**Universidade de Aveiro** Departamento de Ambiente e Ordenamento
**2013**

**Inês Adriana Patrunilho Efe Macário**

# Stepwise strategy for monitoring cyanobacterial blooms

# Estratégia de monitorização faseada para blooms cianobacterianos

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Gestão e Políticas Ambientais, realizada sob a orientação científica da Doutora Daniela Rebelo de Figueiredo, Bolseira de Pós-Doutoramento do Departamento de Biologia e CESAM, Universidade de Aveiro, e da Professora Doutora Maria Isabel Nunes, Professora Auxiliar do Departamento de Ambiente e Ordenamento da Universidade de Aveiro

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**palavras-chave**

*Blooms* de cianobactérias, saúde pública, águas recreativas, planos de monitorização, ficocianina

**resumo**

O desenvolvimento de *blooms* cianobacterianos pode representar um risco para a saúde pública e, num contexto de alterações climáticas, o desenvolvimento de grandes concentrações destes organismos pode ser potenciado. Portugal, à semelhança de outros países, adoptou a metodologia para a monitorização de cianobactérias proposta pela Organização Mundial de Saúde, que considera a clorofila *a* e a densidade celular, como indicadores da presença de cianobactérias. Contudo, tendo em conta as limitações destes indicadores, o presente trabalho visa propor uma estratégia rápida, simples e menos onerosa, a médio prazo, para a monitorização de *blooms* cianobacterianos em sistemas de água doce utilizados para uso recreativo. Esta estratégia propõe uma abordagem sequencial: i) fluorimetria (análise de pigmentos); ii) determinação da clorofila *a*, observação microscópica e enumeração das espécies cianobacterianas dominantes; iii) técnicas moleculares, baseadas em PCR, para avaliação do potencial tóxico; e v) quantificação das cianotoxinas, apenas quando necessário. O trabalho incluiu a determinação de valores de referência para ficocianina, através de fluorimetria, por forma a poder aplicá-los na estratégia de monitorização proposta. Para esta determinação foram utilizadas as espécies *Microcystis aeruginosa, Nostoc muscorum* e *Cylindrospermopsis raciborskii*. Para validação da estratégia de monitorização proposta, foram amostrados nove sistemas lênticos de água doce, do norte, centro e sul de Portugal. Os dados de parâmetros físico-químicos e biológicos das amostras foram integrados, de modo a obter informação acerca do contexto ecológico do desenvolvimento de *blooms*. A determinação dos valores de referência de ficocianina mostrou diferenças inter-específicas, suportando a necessidade de existirem vários valores de referência, que possam ser utilizados de acordo com a espécie cianobacteriana dominante num *bloom*. A metodologia proposta, ao ser faseada, permitiu ir excluindo amostras à medida que o perigo de desenvolvimento de um *bloom* tóxico foi sendo descartado, tendo sido detetadas apenas 2 amostras tóxicas, na Lagoa da Vela e na albufeira da barragem da Aguieira (ambas com blooms de *Microcystis* spp.). Esta estratégia de monitorização provou ser uma mais-valia para o desenvolvimento de planos de gestão de prevenção e controlo do crescimento excessivo de cianobactérias em lagos e albufeiras.

**keywords** Cyanobacterial blooms, public health, recreational waters, monitoring programmes, phycocyanin

**abstract**

The massive growth of cyanobacteria into blooms has risks to the public health and, under a context of climate change, the development of these organisms may be enhanced. Portugal, like other countries, has adopted the methodology proposed by the World Health Organization which uses chlorophyll *a* and cell density as indicators for cyanobacterial density assessment. However, these indicators have several limitations. Therefore, this study aims to propose a strategy for a faster, easier and more cost-effective monitoring of cyanobacterial blooms in lentic freshwater bodies used for recreation purposes. This strategy considers several steps, proposed sequentially: i) fluorometry (pigment analysis); ii) chlorophyll *a* determination, microscopic observation and enumeration of dominant cyanobacterial species; iii) PCR-based methodologies to screen the potential for cyanotoxins production; and v) cyanotoxins quantification, only if required. The study included the determination of phycocyanin thresholds though fluorometry in order to use them in the proposed monitoring strategy. The species used for this determination included *Microcystis aeruginosa, Nostoc muscorum* and *Cylindrospermopsis raciborskii*. For the validation of the monitoring strategy proposal, nine lentic freshwater bodies from northern, central and southern Portugal were sampled. The physico-chemical and biological parameters were integrated in order to provide information about the ecological context for the recorded cyanobacterial blooms. The determined phycocyanin thresholds showed inter-specific differences, highlighting the need for the proposal of not just a general threshold value for phycocyanin, but specific values, according to the dominant bloom-forming cyanobacterial species. The sequential stepwise strategy, allowed the exclusion of samples as the danger of the development of a toxic bloom is being discarded, remaining only two toxic samples at Vela Lake and Aguieira reservoir (both with *Microcystis* spp. blooms). This monitoring strategy proved to be a valuable and cost-effective tool for an early warning of cyanobacterial blooms, which may be important for the development of effective management plans to prevent and control the massive growth of cyanobacteria in target water bodies.

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# Chapter I

## General Introduction

# 1 General Introduction

## 1.1 Motivation and relevance

Climate change are increasing the frequency of episodes of extreme precipitation and drought, endangering the water quality in freshwater bodies (BOND et al., 2008). In recent years, in marine and in freshwater environments, the global increase in frequency, severity and duration of harmful algal blooms has been causing concern (CODD et al., 2005). The reasons behind this expansion are still not clear, but the global warming and the anthropogenic degradation of the water quality are pointed out as two main causes (CHORUS et al., 1999, CODD et al., 2005). Human activities (e.g. agricultural and roads runoff, inadequate sewage treatment) lead to eutrophication of many water bodies (the so-called cultural eutrophication) (CARMICHAEL, 2008) and potentiate excessive proliferation of algae and cyanobacteria which have a considerable impact upon water quality (WHO, 2003). Also, in temperate regions, cyanobacterial dominance is most pronounced during the summer months, which coincides with the period when the demand for recreational water is higher (WHO, 2003), representing health risks due to the production of toxins by many cyanobacteria (CHORUS et al., 1999, CODD et al., 2005, DE FIGUEIREDO et al., 2004). In Portugal, the occurrence of cyanobacterial blooms has been frequently recorded over the past decades (VASCONCELOS, 2001). In 1994, Vasconcelos presented a survey in 30 Portuguese freshwater bodies where 60% of the blooms were toxic (VASCONCELOS, 1994). This highlights the risks for human health during the development of a cyanobacterial bloom in recreational water bodies. Other consequences from blooms' occurrence are the reduction of biodiversity and death of many organisms due to deoxygenation of the water column, loss of fishing opportunities and costs associated with human illness, leading to declassification of tourism spots (MEREL et al., 2013). Moreover, cyanobacteria are very dynamic which makes it difficult to predict the beginning of a bloom. Besides nutrients (phosphorus, P and nitrogen, N) availability, growing evidence suggests that variables related to climate, such as temperature and water-column stability, can also enhance the blooms' development (TARANU et al., 2012). Beside this difficulty to predict cyanobacterial blooms due to their dynamic and unpredictable nature, literature also demonstrates that the response of cyanobacteria to climate and nutrients is not straightforward. According to Taranu *et al.* (2012), conflicting results have been presented between dimictic lakes (i.e., where water column is stratified for most time of the year) and polymictic lakes (i.e., where water column experiences multiple mixing periods). Moreover, as cyanobacterial blooms may suffer highly dynamic changes not only due to seasonality but also during the day (IZYDORCZYK et al., 2005), it is very important to optimise the monitoring strategies to be able to assess in proper time the risk of contamination of a water body concerning cyanobacterial blooms.

The facts present before, point out that the establishment of water management strategies is indispensable. Several methods have been used for phytoplankton quantification (cyanobacteria included) but the concentration of chlorophyll *a* is the most common, along with taxonomic analysis and cell counts obtained through microscopy. However, these methods are time-consuming, require a specialist (in particular, for the taxonomic identification) and results are usually available several days after sampling (GREGOR et al., 2005). Moreover, chlorophyll *a* may not be the most adequate parameter for assessing a cyanobacterial bloom, since this pigment is not exclusive for this group and it is also present in other photosynthetic organisms such as chlorophytes, diatoms or dinoflagellates. Furthermore, chlorophyll *a* concentration does not always increase in a linear relationship with cyanobacterial cell number, even though the current alert system based on such assumption (CHORUS et al., 1999). Therefore, it is important to develop a more simple and objective method, at least for an early stage, replacing chlorophyll *a* determination and the traditional cell counting technique.

Thus, monitoring programs for assessing cyanobacteria in recreational waters could be improved, in order to obtain a more reliable and fast information about the danger of a bloom development. This dissertation aims to test a monitoring strategy for recreational waters by adding phycocyanin determination to the first set of preliminary analyses, which could be easily measured by fluorometry and that does not require a great deal of dexterity or expensive equipment for its determination. Moreover, it is a more effective representation for cyanobacterial biomass than chlorophyll *a* (LEE et al., 1995). Although phycocyanin is an interesting pigment to use in monitoring programmes, thresholds and guidelines for this pigment are still not defined. In the present study, an attempt for determination of phycocyanin threshold values will be performed, corresponding to the different alert levels defined by World Health Organization (WHO) for recreational water bodies.

## 1.2 Dissertation objectives and layout

This work aims to propose a strategy for a faster, easier and more cost-effective monitoring of cyanobacterial blooms, in Portuguese lentic freshwater bodies, particularly those used for recreational purposes. This may be valuable for the development of effective management plans to prevent and control the massive development of cyanobacteria.

The incorporation of phycocyanin in monitoring programmes requires the establishment of phycocyanin thresholds, which complement the defined chlorophyll *a* and cell counts thresholds defined by WHO for the alert levels. This would allow a more effective monitoring of the blooms' occurrence and development. Several laboratory assays were conducted to evaluate these levels, particularly for different bloom species.

Taken into account the limitations of the current cyanobacterial monitoring programmes, a stepwise strategy is proposed using sequential methodologies: i) fluorometry (for phycocyanin

determination) at an early stage; ii) chlorophyll *a* determination, microscopic observation and enumeration of dominant cyanobacterial species; iii) Polymerase Chain Reaction (PCR)-based methodologies to screen the potential for cyanotoxins production and iv) cyanotoxin quantification, only when necessary. In order to test this methodological strategy, nine lentic freshwater bodies from Northern, Central and Southern Portugal were sampled and analysed using this approach.

During the work development, a review on the legislation and implementation of quality standards regarding cyanobacterial blooms was discussed, including national and international scenarios.

The present dissertation begins with an introduction chapter (Chapter I) with the scope and main objectives of the work, following the state of the art with the information on cyanobacterial blooms ecology and on what is being proposed and implemented to deal with the blooms development. Chapter II will focus on the determination and proposal of phycocyanin thresholds. A third chapter (Chapter III) describes the conceptual design of the proposed strategy and its application to nine case studies. The Chapter IV includes the integration of all collected data for an ecological assessment. The main conclusions of the work are assembled and exposed in one the last chapter (Chapter V).

## 1.3 State of the art

### 1.3.1 Cyanobacteria and factors influencing their growth

According to fossil records, cyanobacteria exist for approximately 3.5 billion years (PAERL et al., 2012). Most cyanobacteria have a characteristic bluish-green colour due to the phycocyanin pigment contained in their cells and this is why they have been named cyanobacteria or blue-green algae. However, these organisms are not algae, but gram-negative bacteria containing chlorophyll and performing photosynthesis (COALITION et al., 2009). In the past, cyanobacteria have been also known as Cyanophyceae, Myxophyceae or blue-greens (CARMICHAEL, 2008).

The life cycle of these organisms only requires water, carbon dioxide, inorganic substances (such as phosphorus and nitrogen) and light. Photosynthesis is their primarily energy metabolism, where sunlight and carbon dioxide are used to produce energy-rich molecules and oxygen. However, some species can survive in complete darkness while others have heterotrophic abilities (FAY, 1965). These characters allow them to live nearly anywhere on earth, in every continent, from freshwater to salt and brackish waters, rainforests, deserts and other terrestrial habitats, and even in the air. Therefore, these adaptable organisms can survive under harsh conditions in regions affected by drought and climate change (CHORUS et al., 1999).

Cyanobacteria can occur singly or grouped in colonies and can increase to such large numbers that colour the water and form highly visible thick scums. A complex interaction of environmental factors has been shown to contribute to this cyanobacterial growth, including light intensity, carbon dioxide concentration, nutrient availability (nitrogen, phosphorus, iron, and molybdenum), hydrologic characteristics of the water body and aquatic ecosystem structure and function (MEREL et al., 2013). Cyanobacterial blooms are usually associated with eutrophic water bodies and with low N:P levels, water stability, reduced transparency and increased water temperature, pH and conductivity (CODD, 2000, DE FIGUEIREDO et al., 2006). When compared with other phytoplanktonic organisms, cyanobacteria present slow growth rates and, therefore, require longer retention time periods in calm waters to allow the bloom development. The fact that these organisms have few natural enemies, combined with their ability to avoid sedimentation through buoyancy control, results in a low lost rate of the population, which compensates their slow growth rates (LÜRLING et al., 2013).

Usually nitrogen and phosphorus are the limiting nutrients for algal growth. Thus, they have influence on growth rates, maximum biomass and in phytoplankton species composition (TARANU et al., 2012). Several cyanobacterial genera (e.g. *Anabaena* and *Cylindrospermopsis*) can develop successfully under low nitrogen concentrations (such as nitrates and ammonia) because they possess the capability (diazotrophy) to fix atmospheric nitrogen in specialized cells, called heterocysts (MOISANDER et al., 2012). It is expected that diazotrophic filamentous cyanobacteria, in particular, will be enhanced by the expected global warming, with nutrient inputs playing a major

role (MARKENSTEN et al., 2010). Diazotrophic species such as *Cylindrospermopsis raciborskii, Aphanizomenon* (*Cuspidothrix) issatschenkoi* and *Aphanizomenon aphanizomenoides* have already been considered invasive in Europe (WIEDNER et al., 2007). Additionally, colonial genera such as *Microcystis* can store nitrogen in proteins (cyanophycin and phycocyanin) which can be used during nitrogen-limiting conditions, allowing them to out compete with green algae in nitrogen-poor surface water bodies, under available light (DOKULIL et al., 2000).

Under low phosphate concentrations (few micrograms per litre), cyanobacterial growth and biomass are not limited and many cyanobacteria can store enough phosphorus to perform 2-4 cell divisions (corresponding to a 4-32 fold biomass increase) under phosphate availability (MUR et al., 1999). Therefore, in water bodies were nutrient concentrations are naturally low or have been decreased by remedial actions, cyanobacterial populations may still develop. However, N-fixing filamentous cyanobacteria do not have much success under phosphorus limitation or unavailability (DE FIGUEIREDO et al., 2011).

Another physiological aspect that gives cyanobacteria a competitive edge over phytoplanktonic algae in aquatic ecosystems, particularly in stratified lakes, is the ability to migrate vertically in the water column. This is a mechanism that allows them to choose the best depth for capturing light for optimum growth and the best position to scavenge nutrients from the water column (OLIVER et al., 2000). Many bloom-forming planktonic cyanobacterial species possess intracellular gas vesicles (rigid hollow cylindrical chambers made of proteins containing atmospheric gas) that enable cells to regulate their buoyancy, depending on their stage in daily photosynthetic cycle through the production of carbohydrates from photosynthesis (WALSBY, 1994). However, buoyancy regulation by changing the amount of gas in the vesicles is a slow process and when the weather changes (from stormy to “fine”), the water changes from turbulent to strongly stratified and many excessively buoyant cells or colonies of cells may accumulate at the surface (WHO, 2003). The loss of buoyancy regulation (by photo-oxidation, for example) may lead to a dense accumulation of cells at surface, forming scum. Therefore, species that produce gas-vesicles are the main responsible for surface blooms or scum (e.g. filamentous genera *Anabaena, Aphanizomenon, Anabaenopsis, Nodularia, Cylindrospermopsis, Gloeotrichia, Oscillatoria/ Planktothrix, Spirulina* and genera that form globular colonies like *Microcystis, Gomphosphaeria* or *Coelosphaerium*) (OLIVER et al., 2000). Wind is able to conduct the cells to shores and bays and, in extreme cases, such agglomerations may become very dense and even acquire a gelatinous consistency (CHORUS et al., 2000). Normally, in shallow bays, scum material may take a long time to disperse, as a result of either wave wash or disintegration of the cells. Death and cells lysis release their contents into the water, where pigments may adopt a copper-blue colour and bacterial decomposition leads to rapid putrefaction of the material. This results in in-shore deposits, often repulsive and potentially very toxic. Such situations may change rapidly, within hours, or may remain unchanged for weeks (WHO, 2003).

Within the scope of climate change, another factor that also contributes to the occurrence of cyanobacterial blooms are the summer droughts that cause a rapid decrease in the water levels at shallow water bodies, leading to important changes in the water physical and chemical characteristics, as well as in the composition of the aquatic communities (WIEDNER et al., 2007). Moreover, as these organisms prefer higher temperatures, the temperature rising will enhance bloom development, in addition to nutrient supplies (TARANU et al., 2012). Also, warming of superficial waters will also reduce the vertical mixing intensity and frequency, and increased water temperature will also allow a decrease in water viscosity, which leads to the decrease of water resistance to vertical migration, what confers a competitive advantage to cyanobacteria. According to Pearl *et al.* (2009), some studies suggest that some species are expanding their range due to climate change.

Bloom development occurs through a series of phases: seeding and initial development phase, followed by a rapid or exponential growth phase, a plateau phase and then a die-off phase. If the environmental conditions are appropriate, the plateau can persist for longer periods (COALITION et al., 2009). Normally, cyanobacteria are more abundant in freshwaters environments and, therefore, lakes and reservoirs used for recreational purposes or as sources of drinking water are potential targets (WIEDNER et al., 2008).

Despite the efforts, there is still a big lack of understanding about the complex mechanisms underlying the growth and accumulation of cyanobacterial species into blooms, the fate of cyanotoxins through food chains and the influence of human activities on this process (CODD et al., 2005).

### 1.3.2 Impact and problems associated to cyanobacterial blooms

The occurrence of cyanobacterial blooms causes deterioration of the water quality, with implications for the ecology of the water body and the surrounding area, as well as for the region economy and public health. HAB is the generic name for harmful algal blooms that affect adversely the environment, plants or animal health. In many areas of the world, the greatest public health impact related to HABs are the cyanobacterial blooms (CyanoHABs), both in drinking and recreational waters (BACKER, 2002). CyanoHABs have increased dramatically since the 1960's and as they occur worldwide, they should be treated like a worldwide problem. Since human activity is the primary cause of cultural eutrophication, the patterns of CyanoHABs occurrence and risks are basically the same throughout the world and they normally occur in the warmer and dryer periods of the year (CARMICHAEL, 2008). In freshwater systems, HABs are largely caused by cyanobacteria of the genera *Anabaena, Aphanizomenon, Cylindrospermopsis, Microcystis* and *Oscillatoria*. Among these *taxa*, *Microcystis aeruginosa* is one of the most ecologically damaging species, because it can develop in waters with different nutrient loadings and it is frequently toxic to aquatic and terrestrial organisms (CARMICHAEL, 1992).

Freshwater CyanoHABs have many adverse environmental impacts. From an ecological point of view, a cyanobacterial bloom can lead to the reduction of specific biodiversity at all trophic levels and deterioration of the habitat through increased turbidity and reduction of light levels (below those required for submerged aquatic vegetation to survive), reduction of oxygen concentrations (to levels that can be lethal to some fish and invertebrate species), changes in the organoleptic parameters of the water (with production of substances that give a bad taste and odour to water) or even production of toxins that are noxious to a great variety of organisms (DE FIGUEIREDO et al., 2004, VASCONCELOS, 1995). Phytoplankton community, in particular, is strongly affected by cyanobacterial blooms, not only due to the advantageous competition of cyanobacteria over microalgae (e.g. nitrogen fixation, phosphorus storage and/or buoyancy regulation for achieving better light and nutrients conditions) but also to the effect of cyanotoxins' release as well as a reduced grazing by zooplankton (FIGUEIREDO, 2010). It has been hypothesized that the primary purpose of these toxins is to inhibit the growth of other competing phytoplankton species (through allelopathy) and to decrease losses by killing their grazers (grazer deterrence) (GRANÉLI et al., 2008). Consequentially, economic problems can also arise due to the deterioration of water quality (with bad taste and odour due to the production of compounds such as geosmin and 2-methyl isoborneol (2-MIB) (SMITH et al., 2008) and blockage of water treatment filters in drinking water reservoirs, requiring additional and more effective and expensive water treatment processes to remove cyanotoxins (RAPALA et al., 2006). In recreational waters, the water quality degradation may have also great economic impacts, mainly through the reduction of local tourism (WHO, 2003).

Since cyanobacteria may produce toxins, they could represent a serious human health risk, because toxins produce harmful effects on tissues, cells and/or organisms (DE FIGUEIREDO et al., 2004, METCALF et al., 2012). Thus, cyanotoxins represents a potential hazard in waters used for human and animal drinking-water supplies, aquaculture, agriculture and recreation (RESSOM et al., 1994). Moreover, there is also the health risks associated with bioaccumulation of cyanotoxins through the food chain in food supplements (SAKER et al., 2005) or through crop-spray irrigation (METCALF et al., 2012). Toxic cyanobacteria (see Table 1.1) are found worldwide in inland and coastal water environments and at least 46 species have been shown to cause toxic effects in vertebrates (WHO, 2003). Cyanotoxins can act as genotoxic, tumour-promoting and/or hepato- and neurotoxic agents (METCALF et al., 2012). Also, some cyanobacteria produce a metabolite, β-N-methylamino-L-alanine (BMAA), which may be involved in neurodegenerative diseases (COX et al., 2005). Therefore, cyanotoxins can be classified according to the organs they affect and their chemical structure. According to the toxic effects in mammals, they are classified as neurotoxins, hepatotoxins, cytotoxins, irritants and gastrointestinal toxins. From a chemical point of view, neurotoxins include alkaloids such as anatoxin-a, homoanatoxin-a or saxitoxins and the guanidin methyl phosphate ester; hepatotoxins, the most frequent and dangerous cyanotoxins, include cyclic peptides such as nodularins and microcystins; cytotoxins include the alkaloid cylindrospermopsin;

toxins with irritant and gastrointestinal effects include aplysiatoxin, debromoaplysiatoxin and lyngbyatoxin (which are tumour promoters and cause skin irritation) and lipopolysaccharide endotoxins (LPS) that may enhance inflammatory and gastrointestinal incidents (METCALF et al., 2012)

**Table 1.1** Main cyanobacteria known to produce the major classes of cyanotoxins (adapted from METCALF et al., 2012).

| Toxin | Main producers |
|---|---|
| Microcystin | **Chroococcales:** *Microcystis* spp., *M. aeruginosa, M. viridis*<br>**Oscillatoriales:** *Oscillatoria (Planktothrix) agardhii, Plectonema boryanum, Phormidium corium, P. splendidum, Arthrospira fusiformis*<br>**Nostocales:** *Anabaena* sp., *A. flos-aquae, A. subcylindrica, A. variabilis, Nostoc* sp., *N. spongiaeforme, Anabaenopsis* sp., *Gloeotrichia echinulata, Rivularia biasolettiana, R. haematites, Tolypothrix distorta*<br>**Stigonematales:** *Hapalosiphon* sp. |
| Nodularin | **Nostocales:** *Nodularia spumigena* |
| Anatoxin-a<br>and homoanatoxin-a | **Oscillatoriales:** *Arthrospira fusiformis, Phormidium* sp., *P. formosum, Oscillatoria* sp.<br>**Nostocales:** *Anabaena* spp., *A. flos-aquae, A. planktonica, Aphanizomenon* sp., *Cylindrospermum* sp., *Raphidiopsis mediterranea* |
| Anatoxin-a (S) | **Nostocales:** *Anabaena flos-aquae, A. lemmermannii* |
| Saxitoxin | **Oscillatoriales:** *Lyngbya wollei, Planktothrix* sp.<br>**Nostocales:** *Aphanizomenon flos-aquae, Anabaena circinalis, Cylindrospermopsis raciborskii* |
| Cylindrospermopsin | **Nostocales:** *Cylindrospermopsis raciborskii, Aphanizomenon ovalisporum, Anabaena* sp., *Anabaena lapponica, Raphidiopsis curvata*<br>**Stigonematales:** *Umezakia natans* |

Lipopolysaccharides (LPS) are an integral component of the cell wall of all gram-negative bacteria, including cyanobacteria (STEWART et al., 2006). LPS can cause irritant and allergenic responses, hypertension, inflammatory responses and gastrointestinal upset (METCALF et al., 2012). Besides these negative effects, cyanobacterial LPS are considerably less potent than LPS from Enterobacteriaceae (STEWART et al., 2006) and also less potent as the other cyanotoxins described below (METCALF et al., 2012). Therefore, it is possible that cyanobacterial LPS represent a relatively minor hazard to human health in water contaminated with cyanobacteria (NHMRC, 2008). LPS are synthesized by many brackish and freshwater species of the genera *Anabaena, Aphanizomenon, Nodularia, Oscillatoria* and *Gloeotrichia* (WHO, 2003).

The most potent cyanotoxins are low molecular weight alkaloids and cyclic peptides (METCALF et al., 2012). Most of these cyanotoxins have been found in a wide array of genera and some species may produce more than one toxin. In freshwaters, the most widespread

cyanobacterial toxins are microcystins and neurotoxins (BAKER et al., 2002) and some species contain neurotoxins and microcystins simultaneously (WHO, 2003). While some cyanotoxins (e.g. saxitoxins and lipopolysaccharide endotoxins) are not exclusive products of cyanobacteria, others such as microcystins and anatoxins, are only produced by them (METCALF et al., 2012). However, toxicity is not a specific trait for certain species; rather, most species comprise toxic and nontoxic strains (WHO, 2003). For example, field populations of the most common bloom-forming genus, *Microcystis*, are almost always toxic, but non-toxic strains can occur. The neurotoxins anatoxin-a, anatoxin-a(S) and saxitoxin differ in their mode of action, but they all have the potential to be lethal at high doses by causing asphyxia through paralysis of respiratory muscles (WHO, 2003). However, no human deaths from exposure to neurotoxins associated with recreational use of water are known (NHMRC, 2008). After ingestion of a sublethal dose of these neurotoxins, recovery appears to be complete and no chronic effects have been observed to date (WHO, 2003). The traditional description of microcystins and nodularins as hepatotoxins is no longer sufficient to describe their actions in animals, because microcystins can accumulate in multiple organs and tissues in mammals and fish, such as heart, liver, gonads, lungs, brain and kidney with consequent tissue and cell damage (METCALF et al., 2012). More than 89 microcystins variants are known, being microcystin-LR the most common toxin in the world and the first to be chemically identified (SRIVASTAVA et al., 2013). It is also the most dominant toxin found in Portuguese waters (VASCONCELOS, 1995). Microcystin toxicity is cumulative and in vertebrates, a lethal dose causes death by liver necrosis within hours to a few days, causing both acute and chronic effects in mammals (NHMRC, 2008). In addition, they can also cause cancer promotion through chronic exposure of humans to low microcystin concentrations in drinking water (DE FIGUEIREDO et al., 2004). In Caruaru (Brazil), numerous deaths occurred when 136 dialysis patients were exposed to microcystins in water used for the dialysis (POURIA et al., 1998). A similar case occurred in Portugal, in 1993, during a dry summer, when a cyanobacterial bloom occurred at Monte Novo Reservoir (Évora), used as drinking water supply (OLIVEIRA, 1995).

Nodularin, as well as microcystin, is a hepatotoxin (WHO, 2003) produced by the cyanobacterium *Nodularia spumigena*. This species is regarded as a brackish-water species and is known to form blooms in estuarine lakes in Australia, New Zealand and Europe, although blooms of this species in freshwater are relatively rare (NHMRC, 2008). Moreover, as neurotoxins do not occur in lakes and rivers as frequently as microcystins, high concentrations of this toxic substance in scums will scarcely reach levels acutely neurotoxic to humans (CHORUS et al., 2000). Nevertheless, livestock and pets may be affected because they may drink many litres of contaminated water, as reported in North America, Europe and Australia (WHO, 2003). However, no chronic effects have been observed to date (CHORUS et al., 2000). For this reason, neurotoxins are considered less dangerous than microcystins or cylindrospermopsins that may cause ongoing injury (WHO, 2003).

Cylindrospermopsin was first discovered after a poisoning incident on Palm Island (Queensland, Australia) in 1979, when 148 people, mainly children, were hospitalized with hepatoenteritis due to *Cylindrospermopsis raciborskii* contamination of a drinking water reservoir (GRIFFITHS et al., 2003). This toxin behaves as an hepatotoxin (BAKER et al., 2002) and it is a general cytotoxin that blocks protein synthesis, with acute clinical symptoms like kidney and liver failure, causing widespread organ damage (CHORUS et al., 2000). These clinical symptoms may appear only several days after exposure, so it is difficult to determine a cause-effect relationship. In Australia, patients intoxicated with cylindrospermopsin via drinking water escaped death only through skilled and intensive hospital care (WHO, 2003). Besides the acute symptoms, cilyndrospermopsin is also considered genotoxic and potentially carcinogenic (MESSINEO et al., 2009). Although *Cylindrospermopsis raciborskii* is considered a tropical and subtropical species, it has been reported to be invading temperate regions (PADISÁK, 1997). Global warning associated with climate change may contribute for the spreading of this species in temperate climates such as Portugal (VASCONCELOS, 2006).

A general picture of the frequency of occurrence of cyanotoxins associated with certain cyanobacterial *taxa* is now more complete (see Table 1.1). However, it is less clear what cyanotoxin concentrations may be expected, namely in recreational waters. Few studies have addressed the time and space variability of toxin content during the development of cyanobacterial blooms but this knowledge would be important for risk assessment and management of recreational waters in order to estimate the maximum toxin concentrations to be expected at a given cyanobacterial density (WHO, 2003).

The effects of cyanotoxins can be both acute and chronic, so it is necessary to have protection against both short-term and long-term exposure. Some short-term exposure can lead to health effects from which recovery is complete (e.g. dermal exposure which may lead to skin irritations and allergic reactions) but long-term exposure may result in damage to target organs (FUNARI et al., 2008). Moreover, for chronic poisoning, there are more sensitive groups that require special attention such as B-hepatitis patients, hypersensitive persons and children who tend to play in shallow waters where scum accumulates (CHORUS et al., 2001). Cyanotoxins can entry in the human body through three routes (either as whole cells and/or as dissolved toxins): i) direct contact of exposed parts of the body (i.e. skin, mouth, eyes, ears and throat); ii) accidental swallowing of contaminated water; or iii) inhalation (usually during recreational or occupational exposure of water sprays). Other exposure routes includes: renal dialysis, irrigation water used in crops and possible uptake into the food chain and dietary supplements (CHORUS et al., 1999). Most of the reported cases of human illnesses caused by algae in recreational situations have been due to uptake by ingestion or aspiration of cyanobacterial cells (NHMRC, 2008). In contrast to direct dermal contact, the uptake of cyanobacteria though ingestion or aspiration involves the risk of intoxication (WHO, 2003). Most reported cases of human injury through cyanotoxins involved exposure through drinking-water. People swimming in contaminated lakes have experienced

abdominal pain, nausea, vomiting, diarrhoea, sore throat, dry cough, headache and elevated liver enzymes in the serum, as well as high fever symptoms, dizziness, fatigue, skin and eye irritations, allergic dermatitis, swollen lips, conjunctivitis, earaches and a hay fever-like syndrome. These symptoms are likely to have diverse causes, with several classes of toxins and genera of cyanobacteria involved (BACKER, 2002). Furthermore, chronic effects due to recreational exposure should also be considered because long periods of exposure can occur during summer vacancies with regular swimming in a contaminated water body (CHORUS et al., 1999).

Allergic or irritative dermal reactions may arise from exposure through dermal contact and can vary in severity. Several freshwater cyanobacterial *taxa* (*Anabaena, Aphanizomenon, Nodularia, Oscillatoria* and *Gloeotrichia*) have been reported to cause these reactions after recreational exposure. Bathing suits, particularly wet suits, tend to intensify such effects by accumulating cyanobacterial material, enhancing disruption and release of cell content and it is probable that these symptoms are not due to recognized cyanotoxins but rather to currently largely unidentified substances. Despite those reactions are relatively common, they have been rarely investigated in scientific studies. Furthermore, the low number of reported cases through cyanobacterial intoxication may be also due to the lack of knowledge about the toxicity of cyanobacteria, as neither patients nor doctors associate symptoms with this cause (WHO, 2003).

