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Molecular monitoring of cyanobacteria and their related toxins in Portuguese freshwater systems

Rita Mendes

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Rita Afonso de Moura Mendes

Molecular monitoring of cyanobacteria and their related toxins in Portuguese freshwater systems

Dissertação de Candidatura ao grau de Mestre em Toxicologia e Contaminação Ambientais submetida ao Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto

Orientador – Prof. Agostinho Antunes
Categoria – Professor Auxiliar
Afiliação – Faculdade de Ciências da Universidade do Porto

Co-orientador – Doutora Cristiana Moreira
Categoria – Postdoctoral Researcher
Afiliação – Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR/CIMAR)

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Abstract

In the recent years, the eutrophication processes that are registered in several water systems as led to changes in their physical, chemical and biological properties. In biological terms, the densification of the cyanobacterial community in the water surface has led to the growing production of secondary metabolites, many of them presenting a toxic profile for both the environment and humans. Within this group of secondary metabolites, cyanotoxins are the most frequently produced in the aquatic environment. Due to its high toxicity to the environment and to humans, it is necessary to have a constant monitoring for the presence of cyanobacteria and cyanotoxins, to insure their rapid detection. Therefore, the objective of this study is to use molecular methods as an initial methodology for the detection of cyanobacteria and for identifying the potential of the cyanobacterial community to produce cyanotoxins in freshwater systems located in the North and Center regions of Portugal.

To achieve these goals, environmental samples from seven freshwater points were collected between October and June and analyzed through the amplification of specific genes for the detection of cyanobacteria, as well as genes encoding the biosynthesis of the cyanotoxins in study. Application of molecular techniques allowed the detection of *Microcystis sp*., *C. raciborskii* and *P. agardhii*, as well as the potential for the production of microcystin, cylindrospermopsin, anatoxin-a and saxitoxin, throughout the sampling period and also throughout the several sampling points, located in both North and Center regions of Portugal.

The obtained results demonstrate an increasing need for the creation of more specific and complete monitoring programs for freshwater systems that encompasses the use of molecular methods as an initial screening method for the detection of cyanobacteria and related toxins. It was also possible to observe the high presence of these organisms and their toxic potential in a sampling period characterized by low temperatures and absence of optimal atmospheric conditions for the development of cyanobacteria, this way suggesting the need for extending the monitoring period, in order to include the period sampled in this study.

Keywords: Cyanobacteria, Cyanotoxins, Molecular Methods, Freshwater Systems, Monitoring

Resumo

Nos últimos anos, os fenómenos de eutrofização que se registam em diversos sistemas aquáticos têm levado à alteração das suas propriedades físicas, químicas e biológicas. Em termos biológicos, a densificação das comunidades de cianobactérias na superfície da água tem levado à crescente produção de metabolitos secundários, muitos deles apresentando um perfil tóxico para o ambiente e, em certos casos, para o ser humano. Dentro desse grupo de metabolitos secundários, as cianotoxinas são os mais frequentemente produzidos por cianobactérias quando presentes no meio aquático. Dada a sua elevada toxicidade no meio ambiente e para o ser humano, é necessário haver uma constante monitorização da presença de cianobactérias e cianotoxinas, focada na detecção rápida das mesmas. Assim, o objectivo deste estudo centra-se na utilização de métodos moleculares como ferramenta primária para a detecção de cianobactérias, e da potencialidade da comunidade de cianobactérias de produzir cianotoxinas em sistemas de água doce localizados nas regiões Norte e Centro de Portugal.

Para tal, amostras ambientais de sete sistemas aquáticos foram recolhidas entre Outubro e Junho e posteriormente analisadas com recurso à aplicação de genes específicos para a detecção de espécies de cianobactérias e genes conhecidos por se encontrarem envolvidos na biossíntese das cianotoxinas em estudo. A aplicação das técnicas moleculares permitiu a deteção de *Microcystis sp*, *C. raciborskii* e *P. agardhii*, bem como potencial para a produção de microcistina, cilindrospermopsina, anatoxina-a e saxitoxina, ao longo do período de amostragem, e ao longo dos diversos locais de amostragem, tanto do Norte como do Centro de Portugal.

Os resultados obtidos demonstram a necessidade de criação de planos de monitorização mais específicos e completos, que englobem a utilização de métodos moleculares como método primário para o estudo de cianobactérias e toxinas relacionadas. Foi também possível observar a elevada presença destes organismos e da sua potencialidade tóxica num período de amostragem característico por não possuir as condições atmosféricas propícias para o desenvolvimento óptimo destas espécies, o que sugere a necessidade da extensão do período de monitorização, de modo a abranger também este período amostrado.

Palavras-chave: Cianobactérias, Cianotoxinas, Métodos Moleculares, Sistemas Aquáticos, Monitorização

Table of Contents

List of Tables

List of Figures

List of Abbreviations

BMAA – β-methylamino-$_L$-alanine

BSA – Bovine Serum Albumin

DNA – Deoxyribonucleic Acid

dNTPs – Deoxynucleotide Triphosphates

dsDNA – Double stranded DNA

EDTA – Ethylenediaine Tetraacetic Acid

ELISA – Enzyme-Linked Immunosorbent Assay

*gyr*A – DNA gyrase subunit A

*gyr*B – DNA gyrase subunitB

HPLC – High Performance Liquid Chromatography

IL-1 – Interleukin-1

INAG – Instituto Nacional da Água

IPMA – Instituto Português do Mar e da Atmosfera

LEGE CC – Blue Biotechnology and Ecotoxicology Culture Collection

MS – Mass Spectrometry

NRPS – Nonribosomal Peptide Synthetase

PCR – Polymerase Chain Reaction

PKS – Polyketide Synthase

PSP – Paralytic Shellfish Poison

rRNA – Ribosomal Ribonucleic Acid

RT-PCR – Real-Time PCR

TNF-α – Tumor Necrosis Factor Alpha

WHO – World Health Organization

WWTP – Waste Water Treatment Plant

1. Introduction

In the recent years, the eutrophication of freshwater systems has been recognized as a worldwide problem in terms of environmental contamination (Zhang *et al*., 2009). Characterized by an increase of the nutrient input on water, like nitrogen and phosphorus, the eutrophication of the water systems leads to an intensification of the biomass of some aquatic communities, especially phytoplanktonic communities, at the water surface (Porteiro *et al*., 2005; Gonçalves *et al*., 2006; Nimptsch *et al*., 2008). The eutrophication of freshwater systems is a natural process that can occur in an aquatic system over millennia; however, anthropogenic pressures on the water system can accelerate this process (Porteiro *et al*., 2005). The exploitation of freshwater systems, for socio-economic purposes, creating an unnatural input of nutrient compounds in the water system, alongside with changes in abiotic factors, such as temperature and pH, can provide favorable conditions for the uncontrolled proliferation of cyanobacteria, a common element of phytoplankton communities (Bittencourt-Oliveira, 2003; de Hoyos *et al*., 2004; Quesada *et al*., 2004; Nimpsch *et al*., 2008; Papadimitiou *et al*., 2012).

1.1. Cyanobacteria

Cyanobacteria comprises a group of photosynthetic prokaryotic organisms, that inhabit a wide range of ecosystems, even in extreme conditions, such as tropical and Polar regions (de Hoyos *et al*., 2004; Ionescu *et al*., 2010; Kumar *et al*., 2011; Liu *et al*., 2014). In freshwater systems, the presence of cyanobacteria is detected mostly on aquatic systems classified as eutrophic or mesotrophic, in a huge variety of morphologies, from filamentous to unicellular (Ionescu *et al*., 2010; Liu *et al*., 2014). Cyanobacteria can be classified according their morphology in five distinct orders, according to the Botanical Code Criteria: Order Chroococcales, Order Pleurocapsales, Order Oscillatoriales, Order Nostocales and Order Stigonematales (Henson *et al*., 2002; Komárek, 2003). The presence of cyanobacteria in the water, and its worldwide distribution can be explained in part by its physiology. Some genera possesses gas vesicles, which allow them to aggregate and form blooms at the water surface, as well as regulate its buoyancy and its establishment in the water system, when growth conditions are favorable (WHO, 2003). Formation of cyanobacterial blooms at the water surface make cyanobacteria active competitors against the eukaryotic phytoplankton community for the nutrients present in the water and adsorption of

photosynthetic radiation along the water column (Neilan *et al*., 1999; de Hoyos *et al*., 2004; Quesada *et al*., 2004; Bittencourt-Oliveira *et al*., 2010). For this study, detection of *Microcystis aeruginosa*, *Cylindrospermopsis raciborskii* and *Planktothrix agardhii* will be performed through the use of molecular methods, given their importance in the study of cyanobacteria and potential for cyanotoxin production.

1.1.1. *Microcystis aeruginosa*

Widely present in freshwater systems, *Microcystis aeruginosa* (Kützing) Kützing 1846 is a unicellular cyanobacterium species belonging to the Order Chroococcales. This species exist as a single cell, when cultivated in laboratory however, when in an aquatic environment, *M. aeruginosa* usually presents itself as a colony or aggregate (Bolch and Blackburn, 1996). This tendency to aggregate is suggested to be a phenotypic characteristic of this species to face environmental changes that may interfere with its growing rate (Yang *et al*., 2006). Microcystin potentially-producing species, *M. aeruginosa* is usually the dominant species in a water system, being frequently responsible for the formation of blooms, due to its gas vesicles used for buoyancy in the water column (Saker *et al*., 2005). Molecular detection of *M. aeruginosa* is performed through the amplification of the DNA gyrase subunit B (*gyrB*) sequence specific for this species (Tanabe *et al*., 2007). DNA gyrase, composed by 2 subunits, *gyrA* (DNA gyrase subunit A) and *gyrB*, introduces negative supercoils into double stranded DNA (dsDNA) in an ATP-dependent reaction (Huang, 1996; Stanger *et al*., 2014).

1.1.2. *Cylindrospermopsis raciborskii*

Cylindrospermopsis raciborskii (Woloszynska) Seenayya and Subba Raju, 1972 is a nitrogen fixing filamentous species belonging to the Order Nostocales. Typical from tropical environments, has although a wide geographical distribution, given its invasive behavior (Lagos *et al*., 1999; Bonilla *et al*., 2012). Morphologically, *C. raciborskii* is a heterocystous cyanobacterium, possessing terminal heterocytes, at one or both ends of a trichome (Komárek, 2013). The presence of this cyanobacterium species in a freshwater system can affect the water quality, since its ability to produce both cylindrospermopsin and saxitoxin, which represents a serious threat to Public Health (Lagos *et al*., 1999). At a molecular level, detection of *C. raciborskii* is assessed through the amplification of the DNA-dependent RNA polymerase (*rpoC1*) gene.

Described by Wilson *et al*. (2000), in order to determine the level of diversity among Australian isolates of *C. raciborskii*, this gene encodes the γ subunit of RNA polymerase, being present in the genome as a single copy (Bergsland and Haselkorn, 1991).

1.1.3. *Planktothrix agardhii*

Belonging to Order Oscillatoriales, *Planktothrix agardhii* (Gomont) Anagnostidis and Komárek (1988) is a cyanobacterium species commonly found in hypertrophic temperate freshwater systems (Briand *et al*., 2008). Filamentous cyanobacterium, *P. agardhii* occurs as single trichomes and is characterized by its resilience and shade tolerance, being one of the most common species to form blooms in temperate aquatic systems (Briand *et al.*, 2008; Bonilla *et al*., 2012). *P. agardhii* is an invasive species in freshwater systems and it is capable of synthesizing both microcystins and saxitoxins (Bonilla *et al*., 2012; Pomati *et al*., 2000). Such as *C. raciborskii*, *P. agardhii* is detected, at a molecular level, by the amplification of the rpoC1 gene, through the use of specific primers designed by Churro and collaborators (2012) to be applied, in a first instance, in quantification by Real-Time PCR.

1.2. Cyanotoxins

Proliferation of some genera of cyanobacteria in water systems can lead to the production of secondary metabolites, called cyanotoxins, which can contribute to the degradation of the water quality, and also pose severe threats to humans, animals and environment (Quesada *et al*., 2004). Presenting a wide range of chemical structures and toxicity levels (Kumar *et al*., 2011) cyanotoxins are usually classified according to their mode of action in the target organism. This way, it is possible to classify cyanotoxins in hepatotoxins (microcysins and nodularins), cytotoxins (cylindrospermopsin), neurotoxins (anatoxin-a, anatoxin-a(S), saxitoxin and β-methylamino-$_L$-alanine (BMAA)) and dermatotoxins (lyngbyatoxin and aplysiatoxin) (Bittencourt-Oliveira, 2003; Carmichael and Liu, 2006; Kumar *et al*., 2011). Cyanotoxins are already responsible for high mortality rates of wild and domestic animals, as well as several cases of human intoxications, through consumption or contact with contaminated water (Bittencourt-Oliveira, 2003; Žegura et al., 2011). The majority of cases regarding environmental contamination by cyanotoxins are related to

the presence of four main toxins: microcystins, cylindrospermopsin, anatoxin-a and saxitoxin, all of them evaluated in this study.

1.2.1. Microcystins

Microcystins are a group of cyclic heptapeptides, with more than 90 known structural isoforms (Kumar *et al*., 2011). Variations in the chemical structure of microcystins are originated by the amino acids in the X and Z positions (Figure 1) and despite the number of possible isoforms in the environment, only one or two isoforms dominate in each producing genera (Neilan *et al*., 1999; Bittencourt-Oliveira, 2003; Kumar *et al*., 2011). Mycrocystins can be produced by cyanobacterial species from genera *Microcystis*, *Anabaena*, *Oscillatoria*, *Nostoc* and *Planktothrix* (Xie *et al*., 2005; Kumar *et al*., 2011), being the most reported and widespread toxin produced by cyanobacteria.

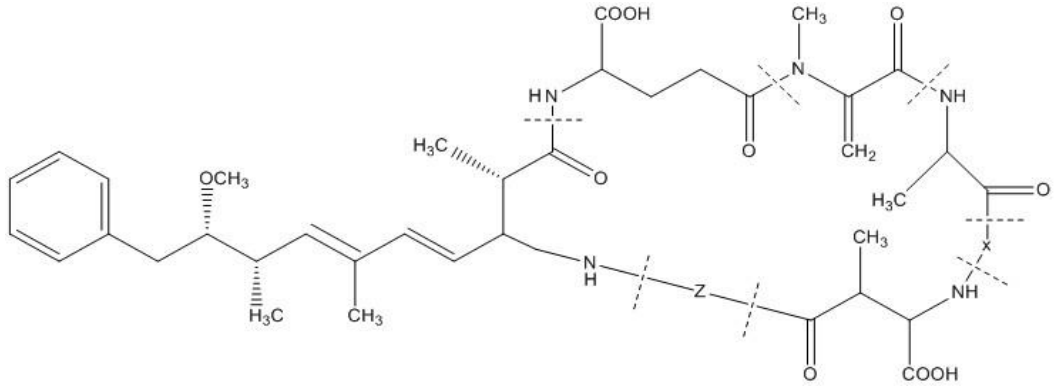

Fig. 1. Chemical structure of microcystins (author's own artwork).

The global presence of microcystins in the water ecosystems raised the necessity to create and optimized methods for its early detection. This toxin possesses a high toxicity, as well a high stability in the water column and thermal resistance, which translates to serious consequences to the environment and the exposed organisms (Neilan *et al*., 1999; Bittencourt-Oliveira *et al.*, 2010; Papadimitriou *et al*., 2012). Nonetheless, microcystin also possesses the capacity to bioaccumulate over several trophic levels in the contaminated ecosytems, representing an even greater threat to organisms (Neilan *et al*., 1999; Bittencourt-Oliveira *et al.*, 2010; Papadimitriou *et al*., 2012).

