced calyptra (Fig. 1L). *P. mougeotii* was very different from the other isolates: the filaments were wider, not attenuated towards the ends and with widely rounded apical cell and exhibiting benthic behavior by attaching to the bottom of culture flasks (Fig. 1N-O).

In an attempt to identify features that could be distinctive between species the surface cell ultrastructure of the filaments was also visualized using a scanning electron microscope (Fig. 2). No major differences were observed between *P. agardhii*, *P. rubescens* and *P. pseudoagardhii*. Nevertheless the two types of filaments ends are clearly visible. In *P. mougeotii* the apical cell is rounded with a smooth area on the top that resembles a calyptra surrounded by pore like structures (Fig. 2G). This structure is also visible in light microscopy (Fig. 1N) and also resembles a small wide hyaline calyptra. It is also visible by SEM the existence of necredia in *P. mougeotii* (Fig. 2H).

The *rpo*C1 phylogenetic analysis revealed that the 32 Portuguese *Planktothrix* isolates distributed themselves throughout three distinctive main clusters: *P.agardhii*/*P.rubescens* cluster, *P. pseudoagardhii* cluster and *P. mougeotii* cluster (Fig. 3), all presenting pp above 0.68.

Microcystin production was detected in six isolates of *P. agardhii* collected from the same reservoir, were the variants of MC-LR and MC-RR were the ones detected (Table 3).

Table 4 - Morphologic characteristics of *Planktothrix* isolates from Portuguese Lakes.

Species	Color	Cell width (µm)	Cell Length (µm)	Thricome end	Apical Cells	Constrictions at Cross-Walls	Calyptra	Necredia
P. agardhii	Blue-green	3,64 ± 0,376	2,32 ± 0,490	Continually attenuated	Narrowed	-	+	-
P. pseudoagardhii	Pale blue-green	3.23 ± 0.266	3.14 ± 0.580	Continually attenuated	Narrowed	-	+	-
P. rubescens	Reddish	4.29 ± 0.813	3.31 ± 0.728	Continually attenuated	Narrowed	-	+	-
P. mougeotii	Dark green	6.27±0.536	3.06±0.467	Filament strait	Rounded	-	+	+

Planktothrix distribution in Portuguese Lakes

The information on *Planktothrix* occurrence was gathered and systematized in order to map *Planktothrix* occurrence and distribution. Figure 4 summarizes the lakes where *Planktothrix* was observed, based on field observations, established cultures, and bibliographic and monitoring records. It can be seen that *Planktothrix* is present across lakes from North to South Portugal and that *P. agardhii* was the most observed/reported species. *P. pseudoagardhii* was only reported in center and south of Portugal and *P. mougeotii* in the center and north near the coast and mainly in waste water treatment plants. In Lakes - Enxoé, Magos, Roxo and S. Domingos - *Planktothrix agardhii* was recurrently present.

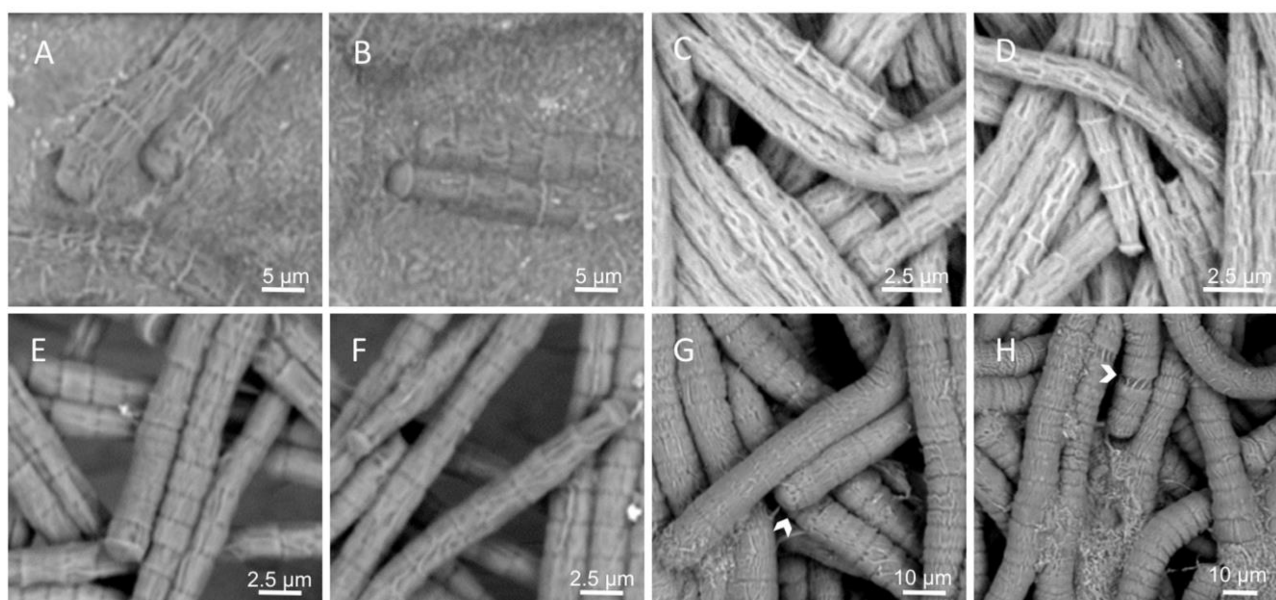

Fig. 2 - Scanning electron micrographs of four *Planktothrix* strains representing the cylindrical shape of the filaments and the surface morphology of the different filament ends. A and B - *P. agardhii* (CHURRO10); C and D – *P. rubescens* (LMECYA203); E and F – *P. pseudoagardhii* (LMECYA162); G and H – *P. mougeotii* (LEGE 06226). The arrow indicates the calyptra in G and the necredia in H.

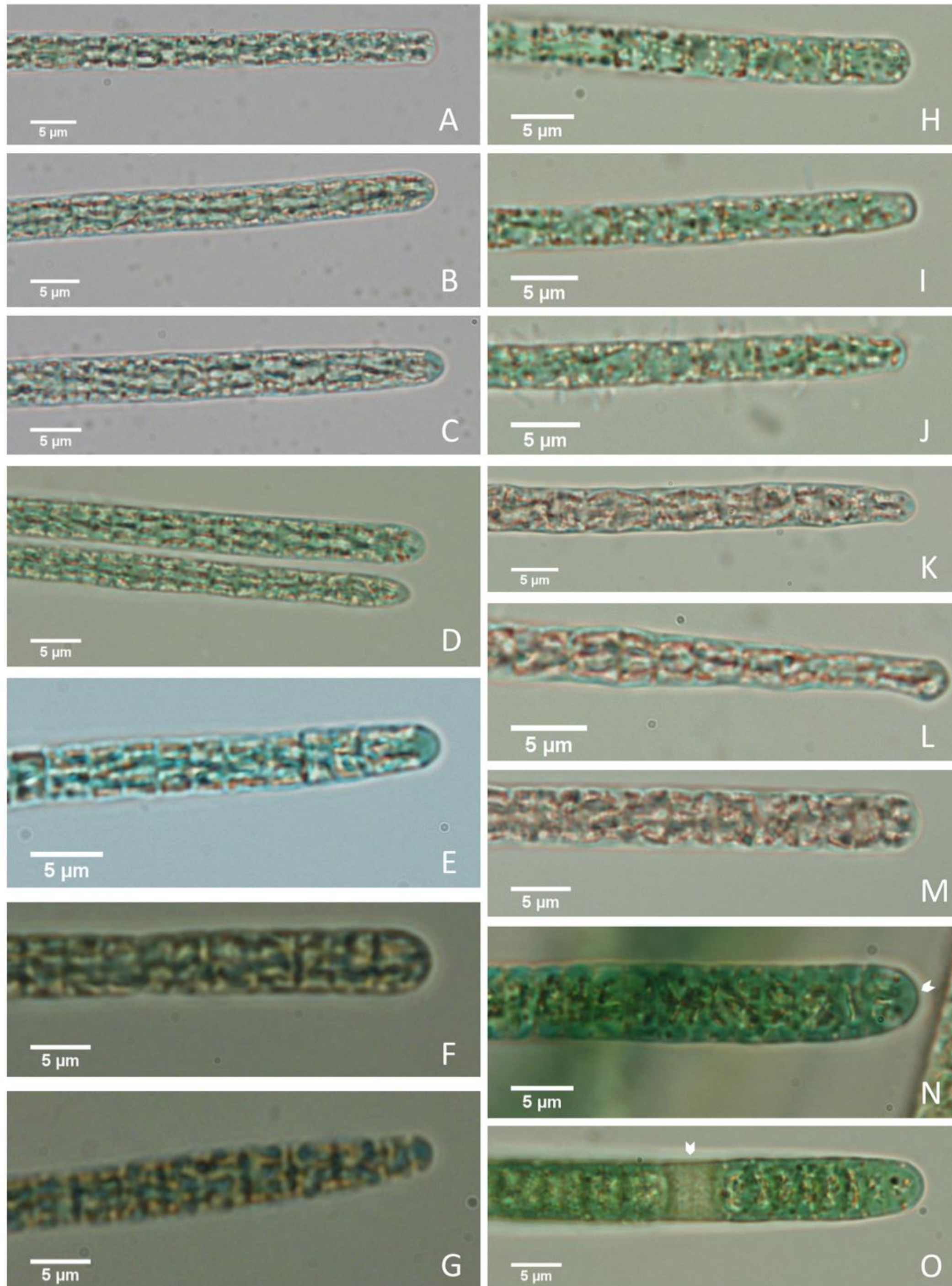

Fig. 1 - Light microscopy photographs of *Planktothrix* strains isolated from Portuguese freshwater reservoirs. A – C: *P. agardhii* CHURRO10; D: *P. agardhii* CHURRO23; E: *P. agardhii* CHURRO29; F-G: *P. agardhii* CHURRO5; H-J: *P. pseudoagardhii* LMECYA162; K-M: *P. rubescens* LMECYA203; N-O: *P. mougeotii* LEGE 06226. The arrow indicates the calyptra in N and the necredia in O.

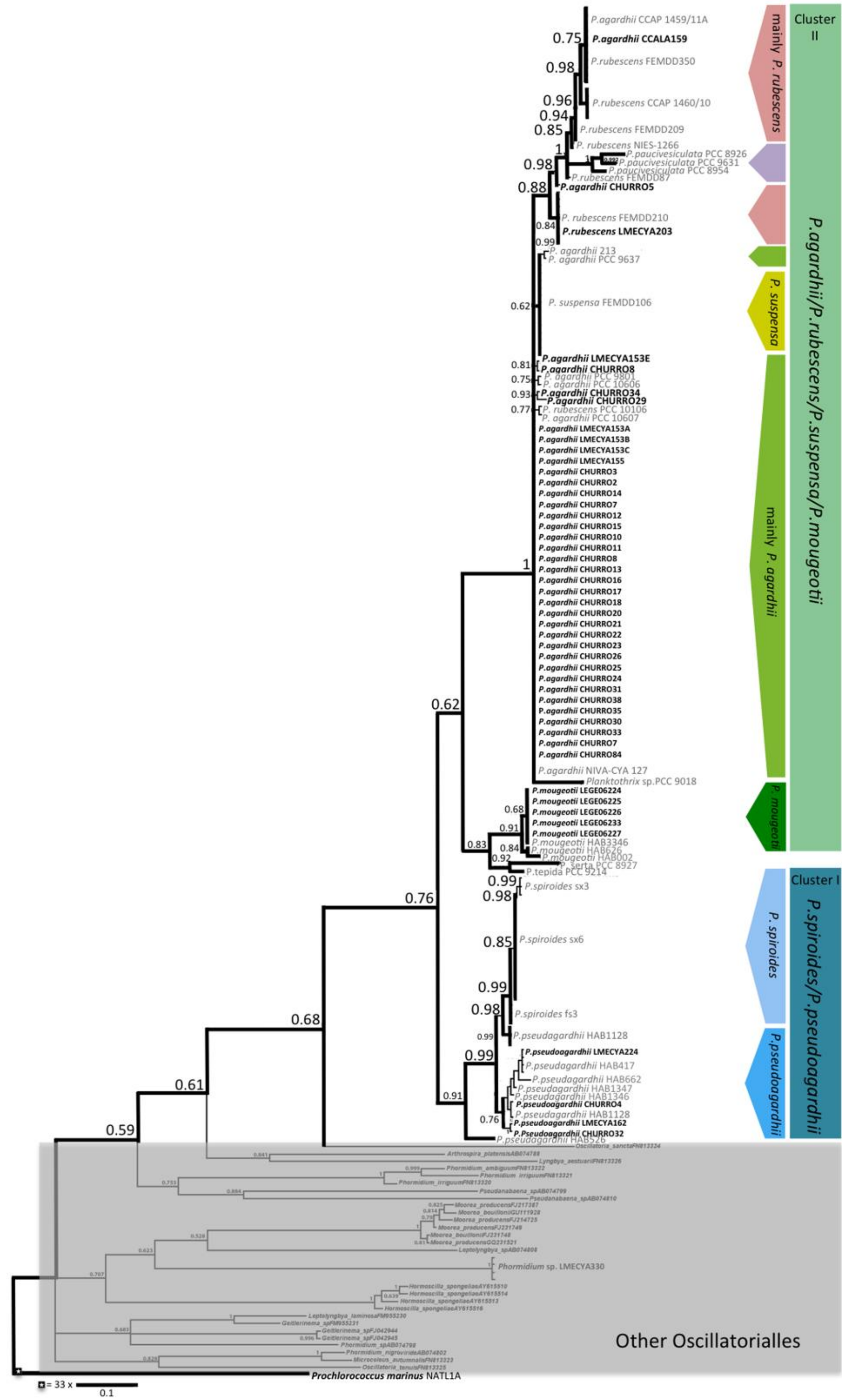

Fig. 3 - Bayesian tree based on *rpo*C1 sequences of *Planktothrix* isolates in this study. Sequences from GenBank are indicated with strain numbers. *Planktothrix* isolates are shown in bold letters.

Fig. 4 - *Planktothrix* occurrence in Portuguese Lakes, based on observations of field samples, established cultures, monitoring and bibliographic records (Vasconcelos and Pereira 2001, Pereira et al. 2001, Galvão et al. 2008; Valério et al. 2008; Paulino et al. 2009; Paulino et al. 2009a, Martins et al. 2010; Bellém et al. 2012; Galvão et al. 2012; Kurmayer et al. 2015, http://acoi.ci.uc.pt). WWTP – Waste Water Treatment Plant.

Discussion

From the fourteen species of *Planktothrix* described worldwide, four – *P. agardhii*, *P. rubescens*, *P. prolifica*, *P. suspensa* - have been reported to cause blooms (Pawlik-Skowroµska et al. 2008, Ernst et al. 2009, Kokocinski et al. 2011, D'alelio and Salmaso 2011, Larson et al. 2014). These bloom-forming species have similar cell dimensions and morphological features. Also, in 16S rRNA and *rpo*C1 phylogenies they belong to the same monophyletic group (Suda et al. 2002, Lin et al. 2010, Gaget et al. 2015, D'alelio and Salmaso 2011). *P. agardhii* and *P. rubescens* are the most commonly found species. They form severe and persistent blooms and are mainly microcystin producers. Although they clustered in the same phylogenetic group the first is green pigmented and second is red pigmented, occupying, different ecological niches. In fact *P. agardhii* is phycocyanin-rich and frequently found in shallow eutrophic to Hypereutrophic lakes all across middle Europe (Poulícková et al. 2004, Catherine et al. 2008, Pawlik-Skowroµska et al. 2008, Farkas et al. 2014). *P. rubescens* is phycoerythrin-rich generally occurring in deeper, stratified meso to oligothrophic and is widely distributed in the north of Europe and southern European Alpine Lakes (Legnani et al. 2005, Halstvedt et al. 2007, Bogialli et al. 2012). *P. agardhii* and *P. rubescens* are rarely found co-occurring (Davis and Walsby 2002, Davis et al. 2003, Halstvedt et al. 2007, Hossain et al. 2012), and some isolates have been found to segregate each other (Oberhaus et al. 2008). One can speculated that these two organisms are undergoing the speciation process and thus constitute two distinct morphotypes not differentiated genetically or the molecular markers that have been used so far to resolve these species phylogeny do not reflect their true genomic differences. *P. prolifica* is very similar to *P. rubescens* and is mainly reported from deep Nordic Lakes (Larson et al. 2014, Rakko and Ott 2014). *P. suspensa* is a morphotype of *P. agardhii* reported to cause blooms in deep Italian Alpine Lakes (D'alelio and Salmaso 2011). The opposite of *P agardhii*/*P. rubescens* is *P. agardhii* and *P. pseudoagardhii*. These species are very similar morphologically but form distinct phylogenetic groups where *P. pseudoagardhii* seem to have diverged earlier in the phylogenetic tree, where it assumes a basal position, being closer related with *P. spiroides*. In this study most of the *Planktothrix* isolates isolated from freshwater lakes were *P. agardhii*, and similarly to other studies (Lin et al. 2010, D'alelio and Salmaso 2011, Gaget et al. 2015) they were closely related with *P. rubescens* and not distinguished in the *rpo*C1 phylogeny. Furthermore, two isolates morphologically identified as *P. agardhii* were closely related to *P. pseudoagardhii*. Similar results were obtained by Lin et al. (2010), when analyzing *Planktothrix* isolates from China, where *P. agardhii* and *P. pseudoagardhii* were

morphologically indistinguishable. The other Portuguese isolates analyzed in this study were *P. mougeotii*. There is some confusion in the description of *P. mougeotii* (see Suda et al. 2002). In this study, the *P. mougeotii* name *sensu* Suda et al. (2002) - Basonym: *Oscillatoria mougeotii* Kutzing ex Lemmermann - was used and attributed to the Portuguese isolates since the description was very similar to Suda et al. (2002) and Lin et al. (2010) and the isolates clustered with *P. mougeotii* isolates from Lin et al. (2010) in the *rpo*C1 phylogeny. Furthermore, the SEM analysis of the *P. mougeotii* isolates from this study revealed a calyptra like structure that has not been described for this species.

Routine monitoring of cyanobacteria is mainly based in morphologic species identification by optical microscopy and *Planktothrix* species identification becomes intricate and complicated since the phenotypic species doesn´t reflect the genetic species and *vice versa*. Recently, Liu et al. (2013) described a coiled filamentous *Arthrospira* like cyanobacterium belonging to the genus *Planktothrix* based on 16S rRNA gene sequence data and that is also a bloom former. These new addition highlights the need for a revision in the description of the genus *Planktothrix* as recently proposed by Gaget el al. (2015). Furthermore, *P. arnoldii*, *P. clathrata*, *P. compressa*, *P. geitleri*, *P. planctonica*, *P. zahidii* have only been described according to their morphology and lack the molecular information to support them.

The definition of species limits is important in order to attribute all sorts of characteristics – toxin production, bloom behavior, ecological niches, etc. – to a particular entity. The systematization of the information will, in turn, help, the monitoring, bloom management and risk assessment.

Regarding our results on *Planktothrix* occurrence and distribution, we found that *P. agardhii* was present in 78% (18/23) of the sampled lakes and was also the main reported species in monitoring and bibliographic records. These results are in accordance with the reported occurrences for other countries in Europe. In France is one of the most frequently found species (Catherine et al. 2008) and was also found as a dominant species in 40% of Czech fishponds (Poulícková et al. 2004). Blooms are also common in Poland, Hungary, Germany and Serbia (Budzyńska et al. 2009, Pawlik-Skowroµska et al. 2008, Farkas et al. 2014, Svirčev et al. 2007). From all the *P. agardhii* isolates isolated during this study the microcystin producing ones were only obtained from one lake. In the monitoring records microcystins were detected in most of the lakes, but there were also other microcystin producing species present, such as, *Microcystis aeruginosa*.

P. pseudoagardhii was mainly detected in the southeast. The information on *P. pseudargardhii* distribution is scarce and as Kurmayer et al. (2015) point out the *P. pseudoagardhii* isolates reported, are from warm areas like East and South Africa, Thailand and China (Kurmayer et al. 2015, Conradie et al. 2008, Suda et al. 2002, Lin et al. 2010). Portugal has a Mediterranean climate and is warmer in the south than in the north. Nevertheless, its distribution might be underestimated since its separation from *P. agardhii* is mainly achieved by molecular information.

P. mougeotii was present in the northwest and reported for wastewater treatment plants and marshes (wetland) which are shallow and have high content of organic matter. Similar to this, the *P. mougeotii* isolates studied by Lin et al. (2010) were mainly isolated from Manmade Wetlands or marshes that are also used for wastewater treatment or storm water runoff.

In Portugal *P. rubescens* was only reported in one lake so far and is a microcystin producing isolate (Paulino et al. 2009). This species was not found during the field surveys in this study. *P. rubescens* is mainly reported in Europe from deep north lakes and alpine lakes characterize by cold temperatures. Nevertheless, recently reports of its occurrence include deep Mediterranean lakes, in Turkey, Greece, Italy and Spain. However, due to its preference/adaptation to deep-water layers, an inadequate water sampling may underestimate its occurrence (Churro et al. 2012, Dokulil and Teubner 2012).

We consider that the available data do not enable yet neither to identify particular niches of *Planktothrix* species nor to map it's spreading throughout Portuguese freshwater reservoirs, since these species are often misidentified and, inclusive, only the genus is referred in many reports. Nevertheless, this report constitutes a comprehensive overview of the current situation of *Planktothrix* occurrence in Portuguese freshwaters. Furthermore, *Planktothrix* was formerly classified into to the genus *Oscillatoria*, which contributed to the undervaluation of *Planktothrix* occurrence overtime (Churro et al. 2012). However, in a survey made by Valério et al. (2008) in southeast Portugal, *Planktothrix* was found to be the main component in the phytoplankton in the lakes Enxoé and Roxo and was not found in the lakes Alqueva, Alvito, Odivelas e Monte Novo. The Alqueva dam became operational in 2002, and is the largest artificial water reservoir in Europe. Since its construction all the pre-existent lakes and the new built ones in an area with about 120,000 hectares are being connected in a pipe network called the “global irrigation system” that is expected to be completed by 2016. In this study *Planktothrix* was widely reported in monitoring records

in Lakes surrounding the Alqueva and was also observed in field surveys in the lakes Alvito, Odivelas and Monte Novo.

What the data gathered in this study can conclude for sure is that the *Planktohrix* genus is a common constituent of phytoplankton, being distributed broadly across Portuguese lakes and its most representative species is *P. agardhii.* Moreover, although most the *Planktothrix* collected from Portuguese freshwaters were non-toxic some of the *P. agardhii* isolates were hepatotoxic.

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Chapter

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ORIGINAL PAPER

Species-specific real-time PCR cell number quantification of the bloom-forming cyanobacterium *Planktothrix agardhii*

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Abstract A species-specific method to detect and quantify *Planktothrix agardhii* was developed by combining the SYBR Green I real-time polymerase chain reaction technique with a simplified DNA extraction procedure for standard curve preparation. Newly designed PCR primers were used to amplify a specific fragment within the *rpoC1* gene. Since this gene exists in single copy in the genome, it allows the direct achievement of cell concentrations. The cell concentration determined by real-time PCR showed a linear correlation with the cell concentration determined from direct microscopic counts. The detection limit for cell quantification of the method was 8 cells μL^{-1}, corresponding to 32 cells per reaction. Furthermore, the real-time qPCR method described in this study allowed a successful quantification of *P. agardhii* from environmental water samples, showing that this protocol is an accurate and economic tool for a rapid absolute quantification of the potentially toxic cyanobacterium *P. agardhii*.

Keywords Cyanobacteria · *Planktothrix agardhii* · Real-time qPCR · *rpoC1* gene

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Introduction

The genus *Planktothrix* comprises several species that are responsible for cyanobacteria blooms in freshwater resources, some of them being able to produce several kinds of toxins (Falconer and Humpage 2005). The presence and quantification of potentially toxic cyanobacteria, for example *P. agardhii*, in fresh
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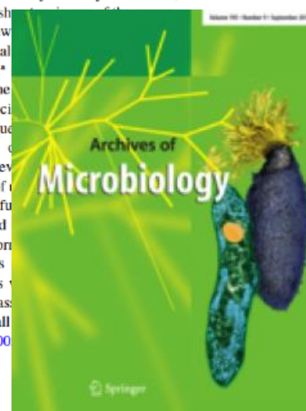

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SPECIES-SPECIFIC REAL-TIME PCR CELL NUMBER QUANTIFICATION OF THE BLOOM FORMING CYANOBACTERIUM *PLANKTOTHRIX AGARDHII.*

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Abstract

A species-specific method to detect and quantify *Planktothrix agardhii* was developed by combining the SYBR-Green I real-time polymerase chain reaction technique with a simplified DNA extraction procedure for standard curve preparation. Newly designed PCR primers were used to amplify a specific fragment within the *rpoC1* gene. Since this gene exists in single copy in the genome, it allows the direct achievement of cell concentrations. The cell concentration determined by real-time PCR showed a linear correlation with the cell concentration determined from direct microscopic counts. The detection limit for cell quantification of the method was 8 cells μL^{-1}, corresponding to 32 cells per reaction. Furthermore, the real-time qPCR method described in this study allowed a successful quantification of *P. agardhii* from environmental water samples, showing that this protocol is an accurate and economic tool for a rapid absolute quantification of the potentially toxic cyanobacterium *P. agardhii*.

Keywords: Cyanobacteria, *Planktothrix agardhii*, Real-time qPCR, *rpoC1* gene.

Introduction

The genus *Planktothrix* comprises several species that are responsible for cyanobacteria blooms in freshwater resources, some of them being able to produce

several kinds of toxins (Falconer and Humpage 2005). The presence and quantification of potentially toxic cyanobacteria, e.g. *Planktothrix agardhii*, in freshwaters is one of the parameters under the Portuguese law to be taken into account when analyzing the water quality (Decreto-Lei nº 306/2007, Diário da República, 1.ª série — N. º 164). This law states that, in cases where the number of cyanobacteria potentially microcystin-producing is higher than 2,000 cells ml^{-1}, the sampling frequency should be increased. Therefore, the quantification of cyanobacteria that are known to be able to produce several kinds of toxins (being therefore potentially toxic) is of utmost importance to know what procedures should be further taken into account. *P. agardhii* is frequently found in shallow eutrophic lakes coexisting with other bloom forming cyanobacteria including other *Planktothrix* species (Davis & Walsby 2002; Halstvedt et al. 2007). Serious water bloom related problems with toxin production associated to *P. agardhii* have been reported in lakes all across Europe (Lindholm et al. 1999; Mbedi et al. 2005; Akcaalan et al. 2006; Yéprémian et al. 2007; Catherine et al. 2008; Mankienwicz-Boczek et al. 2011). In Portugal, the occurrence of several *Planktothrix* species has also been reported (Paulino et al. 2009a, 2009b; Martins et al. 2010); however, the most widespread species and that has been causing prevalent blooms in some reservoirs is *Planktothrix agardhii*.

Conventionally, the surveillance and diagnose of *Planktothrix* species consists mainly in microscopic identification by morphological characters and cell enumeration (Humbert et al. 2010). However, this method is time consuming and requires an expert with practice and significant taxonomic skills. Quantitative polymerase chain reaction (qPCR) for the identification and/or quantification of microorganisms has become a common tool in cyanobacteria studies (Pearson and Neilan 2008; Kurmayer and Christiansen 2009; Martins and Vasconcelos 2011). The main advantage of real-time PCR over conventional PCR is the ability to quantify the target gene copy numbers on a sample, based on a standard curve of known concentrations. The challenge of using qPCR for absolute quantification is the conversion of the real-time PCR cycle threshold (Ct) values or quantification cycle (Cq) values (which is the cycle number where fluorescence passes a set threshold) and the target DNA that is quantified into cell numbers (Bustin et al. 2009; Lefever et al. 2009).

There are some studies that convert the gene copy number into cell equivalents for cyanobacteria quantification, the most common one uses RT-qPCR Taq nuclease assay (TNA) directed for the phycocyanin (PC) gene or its intergenic spacer (Kurmayer and Kutzenberger 2003; Schober and Kurmayer 2006; Schober et al. 2007; Briand et al. 2008; Kurmayer et al. 2011); 16S rRNA (Rinta-Kanto et al. 2005; Doblin et al. 2007;

Davis et al. 2009; Ostermaier and Kurmayer 2009; Baxa et al. 2010) and *rpoC1* gene (Rasmussen et al. 2008; Orr et al. 2010; Moreira et al. 2011) and others use SYBR green and are directed to the phycocyanin gene intergenic spacer (PC-IGS) (Yoshida et al. 2007); 16S rRNA (Tomioka et al. 2008; Al-Tebrineh et al. 2010; Xu et al. 2010) and *rpoC1* gene (Tai and Palenik 2009). Of these two alternatives, the TNA approach although is a very sensitive method, it is more expensive, making it more difficult to be implemented for routine water monitoring.

One of the most common methods employed to convert the Ct values obtained in a qPCR analysis into cell equivalents consists of using an approximation of the genome size (Vaitomaa et al. 2003; Koskenniemi et al. 2007; Ye et al. 2009; Al-Tebrineh et al. 2010). However, this method presents drawbacks since within the same genus and also within the same species there is some genome size variation that cannot be neglected when performing these calculations (Online Resource 1). The other problem is that some genes used as molecular markers in qPCR may exist in multiple copy numbers and also there are quite a few variations of the copy number at least within the same genus (e.g. 16S rRNA or phycocyanin, Online Resource 1). In order to overcome the problem of existing more than one copy of the target gene in the genome, many calibration curves are performed by using log dilutions of a constructed plasmid containing the cloned target gene (Baxa et al. 2010). However, the aforementioned analyses do not enable the conversion of the cell equivalents to cell numbers.

The *rpoC1* gene encodes the unique γ subunit of RNA polymerase, which is not present in the DNA-directed RNA polymerase of other bacteria, and is known to exist as a single copy in the cyanobacterial genome (Scheider and Haselkorn 1988; Bergsland and Haselkorn 1991). The cyanobacterial *rpoC1* gene has been used in several phylogenetic studies of cyanobacteria revealing good species discrimination potential (Fergusson and Saint 2000; Valério et al. 2009). In the case of *Planktothrix* spp. the *rpoC1* gene can be used to distinguish the different *Planktothrix* species (Valério et al., 2009; Lin et al., 2010), however it is not able to discriminate strains/ecotypes (Lin et al., 2010). Taking into account all the aforementioned facts, the purpose of this study was to fulfill a gap in cyanobacteria qPCR analysis by developing a more economical alternative method (with SYBR Green) for cyanobacteria absolute quantification by using a simple and rapid protocol that directly correlates *rpoC1* gene copy numbers with cell numbers in order to simplify and improve sample analysis in water quality monitoring.