Allergic reactions are not exclusive to cyanobacteria, some planktonic algae may cause those reactions as well. However, allergic reactions require high cell densities and mass developments and in freshwaters, they are most frequently due to cyanobacteria than to algae. Furthermore, other groups of algae do not accumulate as surface scum and, therefore, their metabolites will not occur in comparably high concentrations. The first case reported of disease associated with cyanobacteria was presented in London by Farre, in 1844, nevertheless, the first reports of animal deaths were published by Francis, in 1878, in Australia. Since then, different authors have reported deaths of numerous animals (e.g. sheep, cattle, horses, pigs, dogs, fish, rodents, amphibians, waterfowl, bats, zebras and rhinoceroses) over the years (WHO, 2003). A considerable number of human deaths have also been reported by exposure to cyanobacterial toxins through renal dialysis and through drinking-water (POURIA et al., 1998). Health impairments have been also described in several reports of irritations of the skin and/or mucous membranes and in documented cases of illness after exposure through drinking-water as well as accidental swallowing or aspiration of scum material (WHO, 2003).

### 1.3.3 Recreational waters management towards cyanobacterial blooms

Recreational uses of inland and marine waters are increasing worldwide. These uses range from whole-body water contact sports, such as swimming, surfing and slalom canoeing, to non-contact sports, such as fishing, walking, birdwatching and picnicking (WHO, 2003). Although “bloom” does not have an international definition, it is generally considered as a significant production of biomass over a short period of time with consequences in diminution of

phytoplankton diversity (MEREL et al., 2013). For water bodies used for drinking water supply or recreational activities, a bloom can be defined by the cells concentration that causes a problem/nuisance for users (COALITION et al., 2009). In recreational waters, a full range of potential hazards require management, such as: incidents and physical hazards; heat, cold and ultraviolet radiation; microbial contamination; chemical contamination; dangerous or venomous organisms; and toxic algae and cyanobacteria. Exposure to algae and cyanobacteria and their associated toxins are usually considered less of a concern than exposure to pathogenic microorganisms. However, as stated previously, several species of cyanobacteria can be acutely toxic when ingested or absorbed through the skin, or can cause irritation of skin, eyes or mucous membranes, besides the risk that these cyanotoxins can cause through food consumption (NHMRC, 2008). Little is known regarding blooms formation and cyanotoxins variants present in the water (SRIVASTAVA et al., 2013). Thus, for management purposes, it is important to take into account that cyanotoxins are mainly found within cyanobacterial cells and its release into the surrounding water is possible, particularly when cells die and lyse. However, toxins dissolved in water are rapidly diluted and eventually degraded, whereas hazardously high toxin concentrations usually result from the accumulation of cell material as scums (WHO, 2003).

Reservoirs are more prone to bloom formation in temperate climates due to the higher anthropogenic impacts, thermal stratification and pressures from environmental parameters, in particular from floods and droughts. Cyanobacteria may respond quickly to a drought by decreasing significantly their density and toxin production. However, once environmental conditions are stable, their immediate response is to bloom and maximize the toxin quota. Therefore, the water residence time is an important parameter to monitor because it may influence cyanobacterial density and toxin production (VASCONCELOS et al., 2011). Consequently, reservoirs are easier to manage than natural lakes because it may be possible to control their water residence times (TELES et al., 2006).

In cyanobacterial blooms' management, due to the lack of toxicological and epidemiological studies, risk assessment regarding cyanotoxins is very difficult to perform. This is the reason why WHO guidelines have been established only for one group of toxins - the microcystins (CHORUS et al., 1999). It is very complex to create effective measures for the protection of public health and relate them to the risk assessment of toxic cyanobacteria and bloom formation (HEALTH, 2013). As short-term exposure can lead to health effects, it is important to develop and implement standards and monitoring regimes that allow preventive and remedial actions to be taken within realistic timeframes and, at the same time, develop programs for assessing conditions and practices, as well as threshold values that can be used as targets (NHMRC, 2008). Situation assessment can be (HEALTH, 2013):

i) proactive (includes determination if whether a contingency planning is required or if it is needed a long-term action to minimize the formation of blooms);

ii) or reactive (includes the creation of measures to respond to the development of a bloom, such as emergency or incident responses).

Sources of information that support these management options are present in Table 1.2.

**Table 1.2** Proactive and reactive management options and sources of information behind their choice (source HEALTH, 2013).

| Observation | Sources of information | Management options |
|---|---|---|
| Potential for bloom formation | Water quality monitoring data (nutrients, temperature, etc.) | Basis for proactive management |
| History of bloom formation | Cyanobacterial blooms may follow marked seasonal and annual patterns | Can help to create a proactive management |
| Monitoring of cyanobacteria and/or cyanotoxins | Turbidity, discoloration, cell identification, cell counts and toxins analysis | Possible basis for proactive management |
| Scum counting | General public as well as agency staff can report scums in areas with public interest | Possible only during event and enables only reactive management |
| Reporting of animal deaths and human illness | Collecting data through the community | Possible only during event and enables only reactive management |
| Epidemiological detection of disease patterns in the human population | Requires effective reporting and large-scale effects before detection likely | Normally well after an event; can inform future management strategies |

The responsibility for managing risks in recreational waters takes place at two distinct levels (WHO, 2003):

i) society regulators - through central and local government and providers of recreational facilities;

ii) participants in the activities - whether personally or collectively.

This implies a devolvement of responsibilities downward and reporting upwards, in a way that responsibilities for monitoring may be devolved to an environmental agency or local authorities, with analyses being carried out by hospital, public health or university laboratories (WHO, 2003).

#### 1.3.3.1 *Health guidelines*

In 1998, United States Environmental Protection Agency (USEPA) included the group of freshwater cyanobacteria and their toxins on the first Candidate Contaminant List, which is a list of compounds of potential public health concern (BAKER et al., 2002). However, besides cyanotoxins, a vast array of other secondary metabolites including enzyme inhibitors, info- and allelochemicals, have also adverse effects on other biological systems (METCALF et al., 2012). Cyanotoxins such as microcystins, anatoxin-a and saxitoxins are already listed as biological warfare agents and have been considered water threats, since they behave as biotoxins tolerant to chlorine that may be weaponized and used for massive contamination of water bodies and human populations (BURROWS et al., 1999). National and international legislation is starting to include cyanotoxins as potential bioterrorism agents which are, or may become, subject to regulation and control (METCALF et al., 2012). In freshwater environments, the most common potentially cyanotoxic *taxa* are *Microcystis* spp., *Cylindrospermopsis raciborskii, Planktothrix* (*syn. Oscillatoria*) *rubescens, Synechococcus* spp., *Planktothrix (syn. Oscillatoria) agardhii, Gloeotrichia* spp., *Anabaena* spp., *Lyngbya* spp., *Aphanizomenon* spp., *Nostoc* spp., some *Oscillatoria* spp., *Schizothrix* spp. and *Synechocystis* spp., but toxicity cannot be excluded for further species and genera (WHO, 2003). For that reason, Australian Government considers all cyanobacterial blooms as toxic, unless proven otherwise by laboratory analyses (COALITION et al., 2009). This production of toxins is unpredictable and therefore, it is difficult to identify the toxicity of waters and define the restrictions that should be placed on their use (NHMRC, 2008).

Recreational waters may lead to possible negative health effects which makes it important to create guidelines that can be converted into locally (i.e. nationally or regionally) appropriate and applicable standards along with management strategies to ensure a safe, healthy and aesthetically pleasing environment (WHO, 2003). Cyanobacteria should be treated with caution, until the absence of toxicity is confirmed. That is the reason for the creation of the Alert Level Framework (ALF). ALF consists of a monitoring and action sequence that operators and regulators can use for a graduated response to the onset and progress of a potentially toxic cyanobacterial bloom (HEALTH, 2013). Cell counts are normally used in management plans and do not replace toxin analyses, which are required for health risk assessment. The concept of ALF was first developed for algal management in South Australia (1991). It was then adopted and used internationally by the World Health Organization (WHO), as a model system for response to cyanobacterial blooms, and has also been adapted by other users to incorporate recreational and agricultural waters (COALITION et al., 2009). These water-quality guidelines are necessary to protect human health during recreational activities and to preserve the aesthetical appeal of water bodies. Such guidelines should be used in monitoring and managing. They include a range of physical, microbial and chemical characteristics, in order to determine if a water body is suitable for recreational use (NHMRC, 2008). However, it is important to know that any potential adverse effects must be

weighed against the benefits to health and well-being of recreational water use and the positive impacts on local economies that rely on water-associated recreational activities (WHO, 2003).

Guidelines and recommendations range from identifying the need of providing advice to the public, to numerical guidance levels or to a classification system. Therefore, a guideline can be a level of management; a concentration of a constituent that does not represent a significant risk to the health of individual members of significant user groups; a condition under which such concentrations are unlikely to occur; or a combination of the last two. Guidelines are intended to be flexible and should be adapted to suit regional, national and/or local circumstances by taking into consideration socio-cultural, environmental and economic conditions of the site, alongside with knowledge of activities undertaken, routes of exposure and the nature and severity of hazards (WHO, 2003).

***WHO guidelines***

Health impairments from cyanobacteria in recreational waters must be differentiated between the mainly irritative symptoms (caused by unknown cyanobacterial substances) and the more severe risk of exposure to cyanotoxins (as in the case of microcystins). The international discussion on guidelines for cyanotoxins is focusing on microcystins because neurotoxins are not considered to be as hazardous (due to their lack of chronic toxicity) nor as widespread; and cylindrospermopsin, produced mainly by *C. raciborskii*, is a very hazardous toxin, but this species does not form surface scums, representing a higher risk for drinking water than for bathing waters (CHORUS et al., 2000). According to WHO, a single guideline is not appropriate, but rather a series of guidelines associated with incremental severity and probability of adverse health effects at three levels (CHORUS et al., 1999, WHO, 2003). It is very difficult to define “safe” concentrations of cyanobacteria in recreational waters in relation to allergenic effects or skin reactions, because individual sensitivities vary greatly (CHORUS et al., 1999). Each value of the ALF represents a level of risk to public health, and, therefore, indications on each level should go from increasing monitoring frequency, to notification of the relevant health authorities, or cessation of the water areas (COALITION et al., 2009).

WHO recommends the following guidelines:

*Alert Level 1. Relative mid and/or low probabilities of adverse health effects*

This level refers to the protection of health not due to cyanotoxin toxicity, but due to the irritative or allergenic effects of other cyanobacterial compounds. The guideline level corresponds to 20,000 cyanobacterial cells.$mL^{-1}$ (corresponding to 10 µg $L^{-1}$ of chlorophyll *a* under conditions of cyanobacterial dominance), which was derived from the epidemiological study of Pilotto *et al.* (1997); at this density, 2-4 µg $L^{-1}$ of microcystins may be expected if microcystin producing cyanobacteria are dominant; 10 µg $L^{-1}$ is possible to expected with highly toxic blooms. This level is

close to the WHO provisional drinking-water guideline (1 µg $L^{-1}$ for microcystin-LR), which is intended to be safe for lifelong consumption (WHO, 2003). With these values, the health outcomes due to microcystins are unlikely, therefore, it is sufficient to give information for visitors. Besides, it is recommended that the authorities are informed in order to start a further surveillance on the site (CHORUS et al., 1999).

*Alert Level 2. Moderate probability of adverse health effects*

If higher concentrations of cyanobacterial cells are recorded, the probability of irritative symptoms is higher. In these cases, the data used for drinking water provisional guideline value for microcystin-LR may be applied, because swimmers involuntary swallow water (a swimmer can expect to ingest up to 100-200 mL of water in one session whereas sail-board riders and water skiers would probably ingest more). Thus, the guideline level becomes 100,000 cyanobacterial cells.$mL^{-1}$ (corresponding to 50 µg $L^{-1}$ of chlorophyll *a* under conditions of cyanobacterial dominance); at this density, 20 µg $L^{-1}$ microcystins are likely to be expected if there is a bloom of *Microcystis* which has an average toxin content per cell of 0.2 pg or 0.4 µg microcystins per µg chlorophyll *a* (if the bloom mainly consists of *Planktothrix agardhii*, toxic levels may duplicate). Another reason for the increased alert level at 100,000 cells.$mL^{-1}$ is the potential for some species (such as *Microcystis* spp. and *Anabaena* spp., which frequently form scums) to increase local cell density and thus toxin concentration by a factor of 1000 or more in a few hours, changing quickly the risk from moderate to high. Due to the risks of this fast scum formation, monitoring should be performed daily because routine monitoring at the usual time intervals (e.g., 1 or 2 weeks) is unlikely to pick up hazardous maximum levels. In these cases, intervention is recommended to trigger effective information campaigns for the public, in order to educating on avoidance of scum contact. In some cases, restriction of bathing may be appropriate; at the same time monitoring programme should be intensified and health authorities should be notified immediately (CHORUS et al., 1999).

*Alert Level 3. High risk of adverse health effects*

Although evidence exists for potentially severe health hazards associated with scums, no human fatalities have been unequivocally associated with cyanotoxins ingestion during recreational water activities, even though numerous animals have been killed by consuming water containing cyanobacterial scum material. This happens because animals may drink higher volumes of scum-containing water, compared with the small amounts of scum accidentally ingested by humans. Although no reports indicate that these situations occurred, calculations suggest that a children playing in *Microcystis* spp. scums for a prolonged period and ingesting a significant contaminated water volume, could suffer a lethal dose. Therefore, the scum presence is promptly considered an indicator of a high risk of adverse health effects and the recommendation at this level is for “immediate action to control scum contact” and “possible prohibition of swimming and other water-contact activities” (CHORUS et al., 1999).

An initial assessment should be complemented by a risk-benefit approach (qualitative or quantitative). It is due to the advantages of risk-benefit approach, that international standards for recreational water, such as guidelines, are not mandatory limits, but measures for the safety of recreational water environments (WHO, 2003).

***Guidelines from different countries***

Over the last two decades, several frameworks have been designed to aid in the management of episodes of toxic cyanobacteria in drinking water. Finally, several countries are now developing guidelines to assess the concentration of cyanobacteria in recreational waters (CHORUS et al., 2000). Moreover, although WHO has established three guidance levels for recreational waters, some countries follow different guidelines. An extensive review of different guidelines concerning cyanotoxins was recently performed by Chorus (2012). Australia, for example, established thresholds for Alert Level 1 (2,000 cyanobacterial cells.$mL^{-1}$ or 1 µg $L^{-1}$ chlorophyll *a* or a biovolume of 0.2 $mm^3$ $L^{-1}$) and Alert Level 2 (100,000 cyanobacterial cells.$mL^{-1}$ or 50 µg $L^{-1}$ chlorophyll *a* or a biovolume of 10 $mm^3$ $L^{-1}$) (CHORUS et al., 1999) that are much more stringent than the WHO guidelines. However, in Australia, guidelines are not mandatory standards, as they function as recommendations that provide a basis for determining the quality of water to be supplied to consumers. In other countries, guideline levels can be a standard that must be met and compliance monitoring may be required (COALITION et al., 2009). In Korea, the Caution, Warning, and Outbreak Levels are determined by cyanobacterial cell densities of 500, 5,000, and 1,000,000 cells.$ml^{-1}$ and chlorophyll *a* of 15, 25, and 100 µg $L^{-1}$, respectively (AHN et al., 2007). The Massachusetts Department of Public Health (U.S.A.) advise that, if cyanobacteria scum is visible, the local health department, state agency or relevant authority should emit a posting regarding the possible health risks; if cell counts exceeds 50,000 cells.$mL^{-1}$, toxin quantification should be performed, in order to ensure that the guideline of 14 ppb is not exceeded; if either the cell counts exceed 70,000 cells.$mL^{-1}$ or the toxin level exceeds 14 ppb, advisory material should be posted advising not to get in contact with the water (MDPH). In Vermont (U.S.A.), advises include closing the beach and informing the public when cyanobacterial bloom/scum is visible or if microcystin-LR exceeds 6 µg $L^{-1}$, or anatoxin-a exceeds 10 µg $L^{-1}$ (HEALTHVERMONT.GOV). Cylindrospermopsin is starting to become very common in natural waters. For this reason, Humpage and Falconer (2003) suggested a guideline value of 1 µg $L^{-1}$ for drinking water for cylindrospermopsin (HUMPAGE et al., 2003) which is the same value than for microcystins-LR. Brazil and New Zealand already adopted a guideline for this toxin, but for recreational waters, no guideline was proposed (MOREIRA et al., 2013). Portugal only adopted the guideline referent to microcystin-LR for drinking waters.

#### 1.3.3.2 *Monitoring programmes*

In order to support safety in recreational water environments, the responsible management authorities should establish programmes for evaluating existing hazards and monitoring the area to evaluate any changes that may occur (WHO, 2003). Since blooms are easily visible, public health officials can monitor their initiation and development. Therefore, many countries developed programs to monitor cyanobacterial blooms (for example, the "Algae Watch" organization in United States), while in other countries, warning signs are posted when blooms are established (BAKER et al., 2002). Monitoring and sampling strategies can be adapted to the site, according to the particular water use. Hence, knowledge of horizontal distribution of cyanobacteria on the surface during recreational activities is crucial, whereas vertical distributions are essential for placing an intake at depth for the production of drinking water (BRIENT et al., 2008). The development of an appropriate sampling strategy will depend upon the primary objective of the monitoring program, which is determined by the immediate use of the water, which in turn determines the level of confidence required for the monitoring results. For example, if the water is being used directly to supply consumers, it is necessary to have a higher degree of confidence in the monitoring results, than if waters are used only for recreational proposes. Thus, this objective-based approach can be used to design a monitoring programme, considering the level of sampling effort and the resources and costs needed (COALITION et al., 2009).

For recreational waters, monitoring programmes should consider: 1) selection of monitoring sites that ensure that the main public access locations are included, as well as areas prone to accumulate scum; 2) visual inspection and physical checks (water clarity using Secchi discs, location of scums, evidence of benthic populations of cyanobacteria in swimming areas, temperature profiles to determine stratification, prevailing wind direction and weather conditions); 3) collection of samples for algal identification and enumeration, nutrients (phosphates, nitrates, silica, etc.) and toxin quantification (COALITION et al., 2009).

In the specific case of assessment cyanobacteria and algae, WHO suggests that the monitoring programs should consider (WHO, 2003):

1) monitoring of recreational areas should identify the risk of blooms development (taking into account actual or potential accumulation of toxic cyanobacteria and algae);

2) sampling points should be chosen, in order to represent different water bodies types (stratified waters, waters coming from river mouths, etc.) and to assess the sources of nutrients (discharges, upwelling, etc.) and the location of scums;

3) at high risk areas, sampling for phytoplankton should be carried out at least weekly, but during development of blooms, sampling should be intensified to daily;

4) monitoring of toxicity is justified only where there is a reason to suspect that hazards to human health may be significant; samples should only be processed in proper laboratories;

5) temperature, salinity (in marine coastal areas), dissolved oxygen, transparency, water stratification, phytoplankton biomass (chlorophyll), surface current circulation (transport of algae) and meteorological patterns such as seasonal rainfall, storms and special wind regimes should also be considered.

In regulatory monitoring programmes, factors such as frequency of inspection and/or sampling, analytical methods, data analysis, interpretation and reporting, sample site selection and criteria will generally be defined by the regulatory agency (WHO, 2003). The frequency of sampling will be dictated by a number of factors including the category of use, the current alert level status, the monitoring cost, the season and the growth rate of the cyanobacteria. Apart from cost, the most important underlying consideration is the possible health consequences of missing an early diagnosis of a problem. In the case of cyanobacteria, it is extremely important because they have growth rates generally related to seasonal conditions and those can reach 0.1-0.4 $d^{1}$ (equivalent to population doubling times of nearly a week to less than two days, respectively) (COALITION et al., 2009).

One of the simplest and most important ways of monitoring a water body is a regular visual inspection for water discolouration or surface scums of cyanobacteria and the frequency of those visual inspections may vary, depending on seasonal and weather conditions. Blooms or scums are usually most apparent early in the morning following calm days or nights, but as cell concentrations increase, scums may persist at the surface for days or weeks during prolonged periods of calm weather. Scums are visually observed at the downwind end of a reservoir, lake or river reach and also in sheltered backwaters, embayments and river bends; they appear like bright green or olive green “paint” on the surface of the water. Scums only look blue when some or all of the cells are dying, because as the cells die, they release their contents, including all their pigments (chlorophyll, phycobiliproteins, and carotenoids) into the surrounding water. However, cyanobacterial scums should not be confused with scums or mats of filamentous green algae, which appear like hair or spider web material when a gloved hand is passed through the water. The major difference in visual differentiation is the bright green colouring of the green algae, compared with a more olive or blue-green colour of cyanobacteria (COALITION et al., 2009).

Health based monitoring programmes need to be practical and focus on recreational water bodies that present a greater risk to public health. As it is not feasible to sample all water bodies, monitoring programmes need to concentrate on recreational areas that are commonly used for body contact activities such as swimming, and particularly beaches that may be known to have a variable water quality (NHMRC, 2008). Also, as cyanobacteria and cyanotoxins may greatly vary across time, monitoring programmes should be continued in time in order to prevent intoxication episodes (VASCONCELOS et al., 2011).

#### 1.3.3.3 *Information to the public*

Results of monitoring programmes should be available to participants (in a timely manner, so they can make informed decisions) and to regulators (in order to take management decisions). WHO (in "Guidelines for a Safe Recreational Water Environment" (WHO, 2003)) suggests that, as a precaution, the following guidance is recommended for all freshwater-based recreation and should be included in public information: 1) avoid areas with visible cyanobacterial or algal concentrations or scums in the water or on shore (direct contact and swallowing of appreciable amounts of water, represents a high risk of health outcomes); 2) where no scums are visible but the water shows strong greenish discolouration and turbidity, bath should be avoided; 3) where scums and discoloured water are both present, avoid water-skiing due to the potential spraying from algae and cyanobacteria agglomerates; 4) wetsuits for water sports may result in a greater risk of rashes, because cyanobacterial or algal material trapped inside the wetsuit will be in contact with the skin for long periods; 5) sailing and sailboarding may involve swallowing considerable amounts of water, particularly for beginners or in stormy weather; 6) after coming ashore, taking a shower is recommended, in order to remove cyanobacterial or algal material; 7) clothes and equipment should be washed with clean water after any contact with cyanobacterial or algal blooms and scum; 8) if any health effects are experienced, medical advice should be promptly seek and public authorities should be informed.

A review of public perception about this theme indicates that public often perceive the cyanobacterial problem as a high priority, perhaps higher than rational analysis of the available data would indicate (CHORUS et al., 1999). Thus, public participation and communication in relation to cyanobacterial hazards are important aspects of all types of planning, whether for preventive management, contingency planning or response to an incident. Awareness raising and enhancing the capacity for informed personal choice are increasingly seen as important factors in ensuring the safe use of recreational water environments and are important management interventions (WHO, 2003). Public may be informed through local news media, by posting warning notices or by other means; warnings may be supplemented with additional information on other recreational water-quality parameters regularly monitored by the authorities (NHMRC, 2008). Local non-governmental organizations, the tourism industry and local authorities could also contribute to the distribution of information brochures, training consumers on safe conduct and practices, posting warning notices, zoning dangerous areas and providing lifeguards (WHO, 2003). Providing information to consumers and to media is an important aspect for managing water quality problems associated with cyanobacterial blooms. In addition, the information should be prompt and concise with detail about the reasons for changes to supply and explanation for any differences in water quality (COALITION et al., 2009). In the presence of a bloom, a type of message that could be used to inform and involve the population could be: "If you walk into the water up to your knees, carefully, without stirring up sediment, and cannot see your feet because of a greenish discoloration, do not swim and inform the local authority using the following telephone number"

(CHORUS et al., 1999). It is also important to refer the transient nature and variable local distribution of scums, to demonstrate that restrictions on recreational activities are only temporary and often very local; it is also important to inform the public of where acceptable water quality may be found nearby (NHMRC, 2008).

#### 1.3.3.4 *Portuguese situation concerning cyanobacterial blooms occurrence and management*

In Portugal, cyanobacteria commonly occur in natural lakes, reservoirs and large slow flowing rivers (VASCONCELOS, 1999), with the dominance of potentially toxin-producing cyanobacteria such as *Microcystis* spp.*, Aphanizomenon flos-aquae, Aphanizomenon gracile, Planktothrix* sp.*, Anabaena flos-aquae, Anabaena circinalis* and *Cylindrospermopsis raciborskii* (DE FIGUEIREDO et al., 2006, SAKER et al., 2003, VALÉRIO et al., 2005, VASCONCELOS, 2001, VASCONCELOS, 1995). Toxic genera like *Microcystis* and *Cylindrospermopsis* are normally found in freshwaters that are used for captured drinking water or to recreational uses (SAKER et al., 2003). Some studies have shown that *Microcystis* is present in 70% of the rivers, lakes and reservoirs, and in 60% of that samples microcystins are also present (SAKER et al., 2007). Although acute intoxications are rare, human deaths from dialysis have been reported in 1994, at Alentejo, due the contamination of *Aphanizomenon flos-aquae* on drinking water system in Guadiana river (OLIVEIRA, 1995).

In Portugal, as in many countries across the world, the role of risk management frameworks for regulating the safety of drinking-water and the recreational use of water bodies is increasing. For the European Community, the main legislation concerning water management is supported by "Water Framework Directive", which in Portugal is transposed to "Water Law". However, in the case of bathing waters, "Bathing Waters Directive" is the main legal document and in Portugal, it was transposed to Decree-Law (DL) 113/2012. Specifically in case of cyanobacterial contamination, the "Cyanobacteria Monitoring Programme" should be carried out to assess the risk for public health.

*Directive 2006/7/CE Bathing Waters Directive*

Bathing Water Directive (76/160/CEE) of 1976 was one of the first pieces of European environmental legislation. In Portugal, this directive became law in 1998 (DL 236/98, 1st August). However, in 2006, the European Commission (EC) adopted a new directive for recreational bathing waters that called for stricter standards and reduced the number of laboratory tests done in routine beach monitoring from nineteen to two bacterial indicators, (e.g. *Escherichia coli* and intestinal *enterococci*), replacing the policies from the EC Bathing Water Directive, that have existed since 1976 (MANSILHA et al., 2009). Our practice in Portugal is in line with this international development and it is ruled by DL 113/2012, 23rd May.

This directive applies to ‘‘any element of surface water where the competent authority expects a large number of people to bathe and has not imposed a permanent bathing prohibition, or issued permanent advice against bathing’’. The monitoring point is ‘‘the location within the bathing water where most bathers are expected, or the greatest risk of pollution is expected, according to the bathing water profile’’ (2006/7/CE, 2006). With this directive, the monitoring of cyanobacterial proliferation became obligatory. Although it proposes a clear protocol for monitoring the risks related to fecal contamination, it does not fix any precise framework for the monitoring of risks associated with the development of cyanobacteria proliferations (BRIENT et al., 2008).

For recreational waters, most countries use, as guidance values, parameters that reflect cyanobacterial biomass (in order to include symptoms observed in epidemiological studies but not clearly attributable to any of the known cyanotoxins). However, the typically levels (at which such biomass values are set) are also guided by limits for the concentration of microcystins (CHORUS, 2012). However, there is a scientific debate ongoing about the need to include neurotoxins in such considerations (as they are more acutely toxic). Since there is a more actual risk of acute intoxication by neurotoxins rather than microcystins, after accidental ingestion of larger water volumes containing scum material. Also in discussion is the emerging issue of potential intoxication due to benthic mats of cyanobacteria, which may be highly toxic. In fact, New Zealand and Cuba are including benthic cyanobacteria in their approaches to protect recreational site users (CHORUS, 2012).

*DL 113/2012, 23$^{rd}$ May*

As stated above, Directive 2006/7/CE of 15$^{th}$ February, was transposed by DL 135/2009 of 3$^{rd}$ June, which was actualized by DL 113/2012 of 23$^{rd}$ de May. This last regulation, among other changes, defines the Agência Portuguesa do Ambiente, I.P. (APA, I.P.) as the competent authority for the coordination and supervision of the DL. This DL establishes the identification, monitoring and classification of bathing water quality and provides information for the public, to ensure the prevention of human health and the preservation, protection and improvement of the environment. According to this DL, bathing waters are defined as surface waters (inland, coastal or transition) that provide service to a large number of bathers and where bathing has not been banned or discouraged permanently. In waters that were not identified as bathing waters, bathing is not recommended.

It is part of the responsibility of the competent authority (APA, I.P.) to establish monitoring programmes to analyse bathing water quality. These programmes consist on a microbiological evaluation based on the analysis of intestinal *enterococci* and *Escherichia coli* for which the DL 113/2012 shows percentile values, used in the classification of bathing water quality. Based on these monitoring programmes, waters are assessed and classified as having "poor", "acceptable", "good" or "excellent" quality. In addition to these analyses, it is responsibility of the Director of Public Health Department, in conjunction with public health units, to develop health surveillance

activities that complement the assessment of the quality of bathing waters whenever risks may be present. These actions could pass through the evaluation of the presence of cyanobacteria and/or *Salmonella*. It is also his responsibility to perform health surveillance in areas that, in spite of not having been identified as bathing waters, the Health Authority finds health risks due to the number of users or the local conditions (SAÚDE, 2010).

Therefore, regarding cyanobacteria, the Article 13$^{rd}$ of the DL 113/2012 states that when the bathing water profile indicates the potential for cyanobacterial proliferation, appropriate monitoring should be performed. Moreover, this DL considers that moderate health effects (which may progressively worsen) can be expected to occur from 20,000 cells.mL$^{-1}$ or chlorophyll *a* values >10 µg mL$^{-1}$ (with a predominance of cyanobacteria). These values may correspond to microcystins concentrations of 2 to 10 µg L$^{-1}$, depending on the bloom toxicity (LOPES et al., 2007).

*The Cyanobacterial Monitoring Programme*

The Cyanobacteria Monitoring Programme is the responsibility of public health departments (SAÚDE, 2010) and should be implemented only in water bodies where, due to historical or current characteristics, toxic cyanobacterial blooms are expected to occur. This programme can be implemented both for water supply systems for human consumption and inland bathing waters (LOPES et al., 2007). According to this programme, visual observation is an essential aspect, since it is possible to suspect or recognize the presence of cyanobacteria through observation (LOPES et al., 2007): water colour with greenish or blue-green shades; blue, green scum on the shore; or dead animals inside the water or on the shore.

The evaluation for the presence of cyanobacteria should be hold monthly, from May to October. However, when exceptional events happen, this assessment should be strengthened (ARH, 2012). This evaluation considers (LOPES et al., 2007): physico-chemical parameters (transparency, temperature, pH, alkalinity, oxidability, nutrients (ammonia, nitrates and phosphates), odours, colour and wastes); biological parameters (chlorophyll *a* and phaeopigments, identification of the presence and density of cyanobacteria (through microscopy), as well as cyanobacterial identification), and toxicity assessment (whenever analyses reveal that the cell number is higher than 20,000 cells.mL$^{-1}$, toxins should be analysed through a bio-assay).

Therefore, whenever cyanobacteria are visually detected in bathing waters, the risk should be assessed. When that risk is detected, the regional health delegate should immediately inform APA, I.P. which should take the appropriate management measures (MINISTÉRIO DA AGRICULTURA, 2012). The resulting risk should be communicated to the public, which is a responsibility of APA, I.P. and regional health delegates, along with the “Serviço Especial de Proteção da Natureza e Ambiente” (SEPNA), Maritime Authority and with local authorities’ collaboration (MINISTÉRIO DA AGRICULTURA, 2012). As a precaution, the presence of blooms implies the prohibition of bathing area, followed by risk assessment (ARH, 2012).

Shortly measures are only taken when blooms are already established, with potential high risks for human health of the water users. This highlights the need of monitoring plans that may anticipate the occurrence of the cyanobacterial blooms. The use of cyanobacterial specific pigments could help to assess the stage of the development of a bloom in an easier and faster way, and previous knowledge about the environmental and ecological conditions of the water bodies could allow perceiving in which circumstances a bloom may be expected, allowing to water managers to take measures timely.

## 1.4 References


2000/607CE- Directive 2000/60/CE of the European Parliament and of the Council of 23 October 2000 establishing a framework for Community action in the field of water policy. Official Journal of European Communities L 327, 22.12.2000: 1-72. 2000.

2006/7/CE- Directive 2006/7/CE of the European Parliament and of the Council of 15 February (2006) concerning the management of bathing water quality and repealing Directive 76/160/CEE. Official Journal of the European Union. 4.3.2006, L64/37. 2006.