In humans, microcystins interferes on the bile acid transport system, through the hyperphosphorylation of the microfilaments (Neilan *et al.,* 1999; Xie *et al*., 2005;

Papadimitriou *et al*., 2012). Microcystins can also activate the phosphorylase A2 and the cycloooxigenase on hepatocytes, and induce the tumor necrosis factor alpha (TNF-α) and interleukin-1 (IL-1) on macrophytes, this way promoting the occurrence of hepatic tumors (Neilan *et al.,* 1999; Xie *et al*., 2005; Papadimitriou *et al*., 2012). Several cases of intoxication in humans by microcystins have been reported around the globe. One of the most severe cases of intoxication in humans reports to 1996, where 76 patients of a hemodialysis center in the city of Caruaru, Brazil, died due to the dialysis process performed with the use of water contaminated with microcystins (Bittencourt-Oliveira, 2003; Bittencourt-Oliveira *et al*., 2010). In China, the consumption of fish originated from eutrophic lakes with a frequent record of toxic cyanobacterial blooms caused several cases of microcystin contamination through bioaccumulation processes, being already considered a serious case of public health, given the eutrophic state of most of Chinese water resources (Zhang *et al*., 2009).

1.2.2. Cylindrospermopsin

Cylindrospermopsin comprehends a group of tricyclic alkaloid toxins, with hepatotoxic, neurotoxic and cytotoxic effects on the organisms (Pearson *et al*., 2010). This toxin has a low molecular weight (415 Da), and is a polyketide-derived alkaloid, containing a guanidine group and a hydroxide group, connected to a tricyclic-carbon skeleton (Figure 2) (Mihali *et al*., 2008; Pearson *et al*., 2010; Žegura *et al*., 2011; Poniedziałek *et al*., 2012; Gutiérrez-Praena *et al*., 2013).

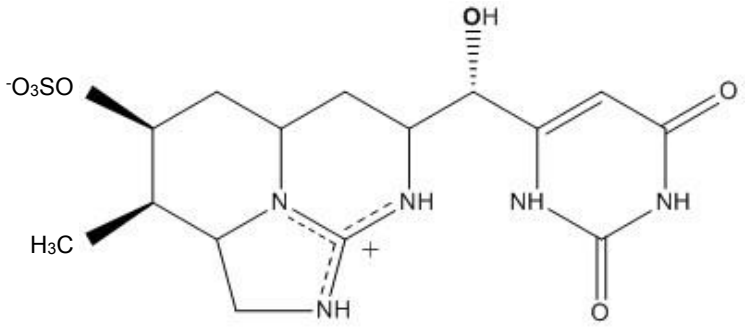

Fig. 2. Chemical structure of cylindrospermopsin (author's own artwork).

Possessing a stable chemical structure, without major variations in their amino acid composition (Aráoz *et al.*, 2010; Žegura *et al*., 2011; Gutiérrez-Praena *et al*., 2013), cylindrospermopsin is produced by freshwater cyanobacterial species like *Cylindrospermopsis raciborskii*, *Umezakia natans*, *Aphanizomenon ovalisporum*,

Raphidiopsis curvata, *Lyngbya wollei*, *Anabaena bergii*, *Aphanizomenon flos-aquae* and *Anabaena lapponica* (Mihali *et al*., 2008; Pearson *et al*., 2010). Changes in cylindrospermopsin' chemical structure leads to the production of this toxin' analogues 7-deoxy-cylindrospermopsin, given the lack of the hydroxyl group at C-7 and 7-epicylindrospermopsin, a toxic minor metabolite of *Aphanizomenon ovalisporum,* due to the occurrence of an epimer at the hydroxyl bridge (Mihali *et al*., 2008).

In the environment, cylindrospermopsin can be characterized as being an extracellular toxin since 90% of the total amount of cylindrospermopsin produced by cyanobacteria is found dissolved in water (Žegura *et al*., 2011). However, given the high stability of its chemical structure, the elimination of cylindrospermopsin from the water is difficult, given its resistance to extreme conditions of temperature, pH and radiation (Saker *et al*., 2004; White *et al*., 2006; Poniedziałek *et al*., 2012; Gutiérrez-Praena *et al*., 2013).

In human organisms, cylindrospermopsin interferes on a cellular level, inhibiting the protein synthesis and interfering on the metabolism of cytochrome p450 (Saker *et al.,* 2004; Aráoz *et al*., 2009; Pearson *et al*., 2010; Gutiérrez-Praena *et al*., 2013). This toxin is also classified as carcinogenic, given its damage caused in the DNA strains (Humpage *et al*., 2000; Shen *et al*., 2002). At a functional level, cylindrospermopsin affects organs as diverse as the kydneys, thymus, liver, lungs, and the immune system (Pearson *et al*., 2010; Žegura *et al*., 2011).

Recently, several Public Health questions has been raised, given the exponencial growth in the number of reported cases of presence of cylindrospermopsin potentially-producing species, and the toxin itself in the water environment (Saker *et al*., 2004; White *et al*., 2006). The wide geographical distribution of cylindrospermopsin and its producing species, its high solubility in water and its high presence in water in its dissolved form, causes humans to be more susceptible to cylindrospermopsin contamination, relatively to other cyanotoxins (Gutiérrez-Praena *et al*., 2006).

1.2.3. Anatoxin-a

Alkaloid toxin chemically analogue to acethylcholine, a neurotransmitter of the organisms, anatoxin-a is an alkaloid toxin composed by a bicyclic secondary amine, highly unstable in the environment (Figure 3) (Teixeira and Rosa, 2006; Osswald *et al*., 2007; Yavasoglu *et al*., 2008). Possessing a high solubility in water and a low molecular weight, when exposed to direct light environments is easily degradated by

aquatic microorganisms and converted in non-toxic products, such as dihydroanatxin-a and epoxyanatoxin-a (Teixeira and Rosa, 2006; Osswald *et al*., 2007; Yavasoglu *et al*., 2008). However, it is to highlight that, despite its high degradation rate when exposed to direct light, its degradation process cannot be classified as a photo-degradation process, since its independent of the presence of oxygen (Osswald *et al*., 2007).

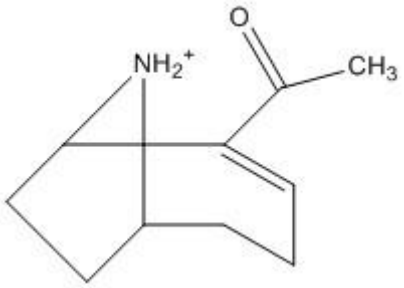

Fig. 3. Chemical structure of anatoxin-a (author's own artwork).

Produced by several phytoplanktonic and benthic species belonging to genera *Anabaena*, *Aphanizomenon*, *Cylindrospermum*, *Oscillatoria*, *Microcystis*, *Raphidiopsis*, *Planktothrix*, *Artrospira*, *Nostoc* and *Phormidium,* anatoxin-a possesses an wide geographical distribution. This, alongside with its high toxicity and instability as raised several questions regarding the effects of anatoxin-a on the environment and to humans (Osswald *et al*., 2007; Ballot *et al*., 2010; Yavasoglu *et al*., 2010; Žegura *et al*., 2011).

Given its chemical structure, anatoxin-a is an acetylcholine cholinergic agonist, having the same mode of action as acetylcholine in the human organism. After entering the organism, anatoxin-a binds irreversibly to acetylcholine neuronal receptors, having higher affinity with those than acetylcholine itself (Teixeira and Rosa, 2006; Yavasoglu *et al*., 2008; Žegura *et al*., 2011). This junction induces the continuous stimulation of the muscle, which posteriorly leads to muscular exhausting, paralysis or even death of the individual, in case of paralysis of the respiratory and/or cardiac muscles (Teixeira and Rosa, 2006; Žegura *et al*., 2011). Recently, it has been raised the necessity for more studies regarding the mode of action of this toxin, since their potential for genotoxic and carcinogenic effects is still unknown (Teixeira and Rosa, 2006; Žegura *et al*., 2011).

1.2.4. Saxitoxins

Possessing over 30 different isoforms, saxitoxins comprehend a group of alkaloid tetrahydro-purines (Sivonen and Jones, 1999; Llewellyn, 2006; Pearson *et al*., 2010), highly stable in the water environment, even in extreme temperature and pH conditions (Llewellyn, 2006). This stability in conferred by its tryciclic structure, in which variations occurs according to the amino acids present in the R positions (Figure 4) (Sivonen and Jones, 1999; Llewellyn, 2006; Pearson *et al*., 2010).

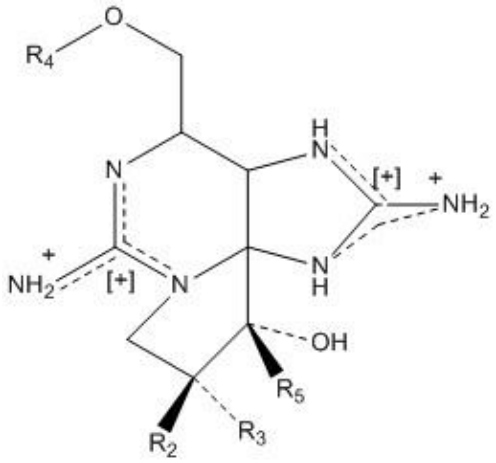

Fig. 4. Chemical structure of saxitoxins (author's own artwork).

Saxitoxins can be produced by both marine dinoflagellates from genera *Alexandrium*, *Gymnodinium* and *Pyrodinium* and by cyanobacteria from genera *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Lyngbya* and *Planktothrix* (Sivonen and Jones, 1999; Landsberg, 2002; Llewellyn, 2006; Pearson *et al*., 2010; Perreault *et al*., 2011). Its wide geographical distribution, in both marine and freshwater ecosystems, leads to extensive reports of contamination of ecosystems and organisms by this toxin, including humans (Pearson *et al*., 2010; Perreault *et al*., 2011).

At a human level, saxitoxins, as well as their derivatives, are the causal agents of PSP - Paralytic Shellfish Poisoning (Sivonen and Jones, 1999). This pathology characterizes itself by the blocking of muscular and nervous sodium channels, stopping this way the influx of sodium to the cells and restricting the signal transmitted between neurons. This can be translated, at an initial stage, in facial numbness, vomits and diarrhea. In more acute cases, it can also cause muscular weakness, ataxia, motor incoordination, incoherence and, sometimes, respiratory insufficiency. When in lethal

doses, this block causes cardiac and respiratory arrest, leading to the death of the individual (Landsberg, 2002; Pearson *et al*., 2010).

1.3. Methods for cyanobacteria and cyanotoxin monitoring

Until recently, cyanobacteria detection and identification was made through the application of microscope methodologies, based on the morphological identification of the observed organisms. However, this methodology presents several limitations, being noteworthy the fact that there are no morphological differences between toxic and non-toxic strains of cyanobacteria, which make the detection of toxic strains of cyanobacteria unfeasible (Bittencourt-Oliveira et al., 2003; Kumar *et al*., 2011). In order to overcome those limitations, the study of toxic and non-toxic cyanobacterial strains have been performed through the use of molecular methods.

Molecular methods are very useful and sensitive tools that allow the detection of cyanobacteria and cyanotoxin potentially-producing species in environmental samples in a reduced number of cells (Baker *et al*., 2002; Saker *et al*., 2007). This detection, usually performed through the application of the Polymerase Chain Reaction (PCR) technique, is made using specific genes that are present in the cyanobacteria genome or involved in the biosynthesis of a specific cyanotoxin (Pearson and Neilan, 2008). However, conventional PCR is only a qualitative method, not allowing the quantification of the resulted amplicon. More recently, the performance of Real-Time PCR (RT-PCT) is being more frequent in monitoring studies (Churro *et al*., 2012; Moreira *et al*., 2011). The method of RT-PCR amplifies the target gene and quantifies the amplicon in gene copies numbers, this way overcoming the limitations of conventional PCR (Moreira *et al*., 2014).

Nevertheless, it is to note that the molecular methods do not allow the quantification of cyanotoxins or give information about the toxicity of a sample or a water ecosystem. The detection and quantification of cyanotoxins is assessed through the performance of analytical methods, which includes chemical and biochemical techniques.

For the detection and quantification of cyanotoxins, chemical methods are the most frequently used in monitoring studies (Moreira *et al*., 2014). Examples of these techniques are the High Performance Liquid Chromatography (HPLC) and Mass Spectrometry (MS) (Msagati *et al*., 2006). Being a highly sensitive method, HPLC is the

most used method for cyanotoxin detection and quantification (Moreira *et al*., 2014). The obtainment of both qualitative and quantitative information of a particular cyanotoxin is made through the analysis of 2 criteria: the retention time and the spectrum of absorption (Ouahid, 2008; Pearson and Neilan, 2008). However, this method presents some disadvantages, such as long extraction process, the need of an expert in order to perform this method and analyze the obtained results, and the high volume of sample needed to perform this method (Rivasseau *et al*., 1999; Rapala *et al*., 2002; Mathys and Surholt, 2004; Sangolkar *et al*., 2006; Pearson and Neilan, 2008; Moreira *et al*., 2014).

Immunological methods, such as ELISA immunological assays, are associated with the biochemical properties presented by cyanotoxins (Moreira *et al*., 2014). ELISA immunological assays are characterized by the use of commercial kits with mono or polyclonal antibodies in its composition, turning this method into a rapid and inexpensive technique for the detection and quantification of cyanotoxins present in environmental samples (Rivasseau *et al.,* 1999; Rapala *et al.,* 2002; Mathys and Surholt, 2004; Sangolkar *et al*., 2006). Comparing with other analytical methods, such as HPLC, the ELISA immunological assays possesses advantages in the use of small amounts of sample, and its lower detection limit (Metcalf and Codd, 2003). However, this method also possesses a high possibility for cross-reactions between antibodies and other variants of the toxin or compounds present in the environmental matrix (Rapala *et al*., 2002; Sangolkar *et al*., 2006).

1.4. Guidelines for cyanobacteria and cyanotoxin detection

The presence of toxic strains of cyanobacteria in freshwater systems, as well as their related toxins, represents a serious threat in terms of Public Health issues. In terms of cyanobacterial frequency in freshwater systems, the Portuguese legislation, under the Decreto-Lei No. 306/ 2007, stated that the frequency of sampling should be increased if the detection, and posterior quantification, of microcystin-potentially producing cyanobacteria is higher than 2000 cells/mL in drinking water and in a transposition of a WHO directive for drinking waters. This guideline, created by WHO in 2003 for microcystin-LR, was based on the study developed by Chorus and Bartram (1999), and was provided given the harmful human effects of cyanotoxins (Lee *et al*., 2017). However, despite the high toxicity of these cyanobacterial metabolites, most countries, including Portugal, still does not have legislated a guideline value for

cyanotoxins other than microcystin, established in 1.0 µg/L for drinking waters and 10 µg/L for recreational waters (Decreto-Lei nº 306/2007). Exceptions are for New Zealand, Australia, United States, Canada and Brazil, with some established guideline values for cyanotoxins, such as cylindrospermopsin, anatoxin-a and saxitoxin (Lee *et al*., 2017).

Regarding the detection and quantification of cyanotoxins, for drinking water, the maximum amount of cylindrospermopsin allowed is legislated in Australia, for the Queensland Region (1.0 µg/L), Brazil (15 µg/L), New Zealand (1.0 µg/L) and United States (3.0 µg/L) (Burch, 2008; Lee *et al*., 2017). Anatoxin-a is legislated in the province of Quebec, Canada (3.7 µg/L), New Zealand (6.0 µg/L) and in the State of Washington, United States (1.0 µg/L) (Burch, 2008; Washington State Department of Health, 2008; Lee *et al*., 2017). Guideline values for saxitoxin were created in Australia, New Zealand and Brazil, where the concentration of saxitoxin should not exceed the 3.0 µg/L (Burch, 2008).