Material and Methods

Primer design and specificity

Species-specific primers within the *rpoC1* gene were designed in this study in order to develop a method that quantifies *P. agardhii* species, using SYBR-Green I real-time qPCR technique. For that purpose 67 *rpoC1* sequences from *Planktothrix* spp., from both microcystin-producing and non-producing strains available from NCBI, were aligned using the BioEdit©v.7.0.5 software (Hall 1999), generating an alignment of 590 nucleotide positions. The forward primer **rpoC1_Plank_F271** (5'-TGTTAAATCCAGGTAACTATGACGGCCTA-3') was located at nucleotide positions 271 to 300 and anneals with *rpoC1* sequences from several *Planktothrix* species; and the species-specific reverse primer **rpoC1_P_agardhii_R472** (5'-GCGTTTTTGTCCCTTAGCAACGG-3') was located at the alignment nucleotide positions 472–495. These two primers provide a target fragment of 201 bp. Furthermore the ability of the primers to generate primer dimers or primer loops was verified using the AutoDimerv.1.0 software (Vallone and Butler 2004). The primers were synthesized by Invitrogen™. A GenBank BLASTn search was made to verify if the primer sequences also paired with sequences other than *Planktohrix agardhii rpoC1* gene. To complete primer specificity analysis, the primers were tested using conventional PCR and genomic DNA of *P. agardhii* strains (LMECYA 153A, 153B, 153C and 155) and two very closely related species, *Planktothrix pseudoagardhii* (LMECYA 162), *Planktothrix rubescens* (LMECYA 203). Cultures are maintained (non-axenic) at the Estela Sousa e Silva algal culture collection and detailed information about the cultures used in this study is available in Paulino et al. (2009b).

Cell counting

The culture cell density was estimated by Light Microscopy (LM) in a Sedgwick-Rafter chamber, according to the method of Laslett et al. (1997). Based on cell numbers inferred by direct counting, samples from *P. agardhii* exponential growing cultures with a cell concentration of 79102 cells μL^{-1} (LMECYA 153B), 74375 cells μL^{-1} (LMECYA 153C) and 81946 cells μL^{-1} (LMECYA 155) were frozen at -20 ºC for at least 24 h and used as standards to construct the calibration curves for real-time PCR analysis.

Preparation of DNA extracts

The development of a DNA extraction method that would be prompt and easy to apply for the standards preparation was one of the purposes of this study. Therefore, we

have developed a protocol for DNA extraction from pure cultures, to be used as standards, which consisted mainly in cell lysis with mechanical treatments. Two equivalent samples (1 mL) from the same culture with known cell concentrations were prepared and frozen. One of the samples was thawn at room temperature and subjected to 20 min of sonication, to disrupt the cells, in an ElmasonicS10-H instrument (Elma®). After sonication the cells were further broken mechanically using glass beads (400-600 µm) with a vortex Vibrax®VXR-IKA at 21460 g for 20 min. The supernatant was immediately used in the PCR and qPCR reactions.

In order to evaluate the success of the DNA extraction protocol described above, the other sample was subjected to an adaptation of the conventional phenol-chloroform method (Franche and Damerval 1988). The method applied was according to Santos & Phillips (2009) with slight modifications. Briefly, after thawing the 1 mL aliquot of *P. agardhii* culture was centrifuged for 10 min at 14500 rpm and resuspended in 500 µL of lyses buffer [10 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA (pH 8.0), 2% Trinton X-100, 1% SDS and freshly added 1% (w/v) polyvinypyrrolidone (PVPP)] and 100 µL of glass beads (400-600 □m). After sonicating the tubes for 5 min, 250 µL of phenol and 250 µL of chloroform were added and the cells were mechanically broken with glass beads by vortex shaking on a Vibrax® VXR-IKA shaker at 1800 rpm for 20 minutes, at room temperature. Samples were then centrifuged at 14500 rpm for 25 min and the aqueous phase transferred to a new tube. The nucleic acids were precipitated by adding an equal volume of cold isopropanol. The pellets were washed with 1 mL of 70% cold ethanol and air dried. The pellet was ressuspended in 50 µL of TE buffer with RNase and incubated at 55 ºC for 15 min and kept at -20ºC.

Standard curve construction

The standard curve construction for real-time PCR was based on cell numbers from *P. agardhii* cultures. Three strains were used for these tests: *P. agardhii* LMECYA 153B, *P. agardhii* LMECYA 153C and *P. agardhii* LMECYA 155. In order to test the sensitivity of our qPCR approach and determine the proper cell number assay range to be used, the DNA extracts prepared with the two different DNA extraction methods were subjected to a dilution series that covered cell abundances over at least ten orders of magnitude, e.g. ranging from 79102×10^{3} to 79102×10^{-6} cells μL^{-1}. The 10-fold serial dilutions prepared above were analyzed in conventional PCR and in triplicate reactions by real-time PCR. Since the *rpoC1* gene is not repeated in the cyanobacteria genome (Online Resource 1), one copy of this gene will correspond to one cell.

Conventional PCR conditions

PCR amplification of *rpoC1* gene fragment was performed in a 25 µL reaction mixture containing: 1 X PCR buffer (Fermentas), 0.05 mM of dNTPs (Invitrogen), 0.2 µM of each primer (rpoC1_Plank_F271 and rpoC1_P_agardhii_R472), 2 mM $MgCl_2$ (Fermentas), 12.5 µL of DNA extract and 1 U of *DreamTaq* DNA polymerase (Fermentas). The amplification was performed in a T Gradient thermocycler (Biometra), consisting of an initial denaturation step at 94 ºC for 3 min, followed by 40 cycles of 20 s at 94 ºC, 20 s at 58 ºC and 20 s at 72 ºC and a final extension step of 5 min at 72 ºC.

The PCR product amplification was separated in 2.5 % agarose gel electrophoresis and visualized by exposure to ultraviolet light after GelRed™ staining.

Real-time PCR conditions

The real-time PCR assays using SYBR green I dye were performed on a Rotor-Gene 6000 (Corbett Research). The following reagents were added in a 12.5 µL reaction mixture: 6.25 µL of TAQurate™GREEN real-time PCR mastermix (Epicentre®), 0.1 µM of the forward and reverse primers, 2 µM of $MgCl_2$ and 4 µL of template DNA. The thermal cycling conditions consisted of an initial preheating step of 3 min at 94 °C followed by 40 cycles of 20 s at 94 °C, 20 s at 58 °C and 20 s at 72 °C. To confirm the specificity of the PCR product amplified, fluorescent melting curve analysis was always performed at the end of the 40 cycles by gradually increasing temperature from 60 to 95 °C by raising 1 °C every 5 s. The threshold line was set at 0.05 of signal fluorescence for all the PCR tests using the Rotor-gene 6000 series software.

Cyanobacteria cultures mixtures preparation

In order to test the effect of background DNA in real-time qPCR quantification of *P. agardhii*, experiments were performed with cyanobacteria mixtures. Several spiked samples containing *P. agardhii* LMECYA 155 at a concentration of 42184 cells μL^{-1} and 1 mL of stationary phase cultures containing non-target DNA from *Anabaena circinalis* LMECYA 123C or *Microcystis aeruginosa* LMECYA 159 or *Synechococcus nidulans* LMECYA 156. The mixed samples were subjected to mechanical DNA extraction, diluted in 10-fold series and analyzed in real-time PCR.

Environmental sample collection and preparation

Six water reservoirs mainly from the center of Portugal were sampled (Fig. 1). The water was collected at several depths using a Van Dorn Bottle sampler resulting in

integrated water column samples. Samples were preserved with Lugol´s iodine solution and *P. agardhii* cells were counted using the Utermöhl chambers under the inverted microscope (Olympus CK40) (Edler and Elbrächter 2010).

Number	Utility	Management Institution	Type	Flooded area	Capacity	Location
#1	• Drinking water supply • Irrigation	Public	Artificial water dam	960 x 1000 m^2	7550 x 1000 m^3	
#2	• Recreational activities • Irrigation	Private	Artificial shallow lake	---	---	
#3	• Recreational activities • Irrigation	Public	Artificial water dam	900 x 1000 m^2	3000 x 1000 m^3	
#4	• Irrigation	Private	Artificial shallow lake	---	---	
#5	• Irrigation	Private	Artificial water dam	1100x 1000 m^2	6200x1000 m^3	
#6	• Drinking water supply • Irrigation	Public	Artificial water dam	11000 x 1000 m^2	99500x 1000 m^3	

Fig. 1 - Information regarding the water reservoirs sampled during this study and their schematic location. (---) information not available.

Prior to the environmental samples collection, the more suitable preservative to fix the cells, which did not interfere with the subsequent treatments and analysis by qPCR, was determined. One of the tested preservatives was lugol's iodine solution (1 drop 2% solution per mL of sample), the most common solution for preserving cyanobacteria samples; the others were formaldehyde (37%), glutaraldehyde (25%), methanol-acetic acid (3:1 v/v) and methanol (100%), using 1 mL of the preservative per 1 mL sample.

For the real-time qPCR analysis, 8 mL samples were fixed in 2 mL of 100% methanol, centrifuged at 10000 g, and the pellets were resuspended/preserved in 2 mL of 100% methanol and stored at -20 ºC until analysis. The DNA from methanol preserved samples was extracted using the phenol-chloroform method, to eliminate possible PCR inhibitors frequently present in environmental samples.

Results

Primer specificity

The results from primer specificity analysis showed that the primer sequences designed in this study did not pair with sequences other than *Planktohrix agardhii rpoC1* gene, since no close matches were found in the GenBank BLASTn search. Furthermore when the primers designed were tested in conventional PCR using genomic DNA of *P. agardhii*, *P. rubescens* and *P. pseudoagardhii* the PCR amplification was only observed for *P. agardhii* genomic DNA. These two results confirmed that the primers **rpoC1_Plank_F271** and **rpoC1_P_agardhii_R472** are specific for *P. agardhii*.

DNA extraction for standards preparation

The detection range in conventional PCR for the samples subjected to mechanical DNA extraction was 79102×10^{3} to 79102×10^{-6} cells μL^{-1}, that corresponded to a range from 98877×10^{4} to 1 cell per reaction, while the samples obtained by chemical DNA extraction the detection range was from 79102×10^{3} to 79102×10^{-4} cells μL^{-1}, corresponding to a range from 98877×10^{4} to 99 cells in a reaction tube for the *P. agardhii* strain LMECYA 153B (data not shown).

The real-time PCR analysis of the 10-fold diluted DNA extracts resulting from the two DNA extraction methods (Fig. 2a) for the *P. agardhii* strain LMECYA 153B showed no significant differences (t-student test, df = 4, $P<0.05$) between the Ct values for the same cell concentration until the standard containing approximately 8 cells μL^{-1}. At this concentration the mechanical extraction method yielded a significant lower Ct value corresponding to a higher DNA template concentration (Fig. 2a). Furthermore, the standard curve achieved for the mechanical extraction was more accurate in slope and amplification efficiency than the one resulting from the chemical extraction method (Table 1). The real-time PCR test for the two DNA extraction methods was repeated using two other *P. agardhii* strains (LMECYA 153C and LMECYA 155) and it was obvious that the availability of target DNA was higher for the samples with the mechanical DNA extraction for all the cell concentrations tested (Fig.2b and Fig.2c).

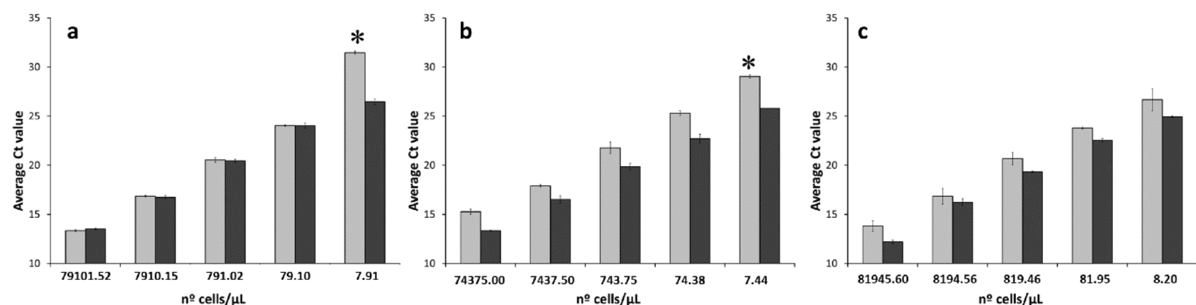

Fig. 2 - Comparison between average Ct values of 2-sets of 10-fold dilutions of the same *P. agardhii* sample subjected to different DNA extraction methods (light grey - phenol-chloroform extraction; dark grey: mechanical treatment) based on predetermined cell concentrations for three *P. agardhii* strains (a) *P. agardhii* LMECYA 153B, (b) LMECYA 153C and (c) *P. agardhii* LMECYA 155. The bars represent the standard deviation. The * denotes the cell concentration that presents significant differences between Ct values for the two DNA extraction methods, t-student test, df = 4, $P<0.05$.

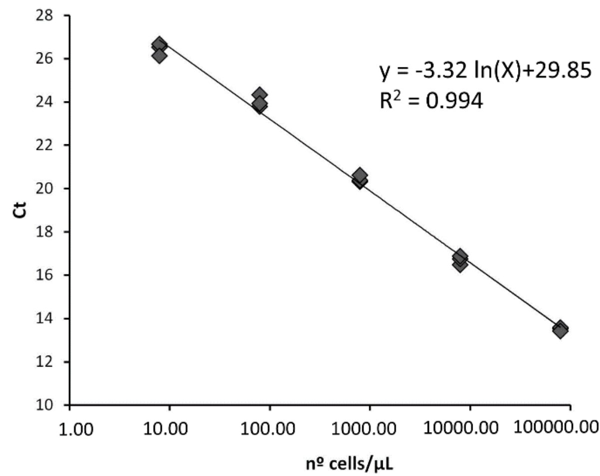

Fig. 3 - Correlation between Ct values and log cell numbers from serial dilutions of DNA extracts from *P. agardhii* strain LMECYA 153B.

Table 1 - Real-time PCR standard curve equations and reaction amplification efficiencies of 2-sets of 10-fold dilutions of the same sample subject to different DNA extraction methods based on predetermined cell concentrations and for three different strains of *P. agardhii*.

	Chemical DNA extraction (phenol-chloroform)	**Mechanical DNA extraction (cell disruption)**
Strain	***P. agardhii* LMECYA 153B**	
Cell range (cells µL^{-1})	79102 to 8	
Standard curve equation	y = -4.28x + 33.73	y = -3.32x + 29.85
Amplification efficiency	0.70	1.00
R^2	0.967	0.994
Strain	***P. agardhii* LMECYA 153C**	
Cell range (cells µL^{-1})	74375 to 7	
Standard curve equation	y= -3.50x+31.90	y= - 3.12x+28.61
Amplification efficiency	0.93	1.09
R^2	0.994	0.996
Strain	***P. agardhii* LMECYA 155**	
Cell range (cells µL^{-1})	81946 to 8	
Standard curve equation	y= - 3.26x+29.87	y= - 3.13x+28.22
Amplification efficiency	1.03	1.08
R^2	0.981	0.991

Real-time qPCR standard curve and detection limit

The mechanical method for DNA extraction of the culture samples to be used as standards was further applied in all the following real-time PCR tests. For standard curve construction several tests were performed and three strains were used for standards preparation (*P. agardhii* LMECYA 153B, LMECYA 153C and LMECYA 155). Furthermore a new standard curve was constructed each time that a PCR run was performed. The following results correspond to the construction of one of these standard curves using the *P. agardhii* strain LMECYA 153B. Values for other standard curves obtained by this method are presented in Table 1. The cell dilutions range considered for standard curve construction was 79102 to 8 cells μL^{-1} and the detection limit of the method was *ca*. 8 cells μL^{-1}, corresponding to 32 cells per reaction. Real-time PCR amplification curves followed a regular exponential growth and the 10-fold cell dilutions were amplified every 3.32 cycles with an efficiency of 1.00 (Fig. 3 and Online Resource 2). Furthermore a linear correlation was obtained between cell numbers inferred by microscopy and those estimated by real-time PCR analysis (Fig. 4).

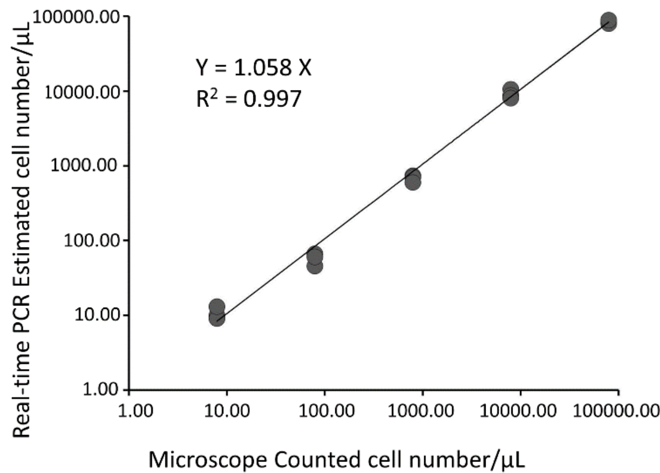

Fig. 4 - Correlation between optical light microscopy cell count numbers and real-time qPCR estimative cell concentration for a serial 10-fold dilutions range between 79102 to 8 cells μL^{-1} of *P. agardhii* strain LMECYA 153B cells.

Effect of background DNA in qPCR analysis

The *P. agardhii* concentration determined by qPCR for the spiked mixtures with *A. circinalis*, *M. aeruginosa* and *S. nidulans* was 46033 cells μL^{-1} ± 1.56×10^4, 44167 cells μL^{-1} ± 1.11 ×10^4 and 45199 cells μL^{-1}± 1.15 × 10^4, respectively. These values were very proximate to the concentration counted under the inverted microscope (42184 cells μL^{-1}). The standard curve equation from *P. agardhii* LMECYA 155 standards for this analysis was y = -3.27x+30.54 with a correlation R^2=0.997 and a reaction efficiency

of 1.02. No PCR inhibition was detected in the samples amplification, since the serial 10-fold dilutions of the samples were amplified every 3.504 cycles with an amplification efficiency of 0.93 and R^2 = 0.983 in the mixture with *A. circinalis*, 3.266 cycles with an amplification efficiency of 1.02 and R^2 = 0.998 in the mixture with *M. aeruginosa* and 3.217 cycles with an amplification efficiency of 1.05 and R^2 = 0.996 in the mixture with *S. nidulans*, which was very similar to the standard curve amplification. Furthermore, the amplification of unspecific products was not detected in melting curve analysis. These tests showed that the presence of non-target DNA from other cyanobacteria, that are also common to exist in environmental water samples, did not interfere with the amplification of target *rpoC1* sequence of *P. agardhii* and consequently with its quantification.

Environmental sample analysis by qPCR

We intended that the preservative to be used in the preservation of environmental samples, besides providing cell preservation, it also should help settled down the cells to minimize the losses that might occur during centrifugation, but it could not interfere in the PCR reactions. So, we first tested several preservatives using pure cultures as samples and verified that the 100% methanol was the most suitable one the other preservatives tested all interfered with the DNA extraction resulting in significant lower quantities of DNA (therefore higher Ct values) when compared with the standards (control treatment) (data not shown). Therefore, the 100% methanol was used to fix the environmental samples.

The environmental samples collected were observed under the inverted microscope and two of the six water reservoirs sampled contained *P. agardhii*, among other cyanobacterial species. The qPCR results obtained for *P. agardhii* quantification in environmental samples for reservoirs #1 and #2 were is in agreement with the microscopically cell quantification (Table 2). Furthermore, for reservoirs # 3, #4, #5 and #6, where *P. agardhii* was not observed in the microscope and also no fluorescence signal was detected in real-time qPCR analysis.

Discussion

The simplicity of the real-time PCR techniques has driven researchers to develop protocols to assess water quality in several microbiological areas and the cyanobacteria field is no exception. It is relevant for water monitoring agencies and public health authorities that the method for detecting potentially toxic cyanobacteria to be simple, fast and cheap to allow a rapid alert of the health hazard and also ensure

that monitoring programs are economically feasible. The studies on cyanobacteria real-time PCR analysis are mainly directed towards the detection and quantification of genes involved in toxin production such as microcystin synthetase genes (Kurmayer and Kutzenberger 2003; Vaitomaa et al. 2003; Furukawa et al. 2006; Baxa et al. 2010; Al-Tebrineh et al. 2011), nodularin synthetase gene (Koskenniemi et al. 2007; Al-Tebrineh et al. 2011) and cylindrospermopsin synthetase genes (Rasmussen et al. 2008; Moreira et al. 2011). These reports are of great use for studding toxic genotype proportion and occurrence in the environment; however, most of them are not species specific. Potentially toxic organism's identification and quantification is also important since the same organism can produce several kinds of toxins (Falconer and Humpage 2005) and therefore represent a health risk.

Generally the standard curves are drawn using serial dilutions of genomic DNA (Koskenniemi et al. 2007; Yoshida et al. 2007; Ostermaier and Kurmayer 2009; Al-Tebrineh et al. 2010) or constructed with plasmids with the target fragment inserted (Rinta-Kanto et al. 2005; Baxa et al. 2010). The method presented in this study to construct the standard curve was based on a serial dilution of simple pure culture extracts that can be maintained frozen, thus representing a simple, rapid and novel method to be applied in qPCR analysis. Furthermore, the cell dilutions range used for qPCR standard curve construction (79102 to 8 cells μL^{-1}; corresponding to 316408 to 32 cells per reaction) are within the limits used by other authors that have used an approach similar to ours (qPCR with SYBR Green) towards *ndaF* gene to quantify *Nodularia* (Koskenniemi et al. 2007) or PC-IGS to quantify *Microcystis* (Yoshida et al. 2007) and the *rpoC1* gene to quantify *Synechococcus* (Tai and Palenik 2009).

The specific primers for the qPCR assay here designed were able to detect and quantify the *rpoC1* target gene for *P. agardhii* with high analytical sensitivity, detecting as few as 32 copies of the target gene per reaction. This value is lower than the one previously reported using TaqMan probes and the *rpoC1* gene for the quantification of *Cylindrospermopsis raciborskii*, where the limit of detection was 100 gene copies per reaction (Rasmussen et al. 2008) and 258 cells per reaction (Moreira et al. 2011).

The classification of *Planktothrix* at species level is a difficult task, even for a skilled taxonomist (Komárek & Komárková 2004). *Planktothrix* was, until recently, included in the genus *Oscillatoria*, however the phylogenetic analyses of the 16S rRNA gene were able to clearly differentiate these two genera into well-defined clades. Furthermore the 16S rRNA phylogenies enable the separation of *Planktothrix* species such as *P. agardhii*, *P. mougeotii* (currently *P. isothrix*) and *P. pseudoagardhii* but not *P. agardhii*

and *P. rubescens* (Suda et al. 2002). Later on, a new species emerged, *P. pseudoagardhii*, which is virtually indistinguishable morphologically from *P. agardhii* (Suda et al. 2002). More recently Lin et al. (2010) analyzed the phylogeny of the 16S rRNA and the *rpoC1* genes of *Planktothrix* spp. and found a good congruence between these two phylogenies. Moreover, the phylogenetic analysis using the *rpoC1* gene was able to differentiate between *P. rubescens* and *P. agardhii* (Valério et al., 2009; Lin et al., 2010).

The use of a species-specific method based on the real-time PCR approach for the detection and quantification of *P. agardhii* bears several advantages over the conventional Utermöhl method such as: is less prone to species identification errors, no need for taxonomy expertise and less time is needed for sample analysis (6h/6 samples vs. 26h/sample).

In conclusion, the method here developed provides a simple and efficient method to achieve quantification of *Planktothrix agardhii* in real-time PCR using the single copy *rpoC1* gene as the molecular target, which allows gene copy numbers to be directly correlated with cell numbers. Furthermore, it combines a simple DNA extraction protocol that is less time consuming for the standards preparation in the real-time qPCR technique. In addition, the method presented was successfully applied to environmental water samples, yielding a *Planktothrix agardhii* quantification that correlates well with the more conventional quantification using the inverted microscope, being therefore an easy-to-use method to be applied in freshwaters monitoring.

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Chapter

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AIL

Applicability of the real-time PCR assay in the amplification of cyanobacterial DNA from preserved samples

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ABSTRACT

Applicability of the real-time PCR assay in the amplification of cyanobacterial DNA from preserved samples

The study and monitoring of cyanobacterial blooms often involves the use of preserved samples to avoid cellular degradation. However, preserved samples may not be suitable for molecular biology studies because preservation methods can interfere with DNA quality/quantity. Real-time quantitative PCR analysis (qPCR) has been widely applied in molecular analysis and is considered a promising method for monitoring purposes. This study intended to evaluate the applicability of the real-time qPCR technique in samples that were subjected to different methods of preservation: (1) 15% Lugol's iodine solution (2) 4% formaldehyde and (3) 25% glutaraldehyde. The ability to amplify and quantify DNA extracted from *Planktothrix agardhii* was assessed using the *rpo*C1 gene as the target fragment in both raw water samples and *in vitro* cultures.
No reliable DNA amplification was obtained from glutaraldehyde-preserved samples. Successful amplification was obtained from Lugol's and formaldehyde-preserved samples. In this case, however, the quantification that was achieved by real-time PCR cannot be used to infer cell numbers, because the Ct values that were obtained from the Lugol's and formaldehyde-preserved samples were significantly higher than the Ct values that were obtained from the unpreserved samples. Therefore real-time PCR can be used for the detection and identification of cyanobacteria in preserved samples but no reliable cell quantification can be performed using this method.

Key words: Cyanobacteria, formaldehyde, glutaraldehyde, lugol's iodine, real-time qPCR, *Planktothrix*.

RESUMEN

Aplicabilidad del qPCR en tiempo real en la amplificación de ADN de cianobacterias pr

El estudio y la vigilancia de las floraciones de cianobacterias implican a menudo el uso de
evitar la degradación celular. Sin embargo, las muestras conservadas pueden no ser adec
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(1) 15% de solución yodoyodurada de Lugol (2) 4% de formaldehído y (3) 25% de g
amplificar y cuantificar el ADN extraído de Planktothrix agardhii *se evaluó utilizando un*
muestras de agua bruta y en cultivos in vitro.
No se obtuvo amplificación fiable de ADN a partir de muestras conservadas en glutaralde
amplificó con éxito en ADN extraído de muestras conservadas en solución yodoyodurad
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elevados que los valores de Ct obtenidos a partir de las muestras sin conservantes. Por lo
utilizar para la detección y la identificación de cianobacterias en muestras conservada
células.

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APPLICABILITY OF THE REAL-TIME PCR ASSAY IN THE AMPLIFICATION OF CYANOBACTERIAL DNA FROM PRESERVED SAMPLES.

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Abstract

The study and monitoring of cyanobacterial blooms often involves the use of preserved samples to avoid cellular degradation. However, preserved samples may not be suitable for molecular biology studies because preservation methods can interfere with DNA quality/quantity. Real-time quantitative PCR analysis (qPCR) has been widely applied in molecular analysis and is considered a promising method for monitoring purposes. This study intended to evaluate the applicability of the real-time qPCR technique in samples that were subjected to different methods of preservation: (1) 15 % Lugol's iodine solution (2) 4 % formaldehyde and (3) 25 % glutaraldehyde. The ability to amplify and quantify DNA extracted from *Planktothrix agardhii* was assessed using the *rpo*C1 gene as the target fragment in both raw water samples and *in vitro* cultures.

No reliable DNA amplification was obtained from glutaraldehyde-preserved samples. Successful amplification was obtained from Lugol’s and formaldehyde-preserved samples. In this case, however, the quantification that was achieved by real-time PCR cannot be used to infer cell numbers, because the Ct values that were obtained from the Lugol’s and formaldehyde-preserved samples were significantly higher than the Ct values that were obtained from the unpreserved samples. Therefore real-time PCR can be used for the detection and identification of cyanobacteria in preserved samples but no reliable cell quantification can be performed using this method.

Keywords: Cyanobacteria, Formaldehyde, Glutaraldehyde, Lugol's Iodine, Real-time qPCR, *Planktothrix*.

Resumen

Aplicabilidad del qPCR en tiempo real en la amplificación de ADN cianobacteriano procedente de muestras fijadas.

El estudio y la vigilancia de las floraciones de cianobacterias implica a menudo el uso de muestras conservadas, con el fin de evitar la degradación celular. Sin embargo, las muestras conservadas pueden no ser adecuadas para los estudios de biología molecular, ya que los métodos de conservación pueden interferir con la calidad/cantidad del ADN.

Este estudio pretende evaluar la aplicabilidad de la técnica de qPCR en tiempo real en muestras sometidas a diferentes métodos de conservación: (1) 15 % de solución yodoyodurada de Lugol (2) 4 % de formaldehído y (3) 25 % de glutaraldehído. La capacidad para amplificar y cuantificar el ADN extraído de Planktothrix agardhii se evaluó utilizando un fragmento del gen rpoC1 en ambas muestras de agua bruta y en cultivos in vitro.

No se obtuvo amplificación fiable de ADN a partir de muestras conservadas en glutaraldehído. El fragmento de gen rpoC1 se amplificó con éxito en ADN extraído de muestras conservadas en solución yodoyodurada de Lugol y formaldehído. En este caso, sin embargo, la cuantificación lograda por PCR en tiempo real no puede utilizarse para inferir el número de células; ya que los valores de Ct obtenidos a partir de muestras conservadas en lugol y formaldehído fueron significativamente más elevados que los valores de Ct obtenidos a partir de las muestras sin conservantes. Por lo tanto-PCR en tiempo real se puede utilizar para la detección y la identificación de cianobacterias en muestras conservadas, pero sin cuantificación fiable de células.