AHN, C.Y. [et al.] - Alternative Alert System for Cyanobacterial Bloom, Using Phycocyanin as a Level Determinant. The Journal of Microbiology. Vol. 45(2) (2007), p. 98-104.

ARH, NORTE - Orientaçoes para a execuçao do programa "Vigilância sanitária das zonas balenares interiores". Ministério da Saúde, 2012.

BACKER, LORRAINE C. - Cyanobacterial Harmful Algal Blooms (CyanoHABs): Developing a Public Health Response. Lake and Reservoir Management. Vol. 18, n.º 1 (2002), p. 20-31.

BAKER, J. A. [et al.] - Monitoring Changing Toxigenicity of a Cyanobacterial Bloom by Molecular Methods. Applied and Environmental Microbiology. Vol. 68, n.º 12 (2002), p. 6070-6076.

BOND, NICHOLAS R.; LAKE, P. S.; ARTHINGTON, ANGELA H. - The impacts of drought on freshwater ecosystems: an Australian perspective. Hydrobiologia. Vol. 600, n.º 1 (2008), p. 3-16.

BRIENT, L. [et al.] - A phycocyanin probe as a tool for monitoring cyanobacteria in freshwater bodies. Journal of Environmental Monitoring. Vol. 10, n.º 2 (2008), p. 248-255.

BURROWS, W.D.; RENNER, S.E. - Biological warfare agents as threat to potable water. Environ Health Perspectives. Vol. 107 (1999), p. 975-984.

CARMICHAEL, W. W. - Cyanobacteria secundary metabolits- the cianotoxins. Review. Journal of Applied Bacteriology. Vol. 72 (1992), p. 445-459

CARMICHAEL, W. W. - A world overview — One-hundred-twenty-seven years of research on toxic cyanobacteria — Where do we go from here? In: HUDNELL, H. K. - Cyanobacterial Harmful Algal Blooms: State of the Science and Research Needs. Advances in Experimental Medicine and Biology, 2008, p. 105-125.

CHORUS, I. - Current approaches to Cyanotoxin risk assessment, risk management and regulations in different countries. Federal Environment Agency (Umweltbundesamt) 2012.

CHORUS, I.; BARTRAM, J. - Toxic Cyanobacteria in Water: A guide to their public health consequences, monitoring and management London: E & FN Spon, 1999.

CHORUS, I. [et al.] - Health risks caused by freshwater cyanobacteria in recreational waters. Journal of Toxicology Environmental Health Part B:Critical Review. Vol. 3, n.º 4 (2000), p. 323-47.

CHORUS, I.; FASTNER, J. - Recreational Exposure to Cyanotoxins. (2001). In: de Figueiredo, D. R. (2010). Caracterização molecular de cianobactérias de sistemas aquáticos portugueses. Biology, Universidade de Aveiro. PhD.

COALITION, GLOBAL WATER RESEARCH; AUSTRALIA, WATER QUALITY RESEARCH - Guidance manual for managemement of toxic cyanobacteria. Gayle Newcombe, 2009.

CODD, G.A. [et al.]- CYANONET A Global Network for Cyanobacterial Bloom and Toxin Risk Management Initial Situation Assessment and Recommendations. Paris: International Hydrological Programme (IHP) of the United Nations Educational, Scientific and Cultural Organization (UNESCO), 2005.

CODD, GEOFFREY A. - Cyanobacterial toxins, the perception of water quality, and the prioritisation of eutrophication control. Ecological Engineering. Vol. 16, n.º 1 (2000), p. 51-60.

COX, P.A. [et al.] - Diverse taxa of cyanobacteria produce B-N-methylamino-L-alanine, a neurotoxic amino acid. Proceedings of the National Academy of Sciences. Vol. 102, n.º 14 (2005), p. 5074 –5078.

DE FIGUEIREDO, D. R. [et al.] - Microcystin-producing blooms - a serious global public health issue. Ecotoxicology and Environmental Safety. Vol. 59, n.º 2 (2004), p. 151-63.

DE FIGUEIREDO, D. R. [et al.] - Differential inter- and intra-specific responses of Aphanizomenon strains to nutrient limitation and algal growth inhibition. Journal of Plankton Research. Vol. 33, n.º 10 (2011), p. 1606-1616.

DE FIGUEIREDO, D. R. [et al.] - The effect of environmental parameters and cyanobacterial blooms on phytoplankton dynamics of a Portuguese temperate lake. Hydrobiologia. Vol. 568, n.º 1 (2006), p. 145-157.

DE FIGUEIREDO, D. R. - Caracterização molecular de cianobactérias de sistemas aquáticos portugueses. Universidade de Aveiro, 2010. PhD.

DOKULIL, M. ; TEUBNER, K. - Cyanobacterial dominance in lakes. Hydrobiologia. Vol. 438 (2000), p. 1-12.

FAY, P. - Heterotrophy and nitrogen fixation in *Chlorogloea fritschii*. In: Chorus, I. and J. Bartram (1999). Toxic Cyanobacteria in Water: A guide to their public health consequences, monitoring and management London, E & FN Spon. (1965).

FUNARI, E.; TESTAI, E. - Human health risk assessment related to cyanotoxins exposure. Critical Reviews in Toxicology. Vol. 38, n.º 2 (2008), p. 97-125.

GRANÉLI, E.; WEBERG, M.; SALOMON, P. S. - Harmful algal blooms of allelopathic microalgal species: The role of eutrophication. Harmful Algae. Vol. 8, n.º 1 (2008), p. 94-102.

GREGOR, JAKUB; MARŠÁLEK, BLAHOSLAV - A Simple In Vivo Fluorescence Method for the Selective Detection and Quantification of Freshwater Cyanobacteria and Eukaryotic Algae. Acta hydrochimica et hydrobiologica. Vol. 33, n.º 2 (2005), p. 142-148.

GRIFFITHS, D. J.; SAKER, M. L. - The Palm Island mystery disease 20 years on: A review of research on the cyanotoxin cylindrospermopsin. Environmental Toxicology. Vol. 18, n.º 2 (2003), p. 78-93.

HEALTH, MINISTERY OF - Guidelines for Drinking-water Quality Management for New Zealand. Wellington, New Zealand Ministry of Health, 2013.

HEALTHVERMONT.GOV- Cyanobacteria (Blue-green algae)-Guidance for Vermont communities

HUMPAGE, A. R.; FALCONER, I. R. - Oral toxicity of the cyanobacterial toxin cylindrospermopsin in male Swiss albino mice: determination of no observed adverse effect level for deriving a drinking water guideline value. Environmental Toxicology. Vol. 18, n.º 2 (2003), p. 94-103.

IZYDORCZYK, K. [et al.] - Measurement of phycocyanin fluorescence as an online early warning system for cyanobacteria in reservoir intake water. Environmental Toxicology. Vol. 20, n.º 4 (2005), p. 425-30.

LEE, T.Y. [et al.] - Quantitative determination of cyanobacteria in mixed phytoplankton assemblages by an *in vivo* fluorimetric method. Analytica Chimica Acta. Vol. 302 (1995), p. 81-87

LOPES, R.; SANTOS, C. - Orientações para a monitorização de cianobactérias em águas superficiais. ARSC/CRSPC, 2007.

LÜRLING, MIQUEL [et al.] - Comparison of cyanobacterial and green algal growth rates at different temperatures. Freshwater Biology. Vol. 58, n.º 3 (2013), p. 552-559.

MANSILHA, C. R. [et al.] - Bathing waters: new directive, new standards, new quality approach. Marine Pollution Bulletin. Vol. 58, n.º 10 (2009), p. 1562-5.

MARKENSTEN, H.; MOORE, K.; PEARSON, I. - Simulated lake phytoplankton composition shifts toward cyanobacteria dominance in future warmer climate. In: de Figueiredo, D. R., A. M. M. Goncalves, et al. (2011). "Differential inter- and intra-specific responses of *Aphanizomenon* strains to nutrient limitation and algal growth inhibition." Journal of Plankton Research. Vol. 33, nº 10 (2010), p. 1606-1616.

MDPH- MDPH Guidelines for cyanobacteria in freshwater recreational water bodies in Massachusetts

MEREL, S. [et al.] - State of knowledge and concerns on cyanobacterial blooms and cyanotoxins. Environment International Vol. 59 (2013), p. 303-27.

MESSINEO, VALENTINA [et al.] - Seasonal succession of *Cylindrospermopsis raciborskii* and *Aphanizomenon ovalisporum* blooms with cylindrospermopsin occurrence in the volcanic Lake Albano, Central Italy. Environmental Toxicology. Vol. 9999, n.º 9999 (2009), p. NA.

METCALF, JAMES S.; CODD, GEOFFREY A. - Cyanotoxins. In: WHITTON, B. A. - Ecology of Cyanobacteria II: Their Diversity in Space and Time. Springer Science+Business Media 2012. p. 651-675.

MINISTÉRIO DA AGRICULTURA, DO MAR, DO AMBIENTE E DO ORDENAMENTO DO TERRITÓRIO-. Diário da Républica, 1º série 2012.

MOISANDER, P. H. [et al.] - Facultative diazotrophy increases *Cylindrospermopsis raciborskii* competitiveness under fluctuating nitrogen availability. FEMS Microbiology Ecology. Vol. 79, n.º 3 (2012), p. 800-11.

MOREIRA, C. [et al.] - Cylindrospermopsin: occurrence, methods of detection and toxicology. Journal of Applied Microbiology. Vol. 114, n.º 3 (2013), p. 605-20.

MUR, L. R.; SKULBERG, R.; UTKILEN, H. - Cyanobacteria in the environment. In: Coalition, G. W. R. and W. Q. R. Australia (2009). Guidance manual for managemement of toxic cyanobacteria, Gayle Newcombe. (1999).

NHMRC - Guidelines for managing Risks in Recreational Water. Australian Government, National Health and Medical Research Council 2008.

OLIVEIRA, F.A.- Effects of cyanobacteria on drinking water and human health: An epidemiological study in Evora, Portugal: Proceedings of the International Symposium on Assessing and Managing Health Risks from Drinking Water Contamination: Approaches and Applications. Rome, Italy, 1995.

OLIVER, R.L.; GANF, G. G. - Freshwater blooms. In: de Figueiredo, D. R. (2010). Caracterização molecular de cianobactérias de sistemas aquáticos portugueses. Biology, Universidade de Aveiro. PhD.

PADISÁK, J. - *Cylindrospermopsis raciborskii* (Woloszynska) Seenayya et Subba Raju, an expanding, highly adaptive cyanobacterium: worldwide distribution and review of its ecology. Archive für Hydrobiologie Suplement Vol. 107 (1997), p. 563– 593.

PAERL, HANS W.; PAUL, VALERIE J. - Climate change: Links to global expansion of harmful cyanobacteria. Water Research. Vol. 46, n.º 5 (2012), p. 1349-1363.

POURIA, SHIDEH [et al.] - Fatal microcystin intoxication in haemodialysis unit in Caruaru, Brazil. The Lancet.. Vol. 352, n.º 9121 (1998), p. 21-26.

RAPALA, J. [et al.] - Removal of cyanobacteria, cyanotoxins, heterotrophic bacteria and endotoxins at an operating surface water treatment plant. In: de Figueiredo, D. R. (2010). Caracterização molecular de cianobactérias de sistemas aquáticos portugueses. Biology, Universidade de Aveiro. PhD

RESSOM, R. [et al.] - Health Effects of Toxic Cyanobacteria (Blue Green Algae). Report to the Environmental Standing Committee of the National Health and Medical Research Council. University of Adelaide/SAHC, 1994.

SAKER, M. L. [et al.] - Variation between strains of the cyanobacterium *Microcystis aeruginosa* isolated from a Portuguese river. Journal of Applied Microbiology. Vol. 99, n.º 4 (2005), p. 749-757.

SAKER, M. L. [et al.] - First report and toxicological assessment of the cyanobacterium *Cylindrospermopsis raciborskii* from Portuguese freshwaters. Ecotoxicology and Environmental Safety. Vol. 55, n.º 2 (2003), p. 243-250.

SAKER, M. L. [et al.] - Molecular techniques for the early warning of toxic cyanobacteria blooms in freshwater lakes and rivers. Applied Microbiology and Biotechnology Vol. 75, n.º 2 (2007), p. 441-9.

SAÚDE, DIREÇÃO-GERAL- Execução do Programa de Vigilância Sanitária das Zonas Balneares Interiores 2010.

SMITH, M. J. [et al.] - Elucidating the factors influencing the biodegradation of cylindrospermopsin in drinking water sources. Environmental Toxicology. Vol. 23, n.º 3 (2008), p. 413-421.

SRIVASTAVA, A. [et al.] - Monitoring approaches for a toxic cyanobacterial bloom. Environmental Scientific Technology. Vol. 47, n.º 16 (2013), p. 8999-9013.

STEWART, I.; SCHLUTER, P. J.; SHAW, G. R. - Cyanobacterial lipopolysaccharides and human health - a review. Environmental Health. Vol. 5 (2006), p. 7.

TARANU, ZOFIA E. [et al.] - Predicting cyanobacterial dynamics in the face of global change: the importance of scale and environmental context. Global Change Biology. Vol. 18, n.º 12 (2012), p. 3477-3490.

TELES, L. O. [et al.] - Time series forecasting of cyanobacteria blooms in the Crestuma Reservoir (Douro River, Portugal) using artificial neural networks. Environmental Management. Vol. 38, n.º 2 (2006), p. 227-237.

VALÉRIO, E. [et al.] - Molecular characterization of *Cylindrospermopsis raciborskii* strains isolated from Portuguese freshwaters. Harmful Algae. Vol. 4, n.º 6 (2005), p. 1044-1052.

VASCONCELOS, V. - Eutrophication, toxic cyanobacteria and cyanotoxins: when ecosystems cry for help. Limnetica. Vol. 25 (2006), p. 425-432.

VASCONCELOS, V. - Freshwater cyanobacteria and their toxins in Portugal. In: CHORUS, I. - Cyanotoxins - Occurrence, Causes, Consequences. Berlin: Springer, 2001. p. 62-67.

VASCONCELOS, V. - Toxic cyanobacteria (blue-green algae) in Portuguese fresh waters. Archiv für Hydrobiologie. Vol. 130 (1994), p. 439-451.

VASCONCELOS, V. M. - Cyanobacterial toxins in Portugal: Effects on aquatic animals and risk for human health. Brazilian Journal of Medical and Biological Research. Vol. 32, n.º 3 (1999), p. 249-254.

VASCONCELOS, V.; MORAIS, J.; VALE, M. - Microcystins and cyanobacteria trends in a 14 year monitoring of a temperate eutrophic reservoir (Aguieira, Portugal). Journal of Environmental Monitoring. Vol. 13, n.º 3 (2011), p. 668-72.

VASCONCELOS, VITOR MANUEL DE OLIVEIRA E - Toxicologia de cianobactérias- Distribuição decianobactérias tóxicas e as suas toxinas em águas doces portuguesas. Bioacumulação em bivalves. Porto: Faculdade de Ciências da Universidade do Porto, 1995. p.43-46 PhD.

WALSBY, A. E. - Gas Vesicles. Microbiological Reviews Vol. 58, n.º 1 (1994), p. 94-144.

WHO - Algae and cyanobacteria in fresh water. In: Guidelines for safe recreational water environments. Geneva, Switzerland: World Health Organization, 2003. p. 136-158.

WIEDNER, C. [et al.] - Seasonal dynamics of cylindrospermopsin and cyanobacteria in two German lakes. Toxicon. Vol. 52, n.º 6 (2008), p. 677-686.

WIEDNER, C. [et al.] - Climate change affects timing and size of populations of an invasive cyanobacterium in temperate regions. Oecologia. Vol. 152, n.º 3 (2007), p. 473-84.

# Chapter II

Determination of phycocyanin thresholds

# Impact of cyanobacterial inter-specific variability on the establishment of threshold guidelines for phycocyanin concentration

(Manuscript in preparation for submission to an indexed journal)

## Abstract

Phycocyanin is a cyanobacterial specific pigment that can be used to assess cyanobacterial concentration in an easy, faster and cost-effective way, using *in vivo* fluorometry. However, the establishment of phycocyanin thresholds is essential, in order to allow the use of this technique in routine monitoring programmes, although there is no consensus on the definition of international guidelines. Cultures of three cyanobacteria (*M. aeruginosa, N. muscorum* and *C. raciborskii*) were used in assays for phycocyanin, chlorophyll *a* and cell density relationships assessment. The potential interference of microalgal blooms (using *P. subcapitata*) and mixtures of different bloom-forming cyanobacteria was also evaluated. Phycocyanin showed a high linear relationship with cyanobacterial cell density, indicated that it as a suitable tool for cyanobacterial assessment, as previously reported. However, results showed a significant impact of inter-specific variation on the phycocyanin content of the tested cyanobacteria (*C. raciborkii* showed the highest phycocyanin content). Therefore, it is proposed not a single threshold but a set of thresholds, depending on the dominant bloom-forming cyanobacterium. The chlorophyll *a* and cell counting guidelines established by World Health Organization (WHO) for recreational waters were used as reference values. The proposed phycocyanin thresholds ranges were 0.8-6 μg $L^{-1}$ and 4-30 μg $L^{-1}$ for level 1 and 2, respectively, for the correspondent cell concentration; and 5.547-8.302 μg $L^{-1}$ and 27.05-41.51 μg $L^{-1}$ for level 1 and 2, respectively, for the correspondent chlorophyll *a.* In the interference evaluation assays, the algal bloom showed no significant effect on the phycocyanin signal, and the mixtures of two different cyanobacteria present high correlation coefficients, between the phycocyanin measured and the phycocyanin expect (obtain through the sum of individual phycocyanin concentrations). This suggests a low interference from these parameters on phycocyanin determination; however, more mixtures (other algae and more cyanobacteria) should be explored in order to obtain a more consistent conclusion.

## Keywords

Cyanobacteria, phycocyanin, fluorometry, thresholds, monitoring

## 2.1 Introduction

The majority of the current alert levels for the assessment of bathing water are based on parameters such as chlorophyll *a* (chl *a*) and cyanobacterial density. However, problems may arise from the use of only these two parameters, when the goal is to avoid the risk for public health, due to cyanobacterial blooms. Chl *a* is not a specific cyanobacterial pigment; and microscopic enumeration of cyanobacterial cells are dependent on the skilfulness and subjective decisions of each counter and therefore may include a substantial error, due to the difficulty of counting large colonies and entangled long filaments (AHN et al., 2007). Hence, it is important to develop a more accurate cyanobacterial bloom alert system which uses cyanobacteria-specific characteristics that can complement or even replace, at an early stage, chl *a* and cells counts determinations. A good option is the use of fluorometry to measure phycobiliproteins; this technique revels to be very effective and does not require a great deal of dexterity or expensive equipment. In case of freshwaters, phycocyanin (PC) is considered the best pigment to determine cyanobacterial concentration (LEE et al., 1995).

Inside phytoplankton cells, light energy is absorbed by light-harvesting pigments and pass on to the photosynthetic reaction centers. When pigments absorb the energy of a photon, they enter into an excited electronic status, and in order to return to the initial status, four competitive processes may occur: photochemical reactions; heat dissipation; transfer the excited energy to adjacent pigments; or emission of a fluorescent photon. Fluorescence analysis depends on this last phenomenon (CAMPBELL et al., 1998). Therefore, *in vivo* fluorescence is inversely correlated with the on-going rate of photosynthesis (LEE et al., 1995).

Fluorescence refers to the molecular absorption of light energy at one wavelength and its nearly instantaneous re-emission at another, usually longer, wavelength. The fluorescent compounds have two characteristic spectra: an excitation and an emission spectrum. These spectra could be referred as a compound's fluorescence signature or fingerprint. Fluorometry is a highly specific analytical technique because it is based on the principle that all compounds have different fluorescence signatures. Following this principle, fluorometers measure the emitted light which is proportional to the analyte concentration to be measured (TURNER).

When compared with other analytical techniques, fluorometry shows a high sensitivity, specificity, simplicity and low cost. Also, there is no special sample handling or processing required, making this technology ideal for monitoring *in situ* or for remote monitoring with *on-line* systems for real-time data collection (SRIVASTAVA et al., 2013).

*In vivo* fluorometry could be used for locating and measure algal densities and it has been used by oceanographers and limnologists for over 30 years (LORENZEN, 1967b). In natural samples, fluorescence signals are specific to photobionts and allow *in situ* measurements of mixed natural populations, in order to quantify phytoplankton pigments (PORYVKINA et al., 2000). As algal classes have their own light-harvesting pigment system, they could be used for spectral discrimination of *in vivo* fluorescence fingerprints (BEUTLER et al., 2002). There have been several studies that tried to achieve a taxonomic identification using *in vivo* bio-optical characteristics of

phytoplankton, like fluorescence excitation and absorption spectra (PORYVKINA et al., 2000). The earlier investigations that used chl *a*/ accessory pigment ratio technique in excitation spectra showed that the identification of the algae class or species in natural mixed phytoplankton population could be difficult. However, if cells can be separated and analyzed individually, fluorescence can be a good method for taxonomic identification (PORYVKINA et al., 2000).

Chl *a* is a pigment that is present in all phytoplankton organisms (GREGOR et al., 2005) and, for that reason, it is normally used to assess total algal biomass (TURNER, 2004). For the estimation of chl *a* there are two main approaches: the earlier and still most used, is the spectrometric analysis, after extraction with organic solvents like methanol or acetone (APHA, 1999); the other is the fluorescence method, which sensitivity is higher than the spectrometric method, requires little amount of water, its faster (HOLM-HANSEN et al., 1965) and can be used *in vivo* with intact cells (LEE et al., 1995). Fluorometry takes into account that chl *a* is mainly located in photosystem II and has an excitation maximum around 440 nm and light emission at 680 nm (LORENZEN, 1967b). However, the analysis of chl *a* fluorescence does not provide great information about the taxonomic composition of the sample (LEE et al., 1995). Although fluorometry can be used for chl *a* determination, in the specific case of cyanobacteria it cannot be applied with precision, since most of the cyanobacterial chl *a* is present in the weakly fluorescent photosystem I. Therefore, its direct excitation by the blue light is not efficient (GREGOR et al., 2005). However, other pigments (so-called accessory pigments) are present in the highly fluorescent photosystem II, and unlike chl *a,* show differences according to the taxonomy of the organisms; but, the majority usually do not fluoresce (GREGOR et al., 2005). Since various accessory pigments are excited by different wavelengths, their fluorescence excitation spectra can be used to differentiate algal groups (BEUTLER et al., 2002). Nevertheless, for specific groups, other pigments can be used, such as PC (blue pigment that exhibit fluorescence), for cyanobacteria (BRIENT et al., 2008).

The photosynthetic system of cyanobacteria is tightly connected to other principal metabolic paths and it is in itself a major metabolic sink for iron, nitrogen and carbon skeletons (CAMPBELL et al., 1998). Their photosynthetic apparatus function in a very similar way to higher plants and algae. However, the light harvesting in cyanobacteria is mostly performed by the accessory pigments, such as phycobilisomes (PBS) which contain phycobiliproteins (PBP) (GLAZER, 1984). Biliproteins act as accessory pigments to collect and transfer the light energy to the reaction center. Based on the position of the absorbed bands, these biliproteins are divided into three classes (in increasing order of wavelength): phycoerythrin (PE), PC, and allophycocyanins (PORYVKINA et al., 2000).

Under different environmental conditions, there are significant variations in the relative content of fluorophores present in cyanobacteria and that should be taken into account when probes are chosen (WLODARCZYK et al., 2012). Also, under certain conditions (e.g., low ionic strength) PBS (which are usually energetically coupled to photosystems) detach and can disassemble into individual PBP (GANTT et al., 1979). The release of these PBS may occur

naturally when the cells suffer lysis, as a consequence of aging or stress conditions (e.g., blooming). More, during some steps of water treatment, cell disruptions may happen (CHORUS et al., 1999) and lysis may represent risks, since there is a correlation between the levels of free PBP and concentrations of toxins released by cyanobacteria. Thus, as the decoupling of PBP from the thylakoids considerably modifies the emission characteristics of the cyanobacterial photosynthetic apparatus, it is important that a monitoring based model considers the form from which PBP are available (WLODARCZYK et al., 2012).

PSP are strongly auto-fluorescent and it acts as the basis of *in vivo* fluorometric distinction of cyanobacteria from other aquatic photosynthetic micro-organisms (BEUTLER et al., 2002). PC is also present in red algae (GROSSMAN et al., 1994); however, in freshwaters, cyanobacteria are the only microorganisms that produce significant quantities of PC and its derivative allophycocyanin (BRIENT et al., 2008). Additionally, in red-algae, PC cannot be measured by fluorometric methods (LEE et al., 1995). Several studies used this property to differentiate cyanobacteria from eukaryotic algae (BASTIEN et al., 2011, GREGOR et al., 2005, IZYDORCZYK et al., 2005, LEE et al., 1995, WATRAS et al., 1988). This was based on the fact that the excitation peak of *in vivo* chl *a* occurs at 440 nm and the emission at 680 nm and these peaks are weak in the cyanobacterial spectra, since the excitation wavelengths of PC occur at 550-650 nm (i.e. in the orange and red band of the spectrum) and the emission peak around 645 nm (LEE et al., 1995).

*In vivo* and *in situ* fluorescence methods for phytoplankton quantification allow the possibility of continuous *on-line* monitoring, with implications for a less frequent discrete sampling and time-consuming laboratory analyses (GREGOR et al., 2005). Several models of submersible probes for monitoring phytoplankton *in situ*, using *in vivo* fluorescence are already commercially available. Some examples of these instruments are: bbe, FluroProbe (a submersible spectrofluorometer with automatic algae class and chl analysis); bbe, Algae Online Analyser (AOA) fluorometer (with *on-line* detection of chl concentration, algal classes and photosynthetic activity); Turner, Fluorometer 10-AU (allows the use of different filters in order to determine the concentration of several pigments, including PC); TriOs, miroFlu-blue (is a low-cost submersible fluorometers for PC detection); and YSI, 6131 BGA-PC (which is a sensor optimized to measured PC in fresh or estuarine waters).

Beside the advantages fluorometry, it is important to take into account that, for the purpose of health risk assessment, it is necessary to determine the concentration of toxins (IZYDORCZYK et al., 2005). However, the fastness of measuring *in vivo* fluorescence provides time to analyze cyanotoxins, without waiting for results from cell counts (BRIENT et al., 2008). Therefore, immediate actions can be implemented. Also, with the measurement of PC fluorescence it is possible to monitor the vertical and horizontal distribution of cyanobacterial cells in a reservoir, allowing optimization of the depth of drinking water captation. In order to forecast potential risks from the generation and translocation of cyanobacterial blooms in a reservoir, monitoring of horizontal distribution may also be useful (IZYDORCZYK et al., 2005). For monitoring bathing waters purposes, horizontal distribution of cyanobacteria on the surface is crucial (BRIENT et al.,

2008). The use of fluorometric determination of PC for the assessment of cyanobacteria can be very useful to provide pre-bloom episode information for water body managers (CHANG et al., 2012). However, one limitation of this technique is to be a qualitative tool rather than quantitative, thus it does not give the exact value of PC concentration. However, as it shows a significant correlation with cell numbers, it can be used as a semi-quantitative tool (BRIENT et al., 2008). Moreover, there have been controversial studies arguing on which values should be adopted as guidelines for PC concentration.

The present study used three cyanobacteria for determining their PC levels and proposes thresholds based on alert guidelines recommended by WHO using chl *a* and cyanobacterial cell concentrations. Three scenarios (isolated cyanobacteria, mixtures with a microalga and mixtures with other cyanobacteria) were created in order to understand how *in vivo* PC is influenced by the presence of algae bloom and other cyanobacteria.

## 2.2 Material and Methods

***Cyanobacteria isolation and cultures establishment***

Three cultures of cyanobacteria (*Nostoc muscorum, Cylindrospermopsis raciborskii* and *Microcystis aeruginosa*) and an alga (*Pseudokirchneriella subcapitata*) were established in laboratory. These strains were isolated from natural shallow water bodies, using a plate isolation approach. Cultures were maintained in erlenmeyers containing sterilized liquid MBL – Woods Hole culture medium (NICHOLS, 1973) and in Petri plates containing agarised MBL medium, in an incubation chamber at 20 ± 2ºC and a 12h L:12h D photoperiod of 1087 LUX provided by cool white fluorescent tubes. The cultures for biomass and pigments analysis were homogenised with a magnetic stirrer.

***Experimental design***

After growing for 7-10 days, cyanobacteria and algae were harvested from cultures at an exponential growth phase. The concentrated inoculum for each cyanobacterium was diluted (according to Table 2.1) and, for each dilution, the parameters cell number, PC and chl *a* concentrations were determined. These determinations were performed in order to assess relationships between these parameters for each cyanobacterial culture and extrapolate PC thresholds, corresponding to cell concentrations and chl *a* guideline values proposed by WHO. The harvested culture at the exponential phase was considered as 100% concentrated and dilutions were performed (ranging from a concentration of 90% to 0.5% or less, according to Table 2.1) in 50 mL Falcon tubes using diluted MBL medium. In all cultures (but particularly for *M. aeruginosa* and *N. muscorum* cultures), a magnetic stirrer was used during the growth phase, in order to homogenise cells in culture, but minimizing the cells lysis. A second part of the experience comprised the assessment of possible interference from microalgal blooms during the measurement of PC (Table 2.1). This was tested using a culture of *P. subcapitata* under different

mixture concentrations of with each cyanobacterium. Mixtures of different cyanobacteria were also tested, in order to evaluate potential overestimation of PC during blooms of more than one dominating cyanobacteria. The same parameters described above were determined for these experiments. Two series of trials were carried out in different temporal occasions, in order to include possible physiological fitness variation between cultures.

PC determination and filtration of water samples for chl *a* determination were performed at the same time and the remaining volume (about 10 mL) was preserved in Lugol for later cells counting.

**Table 2.1** Mixtures and dilutions used in the first and the second trials experiments.

| | **1st Trial** | **2nd Trial** |
|---|---|---|
| | **Isolates** | |
| *M. aeruginosa* | 100%, 90%, 80%, 60%, 50%, 40%, 30%, 20%, 10% | 100%, 90%, 80%, 70 %, 60%, 50%, 40%, 30%, 20%, 10%, 5%, 2.5%, 1%, 0.5% |
| *N. muscorum* | 100%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10% | 100%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10%, 5%, 2.5%, 1%, 0.5% |
| *C. raciborskii* | 100%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10% | 100%, 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10%, 5%, 2.5%, 1%, 0.5% |
| | **Mix (cyanobacterium + alga)** | |
| *M. aeruginosa* / *P. subcapitata* | 90%/10%, 70%/30%, 50%/50%, 30%/70%, 10%/90% | 90%/10%, 70%/30%, 50%/50%, 30%/70%, 10%/90% |
| *N. muscorum* / *P. subcapitata* | 90%/10%, 70%/30%, 50%/50%, 30%/70%, 10%/90% | 90%/10%, 70%/30%, 50%/50%, 30%/70%, 10%/90% |
| *C. raciborskii* / *P. subcapitata* | 90%/10%, 70%/30%, 50%/50%, 30%/70%, 10%/90% | 90%/10%, 70%/30%, 50%/50%, 30%/70%, 10%/90% |
| | **Mix (two cyanobacteria)** | |
| *M. aeruginosa / C. raciborskii* | 90%/10%, 70%/30%, 50%/50%, 30%/70%, 10%/90% | 90%/10%, 70%/30%, 50%/50%, 30%/70%, 10%/90% |
| *C. raciborskii / N. muscorum* | 90%/10%, 70%/30%, 50%/50%, 30%/70%, 10%/90% | 90%/10%, 70%/30%, 50%/50%, 30%/70%, 10%/90% |
| *M. aeruginosa / N. muscorum* | - | 90%/10%, 70%/30%, 50%/50%, 30%/70%, 10%/90% |

### *Phycocyanin determination*

PC was determined through spectral fluorescent signatures (SFS), in the laboratory, using a FLUO-IMAGER™ (SKALAR, Analytical B.V., Breda, The Netherlands), model M53B version 1.0703, which is a fluorometer normally used for detection of photosynthetic pigments in the water. The principal of SFS is based on the measurement of excitation and fluorescent spectra. Excitation wavelength and emission spectra were measured with a Photomultiplier (PMT). After a spectrum is measured, the excitation wavelength shifts 2 nm and the emission spectrum was measured again.

This process continues until the complete excitation range has been scanned. The characteristic spectral structure of SFS is used for substance recognition and the intensity of fluorescence allows the estimation of the analysed substance quantity (Fluo-Imager SKALAR). In general, PE and PC components are included in all live catalogues in mixtures of phytoplankton with blue-green algae. Catalogue TV 17 was used, since it includes the spectra for the peak of PC (excitation at 620 nm and emission at 640 nm). *In vivo* chl *a* was also measured by this method.

