Development of molecular tools for the early warning of potentially toxic cyanobacteria

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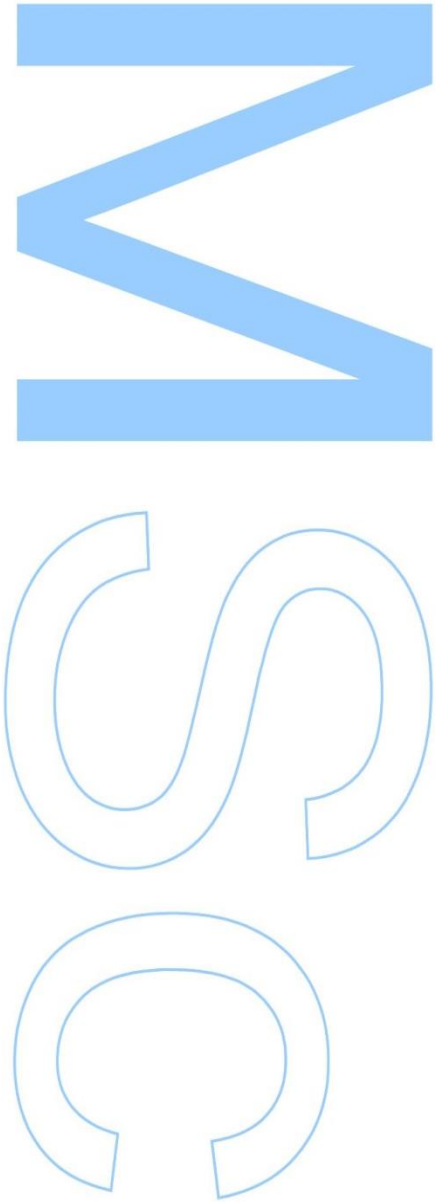

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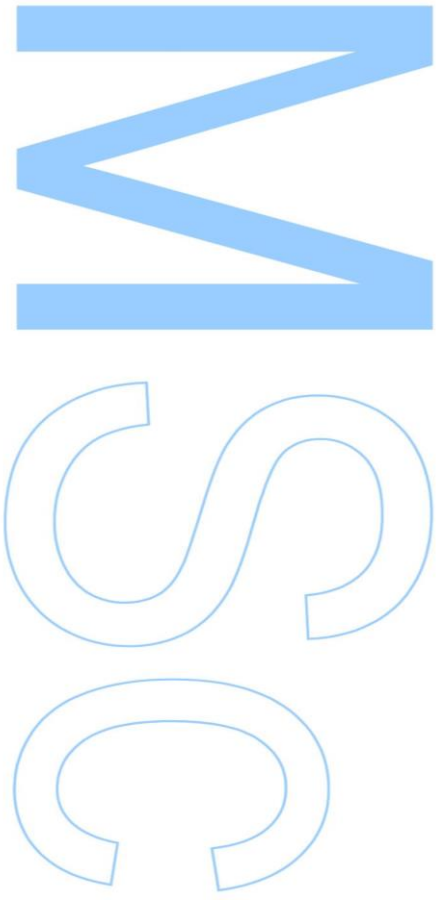

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Abstract

Cyanobacteria are photosynthetic microorganisms that inhabit a wide range of ecological niches. These microorganisms have the capacity to produce secondary metabolites with many biological activities, among them cyanotoxins that represent a human health concern due to their association with cases of human sickness and death, as well as various incidents of animal mortality. Thus, it is necessary to develop new effective tools, such as the molecular methods, for the early warning of water systems for the presence of potentially toxic cyanobacteria species, instead of using solely the traditional microscopy observation and identification. *Microcystis aeruginosa* is known to be widely dispersed in Portuguese freshwater systems, however, the presence of *Cylindrospermopsis raciborskii* and *Planktothrix agardhii* has only been recently described in these water systems. These species can constitute a public health concern due to their potential to form not only blooms but also to produce toxic compounds (cyanotoxins). This study was focused in these three potentially toxic cyanobacteria through the application of molecular techniques for the detection of these species in seven Portuguese freshwater systems located in the North and Centre regions of Portugal, during two consecutive years. In this study, it was also developed a Real-Time PCR protocol for the absolute quantification of *M.aeruginosa* cells in water systems and also specific primers for *Aphanizomenon* genus *rpoC*1 gene since to date they are still unavailable.

The applied techniques allowed the first detection of *C.raciborskii* in North regions of Portugal, emphasizing its invasive behavior in our national territory, as well as the first report of *P.agardhii* in North and Centre regions of Portugal. *M.aeruginosa* was the most dominant species in all the studied water systems. The developed Real-Time PCR protocol for *M.aeruginosa* permitted a detection limit of 55 copies of *gyrB* gene per µL of PCR reaction. Similarly, the preliminary assays with the developed primers for *Aphanizomenon* genus revealed their high specificity and similarity with other sequences of this gene belonging to species of this genus.

In conclusion, this study reinforces not only the need of a continuous monitoring of these three cyanobacteria species in these studied regions but also the need to include other regions of the country. The molecular tools here developed constitute a first step to better characterize the cyanobacteria community in aquatic systems as well as to contribute to the early warning of cyanobacterial blooms.

Keywords: cyanobacteria, molecular tools, freshwater systems, *Microcystis aeruginosa*, *Cylindrospermopsis raciborskii*, *Planktothrix agardhii, Aphanizomenon*

Resumo

As cianobactérias são microorganismos fotossintéticos que habitam uma vasta diversidade de ecossistemas. Um dos grupos de metabolitos secundários mais estudados, produzidos por estes microorganismos, são as cianotoxinas. Estas constituem um perigo ao nível da saúde publica uma vez que estão associadas a casos de morte de animais e intoxicação humana devido ao seu potencial tóxico. Devido a estes factos, é necessário desenvolver novas ferramentas eficazes, tais como os métodos moleculares, para a rápida deteção da presença de espécies de cianobactérias potencialmente tóxicas nos vários ecossistemas de água doce em vez de se usar somente as tradicionais técnicas de microscopia. *Microcystis aeruginosa* é conhecida por ser uma espécie comummente presente nos vários ecossistemas de água doce portugueses contudo *Cylindrospermopsis raciborskii* e *Planktothrix agardhii* só recentemente foram detectadas em Portugal.

Este estudo centrou-se, assim, na monitorização destas três espécies de cianobactérias usando ferramentas moleculares para a sua deteção em sete ecossistemas de água doce localizados em regiões Norte e Centro de Portugal, durante um período de dois anos de amostragem. Neste estudo também foi desenvolvido um protocolo de PCR em tempo real para a quantificação absoluta de células de *M.aeruginosa* em amostras de água bem como o desenho de novos primers específicos para o gene *rpoC1* para o género *Aphanizomenon,* que até ao momento estão indisponíveis.

As técnicas aplicadas permitiram a primeira deteção de *C.raciborskii* nas regiões Norte de Portugal, enfatizando uma vez mais o seu comportamento invasor em território nacional, bem como constitui o primeiro relato de *P.agardhii* em regiões do Norte e Centro de Portugal. *M.aeruginosa* foi a espécie mais dominante nos ecossistemas de água doce estudados. O protocolo de PCR em tempo real desenvolvido para *M.aeruginosa* permite a detecção de até 55 cópias de *gyrB* por µL de reação de PCR. Paralelamente, os testes preliminares para os primers desenvolvidos revelaram elevada especificidade e similaridade para o gene *rpoC1* para espécies do género *Aphanizomenon*. Os resultados obtidos representam não só a necessidade de continuar a monitorização destas espécies de cianobactérias nestas regiões estudadas mas também a necessidade de incluir outras regiões do país.

As ferramentas moleculares desenvolvidas neste estudo constituem o primeiro passo para uma melhor compreensão da comunidade de cianobactérias nos vários ecossistemas aquáticos e contribuem para o alerta precoce de florescências de cianobactérias.

Palavras-Chave: cianobactérias, ferramentas moleculares, sistemas de água doce, *Microcystis aeruginosa*, *Cylindrospermopsis raciborskii*, *Planktothrix agardhii, Aphanizomenon*

Table of Contents

Figures Index

Tables Index

List of Abbreviations

BLAST - *Basic Local Alignment Search Tool*

BMAA - B-methylamino-L-alanine

BSA - Bovine Serum Albumin

DNA - Deoxyribonucleic Acid

dNTP's - Deoxynucleotide Triphosphates

ELISA - Enzyme-Linked Immunosorbent Assay

ERIC-PCR - Enterobacterial Repetitive Intergenic Consensus - Polymerase Chain Reaction

FISH - Fluorescence In Situ Hybridization

gyrA - DNA gyrase subunit A

gyrB - DNA gyrase subunit B

HPLC - High-Pressure Liquid Chromatography

IPMA - Instituto Português do Mar e da Atmosfera

MC-LR - Microcystin-LR

MC-RR - Microcystin-RR

MC-YR - Microcystin-YR

NCBI - National Center for Biotechnology Information

PCR - Polymerase Chain Reaction

PCR-DGGE - Polymerase Chain Reaction - Denaturing Gradient Gel Electrophoresis

PCR-RFLP - Polymerase Chain Reaction - Restriction Fragment Length Polymorphisms

PCR-TGGE - Polymerase Chain Reaction -Temperature Gradient Gel Electrophoresis

PP1 - Protein Phosphatase 1

PP2A - Protein Phosphatase 2A

PSP - Paralytic Shellfish Poisons

Real-Time PCR - Real-Time Polymerase Chain Reaction

1. Introduction

1.1 Cyanobacteria

Cyanobacteria, one of the major phyla of *Bacteria* (Madigan et al. 2010), are probably the most ancient organisms living in the Planet earth since it is believed that its appearance occurred between 2.45 and 2.32 billion years ago, contributing to the Great Oxidation Event and to the increasing levels of oxygen molecules in the atmosphere (Bekker et al. 2004; Schirrmeister et al. 2013). These organisms, classified as Gram-negative bacteria, were the first oxygenic phototrophic organisms (Madigan et al. 2010) and the raise in oxygen molecules was responsible for the evolution and emergence of new species.

Besides oxygen, there is also the production of chlorophyll *a*, carotenoids and phycobilins by these microorganisms. Most of them have a blue-green colour due to the production of phycocyanin, one class of phycobilins, and clorophyll *a*, however some cyanobacteria species may present red or brown colour due to the production of phycoerythrin, a red phycobilin (Madigan et al. 2010).

It is also well known the production of secondary metabolites by cyanobacteria with many biological activities such as anticancer activity, toxic activity and antiprotozoal properties (Tan, 2013). Through extraction and purification methods these compounds have been isolated, characterized and tested to be applied in pharmaceutical and food industries (Liu et al., 2014). However, the secondary metabolites most studied due to their hepatotoxic, neurotoxic, cytotoxic and dermatotoxic potential are the toxic compounds, also known as cyanotoxins.

Cyanotoxins can be organized, by their mode of action, in neurotoxins (anatoxin-a, -as, homoanatoxin-a, B-methylamino-L-alanine and saxitoxins), hepatotoxins (microcystins and nodularins), cytotoxins (cylindrospermopsin), dermatotoxins (lyngbyatoxin and aplysiatoxin) and irritant toxins (lipopolysaccharides) (Carmichael 2001; Cox et al. 2005; Dittmann and Wiegand 2006). The group of hepatotoxins comprises the microcystins and nodularins, being the first frequently detected in the freshwater systems. Several toxic variants of microcystins are known to exist such as MC-LR, MC-RR and MC-YR (Campos and Vasconcelos, 2010). Hepatotoxins have the ability to inhibit serine/threonine-specific protein phosphatases (PP1 and PP2A), which causes phosphorylation of cellular proteins, due to the presence of a rare amino acid (Adda) in their protein composition (Campos and Vasconcelos 2010; Dittmann and Wiegand 2006). The accumulation of these cyanotoxins occurs mainly in liver leading to cellular damages including cytoskeletal disorganization, DNA (deoxyribonucleic acid) fragmentation and intrahepatic bleeding, which can result in the death of organisms, it

is also associated with these cyanotoxins the promotion of tumours (Dittmann and Wiegand 2006). Alternatively, anatoxins and saxitoxins belong to the neurotoxins group. The group of anatoxins includes anatoxin-a, homoanatoxin-a and anatoxin-a(s) which are alkaloid compounds whose toxicological action occurs mainly in the nervous system of the organisms infected. Anatoxin-a is capable of binding to nicotinic acetylcholine receptors of the sodium channel, because it is a nicotinic agonist (Dittmann and Wiegand 2006), altering the electric transmission which may lead to muscle fasciculation, cyanosis and, in severe cases, in death by asphyxiation due to muscular paralysis (Carmichael, 2001). Anatoxin-a(s) may also over stimulate muscles and generate hypersalivation, tremors and ataxia because it is a cholinesterase inhibitor so, when present, acetylcholine is not removed from the nicotinic receptor generating action potentials leading to exhaustion of the nerve cell (Carmichael 2001; Dittmann and Wiegand 2006). Homoanatoxin-a is a homologue of anatoxin-a which mimic the effect of acetylcholine (Carmichael and Li 2006). Moreover, saxitoxins, also known as paralytic shellfish poisons (PSP's), are able to block sodium channels leading to the blocking of nervous transmission which may result in nervousness, ataxia, muscle paralysis and death (van Apeldoorn et al. 2007; Briand et al. 2003; Carmichael 2001). Furthermore, B-methylamino-L-alanine (BMAA), may affect brain and central nervous system causing neurologic damage (Al-Sammak et al. 2014). Cylindrospermopsin besides blocking protein synthesis may induce DNA strand breaks and chromosome loss being described as genotoxic and cytotoxic. After ingestion of this cyanotoxin, the first symptoms are kidney and liver failure (Carmichael, 2001; Humpage et al. 2000). The dermatotoxins are potent tumour promoters, Aplysiatoxin is a protein kinase C activator and Lyngbyatoxin A, besides being a promoter of skin tumour, has caused oral and gastrointestinal inflammations in humans (Carmichael and Li 2006). Cyanobacterial lipopolysaccharides are the group of cyanotoxins less studied however it is associated with cutaneous symptoms, headache, fever and respiratory diseases (Stewart et al. 2006).