Palabras Clave: *Cianobacterias, Formaldehído, Glutaraldehído, solución yodurada de Lugol, qPCR en tiempo real, Planktothrix.*

Introduction

Preserved phytoplankton samples represent a resource of cyanobacterial DNA for molecular studies, regardless of whether they have been preserved for a short or long time. The evaluation of the usefulness of preserved samples in the real-time quantitative PCR (qPCR) method is relevant for the implementation of this technique in water quality monitoring. Several authors have reported the amplification of nucleic acids from preserved phytoplanktonic samples in dinoflagellates (Godhe *et al.*, 2002; Galluzzi *et al.*, 2004), microalgae and other protists (Auinger *et al.*, 2008, Shuang *et al.*, 2013). These studies intend to bypass time-consuming microscopic examinations

(Galluzzi *et al.*, 2004), establish phylogenetic diversity (Godhe *et al.*, 2002; Galluzzi *et al.*, 2004; Qiu *et al.*, 2011; Shuang *et al.*, 2013), establish a direct link between morphological and molecular screening approaches (Auinger *et al.*, 2008) or simply permit the identification and quantification of species that may be overlooked by microscopic identification (Godhe *et al.*, 2002; Lang & Kaczmarska, 2011). In cyanobacteria, reports addressing the availability and PCR amplification of cyanobacterial DNA that was recovered from preserved samples are not available. This study addresses two questions: Can the real-time PCR reaction amplify DNA that is extracted from preserved cyanobacteria samples, and if so, can the real-time qPCR results of preserved samples be used to establish a valid correlation with cyanobacteria densities in environmental samples? Fixatives that are often used for the long-term storage of phytoplankton material include Lugol's iodine (Hötzel & Croome, 1999; Utkilen *et al.*, 1999), formaldehyde and glutaraldehyde solutions (Menden-Deuer *et al.*, 2001; Bertozzini *et al.*, 2005; Karlson *et al.*, 2010). In this study, we evaluated the applicability of the real-time PCR technique in the amplification of a target fragment in DNA that was extracted from samples that were preserved with Lugol's iodine solution, formaldehyde and glutaraldehyde.

Material and methods

Culture and field sample preservation treatments

Cyanobacteria species and genetic marker

To evaluate the amplification by the real-time PCR of cyanobacterial DNA that was extracted from preserved samples, the culture experiments were performed with nonaxenic monoclonal cultures of *Planktothrix agardhii*. The gene that was used for the PCR amplification was the *rpo*C1 that encodes the characteristic cyanobacterial δ subunit of the DNA-dependent RNA polymerase using the primers *rpo*C1_Plank_F271 (5'-TGTTAAATCCAGGTAACTATGACGGCCTA-3') and *rpo*C1_P_agardhii_R472 (5'-GCGTTTTTGTCCCTTAGCAACGG-3'), targeting a fragment of 202 bp (Churro *et al.*, 2012).

Cyanobacteria cultures and field samples

The *P. agardhii* strains (Imecya 153A, 153B and 155) that were used in the experiments were maintained in the Estela Sousa e Silva Algal Culture Collection in the Laboratory of Biology and Ecotoxicology (LBE) at the National Health Institute Dr. Ricardo Jorge, Portugal. Further information about the cultures can be found in Paulino *et al.* (2009). The field sample that was used in the experiments was obtained from an

ongoing monitoring program at the LBE that receives samples periodically from a water reservoir with recurrent blooms of *P. agardhii*.

Sampling

One-millilitre culture samples and 25-mL field samples were preserved separately with three working solutions that were made according with Edler & Elbrächter (2010): 15 % acidic Lugol's iodine solution at pH 3 (final concentration of 1 %); 4 % neutral buffered formaldehyde at pH 7 (final concentration of 0.4 %); and 25 % glutaraldehyde at pH 7 (final concentration of 2 %). The samples were kept in the dark for 24 h or 6 months at room temperature until DNA extraction (Fig. 1).

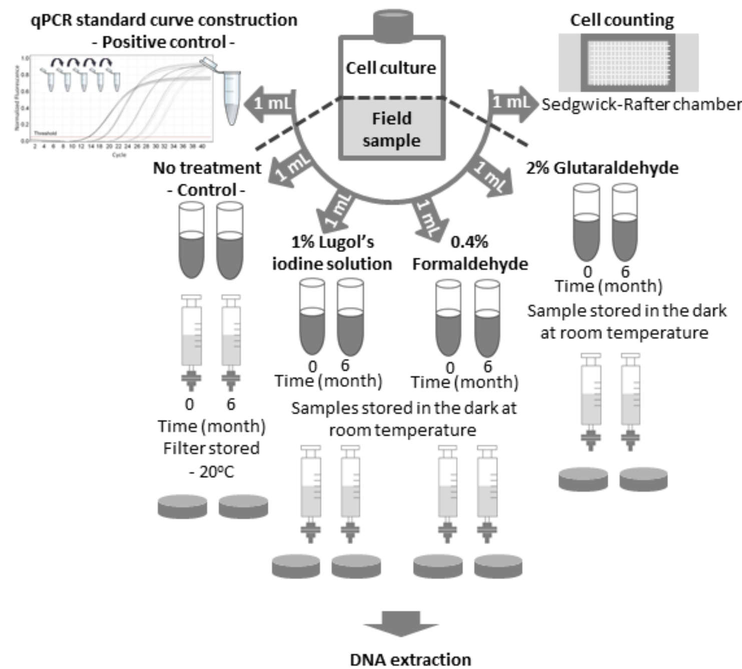

Fig. 1 - Diagram describing the method that was used for culture and field sample preservation. *Esquema del método de preservación utilizado para muestras de cepas y muestras de campo.*

Control samples

Because preserved samples and field samples must be filtered to remove excess fixative solution or field water, a sample with no preservation treatment was used as a control and filtered through a syringe with a Swinnex® (Millipore) holding a paper filter (Fig. 1). To test whether the filtering step influenced DNA attainment, the control sample was compared to the whole-sample positive control, which was also used for standard curve construction (Fig. 1). Both of the non-treatment samples were stored at

-20 ºC for 24 h or 6 months until DNA extraction. No differences were obtained between the filtered (control) and the non-filtered (positive control) samples, which were also stable after 6 months (Fig. 2). Based on these tests, the Ct values that were obtained from the amplification of preserved samples were only compared to the Ct values of the control sample.

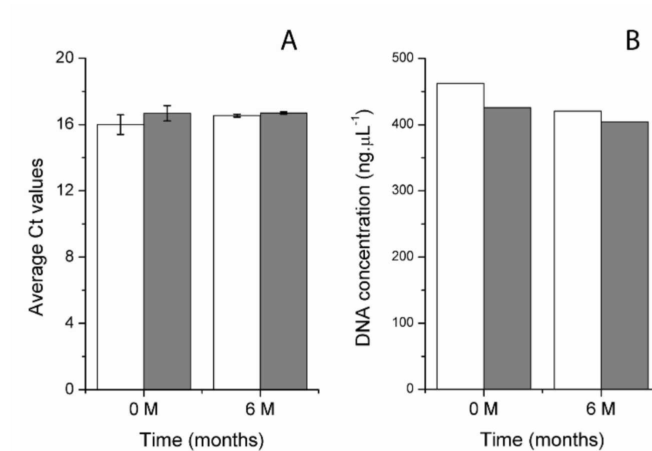

Fig. 2 - Stability of the control samples. (A) Ct values and (B) DNA concentrations at the time of preservation (0 M) and over a 6-month period (6 M). White bar – positive control, grey bar – control (filter with no treatment). The lines represent the standard deviation. No significant differences, Student's *t* test, df = 4, $p < 0.05$. *Estabilidad de las muestras de control. (A) valores de Ct y (B) concentraciones de DNA en el momento de la conservación (0 M) y durante un período de 6 meses (6 M). Barra branca - control positivo, barra gris - control (filtro sin ningún tratamiento). Las líneas representan la desviación estándar. No hay diferencias significativas, prueba t de Student, df = 4, p <0.05.*

gDNA extraction

The preserved samples were filtered through a syringe with a Swinnex® (Millipore) holding a paper filter (Fig. 1). The filter was then placed into a 2-mL Eppendorf tube, and DNA was extracted by phenol-chloroform extraction as described in Churro *et al.* (2012). The DNA of the control and positive control was also extracted by the same method to assure that all of the samples had the same treatment. The gDNA was eluted in 50 µL of DNase-free water and quantified with a spectrophotometer. After quantification, the 50-µL DNA extracts were diluted in 950 µL of DNase-free water.

gDNA quantitation, quality and integrity

After extraction, the DNA concentration and purity were measured using a NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). The DNA was quantified (ng/µL) using the spectrophotometric measurement of UV absorption at a 260-nm wavelength. The DNA purity was determined by the ratios OD260/OD280 and OD260/OD230. Values of the OD260/280 ratio between 1.8 and 2.0 and of the OD260/230 ratio between 2.0 and 2.2 were considered to indicate pure DNA (Vinod,

2004). The integrity of the total gDNA that was extracted from the 20-mL preserved samples of *P. agardhii* (Imecya 153B) culture was checked in a 0.6 % w/v agarose gel. The electrophoresis was performed at 85 V in 0.5× Tris-borate EDTA (TBE) buffer for 45 min. The gDNA migration was visualized by exposure to ultraviolet light after GelRed™ staining.

Real-time qPCR analysis of preserved samples

Standard curve construction

The standard curve construction for real-time qPCR was based on the cell numbers from *P. agardhii* cultures. Because the *rpo*C1 gene exists as a single copy in the cyanobacteria genome, one cell will correspond to one copy of this gene. The culture cell density was estimated by Light Microscopy (LM) using a Sedgwick-Rafter chamber. As previously mentioned, at the time of the culture sampling for preservation, an identical 1-mL sample was taken for the standard curves (Fig. 1). This sample was diluted in 5 serial 10-fold dilutions to construct the calibration curves. The standard curve equations, reaction amplification efficiencies and cell culture densities are presented in table 1.

Table 1- Real-time PCR standard curve equations and reaction amplification efficiencies. *Curva de calibrado y eficiencia de la reacción de la amplificación en la PCR en tiempo real.*

	Strain (Imecya)	Culture cell densities (Cells/mL)	Standard curve equation	Amplification efficiency	R^2	Cell range (cells/reaction)
Experiment I	155	152.07×10^5	y = -3.59log(x)+31.42	0.90	0.997	60 828 to 6
Experiment II	155	231.74×10^5	y = -3.43log(x)+31.19	0.96	0.998	92 698 to 9
Experiment III	153A	474.30×10^5	y = -3.59log(x)+34.09	0.90	0.995	189 692 to 19

qPCR conditions

The real-time qPCR assays were performed on a Rotor Gene Q (Qiagen) using SYBR Green I Dye. The following reagents were added in a 12.5-µL reaction mixture: 6.25 µL of SensiMix™ SYBR NO-ROX kit real-time qPCR MasterMix (Bioline), 0.1 µM forward and reverse primers and 4 µL of template DNA. The thermal cycling conditions consisted of an initial preheating step of 3 min at 94 °C followed by 40 cycles of 20 s at 94 °C, 20 s at 58 °C and 20 s at 72 °C. The specificity of the amplified PCR product was verified by melting curve analysis at the end of the 40 cycles by gradually increasing the temperature from 60 to 95 °C by 1 °C every 5 s. The threshold line was

set at 0.05 of the signal fluorescence for all of the PCR tests using the Rotor-Gene Q series software.

Statistical analysis and replicates

The differences in the amplification were assessed by comparing the cycle threshold values (Ct). The non-treatment control group – control and positive control – was used to determine the expected Ct values. A statistical analysis was performed using Student's *t*-test, and a p value < 0.05 was considered significantly different. All of the samples were run in triplicate in the real-time PCR reaction. The tests were repeated in 3 independent experiments.

PCR inhibition and sensitivity

To test whether the DNA extracts from the preserved samples inhibited the PCR, the reaction efficiency was accessed in the 5 serial 10-fold dilutions of the preserved samples. If the sample contained any PCR inhibitor, the amplification of the 10-fold dilutions would not be linear because the inhibitor would be diluted and the target fragment might be better amplified in low-concentration samples (Wilson, 1997). To compare the sensitivity of real-time PCR with that of conventional PCR in the amplification of the target fragment, the preserved samples were also diluted in 5 serial ten-fold dilutions and amplified in both conventional PCR and real-time PCR.

Conventional PCR conditions

End-point PCR amplification of the *rpo*C1 gene fragment was performed in a 25-µL reaction mixture containing 1× PCR buffer (Invitrogen™), 0.05 mM dNTPs (Invitrogen™), 0.2 µM each primer (*rpo*C1_Plank_F271 and *rpo*C1_P_agardhii_R472), 2 mM $MgCl_2$ (Invitrogen™), 8 µL of DNA extract and 1 U of Taq DNA polymerase (Invitrogen™). The amplification was performed in a TGradient Thermocycler (Biometra) consisting of an initial denaturation step at 94 °C for 3 min, followed by 40 cycles of 20 s at 94 °C, 20 s at 58 °C and 20 s at 72 °C and a final extension step of 5 min at 72 °C. The amplified *rpo*C1 fragments were visualized by exposure to ultraviolet light in a GelRed™-stained 1 % w/v agarose gel after electrophoresis at 85 V in 0.5× Tris-borate EDTA (TBE) buffer for 45 min.

Results

Quantity, quality and integrity of gDNA

The total gDNA that was extracted from the unpreserved samples and samples that were fixed with Lugol's iodine and formaldehyde was mostly greater than 12 kb in length (Fig. 3). Some fragmentation was detected in the control and Lugol's samples

with visible fragments of 1000 and 650 bp (lanes 1 and 2, Fig. 3). In the glutaraldehyde-preserved samples, there was no visible DNA or signs of DNA degradation (lane 4, Fig. 3). Furthermore, a loss in DNA quantity was also visible in the preserved samples compared to that of the control samples (Fig. 3).

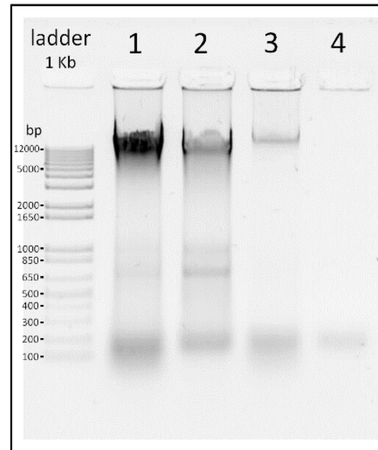

Fig. 3 - Migration of gDNA that was extracted from the preserved and control samples in 0.6 % (w/v) agarose gel electrophoresis at 85 V in TBE buffer for 45 min. (1) Unpreserved sample – control; (2) 15 % Lugol's-iodine-solution-preserved samples; (3) 4 % formaldehyde-preserved samples; and (4) 25 % glutaraldehyde-preserved samples. Ladder: GeneRuler™ DNA Ladder Mix (Fermentas). *Migración de DNA genómico extraído de muestras conservadas y muestra control en gel de agarosa al 0.6 % (w / v) a 85 V en tampón TBE durante 45 min. (1) muestra sin conservantes - control; (2) solución yodoyodurada de Lugol al 15 %; (3) formaldehído al 4 %, (4) glutaraldehído al 25 %.*

At the time of preservation, the DNA quantity was determined spectrophotometrically and decreased in the preserved samples compared to that of the control (Fig. 4). The samples that were preserved in Lugol's iodine showed a DNA loss of between 21 and 35 % more than that of the control. For the formaldehyde-preserved samples, the loss was between 35 and 40 %. In contrast, for the glutaraldehyde-preserved samples, the DNA quantity was much higher than that of the control. This result is most likely unrealistic and misleading, given that no DNA was visible in the total-gDNA electrophoresis. Similar results were obtained in the 3 independent experiments (Fig. 4A, B and C). After 6 months of preservation, the quantity of DNA that was obtained from the preserved samples was substantially low compared to that of both the control and the same preserved samples at the time of preservation (Fig. 4). DNA losses ranged between 58 % and 77 % for Lugol's-iodine-preserved samples and from 63 % to 79 % for formaldehyde-preserved samples.

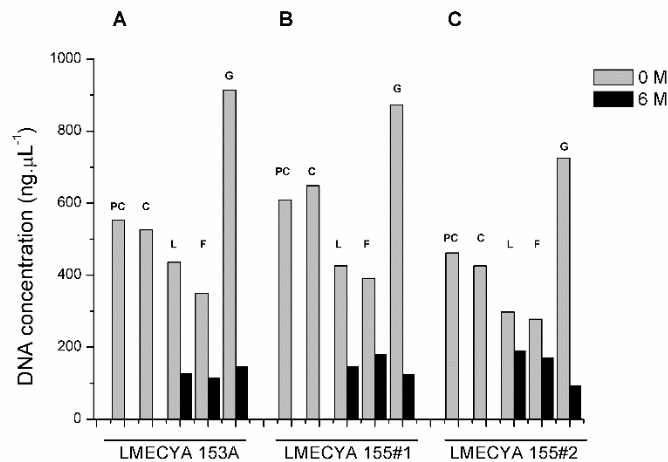

Fig. 4 - Total genomic DNA that was extracted from short-term- (0 months – 0 M grey bars) and long-term- (6 months – 6 M black bars) preserved culture samples in three independent experiments - (A) *Planktothrix agardhii* strain LMECYA 153A, (B and C) *P. agardhii* strain LMECYA 155. PC – positive control (no treatment), C – control (filter with no treatment), L – 15 % Lugol's iodine solution, F – 4 % formaldehyde, G – 25 % glutaraldehyde. *Cantidad total de DNA genómico extraído de muestras de cultivos conservadas poco tiempo (0 meses – 0M barra gris) y en períodos largos (6 meses – 6M barra negra) en tres experimentos independientes - (A) Planktothrix agardhii cepa LMECYA 153A, (B y C) P. agardhii cepa LMECYA 155. PC – controlo positivo (sin tratamiento), C – controlo (filtro sin tratamiento), L - solución yodoyodurada de Lugol al 15 %, F - formaldehído al 4 %, G - glutaraldehído al 25 %.*

The ratios OD260/OD280 and OD260/OD230 indicated good DNA quality from the control and positive control samples (Table 2). The DNA that was extracted from Lugol's- and formaldehyde-preserved samples exhibited greater variability in quality ratios (Table 2). DNA isolated from glutaraldehyde samples showed poor DNA quality (Table 2). The quality of the DNA that was extracted from the short-term-preserved samples was similar to that from the six-month-preserved samples (Table 2).

Table 2 - Genomic DNA quality. The values are present in the range of the OD260/OD280 and OD260/OD230 values that were obtained in the three experiments. The reference ratio values for good-quality DNA are OD260/280 = 1.8 and OD260/230 = 2.0. *Calidad del DNA genómico extraído. Los valores estan apresentados en la gama de OD260/OD280 y OD260/OD230 obtenida en los tres experimentos realizados. Los valores de refencia indicativos de DNA de buena calidad son 1.8 para la razón OD260/OD280 y 2.0 para la razon OD260/OD230.*

Preservation time	0 months		6 months	
Quality ratios	OD260/OD280	OD260/OD230	OD260/OD280	OD260/OD230
Positive control	1.84 - 1.91	2.1 – 2.4	1.85 – 1.93	2.12 – 2.2
Control	1.84 – 2.02	1.9 - 2.36	1.80 – 2.01	1.97 – 2.09
Lugol's iodine	1.70 – 1.87	1.79 - 2.17	1.72 – 1.84	2.05 – 2.16
Formaldehyde	1.44 - 1.92	2.07 - 2.15	1.81 – 1.98	2.06 – 2.17
Glutaraldehyde	1.39 - 1.59	1.45 – 2.17	1.63 – 1.78	2.31 – 2.91

Real-time qPCR amplification of the preserved samples

The DNA that was extracted from the recently preserved samples with the three tested fixatives was successfully amplified by real-time PCR in three independent experiments (Fig. 5A, B and C). For the Lugol's- and formaldehyde-preserved samples, the replicates were consistent, indicating correct target fragment detection in each of the 3 experiments (Fig. 5). Furthermore, a melting curve analysis of the amplified fragments in the Lugol's- and formaldehyde-preserved samples exhibited single melting peaks with high fluorescence, similar to that of the control. The replicate consistency and fragment amplification before PCR cycle 30 for the Lugol's- and formaldehyde-preserved samples caused the Ct values to fall in the standard curve quantification limits and enabled gene copy number quantification for these samples. However, this quantification was not realistic because the Ct values of the preserved samples were significantly higher than those of the control, which resulted in an underestimation of gene copy number (Fig. 5). In the glutaraldehyde-preserved samples, although the replicates were consistent, the Ct values were always measured after PCR cycle 30, except for in one experiment (Fig. 5A). The melting curve analysis for this last sample presented low fluorescence peaks at the melting temperature of the target fragment when compared to that of the control. From the 3 fixatives that were used, the Lugol's-preserved samples presented a lower Ct value, followed by the formaldehyde- and glutaraldehyde-preserved samples (Fig. 5).

After six months of preservation, no reliable Ct values were obtained, except for in the formaldehyde-preserved samples in every experiment (Fig. 5A, B and C) and in the Lugol's-preserved samples in one experiment (Fig. 5C).

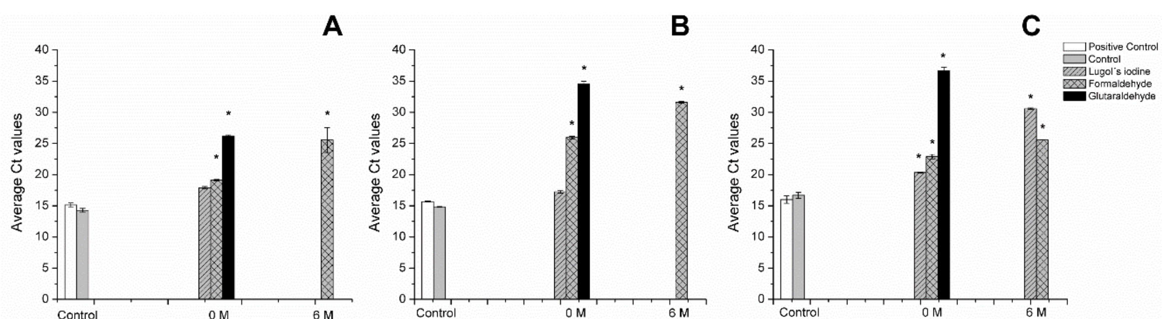

Fig. 5 - Comparison between the average Ct values that were obtained from the real-time amplification of non-preserved and preserved samples with 15 % Lugol's iodine solution, 4 % formaldehyde and 25 % glutaraldehyde in recently and six-month-preserved samples in three independent experiments - (A) *Planktothrix agardhii* strain LMECYA 153A (189 692 cells/reaction) and (B and C) *P. agardhii* strain LMECYA 155 (92 698 and 60 828 cells/reaction, respectively). The asterisk denotes significant differences, Student's t test, df = 4, $p < 0.05$, between the Ct values of the

preserved samples and the Ct values of the control at the time of preservation. The lines represent the standard deviation. *Comparación entre los valores medios de Ct obtenidos de la amplificación por la PCR en tiempo real de muestras sin conservantes y muestras conservadas con solución yodoyodurada de Lugol al 15 %, formaldehído al 4 % y glutaraldehído al 25 % en muestras de culturas conservadas a pronto y a largo en tres experimentos independientes - (A) Planktothrix agardhii cepa LMECYA 153A (189 692 celulas/reacción), (B y C) P. agardhii cepa LMECYA 155 (92 698 and 60 828 celulas/reacción, respectivamente). El asterisco indica diferencias significativas, en la prueba t de Student, df = 4, p <0,05, entre los valores de Ct de muestras conservadas y los valores de Ct del control en el momento de la preservación. Las líneas representan la desviación estándar.*

Real-time PCR inhibition and sensitivity

To test for the presence of co-purified DNA PCR inhibitors, the real-time PCR amplification parameters were analysed in the 5 serial 10-fold dilutions of the preserved samples. In the amplification curves, the replicates of each 10-fold dilution of the preserved sample with Lugol's and formaldehyde solutions were consistent, indicating amplification reproducibility. Furthermore, linearity was obtained for the Lugol's- and formaldehyde-preserved sample dilution series. For Lugol's-preserved samples, the regression equation was y = - 3.596log(x) + 36.47, with r^2 = 0.997 and a reaction efficiency of 0.90. For the formaldehyde-preserved samples, the regression equation was y = -3.197log(x) + 35.69, with r^2 0.977 and a reaction efficiency of 1.05. No regression analysis could be drawn from the log dilutions of the glutaraldehyde samples.

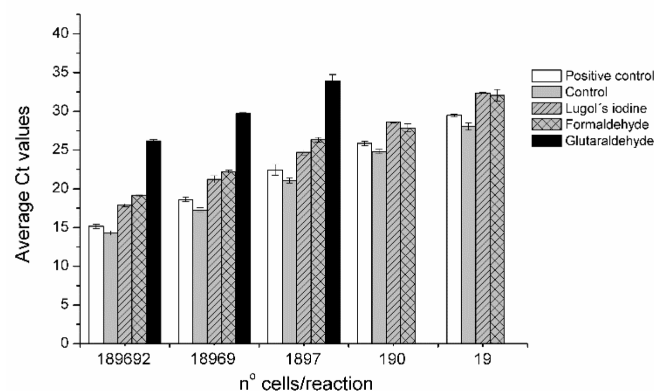

Fig. 6 - Sensitivity of the real-time PCR to target the *rpo*C1 gene in DNA that was extracted from *P. agardhii* strain LMECYA 153A when preserved in 15 % Lugol's iodine solution, 4 % formaldehyde and 25 % glutaraldehyde. The plotted bars represent the average Ct values of 5 serial 10-Logarithmic dilutions for each fixative solution. The lines represent the standard deviation. *Sensibilidad de la PCR en tiempo real para amplificar el gen rpoC1 en DNA extraído a partir de P. agardhii cepa LMECYA 153A conservado con solución yodoyodurada de Lugol al 15 %, formaldehído al 4*

% y glutaraldehído al 25%. Las barras representan los valores medios de Ct obtenidos de 5 series de 10 diluciones logarítmicas seriadas para cada solución de preservación. Las líneas representan la desviación estándar.

The Ct values of the five serial 10-fold dilutions for each preserved sample and the control are presented in figure 6. The amplification fluorescence at all of the concentrations ranged from 189 692 to 190 cells per reaction in the samples that were preserved with Lugol's and formaldehyde solutions (Fig. 6). In the samples that were preserved with glutaraldehyde solution, the amplification products were only detected in the range of 189 692 to 1897 cells per reaction (Fig. 6). Compared to conventional PCR, real-time PCR was able to amplify the target fragment at a lower concentration because the amplification products in conventional PCR were only obtained in the range of 189 692 to 190 cells per reaction for the formaldehyde-preserved samples and 189 692 cells per reaction for the glutaraldehyde-preserved samples (Fig. 7C and D).

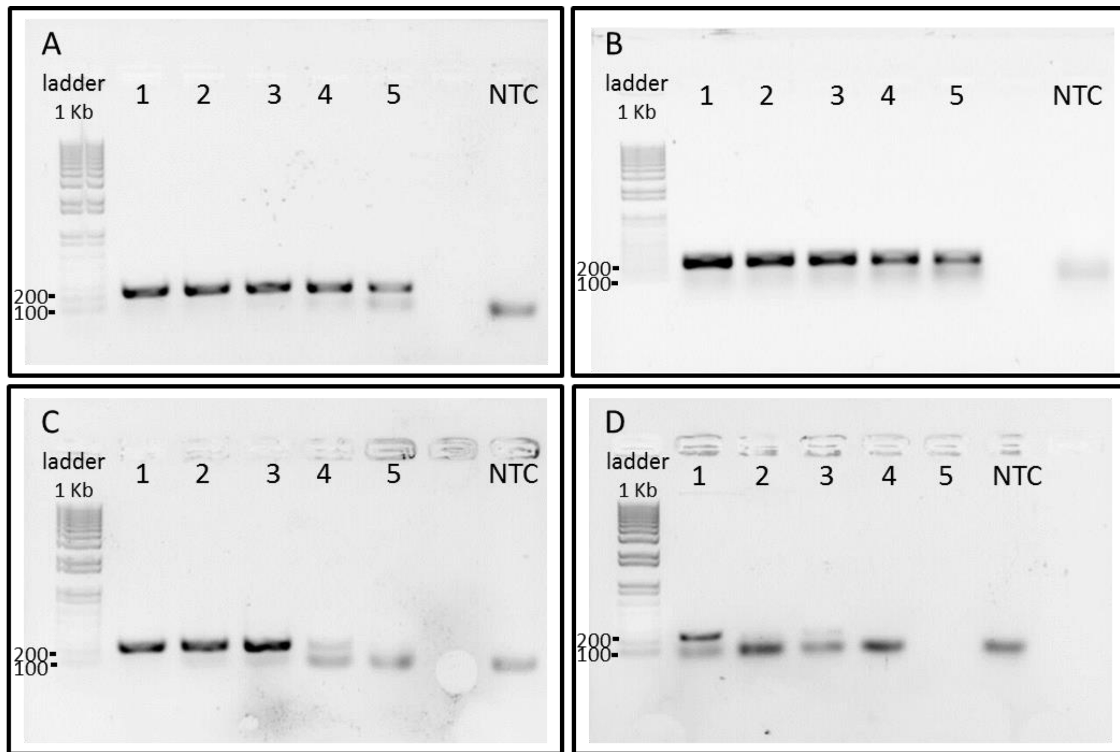

Fig. 7 - Gel image of conventional PCR showing the amplification of the *rpo*C1 gene target sequence of the *P. agardhii* strain LMECYA 153A DNA that was extracted from (A) control samples and samples that were preserved with (B) 15 % Lugol's iodine solution, (C) 4 % formaldehyde and (D) 25 % glutaraldehyde. Lanes 1 to 5 refer to the 5 serial 10-fold dilutions of template DNA equivalent to 379 384 to 38 cells/reaction. Ladder: GeneRuler™ DNA Ladder Mix (Fermentas); NTC: no-template control.*Imagen Gel de la PCR convencional que muestra la amplificación de lo produto de DNA do gen rpoC1 de la cepa P. agardhii LMECYA 153A sin conservates (A) Control y conservado con (B) solución yodoyodurada de Lugol al 15 % (C) formaldehído al 4 % y (D) glutaraldehído al 25 %. Los carriles 1 a 5 se refieren a 5*

series de 10 diluciones logarítmicas seriadas equivalentes a 379 384 a 38 células / reacción. "Ladder": marcador de peso molecular GeneRuler ™ Mix Ladder (Fermentas); NTC: control sin molde.

Applicability in the environmentally preserved samples

To analyse the real-time PCR amplification of the target fragment in the preserved samples with an environmental matrix and cell concentrations, field samples were fixed with Lugol's solution, formaldehyde and glutaraldehyde. The Ct values for the preserved field samples are presented in figure 8. Positive amplification was obtained in the Lugol's- and formaldehyde-preserved field samples, and the *rpo*C1 fragment was successfully detected. Nevertheless, there was a significant difference between the Ct values of the preserved samples and that of the non-treated samples (Fig. 8). The resulting quantification of the target gene copy number in the preserved field sample resulted in an underestimation of the copy number compared to that of the control. Unlike culture experiments, a positive amplification was detected in the Lugol´s-preserved sample after 6 months, and no amplification was obtained from the formaldehyde-preserved sample (Fig. 8).