1.5. Monitoring cyanobacteria and cyanotoxins in Portugal

In Portuguese freshwater systems, the presence of cyanobacteria has been reported since the end of the 20th century (Vasconcelos, 1999). Since that time, several genera and/or species of cyanobacteria have been reported in Portugal. From the target species of this study, *M. aeruginosa* seemed to be the most frequent in Portuguese freshwater systems, being detected in several sampling locations, from North to South of Portugal. Amorim (1994) detected this species in Braça, Vela and Mira Lakes; Pereira and collaborators (2000) reported its presence in Montargil Reservoir; Vasconcelos and Pereira (2001) studied its occurrence in the WWTP of Esmoriz; *M. aeruginosa* presence in the Guadiana River was detected by Moreno *et al.*, (2003); Galvão and collaborators (2008) reported the presence of this species in Alqueva Reservoir, Alvito Reservoir and Odivelas Reservoir; lastly, Martins *et al*., (2009) detected *M. aeruginosa* in samples collected in Braça Lake, Tâmega River, Douro River, Aguieira Reservoir, Bastelos Reservoir and Barrinha de Mira Lake.

The presence of *C. raciborskii* in Portuguese freshwater systems was detected mostly on water ecosystems located on the Center and South regions, being recently detected in one sampling point in the North region. This way, *C. raciborskii* was already reported to be present in Tâmega River, Parque da Cidade do Porto and Mira Lake

(Matos, 2014), 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).

P. agardhii is the species with fewer reports on Portuguese freshwater systems, comparing the reports of the 3 cyanobacteria species to be analyzed in this study. Nonetheless, its presence was detected in the South region of Portugal (Churro *et al*., 2012), and in Torrão Reservoir, Tâmega River, Parque da Cidade do Porto, Vela and Mira Lakes (Matos, 2014).

Regarding the detection of cyanotoxins in freshwater systems, microcystins are widely spread in Portuguese water ecosystems, being detected in several studies throughout the last 2 decades (Vasconcelos, 1993; 1994; 1995; 1999; 2001; Vasconcelos *et al*., 1995; 1996; Freitas, 2009; Morais, 2009; Regueiras, 2009; Mendes, 2014). Detection and quantification of cylindrospermopsin, anatoxin-a and saxitoxin in the freshwater environment was only reported in the study conducted by Mendes (2014), in the sampling sites studied in this work.

1.6. Objectives

Considering the wide distribution and invasive behavior of cyanobacteria, as well as the high toxicity and impacts on both the environment and humans by cyanotoxins in freshwater systems, it is important to establish a monitoring program that encompasses the early detection of cyanobacteria and potential for its production of cyanotoxins. Following this, the main objectives of this work are:

- Application of molecular methods in order to detect the presence of *Microcystis sp*., *M. aeruginosa*, *C. raciborskii* and *P. agardhii*;
- Evaluate the spatial and temporal dynamics of the detected cyanobacteria in the freshwater systems in study;
- Detection of genes that encode the biosynthesis of microcystins, cylindrospermopsin, anatoxin-a and saxitoxin, through the application of molecular methods;
- Estimate the variations in the spatial and temporal dynamics of the detected cyanotoxin potential for production in the several analyzed freshwater systems;
- Establish molecular methods as the ideal screening method for the detection of cyanobacteria and cyanotoxin potential.

2. Materials and Methods

2.1. Study areas

For this study, seven freshwater systems located at North and Center regions of Portugal were chosen. These sampling points were selected due to their socio-economic impact in their geographical area, giving its use for human consumption, recreational and economical activities of the surrounding population. This way, the selected sampling locations in the North regions were Torrão Reservoir, Parque Fluvial do Tâmega and Parque da Cidade do Porto (Lakes 1, 2 and 3) and to represent the Center region, it was chosen Vela and Mira Lakes.

2.1.1. Torrão Resevoir (Tâmega River)

Located on the Tâmega River (41°05'45.7"N, 8°15'15.4"W) (Figure 5), the principal tributary of the Douro River in Portugal, the Torrão Reservoir was created after the construction of Torrão' hydroelectric central, in 1988 (Oliva-Teles *et al*., 2008; Torres *et al*., 2011; Mateus *et al*., 2014). Classified as a eutrophic semi-lentic freshwater system, by the National Water Institute (INAG), the Torrão Reservoir possesses a total water volume of 77 hm^3 and a main depth of 19 m (Oliva-Teles *et al*., 2008; Mateus *et al.,* 2014). This artificial freshwater system is an important water source in the region, being used for a wide range of socio-economical purposes, from human consumption and agriculture, to recreational activities and energy production (Oliva-Teles *et al*., 2008; Torres *et al*., 2011). However, the socio-economic development of the region has led to an increase in the input of wastewaters in the reservoir, affecting the water quality of the Tâmega River and, subsequently, the Torrão Reservoir (Mateus *et al*., 2014). Given the semi-lentic characteristics of the aquatic system and the elevated nutrient input in the water, it has been reported the occurrence of toxin-producing species of cyanobacteria, mainly associated to *M. aeruginosa* and *A. flos-aquae* (Oliva-Teles *et al*., 2008; Torres *et al*., 2011). The existence of cyanobacterial blooms follows a pattern, where their incidence in the water is higher in spring time, followed by a smaller quantity in summertime (Mateus *et al*., 2014).

Fig. 5. Location of Torrão Reservoir (image obtained using Google Earth 7.1.2.2041 software) and sampling site (author's own photography).

2.1.2. Parque Fluvial do Tâmega (Tâmega River)

Inaugurated on December of 2008, in Marco de Canaveses, Parque Fluvial do Tâmega (Figure 6) is situated on Tâmega River (41°11'45.9"N, 8°09'38.2"W), and is included in the reservoir created by the Torrão hydroelectric central, located about 14km downstream. Parque Fluvial do Tâmega is classified as a recreational park, where it is possible the practice of aquatic activities, despite the prohibition of swimming in those waters, given the status of the water, classified as eutrophic, as Torrão Reservoir (Regueiras, 2009).

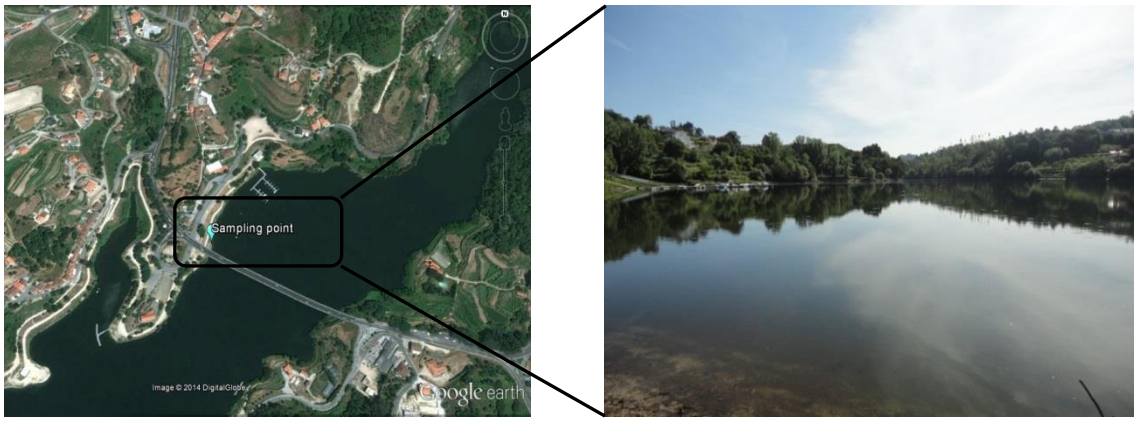

Fig. 6. Location of Parque Fuvial do Tâmega (image obtained using Google Earth 7.1.2.2041 software) and sampling site (author's own photography).

2.1.3. Parque da Cidade do Porto

Being the Portugal's largest urban park, with a total area of 85 ha, Parque da Cidade do Porto (Figure 7) is composed by wooded areas, grassy areas and 4 artificial lakes. Located in the middle of the city of Oporto, this park was inaugurated in 1993, being the stopping point for several species of migratory birds (Morais, 2009). This recreational park possesses an abundant flora and fauna, containing numerous species of tree and shrub species, as well as fishes, wild ducks, geese, frogs, among others inhabiting mostly its artificial lakes (Morais, 2009). Water circulation between the several lakes is made in succession from Lake 1 to Lake 4, through small falls or by underground channeling and, at the same time, supplying the irrigation system of the park (Moreira, 1998; Morais, 2009). At a biological level, previous studies (Morais, 2009; Matos, 2014) detected the presence of several cyanobacteria species, such as *M. aeruginosa*, *C. raciborskii* and *P. agardhii*.

Fig. 7. Location of Parque da Cidade do Porto (image obtained using Google Earth 7.1.2.2041 software) and Parque da Cidade sampling sites (author's own photographies). a) Lake 1 (sampling site 1); b) Lake 2 (sampling site 2); c) Lake 3 (sampling site 3).

Sampling sites of Parque da Cidade, 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°10'01.5N, 8°40'39.8"W) were chosen given the connection between 3 out of the 4 existing lakes in the park (Moreira, 1998; Morais, 2009).

2.1.4. Vela Lake

Natural lentic system located on the West coast of Portugal, Vela Lake (40°16'23.9"N, 8°47'35.1"W) (Figure 8) is a freshwater system of low depth (mean depth: 0.9 m; maximum depth: 2.4 m), with a total water volume of $70x10^4$ cm^3 and a maximum floodable area of 70 ha (Castro *et al.,* 2005; Abrantes *et al*., 2006). Included in Rede Natura 2000 program, this lake has a high conservative interest level, being directly influenced by its geographical proximity to the Atlantic Ocean, in terms of climate (Abrantes *et al*., 2006). Vela Lake is, in part, surrounded by agricultural fields and areas for recreational activities, such as fishing. Alongside with the low depth of the lake and sandy nature of the soil, this freshwater system is highly exposed to constant nutrient inputs, which accelerates the ecosystems' eutrophication process (Castro *et al.*, 2005; Abrantes *et al*., 2006). At a biotic level, this ecosystem is characterized by the presence of a wide range of cyanobacteria species, such as *M. aeruginosa*, *C. raciborskii*, *Aphanizomenon flos-aquae*, *Anabaena flos-aquae* and *P. agardhii* (de Figueiredo *et al*., 2006; Saker *et al*., 2003; Matos, 2014).

Fig. 8. Location of Vela Lake (image obtained using Googl Earth 7.1.2.2041 software) and sampling site (author's own photography).

2.1.5. Mira Lake

Located on the Central coast region of Portugal (40°26'29.8"N, 8°45'07.5"W), Mira Lake (Figure 9) is a small freshwater system of low depth (mean depth: 2.8m; maximum depth: 3.1m) and appeared as a result of the junction of two small streams, running at the surface (Gonçalves *et al*., 1996; Castro *et al*., 2005). Surrounded by agricultural fields and urban areas, this lentic system is exposed to anthropogenic pressures resultant from recreational activities, affluents from urban wastes and nutrient input originated from agricultural activities (Gonçalves *et al*., 1996; Castro *et al*., 2005; Freitas, 2009). Biologically, this eutrophized water system has a low frequency of macrophytes (Castro *et al*., 2005), however, possesses reports of cyanobacterial blooms, namely from *M. aeruginosa, C. raciborskii* and *P. agardhii* (Freitas, 2009; Matos, 2014).

Fig. 9. Location of Mira Lake (image obtained using Google Earth 7.1.2.2041 software) and sampling site (author's own photography).

2.2. Sampling

The monthly sampling was performed from October 2016 to June 2017. This period was chosen given the lack of data regarding the presence of cyanobacteria in Portuguese freshwater systems, as well as their potential for cyanotoxin production, during late autumn, winter and early spring months.

Sampling procedure consisted in collection of water samples from the surface layer of the chosen freshwater systems. The collection was performed through the use of sterilized bottles, with a maximum volume of 2.5L. In all sampling sites, atmospheric temperature values were obtained from Instituto Português do Mar e da Atmosfera

(IPMA) website. Samples were maintained in refrigerated conditions until its processing in the laboratory.

2.3. Sample procedure

Upon arrival to the laboratory, collected water samples were filtered with a vacuum filtration system, using sterile Munktell MGC micro-glass fiber paper filters (47 mm diameter, 1.2 µm porosity) (Munktell™, Sweden). After filtration, the water was discarded and the obtained biomass was maintained at -20°C until further molecular analysis.

2.4. Molecular analysis

2.4.1. DNA extraction

To extract DNA, filters containing the biomass resulted from the filtration of the water samples were scraped using a sterile blade and placed in a sterile Eppendorf tube.

Genomic DNA from the environmental samples was extracted using the PureLink™ Genomic DNA Mini Kit (Invitrogen, CA, USA), following the manufacturers protocol for Gram-negative bacteria. For this protocol, a centrifuge Eppendorf Centrifuge 5415R and a thermomixer plate Eppendorf Thermomixer compact (Eppendorf, Hamburg, Germany) were used. For the elution step, a volume of 50µL of elution solution was used for each DNA sample.

To confirm the presence and integrity of the genomic DNA, it was performed a 1% agarose gel electrophoresis (UltraPure™ Agarose, Invitrogen, CA, USA), stained with a solution of SYBR™ Safe DNA Gel Stain (Invitrogen, CA, USA) in a 1x Tris-Acetate EDTA buffer (Invitrogen UltraPure™ 10x TAE Buffer – 400mM Tris-Acetate, 10mM EDTA, pH 8.3 ± 0.10). For electrophoresis, a constant voltage of 80V, during 30 min, was applied. In each gel well, 10µL of DNA samples, along with 1µL of 1x gel loading buffer (Nucleic acid sample loading buffer 5x, BioRad – 50mM Tris-HCL, pH 8, 25% Glicerol, 5mM EDTA, 0.2% Bromophenol Blue and 0.2% Xylene FF) were loaded. Genomic DNA was visualized and photographed using a transilluminator CSL-MICCRODOC System, under UV light, coupled with a Cannon PowerShot G9 camera system.

2.4.2. PCR amplifications

For PCR amplifications, the Promega GoTaq® Flexi DNA Polymerase (Promega, WI, USA) reagents were used, for a final volume of 20µL. Each PCR reaction mix contained 4µL of 5x Green GoTaq® Flexi Buffer, 2µL of 25mM $MgCl_2$ solution, 2µL of primer forward, 2µL of primer reverse, 1µL of 2.5 mM of deoxynucleotide triphosphate (dNTP's) mix, 0.5µL of Bovine Serum Albumin (BSA) (10 mg/mL), 0.1µL of GoTaq® DNA Polymerase (5u/µL) and 1µL of DNA sample. BSA usage in the PCR mix is related to its known capability to bind the majority of the PCR inhibitors usually present in environmental samples and it is capacity to stabilize the DNA polymerase used in this procedure (Kreader, 1996; Farell and Alexandre, 2012).

Confirmation of the presence of cyanobacterial DNA in the extracted samples was made through the amplification of the 16S rRNA gene, using the 27F/809R and 740F/1494R primer sets and through the amplification of the phycocyanin operon, using the PCβF/ PCβR primer set (Table 1).

Table 1. Primer sets applied in conventional PCR for detection of cyanobacteria.