It is worthy to note that each toxin can be produced by more than one cyanobacterium genus or species which means that the production of cyanotoxins is not strain-specific and that the same cyanobacteria genera or species are able to produce more than one type of cyanotoxin (Table 1). There are many hypotheses about the reason why these compounds are produced, some authors suggest the role of these metabolites as iron scavenging molecules (Utkilen and Gjolme 1995), infochemicals (Kaebernick et al. 2000), growth regulators (Sedmak and Kosi 1998) or even as a protective role (Tran et al. 2013). However, there are species which do not express the cyanotoxins coding genes being known as non-toxigenic species, these

are morphologically indistinguishable from the toxin-producing cyanobacteria (Fastner et al. 2001).

Table 1 – Main cyanobacteria genera producers of cyanotoxins. Adapted table from Carmichael (2001).

Cyanotoxin	Producers
Neurotoxins	
Anatoxin-a	*Anabaena, Aphanizomenon, Oscillatoria, Planktothrix*, *Microcystis*
Homoanatoxin-a	*Anabaena, Aphanizomenon, Planktothrix*
Anatoxin-a(s)	*Anabaena, Planktothrix*
Saxitoxin	*Anabaena, Aphanizomenon, Cylindrospermopsis, Lyngbya, Planktothrix*
Hepatotoxins	
Microcystins	*Anabaena, Aphanocapsa, Hapalosiphon, Microcystis, Nostoc, Oscillatoria, Planktothrix*
Nodularins	*Nodularia*
Cytotoxins	
Cylindrospermopsin	*Aphanizomenon, Cylindrospermopsis, Raphidiopsis, Umezakia*
Dermatotoxins	
Lyngbyatoxin	*Lyngbya*
Aplysiatoxin	*Schizothrix*

This group of microorganisms is characterized by a huge variety of morphologies, existing unicellular as well as filamentous forms which are widespread across the world, from the tropical to the Polar regions, either in aquatic or in terrestrial habitats (Ionescu et al. 2010; Liu et al. 2014). This diversity of morphologies led to the division of the cyanobacteria into five orders according to the Botanical Code criteria (Henson et al. 2002). The first (Order Chroococcales) comprises colonial or coccoid cyanobacteria whose division is through binary fission. Order Pleurocapsales includes coccoid and pseudofilamentous cyanobacteria which has a combined cell division by multiple and binary fission whereas in the Order Oscillatoriales are grouped filamentous cyanobacteria with absence of heterocytes and akynetes. Filamentous cyanobacteria with heterocytes, akynetes and false branching belong to Order Nostocales whilst Order Stigonematales comprises filamentous cyanobacteria, facultative heterocytes and akynetes and true branching (Komárek 2003).

Furthermore, some cyanobacteria possess cellular characteristics favourable to its dispersion and maintenance in water systems. Many cyanobacteria species have gas vesicles which permit them to regulate its buoyancy and adjust its presence in the water column allowing the establishment in optimum growth conditions. This characteristic is responsible for the occurrence of blooms when cyanobacteria accumulate at water surface (WHO 2003). There is also the presence of heterocytes (specialized cells) which occurs only in filamentous cyanobacteria and they are related with nitrogen fixation under anaerobic conditions and concentration of nutrients.

Akynetes, also another type of specialized cell in filamentous cyanobacteria, enable survival under unfavourable conditions (i.e. dry seasons) because they are capable of long-term sustained dormancy (Komárek 2013).

When the optimum conditions for growth and proliferation of cyanobacteria are present (see section 1.2), it is very likely the occurrence of blooms (increase of cyanobacteria growth rate). These events represent an important public health and biological concern due to the presence of potentially toxic cyanobacteria, whose effects and toxicological mechanisms were described above, alterations which may be induced in the present community as well as in the water quality of the contaminated system (Madigan et al. 2010). However, the increase of nutrients (eutrophication) in the water systems due to anthropogenic activity, discharge of pollutants from diverse industrial sources and from agriculture in the water, lead to the development of blooms which are becoming more frequent (Falconer and Humpage 2005).

The existence of cyanobacteria species until these days as well as its enormous dispersion in the aquatic systems may be explained by the mentioned characteristics of these organisms in this chapter.

1.2 Portuguese Freshwater Cyanobacteria Community

Worldwide, some of the cyanobacteria most frequent in freshwater systems are *Microcystis* spp., *Cylindrospermopsis raciborskii*, *Planktothrix rubescens*, *Planktothrix agardhii*, *Anabaena* spp., *Lyngbya* spp., *Aphanizomenon* spp. and *Nostoc* spp. (WHO 2003). Since the beginning of 20th century that it has been reported the presence of cyanobacteria in Portuguese freshwater systems (Sampaio, 1933; Vasconcelos, 1999). However, the studies have also focused the marine habitats, mainly due to the cyanobacteria species present with a high potential of bioactive secondary metabolites biosynthesis (Leão et al. 2013).

Until now, in the studies performed to investigate the diversity of cyanobacteria present in the Portuguese freshwater systems, *Microcystis aeruginosa* is frequently detected being in most studies the dominant species. It has been detected from the North to the South regions of Portugal such as in Braça Lake (Amorim 1993; Martins et al. 2009), Vela Lake (Amorim, 1993; de Figueiredo et al., 2006), Mira Lake (Amorim, 1993), Montargil Reservoir (Pereira et al., 2000), Alqueva Reservoir, Alvito Reservoir and Odivelas Reservoir (Galvão et al., 2008), WWTP of Esmoriz (Vasconcelos and Pereira 2001), Guadiana River (Moreno et al. 2003), Tâmega River, Douro River, Aguieira Reservoir, Bastelos Reservoir and Barrinha de Mira Lake (Martins et al. 2009). Other species have been detected in these water systems, such as

Aphanizomenon flos-aquae in Montargil Reservoir and in Vela Lake (de Figueiredo et al. 2006a; Pereira et al. 2000), Alvito Reservoir (Galvão et al. 2008) and Guadiana River (Moreno et al., 2003), *Microcystis wesenbergii* in Odivelas reservoir (Galvão et al. 2008), *Anabaena circinalis* in Alqueva reservoir (Galvão et al. 2008), *Anabaena* spp. in Guadiana River (Moreno et al. 2003), *Planktothrix* spp. was detected in Enxoé Reservoir (Galvão et al. 2008), *P. rubescens* in Beliche reservoir (Paulino et al. 2009), *Planktothrix mougeotii* and *Pseudoanabaena mucicola* in WWTP of Esmoriz (Vasconcelos and Pereira 2001) and *P. agardhii* was detected in South regions of Portugal (Churro et al. 2012). More recently, *C. raciborskii* has been detected essentially in the Centre and South regions of Portugal such as in Vela Lake (Moreira et al. 2011), Odivelas Reservoir, Ardila River, Caia Reservoir, Maranhão Reservoir, Montargil Reservoir, Agolada Reservoir, Bufo Reservoir, Mértola Reservoir and Patudos Reservoir (Saker et al. 2003).

The occurrence of cyanobacteria is being related with conductivity, high temperature, high light intensity, high nutrient concentration (particularly phosphorus) and low ratio between nitrogen and phosphorus concentrations (de Figueiredo et al. 2006; Vasconcelos and Pereira 2001). It is known that the optimum temperatures which lead to cyanobacteria growth and proliferation are around 25ºC, these high temperatures lead to the vertical migration of the phytoplankton due to the decrease of the water resistance and therefore facilitates the bloom's occurrence by buoyant cyanobacteria (Paerl and Huisman 2009).

These conditions are also reported to be important in the promotion of the cyanobacteria appearance and their dominance in Portuguese freshwater systems (de Figueiredo et al. 2006a; Moreno et al. 2003). However, it is also noteworthy that there is the existence of non-toxic strains which can co-exist with the toxic strains in a bloom event (WHO 2003).

Occurrences of human and animal intoxication by cyanobacteria contact or ingestion have already been reported. In Portugal, it is reported the occurrence of a human intoxication, at Alentejo, related with the consumption of water from Guadiana River which had, at that time, an *Ap. flos-aquae* bloom occurrence (Oliveira 1991). However, worldwide, other cases have been described, a well-known case of human intoxication due to the ingestion of water contaminated with cyanotoxins, particularly with cylindrospermosin, happened in 1979 in Australia (Palm Island) causing gastroenteritis, hepatomegaly and renal insufficiency either in children and in adults (Humpage et al. 2005). Years later, in 1996, in Brazil (Caruaru) at a dialysis center, after a treatment, the patients suffered from visual disturbances, nausea, vomiting and there were also patients who died of acute liver failure. This incident was due to the

use of water contaminated with microcystins that entered the dialysis systems (Jochimsen et al., 1998). Others cases of human exposure to contaminated water by *Microcystis* which led to human deaths, colorectal cancer and primary liver cancer were described in China between 1977 and 1996, these cases are related with ingestion of contaminated water by microcystins (Bláha et al. 2009). In Sweden in 1994, it was also reported a case of human intoxication which caused gastroenteritis, fevers, abdominal and muscular pain due to the ingestion of water contaminated with microcystins produced by *Planktothrix* species (Bláha et al. 2009).

It is important to highlight that cyanotoxins have already been detected in food supplements, as reported by Saker and collaborators, since there are cyanobacteria species as *Ap. flos-aquae* and *Nostoc flagelliforme* that are cultivated for direct consumption or to be included into dietary supplements (Saker et al. 2005). Cyanobacteria habit desert environments where it was also reported the presence of microcystins and anatoxin-a representing a risk to human health, through soil dust inhalation (Metcalf et al. 2012).

In Portugal, as previously mentioned, it is well known the existence of potentially toxic cyanobacteria in freshwaters systems, in this way the presence and quantification of these species in these water systems is one of the parameters taking into account in the water quality analyses (Decreto-Lei No. 306/ 2007, Diário da República, 1.ª série — No. 164). This parameter states that in case of the detection of cyanobacteria potentially microcystin – producing higher than 2000 cells/mL the frequency of sampling should be increased. This fact suggests the need for a constant screening of freshwaters systems.

1.3 Detection Methodologies

Traditionally, the detection and identification of cyanobacteria species in a water system was performed using microscope techniques and morphological characteristics able to distinguish species such as cell size, cell fission type, presence and characteristics of specialized cells. However, this method requires a person with taxonomic skills because despite the taxonomic guides available that can be used to identify an organism, there are species extremely similar in its morphologic characteristics and in the case of small cells they can be undetected or they can be near the limits of resolution of the microscope so it is necessary extreme caution and precision when these analyses are performed. Besides these facts, using microscopic techniques and morphologic characters, it is very difficult, or even impossible, to distinguish in a sample toxic species from non-toxic species. This method is also time-

consuming compared to the molecular methods that exist nowadays which enable the early warning of the cyanobacteria species and its cyanotoxins in the analysed systems. Therefore, the molecular techniques are a useful help in the rapid identification and detection of the cyanobacteria and in the increase of knowledge about their molecular biology, genetic diversity and evolution. Since the development of molecular tools (primers) that conventional Polymerase Chain Reaction (PCR) is the most frequent molecular technique in which these tools, nucleotide sequences which will hybridize to a complementary target sequence, are applied to amplify a specific DNA sequence. In this reaction, occurs the denaturation by heating of the double stranded DNA (template) followed by the annealing step where the primers (forward and reverse) bind to specific DNA sequence to posteriorly be performed the extension step, it is in this step that the addition of dNTP's (deoxynucleotide triphosphates) by *Taq* DNA Polymerase, heat resistant enzyme, happens. This process occurs repeatedly in a number of cycles previously established so the products of one cycle are templates for the next, therefore the amount of the original target DNA doubles each cycle. At the end of the reaction, the amplified sequence (amplicon) will be accumulated in a high number of copies (Madigan et al. 2010). Particularly, in Portugal since the earlier years of 2000 that molecular techniques have been applied, in the study of bacterial community and species detection in freshwater systems, mainly involving PCR based techniques as PCR - Restriction Fragment Length Polymorphisms (PCR-RFLP) (Saker et al. 2007), a technique that comprises a restriction assay with specific enzymes of the PCR products and the analysis of the results may provide signature profiles specific to the genus, species or even strain (Valério et al. 2009), in this technique, usually, it is obtained several fragments that may difficult the analyses of RFLP profiles. On the other hand, there are studies which prefer the use of conventional PCR (de Figueiredo et al. 2007; Lopes et al. 2012; Martins et al. 2009; Moreira et al. 2011; Paulino et al. 2009; Saker et al. 2005; Saker et al. 2003, 2007). Enterobacterial Repetitive Intergenic Consensus (ERIC-PCR) (Valério et al. 2005), is a technique applied to differentiate cyanobacterial genera based on highly repetitive intergenic sequences (Lyra et al. 2001). Moreover, Real-Time Polymerase Chain Reaction (Real-Time PCR) is becoming a technique highly applied in water monitoring programs (Churro et al. 2012; Moreira et al. 2011; Martins et al. 2011). This method overcomes the limitations of conventional PCR because besides the amplification of the target gene it is possible the quantification of the amplicon in gene copies numbers. This is a more reliable way to monitor the dynamics of cyanobacteria in the water systems and it is more sensitive than the conventional PCR (Moreira et al. 2014).