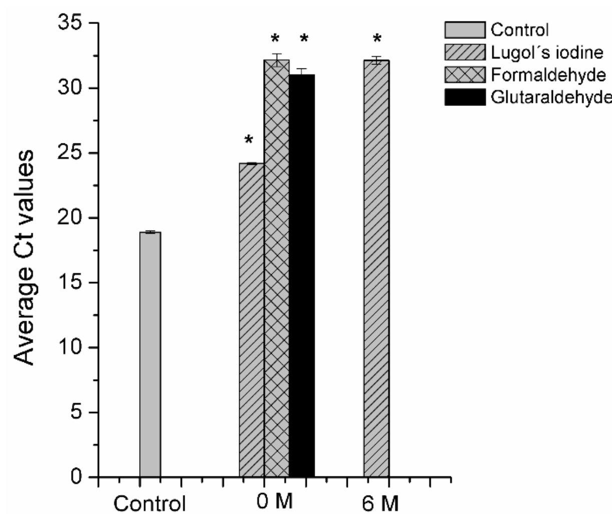

Fig. 8 - Comparison of the average Ct values that were obtained from the real-time amplification of environmental samples that were not preserved or preserved with 15 % Lugol's iodine solution, 4 % formaldehyde and 25 % glutaraldehyde in recently (0 M) and six-month-(6 M) preserved samples. The asterisk denotes significant differences, Student's *t* test, df = 4, $p < 0.05$, between the Ct values of the preserved samples and the Ct values of the control at the time of preservation. The lines represent the standard deviation. *Comparación entre los valores medios de Ct obtenidos de la amplificación por la PCR en tiempo real de muestras de campo sin conservantes y muestras conservadas con solución yodoyodurada de Lugolal 15 %, formaldehído al 4 % y glutaraldehído al 25 % en muestras de culturas conservadas a pronto y a largo. El asterisco indica diferencias significativas, en la prueba t de Student, df = 4, p <0,05, entre los valores de Ct de muestras coservadas y los valores de Ct del control en el momento de la preservación. Las líneas representan la desviación estándar.*

Discussion

The intent of sample preservation is to maintain cell integrity and morphology, and the most common fixatives that are used clearly fulfil this function. These preservatives began to be used a long time ago when nucleic acid availability was not yet a concern. In fact, fixatives such as formaldehyde and glutaraldehyde cross-link and coagulate proteins to maintain cellular and organellar structures, consequently trapping the DNA in the cell and making the DNA unavailable for PCR amplification (Douglas & Rogers, 1998; Kiernan, 2000; Srinivasan *et al.*, 2002). In addition, another reason for the lack of DNA availability from preserved samples includes cell loss during the preservation process. Several studies using formaldehyde-ciliate-fixed samples have reported more than 70 % cell loss compared to that in Lugol´s-preserved samples (Stoecker *et al.*, 1994; Pitta *et al.*, 2001; Modigh & Castaldo, 2005). Furthermore, the fixative inclusion in the cells requires some time, and during the fixation process, the DNases that are present in the cells may destroy DNA (Noguchi *et al.*, 1997; Srinivasan *et al.*, 2002).

The results presented here demonstrate that cyanobacterial DNA can be amplified by real-time PCR using Lugol's- and formaldehyde-preserved culture samples and in samples with a complex matrix, such as environmental samples. High molecular weight gDNA was obtained from Lugol's- and formaldehyde-preserved samples, and the observed fragmentation was also present in the DNA from the non-preserved sample, indicating that this fragmentation was not caused by the preservation treatment. However, the difference in the intensity of gDNA bands from these samples compared to that of the control suggests that the DNA losses were higher in the formaldehyde-preserved samples. In fact, the DNA concentrations measurements from the Lugol's- and formaldehyde-preserved samples also indicated severe DNA losses that increased with the time of preservation. The quality of the DNA that was extracted from the preserved samples was also inferior to that of the sample without treatment, suggesting that fixation decreases the DNA quality. Despite these drawbacks, the target fragment was successfully amplified in samples that were preserved in Lugol's and formaldehyde solutions for a short time and for six months, thus making real-time PCR analysis suitable for fragment detection in preserved samples. However, due to the increased Ct values, the quantification resulted in an underestimation of the gene copy number, meaning that, although the sample can be quantified, the results of that quantification are not realistic and cannot be related to the concentrations prior to fixation. Nevertheless, the target fragment amplification after six months of preservation presented variability in the results between experiments, which may be related to the fixative stability and sample degradation over time. To understand

whether the shift in Ct values was also due to the inhibition of the PCR reaction and not only to DNA loss, the Lugol's- and formaldehyde-preserved samples were diluted, and the serial logarithmic dilutions amplified linearly with efficiencies close to 1, indicating that no PCR inhibitors were present in the reaction. In the literature, the amplification of DNA that is extracted from Lugol´s-preserved phytoplankton samples has been performed mainly with eukaryotic dinoflagellates, and no information could be obtained regarding DNA amplification from preserved cyanobacteria samples. In experiments using dinoflagellates and microalgae DNA from Lugol's-preserved samples, Godhe *et al.* (2002) and Marín *et al.* (2001) were unable to obtain amplification with conventional PCR. In fact, positive results with conventional PCR have only been obtained with single-cell PCR experiments (Bertozzini *et al.*, 2005; Auinger *et al.*, 2008; Godhe *et al.*, 2008; Henrichs *et al.*, 2008; Kavanagh *et al.*, 2010). However, when the real-time PCR technique was applied, successful amplification was reported for the amplification of dinoflagellate DNA (Bowers *et al.*, 2000; Tengs *et al.,* 2001; Galluzzi *et al.*, 2004; Penna *et al.*, 2007; Miyaguchi *et al.,* 2008). Penna *et al.* (2007) and Galluzzi *et al.* (2004) reported that Lugol's solution did not induce DNA loss in preserved samples with up to 15 months of preservation and that preserved samples were suitable for real-time PCR. However, similar to our results, previous reports in dinoflagellates have reported increased Ct values in Lugol's-preserved samples, reflecting a loss in the log cell concentration of 10-fold compared to that of unpreserved DNA; this decrease was also time dependent (Bowers *et al.*, 2000; Miyaguchi *et al.*, 2008). The reports regarding formaldehyde fixation in human tissue have also described the poor preservation of high-molecular-weight DNA and have reported that up to 30 % of nucleic acids could be lost during fixation (Srinivasan *et al.*, 2002). An identical result was also reported by Bertozzini *et al.* (2005), in which DNA recovery from formaldehyde-preserved phytoplankton samples was lower compared to that of un-preserved and Lugol's-preserved samples. Similar to our results, Miyaguchi *et al.* (2008) and Hosoi-Tanabe & Sako (2005) also reported the successful target amplification of formaldehyde-preserved samples in real-time PCR and that fragment detection was inferior in formaldehyde-preserved samples compared to that in Lugol's-preserved and unpreserved samples. Based on our results, no conclusions can be drawn from the amplification of DNA from glutaraldehyde-preserved samples. No visible DNA was present in the total-gDNA gel migration, and the DNA quantification values were unrealistic and of poor quality. Most likely, glutaraldehyde was carried over during DNA extraction and interfered with the quality ratios, resulting in low OD260/OD280 and OD260/OD230 values, as glutaraldehyde has one absorbance peak at 235 and another at 280 nm (Jones, 1974; Gillett & Gull, 1972; Hopwood *et al.*,

1975). Consequently, the obtained Ct values were high, reflecting low-concentration DNA, and the observed amplification can be misleading because the signal is too close to residual fluorescence. In addition, no regression analysis could be drawn from the log dilutions of the glutaraldehyde samples. These results suggest that most of the DNA was lost during the preservation process, indicating that a much higher concentration of starting material is necessary for successful amplification, making the cell concentration in the context of preserved cyanobacteria samples unrealistic. Information about the use of DNA that is extracted from phytoplankton when preserved in glutaraldehyde is scarce. Using conventional PCR, Marín *et al.* (2001) were unable to obtain a positive PCR result with DNA from glutaraldehyde-preserved samples, while using real-time PCR, Hosoi-Tanabe & Sako (2005) were able to amplify DNA from glutaraldehyde-preserved samples and reported that the detection level was lower than that of untreated samples. In contrast to our results, studies of preserved samples of plant and fungi obtained high-molecular-weight DNA from glutaraldehyde-preserved samples and DNA yields that were similar to those of non-preserved samples, with positive PCR amplification (Douglas & Rogers 1998).

Conclusions

In summary, Lugol's-iodine- and formaldehyde-preserved cyanobacterial samples can be analysed using real-time PCR without these fixatives interfering with the reaction. The main factor jeopardizing the amplification of DNA from preserved samples seems to be the DNA loss during the fixation process, rather than fragmentation or PCR inhibition. This DNA loss occurs soon after fixation and increases over time, meaning that to overcome this issue, the preserved samples would have to be highly concentrated to minimize the effects of DNA loss. In this study, we used 25 mL of filtered field samples from a *P. agardhii* bloom and obtained a good fluorescence signal in real-time PCR. In field surveys, usually 100- to 1000-mL samples are preserved for monitoring proposes. Such volumes should be more than adequate for proper real-time PCR detection. Furthermore, it is noteworthy that we have used a target gene that exists in single copy in the cyanobacteria genome; for genes presenting more than one copy in the cell, such as the 16S rRNA and microcystin synthetase genes, the availability of the target fragment could be higher.

This study shows that it is possible to use the real-time PCR technique in preserved cyanobacteria samples, thus providing access to molecular information that might be otherwise discriminated. Moreover, the high sensitivity of this technique, in contrast to that of conventional PCR, can be very useful for the fragment target detection of low-concentration samples that may result from the preservation process. However, the

quantification that is achieved by the real-time PCR of the preserved samples cannot be related to or used to infer copy gene numbers of non-preserved samples. The use of this analysis to detect a specific species or a specific genotype in samples that arrive as fixed to the laboratory or that are preserved for some time can greatly increase the knowledge of bloom occurrence, specificity and toxicity and thus open a time capsule into water reservoir history.

Acknowledgments

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Chapter

06_

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Evaluation of methanol preservation for molecular and morphological studies in cyanobacteria using *Planktothrix agardhii*

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Abstract Molecular studies on cyanobacteria often involve filtering and freezing of samples leading to loss of cell morphological features. Methanol is often used in preservation of biological materials in association with other fixatives. This study evaluates the application of methanol in the preservation of DNA for molecular studies as well as for the preservation of cell morphology for morphometric analysis in filamentous cyanobacteria. In the present study, both culture and environmental bloom samples were preserved using a cold methanol dehydration series (50, 70, and 100 %) and stored at −20 °C for up to 2 years. The DNA quantity and quality, nucleotide sequence retrieval, and real-time PCR quantification were analyzed over time. Morphometric cell analysis was performed on preserved samples. Results show that the DNA extracted from samples preserved up to 6 months was successfully quantified by real-time PCR. After that period, the DNA quantity decreased with the preservation time. Nevertheless, we were able to detect/amplify the target fragment in samples preserved up to 2 years. The DNA sequence and cell morphology were also maintained during the preservation time. Thus, methanol preservation is an adequate method to preserve molecular information and morphological features after long storage periods.

Keywords Cyanobacteria · Lugol's iodine · Methanol · Preservation · Real-time qPCR · *rpo*C1

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Introduction

Cyanobacteria are common constituents of phytoplankton. Recognized for a long time as bloom formers and toxin producers in freshwaters, these organisms endanger water quality and human health across the globe (Merel et al. 2013). Many countries have regulatory programs for the monitoring of cyanobacteria and cyanotoxins (see Churro et al. 2012a and references therein). The routine monitoring of cyanobacteria in freshwaters mainly consists of quantification of cell density, identification of cyanobacterial species, and toxin analysis (Lawton et al. 1999). Together with cell quantification, their identification is needed using morphologic features of cyanobacteria, thus providing detailed species composition of a sample. Furthermore, l... the microscope are also us... provides an estimate of the ... to the overall biomass (Ha... 1999; Lawton et al. 1999).

Over the past 20 years, ... increased application in cy... ground also in cyanobact... technique—the real-time ... involving cyanobacteria ... 2010; Tai and Palenik 200...

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EVALUATION OF METHANOL PRESERVATION FOR MOLECULAR AND MORPHOLOGICAL STUDIES IN CYANOBACTERIA USING *PLANKTOTHRIX AGARDHII*.

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Abstract

Molecular studies on cyanobacteria often involve filtering and freezing of samples leading to loss of cell morphological features. Methanol is often used in preservation of biological materials in association with other fixatives. This study intends to evaluate the application of methanol in the preservation of DNA for molecular studies as well as for the preservation of cell morphology for morphometric analysis in filamentous cyanobacteria. In the present study, both culture and environmental bloom samples were preserved using a cold methanol dehydration series (50, 70 and 100%) and stored at -20 ºC up to two years. The DNA quantity and quality, nucleotide sequence retrieval and real-time PCR quantification were analyzed overtime. Morphometric cell analysis was performed in preserved samples. Results show that the DNA extracted from samples preserved up to six months was successfully quantified by real-time PCR. After that period, the DNA quantity decreased with the preservation time. Nevertheless, we were able to detect/amplify the target fragment in samples preserved up to two years. The DNA sequence and cell morphology was also maintained during the preservation time. Thus, methanol preservation is an adequate method to preserve molecular information and morphological features after long storage periods.

Keywords: Cyanobacteria, Lugol´s iodine, Methanol, Preservation, Real-time qPCR, *rpo*C1.

Introduction

Cyanobacteria are common constituents of phytoplankton. Recognized for a long time as bloom formers and toxins producers in freshwaters these organisms endanger water quality and human health across the globe (Merel et al. 2013). Many countries have regulatory programs for the monitoring of cyanobacteria and cyanotoxins see Churro et al. (2012a) and references therein. The routine monitoring of cyanobacteria in freshwaters mainly consists in quantification of cell density, identification of cyanobacterial species and toxin analysis (Lawton et al. 1999). Together with cell quantification, their identification is needed using morphologic features of cyanobacteria, thus providing detailed species composition of a sample. Furthermore, linear dimensions measured in the microscope are also used to calculate biovolume that provides the contribution of a particular species to the overall biomass (Hawkins et al. 2005; Hillebrand et al. 1999, Lawton et al. 1999).

Over the past twenty years, molecular techniques have found increased application in cyanobacteria research and gained ground also in cyanobacteria monitoring. One particular technique – the real-time qPCR – has been used in studies involving cyanobacteria population dynamics (Orr et al. 2010; Tai and Palenik 2009), detection and quantification of toxic genotypes (Al-Tebrineh et al. 2010; Hautala et al. 2013) and detection and quantification of cyanobacteria species (Churro et al. 2012b; Rueckert et al. 2007), with promising results to simplify cyanobacteria identification and enumeration in routine monitoring programs (Humbert et al. 2010; Martins and Vasconcelos 2011).

In general, the cyanobacteria sample preparation for molecular and morphological purposes follows different workflows. For molecular studies the DNA is extracted either from fresh or frozen samples. In both situations the morphological information of the organisms is lost and the long time maintenance of the sample is performed by freezing purified DNA. For morphological studies the samples are typically preserved in fixative solutions such as Lugol´s Iodine, glutaraldehyde or formaldehyde (Utkilen et al. 1999). Although it is possible to obtain DNA from Lugol´s Iodine and formaldehyde preserved samples the nucleic acid retrieval is poor (Churro et al. 2015, Zimmermann et al. 2008).

Due to the importance that molecular and morphologic analysis represent to cyanobacteria studies, and more important the conciliation of both approaches, it is necessary to preserve not only the morphology but also the nucleic acids integrity.

Therefore a preservation method that serves both purposes is important. The necessity of a conciliator preservation method for multidisciplinary studies has been previously pointed out by researchers and is transversal to several areas of research such as: pathology (Noguchi et al. 1997; Staff et al. 2013), bumblebees (Moreira et al. 2013), nematodes (Fonseca and Fehlauer-Ale 2012; Yoder et al. 2006), macroinvertebrates (Stein et al. 2013) and dinoflagellates (Godhe et al. 2002).

Methanol is a low molecular weight primary alcohol, often used for morphologic preservation purposes in several types of biological materials in conjunction with other fixatives (Dimulescu et al. 1998; Fonseca and Fehlauer-Ale 2012; Godhe et al. 2002; Puchtler et al. 1970). Methanol is highly permeable, due to its high polarity, and acts by changing the hydration state of the cells (Bacallao et al. 2006). Methanol is also used as a cryoprotectant in phytoplankton - to reduce cell damage and increase survival - with promising results (Park 2006; Rastoll et al. 2013; Tzovenis et al. 2004; Wagner et al. 2007). The low molecular weight and rapid tissue penetration of methanol contributes to uniform tissue preservation and alcoholic reagents always yield superior results as nucleic acids fixatives than aldehydes (Noguchi et al. 1997; Srinivasan et al. 2002; Tsai 2006). Due to its characteristics methanol is likely to facilitate preservation of nucleic acids while also preserving cell morphology in cyanobacteria.

The present study aimed to determine whether cold methanol at 100% could preserve DNA for molecular analysis and also maintain morphological features of filamentous cyanobacteria.

Methods

Cyanobacterial cultures and field sample

Four cyanobacterial cultures of *Planktothrix agardhii* (LMECYA153A, 153B, 155 and 256) were used in the present study. These are maintained in the Estela Sousa e Silva Algal Culture Collection in the Laboratory of Biology and Ecotoxicology (LBE) at the National Institute of Health Doutor Ricardo Jorge, Portugal. Cultures are cultivated in Z8 medium (Staub 1961). The culture chamber programmed for a 16:8 h light: dark cycle with a light intensity of 15 μmol.photons.m^{-2}.s^{-1}, and a constant temperature of 20 ± 1 ^{o}C.

One field sample was also used with intent of testing the applicability of the methanol preservation on environmental samples. The field sample was collected from the

surface of an artificial lake in the center of Portugal - Magos Lake - during a bloom consisting mainly of *Microcystis aeruginosa* and *P. agardhii*.

Fixation procedure

Three aliquots (1 mL) were harvested from a 15 days growing culture of each of the *P. agardhii* strains and preserved in a dehydration cold methanol (CH_3OH, J.T.Baker®-HPLC grade) dilution series (50, 70 and 100 %) intercalated with centrifugation steps of 843 *x*g for 10 minutes. Prior to fixation the 100% methanol solution was kept at -20 ºC and the fixation procedure was conducted at room temperature. After fixation the samples preserved in 100% methanol were kept at -20 ºC until analysis. From the three samples taken one was filtered after 24 h of preservation and proceed for analysis and the other two were stored for 6 to 24 months at -20 ºC. For control comparison purposes, one additional 1mL sample was taken at the time of sampling for preservation, from both culture and field sample and was not subjected to preservation. The fresh samples were filtered and proceed for DNA extraction. For reproducibility purposes the procedure was repeated three times with three different cultures (biological replicates). The same preservation treatment was applied to the bloom sample.

Genomic DNA extraction

Prior to DNA extraction the samples were filtered through a syringe with a syringe filter unit (Swinnex®, Millipore) holding a paper filter (1 cm of diameter). The genomic DNA was extracted from the filters using the phenol-chloroform method as described in Churro et al. (2012b). Briefly, the paper filters with the filtered biomass were placed in 2 mL Eppendorf™ tubes and 500 µL of lysis buffer [10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA (pH 8.0), 2% Triton X-100, 1% SDS and freshly added 1% (w/v) polyvinypyrrolidone (PVPP)] was added together with 100 µL of glass beads (400-600 µm, Sigma™). After sonication for 5 min, 250 µL of phenol plus 250 µL of chloroform were added. The extracts were then vortexed for 20 min at room temperature and centrifuged at 16,000 *x*g for 25 min at 4 ºC. After centrifugation the aqueous phase was transferred to a new 1.5 mL tube and the nucleic acids were precipitated by adding an equal volume of cold isopropanol. The pellets were washed with 1 mL of 70 % cold ethanol and air dried. The DNA pellet was ressuspended in 1 mL DNase/RNase-free distilled water (Gibco®).

Genomic DNA quantification and quality

Total DNA concentration was quantified using a NanoDrop® ND-1000 (Thermo Fisher Scientific®) spectrophotometer by measuring UV absorbance at 260 nm. The purity of genomic DNA was evaluated by UV absorption ratio at OD260/280 and OD260/230 nm.

Conventional PCR fragment amplification and sequencing

To test the success of PCR amplification in the DNA from non-preserved and preserved methanol samples, a fragment of 224 bp, counting with primers, within the *rpo*C1 gene was amplified with the primers rpoC1_Plank_F271 (5'-TGTTAAATCCAGGTAACTATGACGGCCTA-3') and rpoC1_P_agardhii_R472 (5'-GCGTTTTTGTCCCTTAGCAACGG-3') (Churro et al. 2012b). The PCR amplification was performed in a 25 µL reaction mixture containing: 1×PCR buffer (NZYTech™), 0.05 mM dNTPs (GE Healthcare®), 0.2 µM of each primer (rpoC1_Plank_F271 and rpoC1_P_agardhii_R472), 2 mM $MgCl_2$ (NZYTech™), 10 µL of DNA extract and 1 U of proofreading Taq DNA polymerase (NZYSpeedy Proof DNA polymerase, NZYTech™). The amplification was performed in a TGradient Thermocycler (Biometra®) with a program consisting of an initial denaturation step at 94 °C for 3 min, followed by 35 cycles of 20 s at 94 °C, 20 s at 58 °C and 20 s at 72 °C and a final extension step of 5 min at 72 °C.

Electrophoretic analysis of the PCR products was performed in 0.8 % w/v agarose gel with GelRed™ DNA staining (Biotium®), at 80 V in 0.5× Tris-borate EDTA (TBE) buffer for 40 min. The fragments were visualized by exposure to ultraviolet light. The amplified PCR products were purified using the PCR DNA and gel band purification kit ilustra™ GFX™ (GE Healthcare®).

Purified PCR products were sequenced in both directions using BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems®). Dye terminators were removed from sequencing reactions with DyeEx 96 plates (QIAGEN®) according to the manufacturer´s instructions; the eluted products were dried at 70ºC in a thermocycler and subsequently ressuspended in 15 µl HiDi formamide (Applied Biosystems®). Electrophoretic separation of sequencing products was performed using POP-7 polymer on a 3130xl Genetic Analyzer equipped with a 50 cm-capillary array (Applied Biosystems®).

Analysis of DNA sequencing quality metrics

Analysis of raw sequencing data was conducted using Sequencing Analysis software v5.3.1 integrated with KB Basecaller software v1.4 (Applied Biosystems®). Processed sequences were evaluated according to various sequencing quality metrics based on the quality value (QV) of individual nucleotides. The QV of a base-call is given by $QV = -10\log_{10}(p)$ where and *p* is the estimated probability of base calling error (Ewing and Green 1998). For instance, a QV=20 for a given base-call corresponds to a probability of error of 1.0%. In the software nucleotides with QV<15, 15≤QV<20 and QV≥20 were classified as low, medium and high QV. To determine the quality of sequencing data, the average QV of the entire read (sample score) and the length-of-read (LOR) were compared between samples sequenced with the same primer. The LOR represents the total number of sequenced nucleotides for each read wherever a sliding window of 20 bases shows an average QV≥20. The LOR for each read was also expressed as a percentage of the number of nucleotides comprised between the 3´ end of the primer and the end of the fragment (i.e., the total number of possible sequenced bases for that fragment). Similar metrics are used to evaluate the quality of DNA sequencing in international external quality assessment schemes (Patton et al. 2006).

The nucleotide sequences retrieved from non-preserved and methanol preserved samples were analysed using the BioEdit© v.7.0.5 software (Hall 1999) and aligned with ClustalW multiple alignment application. The *rpo*C1 sequence of *P. agardhii* NIVA-CYA 127 with the accession number: AY425002.1 available from NCBI was used as a reference.

Real-Time qPCR analysis

The standard curves for the real-time qPCR runs were constructed with 10-fold serial dilutions of DNA extracted from 1 mL *P. agardhii* cultures with gene copy numbers based on culture cell density as described in Churro et al. (2012b). Dilutions were prepared immediately prior to each experiment from one aliquot of culture stored at -20 °C. The amplification efficiencies of the real-time qPCR reactions were between 1.02 and 1.05 with r^2 between 0.996 and 0.997. The *rpo*C1 gene product size was 224 bp and the primers used for the amplification were the same as described above for conventional PCR. The real-time qPCR assays were performed on a Rotor Gene Q (Qiagen®) using SYBR Green I Dye. The following reagents were added in a 12.5 µL reaction mixture: 6.25 µL of SensiMix™ SYBR NO-ROX kit real-time qPCR MasterMix (Bioline®), 0.1 µM forward and reverse primers and 4 µL of DNA. The thermal cycling conditions consisted of an initial preheating step of 3 min at 94 °C followed by 40

cycles of 20 s at 94 °C, 20 s at 58 °C and 20 s at 72 °C. The specificity of the amplified PCR product was verified by melting curve analysis at the end of the 40 cycles by gradually increasing the temperature from 60 to 95 °C by 1 °C every 5 s. All reactions were run in triplicate. The threshold line was set at 0.05 of the signal fluorescence for all of the PCR tests using the Rotor-Gene Q series software. Real-time cycle threshold values (Ct) were used to compare fragment amplification between non-preserved and preserved samples. Statistical differences between treatments were calculated using Student's *t*-test and a p value < 0.05 was considered significantly different.

Cell quantification

The Utermöhl sedimentation technique was used for cell quantification in order to evaluate if the methanol preservation affected the cell density. Two equal samples were taken from *P. agardhii* culture LMECYA 256. One sample was preserved with methanol as described above and the other preserved with Lugol's Iodine solution following the procedure of the Utermöhl technique described in the European Standard EN15204. After preservation the samples were concentrated by sedimentation for 24 h in Utermöhl chambers and the number of cells were counted using an inverted microscope Olympus® CK40.

Morphologic and morphometric analysis

The morphology of the *P. agardhii* filaments was analyzed in both non-preserved and cells preserved in methanol and Lugol's, using the same samples previously used for the cell quantification. Microphotographs were taken in a light microscope Olympus® BX60 with Olympus® DP11digital camera under 1000x magnification. The cell dimensions (width and length) were measured in at least 50 cells and the respective mean and standard deviation were calculated. *P. agardhii* biovolume (V) was calculated from the length (l) and width (w) of cells in the filament assuming that the cell has a cylindrical shape $V = \frac{\pi}{4} \times w^2 \times l$ (Hillebrand et al. 1999). The cell dimensions and biovolume were compared between preserved and non-preserved samples using a z-test, and a p value < 0.01 was considered significantly different.

Results

Genomic DNA quality and quantity

In general, good quality gDNA was obtained from methanol-preserved samples, as shown by the values of UV absorption ratios OD260/280 and OD260/230 nm (Table 1).

Although the OD260/280 nm ratio was lower for the culture sample preserved for 24 months, the values of the quality ratios were within the accepted variability.

Table 1 - Genomic DNA quality determination by means of optical density ratios of non-preserved and preserved methanol samples. The reference ratio values for good-quality DNA are OD260/280 = 1.8 and OD260/230 = 2.0.

P. agardhii	Absorbance ratios	Non-preserved	Time preserved (months)				
			0	6	12	18	24
LMECYA 155	OD260/280	1.52	1.59	1.47	1.51		
	OD260/230	2.09	1.83	1.96	2.03		
LMECYA 153A	OD260/280	1.91	1.74	1.93		1.87	
	OD260/230	2.10	2.23	2.28		2.27	
LMECYA 153B	OD260/280	1.91	2.02	1.84			1.61
	OD260/230	2.40	2.36	1.79			1.97
Bloom Sample	OD260/280	1.89	1.65	1.88			1.52
	OD260/230	1.82	2.26	2.31			2.01

The quantity of gDNA decreased with preservation time for the three culture experiments (Fig. 1). In the six months preserved samples the DNA loss ranged between 7.9 % and 11.25 % when compared to the control. The DNA extracts from non-preserved and 24 h preserved methanol samples were similar and yielded the highest DNA concentrations, while long-time preservation (12, 18 and 24 months) yielded the lowest DNA concentrations (Fig. 1), representing a DNA loss of 25 %, 41 % and 43 % respectively.

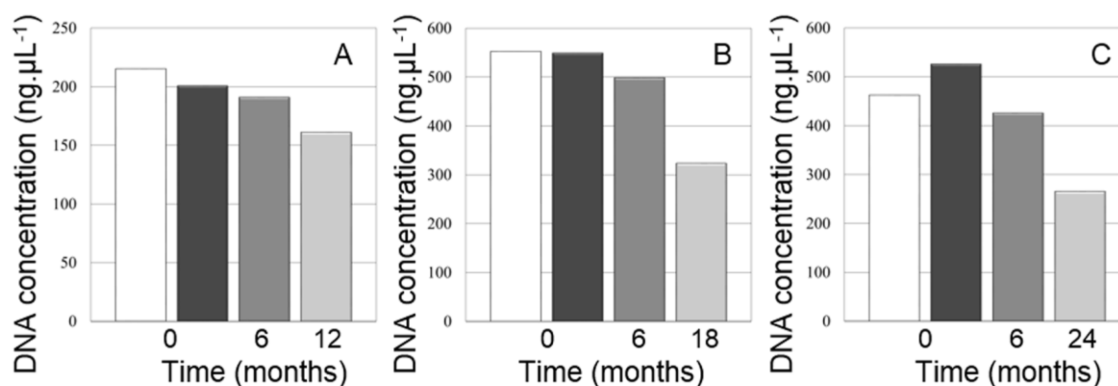

Fig. 1 - Comparative graphical representation of the total genomic DNA concentration obtained for methanol preserved and non-preserved samples in the three independent culture experiments over time. Non-preserved samples – white bars; samples preserved in 100% methanol methanol (24 h preservation - dark grey; 6 months after – soft grey; 12, 18 and 24 months after – light grey). A – *P. agardhii* culture LMECYA 155; B - *P. agardhii* culture LMECYA 153A; C - *P. agardhii* culture LMECYA 153B

Fragment amplification in conventional PCR

Figure 2 presents the results of conventional PCR amplification of the *P. agardhii* *rpo*C1 fragment from both non-preserved and preserved culture samples. Gel electrophoresis bands were visible for the 224 bp *rpo*C1 fragment in both *P. agardhii* cultures preserved for 6 months (Fig. 2, lane 2 and lane 5), 18 months (Fig. 2, lane 3) and 24 months (Fig. 2, lane 6). Thus, successful fragment amplification was obtained from DNA preserved in methanol for up to two years (Fig. 2). Nevertheless, the product yield in 24 months pr eserved sample was lower (Fig. 2, lane 6).