Target gene	***Primer***	***Primer sequence 5'-3'***	***Product length (bp)***	***Reference***
Cyanobacterial 16S rRNA	27F	AGAGTTTGATCCTGGCTCAG	780	Neilan *et al*., 1997
	809R	GCTTCGGCACGGCTCGGGTCGATA		Jungblut *et al*., 2005
	740F	GGCYRWAWCTGACACTSAGGGA	754	Neilan *et al*., 1997
	1494R	TACGGTTACCTTGTTACGAC		
Phycocyanin operon	PCβF	GGCTGCTTGTTTACGCGACA	700	Neilan *et al*, 1995
	PCβR	CCAGTACCACCAGCAACTAA		
C. raciborskii rpoC1	Cyl2	GGCATTCCTAGTTATATTGCCATACTA	247	Wilson *et al*.,2000
	Cyl4	GCCCGTTTTTGTCCCTTTCGTGC		
P. agardhii rpoC1	rpoC1_Plank_F271	TGTTAAATCCAGGTAACTATGACGGCCTA	201	Churro *et al*., 2012
	rpoC1_P_agardhii_R472	GCGTTTTTGTCCCTTAGCAACGG		
M. aeruginosa gyrB	gyrF	GGACGTTTACGAGAACTAGCCTA	416	Tanabe *et al*., 2007
	gyrR	GGTCTTGGTTTGTCCCTCAA		
Microcystis sp. 16S rRNA	Micr 184F	GCCGCRAGGTGAAAMCTAA	220	Neilan *et al*. 1997
	Micr 431R	AATCCAAARACCTTCCTCCC		

2.4.2.1. Cyanobacteria

Presence of toxic cyanobacteria was performed through the detection of *rpoC1* gene sequence from *C. raciborskii*, *rpoC1* gene sequence from *P. agardhii, gyrB* gene sequence from *M. aeruginosa* and 16S rRNA sequence from *Microcystis sp*. in the extracted samples. For this assessment, primer sets cyl2/cyl4, rpoC1_Plank_F271/rpoC1_P_agardhii_R472, gyrF/gyrR, and Micr184F/ Micr431R respectively, were used (Table 1).

2.4.2.2. Cyanotoxins

For the determination of the cyanotoxin potential, fragments of the genes involved in cyanotoxin biosynthesis were amplified according to the cyanotoxin in study. For the study of microcystin, primer sets for the *mcy* cluster, *mcy*A-E and *mcy*G, as indicated in Table 2 were used. Identification of the genes encoding cylindrospermopsin biosynthesis (*cyr*) was performed through the use of *cyr*A, *cyr*B, *cyr*C and *cyr*J primer sets (Table 2). Detection of genes implicated in anatoxin-a biosynthesis was made through the amplification of *anaC* gene cluster, with the use of anaC-genF/ anaC-genR, for general amplification, and anaC-anabF/ anaC-anabR, specific for the genus *Dolichospermum* (Table 2). Genes involved in saxitoxin synthesis are detected with the use of primer sets sxtA855_F/ sxtaA1480_R (*sxtA*), sxtG432_F/ sxtG928_R (*sxtG*) and sxtI682F/ sxtI877R (*sxtI*), for amplification of *sxt* gene cluster (Table 2).

For positive controls, DNA from strains of the Blue Biotechnology and Ecotoxicology Culture Collection (LEGE CC) were used. In the hepatotoxin study, both for *Microcystis sp*. specific PCR and microcystin, a *M. aeruginosa* strain LEGE 00063 was used as positive control; for *C. raciborskii* specific PCR and cylindrospermopsin, a positive control was obtained from an extract of a pure culture of a *C. raciborskii* strain LEGE 97047. For the study of anatoxin-a potential, an extract of an *Anabaena sp*. strain LEGE X-002 was used; an extract from a pure culture of an *A. gracile* strain LMECYA 040 was used as a positive control in the study of saxitoxin potential. Analysis of *P. agardhii* was made without the use a positive control, since the unavailability of an extract of a pure culture in the Culture Collection. A previous study (Matos, 2014), confirmed the capability of the primer set rpoC1_Plank_F271/rpoC1_P_agardhii_R472 to detect amplified fragments, in the expected fragment size, of *P. agardhii* in environmental samples confirming the specificity of this primer set.

Table 2. Primer sets applied in conventional PCR for detection of cyanotoxin potential.

Target gene	*Primer*	*Primer sequence 5'-3'*	*Product length (bp)*	*Reference*
*mcy*A	mcyA-CD1F	AAAATTAAAAGCCGTATCAAA	297	Hisbergues *et al*., 2003
	mcyA-CD1R	AAAAGTGTTTTATTAGCGGCTCAT		
*Microcystis sp. mcy*A	MSF	ATCCAGCAGTTGAGCAAGC	1300	Tillett *et al*., 2001
	MSR	TGCAGATAACTCCGCAGTTG		
*Microcystis sp. mcy*B	mcyB 2156-F	ATCACTTCAATCTAACGACT	955	Mikalsen *et al*., 2003
	mcyB 3111-R	AGTTGCTGCTGTAAGAAA		
*Microcystis sp. mcy*C	PSCF1	GCAACATCCCAAGAGCAAAG	674	Ouahid *et al.*, 2005
	PSCR1	CCGACAACATCACAAAGGC		
*Microcystis sp. mcy*D	PKDF1	GACGCTCAAATGATGAAAC	647	Ouahid *et al*., 2005
	PKDR1	GCAACCGATAAAAACTCCC		
*Microcystis sp. mcy*E	PKEF1	CGCAAACCCGATTTACAG	755	Ouahid *et al*., 2005
	PKER1	CCCCTACCATCTTCATCTTC		
*Microcystis sp. mcy*G	PKGF1	ACTCTCAAGTTATCCTCCCTC	425	Ouahid *et al*., 2005
	PKGR1	AATCGCTAAAACGCCACC		
*cyr*A	AMT Fw	ATTGTAAATAGCTGGAATGAGTGG	1105	Kellmann *et al*., 2006
	AMT Rev	TTAGGGAAGTAATCTTCACAG		
*cyr*B	M13	GGCAAATTGTGATAGCCACGAGC	597	Schembri *et al*., 2001
	M14	GATGGAACATCGCTCACTGGTG		
*cyr*C	K18	CCTCGCACATAGCCATTTGC	422	Fergusson *et al*., 2003
	M4	GAAGCTCTGGAATCCGGTAA		Schembri *et al*., 2001
*cyr*J	cynsulfF	ACTTCTCTCCTTTCCCTATC	578	Mihali *et al.*, 2008
	cylnamR	GAGTGAAAATGCGTAGAACTTG		
*ana*C	anaC-genF	TCTGGTATTCAGTCCCCTCTAT	366	Rantala-Yilmen *et al.*, 2011
	anaC-genR	CCCAATAGCCTGTCATCAA		
Dolichospermum sp. *ana*C	anaC-anabF	GCCCGATATTGAAACAAGT	263	Rantala-Yilmen *et al*., 2011
	anaC-anabR	CACCCTCTGGAGATTGTTTA		
*sxt*A	sxtA855_F	GACTCGGCTTGTTGCTTCCCC	648	Savela *et al*., 2015
	sxtA1480_R	GCCAAACTCGCAACAGGAGAAGG		
*sxt*G	sxtG432_F	AATGGCAGATCGCAACCGCTAT	519	Savela *et al*., 2015
	sxtG928_R	ACATTCAACCCTGCCCATTCACT		
*sxt*I	sxiI 682F	GGATCTCAAAGAAGATGGCA	195	Lopes *et al*., 2006
	sxtI 877R	GCCAAACGCAGTACCACTT		

PCR reactions were carried out on one of these thermocyclers: Biometra Professional Thermocycler (Biometra, Germany) and Veriti™ 96-Well Thermal Cycler (Applied Biosystems, CA, USA). PCR conditions are listed below o´n Tables 3 and 4. Confirmation of the presence of amplified fragments was made with an agarose gel electrophoresis at 1%, in the same conditions applied for the confirmation of genomic DNA (see section 2.3.1).

Table 3. PCR programs applied in conventional PCR for detection of cyanobacteria.

Target gene	***Initial Denaturation***		***Denaturation***		***Annealing***		***Extension***		***Final Extension***	
	Temp.	**Time**	**Temp.**	**Time**	**Temp.**	**Time**	**Temp.**	**Time**	**Temp.**	**Time**
Cyanobacterial 16S rRNA	92 °C	2 min	92 °C	20 sec	50 °C	30 sec	72 °C	1 min	72 °C	5 min
			35 cycles							
Phycocyanin operon	94 °C	3 min	94 °C	30 sec	50 °C	30 sec	72 °C	30 sec	72 °C	5 min
			35 cycles							
C. raciborskii rpoC1	95 °C	2 min	95 °C	90 sec	45 °C	30 sec	72 °C	50 sec	72 °C	7 min
			35 cycles							
P. agardhii rpoC1	94 °C	3 min	94 °C	20 sec	58 °C	20 sec	72 °C	20 sec	72 °C	5 min
			40 cycles							
*M. aeruginosa gyr*B	94 °C	3 min	94 °C	1 min	60 °C	1 min	72 °C	30 sec	72 °C	5 min
			40 cycles							
Microcystis sp. 16S rRNA	95 °C	2 min	95 °C	90 sec	56 °C	30 sec	72 °C	50 sec	72 °C	7 min
			35 cycles							

Table 4. PCR programs applied in conventional PCR for detection of cyanotoxin potential.

Target gene	***Initial Denaturation***		***Denaturation***		***Annealing***		***Extension***		***Final Extension***	
	Temp.	**Time**	**Temp.**	**Time**	**Temp.**	**Time**	**Temp.**	**Time**	**Temp.**	**Time**
*mcy*A	95 °C	2 min	95 °C	90 sec	56 °C	30 sec	72 °C	50 sec	72 °C	7 min
			35 cycles							
*Microcystis sp. mcy*A	95 °C	2 min	95 °C	90 sec	60 °C	30 sec	72 °C	50 sec	72 °C	7 min
			35 cycles							
*Microcystis sp. mcy*B	94 °C	5 min	95 °C	1 min	52 °C	30 sec	72 °C	1 min	72 °C	7 min
			35 cycles							
*Microcystis sp. mcy*C	94 °C	5 min	95 °C	1 min	52 °C	30 sec	72 °C	1 min	72 °C	7 min
			35 cycles							
*Microcystis sp. mcy*D	94 °C	5 min	95 °C	1 min	52 °C	30 sec	72 °C	1 min	72 °C	7 min
			35 cycles							
*Microcystis sp. mcy*E	94 °C	5 min	95 °C	1 min	52 °C	30 sec	72 °C	1 min	72 °C	7 min
			35 cycles							
*Microcystis sp. mcy*G	94 °C	5 min	95 °C	1 min	52 °C	30 sec	72 °C	1 min	72 °C	7 min
			35 cycles							
*cyr*A	94 °C	3 min	94 °C	10 sec	50 °C	20 sec	72 °C	1 min	72 °C	7 min
			30 cycles							
*cyr*B	95 °C	2 min	95 °C	90 sec	55 °C	30 sec	72 °C	50 sec	72 °C	7 min
			35 cycles							
*cyr*C	95 °C	2 min	95 °C	90 sec	55 °C	30 sec	72 °C	50 sec	72 °C	7 min
			35 cycles							
*cyr*J	94 °C	3 min	94 °C	10 sec	57 °C	20 sec	72 °C	1 min	72 °C	7 min
			30 cycles							
*ana*C	94 °C	2 min	94 °C	30 sec	58 °C	30 sec	72 °C	30 sec	72 °C	5 min
			35 cycles							
*Dolichospermum sp. ana*C	94 °C	2 min	94 °C	30 sec	60 °C	30 sec	72 °C	30 sec	72 °C	5 min
			35 cycles							
*sxt*A	98 °C	30 sec	98 °C	5 sec	62 °C	5 sec	72 °C	10 sec	72 °C	1 min
			35 cycles							
*sxt*G	98 °C	30 sec	98 °C	5 sec	62 °C	5 sec	72 °C	10 sec	72 °C	1 min
			35 cycles							
*sxt*I	94 °C	3 min	94 °C	10 sec	52 °C	20 sec	72 °C	1 min	72 °C	7 min
			35 cycles							

3. Results

3.1. Atmospheric temperature

Atmospheric temperature values, corresponding to the minimum and maximum temperature registered on collection day, were recorded from the IPMA website. The recorded data, Figures 10 and 11, represents the temperature registered on the nearest locations from the studied sampling points: Marco de Canaveses (for Torrão Reservoir and Parque Fluvial do Tâmega), Porto (for Parque da Cidade do Porto), Figueira da Foz (for Vela Lake) and Mira (for Mira Lake).

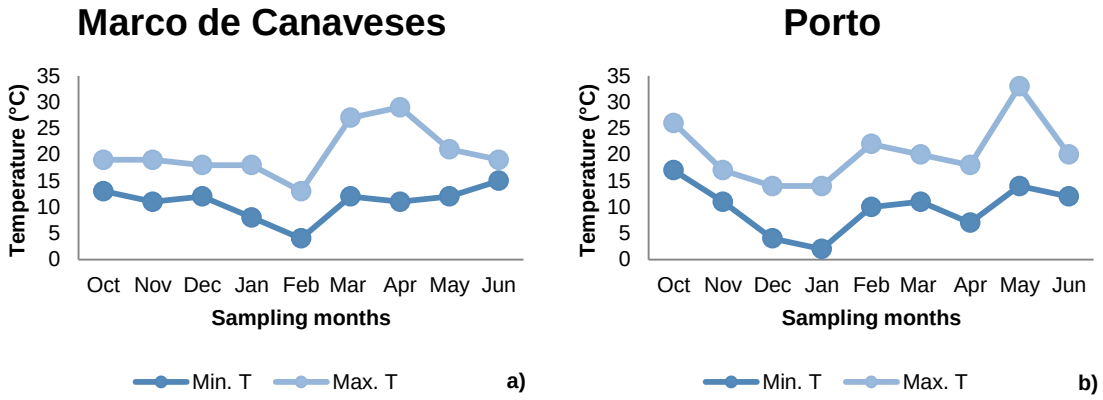

Fig. 10. Minimum (Min. T.) and maximum (Max. T.) values recorded in the sampling months in Marco de Canaveses (a) and Porto (b).

In regard to the North sampling regions, the minimum and maximum temperature data recorded in both sampling points, showed slightly differences between these points. Comparing data, in Marco de Canaveses (Figure 10a), the temperature decreased until February, where it was verified its minimum value, and then increased during the remaining sampling dates; the maximum temperature verified in this sampling point had a sudden increase between February and March, registering its higher value in April. Regarding the temperature data registered in Porto (Figure 10b), temperature values reduced until January, where it was recorded its minimum, with an increase in the remaining sampling dates, with exception for April; about the maximum temperature data, it was recorded a similar curve to the one registered for the minimum temperature, with a sudden peak in April, where it was observed its highest value.

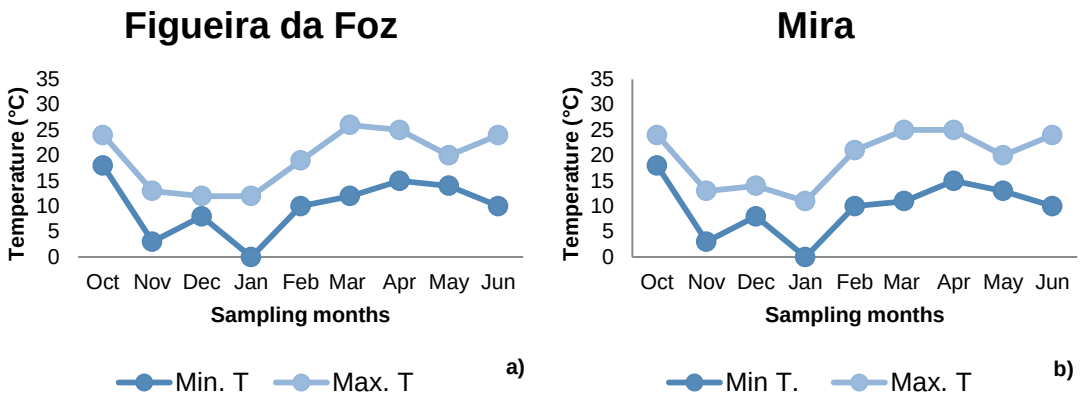

Fig. 11. Minimum (Min. T.) and maximum (Max. T.) temperature values recorded in the sampling dates in Figueira da Foz (a) and Mira (b).