The principle of this technique is the use of a DNA-binding dye which exhibits fluorescence when it binds to DNA, in this way the fluorescence signal increases at the same time that the target gene is amplified, the fluorescence signal reflects the amount of product formed (Kubista et al. 2006). After the amplification cycles, it is performed a melt curve that is the increase of temperature in small increments and the fluorescent signal is monitored at each cycle until reach the temperature at which 50% of the base pairs of a DNA duplex are separated (melting temperature (TM)) appearing a melting peak and the fluorescent signal decrease because occurs the unbinding of the dye. It can be applied sequence specific probes or non-specific labels as reporters. SYBR® Green, a dsDNA binding dye, is one of the most used. To quantify the number of copies amplified, it is performed a standard curve with standard dilution series of known concentrations through which is calculated the number of amplicons obtained and the efficiency of the reaction (Kubista et al. 2006).

When the main objective of the work is to investigate and study the microbial community and determine the genetic diversity present, it is used PCR-denaturing gradient gel electrophoresis (PCR-DGGE) or PCR-temperature gradient gel electrophoresis (PCR-TGGE) (de Figueiredo et al. 2007; Martins et al. 2010), this method consists in the amplification of a universal genetic marker for the group of organisms in study (i.e. 16S rRNA) obtaining an allelic mixture of fragments of the same length but heterogeneous sequence. The PCR products obtained are visualized by a denaturing gradient gel electrophoresis (PCR-DGGE) or temperature gradient gel electrophoresis (PCR-TGGE) based in the principle that differences in base sequence cause differences in the denaturing profile (Madigan et al., 2010; Muyzer, 1999). Multiplex PCR is also applied (Saker et al. 2007; Valério et al. 2010), it is a technique where two or more genes are amplified in the same reaction, for this reason it is necessary the use of more than one primer pair (Henegariu et al. 1997).

The methods described above are PCR-based techniques however methods such as FISH (Fluorescence *In Situ* Hybridization), based in the application of fluorescent probes complementary to target organism sequence, and DNA microarrays may also be applied in cyanobacteria detection and in the study of genes expression (Li et al. 2004).

The sequencing reaction is a useful complementary method since it is possible the corroboration of the results previously obtained in the molecular methods performing a nucleotide comparison, BLAST (Basic Local Alignment Search Tool), between the sequences obtained in the sequencing reaction with the sequences present in a public database, NCBI (National Center for Biotechnology Information).

It is worthy to note that these methods do not inform about the toxicity present in a sample, through the application of molecular techniques it is only possible the detection of potentially toxic cyanobacteria. To assess the toxicity present in a sample it is necessary to perform chemical methods such as the High-Pressure Liquid Chromatography (HPLC) and/or biochemical methods such as the Enzyme-Linked Immunosorbent Assay (ELISA). In figure 1, it is represented a scheme of the mentioned methods.

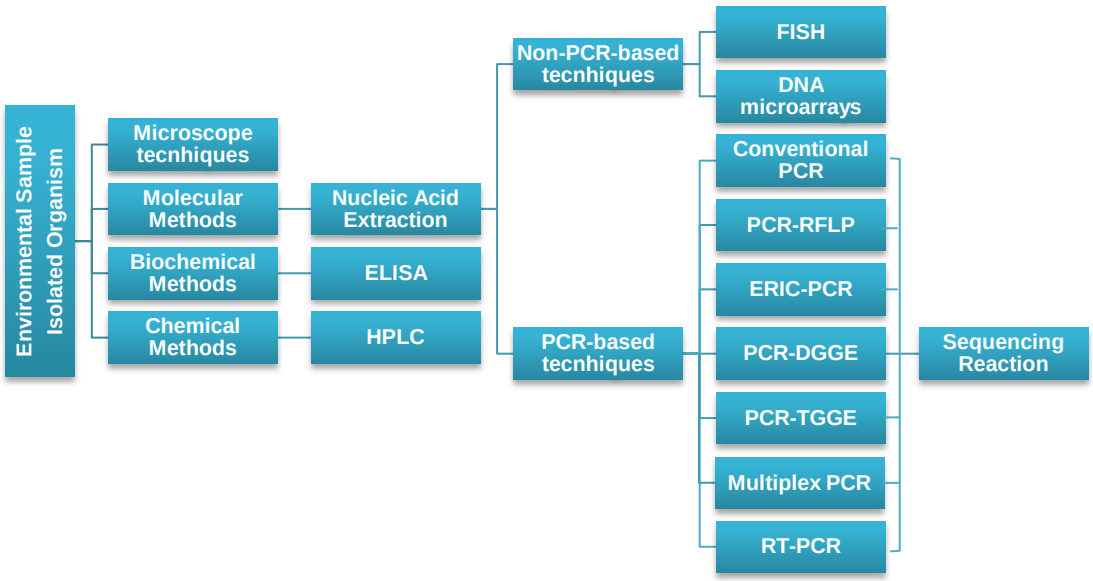

Figure 1 – Representative scheme of the mentioned methods possible to be applied for the detection of cyanobacteria species either in environmental samples or in isolated organisms from laboratory cultures.

1.4 Studied Cyanobacteria Species

This study focused in three potentially toxic cyanobacteria species, *M.aeruginosa*, *P.agardhii* and *C.raciborskii,* and in one cyanobacterium genus, *Aphanizomenon*. Due to the potential production of cyanotoxins, the occurrence of blooms related with the presence of these mentioned species as well as the recent detection of *C.raciborskii* and *P.agardhii* species in Portuguese freshwater systems characterized by their invasive behaviour increase the necessity of monitoring the occurrence and dispersion of this species in Portugal through the application of molecular techniques, mainly in water systems in which the water is used to recreational and human consumption. In this way, in this study, it was applied molecular tools (primers) already developed to detect *M.aeruginosa*, *C.raciborskii* and *P.agardhii* in environmental samples from North and Centre regions of Portugal (see section 2.1.1). For *M.aeruginosa,* the primers developed by Tanabe et al. (2007) are specific for DNA gyrase subunit B (*gyrB*) sequence of this species. *gyrB* and *gyrA* (DNA gyrase subunit A) are the two subunits

which constitute the bacterial DNA gyrase (Type II DNA topoisomerases) (Huang, 1996). DNA gyrase introduces negative supercoils into double stranded DNA (dsDNA) in an ATP-dependent reaction (Huang 1996; Stanger et al. 2014). The DNA gyrase subunit B is a single-copy gene in all bacteria (Dauga 2002; Holmes et al. 2004). On the other hand, the primers applied to detect *C.raciborskii* were described by Wilson et al. (2000) with the objective to examine the level of diversity among Australian isolates of *C.raciborskii* and are specific for the DNA-dependent RNA polymerase (*rpoC1*) gene. This gene encodes the γ subunit of RNA polymerase and it is present in the cyanobacterial genome as a single copy (Bergsland and Haselkorn 1991). The primers applied to detect *P.agardhii* in environmental samples are also specific to *rpoC1* gene of this species and were developed by Churro et al. (2012) to be applied in Real-Time PCR quantification.

1.4.1 *Microcystis aeruginosa*

M. aeruginosa (Kützing) Kützing 1846 (Order Chroococcales) is a unicellular cyanobacterium which has a tendency to form aggregates or colonies. In laboratory cultures, this species exist as single cells whereas in environmental conditions usually occurs as colonies (Bolch and Blackburn 1996). This fact is suggested to be a phenotypic characteristic of this species to face environmental factors that may inhibit its growth (Yang et al. 2006). *M.aeruginosa* has been detected in many eutrophic freshwater systems being in various studies reported as the dominant species in a bloom as well as the responsible for its occurrence (Saker et al. 2005). As mentioned in section 1.2, *M.aeruginosa* is widely dispersed in Portugal representing a water quality problem due to the potential production of toxic blooms. The presence of gas vesicles for buoyancy, in the *M.aeruginosa* cellular constitution, favour the dominance and the occurrence of blooms by this species in the water systems (Saker et al. 2005). This species is able to synthetize microcystins and other compounds with bioactivity such as aeruginosins (thrombin inhibitor), cyanopeptilins (trypsin inhibitor), anabaenopeptins (carboxypeptidase A inhibitor) and microginin (angiotensin-converting enzyme and leucine amino peptidases inhibitors) (Dittmann et al. 2001; Martins et al. 2009).

1.4.2 *Planktothrix agardhii*

P. agardhii (Gomont) Anagnostidis and Komárek (1988) is a filamentous cyanobacterium which belongs to Oscillatoriales Order, occurs as single trichomes and it is usually found in hypertrophic temperate freshwaters (Briand et al. 2008). This species is known for its resilient and shade-tolerant characteristics as well as for the synthesis of microcystins (Keil et al. 2002) and saxitoxin (Bonilla et al. 2012; Pomati et

al. 2000). It is also reported the production of other secondary metabolites by some strains which may affect fish and crustaceans (Ernst et al. 2001; Keil et al. 2002). Until now, in Portugal this species has only been known to occur in the South regions (Churro et al. 2012). It was also recorded that the production of microcystins per dry weight by *Planktothrix* blooms was higher than the production of these toxins by *Microcystis* blooms (Fastner et al. 1999).

1.4.3 *Cylindrospermopsis raciborskii*

The other species in study is *C. raciborskii* (Woloszynska) Seenayya and Subba Raju, 1972 (Order Nostocales) which is a filamentous and nitrogen fixing species original from tropical environments. It is a heterocystous cyanobacterium having its heterocytes only terminal, at one or both ends of a trichome with rounded-pointed ends (Komárek 2013). It has a worldwide distribution however in Portugal only recently was reported its first detection by Saker et al. (2003) in the South part of the country. Since then, it has been detected in the South and Centre regions of Portugal (Freitas 2009; Valério et al. 2005). This species is known by its invasive behaviour and by the ability to produce cylindrospermopsin and saxitoxin (Lagos et al. 1999) which affect the water quality and represent a public concern since in the past this cyanobacterium species was associated with a human poisoning in Australia in 1979, as mentioned above, and implicated in death of cattle (Thomas et al. 1998).

1.4.4 *Aphanizomenon* genus

Species from *Aphanizomenon* genus (Morren ex Bornet et Flahault 1888) (Order Nostocales) are frequently detected in temperate zones. *Aphanizomenon* is able to produce anatoxin-a, cylindrospermopsin and saxitoxin and some of its analogues (Falconer and Humpage 2005; Stuken et al. 2009). The molecular studies developed, so far to *Aphanizomenon* genus, are not capable to distinguish this genus from *Anabaena* genus. These two genera are genetically very similar, they include species that are neither phylogenetically nor morphologically separable (Stuken et al. 2009) and some authors suggest these two genera are intermixed and polyphyletic (Gugger et al. 2002; Lyra et al. 2001). However, it is necessary the development of molecular tools able to differentiate these two genera.

It is essential a reliable detection and identification of the cyanobacteria and its metabolites present in the water systems in order to guarantee the safety of public health.

1.5 Sampling Sites

Seven sampling points located at North and Centre regions of Portugal were chosen to conduct this study. These sites were all chosen for their huge importance in public health domain since they are used as a water source for consumption and for recreational activities of the local population. In that way, the selected sites to represent the North region were Torrão Reservoir, Tâmega River (Marco de Canaveses) and Parque da Cidade do Porto (Lake 1, Lake 2 and Lake 3) and to represent the Centre region of Portugal the locals were Mira Lake and Vela Lake.

1.6 Objectives

The main objectives of this work were:

- Apply the molecular methods already described to detect the presence of *M.aeruginosa*, *C.raciborskii* and *P.agardhii* in water samples obtained from seven sampling sites, representative of North and Centre regions of Portugal, in a two-year study;
- Analyse the seasonal variation and spatial distribution of the cyanobacteria in study;
- Evaluate the invasive behaviour of *P.agardhii* and *C.raciborskii* in Portuguese freshwater systems located in the North and Centre regions;
- Isolate and cultivate the cyanobacteria from the collected environmental samples;
- Development of a Real-Time PCR protocol to quantify *M.aeruginosa* in environmental samples;
- Develop specific molecular tools (primers) to *Aphanizomenon* genus.

2. Materials and Methods

2.1 Sampling

2.1.1 Description of Sampling Sites

As mentioned above, seven sampling sites from North and Centre regions of Portugal were screened for the presence of the cyanobacterial species in study, the sites were: Torrão Reservoir, Tâmega River (Marco de Canaveses), Parque da Cidade do Porto (Lake 1, Lake 2 and Lake 3), Vela Lake and Mira Lake.

In order to detect the seasonal variation of the species in study, the sampling was performed in two consecutive years, from May to September of 2012 and from May to October of 2013. In August and September of 2012 it was not possible to do the sampling in Torrão Reservoir because this location was inaccessible for users. In 2012, the sampling was performed by Doctor Cristiana Moreira. It was chosen the mentioned months because these are the months in which, usually, it is recorded the highest concentration of cyanobacteria species.

Torrão Reservoir (Figure 2) is situated in the North of Portugal, in Tâmega River, an affluent of the Douro River. The sampling was performed in a leisure park located at this reservoir, Parque de Lazer de Alpendorada e Matos (41°05'45.7"N, 8°15'15.4"W). This reservoir has a total capacity of 77 hm^3 and its watershed is about 3280 km^2 (Mateus et al. 2014). It is known that the water from this reservoir is used in agriculture, as a source of potable water to the inhabitants of Marco de Canaveses and Amarante or in recreational activities by the local population as well as in energy production due to the existence of a hydroelectric dam in this river (Mateus et al. 2014; Torres et al. 2011). It has been previously reported in this local the presence of cyanobacteria species such as *Microcystis* spp. (Martins et al. 2011), *M.aeruginosa* (Vasconcelos et al. 1996) and *Ap. flos-aquae* (Teles et al. 2008) as well as cyanotoxins such as microcystins (Saker et al. 2007). The second sampling site, located in the Tâmega River, was Tâmega River (Marco de Canaveses) (Figure 3) in Parque Fluvial do Tâmega (41°11'45.9"N, 8°09'38.2"W). It is reported that *M.aeruginosa* is the dominant species, between July and September (Saker et al. 2005) however it has been already detected the presence of *Anabaena* sp. and *Aphanizomenon* sp. (Osswald et al. 2009).