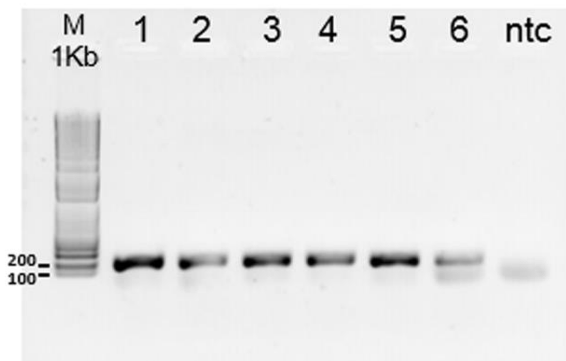

Fig. 2 - Photograph of the electrophoresis gel showing the amplification of the *rpo*C1 gene target sequence in non-preserved and methanol preserved samples. Lanes 1 to 3 *P. agardhii* LMECYA 153A; lanes 4 to 6 *P. agardhii* LMECYA 153B. Lane 1 and 4 – non-preserved sample; lane 2 and 5 – six months preserved samples; lane 3 – 18 months preserved sample; lane 6 – 24 months preserved sample. M – 1Kb Plus DNA Ladder, Invitrogen™; ntc - no-template control.

DNA sequence quality analysis

Analysis of the number of low, medium and high quality bases in reads sequenced with the same primer revealed no relevant differences between non-preserved samples and samples preserved for 6 or 18 months (Table 2). For instance, in culture LMECYA153A, reads sequenced with the forward primer showed that the number of bases with low, medium and high quality ranged between 22-27, 1-3 and 160-165 for all samples. The sample score was also similar in these samples ranging between 37 for the non-preserved sample and 42 for the sample preserved for 6 months. The proportion of each sequence read with a maximum probability of error of 1.0%, as expressed by the %LOR, was identical (93.8%) in the non-preserved sample and in the sample preserved for 18 months. Although the %LOR in the LMECYA153B culture sample preserved for 24 months was similar to the non-preserved sample or the samples preserved for less time, the sample score of reads obtained with either of the

sequencing primers was clearly lower in the 24 month-preserved sample compared to the remaining ones, suggesting lower sequence quality.

Table 2 - Quality assessment of nucleotide sequences of the *rpo*C1 fragment of 224 bp retrieved from non-preserved and preserved methanol samples. The values are given in number of nucleotides, with low quality values (QV), medium QV and high QV, based on base call accuracy. LOR is the length of reading of the nucleotide sequence.

	Sequence direction	Low QV	Medium QV	High QV	Sample Score	LOR	LOR (%)
LMECYA153A							
Non-preserved	fw	27	3	160	37	183	93.8
	rv	14	2	179	44	195	97.0
6 months	fw	23	1	165	42	182	93.3
	rv	14	4	176	42	194	96.5
18 months	fw	22	3	165	41	183	93.8
	rv	13	3	178	44	194	96.5
LMECYA153B							
Non-preserved	fw	27	2	161	40	181	92.8
	rv	16	3	175	42	194	96.5
6 months	fw	26	6	158	41	182	93.3
	rv	17	4	174	45	193	96.0
24 months	fw	31	10	150	26	182	93.3
	rv	27	3	163	33	184	91.5

The alignment of nucleotide sequences showed that no base pair alteration occurred and that the sequence integrity was maintained during preservation on methanol at 100 % (Table S1). However, several sequencing artifacts were obtained in sequences retrieved from samples preserved for 24 months (Table S1).

Real-time qPCR fragment amplification and quantification

The *rpo*C1 fragment amplification was successfully obtained by real-time qPCR and good fluorescence signal was acquired in DNA extracted from samples preserved until 24 months that presented Ct values below the cycle 20 (Fig. 3). The Ct value increased with the time of preservation. After 6 months the Ct values from preserved samples were significantly different from those obtained in non-preserved samples. Nevertheless, for samples preserved for 24 h the Ct values were identical to non-preserved samples. The results were similar for the three cyanobacterial cultures (Fig. 3A, B and C).

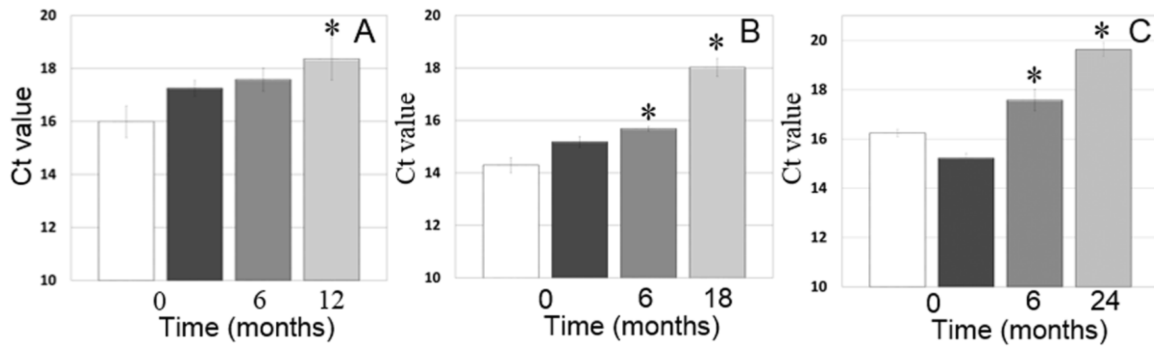

Fig. 3 - Average Ct values obtained from the real-time qPCR amplification of non-preserved and methanol preserved samples in the three independent culture experiments. Non-preserved samples – white bars; samples preserved in 100% methanol – (24 h preservation - dark grey; 6 months after – soft grey; 12, 18 and 24 months after – light grey). A – *P. agardhii* culture LMECYA 155; B - *P. agardhii* culture LMECYA 153A; C - *P. agardhii* culture LMECYA 153B. The asterisk denotes significant differences, Student's t test, df = 4, $p < 0.05$, between the Ct values of the preserved and non-preserved samples.

Cell morphology, morphometry and quantification

Figure 4 present's photographs of non-preserved filaments (Fig. 4A) and filaments preserved with methanol (Fig. 4B) and Lugol´s iodine (Fig. 4C).

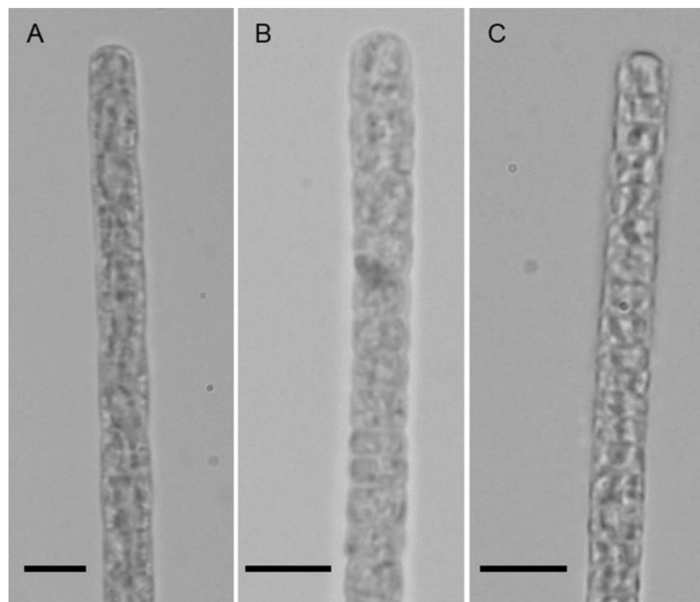

Fig. 4 - Representative morphology of filaments in non-preserved and 24 h preserved samples of *P. agardhii* – LMECYA 256: A - non-preserved sample; B - 100% methanol preserved sample; C - Lugols's iodine preserved sample. Scale bar 5 µm, 1000x magnification.

The filament morphology was maintained in filaments preserved with methanol when compared to non-preserved and Lugol´s iodine preserved filaments. However, there was a color loss in methanol preserved filaments and the cells appeared to be slightly swelled with visible cell divisions (Fig. 4B). Nevertheless the swell effect was not

noticed in the morphometric values (Table 3). In contrast, there was shrinkage of the filaments in both preserved samples (Table 3). The cell width, length and biovolume were significantly lower for preserved when compared to non-preserved samples, particularly in Lugol´s iodine preserved samples (Table 3). There was 16 % shrinkage of the cell width in methanol preserved filaments and 21 % in Lugol´s iodine. The cell length was reduced by 7 % with methanol and 16 % with Lugol´s iodine and the biovolume was 26 % lower in methanol and 42 % in Lugol´s iodine. The Utermöhl cell quantification for methanol preserved samples was 28 577 552 cell.mL^{-1} with a biovolume of 495.98 mm^3.L^{-1} while for Lugol´s iodine preserved samples the cell concentration was 27 689 895 cell.mL^{-1} and the biovolume was 379.74 mm^3.L^{-1}. Regarding cell quantification and biovolume the values in methanol preserved samples were close but higher, to those obtained with Lugol´s iodine preservation that is used in the standard Utermöhl phytoplankton quantification method.

Table 3 - Morphometry of *Planktothrix agardhii* (LMECYA 256) cells in non-preserved samples (fresh sample), 24 h methanol 100% preserved samples and samples preserved with Lugols's iodine solution. The asterisk denotes significant differences between fresh and preserved samples, z-test, $p < 0.01$.

	Cell Width (μm)	Cell Length (μm)	Biovolume (μm^3)
Fresh sample	3.78 ± 0.23	2.34 ± 0.46	23.58 ± 5.67
Methanol 100%	*3.17 ± 0.31	*2.17 ± 0.37	*17.36 ± 4.50
Lugol's Solution	*2.98 ± 0.15	*1.96 ± 0.42	*13.71 ± 3.32

Methanol preservation of the bloom sample

Quantification values of DNA extracted from the bloom sample preserved in methanol showed no alteration until six months and a sharp decline after 24 months of preservation (Fig. 5A). There was a DNA loss of 6 % in six months preserved samples and 57 % after 24 months. Likewise, the lower DNA quality was obtained from samples preserved for 24 months (Table 1). In real-time qPCR amplification there no differences in Ct values from samples preserved in methanol during six months when compared to non-preserved samples (Fig. 5B) and accurate gene copy number was quantified in these samples. After 24 months the Ct values were significantly different from non-preserved samples but the fluorescence signal was good and a correct fragment detection could be obtained (Fig. 5B).

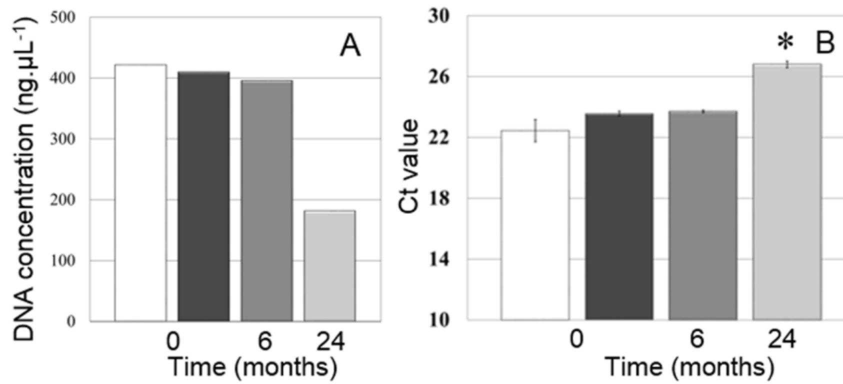

Fig. 5 - Total genomic DNA concentration (A) and average Ct values obtained from the real-time PCR amplification of the *rpo*C1 fragment (B) in the environmental bloom sample. Non-preserved samples – white bars; samples preserved in 100% methanol – (24 h preservation - dark grey; 6 months after – soft grey; 12, 18 and 24 months after – light grey). The asterisk denotes significant differences, Student's t test, df = 4, $p < 0.05$, between the Ct values of the preserved and non-preserved samples.

The filament morphology was also maintained in samples preserved in methanol for 24 months (Fig. 6B). The swell effect, detected previously in culture samples, was not visible in *P. agardhii* filaments observed from the bloom sample and distinctive features of identification, such as the calyptra, were still visible in the apical cells (Fig. 6B). Other cyanobacteria, such as *M. aeruginosa,* also maintained the morphology and could also be identified (Fig. 6C-E). Moreover, the *M. aeruginosa* colonies where still aggregated and *Pseudoanabena* filaments could be seen within the colonies (Fig. 6D).

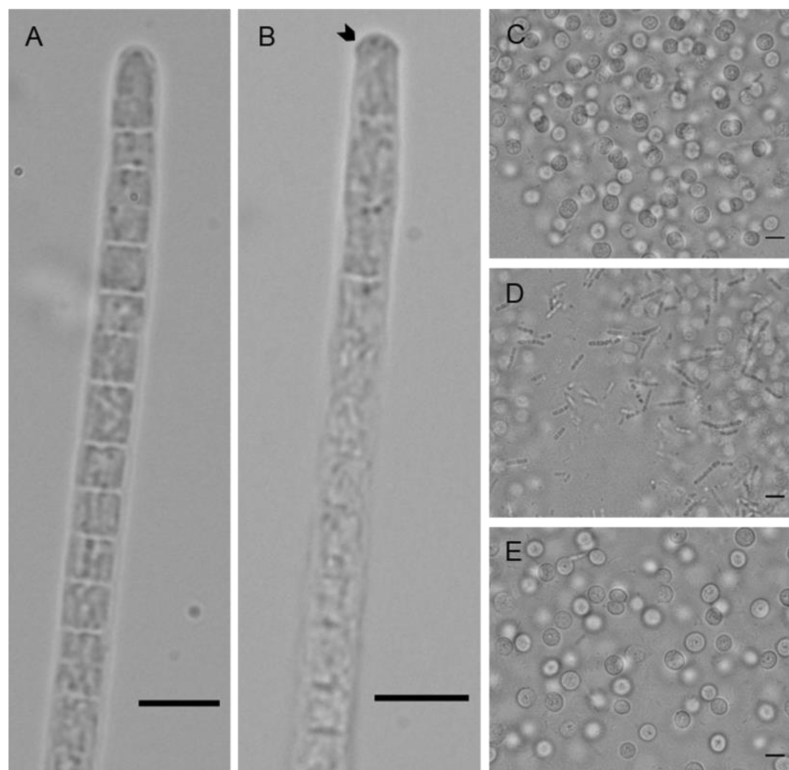

Fig. 6 - Representative morphology of filaments from the bloom sample preserved in 100 % methanol for 24 months. A - *P. agardhii* culture (LMECYA 153B); B – *P. agardhii* from environmental sample. The arrow indicates the calyptra in *P. agardhii* apical cell. C to E – *M. aeruginosa* from environmental sample; D – *Pseudoanabaena mucicola* filaments inside a *M. aeruginosa* colony. Scale bar 5 µm, 1000x magnification.

Discussion

Filamentous cyanobacteria are a large group of organisms that include producers of a wide range of cyanotoxins in both planktonic and benthic habitats (Quiblier et al. 2013). *Planktothrix agardhii* is a widely spread toxic cyanobacterium and was used in this study as a representative of filamentous cyanobacteria.

A dehydration series of methanol until 100% was tested for the preservation of *P. agardhii* used for concerted molecular and morphological analysis. Conventional PCR and real-time PCR based assays were applied to access the usefulness of methanol preserved samples. The genomic DNA quantity and quality, nucleotide sequence and quality were considered to access the quality of the information retrieved from preserved samples. The cell length, width and biovolume were used to evaluate morphometric characteristics and the Utermöhl method for cell quantification of the methanol preserved samples.

Overall, the molecular and morphological information obtained from 24 h methanol preserved samples was very similar to the information obtained from non-preserved samples. The DNA quantity and quality was equivalent to that obtained from non-preserved samples. The conventional PCR amplification and the DNA sequence retrieval from all preserved samples was successful. Similar results were obtained by other authors. Noguchi et al. (1997) reported high-molecular-weight DNA extracted from human tissue and cell lines preserved in 100 % methanol. Also Marín et al. (2000) reported successful DNA fragment amplification in dinoflagellates cells preserved at -20ºC in 100 % methanol. Nevertheless, contrary results were reported by Frampton et al. (2008) who described weak gel bands showing signs of degradation from genomic DNA extracted from bees preserved in 50% and 95 % methanol. In the present study, the long-time preservation of DNA quality was maintained but the DNA quantity declined over the two years of storage. Methanol preservation on higher plants also showed increased DNA degradation after three months of preservation (Flournoy et al. 1996). Nevertheless, in our study DNA sequence quality was maintained during two years of storage and correct fragment detection was achieved in conventional PCR. In dinoflagellates Marín et al. (2000) obtained positive PCR with cells kept for five years in frozen methanol at 100 %.

In conventional PCR amplification the DNA quantity does not have to be equal to the fresh samples because it is an end-point analysis. However, for gene copy number quantification by real-time qPCR the DNA quantity must be as similar as possible to the one from the fresh sample. In this study methanol preserved samples yielded Ct values similar to those obtained from fresh samples, enabling the correct fragment quantification by real-time PCR analysis, in samples stored for up to six months. In previous studies by Churro et al. (2015), with other fixatives and using the same strains as in this study, the DNA retrieval from formaldehyde samples was much lower than from fresh samples and it was unsuccessful for glutaraldehyde preserved samples. As for Lugol´s iodine, the DNA recovery was similar to the fresh samples and the real-time PCR gene copy number quantification was achieved in samples preserved for 24 h but variable between experiments (Churro et al. 2015). In the present study, gene copy number from methanol preserved samples was correctly quantified in 24 h preserved samples and in some samples preserved for 6 months. Furthermore, positive fragment amplification was obtained in real-time qPCR analysis using samples stored up to two years in cold methanol. Therefore, the DNA is maintained in a steadier state during preservation in methanol than in other common phytoplankton fixatives.

There are several types of degradation that DNA can undergo during preservation such as: denaturation, strand breakage, cross-linking of the double strand, and chemical modification within a nucleotide (Brown 1999). Chemical modifications are undesirable because they change the nucleotide sequence (Brown 1999). In our study the nucleotide sequence was maintained during methanol preservation in the sequences retrieved from preserved samples and no base pair alterations where observed. Therefore, reliable genetic information could be retrieved form methanol preserved samples. Although, no base pair alteration was observed, there were non-defined nucleotides in 24 months preserved samples. This is in congruence with low nucleotide quality obtained and was probably due to low template concentration obtained in conventional PCR.

Morphological features were well preserved in methanol samples when compared to fresh and the Lugol´s iodine preserved samples. Lugol´s iodine solution is widely used as a fixative for phytoplankton and methanol fixation showed advantages and disadvantages in relation to this fixative. The morphometric analysis showed significant differences between the measurements obtained in methanol and Lugol´s iodine preserved samples when compared to fresh samples. However the difference was lower for methanol than for Lugol´s iodine preserved samples and therefore the measurement was more accurate. Cell shrinkage has been reported for both Lugol´s

iodine and methanol preservation. In Lugol´s iodine preservation, cell shrinkage was described for ciliates (Stoecker et al. 1994) and cyanobacteria with a reduction of 30 to 40 % in biovolume when compared to non-preserved samples (Hawkins et al. 2005). In methanol preserved samples shrinkage has been described for several types of material and the reduction in dimensions is always lower than with other fixatives (Bacallao et al. 2006; Noguchi et al. 1997; Talbot and White 2013). These reports are in congruence with the results obtained in this study where Lugol´s iodine caused 42% reduction in the biovolume whereas methanol only 26%.

There was also a cell discoloration in preserved methanol samples because pigments tend to be extracted during the dehydration process. However, no color information is also obtained from Lugol´s iodine samples because iodine stains the samples brown. Nevertheless, Lugol´s iodine provides contrast and cellular inclusions and organelles in phytoplankton cells are made visible. The methanol fixation fails in giving this contrast. The methanol preservation also caused a wrong perception of cell swelling which made cell divisions visible in *Planktothrix* filaments. While this ballooning artifact caused by the methanol preservation may be useful for cell counting it also may impair proper species identification since taxonomists are not expecting the cell divisions to be visible in *Planktothrix*.

The cell concentration determined by the Utermöhl technique was similar between methanol and Lugol´s iodine preserved samples. Therefore, methanol preservation didn´t influence the determination of cell quantity. Moreover, the Lugol´s iodine solution is used in the Utermöhl technique because, besides contrast, it also confers density to cells, which facilitates their sedimentation (Lawton et al. 1999). In methanol preserved samples the filaments also settled down in the chamber with no difficulties. This is an important characteristic of methanol preserved samples because fresh samples of cyanobacteria are usually very difficult to concentrate by centrifugation and methanol fixation helps the pelleting of cyanobacteria.

In this study cold methanol at 100 % revealed to be better in DNA preservation than most phytoplankton preservatives used until now and showed suitability for combined molecular and morphological studies on filamentous cyanobacteria. Methanol maintains its liquid state at subzero temperatures which prevents the formation of ice crystals that could destroy the cells, being therefore able to maintain useful morphologic information. Furthermore, methanol fixation enables the maintaining of the DNA quality for 18 months and the DNA quantity for 6 months.

Preserved samples are an important resource and in the future, at the time of sample preservation, not only the preservation of morphology have to be cared for but also the maintenance of genetic information will have to be taken into account. The need for improved preservation methods is an ongoing concern and the search for enhanced preservatives that preserve both molecular and morphologic deserves further developments.

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Chapter

07_

Monitoring the variability of mcyA gene and microcystin concentration of a *Planktothrix agardhii* bloom – Do chytrid parasitism play a role on bloom dynamics?

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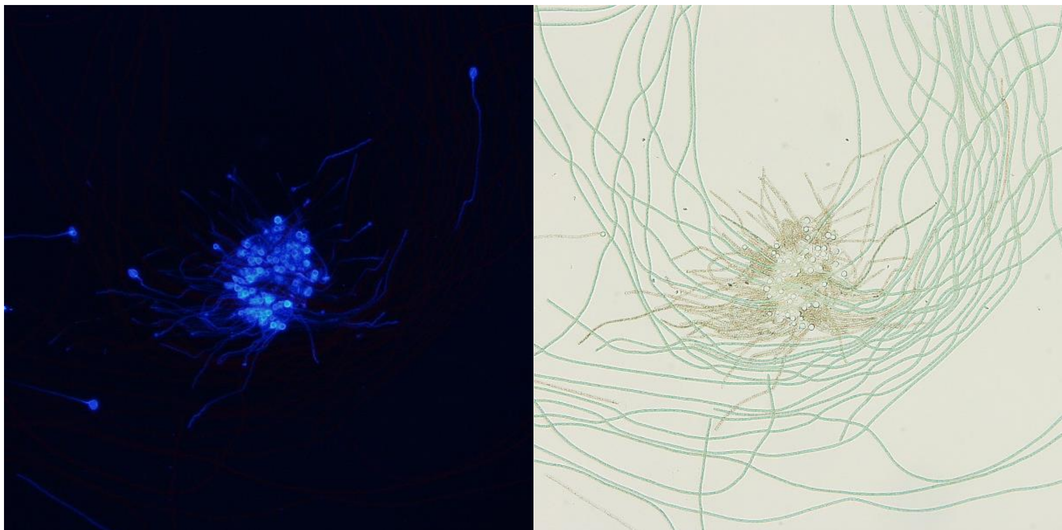

MONITORING THE VARIABILITY OF THE *MCY*A GENE AND MICROCYSTIN CONCENTRATION IN A *PLANKTOTHRIX AGARDHII* BLOOM – DO CHYTRID PARASITISM PLAY A ROLE ON BLOOM DYNAMICS?

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Abstract

The Cyanobacteria *Planktothrix agardhii* form persistent blooms in freshwater reservoirs and is often associated with the presence of microcystins. However, high cell densities of *P. agardhii* not always correspond to high levels of microcystin and *vice versa*. The *Planktothrix* blooms are constituted by toxic and non-toxic strains that are visually indistinguishable. Nevertheless, toxic strains can be quantified molecularly since they possess a number of genes involved in microcystin synthesis, which includes the *mcy*A gene.

In this work, a perennial bloom of *P. agardhii* was monitored for two years (2012-2014) with the aim of characterize the temporal variability of toxic genotypes and concentration of microcystins. Phytoplankton species were identified and quantified microscopically. The total concentration of microcystin in water was measured by

ELISA. The number of copies of the genes *mcy*A, *rpo*C1 and 18SrDNA of chytrid parasites was quantified by real-time PCR. Physicochemical (nitrates, total phosphorus, pH and conductivity) parameters were also measured.

The results showed that the amount of *mcy*A gene and the total concentration of microcystin vary over time and that are correlated (Spearman rank correlation coefficient of 0.84). The period with the highest concentration of microcystin and *mcy*A gene was higher coincided with the presence of chytrid parasites. The amount of the 18SrDNA gene correlated with *mcy*A gene copy numbers (Spearman rank correlation coefficient of 0.83) and microcystin concentration (Spearman correlation coefficient 0.82). There was no correlation between physicochemical parameters and the concentration of *mcy*A gene and microcystins.

These results raises some questions to be explored: What is the influence of chytrid parasites in shaping the density and toxicity of *P. agardhii* blooms? Does the presence of these parasites stimulate the development of toxic blooms? What factors influence the chytrids-*Planktothrix* parasitic relationship?

Keywords: Cyanobacteria, Real-time qPCR, *Planktothrix agardhii*, chytrids, *mcy*A, microcystins, *Rhizophydium megarrhizum*.

Introduction

The development of toxic cyanobacteria blooms in water supplies is frequent worldwide and represents a serious threat to human health and to the quality and sustainability of freshwater resources.

Cyanobacterial blooms are a complex phenomenon. These toxic events are often composed of different species that may have varying toxin production abilities. This implies that in the same bloom may co-exist toxic and non-toxic cyanobacteria from the same and/or different species as well as diverse toxins (Otsuka et al., 1999, Kardinaal et al., 2007, Yéprémian et al. 2007, Ostermaier et al. 2012). Since the coexisting toxic and non-toxic strains of the same species are morphologically indistinguishable, they are often recognized as the same population (Yéprémian et al. 2007, Ostermaier et al. 2012). This fact has been point out as the reason why high biomass or cell concentration doesn´t always indicate high concentrations of toxin and vice-versa (Briand et al. 2002, Yéprémian et al. 2007, Catherine et al. 2008). All these factors contribute for the difficulty in studying the conditions that favor bloom development and

toxin production and ultimately impair the prediction of toxic blooms and the risk management on surface freshwater supplies.

Cyanotoxins can be classified into different groups according to the primary target organ, with the hepatotoxic microcystins as the most commonly found in freshwaters. A well-known producer of these hepatotoxins is the cyanobacterium *Planktothrix agardhii*. This is frequently found blooming in temperate regions and has been reported to have high levels of toxins per biomass (Fastner et al. 1999). This cyanobacterium tolerates a wide range of temperatures and light intensities so it can prevail all year around. It is generally mixed in the water but it can also form metalimnetic blooms, survive under ice-covered lakes and tolerate shade from other phytoplankton under eutrophic conditions (Hašler and Poulícková 2003, Bonilla et al. 2012, Rücker et al. 1997, Pawlik-Skowroµska et al. 2008, Halstvedt et al. 2007).

The bloom development and constrain have been attributed to many variables such as availability of light and nutrients, temperature, stability of the water column and grazing. But other factors may also influence bloom dynamics such as the chytrid parasites. These zoosporic fungi are ubiquitous in aquatic systems and the infection of phytoplankton by these fungi is common in freshwaters. They can affect various types of phytoplankton species including bloom-forming cyanobacteria (Gerphagnon et al. 2013). *Planktothrix* has been observed to be parasitized by the chytrid *Rhizophydium megarrhizum* (Rohrlack et al. 2013, Sønstebø and Rohrlack 2011). However, there is little information on the effect of these infections on the occurrence, frequency and toxicity of overall cyanobacterial blooms.

P. agardhii settled in a Portuguese water supply and is found blooming in this reservoir since 2006 with high cell and microcystins concentration recorded (Churro et al. 2012a, Churro et al. 2013). However high *P. agardhii* densities don't always coincide with high microcystin concentrations and during 2014 chytrid infections were observed in *P. agardhii* filaments from this reservoir (Churro et al. 2014).

The aims of this study were to characterize the seasonal abundance of toxic and non-toxic *P. agardhii* strains, to characterize the variability of microcystin content over time and to understand the conditions underlying the proliferation of these strains. For these purposes, a *P. agardhii* perennial bloom was studied for two years. The abundance of toxic and non-toxic *Planktothrix* strains was followed by gene copy number quantification using the *rpo*C1 gene specific for *Planktothrix* (Churro et al. 2012), the microcystin synthetase gene *mcy*A specific for *Planktothrix* (Briand et al. 2008) and the *mcy*A general for several microcystin producing cyanobacteria (Hisbergues et al.

2003). Molecular quantification of the chytrid fungi was also assessed using the 18S rDNA (Lefèvre et al. 2010).

Methods

Sampling and study site

The studied freshwater reservoir is located in the central region of Portugal and its primary use is the supplying of drinking water for a population of about 20 000 inhabitants. It has a water storage capacity of about 7 million m^3, a surface area of 10 km^2 and a catchment area of 40 km^2. The reservoir has an average depth of 37 m and a maximum depth of 43 m.

In this work, a perennial bloom of *P.agardhii* was monitored during two years (2012-2014). The water reservoir was sampled monthly or weekly during periods of unusual high cyanobacteria densities. Integrated water column samples were collected with a Van Dorn bottle at several water depths and transported to the laboratory under refrigerated conditions.

Molecular analysis

Environmental samples preparation

Aliquots of 2 mL of fresh samples collected at the reservoir were taken and frozen at -20ºC until real-time qPCR analysis. The DNA from these aliquots was extracted by mechanical cell disruption that consisted in the addition of glass beads (400-600 µm) with further sonication for 10 min. plus vortex for 10 min; 4 µL of the supernatant was immediately used in the qPCR reactions. The copy numbers of the target genes were quantified in environmental samples based on standard curves in real-time PCR.