Through the analysis of Figure 11, it is possible to observe that the temperature data is very similar at both locations (Figueira da Foz and Mira). For the minimum temperature, values were higher in October and April, being the minimum value recorded in November and January, for both sampling points. In the case of the maximum temperature, the data revealed that the highest values were observed on October, March, April and June, and the lowest values were registered between November and January. In general, a sudden decrease was observed in both minimum and maximum temperatures between October and November and then a more expressive increase in both values between January and February, in both sampling locations.

3.2. Molecular results

The presence of cyanobacterial DNA was confirmed through the amplification of both 16S rRNA gene (primer sets 27F/809R and 740F/1494R) and phycocyanin operon (primer set PCβF/PCβR) in the extracted samples. After the confirmation of the presence of cyanobacterial DNA in all samples, with exception of the sample from Lake 3 of Parque da Cidade do Porto collected at February of 2017, it was performed the analysis for the presence of genes involved in both cyanobacteria presence and cyanotoxin biosynthesis. Results from the PCR amplification are summarized on Tables 5-20.

3.2.1. Cyanobacteria

Detection of the presence of *Microcystis sp*., *M. aeruginosa*, *C. raciborskii* and *P. agardhii* in the studied freshwater samples was performed using specific primer sets for the detection of each species. The obtained results are described in Tables 5-8.

3.2.1.1. Detection of *Microcystis sp.*

PCR results obtained from the amplification of the gene fragments allowed the detection of *Microcystis sp*. and, specifically, *M. aeruginosa* in the analyzed freshwater systems, which are described below in Tables 5 and 6.

Table 5. Detection of amplified fragments related to the presence of *Microcystis sp*. in the analyzed freshwater systems, using the Micr 184F/Micr 431R primer set. The mark (+) indicates the presence of the amplified fragment and the mark (-) indicates the absence of the fragment in the analyzed sample, through PCR amplification. The diagonal line (/) means the absence of cyanobacterial DNA in the sample.

	Torrão Reservoir	Parque Fluvial do Tâmega	Parque da Cidade do Porto Lake 1	Parque da Cidade do Porto Lake 2	Parque da Cidade do Porto Lake 3	Vela Lake	Mira Lake
October	+	+	+	+	+	+	+
November	+	+	+	+	-	+	+
December	+	+	+	+	-	+	+
January	+	+	+	+	+	+	+
February	+	+	+	+	/	+	+
March	-	+	+	-	-	+	+
April	+	+	+	+	+	+	+
May	+	+	+	+	+	+	+
June	+	+	+	+	+	+	+

After observation of Table 5, regarding the analysis of the 16S rRNA specific for *Microcystis sp*., it is possible to perceive that the presence of this species is registered in all sampling points. Concerning the temporal occurrence of this species, samples collected in March in Torrão Reservoir, and Lakes 2 and 3 of Parque da Cidade do Porto did not showed the presence of the gene. In Lake 3 of Parque da Cidade do Porto, the gene was also absent in the samples collected in November and December.

Table 6. Detection of amplified fragments related to the presence of *M. aeruginosa* in the analyzed freshwater systems, using the gyrF/gyrR primer set. The mark (+) indicates the presence of the amplified fragment and the mark (-) indicates the absence of the fragment in the analyzed sample, through PCR amplification. The diagonal line (/) means the absence of cyanobacterial DNA in the sample.

	Torrão Reservoir	Parque Fluvial do Tâmega	Parque da Cidade do Porto Lake 1	Parque da Cidade do Porto Lake 2	Parque da Cidade do Porto Lake 3	Vela Lake	Mira Lake
October	+	+	+	+	+	+	+
November	+	+	+	+	+	+	+
December	+	+	+	+	+	+	+
January	+	+	+	+	+	+	+
February	+	+	+	+	/	+	+
March	+	+	+	+	+	+	+
April	+	+	+	+	+	+	+
May	+	+	+	+	+	+	+
June	+	+	+	+	+	+	+

Through the analysis of Table 6, it is possible to perceive that *M. aeruginosa* is present in the total amount of the tested samples, with exception of the sample collected in February from Lake 3 of Parque da Cidade do Porto, since the non-detection of cyanobacterial DNA on that sample.

3.2.1.2. Detection of *Cylindrospermopsis raciborskii*

PCR results obtained from the amplification of the gene fragment that allow the detection of *C. raciborskii* in the analyzed freshwater systems are described below in Table 7.

Table 7. Detection of amplifed fragments related to the presence of *C. raciborskii* in the analyzed frehwater systems, using the cyl2/cyl4 primer set. The mark (+) indicates the presence of the amplified fragment and the mark (-) indicates the absence of the fragment in the analyzed sample, through PCR amplification. The diagonal line (/) means the absence of cyanobacterial DNA in the sample.

	Torrão Reservoir	Parque Fluvial do Tâmega	Parque da Cidade do Porto Lake 1	Parque da Cidade do Porto Lake 2	Parque da Cidade do Porto Lake 3	Vela Lake	Mira Lake
October	-	-	-	-	-	-	-
November	-	-	+	+	-	+	-
December	-	-	-	-	-	-	+
January	-	-	-	-	-	-	-
February	-	-	-	+	/	-	-
March	-	-	+	+	+	-	-
April	-	-	-	-	-	-	-
May	-	-	-	-	-	+	+
June	-	+	-	+	-	-	-

The presence of *C. raciborskii* was observed in 12 out of 62 samples, in 6 of the 7 freshwater systems in study (Table 7). Through the sampling period, *C. raciborskii* was not detected in Torrão Reservoir, being its presence also less expressive in Parque Fluvial do Tâmega, with one detection in June, and in Lake 3 of Parque da Cidade do Porto, with one positive amplification in March. The presence of this cyanobacterial species was more pronounced on Lake 2 of Parque da Cidade do Porto, with the presence of amplified fragments in samples collected in November, February, March and June. Through the analysis of Table 7, it is also possible to observe that the presence of *C. raciborskii* was not detected in none of the sampling points in October, January, and April, and had an higher incidence in November (Lakes 1 and 2 of Parque da Cidade do Porto and Vela Lake) and March (Lakes 1, 2 and 3 of Parque da Cidade do Porto).

3.2.1.3. Detection of *Planktothrix agardhii*

PCR results obtained from the amplification of the gene fragment that allow the detection of *P. agardhii* in the analyzed freshwater systems are described below in Table 8.

Table 8. Detection of amplified fragments related to the presence of *P. agardhii* in the analyzed freshwater systems, using the rpoC1_Plank_F271/rpoC1_Plank_R472 primer set. The mark (+) indicates the presence of the amplified fragment and the mark (-) indicates the absence of the fragment in the analyzed sample, through PCR amplification. The diagonal line (/) means the absence of cyanobacterial DNA in the sample.

	Torrão Reservoir	Parque Fluvial do Tâmega	Parque da Cidade do Porto Lake 1	Parque da Cidade do Porto Lake 2	Parque da Cidade do Porto Lake 3	Vela Lake	Mira Lake
October	-	+	-	-	-	+	+
November	+	+	-	-	-	-	+
December	-	-	-	-	-	-	+
January	-	-	-	-	-	-	-
February	-	-	-	-	/	+	-
March	-	-	-	-	+	+	-
April	-	-	+	+	+	-	-
May	-	-	+	+	+	-	-
June	-	-	+	+	+	-	-

Analyzing the obtained results, expressed in Table 8, *P. agardhii* was detected in all sampling points. In terms of sampling dates, *P. agardhii* was not detected in any sample collected in January, being more frequent in samples collected in October,

November, April, May and June. Regarding to the spatial distribution, *P. agardhii* was mostly present in the 3 sampled lakes from Parque da Cidade do Porto, Vela Lake and Mira Lake, and less present in Torrão Reservoir and Parque Fluvial do Tâmega. It is also observable that the presence of *P. agardhii* in Torrão Reservoir, Parque Fluvial do Tâmega and Mira Lake is only detected in the early dates of the sampling, contrary to what is noticeable in the Parque da Cidade do Porto lakes, where the presence of this specie is only detectable in the final months of the sampling period.

3.2.2. Cyanotoxins

Assessment of the presence of the potential for cyanotoxin production in the analyzed freshwater systems was executed through the use of specific primers from genes that encode the biosynthesis of the studied cyanotoxins. The obtained results are described below in Tables 9-20.

3.2.2.1. Detection of microcystin potential

PCR results obtained from the amplification of the gene fragments implicated in the biosynthesis of microcystin in the analyzed freshwater systems are described below from Tables 9 to 14. The results for *mcy*G primer set are not described, since they were negative for all tested samples.

Table 9. Detection of amplified fragments related to the presence of microcystin potential in the analyzed freshwater systems, using the *mcy*A CD1F/CD1R primer set. The mark (+) indicates the presence of the amplified fragment and the mark (-) indicates the absence of the fragment in the analyzed sample, through PCR amplification. The diagonal line (/) means the absence of cyanobacterial DNA in the sample.

	Torrão Reservoir	Parque Fluvial do Tâmega	Parque da Cidade do Porto Lake 1	Parque da Cidade do Porto Lake 2	Parque da Cidade do Porto Lake 3	Vela Lake	Mira Lake
October	+	+	+	+	+	+	-
November	+	+	+	-	-	-	-
December	+	+	+	-	-	+	-
January	+	+	+	-	+	+	-
February	-	-	+	-	/	-	-
March	-	-	-	-	-	-	-
April	+	+	+	-	+	-	-
May	-	+	+	+	-	+	-
June	+	+	+	+	-	-	-

The presence of the amplified fragments of *mcy*A gene was observed in 6 of the 7 freshwater systems analyzed (Table 9). Through the sampling period, the *mcy*A gene was not detected in any sample collected in Mira Lake, being its presence also less expressive in Lakes 2 and 3 of Parque da Cidade do Porto and Vela Lake. The incidence of this gene was more pronounced in Lake 1 of Parque da Cidade do Porto, with the presence of amplified fragments in almost every collected sample, with exception for the sample collected in March. Through the analysis of Table 9 it is possible to observe that, in terms of temporal distribution, the presence of *mcy*A gene was not possible in any sample collected in March. The presence of *mcy*A gene was also less frequent in samples collected in February, with just one detection, in Lake 1 of Parque da Cidade do Porto, and November, with 3 detections, in Torrão Reservoir, Parque Fluvial do Tâmega and Lake 1 of Parque da Cidade do Porto.

Table 10. Detection of amplified fragments related to the presence of microcystin potential in the analyzed freshwater systems, using the *mcy*A MSF/MSR primer set, specific for *Microcystis sp*. The mark (+) indicates the presence of the amplified fragment and the mark (-) indicates the absence of the fragment in the analyzed sample, through PCR amplification. The diagonal line (/) means the absence of cyanobacterial DNA in the sample.

	Torrão Reservoir	Parque Fluvial do Tâmega	Parque da Cidade do Porto Lake 1	Parque da Cidade do Porto Lake 2	Parque da Cidade do Porto Lake 3	Vela Lake	Mira Lake
October	+	-	+	+	+	-	-
November	-	-	-	-	-	-	-
December	-	-	-	-	-	-	-
January	-	-	-	-	-	-	-
February	-	-	+	-	/	-	-
March	-	-	-	-	-	-	-
April	+	-	+	-	-	-	-
May	-	-	+	+	-	-	-
June	-	-	-	-	-	+	-

Through the analysis of Table 10, it is possible to perceive that the presence of amplified fragments, resultant of the analysis of MSF/MSR primer set, specific for *Microcystis sp*., is more recurrent in samples collected in points located in the North Region of Portugal, rather than in points located in the Center Region. Regarding the spatial distribution, the presence of *mcy*A gene specific for this species was not detectable in any of the samples collected in Parque Fluvial do Tâmega and Mira Lake. For Lake 3 of Parque da Cidade do Porto and Vela Lake, this gene was only detected in one sample, collected in October and June, respectively. The incidence of this gene was more expressive in Lake 1 of Parque da Cidade do Porto, with detection in 4 out of 9 samples (October, February, April and May). Concerning the temporal distribution,

the gene was mostly detected in samples collected in October, without any record of its presence in samples collected in November, December, January and March.

Table 11. Detection of amplified fragments related to the presence of microcystin potential in the analyzed freshwater systems, using the *mcy*B 2156-F/3111-R primer set, specific for *Microcystis sp*. The mark (+) indicates the presence of the amplified fragment and the mark (-) indicates the absence of the fragment in the analyzed sample, through PCR amplification. The diagonal line (/) means the absence of cyanobacterial DNA in the sample.

	Torrão Reservoir	Parque Fluvial do Tâmega	Parque da Cidade do Porto Lake 1	Parque da Cidade do Porto Lake 2	Parque da Cidade do Porto Lake 3	Vela Lake	Mira Lake
October	+	+	+	+	+	+	-
November	+	-	+	-	-	-	-
December	+	+	-	-	-	-	-
January	-	-	-	-	-	-	-
February	-	-	-	-	/	-	-
March	-	-	-	-	-	-	-
April	+	-	+	-	-	-	-
May	-	-	+	+	-	-	-
June	-	-	+	+	-	-	-

Examining the obtained results, expressed on Table 11, it is possible to see that the presence of the *mcy*B gene is higher in samples collected in sampling points located in the North Region rather than in samples collected in sampling points located in the Center Region. In the sampling locations located in the Center Region, the presence of this gene was only detected in a sample collected in October on Vela Lake, not being in this way any record of its presence in Mira Lake. Regarding the presence of *mcy*B gene in samples collected in the sampling points from the North Region, its presence is more expressive in Lake 1 of Parque da Cidade do Porto, and Torrão Reservoir, since Lake 3 of Parque da Cidade do Porto only registered the presence of *mcy*B gene in one sample collected in October. Temporally, the majority of detections were registered on October, being the remaining months with record of two detections each, with exception for the period between January and March, in which none of the collected samples demonstrate the presence of the gene.

Table 12. Detection of amplified fragments related to the presence of microcystin potential in the analyzed freshwater systems, using the *mcy*C PSCF1/PSCR1 primer set, specific for *Microcystis sp*. The mark (+) indicates the presence of the amplified fragment and the mark (-) indicates the absence of the fragment in the analyzed sample, through PCR amplification. The diagonal line (/) means the absence of cyanobacterial DNA in the sample.

	Torrão Reservoir	Parque Fluvial do Tâmega	Parque da Cidade do Porto Lake 1	Parque da Cidade do Porto Lake 2	Parque da Cidade do Porto Lake 3	Vela Lake	Mira Lake
October	+	+	+	+	+	+	-
November	+	+	+	-	-	-	-
December	-	+	-	-	-	-	-
January	-	-	+	-	+	+	-
February	-	-	+	-	/	-	-
March	-	-	+	-	-	-	-
April	+	-	+	+	-	-	-
May	-	-	+	+	-	-	-
June	+	+	+	+	+	-	-

The presence of *mcy*C gene, associated with the microcystin potential for production by *Microcystis sp*., was detected in 6 out of the 7 freshwater systems analyzed in this study. In Table 12, it is possible to observe that in Mira Lake there are no records of the presence of this gene in any of the samples collected during the sampling period. Its presence was also less expressive in Vela Lake, with only two detections in October and January, and Lake 3 of Parque da Cidade do Porto, with detections in October, January and June. In Lake 1 of Parque da Cidade do Porto, the presence of *mcy*C gene is detectable through the entire sampling period, with exception for the sample collected in December, in which this gene was absent. Regarding the temporal distribution, the presence of *mcy*C was less frequent in December, February and March, with one detection in Parque Fluvial do Tâmega and Lake 1 of Parque da Cidade do Porto (Table 12). In October and June, the presence of the studied gene was more frequent in the respective collected samples.