In this river park is situated a children playground, a nautical club and reserved areas for fishing and for sport activities. The distance between these two sampling points, Torrão Reservoir and Tâmega River (Marco de Canaveses), is around 14 km, being this last site situated more upstream of the Tâmega River.

Figure 2 - Sampling site at Torrão Reservoir.

Figure 3 - Sampling site at Tâmega River (Marco de Canaveses).

The other three sampling sites located in the North region of Portugal were situated at Parque da Cidade do Porto (Figure 4) which consisted of three consecutive lakes: Lake 1 (41°10'07.1"N, 8°40'20.5"W), Lake 2 (41°10'04.5"N, 8°40'25.6"W) and Lake 3 (41°10'01.5"N, 8°40'39.8"W), (these names were attributed according to its northernmost entrance of the park). Parque da Cidade do Porto is a recreational park located in the Centre of Porto City and it is composed by four artificial lakes and 83 ha of green area. The water circulation is in succession from the Lake 1 to the Lake 4 and these lakes supply the irrigation system of this park. Lake 4 it was not included in this study because it is the less studied. There is the presence of numerous bird species, fishes and its flora is also very extensive, containing numerous species of arboreal and shrub species (Morais 2009; Moreira 1998). Cyanobacteria species that have been detected in these lakes are *Microcystis* sp., *M.aeruginosa* and *Planktothrix* sp. (Morais 2009; Moreira 1998).

Figure 4 - Sampling sites at Parque da Cidade do Porto. In the largest picture it is possible to observe a satellite view of the lakes (1, 2, 3 and 4) and its distribution in the Park. The smallest pictures are photographs of the Lake 1 (1), Lake 2 (2) and Lake 3 (3) taken at the sampling collections.

Vela Lake and Mira Lake were the chosen sampling sites to represent the Centre region of Portugal. Vela Lake (40°16'23.9"N, 8°47'35.1"W) (Figure 5) is an eutrophic shallow freshwater body which belongs to a system of interconnected reservoirs, being this the largest one, located at Figueira da Foz (Antunes et al. 2002) and it is embedded in a protected area, Ecological European Net - Rede Natura 2000. It has around 70 ha and it is frequently observed the occurrence of blooms and some cyanobacteria species associated with it, such as *M.aeruginosa*, *C.raciborskii*, *Ap. flos-aquae* and *Anabaena flos-aquae* (de Figueiredo et al. 2006; Saker et al. 2003). This lake is surrounded by agricultural fields, in which its water is applied, and by a pine forest. It is common the practice of recreational fishing in this Lake (Abrantes et al 2006).

Mira Lake (40°26'29.8"N, 8°45'07.5"W) (Figure 6), the second sampling site in the Centre region, has around 40 ha of area and the distance between these two sampling sites is around 10 km (Gonçalves et al. 1996). At this Lake, it was already detected the presence of cyanobacteria blooms co-dominant with *Actinobacteria* (de Figueiredo et al. 2007). This lake is also located in agricultural areas being its water used for the irrigation of crops. In the opposite border to this site it is situated a touristic house.

Figure 5 - Sampling Site at Vela Lake.

Figure 6 - Sampling Local at Mira Lake.

2.1.2 Sampling Procedure

The sampling consisted in collecting water samples from the surface water column, to avoid the collection of sediments, in plastic bottles previously sterilized, with 2L of maximum volume. Besides the water samples collection, it was also performed a drag, using a plankton net with a 55-µm-mesh, to collect the phytoplankton present in the water systems to posteriorly be isolated at the laboratory.

It was also measured, with a pH meter (WTW MultilineP3 – WTW, Germany) the pH of the water systems sampled. However, the water temperature measurement was not possible due to a technical problem. It was necessary to request the atmospheric temperature registered in those sampling sites to IPMA (Instituto Português do Mar e da Atmosfera).

All the samples were maintained in refrigerated conditions until its processing at the laboratory which was performed within the 24 hours after the collection of samples.

2.1.3 Sample Treatment

In the laboratory, the water samples were filtered with a vacuum filtration system using a Glass-Microfiber filters (grade MG C, 47 mm diameter, 1.2 µm porosity) (Munktell®, Sweden). The obtained biomass was maintained at -20ºC until further DNA extraction.

2.2 Isolation and Culturing

In the laboratory, the phytoplankton samples collected from each sampling site were visualized with an optic microscope (Leica DMLB) and isolated, using the micromanipulation technique, with a Pasteur pipette. It was isolated either colonial or filamentous cyanobacteria. To guarantee that it was only a single cyanobacterium species that was being isolated it was performed washes in distilled water until obtain the isolated organism and transferred it into culture tubes, supplemented with 5 mL of Z8 culture medium (Kotai, 1972) to promote the cyanobacteria growth. The isolated microorganisms were maintained in a culture room under 25ºC temperature with a photoperiod of 14h:10h light-dark and 25 μEm^2s of light intensity without aeration.

After the incubation period, the cultures were visualized with an optic microscope (Olympus BX 41) and from the cultures, which did not present any contaminating organisms, it was transferred around 1 mL of culture to 50 mL culture flasks with filter caps (Orange Scientific, EU) supplemented with 25 mL of Z8 medium, in order to maintain the aseptic conditions of the cultures, this procedure was performed under a flame inside a laminar flow chamber (Cruma, Spain). The cultures were afterwards maintained in the same growth conditions as mentioned above. When these cultures reached its exponential phase of growth, they were visualized at the Olympus BX41 optic microscope coupled with a Olympus DP72 photograph machine and using Olympus Cell^B software for image acquisition of the isolated cyanobacteria cultures. It was also collected 1 mL of the culture for DNA extraction to confirm through PCR the organism present in the culture.

2.3 DNA Extraction

DNA was extracted from the environmental samples and from the isolated microorganisms. In the case of the isolated cyanobacterial cultures when the sample was collected from the culture flask, it was centrifuged for 5 minutes at room temperature at a maximum velocity of 17000*g* (VWR MicroStar 17R, UK). The supernatant was discarded and the pellet was maintained at -20ºC for 48 hours to enhance the cells to break. To extract genomic DNA from the environmental samples it was necessary, to scrape with a sterile blade, the biomass present in the filters, that

resulted from the water filtration. Genomic DNA, from the environmental samples and from the isolated cyanobacterial cultures, was extracted using the PureLink™ Genomic DNA Kit (Invitrogen, Carlsbad, CA, USA), following the protocol for Gram-negative bacteria. DNA was eluted in 50 µL of Elution Buffer.

To confirm the presence of the total genomic DNA, it was performed a 1% (w/v) agarose (UltraPure™ Agarose, Invitrogen Life Technologies, UK) gel electrophoresis (see section 2.4). The DNA was stored at -20ºC until further molecular analysis.

2.4 Agarose gel electrophoresis

The presence of total genomic DNA and amplified DNA was confirmed through a 1% (w/v) agarose (UltraPure™ Agarose, Invitrogen Life Technologies, UK) gel electrophoresis, stained with 3 µL of ethidium bromide (10 mg/mL, Biorad, USA) in 1X TAE buffer (0.4M Tris-acetate, 0.01M EDTA, pH 8.3 ± 0.1, Invitrogen™, UK), at 100 volts for 30 minutes. In each gel was loaded genomic and amplified DNA with 1 µL of Nucleic Acid Loading Buffer (5x, BioRad – 50 mM Tris-HCl, pH 8, 25% Glicerol, 5mM EDTA, 0.2% Bromophenol Blue and 0.2% Xylene FF) along with 1Kb plus DNA ladder at 1 µg/µL concentration (10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 50 mM NaCl, Invitrogen™, UK). The gel was photographed, under a UV transilluminator (Cleaver Scientific, Warwickshire, UK), coupled with a Cannon PowerShot G9 photograph machine.

2.5 DNA Quantification

The quantification of total genomic DNA was performed using the Gen5™ Version 2.0 Data Analysis Software using BioTek's take 3 microplate (Synergy HT, BioTek Instruments, Winooski VT, USA).

2.6 Molecular Methods

2.6.1 Primers development

As mentioned in section 1.6, one of the main aims of this study was to design specific primers, for *Aphanizomenon* genus, to be applied by conventional PCR. To achieve this aim, it was performed a search in the NCBI database for nucleotide sequences belonging to *rpoC*1 gene sequences of this genus. The sequences were imported to the Molecular Evolutionary Genetics Analysis 5 (MEGA 5) software (Tamura et al. 2011) and aligned using the ClustalW algorithm**.** It was performed a BLAST of the alignment consensus sequences comprehending 20-25bp to confirm the primer specificity.

2.6.2 Polymerase Chain Reaction

In all of the PCR reactions, it was applied the GoTaq® protocol from Promega (USA). Each PCR mixture was performed in a volume of 20 µL containing 5-10 ng of genomic DNA, 1 X PCR Green GoTaq® Flexi Buffer, 2.5 mM $MgCl_2$, 250 µM of each deoxynucleotide triphosphate (dNTP's), 10 pmol of each primer, 10 mg/mL (w/v) of Bovine Serum Albumin (BSA) and 0.5 U of GoTaq® DNA polymerase (Promega, USA). BSA was used because it is known that the majority of the PCR inhibitors present in the environmental samples are capable to bind to this reagent (Kreader, 1996), it is also reported that BSA is able to stabilize the DNA polymerase used in PCR (Farell and Alexandre, 2012).

Initially, it was performed a PCR in order to detect the presence of cyanobacteria in all the DNA samples, using the 27F and 809R and 740F and 1494R primer pairs for the amplification of the 16S rRNA cyanobacteria sequence. For the amplification of the *rpoC*1 gene sequence from *C.raciborskii*, *gyrB* gene sequence from *M.aeruginosa* and *rpoC*1 gene sequence from *P.agardhii,* the primers applied were cyl 2 and cyl 4, gyrF and gyrR, rpoC1_Plank_F271 and rpoC1_P_agardhii_R472, respectively. The sets of primers, used in this study, were already described in previous studies and they were designed to amplify specific sequences of the species in study (Table 2). LEGE Culture Collection strains were used as positive control in the PCR reactions, IZ41 (*Microcystis aeruginosa* LEGE 91351) and AQS (*Cylindrospermopsis raciborskii* LEGE 97047) for *M.aeruginosa* and *C.raciborskii* specific PCR, respectively. *P.agardhii* strain was not available, in the laboratory, to be applied as positive control in specific PCR for this species.

The PCR conditions are listed in table 3 and all the reactions were carried out in a TProfessional Thermocycler (Biometra, Germany) or in a Bio-Rad MyCycler™ (Bio-Rad, Hercules, CA, USA). All PCR products were visualized in a 1% (w/v) agarose (UltraPure™ Agarose, Invitrogen Life Technologies, UK) gel electrophoresis (see section 2.4).

For DNA sequencing, it was performed a PCR in a volume of 40 µL and instead of using 1 X PCR Green GoTaq® Flexi Buffer (Promega, USA) it was used 1 X PCR Colourless GoTaq® Flexi Buffer (Promega, USA). The amplified products were purified using the Cut & Spin Gel Extraction Kit (GRiSP, Portugal) in which the DNA band, corresponding to the expected DNA product, was excised from the agarose gel, and it was placed on the top of the column media to be centrifuged at 6200 g for 10 minutes at room temperature in a VWR MicroStar 17R centrifuge. The column was discarded

and the samples were maintained at refrigerated conditions (-20ºC) until be sent for direct sequencing.

The sequencing results were analysed using FinchTV 1.4.0 (Geospiza, Inc.; Seattle, WA, USA; http://www.geospiza.com) and Multalin (Florence 1988). To confirm the amplicons it was performed a BLAST search (Altschup et al. 1990).

Table 2 - Primers applied in conventional PCR and their characteristics.

Target Gene	Primer	Sequence (5'-3')	Product Length (bp)	Reference
C.raciborskii rpoC1	Cyl2	GGCATTCCTAGTTATATTGCCATACTA	247	(Wilson et al. 2000)
	Cyl4	GCCCGTTTTTGTCCCTTTCGTGC		
M.aeruginosa gyrB	gyrF	GGACGTTTACGAGAACTAGCCTA	416	(Tanabe et al. 2007)
	gyrR	GGTCTTGGTTTGTCCCTCAA		
P.agardhii rpoC1	rpoC1_Plank_F271	TGTTAAATCCAGGTAACTATGACGGCCTA	201	(Churro et al. 2012)
	rpoC1_P_agardhii_R472	GCGTTTTTGTCCCTTAGCAACGG		
Cyanobacterial 16S rRNA	27F	AGAGTTTGATCCTGGCTCAG	780	(Jungblut et al. 2005; Neilan et al. 1997)
	809R	GCTTCGGCACGGCTCGGGTCGATA		
	740F	GGCYRWAWCTGACACTSAGGGA	754	(Neilan et al. 1997)
	1494R	TACGGTTACCTTGTTACGAC		

Table 3 - PCR programs applied to detect the species in study

Target Gene	Initial Denaturation		Denaturation		Annealing		Extension		Final Extension	
	Temperature	Time	Temperature	Time	Temperature	Time	Temperature	Time	Temperature	Time
C.raciborskii rpoC1	95 ºC	2 min	95 ºC	90 sec	45 ºC	30 sec	72 ºC	50 sec	72 ºC	7 min
			35 cycles							
M.aeruginosa gyrB	94 ºC	3 min	94 ºC	1 min	60 ºC	1 min	72 ºC	30 sec	72 ºC	5 min
			40 cycles							
P.agardhii rpoC1	94 ºC	3 min	94 ºC	20 sec	58 ºC	20 sec	72 ºC	20 sec	72 ºC	5 min
			40 cycles							
Cyanobacterial 16S rRNA	92 ºC	2 min	92 ºC	20 sec	50 ºC	30 sec	72 ºC	1 min	72 ºC	5 min
			35 cycles							

2.6.3 Real-Time Polymerase Chain Reaction

One of the main objectives of this work was to develop a Real-Time Polymerase Chain Reaction specific for *M.aeruginosa*, to be possible its future absolute quantification mainly in water systems. To achieve this goal, it was necessary to perform a specificity assay and a sensitivity assay.