### *Cell counts*

Cell counts were performed in a Sedgwick-Rafter chamber under an inverted microscope (Olympus CKX41), according to a standard procedure (BELLINGER et al., 2010). After preservation in Lugol, 1 mL of sample was placed into the Sedgwick-Rafter chamber and left to settle for 10 min. Cell counts were performed in the 100% culture cell concentration of the exponentially grown cultures of *M. aeruginosa, N. muscorum, C. raciborskii* and *P. subcapitata*; at least 2000 cell were counted from each sample and the counting was considered valid when a stabilized coefficient of variance was obtained. Cell counts for the dilutions were extrapolated from the value obtained for the 100% concentrated sample. This decision was taken after preliminary counts of the samples showed relatively low correlation coefficients for some cultures (*M. aeruginosa* $r$=0.903, $n$=9, $p < 0.05$; *N. muscorum* $r$=0.860, $n$=10, $p < 0.05$, *C. raciborskii* $r$=0.961, $n$=10, $p < 0.05$ and *P. subcapitata* $r$=0.626, $n$=6, $p < 0.05$), which negatively influenced the remaining correlation between cell densities and pigment concentration.

### *Chlorophyll a determination*

For chl *a* determinations, samples were filtered through Whatmann GF/C filters (1.2 µm), and then frozen (-20ºC) until further processing. Chl *a* was determined using the trichromatic method (APHA, 1999 Section 10200H.2c), however, the monochromatic method (LORENZEN, 1967a) was also used to determine the influence of phaeopigments in the overall measured chl *a*. Chl *a* was extracted with 90% acetone and left at 4ºC overnight; tubes were covered in order to avoid chl degradation. After this period, tubes were centrifuged for 15 min at 4000 rpm, and the absorbance of the supernatant read at 750, 664, 647 and 630 nm in a Shimadzu UV-1800 Spectrophotometer, to allow determination of chl *a*, *b* and *c* through trichromatic equations. For the monochromatic method, absorbance values were measured at 750 and 665 nm with and without acidification (0.1 N HCl).

Since phaeopigments did not show influence (all values were negative), only the chl *a* determination resulting through the trichromatic method was used.

### *Statistical analyses*

Simple linear regressions between PC, cell density and chl *a* were obtained using Sigma Plot® software. Analysis of covariance (ANCOVA) was used to test homogeneity of slopes, in the relationships PC and chl *a* versus cell density, among species. A similar approach was used to

determine the influence of *P. subcapitata* in the PC signal of mixtures of cyanobacteria + alga, ANCOVAs were performed, with Minitab (v16)®. For mixtures with two cyanobacteria, linear regressions of expected PC (corresponding to the sum of individual PC concentrations) and measured PC were tested and the significance of each regression and confidence intervals of the slope was assessed using analysis of variance (ANOVA), with Excel. The choice and interpretation of the statistical analyses performed was supported by bibliography of reference (ZAR, 1996).

## 2.3 Results and Discussion

### *Assays using isolated cyanobacteria*

The chl *a* concentration results for each culture of cyanobacteria (*N. muscorum, C. raciborskii* and *M. aeruginosa*) are presented in Figure 2.1.

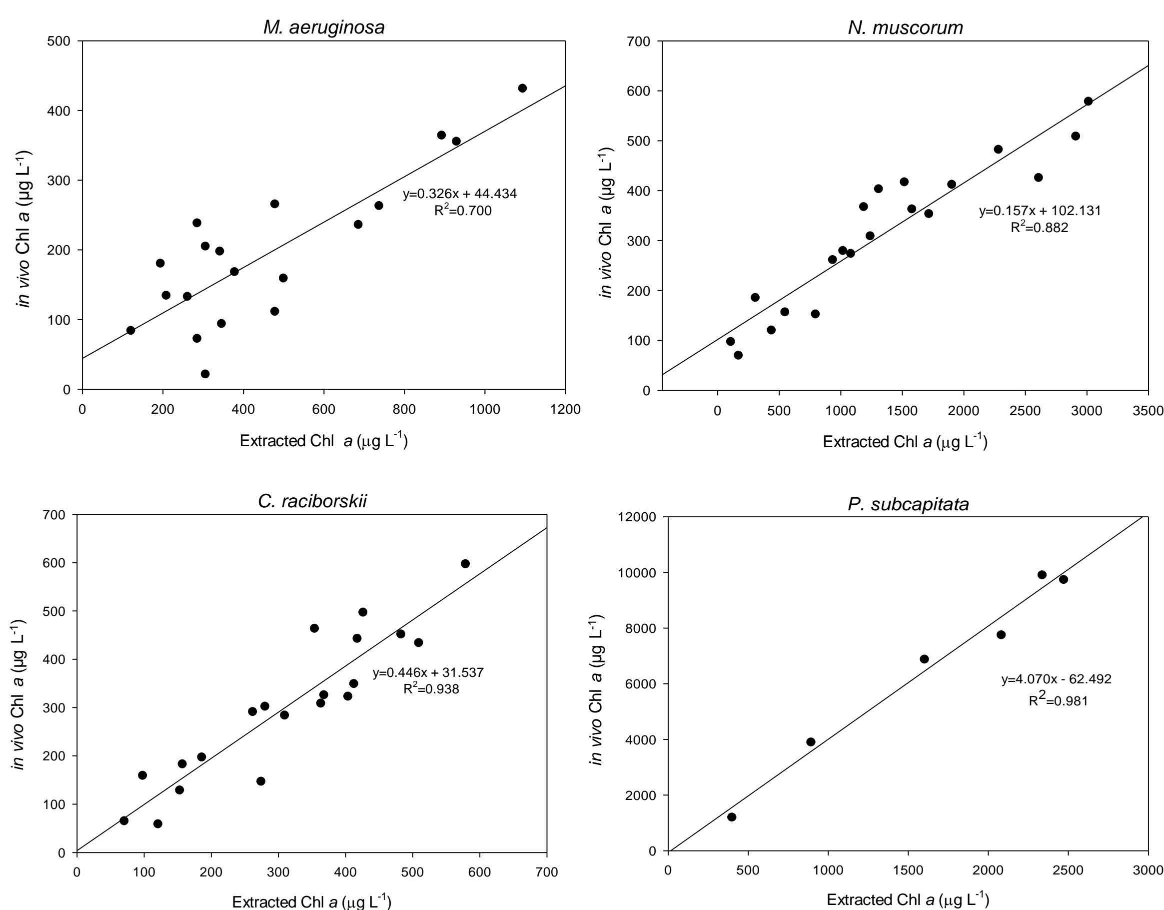



**Figure 2.1** Linear regression between *in vivo* chl *a* (µg $L^{-1}$) and chl *a* extracted and read by spectrophotometry (µg $L^{-1}$) for the cyanobacteria *M. aeruginosa* (*n*=19), *N. muscorum* (*n*=20), *C. raciborskii* (*n*=21) and alga *P. subcapitata* (*n*=6). For all figures $p < 0.001$.

The *in vivo* chl *a* measured by fluorometry and the extracted chl *a* measured through spectrophotometry exhibited a linear relationship. However, for the three cyanobacterial species, *M. aeruginosa*, (*d.f.*=1, 17, F=39.6, $p < 0.001$), *N. muscorum* (*d.f.*=1, 18, F=134.9, $p < 0.001$) and *C. raciborskii*, (*d.f.*=1, 19, F=286.8, $p < 0.001$), this correlation was smaller than for alga *P. subcapitata*, (*d.f.*=1, 4, F=204.1, $p < 0.001$). This linear relationship was already been described (ZAMYADI et al., 2012) and the lower correlation coefficients observed (in cyanobacteria comparatively to algae) may be justified by the fact that the majority of cyanobacterial chl *a* (80-90%) is located in the photosystem I, which is very efficient and therefore weakly fluorescent. The remaining chl *a* is located in photosystem II and only this portion is detected by fluorometry. Therefore, fluorometry allows a good detection of the algal chl *a* (although eukaryotic algae emitted a weaker fluorescence per unit of chl *a* than cyanobacteria (LEE et al., 1995) but may underestimate the cyanobacterial chl *a* (ZAMYADI et al., 2012). For this reason, Bowling *et al.* (2012), considered chl *a* measured by fluorometry as a poor predictor of the amount of total cyanobacterial biovolume in environmental samples ($R^2$=0.015) and Zamyadi *et al.* (2012), also in environmental samples, recorded a poor correlation between *in vivo* chl *a* fluorescence and the extracted chl *a* ($R^2$=0.23). On the other hand, Izydorczyk *et al.* (2009) found a good correlation between *in vivo* chl *a* fluorescence and the extracted chl *a* ($R^2$=0.92). These different results may be explained by the different probes with different specificities and excitation and emission measurements used by several authors. Nevertheless, in spite fluorometry may not be a good technique to estimate cyanobacterial chl *a*, it is a very accurate method to measured PC, and several studies show a high correlation between the *in vivo* and the extracted PC, such as Zamyadi *et al.* (2012), with $R^2$=0.96, and Ahn *et al.* (2007), with $R^2$=0.886. This happens because, in cyanobacteria, phycobilin pigments harvest light in the highly fluorescent photosystem II, and therefore are easily measured by fluorometry (CAMPBELL et al., 1998).

In the present study, PC concentration also presented a good correlation with cell counts, as it is shown in Figure 2.2, for all the three cyanobacteria analysed, *M. aeruginosa* (*d.f.*=1, 21, F=423.2, $p < 0.001$), *N. muscorum* (*d.f.*=1, 22, F=639.4, $p < 0.001$) and *C. raciborskii* (*d.f.*=1, 22, F=222.2, $p < 0.001$). This relation has already been described in previous studies (AHN et al., 2002, BASTIEN et al., 2011, BRIENT et al., 2008, IZYDORCZYK et al., 2005). The same analysis was performed to assess the relationship between PC and extracted chl *a* (Figure 2.2), and results showed also a good correlation for all the cyanobacteria analysed, *M. aeruginosa* (*d.f.*=1, 20, F=98.5, $p < 0.001$), *N. muscorum* (*d.f.*=1, 21, F=234.2, $p < 0.001$) and *C. raciborskii* (*d.f.*=1, 21, F=574.5, $p < 0.001$), which corroborates the results of other authors (AHN et al., 2002, ZAMYADI et al., 2012). In environmental samples, PC also showed a good correlation with extracted chl *a*, under cyanobacterial dominance (AHN et al., 2007, SEPPÄLÄ et al., 2007, ZAMYADI et al., 2012), which is a very important aspect allowing the application of this technique to environmental samples and, consequently, in monitoring programmes.

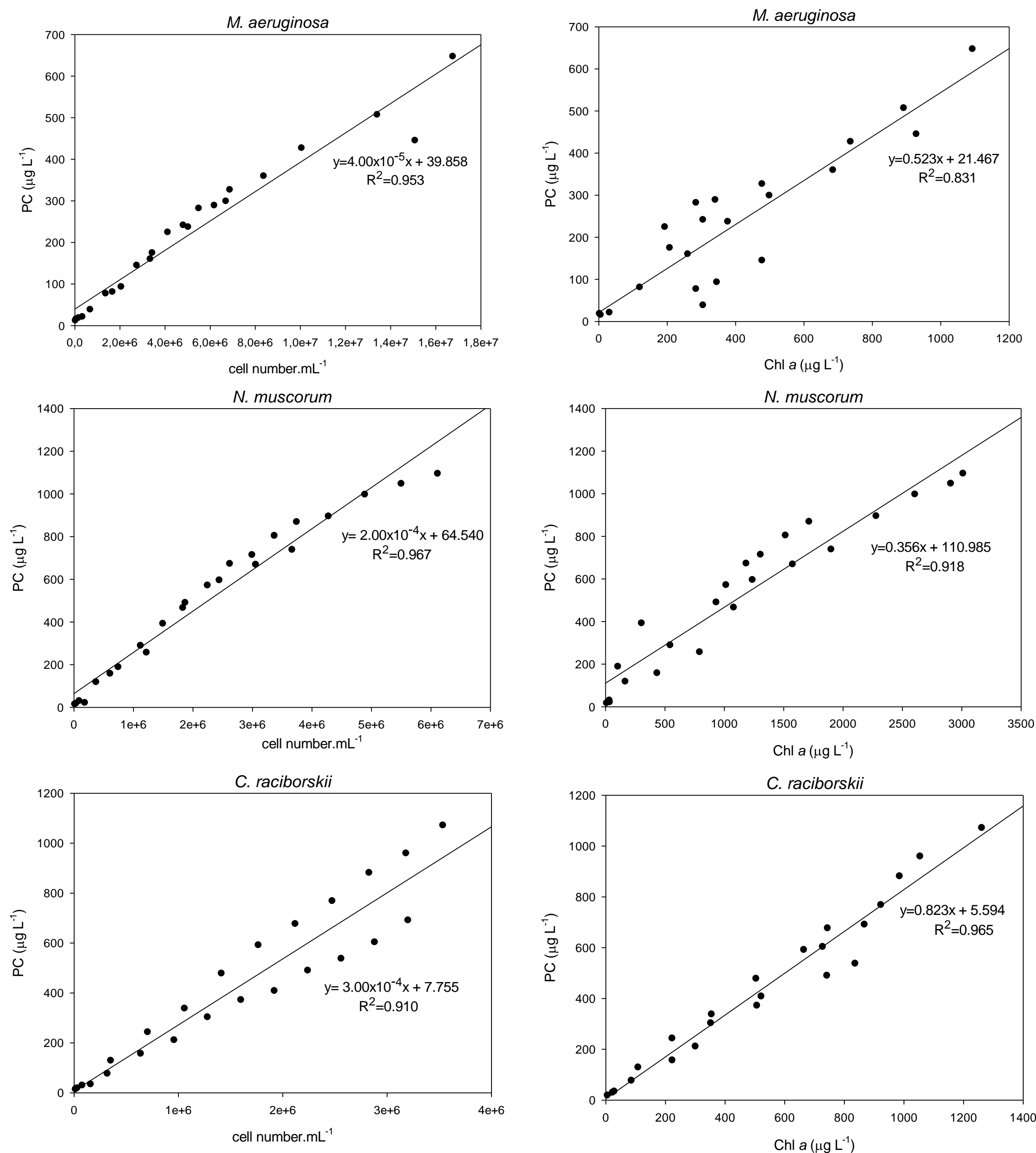



**Figure 2.2** Linear regression between PC (µg $L^{-1}$) and cell density (cell number.$mL^{-1}$) for the three cyanobacterial species, *M. aeruginosa* (*n*=23), *N. muscorum* (*n*=24) and *C. raciborskii* (*n*=24), and between PC (µg $L^{-1}$) and extracted chl *a* (µg $L^{-1}$) for *M. aeruginosa* (*n*=22), *N. muscorum* (*n*=23) and *C. raciborskii* (*n*=23). In all the figures $p < 0.001$.

Current alert systems are based on the assumption that there is a constant linear relationship between cyanobacterial density and chl *a* concentration (WHO, 2003), similarly to the observed in Figure 2.3 (*M. aeruginosa*, (*d.f.*=1, 20, F=153.9, $p < 0.001$); *N. muscorum*, (*d.f.*=1, 21, F=745.6, $p < 0.001$); and *C. raciborskii*, (*d.f.*=1, 21, F=447.4, $p < 0.001$)). However, other studies

refute this relation, as chl *a* content generally decreases with high cell densities, when cells enter the stationary phase (AHN et al., 2007).

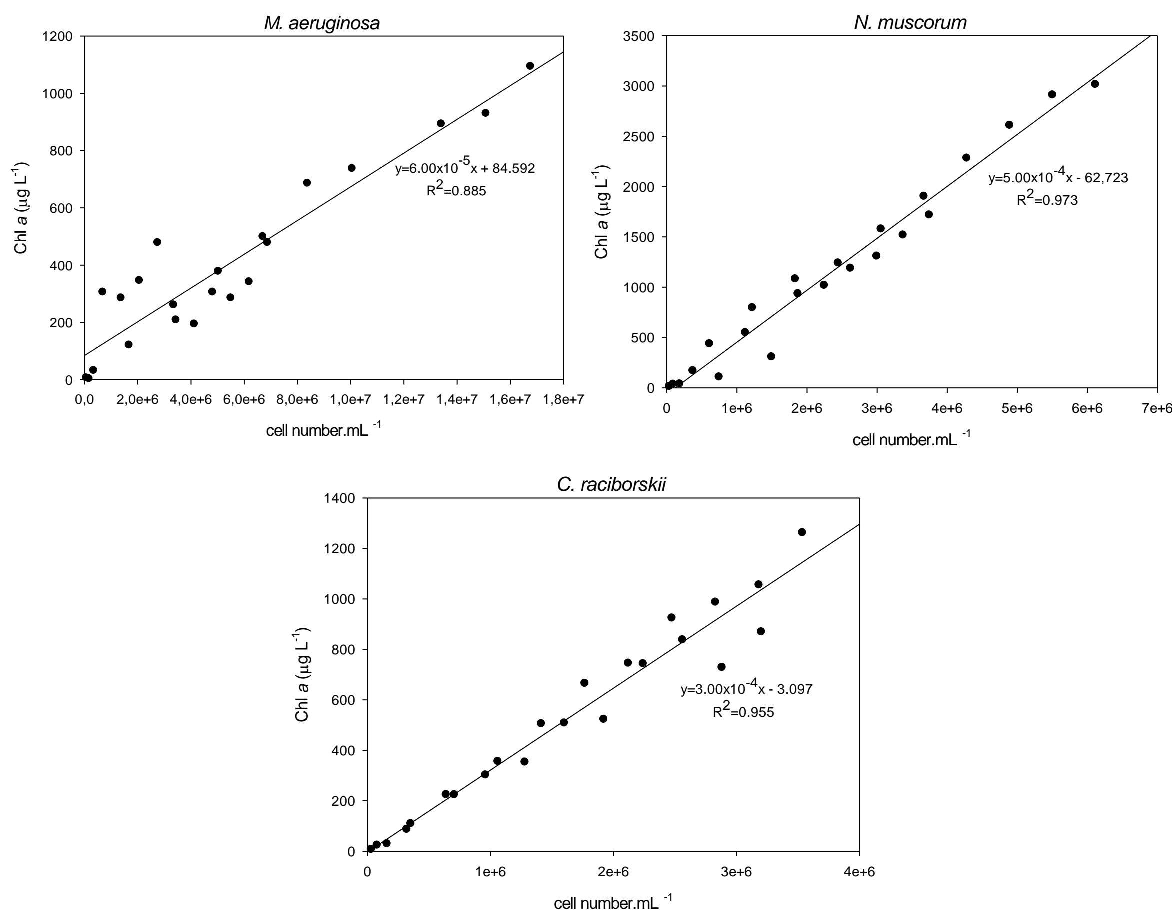



**Figure 2.3** Linear regression between extracted chl *a* (µg $L^{-1}$) and cell density (cell number.$mL^{-1}$) for the cyanobacteria *M. aeruginosa* (*n*=22), *N. muscorum* (*n*=23) and *C. raciborskii* (*n*=23). For all these figures $p < 0.001$.

Different species had different contributions of PC (test of homogeneity of slopes, using ANCOVA: d.f.=2, 65, F=278.8, p < 0.001) (see Figure 2.2) and of chl *a*, (test of homogeneity of slopes, using ANCOVA: d.f.=2, 62, F=489.9, p < 0.001) (see Figure 2.3). These differences between pigments content have already been described in the literature (CHANG et al., 2012, HORVÁTH et al., 2013, IZYDORCZYK et al., 2005, LEE et al., 1995). *M. aeruginosa* presented the lower slope ($4.00x10^{-5}$), which means that its contribution in PC was notoriously smaller than the contribution of the other two cyanobacteria. Filamentous *C. raciborskii* had the highest PC contribution ($3.00x10^{-4}$). In fact, *C. raciborskii* has been already described as a cyanobacterium with high levels of PC, when compared to other filamentous cyanobacteria such as *Anabaena spiroides, Aphanizomenon flos-aquae* or *Aphanizomenon issatschenkoi* (HORVÁTH et al., 2013).

In the case of chl *a, M. aeruginosa* also showed the lowest contribution ($6.00x10^{-5}$), while *N. muscorum* showed the highest ($5.00x10^{-4}$). These different contributions are important when threshold levels are proposed. Chl *a* and PC levels are proportional to the volume of cell and the group to which they belong (BRIENT et al., 2008). Therefore, variations in fluorescence intensity between cyanobacterial species may reflect differences in cell, or filament geometry and intracellular PC dispersion, which will affect the efficiency of light absorption. In addition, this variation may be due to differences in wavelength of the emission peaks (LEE et al., 1995). Therefore, in some species, difficulties in fluorometric determinations can occur, e.g. *Microcystis* sp., which is composed by large colonies. This cell arrangement could hinder the excitation light to penetrate into the cells, which can cause scattering and re-absorption of the fluorescence emission (for colonies larger than 13-18 µm, PC of the inner portion of the colonies will not be excited, or if is excited, the emitted fluorescence will not be detected) (CHANG et al., 2012). Seppälä *et al*. (2007) observed that large colonies produce noisier fluorescence signals, and thus *Microcystis* sp. concentration can be underestimated (CHANG et al., 2012). This may explain the minor PC levels found for this species, when compared to the other two. One way to solve the colonies problem is sonicating samples (GREGOR et al., 2005); however, with this technique, some cells may lyse. In filamentous cyanobacteria, tangling of the filaments can also interfere with fluorometric analysis, causing interference in the distribution of the excitation light (LEE et al., 1995). To overcome this limitations, a magnetic stirrer was used inside the cultures during several days, in order to create a homogeneous culture, avoiding the cells lysis, following the example of Zamyadi *et al*. (2012), since Chang *et al*. (2012) demonstrated that dispersion of the colonies (in case of *M. aeruginosa*) increase PC and cell counts determination.

Phytoplankton *in vivo* fluorescence excitation spectra depends on the taxonomy of algae and on its photoadaptation status (GROSSMAN et al., 1994). Beside differences in the pigment content, variations on the spectra of different species may occur, due to the number and type of different phycobilin chromophores present in each phycobiliprotein, which determine the spectroscopic properties (SEPPÄLÄ et al., 2007). Furthermore, the volume and shape of cyanobacteria, turbidity, growth stage, level of light saturation, aggregation of cells into colonies and historical conditions of light and nutrients can also influence fluorometric analysis (BASTIEN et al., 2011). For example, low light intensity levels can stimulate the synthesis of PC (GROSSMAN et al., 1994) but nitrogen limitations induce PC degradation, since phycobiliproteins are used as a nitrogen reserve (IZYDORCZYK et al., 2005). However, despite the referred limitations, there is a strong correlation between the extracted PC (measured trough quantification methods such as spectrophotometric, liquid chromatographic, capillary and gel electrophoretic, or single-step chromatographic analyses (HORVÁTH et al., 2013)) and the PC measured by fluorometry, which makes this method a reliable tool for PC assessment.

***Evaluation of the interference of mixtures with a microalga***

Since PC showed a good relation with cells number.mL$^{-1}$ (Figure 2.2), it could be used as a good predictor of the cyanobacterial density. According to other authors (CHANG et al., 2012, LEE et al., 1995) algae do not possess PC, as shown by the tested alga, *P. subcapitata*. Under PC wavelength, fluorescence of eukaryotic algae is weak, and Lee *et al.* (1995) observed that using the combination, excitation at 620 nm and emission at 645 nm, it is possible to quantify the concentration of cyanobacteria if green algae and diatoms do not exceed 80% of the total chl *a*. Gregor and Marsalek (2004) also observed that this technique is suitable to be implemented in water bodies with low levels of Chl *a*.

In a mixture, the chl *a* contained within eukaryotic phytoplankton will contribute to the total chl *a* concentration, but not to the PC signal (BOWLING et al., 2012), because *in vivo* fluorescence characteristics of cyanobacteria are different from that of eukaryotic algae (excitation peak of chl *a* occurs at 440 nm and the emission at 680 nm; these peaks are weak in cyanobacterial spectra, because they have a excitation peak ranging 550-650 nm and emission peak around 645 nm) (LEE et al., 1995). Studies have shown that fluorometric interference may affect the correct PC determination, since chl *a* and PC emission wavelengths may overlap (CHANG et al., 2012). However, in all three cases, *P. subcapitata* did not have a significant influence in the PC determination in the mixtures of *M. aeruginosa* + *P. subcapitata* (ANCOVA: *d.f.*=2, 27, F=0.49, *p*=0.615), *N. muscorum* + *P. subcapitata* (*d.f.*=2, 28, F=0.97, *p*=0.393) and *C. raciborskii* + *P. subcapitata* (*d.f.*=1, 30, F=0.52, *p*=0.475) (see Figure 2.4). The relationship between PC and cell density was therefore similar (i.e. had an equal slope) in isolated (mono-specific) cultures or when cyanobacteria were mixed with the green alga.

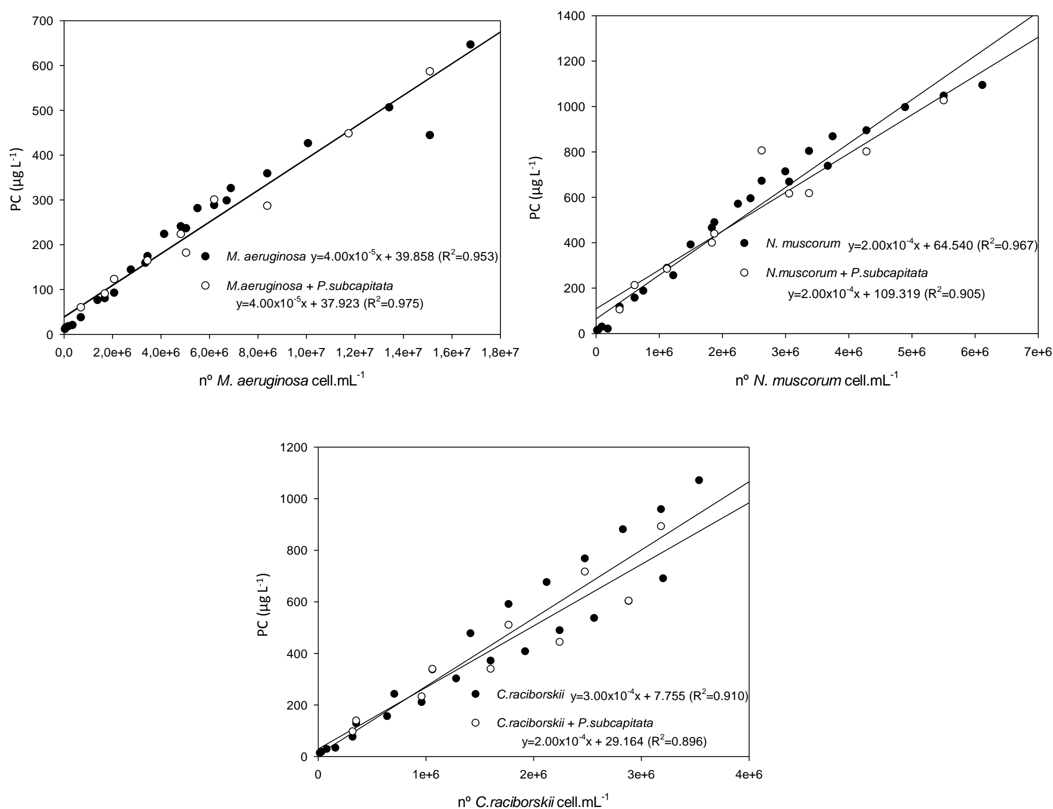



**Figure 2.4** Linear regression between PC (µg $L^{-1}$) and cell density (cell number.$mL^{-1}$) for the isolate species of cyanobacteria *M. aeruginosa* (*n*=23), *N. muscorum* (*n*=24) and *C. raciborskii* (*n*=24), and for the mixtures of cyanobacteria + alga, *M. aeruginosa* + *P. subcapitata* (*n*=10), *N. muscorum* + *P. subcapitata* (*n*=10) and *C. raciborskii* + *P. subcapitata* (*n*=10). For all these figures $p < 0.001$.

Although it was only tested one alga, these data suggest that using the combination (excitation at 620 nm and emission at 640 nm) with a FLUO-IMAGER$^{TM}$ fluorometer, algae do not interfere in the PC signal, even at high concentrations. The assessment of the possible influence of other compounds in *in vivo* PC determination is very important to take into account during the thresholds definition. While *P. subcapitata* did not show influence, in environmental samples, there are other phytoplanktonic species, organic matter and pollutants that could influence PC determination through fluorometry (GREGOR et al., 2005), due to high pigments' variability and concentrations. Therefore, a high variability of results can be generally expected in samples with low amounts of phytoplankton and high diversity of species (GREGOR et al., 2005). Furthermore, when cyanobacterial levels are low, fluorometric analysis of PC could show high variability (BOWLING et al., 2012, GREGOR et al., 2005). For this reason, Bowling *et al.* (2012) considered that false positives may occur with high levels of turbidity caused by non-cyanobacterial cells and with low cyanobacterial concentrations in communities dominated by eukaryotic phytoplankton. Chang *et al.* (2012) also observed that turbidity and chl *a* can cause overestimation of PC

concentration. However, this did not happen in the present study and with proper calibration and a careful choice of excitation and emission bands, this interference can be minimized (IZYDORCZYK et al., 2009). Chang *et al.* (2012) were capable to reduce the error of these interferences up to less than 10%, with proper calibration.

***Evaluation of the interference of mixtures of two cyanobacteria***

In order to assess how PC determination could be affected by blooms of more than one cyanobacterial species, cyanobacteria mixture assays were conducted and the results are shown in Figure 2.5.

In mixtures with more than one cyanobacterium, the measured and the expected PC (using the data from isolated cultures) showed strong significant linear relationships (see coefficients of determination in Figure 2.5): *M. aeruginosa* + *C. raciborskii* (*d.f.*=1, 7, F=120.1 and $p < 0.001$), *M. aeruginosa* + *N. muscorum* (*d.f.*=1, 3, F=210.3 and $p < 0.001$) and *C. raciborskii* + *N. muscorum* (*d.f.*=1, 8, F=100 and $p < 0.001$). Confidence intervals (95%) of the regression slope for *M. aeruginosa* + *N. muscorum* [0.77-1.21] and for *M. aeruginosa* + *C. raciborskii* [0.66-1.02] showed that this slopes were not significantly different from 1. A slope of 1 in these equations means that, for the same cell density, the measured PC corresponds to the sum of the PC of both cyanobacteria, at the correspondent cell density. This means that the contribution of each cyanobacterium in dual species mixtures was additive, which allows easy predictions of the total expected PC, in cases where the individual contribution of each species is known. Concerning *C. raciborskii* + *N. muscorum,* the observed slope was lower than 1 (95% CI [0.60-0.96]), which means that, the PC measured was slightly underestimated. However, in general, total measured PC was reasonably estimated from the species-specific PC - cell density relationships (all the slopes were near to 1). This type of information about the effect of different cyanobacteria in a mixture in PC determination is very important since it constitutes complementary information that should be used when PC thresholds are proposed.