Primers description and specificity tests

Quantification of copy number of the following genes in environmental samples was performed: *rpo*C1 gene specific for *Planktothrix* (Churro et al. 2012), microcystin synthetase gene *mcy*A specific for *Planktothrix* (Briand et al. 2008), *mcy*A general for several microcystin producing cyanobacteria (Hisbergues et al. 2003) and 18S rDNA of chytrid fungi (Lefèvre et al. 2010). The primers and target genes used in this study are described in Table 1. The primers were chosen from available literature and to update their specificity a GenBank BLASTn search in the NCBI database was made. No close hits were obtained from genes other than their targets. To complete primer specificity

analysis, the primers were tested using conventional PCR and genomic DNA of toxic and non-toxic strains of several cyanobacteria species (Table 2).

Table 1 - Target genes, primer sequences and target organisms used in this study. Tann – Primer annealing temperature. The Fragment length includes the primers.

Gene	Oligonucleotide name	Primer sequence 5'- 3'	Tann (ºC)	Fragment size (bp)	Specificity	Reference
*rpo*C1	rpoC1_Plank_F271	TGTTAAATCCAGGTAACTATGACGGCCTA	58	224	*P. agardhii*	Churro et al. 2012
	rpoC1_P_agardhii_R472	GCGTTTTTGTCCCTTAGCAACGG				
*mcy*A	mcyA-Cd 1F	AAAATTAAAAGCCGTATCAAA	60	291 - 297	*Microcystis, Anabaena, Planktothrix, Nostoc*	Hisbergues et al. 2003
	mcyA-Cd 1R	AAAAGTGTTTTATTAGCGGCTCAT				
	MAPF	CTAATGGCCGATTGGAAGAA	60	140	*Planktothrix*	Briand et al. 2008
	MAPR	CAGACTATCCCGTTCCGTTG				
18S rDNA	F-Chyt	GCAGGCTTACGCTTGAATAC	60	310	Order Rhizophydiales	Lefèvre et al. 2010
	R-Chyt	CATAAGGTGCCGAACAAGTC				

Conventional PCR amplifications for each fragment were performed in a 25 µL reaction mixture containing: 1×PCR buffer (invitrogen™), 0.05 mM dNTPs (GE Healthcare®), 0.2 µM each primer, 2 mM MgCl2 (invitrogen™), 2 µL of DNA extract and 1 U of Taq DNA polymerase (Invitrogen™). The amplification was performed in a TGradient Thermocycler (Biometra®) with a thermocycling profile consisting of an initial denaturation step at 94 °C for 3 min, followed by 35 cycles of 20 s at 94 °C, 20 s at annealing temperature (see Table 1) and 20 s at 72 °C and a final extension step of 5 min at 72 °C. Electrophoretic analysis of the PCR products was performed in 0.8 % w/v agarose gel and electrophoresis at 80 V in 0.5× Tris-borate EDTA (TBE) buffer for 40 min. The fragments were visualized by exposure to ultraviolet light after GelRed™ DNA staining (Biotium®). The amplified PCR products were purified using the ExoSAP-IT PCR clean-up Kit USB™ (GE Healthcare®). The sequences from the amplified fragments were obtained by Sanger sequencing using an ABI 3130xl Genetic Analyzer (Applied Biosystems®) and BigDye® Terminator v3.1 solution (Life Technologies®). The sequence identity was cheeked by a BLASTn search in the NCBI database. No amplification was obtained from strains/species other than their targets (Table 2).

Plasmid constructs and Standard curves for real-time qPCR

The standard curves were constructed using DNA plasmids containing cloned sequences of the targeted fragments. The target sequences were obtained from purified DNA of clonal cultures by amplifying the target fragment using the specific primers (Table 1). The *rpo*C1 and *mcy*A gene fragments were obtained from the *P. agardhii* culture LMECYA 256, which is a microcystins producer (Table 2). The 18S rDNA gene fragment was obtained from the *Rhizophydium megarrhizum* chytrid clonal culture that was isolated from the environmental samples (Churro et al. 2014).

Table 2 - Primer specificity. (+) Target DNA fragment amplification, (-) Lack of target DNA fragment amplification, (nt) not tested. MCYS – microcystins, PST – paralytic shellfish poisoning toxins, SXT – Saxitonin, CYLIND – Cylindrospermopsin.

Taxon and species designation	Strain	*rpo*C1 specific for *P. agardhii*	General for *mcy*A	*Planktothrix* spp. specific *mcyA*	Toxin Producer	Method of detection	Reference for toxin production
Microcystis aeruginosa	LMECYA 7	nt	+	-	MCYS-LR		Pereira et al. 2000
M. aeruginosa	LMECYA 81	nt	-	-	Non toxic for MCYS		Valério 2008
M. aeruginosa	LMECYA 92A	nt	+	-	MCYS-LR		Valério et al 2010
M. aeruginosa	LMECYA 110	nt	+	-	MCYS-LR		Valério et al 2010
M. aeruginosa	LMECYA 113	-	+	-	MCYS-LR		Valério et al 2010
M. aeruginosa	LMECYA 127	nt	-	-	Non toxic for MCYS		Valério 2008
M. aeruginosa	LMECYA 136	nt	-	-	Non toxic for MCYS		Valério 2008
M. aeruginosa	LMECYA 142	nt	-	-	Non toxic for MCYS		Valério 2008
M. aeruginosa	LMECYA 147	nt	-	-	Non toxic for MCYS		Valério 2008
M. aeruginosa	LMECYA 151	nt	+	-	MCYS-LR,RR, YR		Valério et al 2010
M. aeruginosa	LMECYA 159	-	+	-	MCYS-RR, YR		Valério et al 2010
Limnothrix redekei	LMECYA 145	-	-	nt	Non toxic for MCYS		Valério 2008
Leptpolyngbya sp.	LMECYA79	-	-	nt	Non toxic for MCYS		Valério 2008
Phormidium sp.	LMECYA 173	-	-	nt	Non toxic for MCYS		Valério 2008
Planktothrix agardhii	LMECYA 153A	+	-	-	Non toxic for MCYS	HPLC	This study
P. agardhii	LMECYA 153B	+	-	-	Non toxic for MCYS	HPLC	This study
P. agardhii	LMECYA 153C	+	-	-	Non toxic for MCYS	HPLC	This study
P. agardhii	LMECYA 155	+	-	-	Non toxic for MCYS	HPLC	This study
P. pseudoagardhii	LMECYA 162	-	-	-	Non toxic for MCYS	HPLC	This study
P. rubescens	LMECYA 203	-	+	+	MCYS	HPLC	This study
P. pseudoagardhii	LMECYA 224	-	-	-	Non toxic for MCYS	HPLC	This study
P. agardhii	LMECYA 229	+	-	-	Non toxic for MCYS	HPLC	This study
P. agardhii	LMECYA 229A	+	-	-	Non toxic for MCYS	HPLC	This study
P. agardhii	LMECYA 230	+	-	-	Non toxic for MCYS	HPLC	This study
P. agardhii	LMECYA 250	+	-	-	Non toxic for MCYS	HPLC	This study
P. agardhii	LMECYA 256	+	+	+	MCYS	HPLC/ELISA	This study
P. agardhii	LMECYA 257	+	-	-	Non toxic for MCYS	HPLC/ELISA	This study
P. agardhii	LMECYA 258	+	+	+	MCYS	HPLC/ELISA	This study
P. agardhii	LMECYA 259	+	+	+	MCYS	HPLC/ELISA	This study
P. agardhii	LMECYA 260	+	-	-	Non toxic for MCYS	HPLC	This study
P. agardhii	LMECYA 269	+	-	-	Non toxic for MCYS	HPLC	This study
P. agardhii	LMECYA 270	+	-	-	Non toxic for MCYS	HPLC	This study
P. agardhii	LMECYA 275	+	-	-	Non toxic for MCYS	HPLC	This study
P. pseudoagardhii	LMECYA 276	-	-	-	Non toxic for MCYS	HPLC	This study
P. agardhii	LMECYA 277	+	-	-	Non toxic for MCYS	HPLC	This study
P. agardhii	LMECYA 280	+	-	-	Non toxic for MCYS	HPLC	This study
P. agardhii	LMECYA 281	+	-	-	Non toxic for MCYS	HPLC	This study
P. agardhii	LMECYA 283	+	-	-	Non toxic for MCYS	HPLC	This study
P. agardhii	LMECYA 284	+	-	-	Non toxic for MCYS	HPLC	This study
P. agardhii	LMECYA 285	+	-	-	Non toxic for MCYS	HPLC	This study
P. agardhii	LMECYA292	+	-	-	Non toxic for MCYS	HPLC	This study
P. agardhii	LMECYA294	+	-	-	Non toxic for MCYS	HPLC	This study
P. agardhii	LMECYA297	+	-	-	Non toxic for MCYS	HPLC	This study
P. agardhii	LMECYA298	+	+	+	MCYS	HPLC	This study
P. agardhii	LMECYA302	+	-	-	Non toxic for MCYS	HPLC	This study
P. agardhii	LMECYA303	+	-	-	Non toxic for MCYS	HPLC	This study
P. agardhii	LMECYA304	+	-	-	Non toxic for MCYS	HPLC	This study
P. agardhii	LMECYA305	+	+	+	MCYS	HPLC	This study
P. agardhii	LMECYA306	+	+	+	MCYS	HPLC	This study
P. agardhii	LMECYA307	+	+	+	MCYS	HPLC	This study
P. agardhii	CCALA159	+	+	+	MCYS	ELISA	This study
P. mougeotii	LEGE 06224	-	-	-	Non toxic for MCYS	HPLC	This study
P. mougeotii	LEGE 06225	-	-	-	Non toxic for MCYS	HPLC	This study
P. mougeotii	LEGE 06226	-	-	-	Non toxic for MCYS	HPLC	This study
P. mougeotii	LEGE 06233	-	-	-	Non toxic for MCYS	HPLC	This study
Aphanizomenon gracile	LMECYA 40	-	-	-	PSP/SXT, Non toxic for MCYS		Pereira et al. 2004, Valério 2008
A. issatschenkoi	LMECYA 31	-	-	-	PSP/SXT , Non toxic for MCYS		Dias et al. 2002, Valério 2008
Cylindrospermopsis raciborskii	LMECYA 238	-	-	-	CYLIND, Non toxic for MCYS		Shalev-Alon et al. 2002,Valério 2008
Nodularia spumigena	LMECYA 247	nt	-	-	Nodularin	ELISA	This study

The amplified fragments were cloned in a plasmid TOPO-TA (invitrogen|) and amplified in *Escherichia coli* DH5α. The inserted fragment was sequenced and its identity was cheeked by a BLASTn search in the NCBI database. The supercoiled plasmids were linearized with restriction enzymes since circular plasmids cause underestimation of gene copy number (Hou et al. 2010, Lin et al. 2011).

The standard curve for each studied gene was constructed with 10-fold serial dilutions of each linearized plasmid containing the cloned fragment. The copy number of each the transformed plasmid was calculated according to its molecular weight and DNA concentration, and then converted into the copy number based on Avogadro's number (1 mol = 6.022 X 1023 molecules). Dilutions were freshly prepared for each experiment from one aliquot of plasmid stock solution stored at - 80°C.

Real-time qPCR assays

The real-time qPCR assays were performed on a Rotor Gene Q (Qiagen®) using SYBR Green I Dye. The following reagents were added in a 12.5 µL reaction mixture: 6.25 µL of SensiMix™ SYBR NO-ROX kit real-time qPCR MasterMix (Bioline®), 0.1 µM forward and reverse primers and 4 µL of DNA/sample lysate. The thermal cycling conditions consisted of an initial preheating step of 3 min at 94 °C followed by 40 cycles of 20 s at 94 °C, 20 s at annealing temperature (Table 1) and 20 s at 72 °C. The specificity of the amplified PCR product was verified by melting curve analysis at the end of the 40 cycles by gradually increasing the temperature from 60 to 95 °C by 1 °C every 5 s. All reactions were run in triplicate. The threshold line was set at 0.05 of the signal fluorescence for all of the PCR using the Rotor-Gene Q series software and the reaction efficiencies as similar as possible. No-template controls were included in each run.

Considering that the DNA was obtained after mechanical cell disruption of environmental samples, an internal control was used to check for reaction inhibition. The internal control (Primerdesign™) was added to the reaction flowing the manufactures instructions.

Microcystin quantification

Microcystin analyses in environmental samples were performed by Enzyme-linked immunosorbent- assay (ELISA), using a specific kit (Abraxis®, ADDA ELISA). The procedure followed the instructions given by the manufacturer.

Microcystin analyses in *Planktothrix* cultures were performed using high-performance liquid chromatography with diode array detection (HPLC-DAD) according to ISO 20179:2005. Briefly, lyophilized cultures were extracted with 70% methanol (10 mL/100 mg dry weight; 2h with agitation). The resulting extracts were sonicated (Sonics Vibra-Cell CV33), centrifuged and the pellets were re-extracted overnight. The combined extracts were subjected to rotary evaporation at 35 ºC (Buchi-R, Flawil) and the resulting aqueous extracts were extracted with SPE cartridges (Sep-Pak C18, 500 mg Waters®). Microcystins were eluted with methanol at 80% (v/v) and the methanolic fraction was evaporated. The resulting aqueous extract was filtered (0,45 um syringe filters) and analyzed by HPLC-DAD. Microcystins were identified by their characteristic absorption maximum at 238 nm and quantified using commercially available MCLR standards (Alexis Biochemicals®).

Phytoplankton identification and quantification

For the identification and enumeration of phytoplankton the samples were preserved with Lugol's iodine solution, settled down in sedimentation chambers and counted using an inverted microscope Olympus® CK40 at 400x magnification following the procedure of the Utermöhl technique described in the European Standard EN15204. The number of cells in *Plankthotrix* filaments was calculated by dividing the measured filament length by the mean cell length.

Measurements of environmental chemical parameters

The following parameters were measured in the water samples: Nitrates, total phosphorus, pH and conductivity. Nitrates were analysed with SKALAR© autoanalyser (SAN system) according to the manufactures instructions. Total phosphorus was determined following the method described by Rodier (1976). The pH was measured using a Mettler© Toledo by the electrometric method 4500 – H+ according to the American Public Health Association (APHA, AWWA, WEF, 1992). The conductivity was measured with a conductimeter GPL31 (Crison©) following the ISO standard method (ISO 7888:1985).

Statistical analysis

The nonparametric Spearman Rank Correlation coefficient was calculated, since assumptions of normality were not met for all variables, to evaluate the correlation between the cell concentrations, microcystins concentration and gene copy numbers.

The two-way Analysis of Variance (ANOVA) was used to determine whether there were any significant differences in environmental parameters between seasons of the year.

Results

Planktothrix agardhii concentration

Two different indicators of *P. agardhii* abundance were used cell counts and *rpo*C1 gene quantification. During the two years of monitoring *Planktothrix* was always present at high concentrations (Fig. 1A). Nevertheless, extraordinary cell densities were present in the period between April and September of 2014 reaching its peak in August of 2014 (2884286 cell.mL^{-1}). Similar to cell counts, high gene copy numbers were obtained of the *rpo*C1 gene during the monitoring period except between May and November of 2013 (Fig. 1B). Furthermore, gene copy numbers were, in general, much higher then cell counts and no correlation was obtained between these two measures.

Microcystin and microcystin synthetase gene (*mcy*A) concentration

Microcystins were always present in the lake during the sampling period and varied between 3.32 and 11.41 ug.L^{-1} during 2012 and 2013. In March of 2014 the total concentration of microcystins started to increase reaching its peak in July of 2014 (134.6 ug.L-1) (Fig. 2).

The microcystin synthetase gene (*mcy*A) targeting toxic *Planktothrix* was also low until April of 2014, reaching high concentrations between April and September of 2014 with a peak in August (5529368 gene copy numbers.mL^{-1}) (Fig. 1C). Furthermore, *mcy*A from *Planktothrix* was positively correlated with microcystins concentration (Spearman rank correlation coefficient of 0.84)

The variability of microcystin synthetase gene (*mcy*A) targeting all microcystin producers was very similar to the variability of *mcy*A targeting *Planktothrix* except from July to October of 2013 (Fig. 1D). Indicating that *Planktothrix* was the major producer of microcystins but also that between July and October 2013 another microcystin producer was present. Similar to the *mcy*A from *Plankthotrix* the general *mcy*A gene was also positively correlated with microcystins concentration (Spearman rank correlation coefficient of 0.77).

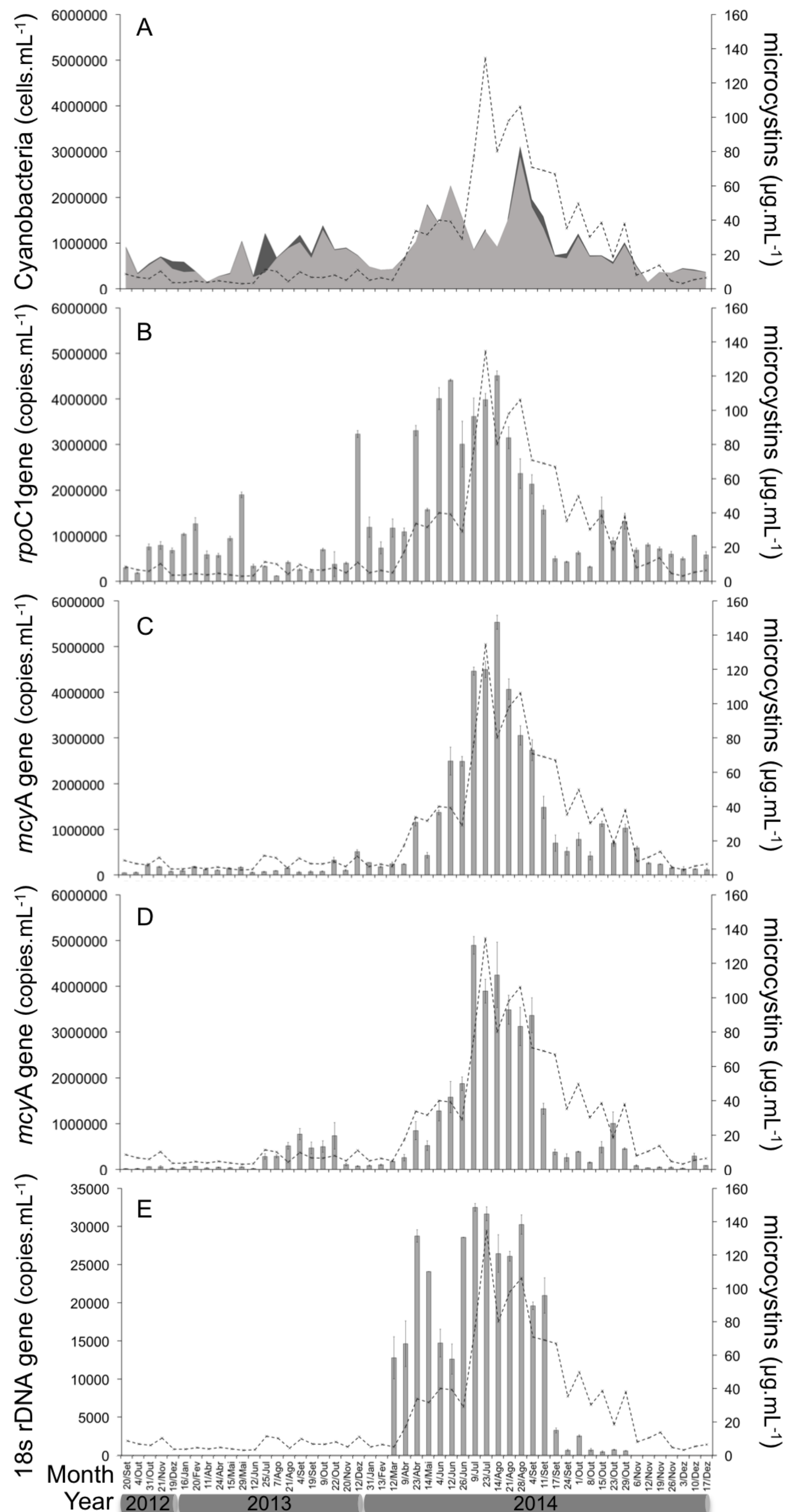

Fig. 1 – *Planktothrix* monitoring. A – Microscopy quantification of total cyanobacteria by the Utermöhl technique (dark grey areas) and *Planktothrix agardhii* (light grey area); dashed line represents microcystin concentrations obtained by ELISA. B – *rpo*C1 gene copy numbers targeting P. agardhii obtained by real-time PCR (ligh grey bars); dashed line

represents microcystin concentrations obtained by ELISA. C – microcystin syntethase gene (*mcy*A) targeting *Planktothrix* obtained by real-time PCR (light grey bars), dashed line represents microcystin concentrations obtained by ELISA. D - microcystin syntethase gene (*mcy*A) targeting microcystin producers obtained by real-time PCR (light grey bars), dashed line represents microcystin concentrations obtained by ELISA. E- 18s rDNA gene targeting chytrid parasites obtained by real-time PCR (light grey bars), dashed line represents microcystin concentrations obtained by ELISA.

18s rDNA of Chytrid parasites concentration

The gene 18s rDNA targeting chytrid parasites was not detected until March 2014. After that, its presence was detected and quantified until October 2014 (Fig. 1E). The 18s rDNA gene concentration was higher between March and September of 2014 and was positively correlated with microcystins (Spearman rank correlation coefficient of 0.82) and both *mcy*A gene copy numbers (*mcy*A *Planktothrix*: Spearman rank correlation coefficient of 0.83; general *mcy*A: Spearman rank correlation coefficient of 0.78).

Phytoplankton Composition

During the all-sampling period the *Planktothrix agardhii* was the most abundant cyanobacterium (Fig. 1A). Other cyanobacteria were also found in lower densities, such as, *Aphanizomenon issatschenkoi*, *Limnothrix redekei* and *Pseudoanabena limnetica*.

Environmental parameters

The physicochemical parameters (total phosphorus, pH and conductivity) were constant during the study period and no significant differences were obtained by the analysis of variances (ANOVA) except for nitrates (Fig. 2). The average values for total phosphorus, pH and conductivity were: 0.14±0.10 mg.L^{-1} of P; 8.3±0.3 and 560.6 ±36.5 µS/m respectively. The variation of nitrates concentration in the water was seasonal, increasing in the autumn and winter.

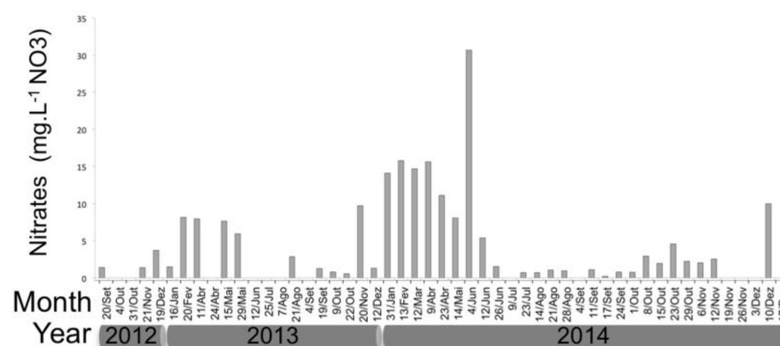

Fig. 2 – Nitrates concentration over the sampling period.

Discussion

Planktothrix blooms associated with microcystin concentrations in the studied freshwater reservoir are a matter of concern for public health authorities since it is used for production of drinking water. The high cell densities within this reservoir were not always indicative of high microcystins concentration (Churro et al. 2012a, Churro et al. 2013). In order to understand the variability of toxic and non-toxic strains, toxic genotypes were monitored by real-time PCR and compared to cell densities and microcystin concentration. During this study, two distinct periods were observed, one from 2012 to 2013 - characterized by moderate microcystin concentrations and moderate bloom densities - and 2014 when high concentrations of microcystins were registered together with an extremely dense bloom. During the study we followed the concentration of toxic genotypes through the quantification of the *mcy*A gene targeting all cyanobacteria containing de *mcy*A and, more specifically, targeting *Planktothrix* containing de *mcy*A. The total population of *P. agardhii* was followed by quantification of the *rpo*C1 gene. The results obtained showed that toxic and non-toxic strains are present in this water reservoir and that the quantity of toxic genotypes is not stable throughout time, since the *rpo*C1 gene was always present in high quantities and the *mcy*A gene from *Planktothrix* greatly increased in April of 2014. Furthermore, other microcystin producer, besides *Planktothrix*, might be present in the water reservoir and contributing to microcystin concentration, since the general *mcy*A was higher during a short period in 2013 where the *mcy*A and *rpo*C1 from *Planktothrix* where lower. Nevertheless, *mcy*A from *Planktothrix* correlated with microcystin concentration indicating that this cyanobacterium is the major responsible for the microcystin concentration. During the sampling period, between 2012 and 2013 the bloom was mainly composed by non-toxic *Planktothrix* strains but from April to October of 2014 something triggered the development of an unusual and highly toxic *Planktothrix* bloom. Despite none of the environmental chemical parameters measured could explain this unusual development. It is generally assumed that the nitrogen and phosphorus are triggering factors of cyanobacteria growth and that each species has its own nutrient requirements and survival strategies (Paerl et al. 2011, Paerl and Paul 2012, Donald et al. 2013). In our study total phosphorous concentrations where constant hover time. However, nitrates concentrations exhibit a seasonal pattern increasing during autumn and winter months when, in general, high rain precipitation occurs which in turn may lead to increase input of nitrates through runoff. Furthermore, nitrates concentrations were higher in the four months before the unusual bloom occurred. Previous studies with *Microcystis* and *Planktothrix* strains and mesocosm

experiments reported that nitrogen increases the growth and toxin production (Sivonen 1990, Orr and Jones 1998, Downing et al. 2005, Donald et al. 2011, Donald et al. 2013, Davis et al. 2015) and that toxic and non-toxic strains respond differently to nutrients load with high nitrogen levels favoring toxic over non-toxic strains of this species (Vézie et al. 2002). Similar to our results, Yoshida et al. (2007) reported the increase of *mcy*A toxic genotypes in a *M. aeruginosa* bloom following an increase in nitrates concentration. The influence of environmental factors on cyanobacterial bloom formation and toxicity has been an issue of extensive research and is not yet fully clarified. Furthermore, the proportion of toxic and non-toxic genotypes and how each one is affected and responds to environmental factors may determine the overall toxicity of blooms (Kurmayer et al. 2002, Kardinaal et al. 2007, Davis et al. 2009).

In our study it was also quantified de gene copy numbers of the 18s rDNA gene from chytrid fungy of the order Rhizophydiales to which the species *Rhizophydium megarrhizum* belongs. *Rhizophydium megarrhizum* was identified parasitizing *Planktothrix* filaments in water samples from the reservoir in study (Churro et al. 2014). The concentration of the 18s rDNA gene was higher during the unusual *P. agardhii* bloom and positively correlated with microcystin concentrations and *mcy*A gene copy numbers. Recent studies on *P.agardhii* chytric parasitism indicate that infection may be dependent on the type of oligopeptides that the cyanobacterium produces and that the production of secondary metabolites such as: microviridins, anabaenopeptins, cyanopeptolins and microcystins can prevent the infection of *P. agardhii* (Rohrlack et al. 2013 and Sønstebø Rohrlack 2011). The zoospores of these parasites find their host by means of chemotaxis (Gleason and Lilje 2009) so the compounds produced by cyanobacteria may indeed be important in the choice of the host by the chytrid parasite. The infection of this type of parasites in phytoplankton results in the dead of the cells. However studies in chytrid parasitism with *Anabena* indicate that these parasites don´t prevent the development of cyanocateria and that the percentage of infection within the population is low (Takano et al. 2008). In our study the cell counts and *rpo*C1 gene copy numbers they were also high during the chytrid parasitism. Considering our results and previous reports (Rohrlack et al. 2013 Sønstebø and Rohrlack 2011) these infections might be exerting a selective pressure on the subpopulations of toxic and non-toxic strains of *P. agardhii*. Thus, we hypothesize that microcystin-producing strains prevent the infection by these parasites, which may cause an imbalance in the density of toxic and non-toxic strains, leading to higher toxicity of blooms by the presence of more toxic strains. Another possibility is that microcystin producing strains might be stimulated by the presence of these parasitic

fungi to produce more toxins. Allelopathic functions of microcystins have been point out (Schatz et al. 2007) but the biological role of these secondary metabolites and their role in food-webs dynamics are not yet fully understood (El-Shehawy et al. 2012). The synthesis of microcystins is energetically demanding and is important to understand if its production is given some advantage over non-toxic strains (El-Shehawy et al. 2012).

The *rpo*C1 gene exists in single copy in the cyanobacterial genome and its quantification showed to relate with cell quantification in controlled culture experiments and environmental samples (Churro et al. 2012). However, the results from the present study show that the quantification of the *rpo*C1 gene yield superior gene copy numbers when compared to microscopic cell quantification. There are several reasons that may justify this discrepancy, especially when environmental samples are used. In this study, the DNA was obtained by cell disruption treatments and all sample lysate was applied directly in the PCR reactions. So, PCR signals could be obtained from freely environmental DNA originated from non-viable cells (Wolffs et al. 2005). Furthermore, when cells are actively dividing the nuclear material is the first to divide and cells may contain more than one copy of the chromosome. Thus, when cell division is not yet completed, and cell membranes are not yet fully developed, individual cells may be underestimated by microscopic counting. Recent studies have also point out that both unicellular and filamentous cyanobacteria can possess multiple copies of their genome per cell (Griese et al. 2011, Al-Tebrineh et al. 2010). Nevertheless, *rpo*C1 gene quantification enabled to successfully monitor *P. agardhii* development in the present study. Regarding *mcy*A copy numbers, it is well known that cyanobacteria have more than one copy of this gene per genome and, recently, Ngwa et al. (2014) found that *mcy*E gene copy number increased when *P. agardhii* was in mixed cultured with Microcystis aeruginosa. In this study the quantification of *mcy*A gene was positively correlated with microcystins concentration. The issue of putative quantification of *mcy*A gene copies from non-viable cells is not a problem in this case because the method used for toxin quantification also included both intra and extracellular microcystins.

This study reports for the first time strong evidence of the influence of chytrid parasites in bloom toxicity and the results obtained raise relevant questions that deserve to be explored: What is the influence of chytrid parasites in shaping the density and toxicity of P. agardhii blooms? Does the presence of these parasites encourage the development of toxic blooms? What factors influence the chytrids-Planktothrix parasitic relationship?