Table 13. Detection of amplified fragments related to the presence of microcystin potential in the analyzed freshwater systems, using the *mcy*D PKDF1/PKDR1 primer set, specific for *Microcystis sp*. The mark (+) indicates the presence of the amplified fragment and the mark (-) indicates the absence of the fragment in the analyzed sample, through PCR amplification. The diagonal line (/) means the absence of cyanobacterial DNA in the sample.

	Torrão Reservoir	Parque Fluvial do Tâmega	Parque da Cidade do Porto Lake 1	Parque da Cidade do Porto Lake 2	Parque da Cidade do Porto Lake 3	Vela Lake	Mira Lake
October	+	+	+	+	+	+	-
November	+	+	+	-	-	+	-
December	+	+	+	-	-	-	+
January	+	+	+	-	-	+	+
February	+	+	+	-	/	-	-
March	+	+	+	-	-	-	-
April	+	+	+	-	+	-	-
May	-	+	+	+	-	-	-
June	-	-	+	+	-	-	-

Analysis of Table 13, concerning the presence of *mcy*D gene, show that the incidence of the presence of amplified fragments of this gene was higher on Torrão Reservoir, Parque Fluvial do Tâmega and Lake 1 of Parque da Cidade do Porto, in comparison with the results achieved in the remaining sampling points. In the first three sampling points, the *mcy*D gene was not detected in samples collected in May in Torrão Reservoir, and in June in Torrão Reservoir and Parque Fluvial do Tâmega. The temporal distribution of the presence of amplified fragments in Lakes 2 and 3 of Parque da Cidade do Porto, and Vela and Mira Lake, does not allow the establishment of an incidence pattern that could relate the obtained data with the seasonal period of this sampling campaign.

Table 14. Detection of amplified fragments related to the presence of microcystin potential in the analyzed freshwater systems, using the *mcy*E PKEF1/PKER1 primer set, specific for *Microcystis sp*. The mark (+) indicates the presence of the amplified fragment and the mark (-) indicates the absence of the fragment in the analyzed sample, through PCR amplification. The diagonal line (/) means the absence of cyanobacterial DNA in the sample.

	Torrão Reservoir	Parque Fluvial do Tâmega	Parque da Cidade do Porto Lake 1	Parque da Cidade do Porto Lake 2	Parque da Cidade do Porto Lake 3	Vela Lake	Mira Lake
October	+	+	+	+	+	+	-
November	+	+	+	-	-	-	-
December	+	+	-	-	-	-	-
January	+	+	+	-	+	+	-
February	-	+	+	-	/	-	-
March	-	-	-	-	-	-	-
April	-	-	-	-	-	-	-
May	-	+	+	+	-	-	-
June	+	+	+	+	-	-	-

Through the study of the results obtained by the analysis of the *mcy*E gene, expressed in Table 14, is possible to observe an absence of positive amplifications in samples collected on Mira Lake, being the highest incidence registered in samples collected in Torrão Reservoir, Parque Fluvial do Tâmega and Lake 1 of Parque da Cidade do Porto. It is also observable that, in both March and April, none of the collected samples had the presence of the *mcy*E gene. In terms of sampling dates, *mcy*E gene was more frequent in samples collected in October, January and June, with fewer incidences in December and February.

3.2.2.2. Detection of cylindrospermopsin potential

PCR results obtained from the amplification of the gene fragments implicated in the biosynthesis of cylindrospermopsin in the analyzed freshwater systems are described below in Tables 15 and 16. The results for *cyr*A and *cyr*J primer set are not described, since they were negative for all tested samples.

Table 15. Detection of amplified fragments related to the presence of cylindrospermopsin potential in the analyzed freshwater systems, using the *cyr*B M13/M14 primer set. The mark (+) indicates the presence of the amplified fragment and the mark (-) indicates the absence of the fragment in the analyzed sample, through PCR amplification. The diagonal line (/) means the absence of cyanobacterial DNA in the sample.

	Torrão Reservoir	Parque Fluvial do Tâmega	Parque da Cidade do Porto Lake 1	Parque da Cidade do Porto Lake 2	Parque da Cidade do Porto Lake 3	Vela Lake	Mira Lake
October	-	-	-	+	-	-	-
November	-	-	-	-	-	-	-
December	-	-	-	-	-	-	-
January	-	-	-	-	-	-	-
February	-	-	-	-	/	-	-
March	-	-	-	-	-	-	+
April	-	-	-	-	-	-	-
May	-	-	-	-	-	-	-
June	-	-	+	-	-	-	-

The presence of amplified fragments corresponding to the gene *cyr*B, detected by using M13/M14 primer set was only observed in 3 samples from 3 freshwater systems in study (Table 15). Over the sampling period, the *cyr*B gene was only detected in a sample from Lake 2 of Parque da Cidade do Porto in October, a sample from Mira Lake in March and a sample from Lake 1 of Parque da Cidade do Porto in June.

Table 16. Detection of amplified fragments relaed to the presence of cylindrospermopsin potential in the analyzed freshwater systems, using the *cyr*C K18/M4 primer set. The mark (+) indicates the presence of the amplified fragment and the mark (-) indicates the absence of the fragment in the analyzed sample, through PCR amplification. The diagonal line (/) means the absence of cyanobacterial DNA in the sample.

	Torrão Reservoir	Parque Fluvial do Tâmega	Parque da Cidade do Porto Lake 1	Parque da Cidade do Porto Lake 2	Parque da Cidade do Porto Lake 3	Vela Lake	Mira Lake
October	-	-	-	-	-	-	-
November	-	-	-	-	-	-	-
December	-	-	-	-	-	-	-
January	+	+	-	-	-	-	-
February	-	-	-	-	/	-	+
March	-	-	-	-	-	-	-
April	+	-	-	-	-	-	-
May	-	-	-	-	-	-	-
June	+	+	-	-	-	+	+

The occurrence of *cyr*C gene in the analyzed samples was observed in 4 of the 7 freshwater systems in study (Table 16). Regarding the sampling locations, the *cyr*C gene was not detected in collected samples from Parque da Cidade do Porto. Its detection occurred only in samples from Torrão Reservoir, where it was noticeable a higher incidence of the presence of this gene, and in samples from Parque Fluvial do Tâmega, Vela Lake and Mira Lake. About the temporal distribution, the presence of this gene was only registered in samples collected in January, February, April and June, being that in February and April, there is only one occurrence in Mira Lake and Torrão Reservoir, respectively.

3.2.2.3. Detection of anatoxin-a potential

PCR results obtained from the amplification of the gene fragment implicated in the biosynthesis of anatoxin-a in the analyzed freshwater systems are described below in Table 17. The results for anaC-anab primer set are not described, since they were negative for all tested samples.

Table 17. Detection of the amplified fragments related to the presence of anatoxin-a potential in the analyzed freshwater systems. The mark (+) indicates the presence of the amplified fragment and the mark (-) indicates the absence of the fragment in the analyzed sample, through PCR amplification. The diagonal line (/) means the absence of cyanobacterial DNA in the sample.

	Torrão Reservoir	Parque Fluvial do Tâmega	Parque da Cidade do Porto Lake 1	Parque da Cidade do Porto Lake 2	Parque da Cidade do Porto Lake 3	Vela Lake	Mira Lake
October	-	-	+	+	-	-	-
November	-	-	-	-	-	-	-
December	-	-	-	-	-	-	-
January	+	+	-	-	-	-	-
February	-	+	-	-	/	-	-
March	-	-	-	-	-	-	-
April	+	-	-	-	-	-	-
May	+	-	+	-	-	-	-
June	-	-	+	-	-	-	-

Analyzing the obtained results, described in Table 17, is observable the presence of *ana*C gene in samples collected in 4 sampling points located in the North Region of Portugal, without any report of its presence in the sampling points from Center Region. Through the sampling period, *ana*C gene was not detected in any sample collected in Lake 3 of Parque da Cidade do Porto, as well as in Vela Lake and Mira Lake. The presence of this cyanotoxin in the analyzed freshwater systems was also less expressive in Lake 2 of Parque da Cidade do Porto, where it was only detected in a single sample collected in October. Detection of anatoxin-a potential was more expressive in samples collected in Torrão Reservoir and Lake 1 of Parque da Cidade do Porto, with 3 positive results, followed by Parque Fluvial do Tâmega and Lake 2 of Parque da Cidade do Porto. In terms of temporal distribution, the *ana*C gene was not identified in any sample collected in November, December and March, although its presence was not relatively dominant in any sampling month.

3.2.2.4. Detection of saxitoxin potential

PCR results obtained from the amplification of the gene fragments implicated in the biosynthesis of saxitoxin in the analyzed freshwater systems are described below in Table 18-20.

Table 18. Detection of amplified fragments related to the presence of saxitoxin potential in the analyzed freshwater systems, using the *sxt*A 855_F/1480_R primer set. The mark (+) indicates the presence of the amplified fragment and the mark (-) indicates the absence of the fragment in the analyzed sample, through PCR amplification. The diagonal line (/) means the absence of cyanobacterial DNA in the sample.

	Torrão Reservoir	Parque Fluvial do Tâmega	Parque da Cidade do Porto Lake 1	Parque da Cidade do Porto Lake 2	Parque da Cidade do Porto Lake 3	Vela Lake	Mira Lake
October	+	-	-	+	-	-	-
November	+	-	-	-	-	+	-
December	-	-	-	-	-	-	-
January	-	-	-	-	-	-	-
February	-	-	-	-	/	-	-
March	-	-	-	-	-	-	-
April	-	-	-	-	-	-	-
May	-	-	-	-	-	-	-
June	-	-	-	-	-	-	-

Detection of *sxt*A gene was positive in 4 samples collected in 3 sampling points in study (Table 18). In terms of spatial distribution, this gene was only present in samples collected in Torrão Reservoir, Lake 2 of Parque da Cidade do Porto and Vela Lake, with more incidence in Torrão Reservoir, with two positive results. Temporally, the *sxt*A gene was only detectable in samples collected in October and November, with no record of positive results in other sampling months.

Table 19. Detection of amplified fragments related to the presence of saxitoxin potential in the analyzed freshwater systems, using the *sxt*I 682F/877R primer set. The mark (+) indicates the presence of the amplified fragment and the mark (-) indicates the absence of the fragment in the analyzed sample, through PCR amplification. The diagonal line (/) means the absence of cyanobacterial DNA in the sample.

	Torrão Reservoir	Parque Fluvial do Tâmega	Parque da Cidade do Porto Lake 1	Parque da Cidade do Porto Lake 2	Parque da Cidade do Porto Lake 3	Vela Lake	Mira Lake
October	+	+	+	+	+	+	-
November	+	+	-	+	-	+	-
December	+	-	-	+	-	+	-
January	-	-	-	-	-	+	-
February	-	-	-	-	/	-	+
March	+	-	-	-	-	-	-
April	+	-	-	-	-	-	-
May	-	-	-	+	-	+	+
June	-	-	-	-	-	+	+

The existence of *sxt*I gene in the analyzed freshwater systems was observed in all sampling points, as described in Table 19. Through the sampling period, the presence of the *sxt*I gene was more expressive in Torrão Reservoir and Vela Lake, being less expressive in Parque Fluvial do Tâmega, and Lakes 1 and 3 of Parque da Cidade do Porto. In terms of temporal distribution, *sxt*I was more frequent in the first months of the sampling period, October and November, being less frequent between January and April, with one detection in each sampling month.

Table 20. Detection of the amplified fragments related to the presence of saxitoxin potential in the analyzed freshwater systems, using the *sxt*G 432_F/928_R primer set. The mark (+) indicates the presence of the amplified fragment and the mark (-) indicates the absence of the fragment in the analyzed sample, through PCR amplification. The diagonal line (/) means the absence of cyanobacterial DNA in the sample.

	Torrão Reservoir	Parque Fluvial do Tâmega	Parque da Cidade do Porto Lake 1	Parque da Cidade do Porto Lake 2	Parque da Cidade do Porto Lake 3	Vela Lake	Mira Lake
October	+	-	-	+	+	+	-
November	+	-	-	+	-	+	-
December	-	-	-	-	-	-	-
January	-	-	-	-	-	-	-
February	-	-	-	-	/	-	-
March	-	-	-	-	-	-	-
April	-	-	-	-	-	-	-
May	-	-	-	-	-	-	-
June	-	-	-	-	-	-	-

Occurrence of *sxt*G gene was reported in 4 samples collected in 4 of the 7 analyzed freshwater systems, as described in Table 20. Its presence is expressed in samples collected in the months of October and November in Torrão Reservoir, Lake 2 of Parque da Cidade do Porto and Vela Lake, and in one sample collected on Lake 3 of Parque da Cidade do Porto, in October. As *sxt*A gene (Table 18), the *sxt*G gene was only detectable in samples collected in October and November, with no record of positive results in other sampling months.

3.2.3. Global analysis – Cyanobacteria and cyanotoxin potential

The results obtained in the detection of the presence of *Microcystis sp*., *M. aeruginosa*, *C. raciborskii* and *P. agardhii*, as well as the detection of the potential for microcystin, cylindrospermopsin, anatoxin-a and saxitoxin production by cyanobacteria were examined and its analysis summarized in the Tables and Figures below.

3.2.3.1. Global analysis of Cyanobacteria

The assessment of the presence of *Microcystis sp*., *M. aeruginosa*, *C. raciborskii* and *P. agardhii* and its frequency through the sampling period is summarized on Table 21 and Figures 12 and 13.

Table 21. Global analysis of the detection of the cyanobacteria species in study for the seven freshwater systems analyzed. The mark (+) indicates the detection of the cyanobacterial species in at least one sampling month and the mark (-) represents the non-detection of the species throughout the entire sampling period.

	Torrão Reservoir	Parque Fluvial do Tâmega	Parque da Cidade do Porto Lake 1	Parque da Cidade do Porto Lake 2	Parque da Cidade do Porto Lake 3	Vela Lake	Mira Lake
Microcystis sp.	+	+	+	+	+	+	+
M. aeruginosa	+	+	+	+	+	+	+
C. raciborskii	-	+	+	+	+	+	+
P. agardhii	+	+	+	+	+	+	+

Analyzing the summarized results, it is possible to observe that Torrão Reservoir was the unique sampling location whose presence of *C. raciborskii* was not detected throughout the entire sampling period. In the remaining sampling points, the detection of *Microcystis sp*., *M. aeruginosa*, *C. raciborskii* and *P. agardhii* was always possible in at least, one sampling month.