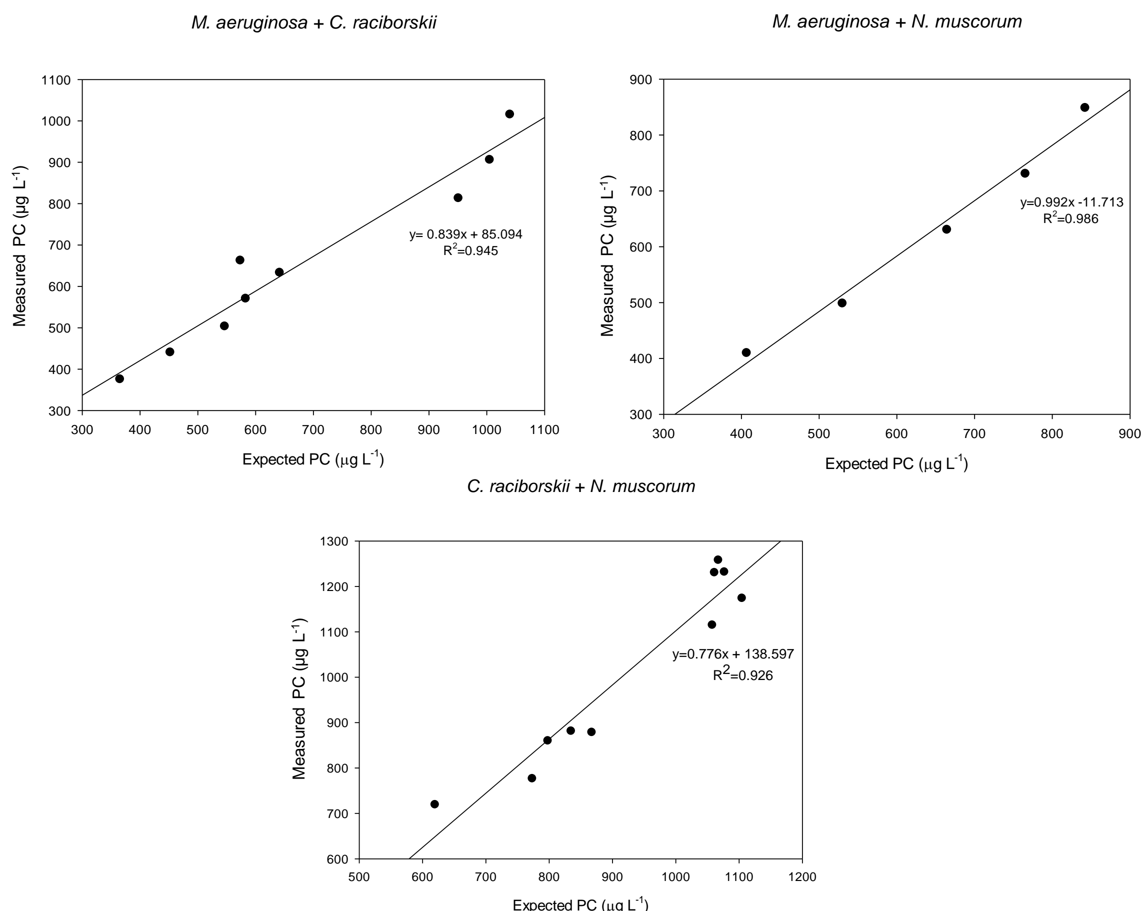



**Figure 2.5** Linear relationship between the measured PC (µg $L^{-1}$) and the expected PC in the mixtures of two cyanobacteria, *M. aeruginosa* + *C. raciborskii* (*n*=9), *M. aeruginosa* + *N. muscorum* (*n*=5) and *C. raciborskii* + *N. muscorum* (*n*=10). For all these figures $p < 0.001$.

### *Phycocyanin thresholds determination*

The use of PC for the assessment of cyanobacterial levels applied in water management requires the establishment of thresholds, which could be used as guidelines, in order to evaluate the risk that these organisms may represents. For these thresholds establishment, laboratory assays with cyanobacterial cultures must be performed, and validated with field samples. In the present work, the resultant equations from the relation between PC and cell density and PC and chl *a* (Figure 2.2) allowed the determination of the PC thresholds, using WHO cyanobacterial guidelines for recreational waters as the basis for calculations (Table 2.2). However, for the purpose of predicting thresholds, we forced the intercept to zero (in the absence of cyanobacterial cells, no PC is detected; and in the absence of chl *a*, no PC is detected). The original regression lines had high intercepts, which would have inflated the PC baseline (when no cyanobacteria were present), therefore overestimated the PC thresholds.

Different cyanobacterial species have different PC contribution and thus originate different thresholds. That is why it is difficult to propose just a consensual threshold value and generalize it

to all cyanobacterial species. It would be more correct to use different thresholds values for common bloom- forming species; this requires microscopic identification for a more accurate choice of the threshold to follow. Since *in vivo* PC fluorescence measurements are qualitative, some authors suggested the use of management thresholds based on PC levels for the determination of the appropriate sampling frequency (BRIENT et al., 2008). Therefore, it may be important to choose a value that could be used as an early warming and that could indicate the need to initiate the following procedures of microscopic identification. This early warning value should be given by the minimum value obtained from the thresholds defined in Table 2.2, in order to follow a precautionary principle. Within the analysed cyanobacteria, this valued should correspond to the first Alert Level for *M. aeruginosa* (PC=0.8 µg $L^{-1}$).

**Table 2.2** PC thresholds calculated in this study and the correspondent WHO guidelines (20,000 cells.$mL^{-1}$ and 10 µg chl *a* $L^{-1}$ for Alert Level 1 and 100,000 cells.$mL^{-1}$ and 50 µg chl *a* $L^{-1}$ for Alert Level 2).

| | **Alert Level 1** | | | |
|---|---|---|---|---|
| | 20,000 cells.$mL^{-1}$ | | 10 µg chl *a* $L^{-1}$ | |
| **Species** | Linear equation | PC (µg $L^{-1}$) | Linear equation | PC (µg $L^{-1}$) |
| *M. aeruginosa* | $y=4.00x10^{-5}$ x | 0.8 | y=0.5574 x | 5.547 |
| *N. muscorum* | $y=2.00x10^{-4}$ x | 4 | y=0.4158 x | 4.158 |
| *C. raciborskii* | $y=3.00x10^{-4}$ x | 6 | y=0.8302 x | 8.302 |
| | **Alert Level 2** | | | |
| | 100,000 cells.$mL^{-1}$ | | 50 µg chl *a* $L^{-1}$ | |
| **Species** | Linear equation | PC (µg $L^{-1}$) | Linear equation | PC (µg $L^{-1}$) |
| *M. aeruginosa* | $y=4.00x10^{-5}$ x | 4 | y=0.5574 x | 27.05 |
| *N. muscorum* | $y=2.00x10^{-4}$ x | 20 | y=0.4158 x | 20.79 |
| *C. raciborskii* | $y=3.00x10^{-4}$ x | 30 | y=0.8302 x | 41.51 |

There are other studies proposing different PC thresholds, but are based on data from environmental samples, instead of laboratory cultures. Moreover, different authors used different probes (Table 2.3) and therefore the results are difficult to compare (ZAMYADI et al., 2012).

**Table 2.3** PC thresholds proposed by different authors.

| Type of sample | Thresholds for PC (µg $L^{-1}$) | Cell number.$mL^{-1}$ | Chl *a* (µg $L^{-1}$) | Fluorometer | Reference |
|---|---|---|---|---|---|
| *M. aeruginosa* | 15 | 15,000 | - | TriOs probe | Bastien *et al.*, (2011) |
| *M. aeruginosa* | 0.8 | 20,000 | 10 | FLUO-IMAGER™ model M53B version 1.0703 | This study |
| | 4 | 100,000 | 50 | | |
| *N. muscorum* | 4 | 20,000 | 10 | | |
| | 20 | 100,000 | 50 | | |
| *C. raciborskii* | 6 | 20,000 | 10 | | |
| | 30 | 100,000 | 50 | | |
| Environmental samples | 20 pM | 20,000 | 10 | Turner 450 | Ahn *et al.*, (2002) |
| Environmental samples | 0.1 | 1,000 | 3 | PerkinElmer Luminescence Spectrometer (LS 45) | Ahn *et al.*, (2007) |
| | 30 | 10,000 | 30 | | |
| | 700 | 100,000 | 100 | | |
| Environmental samples | < 30 ± 2 | < 20,000 | - | TriOS microFlu-blue sensor | Brient *et al.*, (2007) |
| | 90 ± 2 | > 100,000 | - | | |

In 1997, Korea implemented an alert system based in cyanobacterial cell densities and chl *a* to define three levels, Caution Level (500 cells.$mL^{-1}$ and 15 µg $L^{-1}$), Warning Level (5,000 cells.$mL^{-1}$ and 25 µg $L^{-1}$) and Outbreak Level (1,000,000 cells.$mL^{-1}$ and 100 µg $L^{-1}$) (AHN et al., 2007). These authors suggested more strict values for these criteria and introduce correspondent levels of PC: Caution Level (1,000 cells.$mL^{-1}$, 3 µg $L^{-1}$ of chl *a* and 0.1 µg $L^{-1}$ of PC), Warning Level (10,000 cells.$mL^{-1}$, 30 µg $L^{-1}$ of chl *a* and 30 µg $L^{-1}$ of PC) and Outbreak Level (100,000 cells.$mL^{-1}$, 100 µg $L^{-1}$ of chl *a* and 700 µg $L^{-1}$ of PC). In this study, PC thresholds were determined based on the equation resultant from the relation between PC and chl *a.* The same author had already proposed an equivalent value of PC (PC = 20 pM) to 10 µg $L^{-1}$ of chl *a* and 20,000 cells.$mL^{-1}$ (AHN et al., 2002).

In France, levels of PC corresponding to the already established WHO guideline values for cell density were also proposed (BRIENT et al., 2008). A PC signal lower than 30 ± 2 µg $L^{-1}$ would correspond to a density of less than 20,000 cells.$mL^{-1}$ - below this threshold it is recommended to sample every 15 days; above 100,000 cells.$mL^{-1}$, PC equivalent is 90 ± 2 µg $L^{-1}$ - values higher than the first alert level indicates a weekly monitoring and public information; values above the second alert level would point out to a restriction of recreational activities and toxins assessment.

Bastien *et al.* (2011) proposed a PC threshold based on *M. aeruginosa* cultures, of 15 µg $L^{-1}$ of PC corresponding to a density of 15,000 cells.$mL^{-1}$. Since that work is the only one which uses the same species as the present work, the results obtained can be compared each other, despite different probes were used. For 15,000 cells.$mL^{-1}$, correspondent value of PC, in this study, would be 0.6 µg $L^{-1}$, which is a lower value than the 15 µg PC $L^{-1}$ proposed by Bastien *et al.* (2011). This

highlights the variability that can exist between different strains. Brient *et al.* (2008) also reported the variability between probes, in terms of reliability and calibration.

These significant differences among values shows that fluorometric methodologies should be standardized using purified pigments. One of the problems is that the intensity of the fluorescence will vary with the geometry of the instruments and with the characteristics of the filters or the slits used (LEE et al., 1995). This difference may be also due to differences in the fitness of the cyanobacteria, or to the different growth phase, which could also influence the PC content, besides the growth factors (CHANG et al., 2012). Chang *et al.* (2012) performed an experiment to assess differences in PC content during the different phases of growth of three cyanobacteria, *M. aeruginosa, A. circinalis* and *P. raciborskii*. These authors observed that, in the case of *M. aeruginosa*, in the lag and stationary phases PC content was lower and during the logarithmic phase it became higher. Therefore, differences between our results and the results of Bastien *et al.* (2011) could be explained by possible differences in PC determinations in different growth phases of the cultures, or by the use of different strains of *M. aeruginosa*, with different PC content.

## 2.4 Conclusions

PC has shown to be a good cyanobacterial predictor and can be used to effectively assess the cyanobacterial levels in a water body in a first phase (BOWLING et al., 2012, GREGOR et al., 2005, IZYDORCZYK et al., 2009) in a fast and not very expensive way. However, only this pigment analysis is not enough for the assessment of the real risk that these organisms may represent for water managers, concerning the risk for public health. Therefore, field-based assessments of cyanobacterial presence will always need to be confirmed by laboratory analysis using established microscopy methods and toxic analysis.

PC showed a strong linear relationship with cyanobacterial cell densities, which corroborate several previous studies (e.g., (AHN et al., 2002, BASTIEN et al., 2011, BRIENT et al., 2008, IZYDORCZYK et al., 2005)), making it suitable for routine cyanobacterial monitoring. PC thresholds were determined for the three cyanobacterial analysed. *P. subcapitata* did not produce a significant interference in the *in vivo* PC determination, which suggests that this technique applied to this pigment, could be successfully used in the definition of PC thresholds. Moreover, in mixtures of cyanobacterial species, PC measurements did not show significant under or overestimation, which also constitutes evidence that PC thresholds can be correctly applied and used in cyanobacteria monitoring programmes.

Although other authors proposed PC thresholds, the use of different species and environmental samples, besides the use of different probes, makes the comparison between the determined PC values difficult to perform. This highlights the need of creation of standardized fluorometry procedures and calibrations.

Finally, one of the great advantages of PC is the possibility of continuous real-time *in situ* monitoring (IZYDORCZYK et al., 2005) or through remote satellite and airborne sensors (SRIVASTAVA et al., 2013). However, this last option is limited due to its high costs, dependence

on meteorological conditions, and long monitoring intervals, which limits their use for routine monitoring (SRIVASTAVA et al., 2013). Nevertheless, small PC sensors could be applied at lakes and reservoirs for daily collection of data, avoiding the risk of a quickly and unexpected bloom formation and providing a more efficient management.

## 2.5 References

AHN, C. Y.; OH, H. M. - Rainfall, phycocyanin and N:P ratios related to cyanobacterial blooms in a Korean large reservoir. Hydrobiologia. Vol. 474 (1-3) (2002), p. 117-124.

AHN, C.Y. [et al.] - Alternative Alert System for Cyanobacterial Bloom, Using Phycocyanin as a Level Determinant. The Journal of Microbiology. Vol. 45(2) (2007), p. 98-104.

APHA - Standard Methods for the Examination of Water and Wastewater. 20th. Washington, DC: American Public Health Association, 1999.

BASTIEN, C. [et al.] - Performance evaluation of phycocyanin probes for the monitoring of cyanobacteria. Journal of Environmental Monitoring. Vol. 13, n.º 1 (2011), p. 110-8.

BELLINGER, G.E.; SIGEE, D.C. - Freshwater Algae: Identification and Use as Bioindicators. 2º. Spain, 2010. ISBN 978-0-470-05814-5.

BEUTLER, M. [et al.] - A fluorometric method for the differentiation of algal populationsin vivo and *in situ*. Photosynthesis Research. Vol. 72 (2002), p. 39–53.

BOWLING, L. [et al.] - Evaluation of *in situ* Fluorometry to Determine Cyanobacterial Abundance in the Murray and Lower Darling Rivers, Australia. River Research and Applications. (2012).

BRIENT, L. [et al.] - A phycocyanin probe as a tool for monitoring cyanobacteria in freshwater bodies. Journal of Environmental Monitoring. Vol. 10, n.º 2 (2008), p. 248-55.

CAMPBELL, D. [et al.] - Chlorophyll fluorescence analysis of cyanobacteria photosynthesis and acclimatation. Microbiology and Molecular Biology Reviews Vol. 62 (1998), p. 667–683.

CHANG, D. W. [et al.] - Measurement of cyanobacteria using in-vivo fluoroscopy - effect of cyanobacterial species, pigments, and colonies. Water Research. Vol. 46, n.º 16 (2012), p. 5037-48.

CHORUS, I.; BARTRAM, J. - Toxic Cyanobacteria in Water: A guide to their public health consequences, monitoring and management London: E & FN Spon, 1999. ISBN 0-419-23930-8.

GANTT, E. [et al.] - Phycobilisomes from blue-green and red algae: Isolation criteria and dissociation characteristics. Plant Physiology. Vol. 63 (1979), p. 615–620.

GLAZER, A. N. - Phycobilisome – a molecular complex optimized for light energy transfer. Biochimica et Biophysica Acta. Vol. 768 (1984), p. 29–51.

GREGOR, JAKUB; MARŠÁLEK, BLAHOSLAV - A Simple In Vivo Fluorescence Method for the Selective Detection and Quantification of Freshwater Cyanobacteria and Eukaryotic Algae. Acta hydrochimica et hydrobiologica. Vol. 33, n.º 2 (2005), p. 142-148.

GROSSMAN, A.R. [et al.] - The responses of cyanobacteria to environmental conditions:light and nutrients. In: D.A., B. - The molecular biology of cyanobacteria. Dorecht (The Netherlands): 1994. p. 641-675.

HOLM-HANSEN [et al.] - Fluorometric determination of chlorophyll. Journal du Conseil Permanent International pour l'Exploration de la Mer. Vol. 30(1) (1965), p. 3-15.

HORVÁTH, HAJNALKA [et al.] - Extraction methods for phycocyanin determination in freshwater filamentous cyanobacteria and their application in a shallow lake. European Journal of Phycology. Vol. 48, n.º 3 (2013), p. 278-286.

IZYDORCZYK, K. [et al.] - Establishment of an Alert Level Framework for cyanobacteria in drinking water resources by using the Algae Online Analyser for monitoring cyanobacterial chlorophyll *a*. Water Research. Vol. 43, n.º 4 (2009), p. 989-96.

IZYDORCZYK, K. [et al.] - Measurement of phycocyanin fluorescence as an online early warning system for cyanobacteria in reservoir intake water. Environmental Toxicology. Vol. 20, n.º 4 (2005), p. 425-30.

LEE, T.Y. [et al.] - Quantitative determination of cyanobacteria in mixed phytoplankton assemblages by an *in vivo* fluorimetric method. Analytica Chimica Acta. Vol. 302 (1995), p. 81-87

LORENZEN, C.J. - Determination of chlorophyll and pheo-pigments: spectrophotometric equations. Limnology and Oceanography. Vol. 12 (1967a), p. 343-346.

LORENZEN, CARL J. - A method for the continuous measurement of in vivo chlorophyll concentration. Deep Sea Research and Oceanographic Abstracts. Vol. 13, n.º 2 (1967b), p. 223-227.

NICHOLS, H.W. - Handbook of Phycological Methods. Camb. Univ. Press., 1973.

PORYVKINA, L.; BABICHENKO, S.; LEEBEN, A. - Analysis of phytoplankton pigments by excitation spectra of fluorescence. Proceedings of EARSeL-SIG-Workshop LIDAR. (2000), p. 224-232.

SEPPÄLÄ, J. [et al.] - Ship-of-opportunity based phycocyanin fluorescence monitoring of the filamentous cyanobacteria bloom dynamics in the Baltic Sea. Estuarine, Coastal and Shelf Science. Vol. 73, n.º 3-4 (2007), p. 489-500.

SRIVASTAVA, A. [et al.] - Monitoring approaches for a toxic cyanobacterial bloom. Environmental Scientific Technology. Vol. 47, n.º 16 (2013), p. 8999-9013.

TURNER, DESIGNS- Cyanobacteria detection in water using *in vivo* fluorometry. 2004.

TURNER, DESIGNS- Technical Note: An Introduction to Fluorescence Measurments.

WATRAS, C.J; BAKER, A.L - Detection of planktonic cyanobacteria by tandem in vivo fluorometry. Hydrobiologia. Vol. 169 (1988), p. 77-84

WHO - Algae and cyanobacteria in fresh water. In: Guidelines for safe recreational water environments. Geneva, Switzerland: World Health Organization, 2003. p. 136-158.

WLODARCZYK, LUCYNA M.; MOLDAENKE, CHRISTIAN; FIEDOR, LESZEK - Fluorescence as a probe for physiological integrity of freshwater cyanobacteria. Hydrobiologia. Vol. 695, n.º 1 (2012), p. 73-81.

ZAMYADI, A. [et al.] - Monitoring of potentially toxic cyanobacteria using an online multi-probe in drinking water sources. Journal of Environmental Monitoring. Vol. 14, n.º 2 (2012), p. 579-88.

ZAR, J.H. - Biostatistical analysis. 3º edition. 1996.

# Chapter III

Monitoring strategy proposal

# Evaluation of a stepwise strategy for monitoring toxic cyanobacterial blooms in Portuguese lentic water bodies

(Manuscript in preparation for submission to an indexed journal)


## Abstract

The present work proposes a stepwise strategy for monitoring cyanobacteria in recreational waters. This strategy incorporates several techniques (phycocyanin assessment through fluorometry, chlorophyll *a* determination, cyanobacterial identification and cell counts, toxic potential evaluation and cyanotoxins analysis), which allows a more effective monitoring of cyanobacterial blooms. This stepwise approach allows the exclusion of samples which do not represent a risk for the public health. In order to test this methodology, nine study cases were used. All samples showed chlorophyll *a* and cell concentrations above the WHO guideline for level 2 (moderate risk of adverse health effects) and there were found two situations of blooms surpassing the WHO chlorophyll *a* and cyanobacterial concentration guidelines for level 3 (high risk of adverse health effects). *Microcystis aeruginosa*, *Aphanizomenon flos-aquae* and *Cylindrospermopsis raciborskii* were the dominant bloom-forming cyanobacteria but only the *Microcystis* blooms in two sites proved to be toxic. In one of these sites, the recorded Microcystin-LR value was up to 12 µg $L^{-1}$ which represents major risks for water recreational users. Using the present strategy, the nine initial samples were sequentially evaluated and selectively excluded for further analyses, resulting in only two samples with effectively high health risks, contrarily to the eight firstly considered according to WHO guidelines. Therefore, this approach proves to be a long-term cost-effective alternative and a valuable tool to be used in management and monitoring strategies for recreational waters where dense and toxic blooms tend to occur frequently.



## Keywords

Cyanobacterial blooms, phycocyanin, fluorometry, monitoring strategy, health risk assessment

## 3.1 Introduction

A major issue concerning the occurrence of cyanobacterial blooms is the potential for toxins production (DE FIGUEIREDO et al., 2004, METCALF et al., 2012). As cyanotoxins are confined into the cells, they are released to surrounding water only when cells lyse, and water that previously contained a toxic bloom may appear to be free of cyanobacterial cells but may be still contaminated with free toxin (BAKER et al., 2002). An exception appears to occur with cylindrospermopsin, produced mainly by *Cylindrospermopsis raciborskii*, where a significant amount of the toxin is also present in the surrounding water during the development of a bloom (CHISWELL et al., 1999), which represents even a major problem for water treatment strategies.

Moreover, a bloom may consists of a mixture of toxic and nontoxic cyanobacterial strains (which are impossible to distinguish under the microscope), and this is believed to be the reason why the toxicity of a bloom varies over time and between samples (BARON-SOLA et al., 2012). Conditions leading to cyanobacterial proliferation are well understood, but the physiological or biochemical function of toxins of cyanobacteria is still subject of many hypotheses (CHORUS et al., 1999, ELLIOTT, 2012, JANSE et al., 2004). Furthermore, the factors leading to the dominance of toxic strains over non-toxic ones are still not clear, and therefore, more sophisticated analyses are needed to define if a bloom contains toxic species or not (WHO, 2003).

Current alert levels for assessing bathing waters are based in parameters such as chlorophyll *a* (chl *a*) and cyanobacterial density (CHORUS et al., 1999). However, these approaches have limitations when the objective is to assess the health problems that could be associated with a cyanobacterial bloom (AHN et al., 2007). Chl *a* is not a specific cyanobacterial pigment and its concentration does not increase in a linear relationship with cyanobacterial cell numbers (CHORUS et al., 1999). Microscopic identification can have a high level of error, due not only to the fact that different species may be morphologically similar, compromising a correct identification, but also because it is not possible to distinguish between toxic and nontoxic strains (RASMUSSEN et al., 2007). Moreover, cell counts can also include substantial errors because microscopic enumeration of cyanobacterial cells can be hindered by large colonies and entangled long filaments, making cyanobacterial counts very dependent on the skilfulness and subjective decisions of each counter (AHN et al., 2007). In addition, traditional approaches for microscopic identification and cell counting are time-consuming, taking 2-5 days to produce results, since samples are transported to laboratories where trained personnel perform the analysis. The toxin quantification is also an important step in every monitoring programme and it is determined through biological, biochemical and/or physicochemical methods (METCALF et al., 2012, SRIVASTAVA et al., 2013). The Enzyme Linked Immune Substrate Assays (ELISA) is the most common biochemical technique for cyanotoxins screening and it is based on the counting of well plates or test tubes with toxin antibodies (COALITION et al., 2009). It is a relatively inexpensive, simple, fast, sensitive, specific and easy technique (CHORUS et al., 1999) and does not require pre-concentration of samples. However, it does not distinguish toxin variants, which can complicate risk

management decisions due to inter-variant toxicity differences. Although ELISA shows cross variable reactivity, it is effective to determinate the microcystins content in environmental samples (MSAGATI et al., 2006). It is a good screening tool to be incorporated into a suite of routine analyses and could indicate when it is necessary to begin more expensive and time-consuming analyses capable of resolving toxin variants (COALITION et al., 2009). Besides the expensive costs of methodologies based on chromatography and mass spectrometry, bioassays have been also used for toxins assessment but they raise ethical issues (EPA, 2012). Therefore, alternatives that help minimize the number of samples for toxin analysis should be preferred.

Another important aspect to consider is that the non-production of toxins at a given time during a bloom development does not mean that there are no risks for toxic outcomes from that bloom. Many studies have already shown that cyanotoxins production is not constant and it is dependent on many physiological and environmental parameters (SAKER et al., 2005). Therefore, the toxin quantification assays, by themselves, are not also a definitive assay for toxicity safety, as variation on intrinsic or environmental conditions may trigger the toxin production at any time during the bloom development and toxic and nontoxic cyanobacteria may be present in the same water bloom (RASMUSSEN et al., 2007). Under this scope, molecular approaches can play an important role as they are simple, rapid, cost effective, sensitive and specific, allowing the simultaneous analysis of several target gene products (PEARSON et al., 2008). The toxicity potential assessment of isolated strains or environmental samples can be easily measured by Polymerase Chain Reaction (PCR), using primers targeting regions of the operons involved in the synthesis of microcystins (TILLETT et al., 2001); cylindrospermopsin (SCHEMBRI et al., 2001), saxitoxins (AL-TEBRINEH et al., 2010), anatoxins (WOOD et al., 2010) and nodularins (KOSKENNIEMI et al., 2007). For routine monitoring programmes, it is also important to evaluate the toxic potential before/ along with assessing toxins concentrations. By determining the potential for toxin production it is immediately determined if there is a risk for toxic outcomes, even if the toxin(s) is(are) not being produced at that time, under those particular conditions, as stated above. Moreover, molecular approaches can be also very useful in cyanobacterial blooms monitoring strategies to provide a correct taxonomical identification of the cyanobacterial community present in the water body. For community taxonomical studies, culture-independent molecular methodologies, such as Denaturing Gradient Gel Electrophoresis (DGGE), have proven to be suitable for a general and rapid characterization of bacterial assemblages in natural aquatic environments, allowing to compare temporal or/and spatial patterns (MUYZER et al., 1998). This is particularly useful to assess spatial distribution and dispersal of a bloom and determine hotspots with higher risks for health. However, in a near future, modern massive sequencing analyses will become more accessible and may be a strategic instrument for the characterization of samples not only in terms of diversity and spatial/ temporal patterns but also with taxonomic information for an overview of the dominant cyanobacterial community (STEFFEN et al., 2012). On the other hand, community analyses can help to interpret some results, because they allow to extract qualitative and semi-

quantitative estimations of diversity of predominant phylotypes in target communities (DORIGO et al., 2005), allowing a more holistic view of what is going on with the water communities and also help to predict the occurrence of similar blooms in the future.

As discussed above, all monitoring methods provide important information but have also limitations, suggesting that no single approach is sufficient to accurately assess the risk associated with cyanobacterial blooms (SRIVASTAVA et al., 2013). Consequently, it is important to establish a more accurate cyanobacterial blooms alert system using cyanobacteria-specific factors, at an early stage. Phycobiliprotein measurements through fluorometry proved to be very effective and phycocyanin (PC), in particular, shows several attractive characteristics comparatively to chl *a* and cell concentrations, since it is a cyanobacterial specific pigment and does not require a great deal of dexterity or expensive equipment (LEE et al., 1995). Another advantage of that technique is the *on-line* monitoring and real-time data collection option. Moreover, it shows high sensitivity, specificity, simplicity, low cost and no special sample handling or processing requirements (SRIVASTAVA et al., 2013). Several studies have been using this technique for taxonomic assays using *in vivo* bio-optical characteristics of phytoplankton, like fluorescence excitation and absorption spectra of some pigments (PORYVKINA et al., 2000). Besides chl *a*, other pigments (so-called accessory pigments) could also be present in photosystem II, but unlike chl *a*, they show differences according to the taxonomy of the organisms and usually do not show fluorescence (GREGOR et al., 2005). However, in cyanobacteria, PC gives fluorescence and several studies used this property to differentiate cyanobacteria from eukaryotic algae (BASTIEN et al., 2011, GREGOR et al., 2005, IZYDORCZYK et al., 2005, LEE et al., 1995, WATRAS et al., 1988). Those studies are based on the fact that the excitation spectra of *in vivo* chl *a* (excitation at 440 nm and the emission at 680 nm) is weak in the cyanobacterial spectra, because the excitation wavelengths of PC happens at 550-650 nm and the emission peak around 645 nm (LEE et al., 1995). However, in spite of the advantages of this methodology, it is important to take into account that, for the purpose of a monitoring strategy, it does not assess other parameters such as toxin concentrations (IZYDORCZYK et al., 2005). This fact highlights, once more, the need for integration of several methodologies within a same monitoring strategy. Nonetheless, the rapid measurement of *in vivo* fluorescence gives insights on the cyanobacterial density, saving the time required for cell counts results before cyanotoxins analyses (BRIENT et al., 2008), which gives the possibility for implementing immediate actions (SRIVASTAVA et al., 2013).

Considering the advantages and the limitations of the methods described above, the present work proposes a stepwise strategy for cyanobacterial monitoring in recreational water bodies combining several techniques (from fluorometry to molecular approaches), in order to allow the technicians to be able to exclude samples as the risk of a toxic bloom occurrence is being discarded.

## 3.2 Material and Methods

***The stepwise strategy proposal***

The strategy proposal for monitoring cyanobacterial blooms combines several techniques used sequentially in order to optimize resources (Figure 3.1). It was designed to be preferentially used in lentic recreational water bodies where dense cyanobacterial blooms tend to occur.

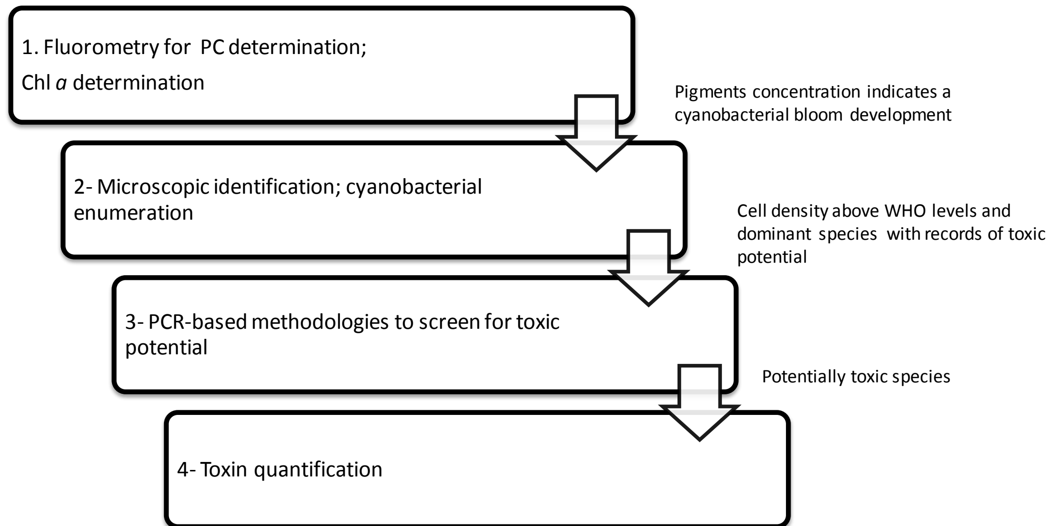



**Figure 3.1** Proposed cyanobacterial monitoring methodology**.**

The first step of the proposed strategy is the fluorometric determination of PC, to be used as a complement to the World Health Organization (WHO) guidelines for chl *a* (1. Alert Level - chl *a* > 10 µg $L^{-1}$; 2. Alert Level - chl *a* > 50 µg $L^{-1}$; and 3. Alert Level - presence of scum) (CHORUS et al., 1999). As shown in Chapter 2 of this dissertation, different cyanobacterial blooms have different PC contents and different thresholds should be used for different dominant bloom-forming cyanobacteria (*Microcystis aeruginosa* (0.8 µg $L^{-1}$ for Alert Level 1 and 4 µg $L^{-1}$ for Alert Level 2), *Nostoc muscorum* (4 µg $L^{-1}$ for Alert Level 1 and 20 µg $L^{-1}$ for Alert Level 2) and *Cylindrospermopsis raciborskii* (6 µg $L^{-1}$ for Alert Level 1 and 30 µg $L^{-1}$ for Alert Level 2)), corresponding to the cell counts guidelines defined by WHO. Therefore, PC analysis should to be complemented with cyanobacterial identification, in order to identify the dominant species or genus/genera, to know what threshold should be more suitable for each case. However, if no PC is detected, samples can be excluded from subsequent analyses, even without cyanobacterial identification. This may reduce substantially the number of samples for further processing. However, if the analysis of these pigments reveals the presence of a cyanobacterial bloom, microscopic observation and enumeration of dominant cyanobacterial species will be performed. If Cyanobacteria is the dominant group and it appears in higher number than the WHO guidelines, it

is important to assess whether the dominant species have historical records of toxicity. PCR-based methodologies to screen the potential for cyanotoxins production should be carried out if the species identified have historical records of toxicity. This is an important step because, although a species possesses a historical record of toxicity, not all the strains are toxic and may be producing the toxin or not, according to the environmental conditions (JANSE et al., 2004). For the last step, only for the samples where toxic potential was detected, toxin quantification will be performed through biochemical methods.