2.6.3.1 Specificity assay

To perform this assay it was applied twelve *M.aeruginosa* strains representing different geographic origins (Table 4) in order to test the primers specificity as well as the Real-Time PCR protocol developed. In this experiment, the primers applied were the same primers applied in conventional PCR, gyrF and gyrR, at 10 *p*mol, 1x iTAQ™ Universal SYBR Green SuperMix (Bio-Rad, Hercules, CA, USA), 2 µL of DNA to perform the final volume of 10 µL. The Real-Time PCR protocol tested consisted in an initial denaturation of 10 minutes at 95ºC, followed by 35 cycles of 15 seconds at 94ºC, 15 seconds at 60ºC and 50 seconds at 72ºC. It was also tested a Real-Time PCR protocol which consisted in an initial denaturation of 10 minutes at 95ºC, followed by 35 cycles of 15 seconds at 94ºC, 20 seconds at 60ºC and 50 seconds at 72ºC. Data acquisition was monitored after each extension step with temperature increments of 0.5 ºC between 55ºC and 95ºC. Melting temperatures of the products were determined with iQ™5 Optical System Software Version 2.1 (Bio-Rad, USA).

Amplifications were performed in the iQ™5 Multicolor Real-Time PCR Detection Systems (Bio-Rad, USA) and the results analyzed with iQ™5 Optical System Software Version 2.1 (Bio-Rad, USA).

Table 4 - *Microcystis aeruginosa* strains applied in Real-Time PCR

Strain	Continent	Country
PCC 786	Europe	Netherlands
PCC 7005	America	USA
NIVA-CYA 31	America	Canada
Ed08	Africa	Uganda
Ma Viet	Asia	Viet Nam
MN	Africa	Tunisia
27M	America	Mexico
19M	America	Mexico
IZ56	Europe	Portugal
M6	Europe	Portugal
90M	America	Mexico
82M	America	Mexico

2.6.3.2 Sensitivity assay

To perform this assay, it was necessary to obtain standards with known concentrations in terms of gene copy numbers to determine the minimum number able to detect in a given reaction. In this way, it was performed an amplification of a *M.aeruginosa* strain (M6 strains; see table 4) through a conventional PCR with a final volume of 100 µL. The PCR product was sent to Dr. Catarina Churro from the Instituto Nacional de Saúde Dr. Ricardo Jorge (Lisbon, Portugal) for further development of the standards based in a protocol already described by Baxa et al., (2010).

Once the standard DNA arrived at the Laboratory, it was performed a tenfold serial dilutions of the standard in molecular grade water, ranging from $5.5x10^8$ copies/µL to $5.5x10^0$ copies/µL. Posteriorly, they were tested in Real-Time PCR, in triplicate, with the developed program to detect the sensitivity limit. The reagents and its concentrations applied in this reaction were mentioned in the section 2.6.3.1. Amplifications were performed in the iQ™5 Multicolor Real-Time PCR Detection Systems (Bio-Rad, USA) and the results analyzed with iQ™5 Optical System Software Version 2.1 (Bio-Rad, USA).

2.7 Statistical Analyses

Shapiro–Wilk normality test was used to test the normal distribution of maximum and minimum atmospheric temperature data and Levene's test was used for the analysis of the homogeneity of variance. Statistically significant differences of the maximum and minimum temperatures registered between North and Centre regions of Portugal were calculated using a Student's t-test with 95% confidence level. The statistical analyses of this work were performed with IBM Statistical Package for the Social Sciences (SPSS Inc.) software version 22 (IBM Corporation, Armonk, NY, USA).

3. Results

3.1 Physical and Chemical Parameters

At each sampling campaign the pH values of the freshwater systems analysed were recorded and the atmospheric temperature values were requested to IPMA. Both pH and temperature values registered at each sampling site in the two consecutive sampling years are represented in the figures 7 to 9 and in figures 10 and 11, respectively. IPMA provided the atmospheric minimum and maximum temperatures registered in the weather stations that were nearest to the sampling sites: Parque da Cidade do Porto (Porto/Pedras Rubras weather station), Torrão Reservoir and Tâmega River (Marco de Canaveses) (Luzim weather station), Mira Lake (Dunas de Mira weather station) and Vela Lake (Figueira da Foz weather station).

3.1.1 pH

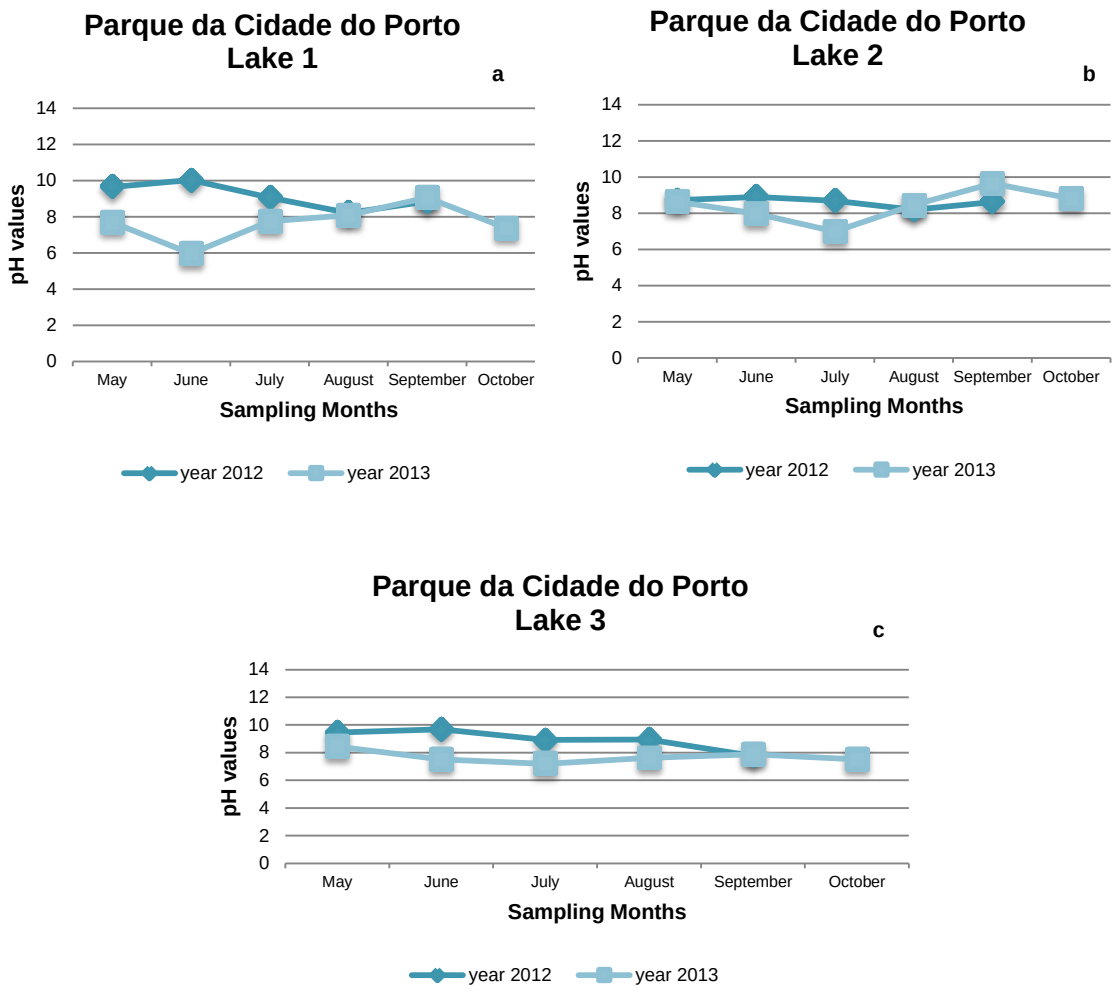

Figure 7 - pH values registered during the sampling dates in Lake 1 (a), Lake 2 (b) and Lake 3 (c) from Parque da Cidade do Porto in the two sampling years (2012 and 2013).

In the first sampling year, in the three sampled Lakes of Parque da Cidade do Porto, the pH values achieved the highest values in May and June, starting to decrease in the subsequent sampling months. In contrast, during 2013, the highest pH values were obtained in August and September, being June and July the months in which it was recorded the lowest pH values (Figure 7).

In 2012, it was reported, with exception of September, in Lake 2 of Parque da Cidade do Porto the lowest pH values and the highest values were recorded in Lake 1, with exception of August. On the other hand, in 2013, the highest pH values, with exception of July, were registered in Lake 2 and the lowest values were registered either in Lake 1 in May, June and October and in Lake 3 in July, August and September.

The three sampled Lakes registered higher pH values in 2012 than in 2013, with the exception in the month of September. In general, in 2012 the pH values were more alkaline than the pH values registered in 2013. Through the analysis of figure 7, it is possible to observe that between the two sampling years it was detected more similarity of the pH values between Lakes 2 and 3.

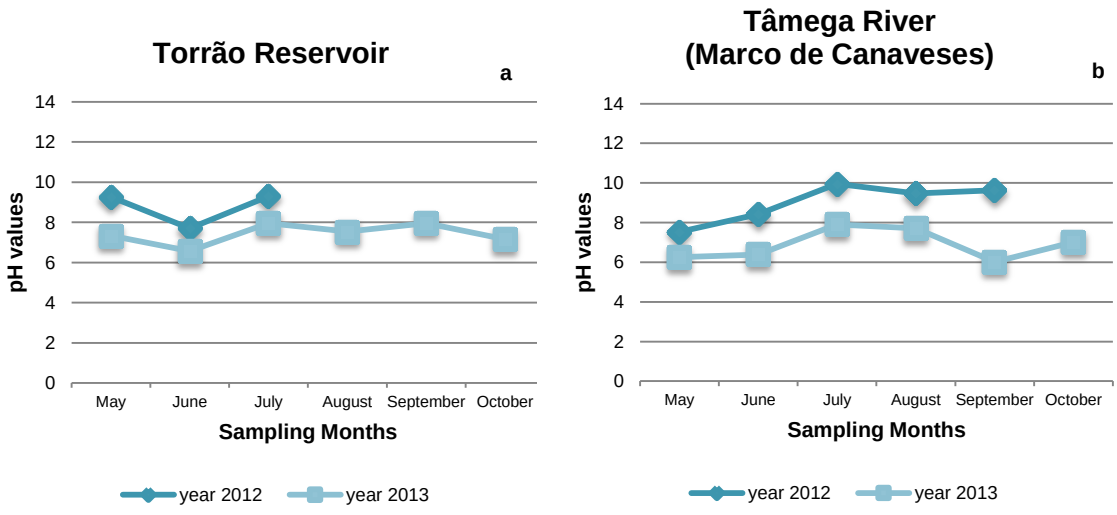

Figure 8 - pH values registered at the sampling dates in Torrão Reservoir (a) and Tâmega River (Marco de Canaveses) (b) in the two sampling years, 2012 and 2013.

It is possible to verify that in the Tâmega River (Marco de Canaveses), in 2012, the water was more alkaline than in the second year, the pH values were higher in 2012 than in 2013 (Figure 8b). In both sampling years, it was observed a similar variation of the pH values between the sampling months, with exception of September. In 2012 the lowest value was registered in May and the highest in July. In 2013, July had the highest value and September the lowest value recorded.

It is only possible to compare the pH values between the sampling years from May to July, in Torrão Reservoir (Figure 8a). However, the same record is observed at this sampling station compared to Tâmega River (Marco de Canaveses), it was in 2012 that the highest values were recorded and it was July that registered the most alkaline value. In 2013, July was also the point with the highest pH value. In both years, between the sampling months the pH showed a similar variation. In general pH values were more alkaline in 2012 than in 2013.

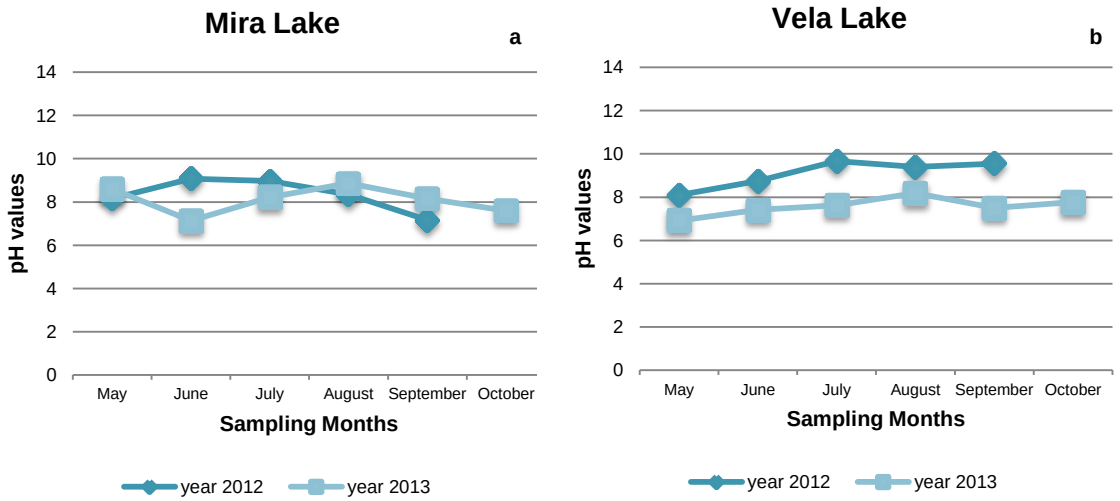

Figure 9 - pH values registered at the sampling dates in Mira Lake (a) and Vela Lake (b) in the two sampling years, 2012 and 2013.