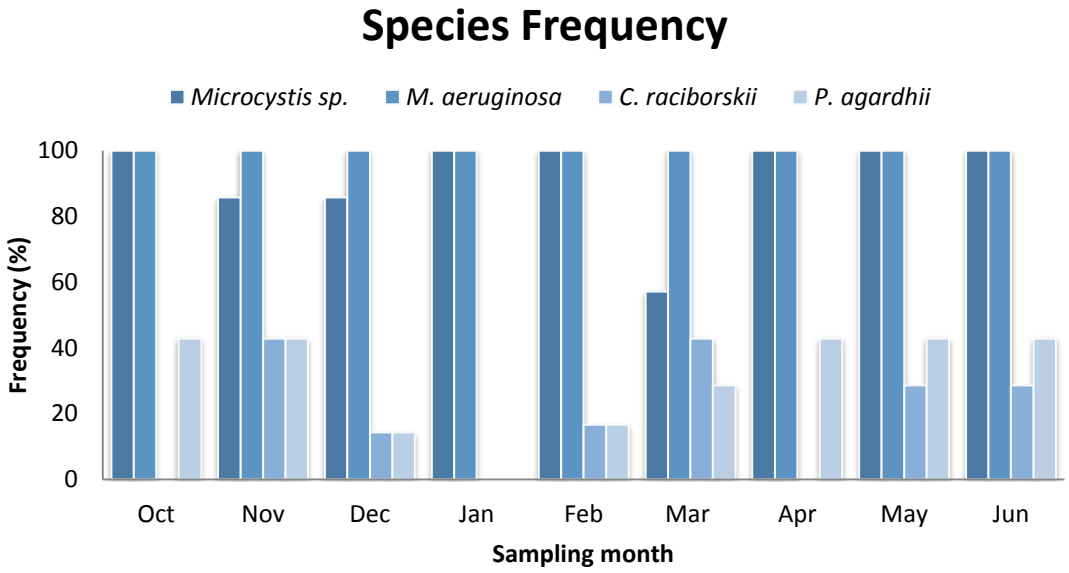

Fig. 12. Frequency of detection of *Microcystis sp*., *M. aeruginosa*, *C. raciborskii* and *P. agardhii*, per sampling month.

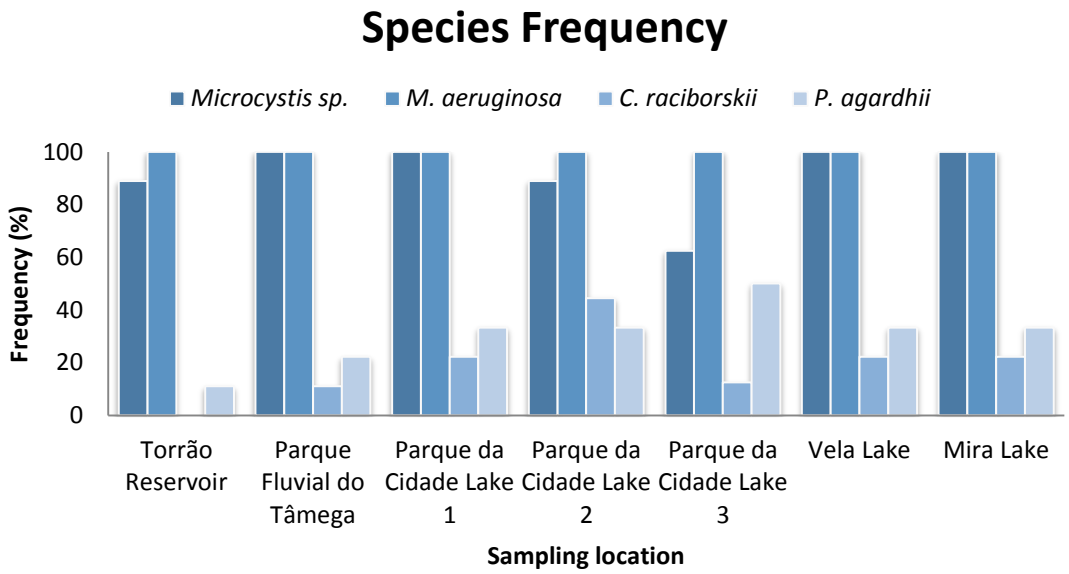

Fig. 13. Frequency of detection of *Microcystis sp*., *M. aeruginosa, C. raciborskii* and *P. agardhii*, per sampling location.

Figures 12 and 13 represent the variation in the frequency of the cyanobacterial species in study throughout the sampling period and sampling location.

Temporally, *Microcystis sp*. and *M. aeruginosa*, present themselves as the most dominant genera and species through the entire sampling period (Figure 12). Exception goes for March, where the frequency of *Microcystis sp*. was almost half of the frequency of *M. aeruginosa*. In this sampling period, *C. raciborskii* was not detected in any sampling point in samples collected on October, January and April. Otherwise

November and March were the months that registered the highest frequency of this cyanobacterial species in the studied freshwater points. For the study of *P. agardhii*, it is possible to observe that its frequency was almost constant through the entire sampling period, with exception for January, where this species was not detected, and December and February, where it registered its lowest frequency in the entire sampling period.

Geographically, *M. aeruginosa* was the most dominant species, with record of its presence in all samples collected in every sampling location in study (Figure 13). Analysis of *Microcystis sp*., showed that this was the dominant genera, with presence in almost every sample collected in all sampling points. Exception was in Lake 3 of Parque da Cidade do Porto, where its detection had its lowest frequency, comparing with *M. aeruginosa*. For *C. raciborskii*, the results showed that in Torrão Reservoir this species was not detected in any of the collected samples through the entire sampling period, otherwise registering its highest frequency in Lake 2 of Parque da Cidade do Porto, with 4 detections through the sampling period. The study of spatial distribution of *P. agardhii* pointed out that its presence was greater in Lake 3 of Parque da Cidade do Porto, in comparison with other sampling points. In Torrão Reservoir, *P. agardhii* was only detected in one sampling during the entire sampling period, being the record of detection of this species constant in the remaining sampling points.

3.2.3.2. Global analysis of cyanotoxin potential

The results obtained for the detection of gene clusters associated with the production of microcystin, cylindrospermopsin, anatoxin-a and saxitoxin and its frequency through the sampling period are summarized on Table 22 and Figures 14 and 15. The co-occurrence of gene clusters for the production of hepatotoxins and neurotoxins is summarized on Table 23.

Table 22. Global analysis of the detection of the cyanotoxin potential for production in study for the seven freshwater systems analyzed. The mark (+) indicates the detection of the gene in at least one sampling month and the mark (-) represents the non-detection of the gene throughout the entire sampling period.

	Torrão Reservoir	Parque Fluvial do Tâmega	Parque da Cidade do Porto Lake 1	Parque da Cidade do Porto Lake 2	Parque da Cidade do Porto Lake 3	Vela Lake	Mira Lake
mcy	+	+	+	+	+	+	+
cyr	+	+	+	+	-	+	+
anaC	+	+	+	+	-	-	-
sxt	+	+	+	+	+	+	+

The results, expressed in Table 22, show that *mcy* and *sxt* genes were detected in all sampling sites, at least once during the sampling period. Detection of *cyr* gene was possible in 6 out of 7 sampling locations, with exception for Lake 3 of Parque da Cidade do Porto, where this gene was not detected in any sample collected during this sampling period. Regarding the study of *anaC* gene, this gene was only detected in samples collected in Torrão Reservoir, Parque Fluvial do Tâmega and Lakes 1 and 2 of Parque da Cidade do Porto, having no record of detection in samples collected in the remaining sampling sites.

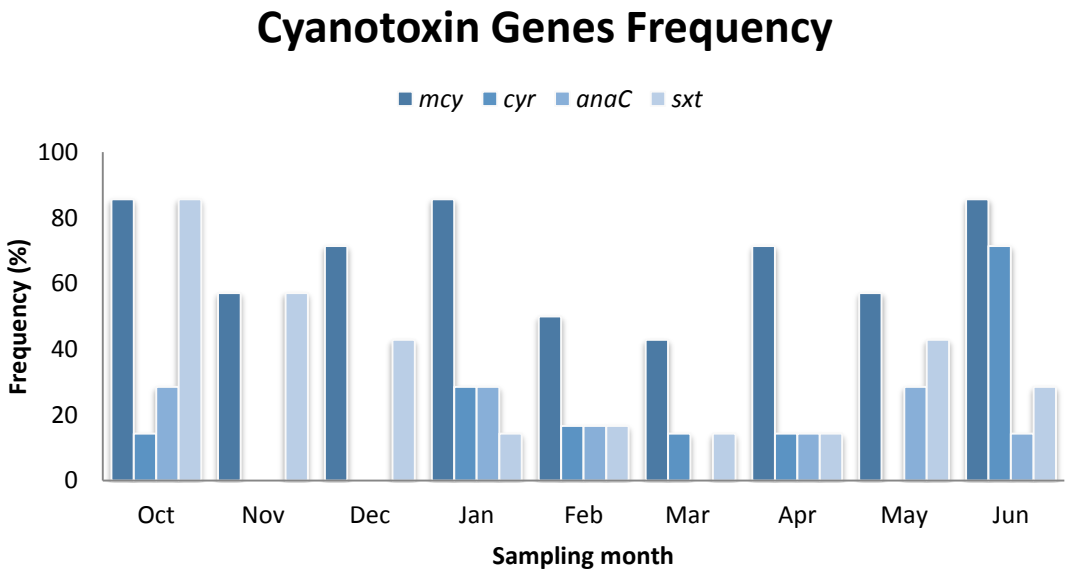

Fig. 14. Frequency of detection of *mcy*, *cyr*, *anaC* and *sxt* genes, per sampling month.

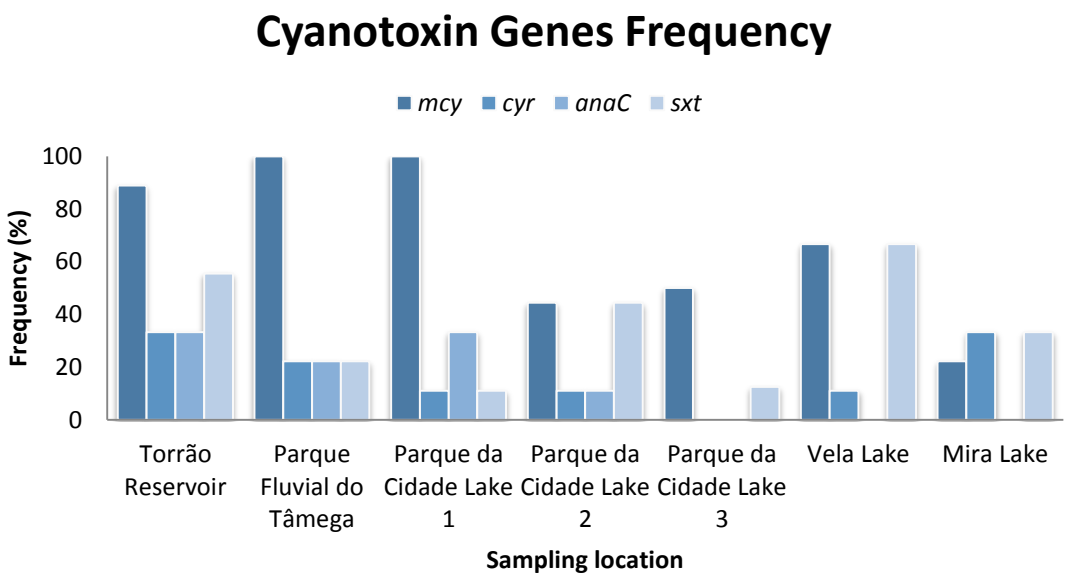

Fig. 15. Frequency of detection of *mcy*, *cyr*, *anaC* and *sxt* genes, per sampling location.

Data shown in Figures 14 and 15 represent the variation in the frequency of the genes related to cyanotoxin biosynthesis throughout the sampling period and sampling location.

In terms of sampling period (Figure 14), the *mcy* gene was the most dominant gene detected in the collected samples, being detected in at least half the samples collected in each sampling month. Its incidence was higher in samples collected in October, January and June, registering its lowest frequency in February and March. Regarding the presence of *cyr* gene, this was only detectable in samples collected in October, in the period comprehended between January and April, registering a sudden increase in its frequency in June, not having records of its detection in November, December and May in any of the collected samples. The study of *anaC* gene showed that its presence was not detectable in any sample collected in November, December and March, having an almost constant frequency in the remaining sampling months. Results for *sxt* gene demonstrate that this gene was usually the second most dominant gene present in the analyzed samples. This gene had its highest frequencies in the first 3 months of the sampling period, and also in May, with record of its presence in collected samples in all sampling months. It is also possible to observe that *sxt* was only surpassed in its frequency by *anaC* and/or *cyr* gene in January and June.

Regarding the geographic distribution of cyanotoxin genes (Figure 15), the results show that these genes had a high incidence in samples collected in Torrão Reservoir, especially the *mcy* and *sxt* genes. The presence of *mcy* genes was lower in samples

collected in Mira Lake, dominated, in a certain way, by genes related to neurotoxic potential. Observing the results obtained for the detection of the *cyr* gene, it is possible to perceive a decrease in the frequency of this gene throughout the sampling locations, until there is no record of its presence in Lake 3 of Parque da Cidade do Porto, and then an increase in the frequency recorded in Vela and Mira Lake. Results for *anaC* gene show that the presence of this gene was only registered in sampling points located in the North Region of Portugal, with exception for Lake 3 of Parque da Cidade do Porto, with no record of the presence of this gene throughout the sampling period. The presence of *sxt* gene was recorded mostly in samples collected on Vela Lake, Torrão Reservoir and Lake 2 of Parque da Cidade do Porto, having its lowest frequency on Lakes 1 and 3 of Parque da Cidade do Porto. Despite this fact, the presence of this gene was detected in all the studied sampling sites.

Table 23. Global analysis of the co-occurrence of genes for hepatotoxin and neurotoxin production in study for the seven freshwater systems analyzed. The mark (+) indicates the detection of the analyzed genes in at least one sampling month and the mark (-) represents the non-detection of the gene throughout the entire sampling period.

	Torrão Reservoir		Parque Fluvial do Tâmega		Parque da Cidade do Porto Lake 1		Parque da Cidade do Porto Lake 2		Parque da Cidade do Porto Lake 3		Vela Lake		Mira Lake	
	Hep	Neu	Hep	Neu	Hep	Neu	Hep	Neu	Hep	Neu	Hep	Neu	Hep	Neu
October	+	+	+	+	+	+	+	+	+	+	+	+	-	-
November	+	+	+	+	+	-	-	+	-	-	+	+	-	-
December	+	+	+	-	+	-	-	+	-	-	+	+	+	-
January	+	+	+	+	+	-	-	-	+	-	+	+	+	-
February	+	-	+	+	+	-	-	-	-	-	-	-	+	+
March	+	+	+	-	+	-	-	-	-	-	-	-	+	-
April	+	+	+	-	+	-	+	-	+	-	-	-	-	-
May	-	+	+	-	+	+	+	+	-	-	+	+	-	+
June	+	-	+	-	+	+	+	-	+	-	+	+	+	+

Co-occurrence of genes related to cyanotoxin production was registered in all sampling sites throughout the sampling period (Table 23). Comparing data referent to hepatotoxins (considering, for this case, cylindrospermopsin as a hepatotoxin) and neurotoxins, it is possible to observe that the occurrence of both groups of genes is recurrent through the several months. This situation is highlighted when analyzing the data obtained in both Torrão Reservoir and Vela Lake, where in 6 out of the 9 months of sampling period is registered the co-occurrence of both genes associated with the production of hepatotoxins and neurotoxins. This situation is less frequent on Lakes 2 and 3 of Parque da Cidade do Porto and Mira Lake, where the co-occurrence is only registered 1 or 2 times during the entire sampling period. In terms of temporal

distribution, the simultaneously presence of genes related to both hepatotoxic and neurotoxic potential is most visible in October, where is registered in 6 out of 7 sampling sites. In the remaining sampling months, the situation of co-occurrence decrease through the sampling period, registering its lower frequency in March and April, with only one occurrence, and then increasing again its frequency in May and June.