At the same time these analyses are performed, ecological data (environmental parameters and phytoplanktonic/cyanobacterial community) should be recorded (see Chapter 4 of the present dissertation), in order to help the development of wider water management strategies to mitigate and even prevent cyanobacterial blooms appearance.

***Sampling and sites description***

The proposed methodology was applied for nine lentic Portuguese water bodies: three lakes (Vela, Mira and Barrinha de Mira) and six reservoirs (Aguieira, Crestuma, Alvito, Odivelas, Monte Novo and Montargil) (Table 3.1). These freshwater bodies were chosen given their historical records on cyanobacterial blooms' occurrence and their use as recreational water bodies. During the late summer period (from end of September to beginning of October 2012) sampling was performed, since early summer and late autumn are the seasons with better environmental conditions for cyanobacterial blooms development (ELLIOTT, 2012).

**Table 3.1** Site description with the reports of the cyanobacterial species present in the lake/reservoir.

| Hydrographic River Basin | Location | Original water body | Reservoir/Lake (Code) | Occurrence of cyanobacterial spp. | References |
|---|---|---|---|---|---|
| Mondego | Quiaios (Figueira da Foz) (40º16'N; 8º47'W) | - | Vela Lake (LV) | *Microcystis aeruginosa*, *Aphanizomenon flos-aquae* | (DE FIGUEIREDO et al., 2006) |
| | | | | *Aphanizomenon gracile*, *Aphanizomenon aphanizomenoides*, *Aphanizomenon issatschenkoi* | (DE FIGUEIREDO et al., 2010) |
| | | | | *Chroococcus limneticus* | (LOPES et al., 2009) |
| | | | | *Cylindrospermopsis raciborskii* | (MOREIRA et al., 2011) |
| Vouga | Mira (Mira) (40º26'N; 8º45'W) | - | Mira lake (LM) | *Microcystis aeruginosa* | (VASCONCELOS et al., 1993a) |
| | | | | *Microcystis wesenbergii, Anabaena scheremetievi, Chroococcus dispersus* | (VASCONCELOS, 1995) |
| | | | | *Anabaena flos-aquae* | (VASCONCELOS et al., 1996) |
| | | | | *Aphanizomenon flos-aquae* | (SAKER et al., 2007) |
| | | | | Chroococcales spp. | (DE FIGUEIREDO et al., 2007) |
| Vouga | Mira (Mira) (40º27'N; 8º48'W) | - | Barrinha de Mira Lake (LB) | *Anabaena fos-aquae, Anabaena scherenetievi, Anabaena spiroides, Chroococcus limneticus, Chroococcus turgidus,* Chroococcales, *Microcystis aeruginosa* | (VASCONCELOS, 1995) |
| Mondego | Aguieira (Penacova) (20º40'N; 8º9'W) | Mondego River | Aguieira Reservoir (BAG) | *Anabaena flos-aquae, Anabaena spiroides, Aphanizomenon flos-aquae, Aphanizomenon issatschenkoi, Dactylococcopsis irregularis, Dactylococcopsis smithii, Gomphosphaeria aponina, Gomophosphaeria lacustris, Merismopedia tenuissima, Microcystis wesenbergii, Microcystis aeruginosa, Oscillatroria limnetica, Oscillatoria planctonica, Oscillatoria tenuis, Phormidium mucicola, Pseudoanabaena mucicola* | (VASCONCELOS et al., 2011) |
| Douro | Crestuma (Porto) (41º4'N; 8º28'W) | Douro River | Crestuma Reservoir (BCR) | *Microcystis aeruginosa* | (VASCONCELOS et al., 1993b) |
| | | | | *Aphanizomenon flos-aquae* | (FERREIRA et al., 2001) |
| Sado | Vila Alva (Beja) (38º17'N; 7º54'W) | Ribeira de Odivelas | Alvito Reservoir (BALV) | *Microcystis aeruginosa, Anabaena circinalis*, *Aphanizomenon flos-aquae* | (GALVÃO et al., 2008) |
| Sado | Ferreira do Alentejo (Beja) (38º11'N; 8º6'W) | Ribeira de Odivelas | Odivelas Reservoir (BDV) | *Anabaena* sp., *Aphanizomenon flos-aquae,* Chroococcales | (VASCONCELOS, 1995) |
| | | | | *Cylindrospermopsis raciborskii* | (VALÉRIO et al., 2005) |
| | | | | *Microcystis* spp. | (VALERIO et al., 2010) |
| Guadiana | Évora (Évora) (38º30'N; 7º42'W) | Degebe River | Monte Novo Reservoir (BMN) | *Anabaena scheremetievi, Anabaena spiroides, Aphanizomenon flos-aquae, Microcystis aeruginosa, Oscillatoria planctónica* | (VASCONCELOS, 1995) |
| | | | | *Aphanizomenon gracile* | (DE FIGUEIREDO et al., 2010) |
| Tejo | Ponte de Sôr (Portalegre) (39°3'N; 8°10'W) | Ribeira de Sôr | Montargil Reservoir (BMT) | *Anabaena spiroides, Aphanizomenon flos-aquae, Oscillatoria* sp., *Pseudoanabaena catenata* | (VASCONCELOS, 1995) |
| | | | | *Aphanizomenon issatschenkoi, Microcystis aeruginosa* | (VALÉRIO et al., 2005) |
| | | | | *Cylindrospermopsis raciborskii* | (SAKER et al., 2003) |

### ***Pigments analysis***

For pigments determinations, in each sampling site, 1L of water sample was collected, kept under dark and in cold during transportation.

*Chlorophyll a*

In laboratory, the samples were filtered through Whatmann GF/C filters (1.2 µm) which were frozen (-20ºC) until further processing. Chl *a* was determined using the trichromatic method (APHA, 1999 Section 10200H.2c) but the monochromatic method (LORENZEN, 1967) was also used to determine the influence of phaeopigments in the overall measured chl *a*. Chl *a* was extracted with 90% acetone and left at 4ºC overnight; tubes were covered in order to avoid chl degradation. After this period, tubes were centrifuged for 15 min at 4000 rpm and the absorbance of the supernatant measured at 750, 664, 647 and 630 nm in a Shimadzu UV-1800 Spectrophotometer. The absorbance values are used to determine chl *a*, *b* and *c* using trichromatic equations. For the monochromatic method, the absorbance was measured at 750 and 665 nm with and without acidification (0.1 N HCl).

*Phycocyanin*

PC concentration was determined in laboratory, by fluorometry - spectral fluorescent signatures (SFS) - using the FLUO-IMAGER$^{TM}$ (SKALAR, Analytical B.V., Breda, The Netherlands), model M53B version 1.0703. This fluorometer is normally used for detection of photosynthetic pigments in the water. SFS is based upon the measurement of excitation spectra and fluorescent spectra: at given excitation wavelength, an emission spectrum is measured with a Photomultiplier (PMT), then the excitation wavelength shifts 2 nm and the emission spectrum is measured again. This process continues until the complete excitation range has been scanned. In general, phycoerythrin and PC components are included in all live catalogues mixtures of phytoplankton sample with blue-green algae. Catalogue TV 17 was used for samples with cyanobacteria, because it includes the spectra for the peaks of PC (excitation at 620 nm and emission at 640 nm).

### ***Identification and enumeration of dominant cyanobacteria and cell counting***

*Microscopic identification of cyanobacteria*

Taxonomic identification was made attending to morphologic characters like shape, type, dimension and arrangement of the colonies/filaments disposition and dimension of the vegetative cells, presence/absence of sheath, and the presence/absence of specialized cells and their shape (heterocysts and akinetes) (BELLINGER et al., 2010, CRONBERG et al., 2006). Identification was performed using *in vivo* samples and Lugol’s preserved samples.

*Quantification of cyanobacterial cells*

Environmental samples for cell counting were preserved with Lugol and kept in the dark until further processing. Phytoplankton cells counting was carried out using the Utermöhl technique on a 5 mL sub-sample, which was sedimented in an Utermöhl chamber over a period of 4 hours per centimetre of the liquid column (INAG, 2009). Counts were performed under an inverted microscope (Olympus CKX4). Two perpendiculars transects were counted and phytoplankton diversity was calculated according to the equation (1) proposed by the Water Framework Directive (INAG, 2009):

$$N = X\frac{A\ d}{a\ V} \qquad (1)$$

$N$ represents cells number.mL$^{-1}$, $X$ the number of cells for transept, $A$ the sedimentation chamber area, $d$ the dilution factor, $a$ optic field area and $V$ sample volume in the sedimentation chamber.

***Toxic potential assessment by PCR***

Water samples were collected in 2L sterile bottles and maintained in dark and cold conditions until further processing at the laboratory. For total DNA extraction, samples were filtered with 0.22 µm polycarbonate sterile filters (Poretics Products, Livermore, USA) until they clogged (the filtered volumes ranged from 30 to 1000 mL, depending on the water turbidity). Then, filters were washed with 2 mL of TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0). After centrifugation, lysozyme was added after resuspension in 200 µL of TE following incubation at 37ºC for 1h. The following DNA extraction and purification were carried out using the Genomic DNA Purification Kit (MBI Fermentas, Vilnius, Lithuania). DNA was finally suspended in TE buffer and stored at -20 ºC.

The amplification of *Microcystis* spp. partial sequences of *mcyA* regions of microcystin synthetase was achieved through PCR using the primers QmetF (5'-TTA TTC CAA GTT GCT CCC CA-3') and QmetR (5'-GGA AAT ACT GCA CAA CCG AG-3') (WILSON et al., 2005). These primers target fragments of 220 bp and have the following amplification program: initial denaturation step at 95°C for 2 min followed by 30 cycles of 10s at 94°C, 20s at 45°C and 30s at 72°C, and a final extension step at 72°C for 10 min. For assessing the cylindrospermopsin production potential, a set of primers targeting regions coding for the cylindrospermopsin synthetase was used: PS specific primers M13(Fw) (5'-GGCAAATTGTGATAGCCACGAGC-3') and M14(Rv) (5'-GATGGAACATCGCTCACTGGTG-3') (SCHEMBRI et al., 2001); PKS specific primers M4(Fw) (5'-GAAGCTCTGGAATCCGGTAA-3') and M5(Rv) (5'-AATCCTTACGGGATCCGGTGC-3') (SCHEMBRI et al., 2001); and AMT specific primers CYLATF (5'-ATTGTAAATAGCTGGAATGAGTGG-3') and CYLATR (5'-TTAGGGAAGTAATCTTCACAG-3') (KELLMANN et al., 2006), which amplify fragments of 597 bp, 650 bp and 1105 bp, respectively. The amplification program for PS and PKS consists of an initial denaturation step at 95°C for 2 min

followed by 35 cycles of 90s at 95°C, 30s at 55°C and 50s at 72°C, and a final extension step at 72°C for 7 min; and for AMT primers, an initial denaturation step at 94°C for 3 min followed by 30 cycles of 10s at 94°C, 20s at 50°C and 60s at 72°C, and a final extension step at 72°C for 7 min, were performed. Negative (without any template DNA) and positive controls reactions were performed simultaneously. The PCR amplicons were electrophoresed (Bio-Rad Power-Pac Power Basic) in a 1.5% agarose gel, stained with RedSafe (iNtRON) at 80 V for 50 min and compared with a molecular weight marker (VC 100bp PLUS) (Fermentas) and the gel visualized in a Bio Rad Molecular Imager Gel Doc$^{TM}$ XR+ with Image Lab Software.

***Toxins quantification***

For the potential revealed by PCR, quantification of the corresponding toxins with positive results was performed using the corresponding ELISA kits. In this study, only a MC ELISA kit was used for microcystins (MC) assessment. This assay targets MC-LR, the most common variant of microcystins (CHORUS et al., 1999).

## 3.3 Results and Discussion

***Pigments analysis***

The proposed methodology was applied to nine Portuguese water bodies (lakes and reservoirs) and only at Crestuma Reservoir PC levels were not detected (Figure 3.2), since no signal was detected in the PC spectra used (excitation at 620 nm and emission at 640 nm). However, according to the WHO guidelines, all samples showed chl *a* levels corresponding to the level 1 for health risks outcomes (CHORUS et al., 1999). Montargil Reservoir was also above level 2 and Vela Lake and Aguieira Reservoir were within the third alert level, due to the presence of scum.

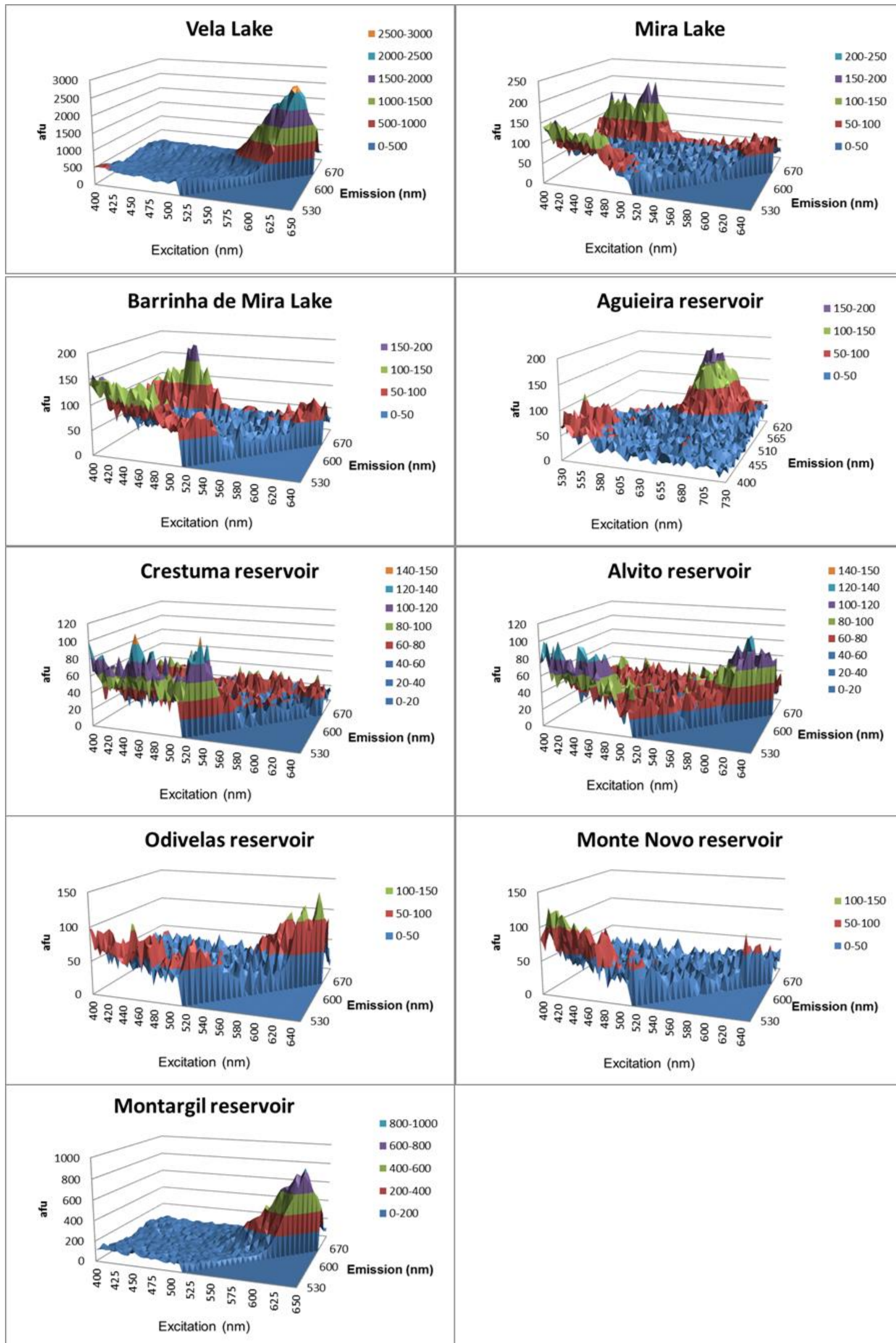



**Figure 3.2** Spectral fluorescent signatures (SFS) in collected samples.

From Figure 3.2, it is clear where there was a dominance of cyanobacteria in water samples (Vela Lake and Aguieira, Alvito, Odivelas and Montargil reservoirs) within the graph area corresponding to the excitation at 620 nm and emission at 640 nm. The chl *a* concentrations presented in Figure 3.3 were obtained using the trichromatic method, since the contribution of phaeopigments was very low and both methods showed to be highly correlated ($R^2$=0.999, $n$=9, $p < 0.001$).

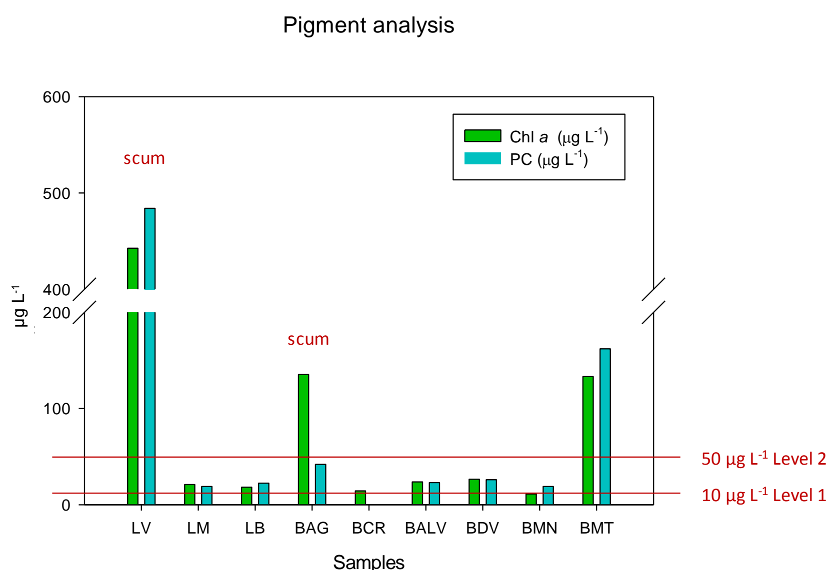



**Figure 3.3** Chl *a* and PC concentrations in the field samples. Red lines represent the WHO alert levels, concerning chl *a* concentration. Sample codes correspond to LV (Vela Lake), LM (Mira Lake), LB (Barrinha de Mira Lake), BAG (Aguieira Reservoir), BCR (Crestuma Reservoir), BALV (Alvito Reservoir), BDV (Odivelas Reservoir), BMN (Monte Novo Reservoir) and BMT (Montargil Reservoir).

The analysis of chl *a* revealed the potential for bloom development in all samples, with concentrations above the WHO guidelines (CHORUS et al., 1999). Therefore, if only chl *a* was considered, it could not be excluded any site for subsequent analyses. However, when PC results are added, it is clear that chl *a* concentration at Crestuma Reservoir was due to the presence of other photosynthetic organisms, rather than cyanobacteria. Without the analysis of these cyanobacteria specific pigment would not be possible to suspect of the dominating taxonomic group in the water bodies. Therefore, PC reveals a great potential, avoiding false positives at an initial stage, without further samples processing. Figure 3.4 shows PC concentration and the lines that define the threshold values. In the present study, at Aguieira Reservoir, *Microcystis* spp. was the dominant cyanobacteria and, in Vela Lake, this was also one of the dominant species, while at Montargil Reservoir, *Cylindrospermopsis raciborskii* was the dominant species (see next section – Figures 3.5 and 3.6). Therefore, these samples were used to validate the PC thresholds determined in Chapter 2 of this dissertation. Vela Lake showed the highest chl *a* (442.7 µg $L^{-1}$) and PC concentrations (482 µg $L^{-1}$ in water and 1440 µg $L^{-1}$ in scum). For Vela Lake and Aguieira Reservoir the threshold correspondent to alert level 2 (4 µg $L^{-1}$) for *M. aeruginosa* was largely

exceeded. Aguieira Reservoir also showed high levels of chl *a* (135.29 µg $L^{-1}$) and PC (42 µg $L^{-1}$ on water and 1047 µg $L^{-1}$ on scum), indicating the presence of a strong bloom. At Montargil Reservoir, the proposed alert level 2 for *C. raciborskii* (30 µg $L^{-1}$) was exceeded, showing high concentrations of chl *a* (133.16 µg $L^{-1}$) and PC (162 µg $L^{-1}$). All the remaining samples showed moderate values for chl *a* and PC (all below 41 µg $L^{-1}$ and 26 µg $L^{-1}$, respectively), however all of them are above level 2 threshold for *M. aeruginosa* and level 1 threshold for *C. raciborskii.* This highlights that, although these thresholds were able to assess safety the cyanobacterial blooms recorded, further laboratory assays using other bloom-forming cyanobacterial species and validation with environmental samples should be performed.

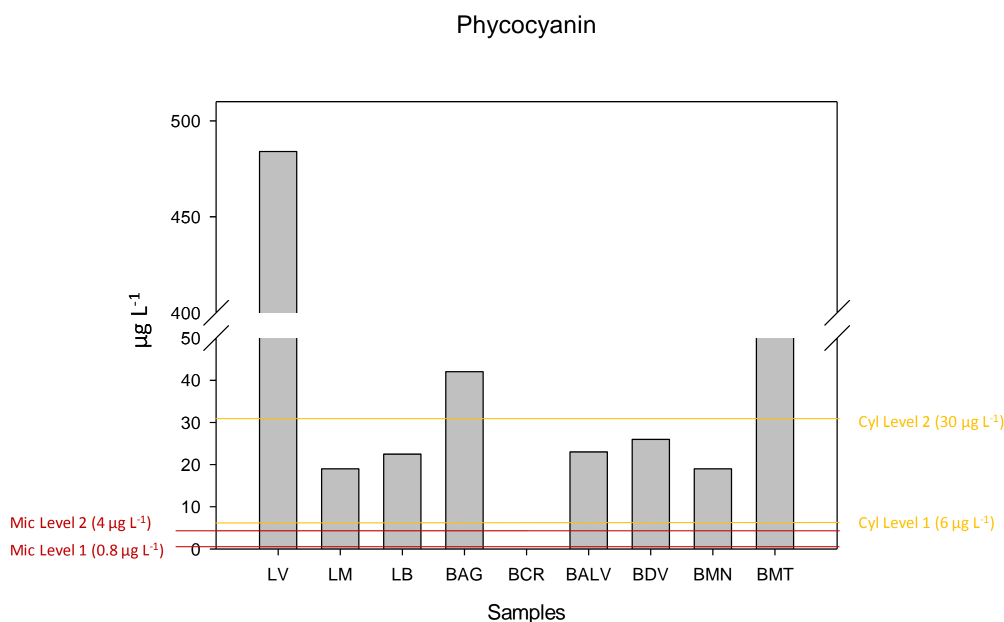



**Figure 3.4** PC concentrations for the field samples. Red lines represent the defined thresholds for *M. aeruginosa* and the orange lines the thresholds for *C. raciborskii*. Sample codes correspond to LV (Vela Lake), LM (Mira Lake), LB (Barrinha de Mira Lake), BAG (Aguieira Reservoir), BCR (Crestuma Reservoir), BALV (Alvito Reservoir), BDV (Odivelas Reservoir), BMN (Monte Novo Reservoir) and BMT (Montargil Reservoir).

Besides the difference in contents of chl *a* and PC in different species, factors such as the phytoplankton composition or the presence of suspended particles in the water can also cause interference with the estimation of pigment concentrations (SRIVASTAVA et al., 2013). The colony shape (LEE et al., 1995), the colony growth stage, and historical conditions of light and nutrients may also have influence over the *in vivo* fluorometry (BASTIEN et al., 2011). For example, while low light intensity levels could stimulate the synthesis of PC (GROSSMAN et al., 1994), nitrogen limitations can induce PC degradation, since phycobiliproteins are used as a nitrogen reserve (IZYDORCZYK et al., 2005). Moreover, the growth stage may also represents a high source of variability, since more or less PC concentration is associated with different phases, in different species (CHANG et al., 2012) and with environmental samples, this aspect cannot be controlled.

Nevertheless, fluorometric probes could be used as a rapid tool for assessing the development of cyanobacterial blooms.

### *Identification and enumeration of dominant cyanobacteria and cell counting*

Microscopic identification and enumeration of phytoplankton are shown in Figure 3.5 (for all sampled sites) and in Figure 3.6 (for all sampled sites except Vela Lake).

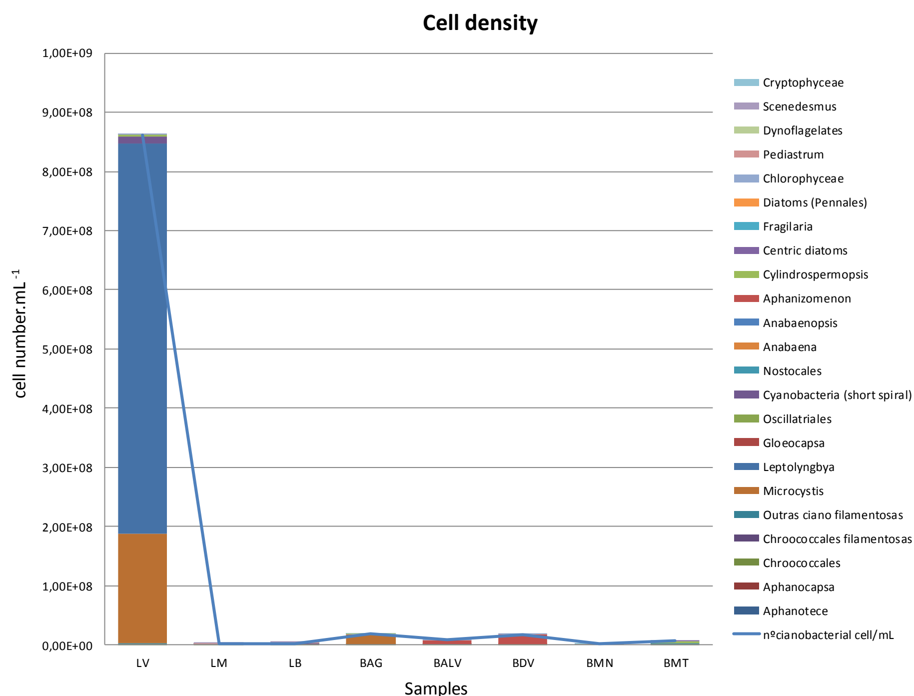



**Figure 3.5** Cell concentrations in collected samples. Codes correspond to LV (Vela Lake), LM (Mira Lake), LB (Barrinha de Mira Lake), BAG (Aguieira Reservoir), BALV (Alvito Reservoir), BDV (Odivelas Reservoir), BMN (Monte Novo Reservoir) and BMT (Montargil Reservoir). The line represents only the number of cyanobacterial cells.mL$^{-1}$, while the bars represent the total cell number.mL$^{-1}$.

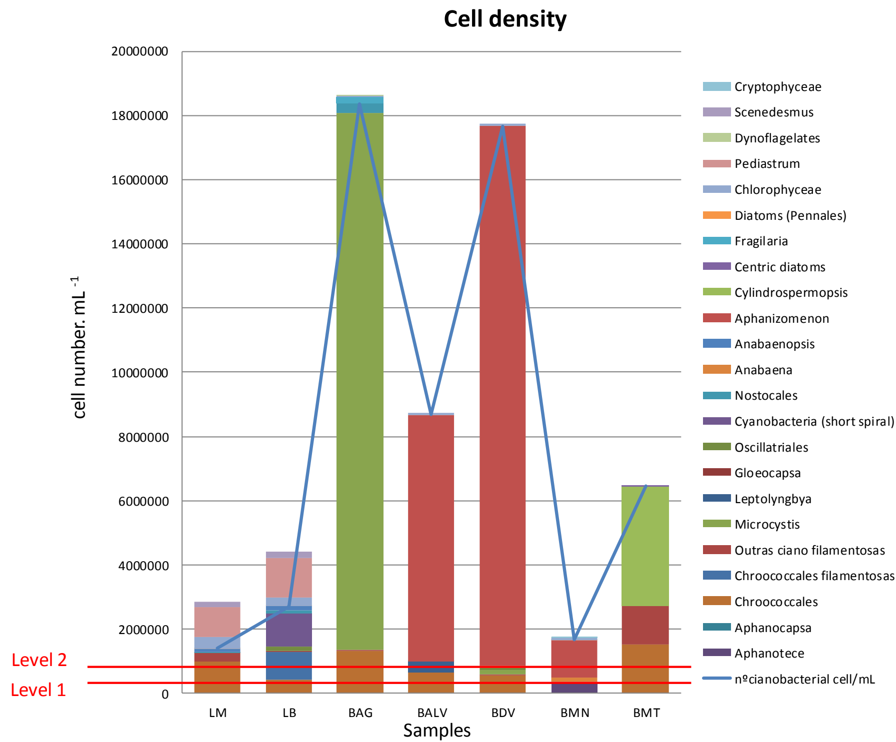



**Figure 3.6** Cell concentrations in collected samples. Codes correspond to LM (Mira Lake), LB (Barrinha de Mira Lake), BAG (Aguieira Reservoir), BALV (Alvito Reservoir), BDV (Odivelas Reservoir), BMN (Monte Novo Reservoir) and BMT (Montargil Reservoir). The line represents only the number of cyanobacterial cells.mL$^{-1}$, while the bars represent the total cells number.mL$^{-1}$. Red lines represent the WHO guideline values for alert levels concerning cyanobacterial cell density (Level 1 > 20,000 cyanobacterial cells.mL$^{-1}$; Level 2 > 100,000 cyanobacterial cells.mL$^{-1}$)

Cell counts showed that in the eight samples considered in this step, cyanobacteria was the dominant group in all samples, except in Mira Lake (Figure 3.7). However, organisms from the Bacillariophyceae, Chlorophyta, Dynoflagellata and Cryptophyceae groups were also identified. Although cell counts give a direct assessment of the organisms present in the water, without requirement for elaborate equipment, it has limitations, namely it is a very laborious and time-consuming technique and requires skilled analysts for species identification. Moreover, colonies of *Microcystis* spp., for example, which consist in a large number of cells, may easily interfere with the accurate estimation of cell density (SRIVASTAVA et al., 2013).