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Chapter

08_

FINAL CONSIDERATIONS

_The most common toxic bloom forming species in freshwaters reservoirs across Europe are *Planktothrix agardhii* and *Microcystis aeruginosa*. Although, *M. aeruginosa* has been recurrently studied in Portuguese freshwaters there was a lack of information about *P. agardhii*. The genus *Planktothrix* has some particularities that set them apart from other cyanobacteria with implications for its monitoring (Churro et al. 2012 - Chapter 2). In this study the field survey and the gathering of monitoring and bibliographic records revealed that *Planktothrix* is a widely distributed genus in Portuguese freshwaters and that *P. agardhii* is the most commonly found (Chapter 3). This species can be found forming blooms in several lakes and microcystin producing strains were isolated from Portuguese waters (Churro et al. 2012 - Chapter 2; Chapter 3; Churro et al. 2013 – Appendix A, Pag. 187). The real-time PCR monitoring of a persistent *P. agardhii* bloom (Churro et al. 2012 – Chapter 2, Churro et al. 2013 – Appendix A, Pag. 187) revealed that the bloom consisted of microcystin producing and non-producing strains. The strains density was not constant over time and that they may bloom in different periods (Chapter 7). During the monitoring of this reservoir chytrid parasites infecting *P. agardhii* where observed (Churro and Pereira 2014 – Appendix A, Pag. 191). The abundance of chytrid parasites was also quantified by real-time PCR and it showed that correlated with *P. agardhii* toxic strains and microcystins (Chapter 7). Chytrid infections in phytoplankton and cyanobacteria have been reported for a long time but the effects of this parasitism on toxic cyanobacterial blooms are not well understood and the evidence of influence in toxicity of *P. agardhii* blooms as described in this study has never been reported (Chapter 7).

The simplicity of the real-time PCR techniques has driven researchers to develop protocols to assess water quality in several microbiological areas and the cyanobacteria field is no exception. It is relevant for water monitoring agencies and public health authorities that the method for detecting potentially toxic cyanobacteria to be simple, fast and cheap to allow a rapid alert of the health hazard and also ensure that monitoring programs are economically feasible. The studies on cyanobacteria real-time PCR analysis are mainly directed towards the detection and quantification of genes involved in toxin production such as microcystin synthetase genes (Kurmayer and Kutzenberger 2003; Vaitomaa et al. 2003; Furukawa et al. 2006; Baxa et al. 2010; Al-Tebrineh et al. 2011), nodularin synthetase gene (Koskenniemi et al. 2007; Al-Tebrineh et al. 2011), cylindrospermopsin synthetase genes (Rasmussen et al. 2008;

Moreira et al. 2011) and saxitoxin synthetase genes (Al-Tebrineh et al. 2010). These reports are of great use for studying toxic genotype proportion and occurrence in the environment and have been proven to correlate with toxin concentration. Nevertheless, and considering that the same organism can produce several kinds of toxins (Falconer and Humpage 2005), the identification and quantification of the organism's is still an important parameter in the risk assessment of cyanobacterial toxic episodes. Therefore, the traditional procedures in the monitoring of cyanobacteria still uses the analysis of toxins as well as the cell counting and identification, which are moderately costly, time consuming and highly operator dependent. So, species-specific real-time PCR analysis is also useful in cyanobacteria quantification. The most common real-time PCR methods for quantification and identification of particular species uses qPCR Taq nuclease assay (TNA) directed for the phycocyanin (PC) gene or its intergenic spacer (Kurmayer and Kutzenberger 2003; Schober and Kurmayer 2006; Schober et al. 2007; Briand et al. 2008; Kurmayer et al. 2011), 16S rRNA (Rinta-Kanto et al. 2005; Doblin et al. 2007; Davis et al. 2009; Ostermaier and Kurmayer 2009; Baxa et al. 2010) and *rpo*C1 gene (Rasmussen et al. 2008; Orr et al. 2010). Others use SYBR green and are directed to the phycocyanin gene intergenic spacer (PC-IGS) (Yoshida et al. 2007), 16S rRNA (Tomioka et al. 2008; Al-Tebrineh et al. 2010; Xu et al. 2010) and *rpo*C1 gene (Tai and Palenik 2009; Moreira et al. 2011). The difference between TNA and SYBR green assays relies on the type of fluorescence signal. The TNA uses one primer pair and one probe with fluorophores, the fluorescence is emitted only if the three sequences bind to the target DNA. This brings high specificity to the assay and diminishes the possibility of false-positives. The SYBR green binds to double strand DNA and emits fluorescence, so the primer pair used for the target sequence has to be very specific and selected carefully to avoid non-specific binds. The advantage in SYBR green over TNA is that the first is significantly cheaper which is important in a method for routine monitoring. The aforementioned studies are sensitive with low detection limits. In this study the real-time PCR assay developed also prove to be useful in the specific detection and quantification of *Planktothrix agardhii* with a detection limit of 8 gene copies per reaction (Churro et al. 2012a – Chapter 4).

Another aspect of cyanobacteria monitoring is the preservation of samples with chemicals whether for improved morphological observation, to avoid sample degradation during transport or for maintaining the sample for confirmation analysis. So the use and availability of preserved samples in a routine monitoring cyanobacteria laboratory is common. To understand whether or not the real-time PCR is able to amplify and detect target sequences from those samples is important for cyanobacteria

research and monitoring since it enables to analyze the samples already in use in the laboratories and also gives the opportunity to compare present data with retrospective data, which, in turns, contribute to a better understanding of toxic cyanobacterial bloom/species dynamics within reservoirs. In this study the real-time PCR was able to amplify target sequences from samples preserved in the most common fixator used – Lugol's iodine solution (Churro et al. 2015 – Chapter 5). However, Lugol's iodine solution interferes with DNA availability so, to overcome this, a method suitable for DNA preservation that don't interfere with real-time PCR analysis and also maintained morphological features of cyanobacteria was achieved with 100% methanol preservation (Churro et al. 2015a – Chapter 6). The real-time PCR technique also enabled to reveal the pressure that chytrid parasitism exerts in cyanobacterial blooms (Chapter 7) being a helpful tool in studying this poorly understand parasitic relation and quantification of uncultured organisms in the environment.

Considering those facts, we may ask why isn't the real-time qPCR currently applied in routine monitoring programs? One of the main reasons is that there are no available commercial kits with the standards for calibrating real-time PCR for cyanobacteria as they are for pathogenic bacteria for example: *Legionella* (iQ-Check® Legionella Real-Time PCR Kits, BioRad), *Escherichia coli* (resDNASEQ® Quantitative E. coli DNA Kit, ThermoFisher), *Clostridium* (foodproof® Clostridium botulinum Detection LyoKit, Biotecon Diagnostics) and *Salmonella* (TaqMan® Salmonella Enteritidis Detection Kit, ThermoFisher). This fact discourages laboratories in the introduction of real-time PCR in their routine, since all the reaction set up has to be custom made and real-time PCR standards are made through cloning which is time consuming and costly. Recently, Neilan and his colleagues took one step forward and are already commercializing standards for the quantification of microcystin, nodularin, cylindrospermopsin and saxitoxin genes (Phytoxigene™, Diagnostic Technology).

Another important reason is that the real-time PCR quantifies gene copy numbers in a solution and one of the challenges is to relate gene copy numbers with cell counts, since all the guideline values concentrations are based in toxin and/or cell concentrations (Falconer et al. 1999, Decreto-Lei nº 306/2007, Diário da República, 1. ª série – N. º 164). The difficulty in this relation is inherent to cyanobacteria biology since there could be several copies of the same gene in the genome (Churro et al. 2012a – Chapter 4: Appendix B, Kurmayer and Christiansen 2009) and also several copies of the genome in the cell (Griese et al. 2011, Al-Tebrineh et al. 2010). So, similar to what was done to measure the amount of toxin per cell, that originated the safety toxin guidelines we use today, we should also do similar exercise to gauge the

number of gene copies that corresponds to a certain level of toxin that constitutes a risk. This will certainly help to introduce the real-time PCR technique in routine monitoring programs.

Furthermore, in order for the real-time PCR to be used in cyanobacteria monitoring, normalization in the experiment set up and analysis has to be achieved between researchers and laboratories. Starting with standardization in data report and reaction quality assessments (Bustin et al. 2009, Lefever et al. 2009), DNA extraction, molecular markers and standard curve construction methods, limits of detection and quantification. Finally, intercalibration studies should be performed to assure reproducibility of the results (Schober et al. 2007).

The real-time PCR technique will still be useful from years to come. New generation sequencing techniques are revealing new sequences from new organisms and new compounds and for certain the real-time PCR will be useful in the study and quantification of these new sequences.

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APPENDIX

A

Other publications within the scope of this thesis.

C.Churro, E.Dias, S.Paulino, E.Alverca, P.Pereira (2013). Importância da monitorização de cianobactérias em albufeiras portuguesas. Boletim Epidemiológico Observações 2(4): 18-20, ISSN: 2182-8873, Instituto Nacional de Saúde Dr. Ricardo Jorge (Ed.).

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artigos breves_ n. 6

_Importância da monitorização de cianobactérias em albufeiras portuguesas

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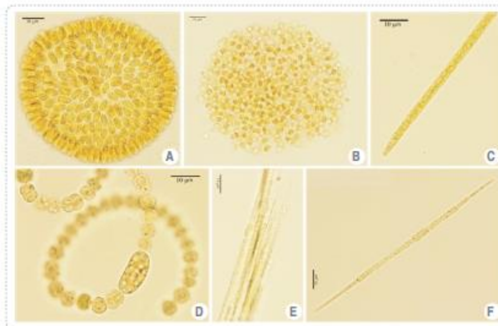
_Cerca de 60 % da população portuguesa consome água potável proveniente de reservas superficiais (1). Segundo o Instituto Nacional da Água (INAG), 42 % das albufeiras portuguesas estão eutrofizadas e muitas outras apresentam sinais de progressiva degradação do estado trófico (2).

_As cianobactérias são constituintes naturais do fitoplâncton que em águas eutrofizadas se desenvolvem excessivamente constituindo populações muito densas denominadas florescências *(Figura 1)*. Estas florescências são por vezes acompanhadas pela produção de toxinas (cianotoxinas), constituindo um risco para a saúde pública.

Figura 1: ***Cianobactérias mais comuns nas albufeiras portuguesas.***



A – Woronichinia, B – Microcystis, C - Planktothrix, D- Dolichospermum, E – Aphanizomenon, F – Cuspidothrix.

_As microcistinas são hepatotoxinas e têm sido implicadas em episódios de intoxicação humana (3). A baixas concentrações atuam como promotores tumorais e estão classificadas pela Agência Internacional para a Investigação do Cancro (IARC) como agentes potencialmente cancerígenos para o Homem (4). No Decreto-Lei 306/2007 está estabelecido o valor paramétrico de referência para as microcistinas de 1ug.l^{-1} de microcistina-LR$_{equiv}$ em águas de consumo (5).

_O Laboratório de Biologia e Ecotoxicologia (LBE) do Instituto Nacional de Saúde Doutor Ricardo Jorge (INSA) assegura, desde 1996, a monitorização de cianobactérias e toxinas associadas em vários reservatórios de água doce superficial, de norte a sul do país. Exemplificamos seguidamente os resultados dessa monitorização numa albufeira que tem como uso primário a produção de água para consumo humano, abastecendo uma população de cerca de 20 mil pessoas. Nesta albufeira foram colhidas amostras com uma periodicidade mensal ou quinzenal, em cinco pontos de colheita: na albufeira, à entrada da Estação de Tratamento de Água (ETA) (água bruta – não tratada), ao longo das fases sucessivas de tratamento (decantação e filtração) e à saída da ETA (água tratada). Cada amostra foi analisada para identificação e quantificação de fitoplâncton ao microscópio e quantificação de microcistinas por ensaio imunoenzimático (ELISA).

_Os dados apresentados *(Gráfico 1)* revelam a presença de densidades elevadas de fitoplâncton ao longo de todo o período analisado (2009-2010), com predomínio de cianobactérias filamentosas do género *Planktothrix*. Associada a esta presença constante e massiva de cianobactérias, observou-se também a contaminação persistente da água bruta com microcistinas, em valores significativamente elevados. Nas amostras em que a densidade fitoplanctónica na água bruta foi extremamente elevada, a eficiência da ETA ficou comprometida, observando-se a presença de um número elevado de cianobactérias na água tratada *(Gráfico 2)*.

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Gráfico 1: **Densidade de fitoplâncton e cianobactérias e concentração de microcistinas** ao longo de dois anos (2009 e 2010) **na água da albufeira.**

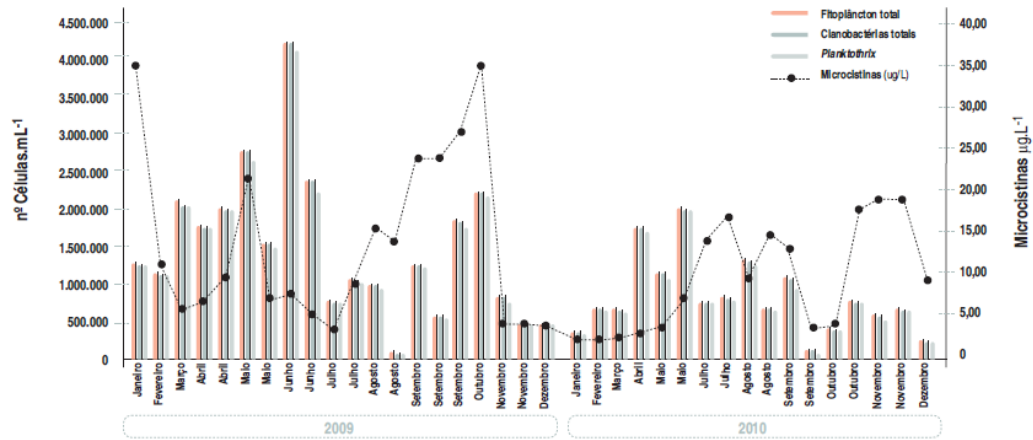
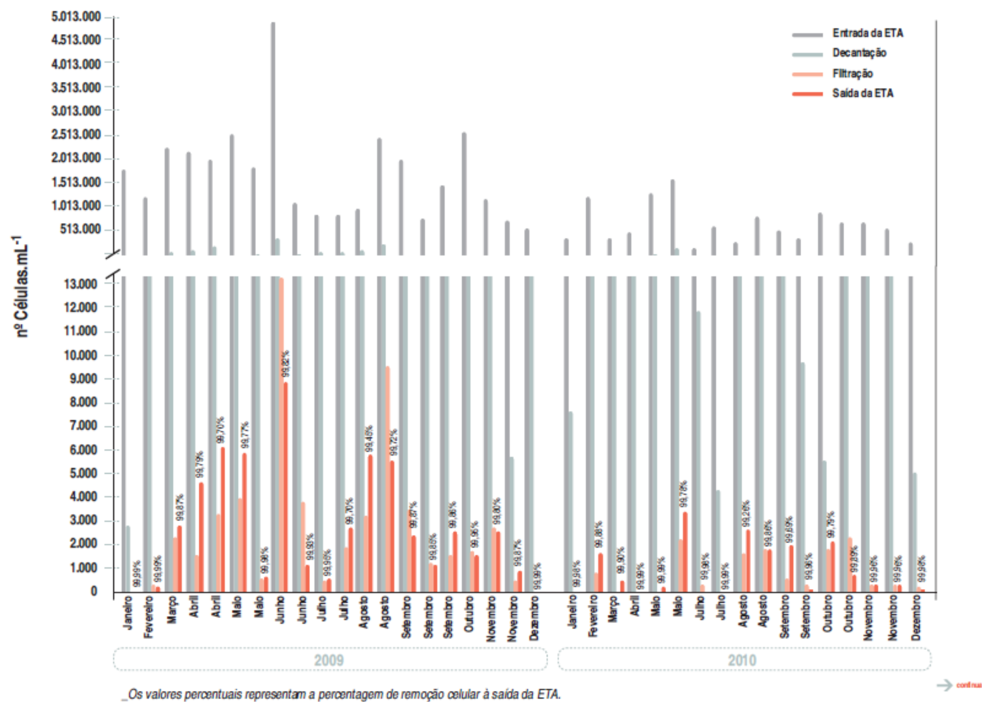


Gráfico 2: **Densidade de cianobactérias totais nas amostras provenientes das várias fases de tratamento da água da ETA** ao longo de dois anos de monitorização (2009 e 2010).



_Os valores percentuais representam a percentagem de remoção celular à saída da ETA.

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_Os dados apresentados mostram que a presença de cianobactérias e cianotoxinas pode ser persistente em águas doces superficiais, havendo riscos de exposição prolongada a compostos com efeitos crónicos ainda mal conhecidos. Destacam-se também as grandes oscilações na densidade celular e na concentração de toxinas (microcistinas), em que a ausência de padrão sazonal ou de regularidade revela o caracter imprevisível destas ocorrências e a necessidade de monitorização sistemática. O acompanhamento destas situações, associado a informação epidemiológica, constitui um contributo importante para o conhecimento, ainda muito limitado, acerca dos efeitos para saúde decorrentes da exposição humana a cianotoxinas. Importa também referir que a eficiência e custos do tratamento da água dependem essencialmente da qualidade da água na captação pelo que é fundamental preservar os recursos hídricos, minimizando as fontes de poluição a que esses sistemas estão sujeitos.

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_Fatores de Risco em Ambientes Hídricos

_Parasita fúngico de cianobactérias isolado de uma albufeira portuguesa: possíveis implicações no controlo de florescências

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_Introdução

Os parasitas fúngicos zoospóricos são ubíquos nos sistemas aquáticos e a infeção de fitoplâncton por estes fungos é um fenómeno comum em águas doces. Os fungos parasitas de fitoplâncton pertencem principalmente ao filo Chytridiomycota (quitrídeos) e distinguem-se de outros fungos por produzirem zoósporos flagelados, o que lhes confere mobilidade e particular adaptação ao meio aquático [1-3]. Os quitrídeos são parasitas obrigatórios dependentes do seu hospedeiro para a sua nutrição e desenvolvimento, pelo que após a infeção, as células hospedeiras ficam irreversívelmente danificadas, o que se traduz na morte do organismo infetado [1].

Estes fungos parasitas podem infetar vários tipos de fitoplâncton incluindo espécies de cianobactérias formadoras de florescências [1]. Algumas destas florescências são tóxicas para o Homem e daí o impacto que estes fungos podem ter na saúde humana [4]. Apesar da sua importância ser reconhecida, existe pouca informação sobre o efeito destas infeções na ocorrência, periodicidade e toxicidade das florescências cianobacterianas. A falta de informação deve-se, em parte, ao facto de a ocorrência destas infeções poder passar despercebida, mas é sobretudo a dificuldade de cultivar estes organismos e de obter culturas puras que impede o estudo aprofundado destas interações [2, 3]. Alguns quitrídeos já foram isolados com sucesso [2, 10] mas ainda assim são considerados na sua maioria como um grupo de fungos não cultiváveis.

O desafio no cultivo destes organismos prende-se com o facto de serem parasitas obrigatórios, pelo que têm de ser co-cultivados com o seu hospedeiro sendo imprescindível manter o parasita e o hospedeiro simultaneamente saudáveis.

_Objetivo

Neste estudo descrevemos o isolamento e manutenção em cultura monoclonal de um parasita quitrídeo recolhido de uma florescência da cianobactéria *Planktothrix agardhii*.

_Material e métodos

Em junho de 2014 foi observada uma infeção fúngica em células de *P. agardhii* em duas amostras provenientes de uma albufeira com uma florescência. Uma pequena quantidade (1mL) de cada amostra foi transferida para culturas puras de *P. agardhii*-Imecya230 isolado da mesma albufeira em maio de 2007 e mantido na coleção de algas Estela Sousa e Silva no Laboratório de Biologia e Ecotoxicologia, Instituto Nacional de Saúde Doutor Ricardo Jorge (INSA).

As culturas foram examinadas diariamente para observação da propagação da infeção. Ao fim de sete dias foi isolado, com uma pipeta capilar, um único filamento de *P. agardhii* infetado com apenas um esporângio. Este filamento infectado foi transferido para uma nova cultura pura de *P. agardhii*-Imecya230 para se obter culturas monoclonais deste fungo. As culturas inoculadas com o par parasita-hospedeiro foram mantidas a uma temperatura constante de 20 ± 1°C, com uma intensidade de luz de 20 μmol de fotões m^{-2} s^{-1} e um ciclo de 14 h de luz e 10 h de escuro.

_Resultados

Duas culturas monoclonais de fungos quitrídeos foram obtidas com sucesso. De acordo com a descrição morfológica de Letcher e Powell (2012) [12] os quitrídeos isolados pertencem à ordem Rhizophydiales espécie *Rhizophydium megarrhizum* já descrita como parasita da cianobactéria *P. agardhii*.

O processo infecioso está representado na figura 1: os zoósporos ligam-se ao filamento de *P. agardhii* (figura 1A) e desenvolvem os seus rizóides no interior das células penetrando ao longo do

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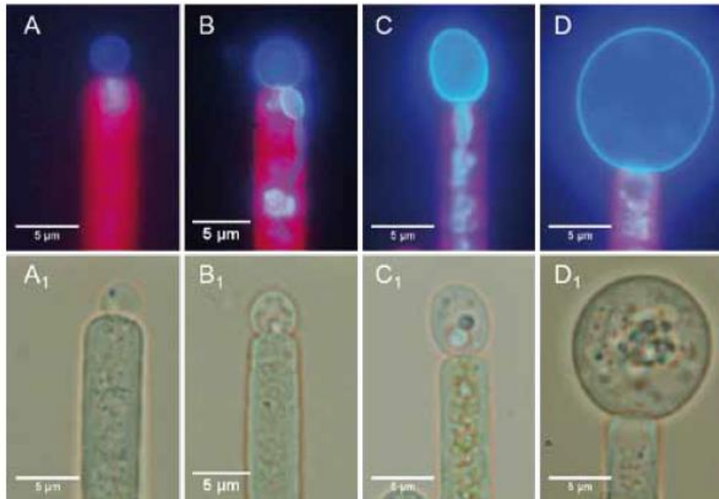
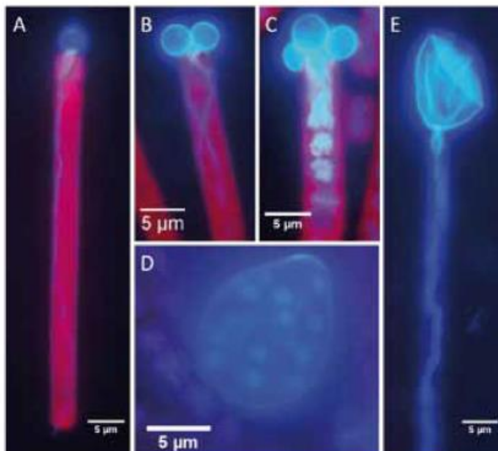
Figura 1: Desenvolvimento do esporângio do parasita *Rhizophydium megarrhizum* em filamentos da cianobactéria *Planktothrix agardhii.*



(A e A_1) zoósporo recentemente ligado ao filamento e início da formação do rizóide; (B e B_1) início da expansão do esporângio e do rizóide que parasita o filamento; (C e C_1) esporângio em expansão já com o rizóide intracelular bem desenvolvido; (D e D_1) esporângio maduro; (A-D) imagens de microscopia de fluorescência sob luz UV que evidenciam a parede quitinosa do esporângio e dos rizóides corados com calcofluor; a cianobactéria é visível através da autofluorescência vermelha; (A_1-D_1) imagens de microscopia óptica de campo claro. Ampliação 1000x, escala de 5 µm.

Figura 2: Parasitismo do fungo quitrídeo *Rhizophydium megarrhizum* em filamentos da cianobactéria *Planktothrix agardhii.*



filamento (figura 1B-D), o esporângio é formado no exterior no qual são produzidos e libertados novos zoósporos (figura 2D). A ligação é feita sempre pelo topo do filamento e os rizóides perfuram todo o filamento (figura 2A), mesmo que o filamento seja longo, podendo os rizóides atingir 140 µm. O mesmo filamento pode ser infetado por vários zoósporos, tendo sido observados até quatro esporângios por filamento (figura 2B,C). A infeção resulta na morte da cianobactéria (figura 2E).

(A) filamento infetado em toda a sua extensão; (B) filamento infetado com dois esporângios; (C) filamento infetado com três esporângios; (D) esporângio maduro com zoósporos no seu interior; (E) esporângio vazio e rizóide num filamento já morto. Imagens de microscopia de fluorescência sob luz UV; a parede quitinosa do fungo foi corada com calcofluor emitindo fluorescência azul; a cianobactéria é visível através da autofluorescência vermelha; os zoósporos foram corados com NucBlue® emitindo fluorescência verde. Ampliação 1000x, escala de 5 µm.

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_Discussão

As culturas de *R. megarrhizum* obtidas são provenientes de uma albufeira que é monitorizada relativamente à presença de cianobactérias e toxinas associadas no laboratório de Biologia e Ecotoxicologia do INSA desde 2003. Esta albufeira tem uma florescência persistente de *P. agardhii* associada à presença de hepatotoxinas (microcistinas) desde 2006. Os isolamentos do fungo parasita foram efetuados em duas amostras colhidas durante o mês de junho de 2014 (dia 4 e 12). As amostras continham uma grande densidade de *P. agardhii* (14.14762 e 22.50476 céls.mL^{-1} respetivamente) assim como uma elevada concentração de microcistinas (40,05 e 39,32 µg.L^{-1} respetivamente). Um mês depois, a 23 de julho de 2014, registou-se a concentração de microcistinas mais elevada (134,60 µg.L^{-1}) para esta albufeira desde o início da sua monitorização em 2003. Em junho de 2014 foi a primeira vez que foi observada a infeção fúngica da cianobactéria *P.agardhii* nesta albufeira. Desconhece-se se nesta albufeira as infeções fúngicas de *P. agardhii* já decorrem há algum tempo ou se surgiram agora, assim como, é desconhecida a sua influência na população da cianobactéria *P. agardhii*.

Estudos recentes em parasitismo quitrídico em *P. agardhii* indicam que a infeção pode ser dependente do tipo de oligopéptidos produzidos e que a produção de microviridinas, anabaenopeptinas, cianopeptolinas e microcistinas pode prevenir a infeção de *P. agardhii* [10, 11]. Os zoósporos encontram o seu hospedeiro por meio de quimiotaxia [13] pelo que os compostos produzidos pelas cianobactérias podem de facto ser importantes na escolha do hospedeiro pelo fungo. Os resultados destes estudos indicam que a infeção pode exercer uma forte pressão seletiva sobre a população e resultar em subdivisão da mesma [10,11]. As florescências cianobacterianas são frequentemente compostas por estirpes produtoras e não produtoras de toxinas, dentro de uma mesma espécie, que competem pelos mesmos recursos. Assim, coloca-se a hipótese de que o facto de as estirpes serem produtoras de microcistinas impede a infeção pelo fungo, pode causar um desequilíbrio na densidade de estirpes tóxicas e não tóxicas podendo conduzir a uma toxicidade mais elevada das florescências. Outro fator ainda pouco estudado é se a presença destes fungos parasitas estimulará as estirpes a produzir maior quantidade de toxina. Do ponto de vista da saúde pública, sendo a albufeira em estudo usada para produção de água para consumo humano, é importante perceber a influência da infeção fúngica na frequência, densidade e toxicidade das florescências da cianobactéria *P. agardhii*.

_Conclusões

As culturas de *R. megarrhizum* obtidas vão possibilitar a realização de trabalho experimental como por exemplo acesso ao ADN fúngico para estudos moleculares e o estudo de diversos fatores envolvidos neste parasitismo. Estes estudos laboratoriais permitirão compreender a influência destes parasitas na população de *P. agardhii* e perceber se a sua presença possibilita o aparecimento de florescências mais tóxicas com um elevado risco para a saúde humana.

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_Abordagem multidisciplinar na identificação e monitorização de cianobactérias potencialmente tóxicas

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_Introdução

O que são cianobactérias?

Cianobactérias são organismos procariotas fotossintéticos, e como constituintes naturais do fitoplâncton são uma componente essencial na produção primária e cadeia alimentar em ambientes de água superficiais. Contudo são também responsáveis pela eutrofização dos recursos hídricos, uma vez que algumas espécies podem desenvolver-se rapidamente e formar grandes acumulações chamadas de florescências ou *blooms* (figura 1). Este desenvolvimento anómalo afeta adversamente a qualidade das águas superficiais que são usadas para captação de água para consumo humano, atividades recreativas e agricultura [1].

Porquê a monitorização de cianobactérias?

O risco que as florescências cianobacterianas representam para a saúde humana advêm do facto destes desenvolvimentos excessivos estarem frequentemente associados à produção de cianotoxinas (quadro 1) [1]. As principais vias de exposição para o homem são através de água potável contaminada, diálise, consumo de

Figura 1: Florescência das cianobactérias *Microcystis aeruginosa* e *Planktothrix agardhii* em uma albufeira portuguesa usada para fins recreativos.

peixe e marisco contaminado e atividades recreativas [2]. A toxicidade destes compostos é elevada, como pode ser constatado no gráfico 1 em que está representada a comparação da toxicidade, com base na dose-letal (LD50%) em murganhos, entre as cianotoxinas e algumas das toxinas mais conhecidas em relação ao cianeto [3].

_Métodos

Identificação e quantificação tradicional de cianobactérias

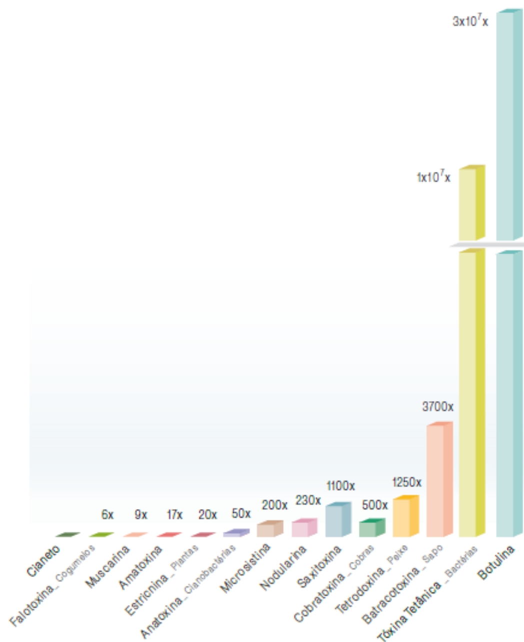
As cianobactérias são identificadas por taxonomia clássica usando microscopia ótica, com base em características morfológicas que são utilizadas para classificar as várias espécies. A quantificação celular é determinada pela contagem de células individuais em câmaras de sedimentação com volume conhecido usando o método de Utermöhl [4]. Este processo é dependente do operador, sendo necessário uma pessoa com experiência e conhecimentos apro-

Quadro 1: Cianotoxinas produzidas por cianobactérias comuns nas albufeiras portuguesas.