4. Discussion

The detection of cyanobacteria and genes related to the production of cyanotoxins in the several sampling sites in study shows that the temperature conditions were appropriated for the proliferation of cyanobacteria. According to the literature, cyanobacteria have their most favorable growth phase when temperatures are between 15°C and 30°C. It should be noted that variations in the physical/chemical parameters of the water systems may originate large variations on the cyanobacteria community (Vasconcelos, 1993; Vale, 2005; Funari and Testal, 2008). In this study, the atmospheric temperature registered was often below 15°C, especially during the winter months, however, it was possible the detection of cyanobacteria and genes associated with cyanotoxin production. Although the temperature data presented in this work refer to the atmospheric temperature registered at the sampling day, it is demonstrated that the atmospheric temperature influences directly the water temperature (Vale, 2005).

The application of molecular methods in the detection of cyanobacteria and cyanotoxin production related genes allows the analysis of the cyanobacterial community in a freshwater system. Confirmation of the presence of cyanobacterial DNA in the collected samples was made through the amplification of the 16S rRNA and phycocyanin genes, which revealed to be positive for every sample except the one collected in February 2017 in Lake 3 of Parque da Cidade do Porto. The non-detection of cyanobacterial DNA in this sample may be possibly due to a concentration of cyanobacterial biomass present in the water system that is below the detection limit of the PCR technique. Another reason may be due to the absence of aggregates or colonies of cyanobacteria in the collected sample, since the water in that sampling point on February was clearer than in the other months and collection points (*in situ* observation).

Microcystis sp., and particularly *M.aeruginosa*, is a cyanobacterium species with a global distribution (van Gremberghe *et al*., 2011). In this study, *M. aeruginosa* was present in all sampling sites throughout the entire sampling period, this way confirming its global distribution and dominance in freshwater systems. Its presence, even when the temperature is below the optimal temperature for cyanobacterial development, can be explained by the physiology of the species itself. *M. aeruginosa*, at a cellular level, possesses thick cell walls, intracellular gas vesicles for buoyancy and also possesses the capacity to form colonies or aggregates (Tanabe *et al*., 2007; van Germberghe *et al*., 2011). When forming aggregates, these are protected by a mucilage layer, which

also facilitates its dispersions and capacity to tolerate more extreme conditions, such as low temperatures (Wu *et al*., 2008). According to Bittencourt-Oliveira et al. (2001), the occurrence of several *M. aeruginosa* genotypes in the same water system can explain its high dispersion potential and dominance, in comparison with other cyanobacterium species. In Portugal, the presence of this species had already been reported on the studied freshwater systems, in Torrão Reservoir and Parque Fluvial do Tâmega (Regueiras, 2009; Matos, 2014), the three sampled lakes of Parque da Cidade do Porto (Morais, 2009; Matos, 2014), Vela and Mira Lakes (de Figueiredo *et al*., 2006; Freitas, 2009; Matos, 2014), maintaining its report in this work in all the analyzed sampling sites.

In this work, the presence of *C. raciborskii* in Portuguese freshwater systems was the least frequent of the three cyanobacterium species in study. Despite this fact, its presence was detected mostly on samples collected on Lake 2 of Parque da Cidade do Porto, with a punctual presence in some sampling sites, already described in the results section. Its presence had already been reported in all the analyzed sampling locations (Freitas, 2009; Matos, 2014), with exception for Torrão Reservoir, which still does not have a report of the presence of this species. However, it is to highlight that the non-detection of this species, or its low frequency, does not mean its absence from the water system. This non-detection can be related to a low cell number, below the detection limit of the PCR method, or the occurrence of blooms of other cyanobacterial species, usually from the genera *Microcystis*, frequently dominant in freshwater systems (Huisman *et al*., 2005). Also, the low temperatures registered during the sampling period may have influenced the frequency of this species in the diverse freshwater systems, despite its capability to proliferate at temperatures as low as 11°C (Bonilla *et al*., 2012). This species was not detected in any sample collected in October, January and April, being noteworthy that, in January the registered temperatures were close to 0°C. Nonetheless, it is to mention that other parameters, not evaluated in this study, such as pH, nutrient concentration or oxygen availability can directly influence the frequency of this cyanobacterial species in the water system (Sze, 1986).

Detection of *P. agardhii* was possible in all sampling sites, contrary to what happened with *C. raciborskii*, other invasive species in Portuguese freshwater systems. Despite not being as frequent as *M. aeruginosa*, its presence was registered throughout the sampling period, with exception for samples collected in January, where its presence was not detected in any of the studied sampling locations. *P. agardhii* was detected mostly on the sampling sites from the Center Region, especially in the

beginning of the sampling period in Mira Lake and in the 3 lakes of Parque da Cidade do Porto, on the final months of the sampling period. In Portugal, the presence of strains of *Planktothrix sp*. had been reported in samples collected in Parque da Cidade do Porto (Morais, 2009). However, the presence of *P. agardhii* in Portuguese freshwater systems had only been reported in the study conducted by Matos (2014), analyzing the same locations as this study, and in the South Region of Portugal (Churro *et al*., 2012). Despite the optimal temperature for cyanobacteria ranges between 15°C and 30°C (Vale, 2005), *P. agardhii* can grow in temperatures as low as 10°C (Bonilla *et al*., 2012), which can explain its presence in sampling months where the range of temperatures registered was below the stipulated optimal temperature range. This can be observed in the frequency of *P. agardhii* registered throughout the sampling period, despite its presence decreased in the sampling months where the temperatures were below 10ºC

The study of genes related to cyanotoxin production, through PCR analysis, is based on the amplification of fragments of the peptide chain that is involved in the biosynthesis of cyanotoxins (Pearson and Neilan, 2008). In this study, it was assessed the presence of genes involved in the biosynthesis of microcystin (*mcy*), cylindrospermopsin (*cyr*), anatoxin-a (*anaC*) and saxitoxin (*sxt*).

For the study of the potential for microcystin production, the amplification of the *mcy* gene, *mcy*A-E and *mcy*G, was performed. The results showed the presence of at least one gene in all sampling sites, throughout the entire sampling period, with exception for *mcy*G, whose results were negative in all tested samples. The incidence of *mcy* genes, in general, was higher in Torrão Reservoir, Parque Fluvial do Tâmega and Lake 1 of Parque da Cidade do Porto, and temporally, in samples collected in October and, in the detection of *mcy*A, *mcy*D and *mcy*E, January. In terms of genes, the incidence was higher for the detection of *mcy*A, a nonribosomal peptide synthetase (NRPS) module, and *mcy*D, a Polyketide Synthase (PKS) module (Dittmann *et al*., 2013), in the analyzed samples. The positive amplification of the *mcy*B-E gene, whose presence is specific in *Microcystis sp.* genome, in most of the analyzed samples shows the high presence of microcystin encoding genes in the water ecosystems. These results are in agreement with the results obtained in the analysis of the presence of *Microcystis sp*. and *M. aeruginosa*, which showed the dominance of these genera in the analyzed freshwater systems. The *mcy*C gene is a NRPS module linked to the incorporation of amino acids in the peptide chain when occurs the biosynthesis of microcystin (Dittmann *et al*., 2013), like *mcy*A and *mcy*B gene. The *mcy*E gene is a PKS module related to the formation of the skeleton of Adda amino acid and also

related to the incorporation, in this amino acid, of side chain modifications (Dittmann *et al*., 2013). The presence of this gene is related to the toxicity presented by microcystins when produced (Ouahid *et al*., 2008). However, its presence does not necessarily mean that the production of microcystin is occurring in the analyzed freshwater systems. In Portugal, the presence of genes related to microcystin biosynthesis in the freshwater systems analyzed in this work is well documented and the presence of microcystin in these freshwater systems was also reported in several studies (Vasconcelos 1995; 1999; 2001; Vasconcelos *et al*., 1996; Saker *et al*., 2007; Freitas, 2009; Morais, 2009; Regueiras, 2009; Mendes, 2014). The present study, confirms once more, the dominance of the presence of the microcystins encoded genes in Portuguese freshwater systems which highlights the need of the guidelines establishment for this toxin.

The identification of *cyr* gene showed that this gene was present in almost every sampling sites studied, with the exception of Lake 3 from the Parque da Cidade do Porto. This detection was performed through the use of 2 genes, *cyr*B and *cyr*C, with non-coincident results between them. For *cyr*B gene, related to a NRPS/PKS hybrid module (Moreira et al., 2012), its presence was detected in Lakes 1 and 2 of Parque da Cidade do Porto, and in a sample collected in March in Mira Lake. In the study of *cyr*C gene, a PKS module linked to the elongation of the peptide chain with an acetate molecule (Moreira *et al*., 2012), its frequency was higher than the one reported in the study of *cyr*B gene. Results of the *cry*C analysis show that its presence was only detected in samples collected in Torrão Reservoir, Parque Fluvial do Tâmega, Vela and Mira Lakes. Temporally, it is to highlight that the first detection of this gene was observable in samples collected in January and February, when it was registered the lowest temperature of the sampling period. This detection could be explained by the presence of a cyanobacterial strain that possesses the *cyr* gene and may resist to more extreme environment conditions such as low temperature. Another explanation for these results could be the senescence of the biomass present in the water system, which may have released its genetic material into the water that was later collected for analysis. In Portuguese freshwater systems, the presence of a *cyr* gene was already detected in samples collected from Vela Lake (Moreira *et al*., 2011; Mendes, 2014). Detection of cylindrospermopsin was only possible in the study conducted by Mendes (2014), in samples collected in Vela Lake in 2012, through chemical and biochemical methods.

The presence of anatoxin-a biosynthesis related gene – *anaC* - was only detected in sampling sites located in the North Region of Portugal. The *anaC* gene is

responsible for the initial step in the biosynthesis of anatoxin-a, the proline adenylation, with the encoding of the AnaC protein (Rantala-Yilmen *et al*., 2011). In this study, the presence of this gene was detected in Torrão Reservoir, Parque Fluvial do Tâmega and Lakes 1 and 2 of Parque da Cidade do Porto, despite its low incidence throughout the sampling period. In Portugal, the study of anatoxin-a and genes related to its production is still scarce, being only described the presence of genes related to anatoxin-a biosynthesis in the work developed by Mendes (2014). Presence of anatoxin-a in freshwater systems is reported in studies conducted by Osswald *et al*. (2009) and Mendes (2014).

Amplification of *sxt* gene clusters was reported in all sampling sites during this sampling period. According to the literature, the *sxt*A gene has a PKS-like structure characterized by four catalytic domains and it is considered a starting gene of the synthesis of saxitoxin in cyanobacteria (Perini *et al*., 2014). The *sxt*G gene is known to encode the amidinotransferase, that later incorporate an amidino group from an arginine molecule into the saxitoxin intermediate (Perini *et al*., 2014). For *sxt*I, this gene is known to encode a carbamoyltranferase and to be present in PSP (Paralytic Shellfish Poison) toxin producing cyanobacteria (Ballot *et al*., 2016). The results showed that, in the amplification of the *sxt*A and *sxt*G genes, its presence was only detected in samples collected in October and November, not being detected in other sampling months. Regarding the study of *sxt*I gene, results show fragment amplification in diverse samples collected in the several sampling points. Geographically, the incidence of *sxt*I gene was higher in Torrão Reservoir, Lake 2 of Parque da Cidade do Porto and Vela Lake, in comparison with the remaining sampling points. In terms of temporal distribution, this gene had a higher occurrence rate in the first 2 months of the sampling period, October and November, having a lower incidence between February and April. In Portugal, the study of the *sxt* gene is mostly limited to the amplification of the *sxt*I gene, as shown by the literature (Lopes *et al*., 2012; Mendes, 2014). Saxitoxin presence in Portuguese freshwater systems has been the objective of several studies that were able to detect this toxin in the water ecosystems (Pereira *et al*., 2000; Ferreira *et al*., 2001; Mendes, 2014).

In general, molecular methods have proven themselves effective in the detection of cyanobacteria and genes encoding the biosynthesis of cyanotoxins, being ideal as an early warning method in a monitoring program for freshwater systems. However, it is to highlight that the presence of genes associated with the biosynthesis of cyanotoxins in a cyanobacterium species does not necessarily means that its production is occurring in the water ecosystem. Previous studies (cited in Bittencourt-

Oliveira *et al*., 2010 and Kumar *et al*., 2011) found out that some cyanobaterium species, despite possessing all the gene clusters that encode the biosynthesis of a specific cyanotoxin, are not able to produce them. It is speculated that this incapability is related to an inactivation of the genes encoding the biosynthesis of a specific cyanotoxin or related to a substitution, on a functional level, of the produced toxins by another peptide (Bittencourt-Oliveira *et al.,* 2010; Kumar *et al*., 2011). In this way, and despite the efficacy of molecular methods, the use of analytical methods is still needed in a monitoring program for the detection and quantification of cyanotoxins.

The Portuguese freshwater systems sampled in this study have a high socio-economic impact in the geographical area in which they are located. Its exploitation, in terms of human consumption, economical and recreational purposes, leads to a high human exposure to the presence of cyanobacteria and cyanotoxins that may be present in those freshwater systems. The Portuguese legislation only regulates cyanobacteria and microcystin analysis, through cell counting and microcystin detection and quantification through analytical methods (Decreto-Lei nº 306/2007 de 27 de agosto). However, the elevated toxicity of other cyanotoxins and the wide distribution of several cyanobacterial species create the necessity of more monitoring studies, encompassing several detection and quantification methods, in order to prevent risks for both human health and the environment.

5. Conclusion

The data obtained throughout this study demonstrate the wide distribution of *Microcystis sp*, in specific *M. aeruginosa*, as well as *C. raciborskii* and *P. agardhii* across the several freshwater systems analyzed, located in both North and Center regions of Portugal. Given the invasive behavior of *C. raciborskii* and *P. agardhii*, it is to highlight its presence in every freshwater system studied, with the exception of the Torrão Reservoir, where *C. raciborskii* was not detected through the entire sampling period. Regarding the study of the potential for cyanotoxin production, analyzed through the use of specific primer sets, the results achieved through this work determine that the presence of gene clusters encoding the biosynthesis of the cyanotoxins in study had an elevated frequency for both hepatotoxins and neurotoxins. It is important to highlight that the presence of both cyanobacteria and gene clusters related to cyanotoxin production was observed, in general, throughout the entire sampling period. It is also noteworthy the presence of this cyanobacterial species and gene clusters for cyanotoxin production in several freshwater systems during sampling months with record of lower temperatures than the ones observed during the established sampling period for the study of cyanobacteria, usually with warmer temperatures.

These results also show the growing need for the creation of complete monitoring programs that includes molecular methods as an initial screening method. Their rapidness and accuracy in the detection of cyanobacteria and potentially toxic strains makes these methods the ideal tools for a preliminary study of freshwater ecosystems.

6. Future Perspectives

As future work, it is suggested the performance of analytical methods for the detection and quantification of cyanotoxins. It would be interesting the analysis of freshwater samples using methods such as HPLC and ELISA, especially in the study of neurotoxins (anatoxin-a and saxitoxin), in order to compare with the data obtained from the molecular analysis. It is also suggested the sequencing of the amplified fragments achieved by PCR, for determination of the producing genera of the detected cyanotoxin potential for production. Given the obtained results in this work, it is proposed the extension of this study to other Portuguese freshwater systems, especially in the South Region of Portugal, as well as an extension of the sampling period, that includes the period between October and May. Finally, it would be very interesting to establish a monitoring program that aim the detection of key cyanobacteria species and their related toxins, encompassing molecular, chemical and analytical methods, during the entire year, and not only during the period between May and October, where is usually recorded higher temperatures and greater light exposure.

7. Communications

Rita Mendes, Cristiana Moreira, Vítor Vasconcelos and Agostinho Antunes (2017). Neurotoxicity in Portuguese freshwater systems: a molecular approach.
Oral communication in 5CIC – 5º Iberian Congress of Cyanotoxins and 1º Iberoamerican Congress of Cyanotoxins, Lugo, Spain, July of 2017.

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