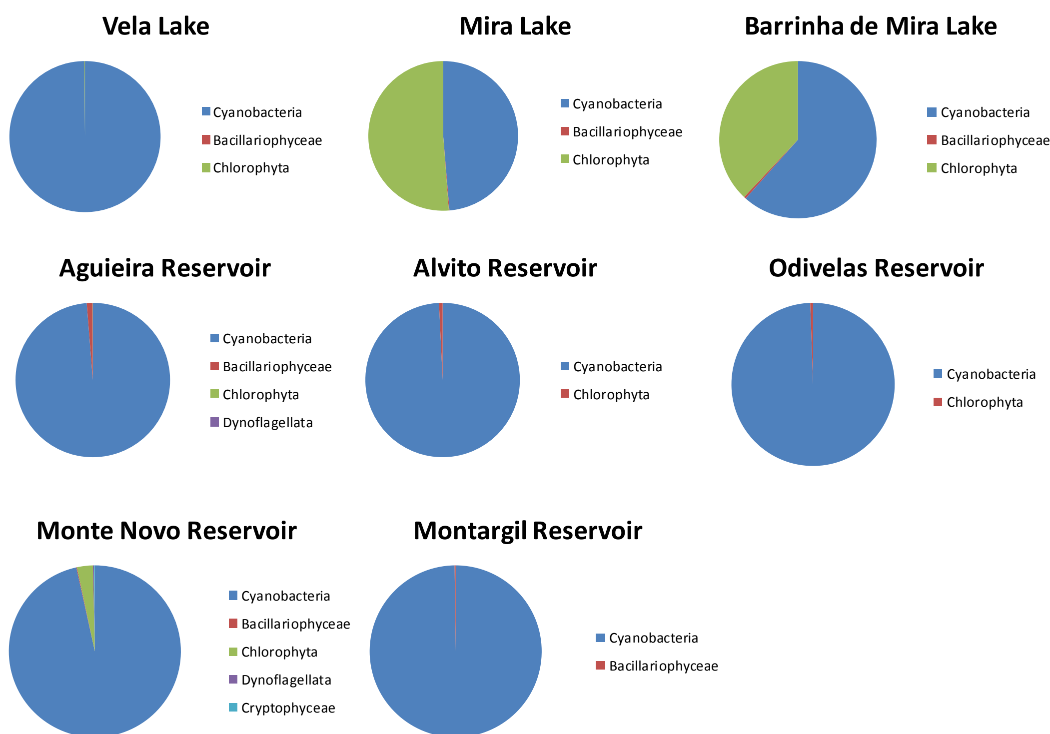



**Figure 3.7** Dominant groups found at the sampled places.

Figure 3.5 clearly show that Vela Lake had the highest cell number, being the majority composed by cyanobacteria cells ($8.62x10^8$ cells.mL$^{-1}$), corresponding to 442.7 µg chl *a* L$^{-1}$ and 482 to 1440 µg PC L$^{-1}$. *Leptolyngbya* sp. was the most abundant *taxa*, along with *Microcystis* sp. This is not a surprise, as over the past years, at this lake, cyanobacteria responsible for blooms have been identified as belonging to *Microcystis* spp. as well as to some filamentous forms belonging to the species *Anabaena (A. flos-aquae*) and *Aphanizomenon (A. flos-aquae, A. gracile* and *A. aphanizomenoides*) (DE FIGUEIREDO et al., 2006, DE FIGUEIREDO et al., 2010). Aguieira Reservoir also presented scum and *Microcystis* sp. was the dominant cyanobacteria (Figure 3.6) with 135.29 µg chl *a* L$^{-1}$ and 42 to 1047 µg PC L$^{-1}$, supporting the presence of a bloom. Blooms of this genus have also been recorded in this reservoir by other authors (VASCONCELOS, 1995) as well as other potentially toxic species such as *Anabaena flos-aquae, Anabaena spiroides, Aphanizomenon flos-aquae* and *Aphanizomenon issatschenkoi* (VASCONCELOS et al., 2011). Odivelas Reservoir showed moderate values for chl *a* (26.54 µg L$^{-1}$) and PC (26 µg L$^{-1}$), but showed a high cyanobacterial density ($1.8x10^7$ cells.mL$^{-1}$) with *Aphanizomenon* as the dominant genus (Figure 3.6), as previously observed by Vasconcelos (1995). This discrepancy between chl *a* and cell density highlights, once more, the need of several complementary approaches to assess the risk of bloom development as different bloom-forming cyanobacteria may show different PC:Chl *a* ratios. On the other hand, in spite Montargil Reservoir showed high concentrations of chl *a* (133.16 µg L$^{-1}$) and PC (162 µg L$^{-1}$), a lower cyanobacterial

concentration was recorded ($6.4 \times 10^{6}$ cells.mL$^{-1}$) when compared to other samples with lower pigment values; the dominant species was *C. raciborskii*. In fact, *C. raciborskii* has proven to have a higher PC content comparing to other cyanobacterial species (HORVÁTH et al., 2013) (see also Chapter 2 of this dissertation). Moreover, high levels of *Aphanizomenon* spp. and Chroococcales were also recorded which may also explain the high PC levels in this sample. Historical records of *C. raciborskii* and of other toxic genera have already been described for Montargil Reservoir (SAKER et al., 2003, VALÉRIO et al., 2005). *C. raciborskii* was first classified as a tropical species due to its affinity to warm water temperatures, but has now a worldwide distribution with records in all continents, corroborating the theory of invasive species to temperate regions (PADISÁK, 1997). The appearance of this species in temperate climates such as Portugal may be related to global warning associated with climate change (VASCONCELOS, 2006).

According to WHO recommendations, chl *a* and cyanobacterial density should be the parameters used to assess the risk of cyanobacterial blooms and eight of the nine samples (excluding Crestuma Reservoir because of its undetectable PC) would be classified within the 2$^{nd}$ alert level (Barrinha de Mira and Mira Lake, and Alvito, Odivelas, Monte Novo and Montargil reservoirs) or the 3$^{rd}$ alert level (Vela Lake and Aguieira Reservoir). However, using the strategy proposed in the present study, it is also important to assess if the dominant cyanobacteria have historical records of toxicity. Only the potential for microcystin (MC) and cylindrospermopsin (CYL) production was assessed, after the evaluation of dominant *taxa*. International discussion on guidelines for cyanotoxins focus mainly on MC because neurotoxins are not considered to be as hazardous (due to their lack of chronic toxicity) nor as widespread; and *Cylindrospermopsis*, whose toxin is very hazardous, does not form surface scums, and thus represents a higher risk for drinking water than for bathing waters (CHORUS et al., 2000). However, in this study, CYL was also considered because of the high density of species producers of this toxin in some samples.

Although all samples have cyanobacterial densities higher than those proposed by WHO alert levels, at Mira and Barrinha de Mira lakes the dominant species did not have historical records of toxicity (see Table 3.2). In spite of, in these samples, chl *a* and cyanobacterial concentrations were higher than those recommended by WHO, those blooms were not worrying for public health and, therefore, did not require prosecution for the subsequent analyses. However, in the remaining samples, there were identified species known to be able produce the cyanotoxins MC and/or CYL. These two toxins are the most associated to poisoning reports and, therefore these samples need posterior analyses in order to assess if there is the potential for toxin production. This analysis is necessary because toxic and nontoxic cyanobacteria may be present in a same water bloom (BARON-SOLA et al., 2012). Moreover, toxic cyanobacterial may not be always producing the toxins which can mislead results to unsafe risk assessment.

**Table 3.2** Dominant cyanobacteria identified in samples and corresponding historical record of toxicity. Legend: MC (microcystins) and CYL (cylindrospermopsin).

| Samples | Dominant cyanobacteria (cell density) | Historical records of toxicity |
|---|---|---|
| Vela Lake | *Microcystis* spp. ($1.8 x 10^8$) | Yes (MC) |
| | *Leptolyngbya* spp. ($6.6 x 10^8$) | No |
| Mira Lake | Chroococcales ($9.8 x 10^5$) | No |
| Barrinha de Mira Lake | Chroococcales ($1.0 x 10^6$) | No |
| Aguieira Reservoir | *Microcystis* spp. ($1.7 x 10^7$) | Yes (MC) |
| Alvito Reservoir | *Aphanizomenon flos-aquae/gracile* ($7.7 x 10^6$) | Yes (MC), Yes (CYL) |
| Odivelas Reservoir | *Aphanizomenon flos aquae/issatschenkoi* ($1.2 x 10^6$) | Yes (MC), Yes (CYL) |
| Monte Novo Reservoir | *Aphanizomenon flos-aquae/gracile* ($1.7 x 10^7$) | Yes (MC), Yes (CYL) |
| Montargil Reservoir | *Cylindrospermopsis raciborskii* ($3.7 x 10^6$) | Yes (CYL) |

***Toxic potential assessment by PCR***

Results from PCR using primers for the assessment the single N-methyltransferase (NMT) domain encoded by the MC synthetase gene *mcyA* (TILLETT et al., 2001) showed the presence of the targeted fragment (220 bp) in samples from Vela Lake and Aguieira Reservoir (Figure 3.8), where blooms of *Microcystis* spp. were recorded. Although caution should be taken, as Tillet *et al*. (2001) found two nontoxic strains of *Microcystis* which contained the NMT module of *mcyA,* field populations of *Microcystis* spp. are almost always toxic (CARMICHAEL, 1995) and thus, those two samples shows the potential for MC production. *Microcystis* sp. is the most frequent cyanobacteria in freshwaters worldwide and it is the major producer of the hepatotoxin MC. In Portugal, *M. aeruginosa* represents a major problem in water bodies used for drinking water supply and recreation during summer months and MC can be commonly found in those waters (SAKER et al., 2005). Knowledge regarding the complex interactions between blooms development and MC synthesis regulation is still limited. For *Microcystis*, it has been shown that the toxicity of a strain depends on whether or not it contains the genes operon for MC production and that field populations are a mixture of both genotypes with and without this gene (SRIVASTAVA et al., 2013). Biochemical and genetic studies have suggested a mixed polyketide synthase (PKS)/nonribosomal peptide synthetase (NRPS) origin for the MC (TILLETT et al., 2000).

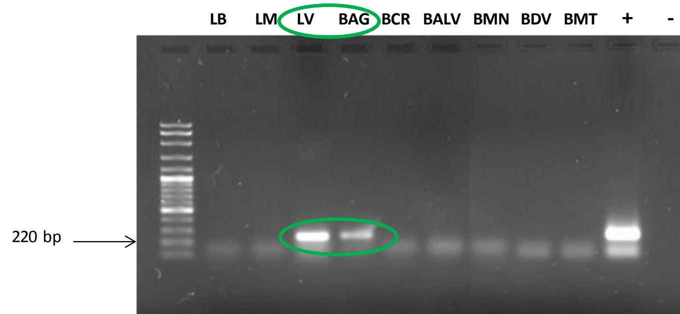



**Figure 3.8** Agarose gel with the product of PCR amplification of gene *mcyA* with Qmet primers. Codes correspond to LB (Barrinha de Mira Lake), LM (Mira Lake), LV (Vela Lake), BAG (Aguieira Reservoir), BCR (Crestuma Reservoir), BALV (Alvito Reservoir), BMN (Monte Novo Reservoir), BDV (Odivelas Reservoir) and BMT (Montargil Reservoir).

A similar PCR methodology was applied for the assessment of CYL producing potential, but PCR shows no amplification of the target fragments (597, 650 and 1105 bp) of the *aoaA, aoaB* and *aoaC* genes, respectively. Although the genes responsible for the production of CYL have not been completely elucidated yet, three genes were identified (*aoaA, aoaB* and *aoaC*) in *A. ovalisporum* and in *C. raciborskii*; sequence homology suggested that these genes encoded an amidinotransferase, a mixed peptide synthetase ⁄ polyketide synthase and a polyketide synthase, respectively, which are involved in CYL biosynthesis (RASMUSSEN et al., 2007). CYL is after MC, the most widely occurring cyanotoxin produced by cyanobacteria (MOREIRA et al., 2011) and records of this toxin are becoming more and more common in several countries (MOREIRA et al., 2013). The tendency of *C. raciborskii* to form dense blooms leads to global concerns for drinking water quality and requires the monitoring of water reserves for the presence of CYL producers (MIHALI et al., 2008). CYL is usually produced by *C. raciborskii* but other species can also produce this toxin, such as *Aphanizomenon flos-aquae* (PREUSSEL et al., 2006) which was, in the present study, recorded as a dominant species at Alvito, Odivelas and Monte Novo reservoirs. In Portugal, there are reports for the presence of *C. raciborskii* in many reservoirs and lakes used for recreational activities and for drinking water, in the center and south of the country (SAKER et al., 2003). However, CYL was never detected (MOREIRA et al., 2011).

At the end of this step, only two samples (Vela Lake and Aguieira Reservoir) showed toxic potential and the need to continue to the next step, which is cyanotoxin quantification.

#### ***Toxins quantification***

This is the last (and may become also the most expensive) step of the proposed methodology. According to the Portuguese Cyanobacterial Monitoring Programme, for values of cyanobacterial cell density > 20,000 cells.mL$^{-1}$ (which correspond to the first alert level of WHO), bioassays are required. However, ethical issues are leading to the suppression of this kind of

bioassays and replacement by analytical methodologies, whenever it is possible (EPA, 2012). According to this programme, all the eight initial samples would have needed cyanotoxins quantification. However, according to the proposed strategy, only two showed actually true danger of having a toxic bloom, and therefore, only for these two samples it would be important to assess cyanotoxins content. This reduction of sites allows a big reduction of the money invested in methodologies, personnel and/or equipment to analyze cyanotoxins which are relatively complex, expensive, time-consuming, and require high concentration of cells or toxins (BARON-SOLA et al., 2012).

For the analysis of MC, the ELISA assay showed detectable values of MC content between 1.4 and >12.3 µg $L^{-1}$ for Aguieira Reservoir and Vela Lake, respectively (Table 3.3). The remaining samples, as expected, did not show detectable levels of MC. Moreover, the presence of scum normally implies a high concentration of toxins, and thus represents a high risk for public health (CHORUS et al., 1999).

**Table 3.3** Compilation of the results from the analysis of the proposed methodology. Grey cells represent the samples which do not require further analysis.

| Sample | PC (µg $L^{-1}$) | Chl *a* (µg $L^{-1}$) | Number cianob cells.$mL^{-1}$ | Dominant cyanobacteria (cell density) | History of toxicity | Potential for toxin production | Toxin level (MC µg $L^{-1}$) |
|---|---|---|---|---|---|---|---|
| LV | 484<br>1440 (scum) | 442.7 | $8.6x10^8$ | *Leptolyngbya* ($6.6x10^8$)<br>*Microcystis* ($1.8x10^8$) | No<br>Yes (MC) | Yes | > 12.3 |
| LM | 19 | 21.0 | $1.4x10^6$ | *Chroococcales* ($9.8x10^5$) | No | | |
| LB | 22.5 | 18.3 | $2.7x10^6$ | *Chroococcales* ($1.0x10^6$) | No | | |
| BAG | 42<br>1074 (scum) | 135.3 | $1.8x10^7$ | *Microcystis* ($1.7x10^7$) | Yes (MC) | Yes | 1.4 |
| BCR | | 14.4 | | | | | |
| BALV | 23 | 23.7 | $8.7x10^6$ | *Aphanizomenon flos-aquae/ A. gracile* ($7.7x10^6$) | Yes (MC) | No | |
| BDV | 26 | 26.5 | $1.8x10^7$ | *Aphanizomenon flos-aquae/ A. gracile* ($1.7x10^7$) | Yes (MC) | No | |
| BMN | 19 | 11.2 | $1.7x10^6$ | *Aphanizomenon flos-aquae/ A. issatschenkoi* ($1.2x10^6$) | Yes (MC) | No | |
| BMNT | 162 | 133.2 | $6.4x10^6$ | *Cylindrospermopsis raciborskii* ($3.7x10^6$) | Yes (CYL) | No | |

Concerning MC content, Aguieira Reservoir stood near the WHO 1$^{st}$ alert level guideline; at a density of 20,000 cyanobacterial cells.$mL^{-1}$, 2-4 µg $L^{-1}$ of MC may be expected if MC producing cyanobacteria are dominant, with 10 µg $L^{-1}$ being possible to expect with highly toxic blooms

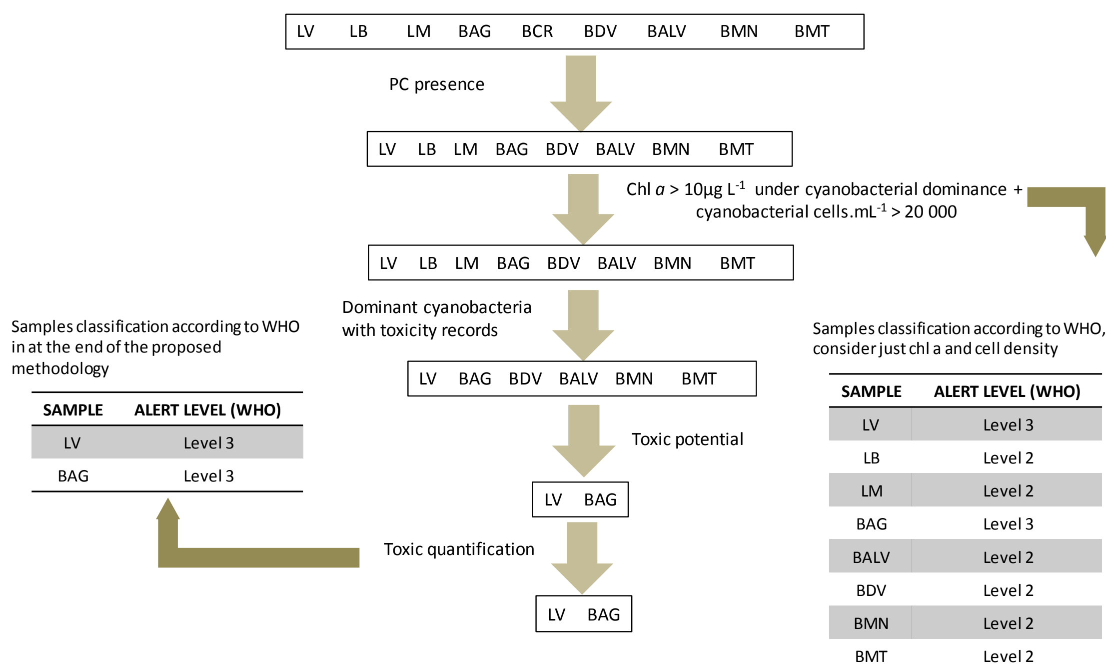
(CHORUS et al., 1999). In this sample, the dominant *taxon* was *Microcystis* sp., which could be a producer of MC. Although the obtained value was a bit smaller than the MC content expected in recreational waters, it exceeds the limit of 1 µg $L^{-1}$ of MC-LR, proposed by WHO for drinking-water supplies. For Vela Lake, *Microcystis* sp. was also one of the dominant species and MC value (>12.3 µg $L^{-1}$) was a bit higher than the 10 µg $L^{-1}$ correspondent to highly toxic blooms. At these places, toxic blooms of *M. aeruginosa* have already been described by Vasconcelos *et al.* (1993, 2011). Therefore, bloom dynamics and toxicity can be very variable, even within blooms of the same genus or even species.

For both Vela Lake and Aguieira Reservoir scum was found. However, although *Microcystis* sp. was one of the dominant species in both places, the appearance of the scum was different. More, difference in MC content (MC quota was higher in Aguieira Reservoir) may be indicator of the presence of two different species of *Microcystis*, which were not easily differentiated by microscopic analysis. The differentiation between different species of *Microcystis* under the microscope is very difficult. Moreover, morphological differences can occur in response to environmental conditions. Therefore, different species with different colony arrangement could create visually differences in scum, as *Microcystis* could change the colony form in field as in cultures (OTSUKA et al., 2000). The capability of MC production is determined genetically, but environmental factors may also affect MC production. The same bloom may have toxic and nontoxic strains and type and cellular content of MC could also differ between strains (JANSE et al., 2004). Thus, these differences in scum appearance and in toxin content may be due to the presence of different *Microcystis* species and different environmental conditions.

## 3.4 Conclusions

The unpredictable nature of cyanobacterial blooms requires management strategies that incorporate several measurements that could function as indicators of the stage of development of a bloom. Determination of chl *a* concentration and cyanobacterial density alone do not reflect the necessary information for the assessment of the risk toxic cyanobacterial blooms. However, complementary techniques such as fluorometry and molecular analysis allow a more accurate and cyanobacterial specific monitoring. Several studies used the determination of *in vivo* PC for assessing cyanobacteria densities, and have proven that it is a very reliable method. The use of fluorometry to assess PC concentration in this work also proved to be a valuable step, since it allows the exclusion of false positive signals given for chl *a* (like happened in Crestuma Reservoir). Also, molecular analysis enables the identification of potentially toxic samples, allowing a safe exclusion of the samples which do not represent a risk to public health (see schematic Figure 3.9). Once again, excluding false positives given by chl *a* and cell counts *per se*. Therefore, following just WHO recommendations, would have eight sites with risks for public health (and requiring the activation for some kind of intervention measures) whereas with the proposed methodology only

two samples were considered effectively hazardous- Vela Lake and Aguieira Reservoir - that should be classified as alert level 3 (due to the existence of scum on the shore).




| SAMPLE | ALERT LEVEL (WHO) |
|---|---|
| LV | Level 3 |
| BAG | Level 3 |

| SAMPLE | ALERT LEVEL (WHO) |
|---|---|
| LV | Level 3 |
| LB | Level 2 |
| LM | Level 2 |
| BAG | Level 3 |
| BALV | Level 2 |
| BDV | Level 2 |
| BMN | Level 2 |
| BMT | Level 2 |

**Figure 3.9** Application of the proposed methodology to the sampled water bodies and classification these according to WHO guidelines at different steps. Codes correspond to LV (Vela Lake), LM (Mira Lake), LB (Barrinha de Mira Lake), BAG (Aguieira Reservoir), BCR (Crestuma Reservoir), BALV (Alvito Reservoir), BDV (Odivelas Reservoir), BMN (Monte Novo Reservoir) and BMT (Montargil Reservoir).

Although this strategy may invest more effort in the methodology, this effort can be compensated after a possible reduction of the number of sites needing intervention (e.g. informing the public and the health authorities, monitoring the site with more frequency or interdiction of use) proving to be a cyanobacterial-specific and cost-effective monitoring approach, allowing a safer recognition of the samples that really represented a risk for the public health. PC fluorometric determination, at an early stage, offers great possibilities for monitoring programmes, allowing saving money and the effort of technicians in unnecessary analyses. However, thresholds of PC are not well defined and more work is important to be developed in that area.

## 3.5 References


AHN, C.Y. [et al.] - Alternative Alert System for Cyanobacterial Bloom, Using Phycocyanin as a Level Determinant. The Journal of Microbiology. Vol. 45(2) (2007), p. 98-104.

AL-TEBRINEH, J. [et al.] - Detection of Saxitoxin-Producing Cyanobacteria and *Anabaena circinalis* in Environmental Water Blooms by Quantitative PCR. Applied and Environmental Microbiology. Vol. 76, n.º 23 (2010), p. 7836–7842.

BAKER, J. A. [et al.] - Monitoring Changing Toxigenicity of a Cyanobacterial Bloom by Molecular Methods. Applied and Environmental Microbiology. Vol. 68, n.º 12 (2002), p. 6070-6076.

BARON-SOLA, A.; OUAHID, Y.; DEL CAMPO, F. F. - Detection of potentially producing cylindrospermopsin and microcystin strains in mixed populations of cyanobacteria by simultaneous amplification of cylindrospermopsin and microcystin gene regions. Ecotoxicology and Environmental Safety. Vol. 75, n.º 1 (2012), p. 102-8.

BASTIEN, C. [et al.] - Performance evaluation of phycocyanin probes for the monitoring of cyanobacteria. Journal of Environmental Monitoring. Vol. 13, n.º 1 (2011), p. 110-8.

BELLINGER, G.E.; SIGEE, D.C. - Freshwater Algae: Identification and Use as Bioindicators. 2º. Spain, 2010. ISBN 978-0-470-05814-5.

BRIENT, L. [et al.] - A phycocyanin probe as a tool for monitoring cyanobacteria in freshwater bodies. Journal of Environmental Monitoring. Vol. 10, n.º 2 (2008), p. 248-55.

CARMICHAEL, W. W. - Toxic *Microcystis* and the environment In: WHO (World Health Organization) (2003). Guidelines for Safe Recreational Water Environments. Volume 1. Coastal and Fresh Waters. WHO, Geneva. (1995).

CHANG, D. W. [et al.] - Measurement of cyanobacteria using in-vivo fluoroscopy - effect of cyanobacterial species, pigments, and colonies. Water Research. ISSN 1879-2448 (Electronic) 0043-1354 (Linking). Vol. 46, n.º 16 (2012), p. 5037-48.

CHISWELL, R.K. [et al.] - Stability of Cylindrospermopsin, the Toxin from the Cyanobacterium, *Cylindrospermopsis raciborskii*: Effect of pH, Temperature, and Sunlight on Decomposition. Environmental Toxicology. Vol. 14 (1999), p. 155-161.

CHORUS, I.; BARTRAM, J. - Toxic Cyanobacteria in Water: A guide to their public health consequences, monitoring and management London: E & FN Spon, 1999. ISBN 0-419-23930-8.

CHORUS, I. [et al.] - Health risks caused by freshwater cyanobacteria in recreational waters. Journal of Toxicology Environmental Health Part B:Critical Review. Vol. 3, n.º 4 (2000), p. 323-47.

COALITION, GLOBAL WATER RESEARCH; AUSTRALIA, WATER QUALITY RESEARCH - Guidance manual for managemement of toxic cyanobacteria. Gayle Newcombe, 2009. ISBN 978–90–77622–21–6

CRONBERG, G.; ANNADOTTER, H. - Manual on aquatic cyanobacteria. A photo guide and a synopsis of their toxicology. Denmark: International Society for the Study of Harmful Algae United Nations Educational, Scientific and Cultural Organisation, 2006. ISBN 87-990827-0-5.

DE FIGUEIREDO, D. R. [et al.] - Microcystin-producing blooms-a serious global public health issue. Ecotoxicology and Environmental Safety. Vol. 59, n.º 2 (2004), p. 151-63.

DE FIGUEIREDO, D. R. [et al.] - Bacterial community composition over a dry winter in meso- and eutrophic Portuguese water bodies. FEMS Microbiology Ecology. Vol. 59, n.º 3 (2007), p. 638-650.

DE FIGUEIREDO, D. R. [et al.] - The effect of environmental parameters and cyanobacterial blooms on phytoplankton dynamics of a Portuguese temperate lake. Hydrobiologia. Vol. 568, n.º 1 (2006), p. 145-157.

DE FIGUEIREDO, DANIELA R. [et al.] - Molecular characterization of bloom-forming *Aphanizomenon* strains isolated from Vela Lake (Western Central Portugal). Journal of Plankton Research. Vol. 32, n.º 2 (2010), p. 239-252.

DORIGO, U.; VOLATIER, L.; HUMBERT, J. F. - Molecular approaches to the assessment of biodiversity in aquatic microbial communities. Water Research. Vol. 39, n.º 11 (2005), p. 2207-2218.

ELLIOTT, J. A. - Is the future blue-green? A review of the current model predictions of how climate change could affect pelagic freshwater cyanobacteria. Water Research. Vol. 46, n.º 5 (2012), p. 1364-1371.

EPA, US- Cyanobacteria and Cyanotoxins: Information for Drinking Water Systems United States Environmental Protection Agency, 2012. ISBN/ISSN EPA-810F11001.

FERREIRA, F. M. B. [et al.] - PSP toxins from *Aphanizomenon flos-aquae* (cyanobacteria) collected in the Crestuma-Lever reservoir (Douro river, northern Portugal). Toxicon. Vol. 39, n.º 6 (2001), p. 757-761.

GALVÃO, H. M. [et al.] - Cyanobacterial blooms in natural waters in southern Portugal: A water management perspective. Aquatic Microbial Ecology. Vol. 53, n.º 1 (2008), p. 129-140.

GREGOR, JAKUB; MARŠÁLEK, BLAHOSLAV - A Simple In Vivo Fluorescence Method for the Selective Detection and Quantification of Freshwater Cyanobacteria and Eukaryotic Algae. Acta hydrochimica et hydrobiologica. Vol. 33, n.º 2 (2005), p. 142-148.

GROSSMAN, A.R. [et al.] - The responses of cyanobacteria to environmental conditions:light and nutrients. In: D.A., B. - The molecular biology of cyanobacteria. Dorecht (The Netherlands): 1994. p. 641-675.

HORVÁTH, HAJNALKA [et al.] - Extraction methods for phycocyanin determination in freshwater filamentous cyanobacteria and their application in a shallow lake. European Journal of Phycology. Vol. 48, n.º 3 (2013), p. 278-286.

INAG, I.P. - Manual para a avaliação da qualidade biológica da água em lagos e albufeiras segundo a Directiva Quadro da Água. Protocolo de amostragem e análise para o Fitoplâncton. Ministerio do Ambiente, do Ordenamento do Território e do Desenvolvimento Regional. Instituto da Água, I.P., 2009.

IZYDORCZYK, K. [et al.] - Measurement of phycocyanin fluorescence as an online early warning system for cyanobacteria in reservoir intake water. Environmental Toxicology. Vol. 20, n.º 4 (2005), p. 425-30.

JANSE, I. [et al.] - Toxic and nontoxic microcystis colonies in natural populations can be differentiated on the basis of rRNA gene internal transcribed spacer diversity. Applied Environmental Microbiology. Vol. 70, n.º 7 (2004), p. 3979-87.

KELLMANN, R.; MILLS, T.; NEILAN, B. A. - Functional modeling and phylogenetic distribution of putative cylindrospermopsin biosynthesis enzymes. Journal of Molecular Evolution. Vol. 62, n.º 3 (2006), p. 267-80.

KOSKENNIEMI, K. [et al.] - Quantitative Real-Time PCR Detection of Toxic *Nodularia* Cyanobacteria in the Baltic Sea. Applied Environmental Microbiology. Vol. 73, n.º 7 (2007), p. 2173-2179.

LEE, T.Y. [et al.] - Quantitative determination of cyanobacteria in mixed phytoplankton assemblages by an *in vivo* fluorimetric method. Analytica Chimica Acta. Vol. 302 (1995), p. 81-87.

LOPES, A. R. [et al.] - Spring pelagic communities of phytoplankton, cyanobacteria, associated heterotrophic bacteria and viruses in an eutrophic shallow temperate lake. Fresenius Environmental Bulletin. Vol. 18, n.º 5b (2009), p. 875-884.

LORENZEN, C.J. - Determination of chlorophyll and pheo-pigments: spectrophotometric equations. Limnology and Oceanography. Vol. 12 (1967), p. 343-346.

METCALF, JAMES S.; CODD, GEOFFREY A. - Cyanotoxins. In: WHITTON, B. A. - Ecology of Cyanobacteria II: Their Diversity in Space and Time. Springer Science+Business Media 2012. p. 651-675.

MIHALI, T. K. [et al.] - Characterization of the gene cluster responsible for cylindrospermopsin biosynthesis. Applied Environmental Microbiology. Vol. 74, n.º 3 (2008), p. 716-22.

MOREIRA, C. [et al.] - Cylindrospermopsin: occurrence, methods of detection and toxicology. Journal of Applied Microbiology. Vol. 114, n.º 3 (2013), p. 605-20.

MOREIRA, C. [et al.] - Application of real-time PCR in the assessment of the toxic cyanobacterium Cylindrospermopsis raciborskii abundance and toxicological potential. Appl Microbiol Biotechnol. Vol. 92, n.º 1 (2011), p. 189-97.

MSAGATI, T.; SIAME, B.; SHUSHU, D. - Evaluation of methods for the isolation, detection and quantification of cyanobacterial hepatotoxins. Aquatic Toxicology Vol. 78, n.º 4 (2006), p. 382-397.

MUYZER, G.; SMALLA, K. - Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie van Leeuwenhoek, International Journal of General and Molecular Microbiology. Vol. 73, n.º 1 (1998), p. 127-141.

OTSUKA, S. [et al.] - Morphological variability of colonies of *Microcystis* morphospecies in culture. Journal of General and Applied Microbiology. Vol. 46(1) (2000), p. 39-50.

PADISÁK, J. - *Cylindrospermopsis raciborskii* (Woloszynska) Seenayya et Subba Raju, an expanding, highly adaptive cyanobacterium: worldwide distribution and review of its ecology. Archive für Hydrobiologie Suplement Vol. 107 (1997), p. 563– 593.

PEARSON, L. A.; NEILAN, B. A. - The molecular genetics of cyanobacterial toxicity as a basis for monitoring water quality and public health risk. Current Opinion in Biotechnology. Vol. 19, n.º 3 (2008), p. 281-288.

PORYVKINA, L.; BABICHENKO, S.; LEEBEN, A. - Analysis of phytoplankton pigments by excitation spectra of fluorescence. Proceedings of EARSeL-SIG-Workshop LIDAR. (2000), p. 224-232.

PREUSSEL, K. [et al.] - First report on cylindrospermopsin producing Aphanizomenon flos-aquae (Cyanobacteria) isolated from two German lakes. Toxicon. Vol. 47, n.º 2 (2006), p. 156-62.

RASMUSSEN, J. P. [et al.] - Development and field testing of a real-time PCR assay for cylindrospermopsin-producing cyanobacteria. Journal of Applied Microbiology. Vol. 104, n.º 5 (2007), p. 1503-15.

SAKER, M. L. [et al.] - Variation between strains of the cyanobacterium *Microcystis aeruginosa* isolated from a Portuguese river. Journal of Applied Microbiology. Vol. 99, n.º 4 (2005), p. 749-757.

SAKER, M. L. [et al.] - First report and toxicological assessment of the cyanobacterium *Cylindrospermopsis raciborskii* from Portuguese freshwaters. Ecotoxicology and Environmental Safety. Vol. 55, n.º 2 (2003), p. 243-250.

SAKER, M. L. [et al.] - Molecular techniques for the early warning of toxic cyanobacteria blooms in freshwater lakes and rivers. Applied Microbiology and Biotechnology. Vol. 75, n.º 2 (2007), p. 441-449.

SCHEMBRI, M.A.; NEILAN, B. A.; SAINT, C. P. - Identification of Genes Implicated in Toxin Production in the Cyanobacterium *Cylindrospermopsis raciborskii*. Environmental Toxicology. Vol. 16 (2001), p. 413-421.

SRIVASTAVA, A. [et al.] - Monitoring approaches for a toxic cyanobacterial bloom. Environmental Scientific Technology. Vol. 47, n.º 16 (2013), p. 8999-9013.

STEFFEN, M. [et al.] - Comparative Metagenomics of Toxic Freshwater Cyanobacteria Bloom Communities on Two Continents. PLoS ONE. Vol. 7(8) (2012).