In the two sampling sites, located at the Centre of Portugal, it was observed in both sampling years an alkaline state of the water (Figure 9). In Mira Lake it was registered the lowest pH value in June and September, in 2013 and 2012, respectively. The highest pH values were recorded in August and June, in 2013 and 2012, respectively. In Vela Lake the pH values were always higher in 2012 than in 2013. On the other hand, in Mira Lake the pH values were more similar between the sampled years and between the sampling months. In Vela Lake, the lowest pH values were obtained in May in the two years of sampling, the highest values were registered in August and July, in 2013 and 2012, respectively. The sampling month with the highest value in 2012 corresponds to the sampling month that registered the lowest pH value in 2013.

3.1.2 Atmospheric Temperature

As previously mentioned, the atmospheric temperature data was requested to IPMA. An independent Student's t test was performed to verify if significant differences occurred in the values of the registered atmospheric temperatures in the North and Centre Regions.

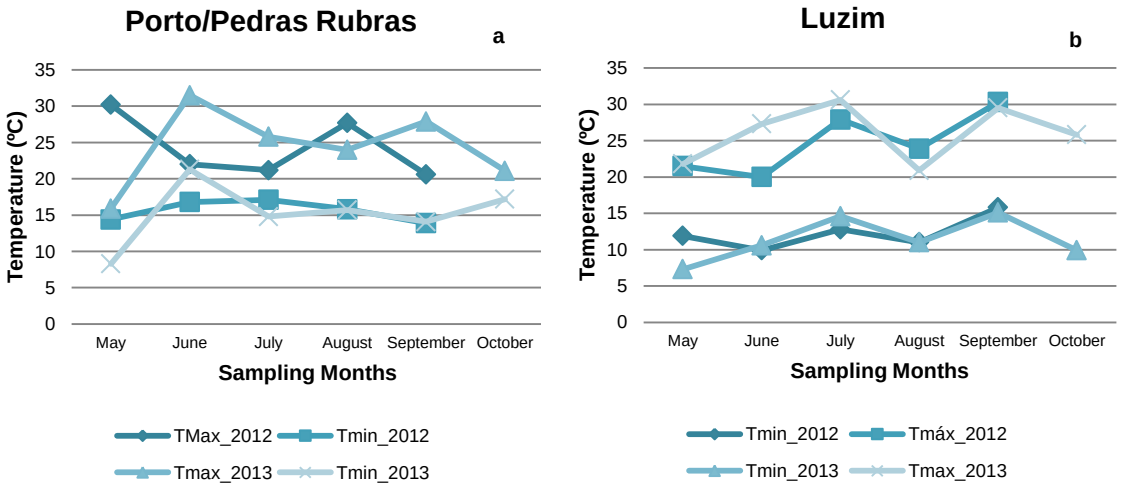

Figure 10 – Maximum and minimum temperature registered in the sampling dates in Porto/Pedras Rubras weather station (a) and Luzim weather station (b) (North regions) in the two sampling years, 2012 and 2013.

Relatively to the North sampling regions, the minimum temperature data registered either between the two sampling years and between the two North weather stations (Porto/Pedras Rubras and Luzim) was very similar. Comparing both sampling years, the maximum temperature registered in 2013 at Luzim weather station (Figure 10b) was higher in June and July than in 2012, in May and September the temperature was similar in the two sampling years and in August and September it was registered in 2012 higher temperature values than in 2013. In 2012 in Porto/Pedras Rubras Station (Figure 10a), the maximum temperature was higher in May and August than the temperature values registered in 2013. Statistically there were no differences between the maximum temperatures (p=0.679) and the minimum temperatures (p=0.735) in 2012 and 2013 in both weather stations.

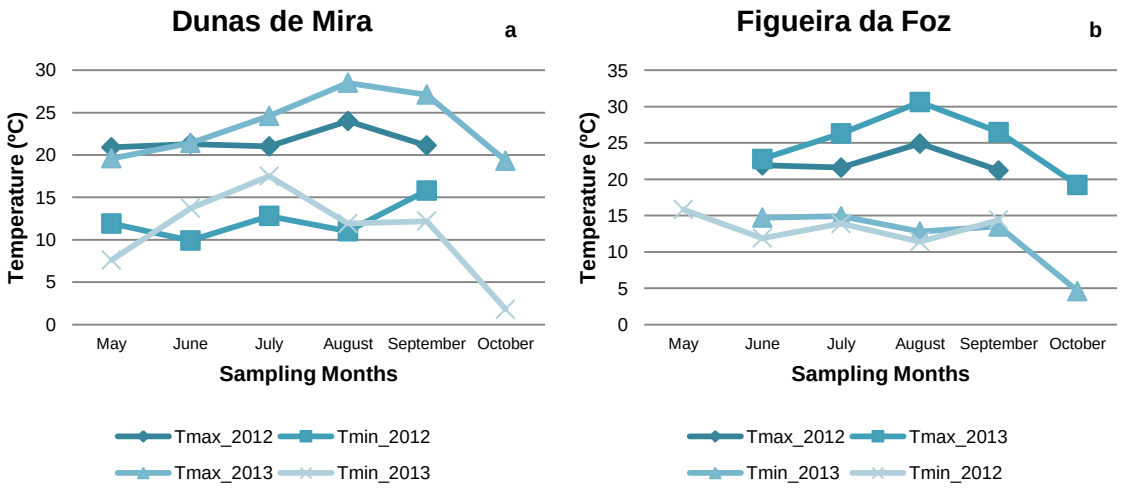

Figure 11 - Maximum and minimum temperature registered in the sampling dates in Dunas de Mira weather station (a) and Figueira da Foz weather station (b) (Centre regions) in the two sampling years, 2012 and 2013.

In the Centre regions, the temperature data was very similar, at both weather stations (Dunas de Mira and Figueira da Foz), in general the maximum temperature was higher in 2013 than in 2012. In the case of the minimum temperature, the data collected at Figueira da Foz station was more similar between 2012 and 2013 than the data collected at Dunas de Mira station whose minimum temperature was more similar between the sampling months in 2012 than the temperature registered in 2013 (Figure 11). Despite these variations, there were no statistical differences between the maximum (p=0.114) and the minimum (p=0.225) temperature values registered in 2012 and in 2013 in these two Centre weather stations.

Between the North and Centre regions, it was also verified if there were statistical differences in the temperature registered in the two sampling years. However it was not detected significant differences in 2012 between the maximum (p=0.091) and minimum (p=0.516) registered temperatures and also in 2013 between the maximum (p=0.584) and the minimum temperatures (p=0.3).

3.2 Molecular Results

To detect the presence of *M.aeruginosa*, *P.agardhii* and *C.raciborskii* in the sampling sites in all the sampling periods, it was performed a specific PCR protocol for the environmental detection of each species. The PCR results obtained in the seven freshwater systems analysed are described in tables 5, 6 and 7.

3.2.1 Detection of *M.aeruginosa*

The PCR results obtained in the seven analysed freshwater systems relative to the detection of *M.aeruginosa* are described in the table 5.

Table 5 - Detection of *M.aeruginosa* in the analysed freshwater systems in the two monitoring programs. The mark (+) indicates the detection of *M.aeruginosa* and the mark (-) indicates the non-detection of this species, through PCR amplification. The diagonal line (/) means that the sampling was not performed in the marked site or month.

Local of Sampling	Parque da Cidade do Porto Lake 1		Parque da Cidade do Porto Lake 2		Parque da Cidade do Porto Lake 3		Tâmega River (Marco de Canaveses)		Torrão Reservoir		Mira Lake		Vela Lake	
Year / Month	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013
May	+	-	+	+	+	+	-	-	+	-	+	+	+	+
June	+	+	+	+	+	+	+	+	+	+	+	+	+	+
July	+	+	-	+	+	+	+	+	+	+	+	+	+	+
August	+	-	+	-	+	-	+	-	/	+	+	+	+	-
September	+	+	+	+	+	+	+	+	/	+	+	+	+	+
October	/	+	/	+	/	+	/	+	/	+	/	+	/	+

Through the analysis of table 5, it is possible to observe that in 2012, *M.aeruginosa* was not only detected in May at Tâmega River (Marco de Canaveses) and in July in Lake 2 of Parque da Cidade do Porto. However, in the second sampling year, there was an increase in the sampling sites and months in which this species was not detected; the detection of this species was not possible in a total of eight of the analysed samples, in contrast with two obtained in 2012.

Tâmega River (Marco de Canaveses) was the unique sampling site whose detection of *M.aeruginosa* was not possible in the same sampling month (May) in the two sampling years. Otherwise, Mira Lake was the unique sampling site whose detection of *M.aeruginosa* species was always positive in both sampling years and at every sampling sites and months.

August was the month in which there was less detection of *M.aeruginosa,* on the other hand, *M.aeruginosa* was always detected in the two sampling years in June. In the month of October its detection was also possible in all the analysed samples however the sampling was only performed in the last sampling year. Overall in both 2012 and 2013, the presence of this cyanobacterium was more prevalent in the water systems from the Centre region of Portugal (Mira Lake and Vela Lake).

3.2.2 Detection of *C.raciborskii*

The PCR results obtained in the seven freshwater systems analysed relative to the detection of *C.raciborskii* are described in the table 6.

Table 6 - Detection of *C.raciborskii* in the freshwater systems analysed in the two monitoring programs. The mark (+) indicates the detection of *C.raciborskii* and the mark (-) indicates the non-detection of this species, through PCR amplification. The diagonal line (/) means that the sampling was not performed in the marked site or month

Local of sampling	Parque da Cidade do Porto Lake 1		Parque da Cidade do Porto Lake 2		Parque da Cidade do Porto Lake 3		Tâmega River (Marco de Canaveses)		Torrão Reservoir		Mira Lake		Vela Lake	
Month \ Year	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013
May	+	-	-	+	+	-	-	-	-	-	-	-	-	-
June	-	-	+	-	+	-	+	-	-	-	+	-	+	-
July	+	-	+	-	+	-	-	-	-	-	+	-	+	-
August	-	-	-	-	-	-	-	-	/	-	-	-	-	-
September	-	-	+	-	+	-	-	-	/	-	+	-	+	-
October	/	-	/	-	/	-	/	-	/	-	/	-	/	+

In 2012, the first year of sampling, this species was detected in 6 of the 7 analysed freshwater systems. In this year, *C.raciborskii* presence was not detected in Torrão

Reservoir sampling site and its presence was also less pronounced in Marco de Canaveses since its detection only occurred in one month (June).

On the other hand, it was in Lake 3 of Parque da Cidade do Porto that the presence of this species was mostly detected in 2012, in this sampling site *C.raciborskii* was only not detected in one sampling month, August. In 2012, the detection of *C.raciborskii* occurred mainly in June, July and September. In 2013, *C.raciborskii* presence was only detected in Lake 2 of Parque da Cidade do Porto in May and in Vela Lake in October.

It was not possible to detect the presence of *C.raciborskii* in Torrão Reservoir, in both sampling years. In general, it was possible the detection of *C.raciborskii* in the North and Centre regions of Portugal, mainly in the three analysed lakes of Parque da Cidade do Porto, Mira Lake and Vela Lake.

3.2.3 Detection of *P.agardhii*

The PCR results obtained in the seven freshwater systems analysed, relative to the detection of *P.agardhii,* are described in the table 7. Since for the *P.agardhii* species-specific PCR there was not a strain available, in our laboratory, to extract DNA to be used as positive control, one of the positive results was sent to direct sequencing to confirm the specificity of the reaction. Through a nucleotide blast search with the obtained sequence it was obtained a 100% of identity with sequences belonging to *rpoC*1 gene sequence of *P.agardhii*.

Table 7 - Detection of *P.agardhii* in the freshwater systems analysed in the two monitoring programs. The mark (+) indicates the detection of *P.agardhii* and the mark (-) indicates the non-detection of this species, through PCR amplification. The diagonal line (/) means that the sampling was not performed in the marked site or month.

Local of sampling	Parque da Cidade do Porto Lake 1		Parque da Cidade do Porto Lake 2		Parque da Cidade do Porto Lake 3		Tâmega River (Marco de Canaveses)		Torrão Reservoir		Mira Lake		Vela Lake	
Year / Month	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013
May	+	-	+	+	+	-	-	-	-	-	-	-	-	-
June	-	+	+	+	+	+	-	+	-	-	-	+	-	-
July	-	-	-	-	+	+	-	-	-	+	-	-	-	-
August	-	-	+	+	+	+	-	-	/	-	-	-	+	-
September	+	-	+	+	-	+	-	-	/	-	-	-	+	-
October	/	-	/	+	/	+	/	-	/	-	/	-	/	-

In the first year of sampling (2012), *P.agardhii* was not detected in Marco de Canaveses, Torrão Reservoir and Mira Lake being its presence mostly detected in the three sampled lakes of Parque da Cidade do Porto. In 2013, *P.agardhii* was only not detected in Vela Lake (Table 7). Analysing the sampling months, in 2012 the presence

of this species was mainly recorded in May, August and September. In 2013, *P.agardhii* detection occurred mainly in June.

Comparing both sampling years and relatively to the spatial distribution of the sampling sites, it is possible to observe that in 2012, *P.agardhii* was essentially detected in the North region of Portugal, particularly in the three sampled lakes of Parque da Cidade do Porto, however, its presence was also detected in the Centre region at Vela Lake. In 2013, this species was detected in every sampling site of the North region but in the Centre region its presence was only recorded in Mira Lake.