Cianotoxina	Atividade	Cianobactéria
Hepatotoxinas		
Microcistinas	Inibição das fosfatases proteicas	*Microcystis, Anabaena, Nostoc, Planktothrix, Anabaenopsis, Phormidium*
Cilindrospermopsina	Inibição da síntese proteica	*Cylindrospermopsis, Aphanizomenon, Anabaena, Raphidiopsis*
Neurotoxinas		
Anatoxina-a	Liga-se aos recetores de acetilcolina nicotínicos	*Aphanizomenon, Anabaena, Raphidiopsis, Oscillatoria, Planktothrix, Cylindrospermum*
Anatoxina-a(s)	Inibe a acetilcolinesterase	*Aphanizomenon, Anabaena*
Saxitoxina	Liga-se aos canais de sódio	*Aphanizomenon, Anabaena, Planktothrix, Cylindrospermopsis, Lyngbya*

Adaptado de Codd 2014) (3) e Merel et al. 2013 (5).

Gráfico 1: Comparação da toxicidade relativamente ao cianeto, com base na dose letal (LD50%) em murganhos, entre as cianotoxinas e algumas das toxinas mais conhecidas.



Adaptado de Metcalf e Codd 2014 (3).

fundados de taxonomia para conseguir uma correta identificação. Contudo, para algumas cianobactérias a distinção entre espécies é uma tarefa difícil mesmo para um taxonomista experiente assim como é difícil de distinguir o limite entre células, o que pode interferir com a sua estimativa.

Deteção e quantificação de cianotoxinas

A deteção e quantificação de muitas cianotoxinas (microcistinas, cilindrospermopsina, anatoxina e saxitoxina) podem ser feitas através de imunoensaios-ELISA ou ensaios de ligação ao recetor-RBA. Estes ensaios detetam e quantificam através do reconhecimento e ligação a anticorpos ou recetores específicos. Estes métodos são rápidos e sensíveis contudo pode haver reações inespecíficas levando a uma sobrestimação da quantidade de toxina presente (5). A cromatografia líquida de alta eficiência-HPLC permite o estudo aprofundado das toxinas e suas variantes. No entanto requer um extenso processamento da amostra e padrões específicos para cada toxina (3,5).

Classificação e identificação molecular de cianobactérias

A obtenção de sequências de ADN de genes envolvidos em diferentes processos celulares, como é o caso dos genes *house-keeping* rRNA 16S, *rpoC1* e *cpcB* providenciam a classificação e identificação molecular das espécies cianobacterianas. A similaridade das sequências destes genes com outras sequências presentes nas bases de dados dá uma indicação da identidade do organismo. A análise filogenética estabelece relações de proximidade e ancestralidade com base na similaridade das sequências de ADN. Recorrendo a uma análise filogenética *multilocus* destes marcadores, aumenta a resolução da identificação dos isolados de cianobactérias até à espécie. Contudo a classificação molecular requer monoculturas ou culturas puras de cianobactérias.

Deteção de genes associados à produção de toxinas

As cianotoxinas só são produzidas pelas estirpes que contêm os genes apropriados para o fazer. Já foram descobertos e sequenciados quase todos os *clusters* de genes que conferem às cianobactérias a capacidade de proceder à síntese da maior parte das cianotoxinas. A sequenciação completa destes *clusters* permitiu o desenvolvimento de sondas moleculares gerais para estirpes tóxicas das diversas toxinas e específicas para algumas espécies produtoras (6). A amplificação destes marcadores indica o potencial de produção de determinada toxina em amostras ambientais.

Quantificação por PCR em tempo-real

A quantificação do número de cópias de um determinado gene em amostras naturais é possível recorrendo à técnica de PCR em tempo-real. Várias sondas foram já desenvolvidas para a quantificação da população total de uma determinada cianobactéria usando o gene rRNA 16S, *cpcB* e *rpoC1* (6,7). Estão também disponíveis várias sondas para os genes envolvidos na produção das várias toxinas o que permite quantificar diversos genótipos e a sua proporção numa determinada amostra.

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Expressão génica

A taxa de produção das cianotoxinas é também influenciada por fatores ambientais, tais como pH, nutrientes, temperatura e intensidade luminosa (8-10). Têm-se realizado vários estudos no sentido de compreender de que forma estes fatores ambientais afetam/alteram a expressão dos genes das cianobactérias, principalmente os genes dos *clusters* responsáveis pela produção de cianotoxinas.

Manutenção de culturas de cianobactérias

O isolamento e manutenção de culturas monoclonais de cianobactérias provenientes de florescências dão um apoio fundamental ao estudo e monitorização destes organismos. A existência dessas culturas permite avaliar a sua toxicidade, traçar o perfil de toxinas que produzem, obter o ADN para testar e desenvolver sondas moleculares e efetuar o estudo aprofundado da sua identificação.

_Conclusões

Desafios na monitorização de cianobactérias e multidisciplinaridade

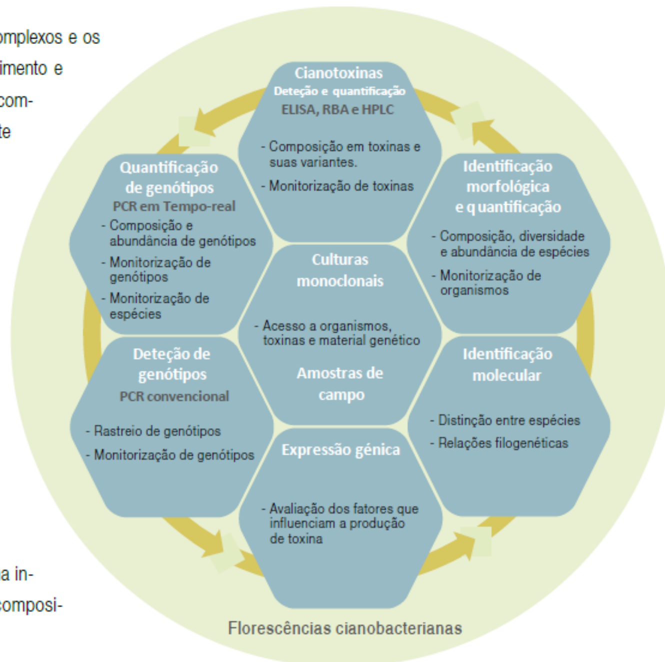
As florescências cianobacterianas são fenómenos complexos e os fatores que regulam o seu aparecimento, desenvolvimento e persistência nos sistemas aquáticos ainda não são completamente conhecidos. Compostas frequentemente por diferentes espécies produtoras e não produtoras de toxinas, acresce que dentro da mesma espécie existem estirpes tóxicas e não tóxicas. Além do mais, a regulação genética e fatores que influenciam a produção de cianotoxinas são ainda um desafio para os investigadores. Várias toxinas podem estar presentes e podem ter origem no mesmo ou em diferentes organismos. Neste contexto, é importante compreender que organismos estão presentes, averiguar se têm o potencial para produzir toxinas, que toxinas podem produzir e se estão ativamente em produção.

Todos os métodos anteriormente descritos dão uma informação diferente e pertinente para o estudo da composição das florescências cianobacterianas (figura 2).

A identificação e enumeração ao microscópio podem ser demoradas e trabalhosas mas fornece informação detalhada sobre a composição de espécies. A deteção imunológica de toxinas é um método rápido e sensível para a quantificação de toxinas na água potável antes e depois do tratamento. O imunoensaio em conjunto com a identificação e quantificação microscópica dá informação útil sobre a composição e abundância da comunidade e quantidade de toxina para as análises de rotina de amostras ambientais.

A deteção molecular de genes responsáveis pela produção de cianotoxinas informa se os organismos presentes na amostra têm ou não o potencial para a sua produção, o que se torna muito útil como ferramenta de rastreio para as várias toxinas que possam estar presentes e direcionando qual o imunoensaio que deverá ser aplicado para a deteção de uma toxina específica.

Figura 2: Multidisciplinaridade na identificação e monitorização de cianobactérias potencialmente tóxicas.



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O PCR em tempo-real faculta a quantificação precisa de um determinado gene, permitindo assim obter informação sobre composição de genótipos tóxicos que aliado ao imunoensaio indica estirpes tóxicas e não tóxicas e se estão em produção ativa de toxina. Esta abordagem é também bastante útil na quantificação de espécies difíceis de identificar e contar por microscopia ótica.

Outros métodos que têm limitações para ser aplicados em rotina podem fornecer informações importantes para complementar e facilitar a monitorização. A análise aprofundada do perfil toxicológico dos isolados por HPLC, ou a identificação das espécies por análise filogenética permite saber que toxinas e que espécies podemos observar nas amostras de campo. O estudo da regulação genética da produção de cianotoxinas por PCR em tempo-real permite compreender em que condições os organismos produzem toxina, e que fatores influenciam a sua produção.

De forma a dar respostas na prevenção da exposição a cianotoxinas em saúde pública, o estudo e monitorização de cianobactérias e cianotoxinas deve recorrer às várias metodologias de uma forma multidisciplinar, em que cada uma delas se complementa e contribui com uma pequena peça do grande *puzzle* que são as florescências cianobacterianas.

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APPENDIX

B

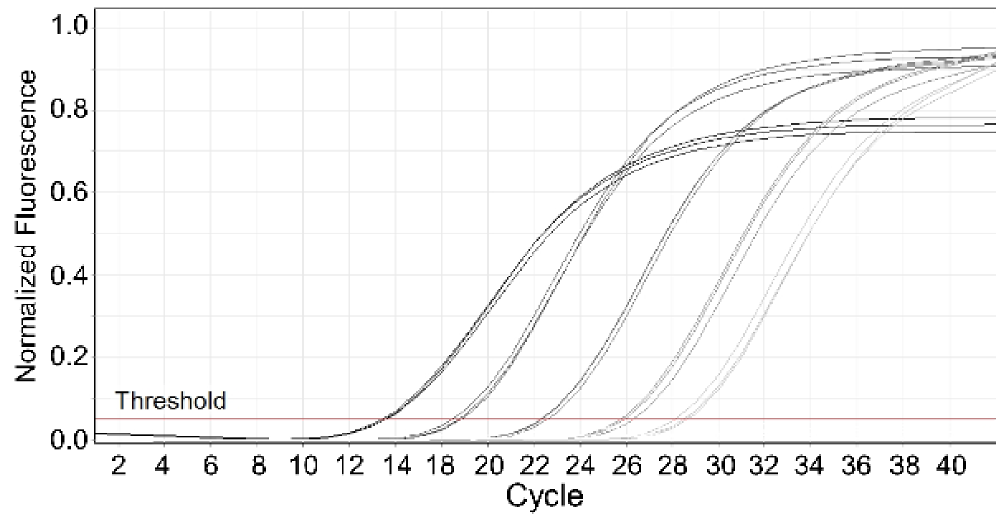
Supplementary material of Chapter 4

Online Resource 1. Number of *rpoC1* , 16S rRNA and phycocyanin gene copies present in cyanobacteria genomes

Lineage	Complete genome of	Chromosome size (nt)	NCBI Acession number	Number of gene copies: *rpoC1* (Locus_tag)	16S rRNA (Locus_tag)	phycocyanin, subunit alpha (*cpcA*) (Locus_tag)	phycocyanin, subunit beta (*cpcB*) (Locus_tag)	Database
Gloeobacterales; Gloeobacter	*Gloeobacter violaceus* PCC 7421	4,659,019	NC_005125	1 gvip556	1 gvir003	2 gvip155 gvip444	2 gvip154 gvip443	NCBI
Chroococcales; Acaryochloris	*Acaryochloris marina* MBIC11017	6,503,724	NC_009925	1 (AM1_3593)	2 (AM1_6417; AM1_6420)	Not found	Not found	NCBI
Chroococcales; Cyanothece	*Cyanothece* sp. ATCC 51142	4,934,271	NC_010546	1 (cce_3836)	2 (cce_RNA045, rrn16Sa; (cce_RNA050, rrn16Sb)	1 cce_2652	1 cce_2651	NCBI
	Cyanothece sp. PCC 7425	5,374,574	NC_011884	1 (Cyan7425_0487)	2 (Cyan7425_R0006; Cyan7425_R0043)	2 Cyan7425_1696 Cyan7425_2872	2 Cyan7425_1697 Cyan7425_2871	NCBI
	Cyanothece sp. PCC 8801	4,679,413	NC_011726	1 (PCC8801_0616)	2 (PCC8801_R0024; PCC8801_R0041)	1 PCC8801_3075	1 PCC8801_3074	NCBI
	Cyanothece sp. PCC 8802	4,669,813	NC_013161	1 (Cyan8802_0632)	2 (Cyan8802_R0042; Cyan8802_R0023)	1 (Cyan8802_3045)	1 (Cyan8802_3046)	NCBI
	Cyanothece sp. PCC 7424	5,942,652	NC_011729	1 (PCC7424_2547)	3 (PCC7424_R0029; PCC7424_R0011; PCC7424_R0038)	1 (PCC7424_0160)	1 (PCC7424_0161)	NCBI
	Cyanothece sp. PCC 7822	6,091,620	NC_014501	1 (Cyan7822_4769)	3 (Cyan7822_R0025; Cyan7822_R0047; Cyan7822_R0039)	2 Cyan7822_1651 Cyan7822_3896	2 Cyan7822_1652 Cyan7822_3895	NCBI
Chroococcales; Microcystis	*Microcystis aeruginosa* NIES-843	5,842,795	NC_010296	1 (MAE_11110)	2 (MAE_r001, rrn16S_1; MAE_r008, rrn16S_2)	2 MAE_24460 MAE_51670	2 MAE_24450 MAE_51680	NCBI
Chroococcales; Synechococcus	*Synechococcus elongatus* PCC 6301	2,696,255		1 (syc2508_d)	2 (sycRNA021_c, rrn16Sa; sycRNA033_d, rrn16Sb)	2 (syc0495_c syc0500_c)	2 (syc0496_c syc0501_c)	Cyanobase NCBI
	Synechococcus elongatus PCC 7942	2,695,903	NC_007604	1 (Synpcc7942_1523)	2 (Synpcc7942_R0052; Synpcc7942_R0004)	2 (Synpcc7942_1048; Synpcc7942_1053)	2 (Synpcc7942_1047; Synpcc7942_1052)	Cyanobase NCBI
	Synechococcus sp. CC9311	2,606,748	NC_008319	1 sync_2357	2 sync_0553 sync_2539	1 sync_0488	1 sync_0489	NCBI
	Synechococcus sp. PCC 7002	3,008,047	NC_010475	1 SYNPCC7002_A2044	2 SYNPCC7002_A1395 SYNPCC7002_A2788	1 SYNPCC7002_A2210	1 SYNPCC7002_A2209	NCBI
	Synechococcus sp. JA-2-3B'a(2-13)	3,046,682	NC_007776	1 CYB_2437	2 CYB_1377 CYB_1957	2 CYB_0940 CYB_2738	2 CYB_0939 CYB_2740	NCBI
	Synechococcus sp. JA-3-3Ab	2,932,766	NC_007775	1 CYA_0410	2 CYA_1089 CYA_2307	2 CYA_0220 CYA_2042	2 CYA_0221 CYA_2043	NCBI
	Synechococcus sp. CB0205	2,427,308	NZ_ADXM00000000	1 SCB02_010100005353	2 SCB02_010100r00022 SCB02_010100r13854	1 SCB02_010100004410	1 SCB02_010100007213	NCBI
	Synechococcus sp. RCC307	2,224,914	NC_009482	1 SynRCC307_1957	1 RNA_8	1 SynRCC307_2064	1 SynRCC307_2063	NCBI
	Synechococcus sp. WH 7803	2,366,980	NC_009481	1 SynWH7803_2061	2 RNA_16 RNA_51	1 SynWH7803_0479	1 SynWH7803_0480	NCBI
	Synechococcus sp. WH 8102	2,434,428	NC_005070	1 SYNW0614	2 RNA_40 RNA_53	1 SYNW2023	1 SYNW2022	NCBI
	Synechococcus sp. CC9605	2,510,659	NC_007516	1 Syncc9605_2066	2 Syncc9605_R0016 Syncc9605_R0051	1 Syncc9605_0420	1 Syncc9605_0421	NCBI
	Synechococcus sp. CC9902	2,234,828	NC_007513	1 Syncc9902_0606	2 Syncc9902_R0046 Syncc9902_R0051	1 Syncc9902_1909	1 Syncc9902_1908	NCBI
	Synechococcus sp. RS9916	2,664,465	NZ_AAUA00000000	1 RS9916_34817	1 RS9916_r40510	1 RS9916_40146	1 RS9916_40151	NCBI
	Synechococcus sp. BL107	2,283,377	NZ_AATZ00000000	1 BL107_16035	1 BL107_r04839	1 BL107_08876	1 BL107_08881	NCBI
	Synechococcus sp. WH 5701	3,043,834	NZ_AANO00000000	1 WH5701_15446	1 WH5701_r06889	2 WH5701_05895 WH5701_05915	4 WH5701_05900 WH5701_05920 WH5701_10599 WH5701_16495	NCBI
Chroococcales; Synechocystis	*Synechocystis* sp. PCC 6803	3,573,470	NC_000911	1 (slr1265)	2 (6803r03,rrn16Sa; 6803r04, rrn16Sb)	1 (sll1578)	1 (sll1577)	Cyanobase NCBI

Chroococcales; Thermosynechococcus	*Thermosynechococcus elongatus* BP-1	2,593,857	NC_004113	1 tll0641	1 rrn16S	1 tlr1958	1 tlr1957	NCBI
Nostocales; Nostoc	*Nostoc punctiforme* PCC 73102	8,234,322	NC_010628	1 (Npun_F4986)	4 (Npun_r020, rrn16Sa; Npun_r067, rrn16Sb; Npun_r075, rrn16Sc; Npun_r084, rrn16Sd)	1 Npun_F5290	1 Npun_F5289	NCBI
	Nostoc punctiforme ATCC 29133	8,234,322		1 (Npun_F4986)	4 (Npun_r020, rrn16Sa; Npun_r067, rrn16Sb; Npun_r075, rrn16Sc; Npun_r084, rrn16Sd)	1 Npun_F5290	1 Npun_F5289	Cyanobase
	Nostoc sp. PCC 7120	6,413,771	NC_003272	1 (alr1595)	4 (allrr01, rrn16Sa; allrr04, rrn16Sb; allrr09, rrn16Sc; allrr12, rrn16Sd)	alr0529	alr0528	NCBI
	Nostoc azollae' 0708	5,354,700	NC_014248	1 Aazo_1015	4 Aazo_R0008 Aazo_R0016 Aazo_R0025 Aazo_R0038	1 Aazo_3488	1 Aazo_3489	NCBI
Nostocales, Cylindrospermopsis	*Cylindrospermopsis raciborskii* CS-505	3,879,030	NZ_ACYA00000000	1 (CRC_01785)	3 (CRC_01246; CRC_01178; CRC_02357)	1 (CRC_01959)	1 (CRC_01958)	NCBI
Nostocales, Raphidiopsis	*Raphidiopsis brookii* D9	3,186,511	NZ_ACYB00000000	1 (CRD_00849)	2 (CRD_01297; CRD_01676)	1 (CRD_01227)	1 (CRD_01226)	NCBI
Nostocales, Anabaena	*Anabaena variabilis* ATCC 29413	6,365,727	NC_007413	1 Ava_4208	4 Ava_R0006 Ava_R0024 Ava_R0035 Ava_R0053	1 Ava_2931	1 Ava_2930	NCBI
Nostocales, Nodularia	*Nodularia spumigena* CCY 9414	5,316,258	NZ_AAVW00000000	1 N9414_14383	4 N9414_r20046 N9414_r17988 N9414_r05922 N9414_r21536	1 N9414_13285	Not found	NCBI
Oscillatoriales; Oscillatoria	*Oscillatoria* sp. PCC 6506	6,676,705	NZ_CACA00000000	1 (OSCI_3100001)	1 (OSCI_16s_rRNA_1)	1 OSCI_2780022	1 OSCI_2780021	NCBI
Oscillatoriales; Trichodesmium	*Trichodesmium erythraeum* IMS101	7,750,108	NC_008312	1 (Tery_2938)	2 (Tery_R0029; Tery_R0014)	1 Tery_5048	1 Tery_5049	Cyanobase NCBI
Oscillatoriales; Microcoleus	*Microcoleus vaginatus* FGP-2	6,698,929	NZ_AFJC00000000	1 (MicvaDRAFT_3208)	1 (MicvaDRAFT_R0025)	1 MicvaDRAFT_4098	1 MicvaDRAFT_4099	NCBI
Oscillatoriales; Lyngbya	*Lyngbya majuscula* 3L	8,389,417	NZ_AEPQ00000000	1 LYNGBM3L_27230	1 LYNGBM3L_r59710	1 LYNGBM3L_16550	1 LYNGBM3L_16560	NCBI
	Lyngbya sp. PCC 8106	7,037,511	NZ_AAVU00000000	1 L8106_05555	2 L8106_r17850 L8106_r12018	1 L8106_02252	1 L8106_02247	NCBI
Oscillatoriales; Arthrospira	*Arthrospira platensis* str. Paraca	4,997,563	NZ_ACSK00000000	1 AplaP_010100004089	1 AplaP_010100r09305	1 AplaP_010100003267	1 AplaP_010100003262	NCBI
	Arthrospira maxima CS-328	6,003,314	NZ_ABYK00000000	1 AmaxDRAFT_0135	1 AmaxDRAFT_R0020	1 AmaxDRAFT_0385	1 AmaxDRAFT_0386	NCBI
Prochlorales; Prochlorococcus	*Prochlorococcus marinus* str. MIT 9211	1,688,963	NC_009976	1 (P9211_16061)	1 (P9211_rrsVIMSS1309438)	Not found	Not found	NCBI
	Prochlorococcus marinus subsp. *pastoris* str. CCMP1986	1,657,990	NC_005072	1 (PMM1484)	1 (RNA_39)	Not found	Not found	NCBI
	Prochlorococcus marinus subsp. *marinus* str. CCMP1375	1,751,080	NC_005042	1 (Pro1639)	1 (Pror01)	Not found	Not found	
	Prochlorococcus marinus str. MIT 9313	2,410,873	NC_005071	1 (PMT1506)	2 (RNA_53; RNA_50)	Not found	Not found	NCBI
	Prochlorococcus marinus str. MIT 9303	2,682,675	NC_008820	1 P9303_04371	2 P9303_rrsVIMSS1309419 P9303_rrsVIMSS1309420	Not found	Not found	NCBI
	Prochlorococcus marinus str. MIT 9215	1,738,790	NC_009840	1 P9215_17531	1 P9215_rrsVIMSS1723409	Not found	Not found	NCBI
	Prochlorococcus marinus str. NATL2A	1,842,899	NC_007335	1 PMN2A_1015	1 PMN2A_R0036	Not found	Not found	NCBI
	Prochlorococcus marinus str. MIT 9301	1,641,879	NC_009091	1 P9301_16751	1 P9301_rrsVIMSS1309398	Not found	Not found	NCBI
	Prochlorococcus marinus str. NATL1A	1,864,731	NC_008819	1 NATL1_18851	1 NATL1_rrsVIMSS1309404	Not found	Not found	NCBI
	Prochlorococcus marinus str. MIT 9515	1,704,176	NC_008817	1 P9515_16641	1 P9515_rrsVIMSS1309392	Not found	Not found	NCBI
	Prochlorococcus marinus str. AS9601	1,669,886	NC_008816	1 A9601_16881	1 A9601_rrsVIMSS1309386	Not found	Not found	NCBI
	Prochlorococcus marinus str. MIT 9312	1,709,204	NC_007577	PMT9312_1577	PMT9312_R0009	Not found	Not found	NCBI

NOTES:
NCBI: http://www.ncbi.nlm.nih.gov/genomes/genlist.cgi?taxid=2&type=0&name=Complete%20Bacteria
Cyanobase: http://genome.kazusa.or.jp/cyanobase/
*rpo*C1 = DNA-directed RNA polymerase subunit gamma



Online Resource 2: Amplification curves by real-time PCR of the serial 10-fold dilutions of DNA extracts from *P. agardhii* strain LMECYA 153B. The gray gradient in amplification curves from left to right corresponds to the cell dilutions range between 79102 to 7.9 cells μL^{-1}.

APPENDIX

C

Supplementary material of Chapter 6

Table S1: Nucleotide alignment of the *rpo*C1 sequences retrieved from non-preserved samples and samples preserved in methanol for 6, 18 and 24 months. The *rpo*C1 sequence of *P. agardhii* NIVA-CYA 127 with the accession number: AY425002.1 was used was a reference. Grey boxes indicates the primer hybridization site; black boxes indicates non-defined nucleotides. Abbreviations: fw – Forward; rv – Reverse; mos. – Months.

***P. agardhii* strain** **Nucleotide sequence 5' – 3'**

NIVA-CYA 127
TGTTAAATCCAGGTAACTATGACGGCCTATCCTATAAACAGTTATTAACCGAAGATACTTGGTTAGAAATTGAAGACCAAATTTATAGTGAAGATTCCACCTTAACCGGAATTGAAGTGGGAATTGGAGCCGAAGCCATTTCCCGTTTGCTCGAAGATATTCCCTTAGAAGAAGAAGCCGAAAGATTACGGGAAGAAATTGCCGTTGCTAAGGGACAAAAACGC

LMECYA 153A no prservation fw --
TTGGTTAGAAATTGAAGACCAAATTTATAGTGAAGATTCCACCTTAACCGGAATTGAAGTGGGAATTGGAGCCGAAGCCATTTCCCGTTTGCTCGAAGATATTCCCTTAGAAGAAGAAGCCGAAAGATTACGGGAAGAAATTGCCGTTGCTAAGGGACAAAAACGC

LMECYA 153B no preservation fw --
TTGGTTAGAAATTGAAGACCAAATTTATAGTGAAGATTCCACCTTAACCGGAATTGAAGTGGGAATTGGAGCCGAAGCCATTTCCCGTTTGCTCGAAGATATTCCCTTAGAAGAAGAAGCCGAAAGATTACGGGAAGAAATTGCCGTTGCTAAGGGACAAAAACGC

LMECYA 153A 6 mos. preservation fw --
TTGGTTAGAAATTGAAGACCAAATTTATAGTGAAGATTCCACCTTAACCGGAATTGAAGTGGGAATTGGAGCCGAAGCCATTTCCCGTTTGCTCGAAGATATTCCCTTAGAAGAAGAAGCCGAAAGATTACGGGAAGAAATTGCCGTTGCTAAGGGACAAAAACGC

LMECYA 153B 6 mos. preservation fw --
TTGGTTAGAAATTGAAGACCAAATTTATAGTGAAGATTCCACCTTAACCGGAATTGAAGTGGGAATTGGAGCCGAAGCCATTTCCCGTTTGCTCGAAGATATTCCCTTAGAAGAAGAAGCCGAAAGATTACGGGAAGAAATTGCCGTTGCTAAGGGACAAAAACGC

LMECYA 153A 18 mos. preservation fw --
TTGGTTAGAAATTGAAGACCAAATTTATAGTGAAGATTCCACCTTAACCGGAATTGAAGTGGGAATTGGAGCCGAAGCCATTTCCCGTTTGCTCGAAGATATTCCCTTAGAAGAAGAAGCCGAAAGATTACGGGAAGAAATTGCCGTTGCTAAGGGACAAAAACGC

LMECYA 153B 24 mos. preservation fw --
TTGGTTAGAAATTGAAGACCAAATTTATAGTGAAGATTCCACCTTAACCGGAATTGAAGNGGGAATTGGAGCCNAANCCATTTCCCGTTTGCTCGAAGATATTCCCTTAGAAGAAGAAGCCGAAAGATTACNGNAAGAAATTGCCGTTGCTAAGGGACAAAAAC---

LMECYA 153A no preservation_rv TGTTAAATCCAGGTAACTATGACGGCCTATCCTATAAACAGTTATTAACCGAAGATACTTGGTTAGAAATTGAAGACCAAATTTATAGTGAAGATTCCACCTTAACCGGAATTGAAGTGGGAATTGGAGCCGAAGCCATTTCCCGTTTGCTCGAAGATATTCCCTTAGAA---

LMECYA 153B no preservation rv TGTTAAATCCAGGTAACTATGACGGCCTATCCTATAAACAGTTATTAACCGAAGATACTTGGTTAGAAATTGAAGACCAAATTTATAGTGAAGATTCCACCTTAACCGGAATTGAAGTGGGAATTGGAGCCGAAGCCATTTCCCGTTTGCTCGAAGATATTCCCTTAGAA---

LMECYA 153A 6 mos. preservation rv TGTTAAATCCAGGTAACTATGACGGCCTATCCTATAAACAGTTATTAACCGAAGATACTTGGTTAGAAATTGAAGACCAAATTTATAGTGAAGATTCCACCTTAACCGGAATTGAAGTGGGAATTGGAGCCGAAGCCATTTCCCGTTTGCTCGAAGATATTCCCTTAGAA---

LMECYA 153B 6 mos. preservation rv TGTTAAATCCAGGTAACTATGACGGCCTATCCTATAAACAGTTATTAACCGAAGATACTTGGTTAGAAATTGAAGACCAAATTTATAGTGAAGATTCCACCTTAACCGGAATTGAAGTGGGAATTGGAGCCGAAGCCATTTCCCGTTTGCTCGAAGATATTCCCTTAGAA---

LMECYA 153A 18 mos. preservation rv TGTTAAATCCAGGTAACTATGACGGCCTATCCTATAAACAGTTATTAACCGAAGATACTTGGTTAGAAATTGAAGACCAAATTTATAGTGAAGATTCCACCTTAACCGGAATTGAAGTGGGAATTGGAGCCGAAGCCATTTCCCGTTTGCTCGAAGATATTCCCTTAGAA---

LMECYA 153B 24 mos. preservation rv -------------CCAGGTNNCTATGACGGCCTATCCTATAAACAGTTATTANCCGAAGATACTTGGTTAGAAATTGAAGNCCAAATTTATAGTGAAGATTCCACCTTAACCGGAATTGAAGTGGGAATTGGAGCCGAAGCCATTTCCCGTTTGCTCGAAGATATTCCCTTAGAA---