Through the analysis of table 7, in an overall analysis of the two sampling years, it is possible to verify that the presence of *P.agardhii* was mainly detected in Parque da Cidade do Porto, essentially in Lake 2 and Lake 3. Analysing all data, independently of the sampling years, May and October were the months whose presence of *P.agardhii* was less detected.

3.2.4 Global Analysis - *M.aeruginosa*, *C.raciborskii* and *P.agardhii*

Table 8 is a summary of the obtained results relative to the detection of the three cyanobacteria species in study.

Table 8 - Overall analysis of the detection of the three cyanobacteria species in the seven freshwater systems analysed. (+) represents the detection of the species in at least one sampling month and (-) represents the non-detection of the species in all the sampling months.

Local of sampling	Parque da Cidade do Porto Lake 1		Parque da Cidade do Porto Lake 2		Parque da Cidade do Porto Lake 3		Tâmega River (Marco de Canaveses)		Torrão Reservoir		Mira Lake		Vela Lake	
Year / Species	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013	2012	2013
M.aeruginosa	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C.raciborskii	+	-	+	+	+	-	+	-	-	-	+	-	+	+
P.agardhii	+	+	+	+	+	+	-	+	-	+	-	+	+	-

This table shows that Lake 2 of Parque da Cidade do Porto was the unique sampling site whose detection of *M.aeruginosa*, *C.raciborskii* and *P.agardhii* species in the two sampling years was always possible. In the other two lakes of Parque da Cidade do Porto the presence of the three species, particularly *C.raciborskii*, was not detected in the two sampling years. *M.aeruginosa* was the only species of the three in this study to be present in all the sampling sites at both sampling years. *P.agardhii* was detected in all the sampling sites even if in 4 of them its presence had only been detected in one of the sampling years. *C.raciborskii* was not detected in Torrão Reservoir in the two sampling years.

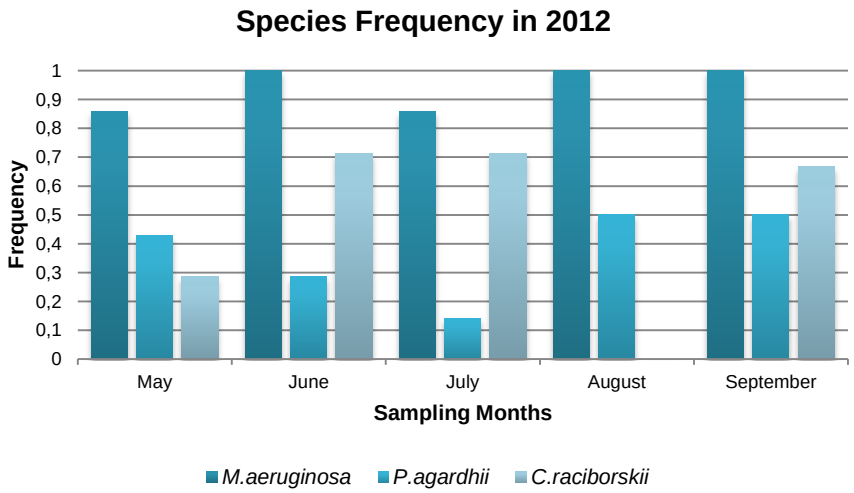

Figure 12 - Frequency of detection, per sampling month, of *M.aeruginosa*, *C.raciborskii* and *P.agardhii* in the first year of sampling (2012), in all of the sampling points, through PCR technique.

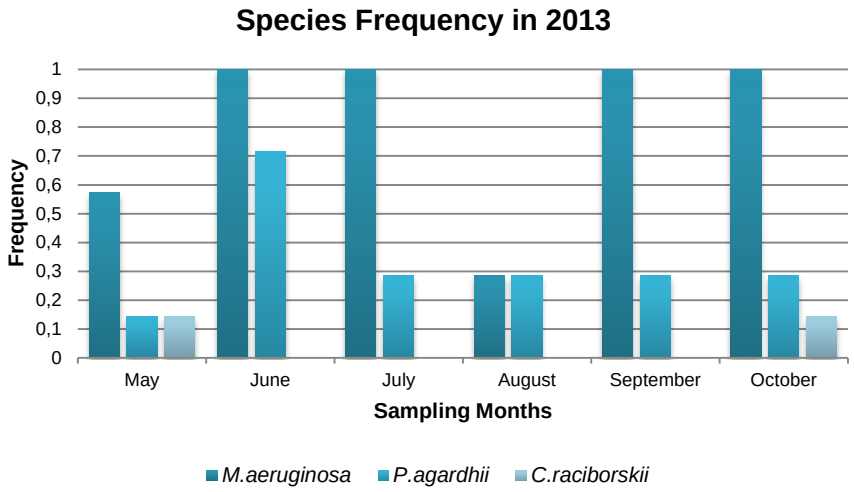

Figure 13 - Frequency of detection, per sampling month, of *M.aeruginosa*, *C.raciborskii* and *P.agardhii* in the second year of sampling (2013), in all of the sampling points, through PCR technique.

The figures 12 and 13 represent the seasonal variation of the cyanobacteria frequency in the two sampling years.

In 2012, of all the three cyanobacteria species studied, *M.aeruginosa* was the most dominant species. In that year, *C.raciborskii* was not detected in August otherwise June and July were the months which registered the highest frequency of detection of this species. In 2012, the frequency of detection of *P.agardhii* was decreasing from May to July, in August and September this species achieved the highest frequency levels of detection. In general, September of 2012 was the month which registered the highest frequency of the three species in study.

In 2013, *M.aeruginosa* was also the species most detected in all the sampling months. *C.raciborskii* was only detected in the first and in the last sampling month. June was the month in which *P.agardhii* had the highest frequency of detection, in the other sampling months its detection remained constant.

3.3 Microscopy Results

Through micromanipulation technique and cellular cultures it was possible to isolate two of the cyanobacteria species in study, *M.aeruginosa* and *C.raciborskii*. The isolated *M.aeruginosa* (Figure 14) is an isolated microorganism from Tâmega River (Marco de Canaveses) and the *C.raciborskii* isolate belongs to Lake 2 of Parque da Cidade do Porto (Figure 15). Besides the observation of the morphological characteristics under the microscope and the consultation of cyanobacteria flora to confirm the isolated species, its identification was also confirmed through specific PCR amplification (Figure 16 and 17).

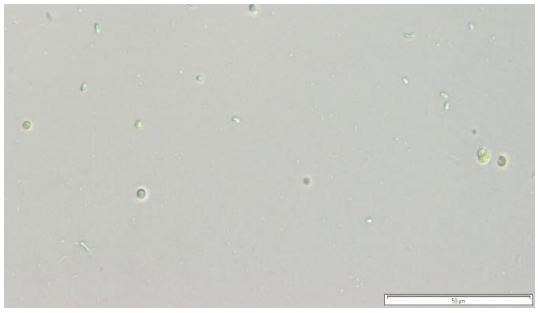

Figure 14 - *M. aeruginosa*, isolated from Tâmega River (Marco de Canaveses).

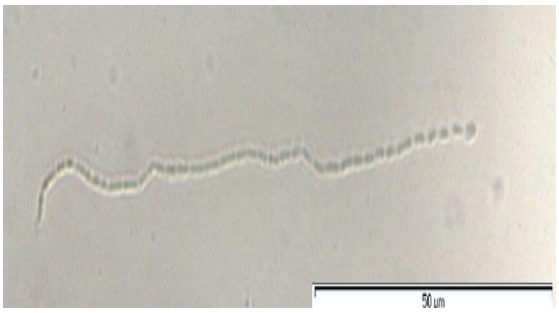

Figure 15 - *C.raciborskii* isolated from Lake 2 of Parque da Cidade do Porto.

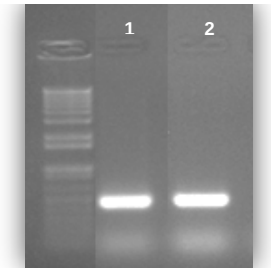

Figure 17 - Result of the PCR performed to confirm the identification of the *M.aeruginosa* isolated from Tâmega River (Marco de Canaveses) (1); positive control (2).

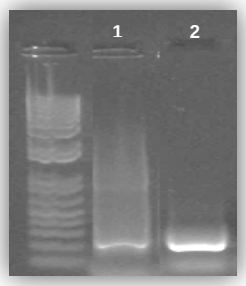

Figure 16 - Result of the PCR performed to confirm the identification of the *C.raciborskii* isolated from Lake 2 of Parque da Cidade do Porto (1); positive control (2).

3.4 Real-Time PCR

It was performed a specificity and a sensitivity assay to test a Real-Time PCR program developed in this work. Since the primers used (previously described) are specific for the DNA gyrase subunit β (*gyrB*) gene sequence which is a single-copy gene, applying this protocol in water systems allows to correlate the gene copy numbers with cell numbers present in the analysed samples.

3.4.1 Specificity assay

The specificity of the developed Real-Time PCR protocol was tested through the amplification of 12 *M.aeruginosa* strains with different geographical origins. The objective was to verify if in all the samples occurred amplification and to identify the melting temperature of the amplicon. The determination of the melting temperature specific of *M.aeruginosa* strains allows the discrimination of this cyanobacterium in a sample with a mixture of microorganisms.

The optimum Real-Time PCR protocol tested consisted in an initial denaturation of 10 minutes at 95ºC, followed by 35 cycles of 15 seconds at 94ºC, 20 seconds at 60ºC and 50 seconds at 72ºC. Data acquisition was monitored after each extension step with temperature increments of 0.5 ºC between 55ºC and 95ºC. Melting temperatures of the products were determined with iQ^{TM}5 Optical System Software Version 2.1 (Bio-Rad, USA). The figure 18 shows the melt curve chart obtained in the assay to test the specificity of the developed program. It is possible to observe that the melting temperatures are similar to all the strains tested and the melting peak occurs between 80ºC ± 0.5.

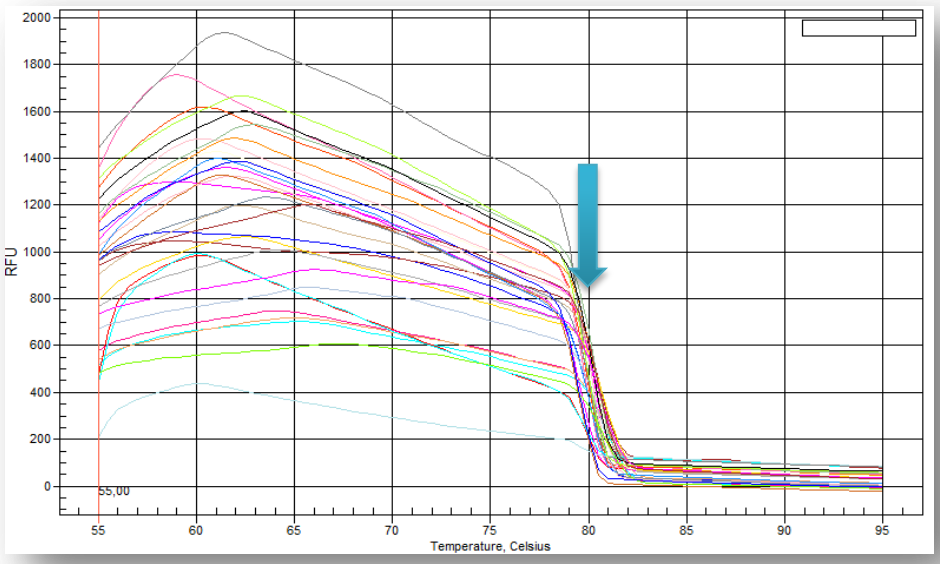

Figure 18 - Melt curve chart of the *M.aeruginosa* strains applied in the Real-Time PCR specificity assay.

3.4.2 Sensitivity assay

To execute the sensitivity assay, it was performed a tenfold serial dilution of the *M.aeruginosa* standards which were also applied in the Real-Time PCR following the developed program in order to verify which was the minimum limit of copy number able

to be detected with this protocol. It was verified that the minimum limit of copy numbers able to be detected in this protocol is $5.5x10^1$ copies/µL.

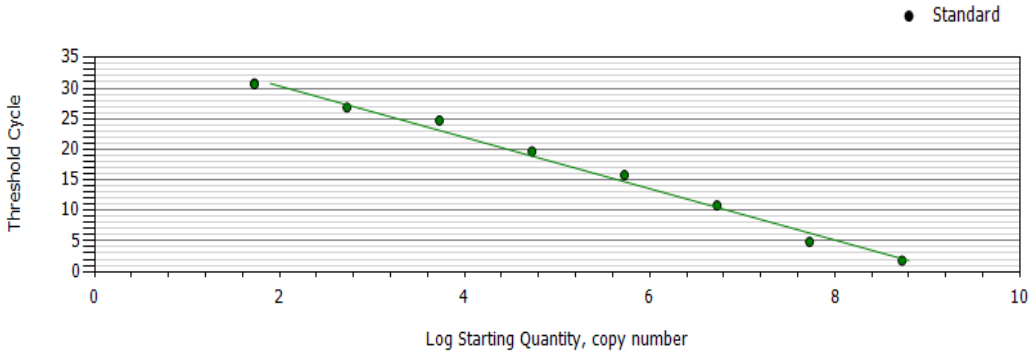

Figure 19 - Standard curve obtained with the application of the tenfold serial dilution of *M.aeruginosa* standards ranging from $5.5x10^8$ copies/µL to $5.5x10^1$ copies/µL.

With the standard constructed curve equation obtained (Figure 19) from the tenfold serial dilution of the *M.aeruginosa* standards it was obtained a correlation (R^2) of 0.991 through its analysis it is also possible to verify that the detection limit was $5.5x10^1$ copies per µL of PCR reaction, the standard amplification of $5.5x10^0$ copies/µL of PCR reaction was not possible.

3.5 Primers Development

In order to develop molecular tools able to detect in a sample specifically the *Aphanizomenon* genus, specific primers were designed to be later applied in a specific PCR detection of this genus. The developed primers for *Aphanizomenon* genus and their characteristics are present in table 9. To test the specificity of the developed tools a Blast search was performed, to verify if the primers corresponded only to *Aphanizomenon* sequences or if other sequences from other microorganisms had similarities with the chosen primers. With this search it was possible to verify that the primers sequence had high similarities with the *rpoC1* nucleotide sequences only from *Aphanizomenon* genus, presenting 100% of identity and query coverage to *rpoC1* sequences belonging to *Aphanizomenon* genus. A maximum score of 48.1 and 44.4 were obtained for primer forward and reverse, respectively.

Table 9 – Developed Primers for *Aphanizomenon* genus and their characteristics.

Primer	Sequence (5'-3')	Gene	Product (bp)	Melting Temperature
AphrpoC1Fw	GCGTACCTTGCCTAATGGACAACT	*rpoC1*	379	57.4 ºC
AphrpoC1Rev	GTTTTAGCGCCTGGTAACGCGG			58.6 ºC

4. Discussion

The values of the physical and chemical parameters registered in this study revealed to be in accordance with the values that previous studies suggested to be the most favourable to cyanobacterial growth with the temperature between 15ºC and 30ºC and pH values between 6 and 9 (Vale, 2005). Though the temperature presented here is the atmospheric temperature, it is well-known that this brings direct influence in the water temperature. In 2012, it was observed more bloom occurrences at the sampling sites studied than in 2013 (*in situ* visualization). It is suggested that higher values of pH are common during periods of cyanobacteria dominance, mainly during blooms (Martins 2007), these facts are in accordance since in 2012 the pH values were more alkaline than the pH values registered in 2013. This parameter is referred by some authors as the only limiting parameter which restricts the distribution of cyanobacteria, since they tend to prefer neutral or basic conditions (Sze, 1986).

M.aeruginosa is currently established to be a cyanobacterium species with a cosmopolitan distribution (van Gremberghe et al., 2011). Recent studies suggest that the origin of this species may have occurred in Africa and posteriorly colonized the European continent where its dispersion to the remaining continents may have occurred (Moreira et al., 2014). In this study, its presence was detected in all of the sampling sites and months analysed in the two sampling years, it was also possible the isolation of this microorganism through microscopy and isolation techniques. The results obtained with this study are in agreement with the previous designation of the cosmopolitan distribution of this species both at a national and worldwide scale (van Gremberghe et al., 2011).

The easy and wide dispersion of *M.aeruginosa* might be explained by its cellular characteristics as it possesses thick cell walls, intracellular gas vesicles for buoyancy and the capacity to form aggregates or colonies (Tanabe et al., 2007; van Gremberghe et al., 2011). These aggregates are surrounded by a mucilage layer which confers protection to this cyanobacterium species and consequently facilitates its dispersion as well as the capacity to resist drought or intestinal conditions, tolerance to low temperatures and darkness (Wu et al., 2008). Gas vesicles confer to cyanobacteria the ability to accumulate on the water surface (Dittmann and Wiegand, 2006), therefore through winds and migratory birds, that are associated with these water systems, the dispersion of cyanobacteria may occur. The high potential for the dispersion, maintenance and dominance of *M.aeruginosa* in the water systems may also be explained by the occurrence of several *M.aeruginosa* genotypes in a single water body as reported by Bittencourt-Oliveira et al. (2001).

However, *M.aeruginosa* was not detected in ten samples, mainly in the second sampling year. The only case where this cyanobacterium was not detected in the same sampling site across the two sampling years was in May at Tâmega River (Marco de Canaveses). The lowest pH values registered in 2013 may suggest a decrease in the concentration of the cyanobacteria in these water systems so the non-detection of *M.aeruginosa* may be related with a decrease in its biomass concentration which made the detection of its presence through simple PCR amplification impossible due to the limited detection limit that this technique carries. In 2012, it was observed that the occurrence of blooms was more frequent than in 2013, this fact may contribute to the possible decrease in the cell number concentration of the sampled freshwater systems since *M.aeruginosa* is one of the most predominant bloom-forming species, already described by the bloom occurrence in Vela Lake in 2001 (de Figueiredo et al., 2006). The presence of this species has been already reported in these sampling sites by previous studies, in the three sampled lakes of Parque da Cidade do Porto (Morais, 2009), Torrão Reservoir and Tâmega River (Marco de Canaveses) (Regueiras, 2009), Vela and Mira Lake (de Figueiredo et al., 2006; Freitas, 2009).

Detection of *C.raciborskii* was not as frequent as the detection of *M.aeruginosa* in the freshwater systems analysed. *C.raciborskii*, known by its invasive behaviour, had only been detected in the Centre and South regions of Portugal (Freitas, 2009; Saker et al., 2003). Previously to this study, *C.raciborskii* had already been detected in one of the sampling sites of this study, Vela Lake (Centre region) (Freitas, 2009). In this study, its presence besides being detected in the Centre region of Portugal (Mira and Vela Lake), it was also detected in the North region (in the three sampled lakes of Parque da Cidade do Porto and Tâmega River (Marco de Canaveses)) being this study the first report of *C.raciborskii* in two separate North regions of Portugal. It was also possible through isolation and microscopy techniques to obtain a *C.raciborskii* isolate, belonging to one of the sampling sites of the North region of Portugal (Lake 2 of Parque da Cidade do Porto). The detection of the presence of *C.raciborskii* in the North regions of Portugal was possible through both molecular and microscopy techniques.

In 2013, the detection of this species decreased drastically in comparison to 2012. However, the non-detection of *C.raciborskii* does not mean its absence, it can be related with the detection limit of the PCR technique, occurrence of blooms of *Microcystis* spp. or other species which may have blocked the light penetration and competition for nutrients (Huisman et al., 2005). The atmospheric temperature registered was similar in the two sampling years. However, as previously mentioned, the pH was more alkaline in 2012 than in 2013 which may be related with the decrease in the detection of *C.raciborskii* in 2013 since cyanobacteria tend to prefer neutral or

basic conditions (Sze, 1986). Nevertheless, it is worthy to mention that other parameters, not measured in this study, such as nutrient concentration, oxygen availability, phosphorus and nitrogen concentration may have influenced the variation in the frequency of this cyanobacterium species.

The possible hypotheses that led to the dispersion and invasion of *C.raciborskii* to the North regions of Portugal may be associated with migratory birds, winds and even human activities as previously described (Kristiansen, 1996). Once the dispersion occurred, the physiological characteristics of this cyanobacterium species along with its migratory capacity in the water column, tolerance to variations in light availability and its ability to fix inorganic carbon at high pH values (Chellappa et al., 2008) may have contributed to its proliferation and establishment in the water systems. Other factors may have contributed to the expansion of *C.raciborskii* to the North regions of Portugal such as the increase of atmospheric temperature, that has been possible to observe in the last years, which is related with water temperature (Wiedner et al., 2007). However, while being described as a tropical species, *C.raciborskii* was already detected in a water system at 11ºC in Uruguay (Bonilla et al., 2012). Other hypotheses that may have contributed to its successful invasion of North regions of Portugal is its phenotypic plasticity (Bonilla et al., 2012; Briand et al., 2004) and existence of ecotypes conferring a wide intraspecific variability to *C.raciborskii* due to the different environmental preferences and tolerances (Chonudomkul et al., 2004; Piccini et al., 2011).

In accordance with what happened with *C.raciborskii*, the detection of *P.agardhii* was not as frequent as the detection of *M.aeruginosa* in the freshwater systems studied. However, its detection frequency did not showed a remarkable change between the two sampling sites as occurred with *C.raciborskii.* To date, the presence of *P.agardhii* had only been reported in the South region of Portugal (Churro et al., 2012). However, the presence of *Planktothrix* sp. was already detected in the lakes of Parque da Cidade do Porto (Morais, 2009). The detection of *P.agardhii,* in this study, was possible in both North and Centre regions of Portugal, mainly in Parque da Cidade do Porto, being this the first report of *P.agardhii* in North and Centre regions of Portugal. Unfortunately through several unsuccessful attempts it was not possible to obtain any isolate of *P.agardhii.*

The previously proposed dispersion vectors of *C.raciborskii* that led to its dispersion and invasion to the North regions of Portugal (migratory birds, winds and human activities) could have also contributed to the occurrence of *P.agardhii* in both the North and Centre regions of Portugal. This filamentous cyanobacterium species can grow in a wide range of temperatures from 10ºC to 31ºC (Bonilla et al., 2012), which favours its expansion, being also frequent a higher development of *P.agardhii* in

turbid waters (Kokociński et al., 2010). There are many factors responsible for the increase of water turbidity as the input of agriculture and sewage residue in the water systems as well as the occurrence of blooms (Chellappa et al., 2008), which may explain the higher detection frequency of *P.agardhii* in comparison with *C.raciborskii*. Occurrence of blooms dominated by *M.aeruginosa* could have led to an increase in the water turbidity favouring the growth of *P.agardhii* since *C.raciborskii* has preference for less turbid waters (Kokociński et al., 2010). Bonilla et al. (2012) reported that between these two filamentous cyanobacterium species, *P.agardhii* has a more limited plasticity.

There were no statistical significant differences in the average temperature values registered in the North and Centre sampling sites. The hypotheses here presented is that when the dispersion of this two species happened, the fact that the temperatures in North and Centre regions were very similar contributed to the growth, proliferation and maintenance of *C.raciborskii* and *P.agardhii* species in these freshwater systems. The alkaline pH values registered may also have contributed to the establishment of these species in the analysed freshwater systems. This parameter is referred by some authors as the only limiting parameter which restricts the distribution of cyanobacteria, since they tend to prefer neutral or basic conditions (Sze, 1986; Summerfield & Sherman, 2008).

With the increase of climate changes, pollution and changes in the geographic distribution of potential toxic cyanobacteria species it is expectable that the occurrence of blooms will become more frequent and in unpredictable sites, representing a huge concern in terms of public health and in the ecosystem stability. Therefore, it is demanding the development of molecular tools for the early warning of toxic cyanobacteria species. In the present study, it was developed a specific Real-Time PCR protocol for absolute quantification of *M.aeruginosa* species and specific primers to be applied in simple PCR amplification in the detection of species belonging to *Aphanizomenon* genus.

With the development of the Real-Time PCR protocol to quantify *M.aeruginosa* cells in the water systems it was possible to detect until approximately 55 gene copy numbers of *gyrB* per µL of PCR reaction. Since this gene is in a single-copy in the *M.aeruginosa* genome, the number of gene copies detected will correspond approximately to the cells number of *M.aeruginosa* present in a given water sample.

To date, it has been only developed and applied in the field, protocols for the quantification of *Microcystis* sp.. The developed protocols so far, for this genus, are based either in the amplification of microcystin synthetase A gene, having a detection limit of 8.8 cells per millilitre (Furukawa et al., 2006), or using the intergenic spacer region within the phycocyanin operon whose detection limit was less than five cells per

millilitre (Kurmayer & Kutzenberger, 2003). Other studies developed a protocol for the quantification of *Microcystis*-specific 16S rRNA fragment with a detection limit of 5 gene copies per millilitre of water (Rinta-Kanto et al., 2005). Since, *M.aeruginosa* is one of the most important bloom forming species it is very important its exact quantification instead trying to extrapolate it from the total quantification of *Microcystis*. Therefore, applying this protocol in regular monitoring programs it will be possible to assess the seasonal variation in terms of exact *M.aeruginosa* species numbers in a water system as well as predict the occurrence of a bloom and therefore increase the monitoring programs to detect and quantify the possible production of cyanotoxins.

The primers developed for *Aphanizomenon* genus represent the first step for obtaining a valuable tool for the detection of species belonging to *Aphanizomenon* genus. The results showed a high specificity for this genus, however, further work would be needed to develop a more specific PCR protocol.

Potentially toxic cyanobacteria are widespread in the Portuguese Freshwater Systems, as demonstrated in this study, the molecular tools here developed contribute to their early warning in these aquatic systems.

5. Conclusion and Future Perspectives

The molecular techniques used in this study allowed a more rapid and accurate analysis of freshwater systems for the presence of potentially toxic producing cyanobacteria species. The obtained results demonstrates the dominance and the wide distribution of *M.aeruginosa* in the North and Centre regions of Portugal in all the sampling period and emphasize once more the invasive behaviour of *C.raciborskii* and *P.agardhii* in the North and Centre Portuguese freshwater systems, which was unforeseen until now. These facts lead to the necessity of the establishment of monitoring programs of these water systems for the presence of these three well-known cyanobacteria species, as well as for the presence of the cyanotoxins that can be potentially produced by them. The development of a Real-Time PCR protocol to quantify *M.aeruginosa gyrB* gene sequences will allow future investigations to easily and rapidly quantify this potentially toxic cyanobacterium and predict the occurrence of blooms, avoiding or minimizing future public health problems that may result from the unexpected increase of this species in water systems.

In the future, it would be interesting to apply the Real-Time PCR protocol described in this study as well the Real-Time PCR protocols previously developed for the quantification of *M.aeruginosa*, *C.raciborskii* and *P.agardhii* in the environmental samples collected between 2012 and 2013 in both the North and Centre regions of Portugal. It would also be interesting to continue the specificity tests of the developed *Aphanizomenon* genus primers and develop a conventional PCR protocol and posteriorly apply it to the environmental samples. Finally, it would be very interesting to perform a phylogenetic analysis with the *C.raciborskii* isolated from Lake 2 of Parque da Cidade do Porto along with the strains from the Centre and South regions to determine the genetic diversity among the national isolates and investigate the possible introduction route of *C.raciborskii* detected in the North regions of Portugal.

Communication

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