TILLETT, D. [et al.] - Structural organization of microcystin biosynthesis in *Microcystis aeruginosa* PCC7806: an integrated peptide-polyketide synthetase system. Chemistry & Biology Vol. 7 (2000), p. 753-764.

TILLETT, D.; PARKER, D. L.; NEILAN, B. A. - Detection of toxigenicity by a probe for the microcystin synthetase A gene (mcyA) of the cyanobacterial genus *Microcystis*: comparison of toxicities with 16S rRNA and phycocyanin operon (Phycocyanin Intergenic Spacer) phylogenies. Applied and Environmental Microbiology. Vol. 67, n.º 6 (2001), p. 2810-8.

VALERIO, E. [et al.] - Multiplex PCR for detection of microcystins-producing cyanobacteria from freshwater samples. Environmental Toxicology. Vol. 25, n.º 3 (2010), p. 251-60.

VALÉRIO, E. [et al.] - Molecular characterization of *Cylindrospermopsis raciborskii* strains isolated from Portuguese freshwaters. Harmful Algae. Vol. 4, n.º 6 (2005), p. 1044-1052.

VASCONCELOS, V. - Eutrophication, toxic cyanobacteria and cyanotoxins: when ecosystems cry for help. Limnetica. Vol. 25 (2006), p. 425-432.

VASCONCELOS, V. M. [et al.] - Toxicidade de estirpes de cianobactérias isoladas a partir das lagoas das Braça, Vela e Mira. Boletim UCA, Universidade do Algarve, UCTRA Vol. 1 (1993a), p. 193-201.

VASCONCELOS, V. M. [et al.] - Isolation of microcystin-LR from a Microcystis (cyanobacteria) waterbloom collected in the drinking water reservoir for Porto, Portugal. Journal of Environmental Science and Health - Part A Environmental Science and Engineering. Vol. 28, n.º 9 (1993b), p. 2081-2094.

VASCONCELOS, V. M. [et al.] - Hepatotoxic microcystin diversity in cyanobacterial blooms collected in Portuguese freshwaters. Water Research. Vol. 30, n.º 10 (1996), p. 2377-2384.

VASCONCELOS, V.; MORAIS, J.; VALE, M. - Microcystins and cyanobacteria trends in a 14 year monitoring of a temperate eutrophic reservoir (Aguieira, Portugal). Journal of Environmental Monitoring. Vol. 13, n.º 3 (2011), p. 668-72.

VASCONCELOS, VITOR MANUEL DE OLIVEIRA E - Toxicologia de cianobactérias- Distribuição decianobactérias tóxicas e as suas toxinas em águas doces portuguesas. Bioacumulação em bivalves. Porto: Faculdade de Ciências da Universidade do Porto, 1995. 43-46 f. PhD.

WATRAS, C.J; BAKER, A.L - Detection of planktonic cyanobacteria by tandem *in vivo* fluorometry. Hydrobiologia. Vol. 169 (1988), p. 77-84

WHO - Algae and cyanobacteria in fresh water. In: Guidelines for safe recreational water environments. Geneva, Switzerland: World Health Organization, 2003. p. 136-158.

WILSON, A. E. [et al.] - Genetic variation of the bloom-forming Cyanobacterium Microcystis aeruginosa within and among lakes: implications for harmful algal blooms. Applied Environmental Microbiology. Vol. 71, n.º 10 (2005), p. 6126-33.

WOOD, S.A. [et al.] - Fine-scale spatial variability in anatoxin-a and homoanatoxin-a concentrations in benthic cyanobacterial mats: implication for monitoring and management. Journal of Applied Microbiology. Vol. 109 (2010), p. 2011-2018.

# Chapter IV

Ecological assessment and integration of data

# Relationship between environmental factors and the occurrence of cyanobacterial blooms in recreational water bodies from Northern, Central and Southern Portugal

(Manuscript in preparation for submission to an indexed journal)

## Abstract

Cyanobacterial blooms are commonly associated with shallow lakes and reservoirs with high retention times. In Portuguese water bodies this phenomenon is becoming more frequent and several blooms of toxic species have already been reported. In order to assess the relationship between environmental and biological variables and phytoplankton species occurrence, nine places (three lakes: Vela, Mira and Barrinha de Mira and six reservoirs: Aguieira, Crestuma, Alvito, Odivelas, Monte Novo and Montargil) were studied. All sites were considered eutrophic; being Vela Lake the most eutrophic place. At Vela Lake and Aguieira Reservoir, toxic *Microcystis* spp. blooms were identified. Alentejo reservoirs showed a high similarity in environmental parameters, possibly due to common features in reservoirs from this region. Multivariate analyses showed the most influent variables for phytoplankton distribution were water temperature, chloride concentration, dissolved oxygen levels and DIN:SRP ratio. This highlights the importance to control and manage nutrient inputs on lentic water bodies, particularly under the scope of the global temperature increase.

## Keywords

Recreational water bodies, water quality, cyanobacterial blooms, multivariate analysis

## 4.1 Introduction

Cyanobacterial blooms are commonly associated with shallow lakes and reservoirs with high retention times (MEREL et al., 2013). Climate change and global warming are also enhancing their occurrence and frequency (WIEDNER et al., 2007). Since blooms of cyanobacteria are associated with water quality degradation and could also represent a danger to public health, management strategies are thus indispensable for dealing with these organisms. However, it is required a previous knowledge about the environmental variables that most affect the communities and their interactions with blooms' occurrence. Therefore, information about physical, chemical and biological parameters is fundamental for the development of management strategies. This information can help in the prediction and assessment of cyanobacterial mass developments into blooms, scums or mats, as well as the type of cyanobacterial bloom and its location. Therefore, historical records are very important to indicate whether a water body has been prone to cyanobacterial blooms development. However, the absence of those records cannot be taken as an assurance for the absence of health risks (CHORUS et al., 1999).

The main water quality monitoring programmes are based on physico-chemical parameters that should have values below the recommended levels for each water use. These parameters, along with chlorophyll *a,* allow assessing the trophic state of aquatic ecosystems (PEREIRA et al., 2005). However, the integration of this information with the taxonomic composition of the phytoplankton community is of major importance for the construction of historical time-series data bases. Therefore, at the beginning and during the development of cyanobacterial blooms, further investigation should be conducted in order to understand which factors are the main responsibles for triggering the cyanobacterial growth into a bloom. Several studies have identified those factors, such as temperature (WIEDNER et al., 2007), light exposure, nutrients concentration (MUR et al., 1999), pH (GAO et al., 2012), etc. However, different dominant bloom-forming species may have different environmental requirements, leading to the occurrence of blooms under different environmental contexts (PEARL, 2008).

Due to climate change, cyanobacterial growth can be enhanced either by temperature increase, or due to summer droughts that cause a rapid decrease of water levels at shallow water bodies, leading to important changes of the water physical and chemical characteristics as well as in the composition of the aquatic communities (WIEDNER et al., 2007). In addition, the warming of superficial waters reduces the vertical mixing and allows a decrease of water viscosity and resistance, enabling the vertical migration of bloom-forming cyanobacteria. These conditions linked to nutrient supplies have allowed the expansion of the range of several cyanobacterial species (PAERL et al., 2009), with negative consequences for water quality, and negative implications to economy, biodiversity and public health (CODD, 2000). When dense blooms die, are decomposed and cause excessive oxygen consumption that leads to hypoxia conditions. This process could be responsible to the decline or elimination of several organisms, such as fish, shellfish, invertebrates and even plant habitats (DIAZ et al., 1999). More, cyanobacterial blooms can often produce a

variety of odour and taste compounds (geosmins, DMIB), besides the toxins production potential, which affect negatively the water quality, reduce the touristic potential and endanger public health (PEARL, 2008).

Over the past decades, several reports on cyanobacterial blooms have been published and there seems to be an intensification and extended duration of these blooms, particularly in shallow water bodies and reservoirs, with relatively high concentrations of primary algal nutrients (nitrogen, phosphorous, and carbon) (MEREL et al., 2013). In Portuguese water bodies, this phenomenon is becoming more frequent and several blooms of toxic species have already been reported in natural lakes, reservoirs and large slow flowing rivers (VASCONCELOS, 1999), with the dominance of potentially toxin-producing cyanobacteria such as *Microcystis* spp.*, Aphanizomenon flos-aquae, Aphanizomenon gracile, Planktothrix* sp.*, Anabaena flos-aquae, Anabaena circinalis* and *Cylindrospermopsis raciborskii* (see Table 3.1 in Chapter 3). Small shallow lakes used for agriculture and recreation have recurrent blooms' formation (e.g. Vela Lake (DE FIGUEIREDO et al., 2006, VASCONCELOS et al., 1993) and Mira Lake (SAKER et al., 2007, VASCONCELOS et al., 1993)) and this may have a high impact on the health of the lake's users, as well as in the consumption of crops from surrounding fields (bioaccumulation of cyanotoxins has been widely reported (CODD et al., 1999, DE FIGUEIREDO et al., 2004, WILLIAMS et al., 1997)).

Humans are potentially exposed to cyanotoxins mainly through recreational activities and consumption of contaminated drinking water. Therefore, efficient management of waters bodies is indispensable (MEREL et al., 2013) and from a management perspective, the identification of the environmental factors which lead to the development of toxic blooms is essential for the establishment of measures that may control and prevent it (PEARL, 2008). In the present study, several lakes and reservoirs with historical occurrence of cyanobacterial blooms were investigated regarding the phytoplankton/cyanobacterial community and environmental parameters, in order to identify the presence of potentially toxic cyanobacterial blooms.

## 4.2 Material and Methods

***Sites description***

Nine lentic Portuguese water bodies were chosen for sampling, due to their historical records on cyanobacterial blooms and their use as recreational water bodies. These included three lakes (Vela, Mira and Barrinha de Mira) and six reservoirs (Aguieira, Crestuma, Alvito, Odivelas, Monte Novo and Montargil) - see further description in Chapter 3 (Table 3.1).

***Sampling and environmental parameters determination in situ***

Three kinds of parameters were measured: physico-chemical, organic load and biological.

The parameters pH, temperature, conductivity and dissolved oxygen (DO) were measured *in situ* using a digital multi meter WTW Multi 3430 Set F. Parameters such biochemical oxygen

demand (BOD), chemical oxygen demand (COD), total organic carbon (TOC), suspended solids, phosphorus, soluble reactive phosphorus (SRP), Kjeldahl nitrogen, ammonia, nitrites+nitrates, sulfate and chloride were analyzed in the laboratory. For the determination of these parameters at laboratory, it was collected 10 L (in polyethylene terephthalate bottles (PET) previously washed with distilled water), except for phosphorus, for which it was sampled 1 L (in glass bottles previously washed with HCl). Bottles for the chemical parameters were previously rinsed three times with the water sample before the final collection. For biological parameters (chlorophyll, phycocyanin and cell density) and molecular analyses, 2 L water samples were collected in sterile bottles.

Water samples were taken sub-superficially at 1 m from the shore, avoiding sediment collection, and immediately stored at 4ºC under dark conditions.

***Environmental parameters determination in laboratory***

At the laboratory, the samples were filtered and preserved according to the target analysis following standard procedures (APHA, 1999). For the analyses of Kjeldahl nitrogen, ammonia and COD, samples were preserved with $H_2SO_4$ until pH < 2 and refrigerated. For the analyses of phosphorus and SRP, samples were frozen without any chemical preservation and the remaining samples were refrigerated until analysis.

Analytical protocols adopted in this study were in accordance with the standard methods described in APHA (1999) and the Application Notes of the apparatus, as shown in Table 4.1. These methods were chosen according to the apparatus and reagent availability of Department of Environment and Planning laboratory and three replicates were performed for each sample.

**Table 4.1** Analysis method for the determined physico-chemical and organic load* parameters.

| Parameter | Analysis method | Reference | Pre-treatment | Preservation |
|---|---|---|---|---|
| Total suspended solids (TSS) | 2540 D Total Suspended Solids Dried at 103-105ºC | (APHA, 1999) | ------------- | Immediately determined |
| Fixed (FSS) and Volatile (VSS) suspended solids | 2540 E Fixed and Volatile Solids Ignited at 550ºC | (APHA, 1999) | ------------- | ---------------------- |
| *$BOD_5$ | 5210 B 5-day BOD Test | (APHA, 1999) | ------------- | Refrigeration |
| *COD total | 5220 B Open Reflux Method | (APHA, 1999) | ------------- | Add $H_2SO_4$ until pH<2 + refrigeration |
| Dissolved organic carbon | 5310 B High-temperature Combustion Method | (APHA, 1999) | ------------- | Refrigeration |
| Particulate organic carbon | 5310 B High-temperature Combustion Method | (APHA, 1999) | Filtration | Refrigeration |
| Kjeldahl N | 4500-Norg B Macro-Kjeldahl Method<br>Tecator Application Note | (APHA, 1999)<br>Tecator AN 86/87 (TECATOR) | ------------- | Add $H_2SO_4$ until pH<2 + refrigeration |
| Ammonia | 4500-$NH_3$ F Phenate Method | (APHA, 1999) | ------------- | Add $H_2SO_4$ until pH<2 + refrigeration |
| $NO_2^-$ + $NO_3^-$ | FOSS Application Note 5210 | FOSS Application Note 5210 (FOSS) | Filtration | Add HCl until pH<2 + refrigeration |

| Phosphorus (total) | 4500-P B 4. Sulfuric Acid-Nitric Acid Digestion and Ascorbic Acid Method | (APHA, 1999) | ------------- | Freeze |
|---|---|---|---|---|
| Soluble reactive phosphorus (SRP) | 4500-P E Ascorbic Acid Method | (APHA, 1999) | Filtration | Freeze |
| Sulfate | 4110 B Ion Chromatography with Chemical Suppression of Eluent Conductivity | (APHA, 1999) | Filtration | Refrigeration |
| Chloride | 4110 B Ion Chromatography with Chemical Suppression of Eluent Conductivity | (APHA, 1999) | Filtration | Refrigeration |

***Suspended solids***

Total suspended solids (TSS) were determined by gravimetry after filtration of a well-mixed sample volume using a glass fibre filter and drying at 105°C. The increase in weight of the filter represents the portion of TSS. Volatile suspend solids (VSS) and fixed suspended solids (FSS) were determined by ignited the residue to a constant weight at 550ºC. The remaining solids represent the fixed solids, while the weight lost on ignition are the volatile solids.

***Biochemical oxygen demand (BOD)***

BOD measures the relative oxygen requirements of waters and it was determined by 5-day BOD Test, which measures the molecular oxygen consumed during a certain incubation period, in this case a period of 5 days, for the biochemical degradation of the organic material. As bacterial growth requires nutrients such as nitrogen, phosphorus, and trace metals, these were added to the dilution water, which was buffered to ensure that the pH of the incubated sample remains in a range suitable for bacterial growth. Samples were neutralized to pH between 6.4 and 7.5 with NaOH and $H_2SO_4$ and then diluted several times, based on residual DO of at least 1 mg $L^{-1}$ and a DO uptake of at least 2 mg $L^{-1}$ after a 5-days incubation. Three Winkler bottles were filling with the diluted sample to overflowing and DO was measured by the membrane electrode method (Section 4500-O G., APHA, 1999); diluted samples, seed controls and dilution water blanks were incubated at 20ºC during 5 days; passed this period, the DO was measured again. Then $BOD_5$ was determined as the difference between initial and 5-day DO content corrected by the dilution factor.

***Chemical oxygen demand (COD)***

Total COD was measured using the dichromate open reflux method. Samples were digested with a solution of $H_2SO_4$ conc. with $Ag_2SO_4$, $K_2Cr_2O_7$ and $HgSO_4$ at 150ºC during 2h, in order to oxidize the organic matter. After digestion, the remaining unreduced $K_2Cr_2O_7$ was determined by tritation with ferrous ammonium sulphate (FAS), using ferroin solution as indicator. The end point of titration were consider the first sharp colour change from blue-green to reddish brown that persists for 1 min or longer.

Two concentration ranges of $K_2Cr_2O_7$ and FAS were used, according to COD levels. In cases where COD values were above 50 mg $O_2$ $L^{-1}$, like verified in Vela Lake and Montargil Reservoir, the concentrations used were: $K_2Cr_2O_7$ 0.0417 M and FAS 0.125 M. For the remaining sites, where

COD values were in the range 5-50 mg $O_2$ $L^{-1}$, the concentrations used were: $K_2Cr_2O_7$ 0.00417 M and FAS 0.01 M.

***Total Organic Carbon (TOC)***

In order to determine TOC, the organic molecules must be broken down and converted to a single molecular form, $CO_2$, that could be measure quantitatively. TOC was analysed by High-Temperature Combustion Method in a Shimadzu analyser with two modules, TOC-$V_{CHP}$ (for liquid combustion) and SSM-5000A (for dry combustion). This method used high temperature, catalysts and oxygen to convert the organic carbon in $CO_2$ and then quantify it through a nondispersive infrared analyser (NDIR). To determine total particulate carbon: samples were filtered in carbon free filters (Whatman GF/C glass-fiber filters), and were analysed in the SSM-5000A module. Samples were oxidized at 900ºC, with ultra-pure oxygen and a catalyst of platinum covered by aluminium oxide, to obtain the final product $CO_2$, which was determining by NDIR. To determine dissolved organic carbon, the filtered samples were injected in the TOC-$V_{CHP}$ module; samples were oxidized at 680ºC, with ultra-pure oxygen and a catalyst of platinum, to obtain the final product $CO_2$ which was also determining by NDIR. TOC was determined by the sum of particulate organic carbon and dissolved organic carbon.

***Nitrogen forms***

*Kjeldahl-N*

Samples were digested following a distillation, according to the Macro-Kjeldahl Method (Section 4500-Norg B, APHA, 1999) and the application note from Tecator, with some changes; samples were digested with $H_2SO_4$ and with one Kjeltab (3.5 g $K_2SO_4$ + 0.175 g HgO) in a Tecator™ Digestor System-FOSS. The digestion started at 150ºC in order to reduce the volume to half; then the temperature was rinsed to 385ºC during 30 to 40 min, until the solution becomes transparent. For the distillation step, the digested samples were diluted in distillate water and then neutralized with NaOH (in order to obtain a pH > 11) and distilled in a *Kjeltec 1002 System* Distilling Unit-FOSS, until have 125 mL of distillate, which was collected into an erlenmeyer containing $H_2SO_4$ 0.04 M. Finally, the potential of the solution was determined using the ammonia-selective electrode method (Section 4500-$NH_3$ D, APAH 1999).

*Ammonia/ammonium ($NH_3$ / $NH_4^+$)*

Ammonia were analysed by the Phenate Method, as an intensely blue compound (indophenol) is formed by the reaction of ammonium, hypochlorite and phenol catalysed by sodium nitroprusside. Samples were putted in beakers and added phenol solution, sodium nitroprusside and an oxidizing solution (alkaline citrate + sodium hypochlorite). Then, samples were covered with Parafilm®, and stored in subdued light for more than 1h, in order to allow the colour developed. Finally, the absorbance of samples was read in a Camspec M501 Single Beam Scanning UV/Visible Spectrophotometer at 640 nm.

*Organic-N*

Organic-N was calculated by the difference between ammonium and Kjeldahl-N contents.

*Nitrite and nitrate ($NO_3^-$ + $NO_2^-$)*

Samples were mixed with a buffer solution and nitrate present in the sample were reduced to nitrite in a cadmium reductor. With the addition of an acidic sulphanilamide solution, nitrite initially present and nitrite formed from reduction of nitrate will form a diazo compound. These compounds were coupled with N-(1-naphtyl)-Ethylene Diamine Dihydrochloride (NED) to form a purple azo dye, which were measured at 540 nm (FOSS). Subsamples of 50 mL were obtained by filtration through a 0.45 µm pore size Milipore© HA membrane filter and the sum of nitrates and nitrites concentrations were measured with a flow injection analyser (FIAstar$^{TM}$ 5000, FOSS-Tecator).

***Phosphorus***

Phosphorus analysis included two steps: the conversion of the phosphorus to dissolved orthophosphate and the colorimetric determination of these dissolved orthophosphate. Therefore, total phosphorus was measured as orthophosphate by the colorimetric ascorbic acid method after acid digestion with $H_2SO_4$ and $HNO_3$. In order to convert phosphorus in orthophosphate, samples were digested with $H_2SO_4$ conc. and $HNO_3$ conc. in a sand bath, to a volume of approximately 1 mL and until the solution becomes colourless, to remove the $HNO_3$. Then samples were cooled and diluted in distillate water; 1 drop of phenolphthalein indicator was added and then added dropwise 1 N NaOH until the solution becomes pink; after that, also dropwise with 5 N $H_2SO_4$ until the solution becomes colourless again. At this time, all the phosphorus was in the form of orthophosphate and could be measured by the colorimetric ascorbic acid method. Samples were read in a spectrophotometer (Camspec M501 Single Beam Scanning UV/Visible Spectrophotometer) at 880 nm, after 15 min of being added the combined reagent (5 N $H_2SO_4$ + potassium antimonyl tartrate solution + ammonium molybdate solution + ascorbic acid solution). SRP is a measure of orthophosphate and it was determined in filtered samples, by the colorimetric ascorbic acid method without the digestion step.

***Anions sulfate, $SO_4^{2-}$ and chloride, $Cl^-$***

Ion chromatography is a common technique used for determination of anions, such as sulfate and chloride, which eliminates the need to use hazardous reagents required for other methods. These anions were analysed by Ion Chromatography with Chemical Suppression of Eluent Conductivity in a DX-100 Ion Chromatograph Dionex, with an eluent solution of sodium bicarbonate-sodium carbonate (0.0017 M $NaHCO_3$ – 0.0018 M $Na_2CO_3$) and a regenerant solution of $H_2SO_4$ 0.025 N. In this method, a water sample is injected into a stream of carbonate-bicarbonate eluent and passed through a series of ion exchangers, where the anions of interest are separated on the basis of their relative affinities for a low capacity, strongly basic anion exchanger. Then the anions are identified on the basis of retention time as compared to standards and the quantification is done by measurement of the peak area or the peak height.

***Biological parameters***

*Chlorophyll a*

In laboratory, the sampled water was filtered through Whatmann GF/C filters (1.2 µm) which were frozen (-20ºC) until further processing. Chlorophyll *a* was determined using the trichromatic method (APHA, 1999 Section 10200H.2c) but the monochromatic method (LORENZEN, 1967) was also used to determine the influence of phaeopigments in the overall measured chlorophyll *a*. Chlorophyll *a* was extracted with 90% acetone and left at 4ºC overnight; tubes were covered in order to avoid chlorophyll degradation. After this period, tubes were centrifuged for 15 min at 4000 rpm, and the absorbance of the supernatant was read at 750, 664, 647 and 630 nm in a Shimadzu UV-1800 Spectrophotometer; for the monochromatic method, absorbance values were measured at 750 and 665 nm with and without acidification (0.1 N HCl).

*Phycocyanin*

Pycocyanin concentration was in determined, in laboratory, through fluorometry, by spectral fluorescent signatures (SFS) using the FLUO-IMAGERTM (SKALAR, Analytical B.V., Breda, The Netherlands), model M53B version 1.0703. This fluorometer is normally used for detection of photosynthetic pigments in the water. SFS based upon the measurement of excitation spectra and fluorescent spectra. At an excitation wavelength, an emission spectrum is measured with a Photomultiplier (PMT); after spectrum is measured excitation wavelength shifts 2 nm and the emission spectrum is measured again. This process continues until the complete excitation range has been scanned. In general, phycoerythrin and phycocyanin components are included in all live catalogues mixtures of phytoplankton sample with blue-green algae. Catalogue TV 17 was used for samples with cyanobacteria, because it includes the spectra for the peaks of phycocyanin (excitation at 620 nm and emission at 640 nm).

*Microbiological analyses*

Microbiological analyses were performed in order to characterize the water quality of bathing waters, according to the Decree Law nº113/2012. Fecal indicator bacteria concentrations were determined using chromogenic substrate tests, as described in Standard Methods Section 9223 B (APHA, 1998). Total coliforms and *E.coli* were quantified using the IDEXX media Colilert 18 (24h at 37ºC), while enterococci were quantified using Enterolert media and *Pseudomonas aeruginosa* concentration was determined by IDEXX media Pseudalert (24h at 38ºC) (IDEXX, Westbrook, Me, USA). All incubations were performed using IDEXX Quanti-Tray 2000.

*Microscopic identification and counting of cyanobacteria*

Taxonomic identification was made attending to morphologic characters like shape, type, dimension and arrangement of the colonies/filaments disposition and dimension of the vegetative cells, presence or absence of sheath, and the presence or absence of specialized cells and their

shape (heterocysts and akinetes) (BELLINGER et al., 2010, CRONBERG et al., 2006). Identification was performed using *in vivo* samples and Lugol's preserved samples.

Environmental samples for cell counting were preserved with Lugol and kept in the dark until further processing. Phytoplankton cells counting was carried out using the Utermöhl technique on a 5 mL sub-sample, which was sedimented in an Utermöhl chamber over a period of 4 hours per centimetre of the liquid column (INAG, 2009). Counts were performed under an inverted microscope (Olympus CKX4). Two perpendicular transects were counted and phytoplankton diversity was calculated according to the equation (1) proposed by the Water Framework Directive (INAG, 2009):

$$N = X\frac{A\ d}{a\ V} \quad (1)$$

$N$ represents cells number.mL$^{-1}$, $X$ the number of cells for transept, $A$ the sedimentation chamber area, $d$ the dilution factor, $a$ optic field area and $V$ sample volume in the sedimentation chamber.

### *Trophic state assessment*

Currently, in Portugal, the quantitative criteria used for eutrophication in lentic systems (lakes and reservoirs) are based on the classification criteria defined by Organization for Economic Co-operation and Development (OECD) (1982), shown in Table 4.2. The trophic state corresponds to the most unfavorable classification obtain for set of referred parameters (PEREIRA et al., 2005).

**Table 4.2** Standard values for assessing trophic state of lakes and reservoirs, according to OECD (1982).

| | Oligotrophic | Mesotrophic | Eutrophic |
|---|---|---|---|
| Total phosphorus (mg P m$^{-3}$) | < 10 | 10 - 35 | > 35 |
| Chlorophyll *a* (mg m$^{-3}$) | < 2.5 | 2.5 - 10 | > 10 |
| Dissolved oxygen (% sat.) | ----- | ----- | < 40 |

For the trophic state classification, at least one sample at each season should be taken at 0.5 m of deep. However, in this study, the analysis was performed once.

### *Toxins quantification*

Toxin quantification was assessed through Enzyme-Linked Immunosorbent Assay (ELISA) kits. In this study, only a MC ELISA kit was used for the assessment of microcystins (MC). This assay targets MC-LR, the most common variant of microcystins (CHORUS et al., 1999).

### *Statistical analyses*

Pearson correlations were performed between chlorophyll *a* and phycocyanin and the environmental and biological parameters (ZAR, 1996), to assess the environmental variables that best correlated with these two parameters.

Analysis between similarities of the sampled sites was performed through a dendrogram with the similarities calculated by Pearson correlation coefficients in the PAST software version 2.12 (HAMMER et al., 2001).

Results obtained from the environmental variables and cyanobacteria/phytoplankton at the sampled sites (excluding Crestuma Reservoir, due to the absence of phycocyanin) were analysed for the assessment of relationships between them through a canonical correspondence analysis (CCA) (TER BRAAK, 1986), using CANOCO 4.5 (Scientia Software). Environmental variables (see Table 4.3) were standardized (by subtracting the mean from each observation and dividing by the corresponding standard deviation); phytoplankton data were not transformed. The statistical significance of the relationship in the final model (including only selected environmental variables), was assessed by a Monte Carlo (unrestricted) permutation test. For the phytoplankton data, species identification and the cell counts performed for Chapter 3 were used (see Chapter 3, Figures 3.5 and 3.6 and Table 3.3).

## 4.3 Results and Discussion

### Water quality

Table 4.3 resumes the results of the environmental and biological parameters from the nine samples, which can be shown in Figures 4.1, 4.2, 4.3, 4.4 and 4.5.

**Table 4.3** Sites description and environmental parameters retrieved from samples (ND – not detected, below detection limit).

| Sampling site (code) coordinates | Temperature (ºC) | Conductivity (µs cm$^{-1}$) | DO (mg L$^{-1}$) | DO (saturation %) | pH | $BOD_5$ (mg L$^{-1}$) | COD (mg L$^{-1}$) | TOC (mg L$^{-1}$) | TSS (mg L$^{-1}$) | VSS (mg L$^{-1}$) | FSS (mg L$^{-1}$) | P total(mg P L$^{-1}$) | SRP (mg P L$^{-1}$) | organic N (mg N L$^{-1}$) | $NH_4^-$ (mg N L$^{-1}$) | $NO_2^-$+$NO_3^-$ (mg N L$^{-1}$) | $SO_4^{2-}$ (mg L$^{-1}$) | $Cl^-$ (mg L$^{-1}$) | Chl *a* (µg L$^{-1}$) | PC (µg L$^{-1}$) | Coliform bacteria (MPN 100mL$^{-1}$) | *E. coli* (MPN 100mL$^{-1}$) | Intestinal enterococcos (MPN 100mL$^{-1}$) | *P. aeruginosa* (MPN 100mL$^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Vela Lake (LV) 40º16'N;8º47'W | 21.8 | 802 | 12.1 | 136.7 | 9.93 | 17 | 339.25 | 56.119 | 156.67 | 156.67 | ND | 0.3 | 0.0137 | 48.763 | 0.0237 | 36.66 | 256.08 | 352.37 | 442.7 | 484 | 2700 | 1 | 45 | 2700 |
| Mira Lake (LM) 40º26'N;8º45'W | 22.2 | 472 | 8.99 | 102.8 | 8.7 | 2.37 | 22.86 | 13.223 | 11.67 | 11.33 | 0.56 | 0.0533 | 0.0229 | 11.347 | 0.0053 | 63.07 | 245.22 | 122.69 | 20.97 | 19 | 2700 | 146 | 150 | 3 |
| Barrinha de Mira Lake (LB) 40º27N;8º48'W | 21.3 | 470 | 9.16 | 103 | 8.65 | 3.78 | 23.22 | 17.96 | 11.14 | 9.86 | 1.29 | 0.07 | 0.0146 | 0.4795 | 0.0105 | 37.83 | 287.9 | 137.32 | 18.3 | 22.5 | 1553 | 36 | 8 | 0 |
| Aguieira Reservoir (BAG) 20º40'N;8º9'W | 21 | 82 | 8.84 | 99.6 | 8.39 | 1.98 | 26.23 | 14.121 | 21.22 | 20.2 | 2.23 | 0.05 | 0.0099 | 0.618 | 0.012 | 117.5 | 23.2 | 39.7 | 135.29 | 42 | 2700 | 0 | 1 | 2700 |
| Crestuma Reservoir (BCR) 41º4'N;8º28'W | 23.9 | 289 | 7.74 | 91 | 8.07 | 0.94 | 8.95 | 5.856 | 3.39 | 2.04 | 1.35 | 0.06 | 0.0387 | 28.885 | 0.0115 | 0.8 | 77.72 | 33.48 | 14.4 | ND | 358 | 5 | 0 | 0 |
| Alvito Reservoir (BALV) 38º17'N;7º45'W | 23.3 | 366 | 11 | 130 | 8.95 | 1.9 | 20.2 | 13.256 | 5.14 | 5.14 | ND | 0.0167 | 0.0022 | 1.998 | 0.002 | 0.13 | 125.53 | 177.42 | 23.75 | 23 | 2420 | 9 | 37 | 0 |
| Odivelas Reservoir (BDV) 38º11'N;8º6'W | 24 | 392 | 10.2 | 120 | 8.88 | 2.7 | 19.76 | 16.304 | 6.99 | 6.09 | 0.52 | 0.02 | 0.0048 | 0.7805 | 0.0095 | 0.17 | 103.42 | 201.37 | 26.54 | 26 | 2700 | 20 | 69 | 5 |
| Monte Novo Reservoir (BMN) 38º30'N;7º42'W | 25.4 | 333 | 13.6 | 165 | 9.46 | 1.94 | 13.05 | 12.082 | 2.82 | 1.95 | 0.87 | 0.0633 | 0.0031 | 1.64 | ND | 0.28 | 123.54 | 156.42 | 11.2 | 19 | 1986 | 19 | 18 | 0 |
| Montargil Reservoir (BMT) 39º3'N;8º10'W | 21.7 | 161 | 10.5 | 120 | 9.51 | 3.81 | 47.45 | 34.177 | 17.43 | 15.14 | 2.29 | 0.03 | 0.0247 | 43.315 | 0.0085 | 0.27 | 24.13 | 85.71 | 133.16 | 162 | 1986 | 8 | 6 | 0 |

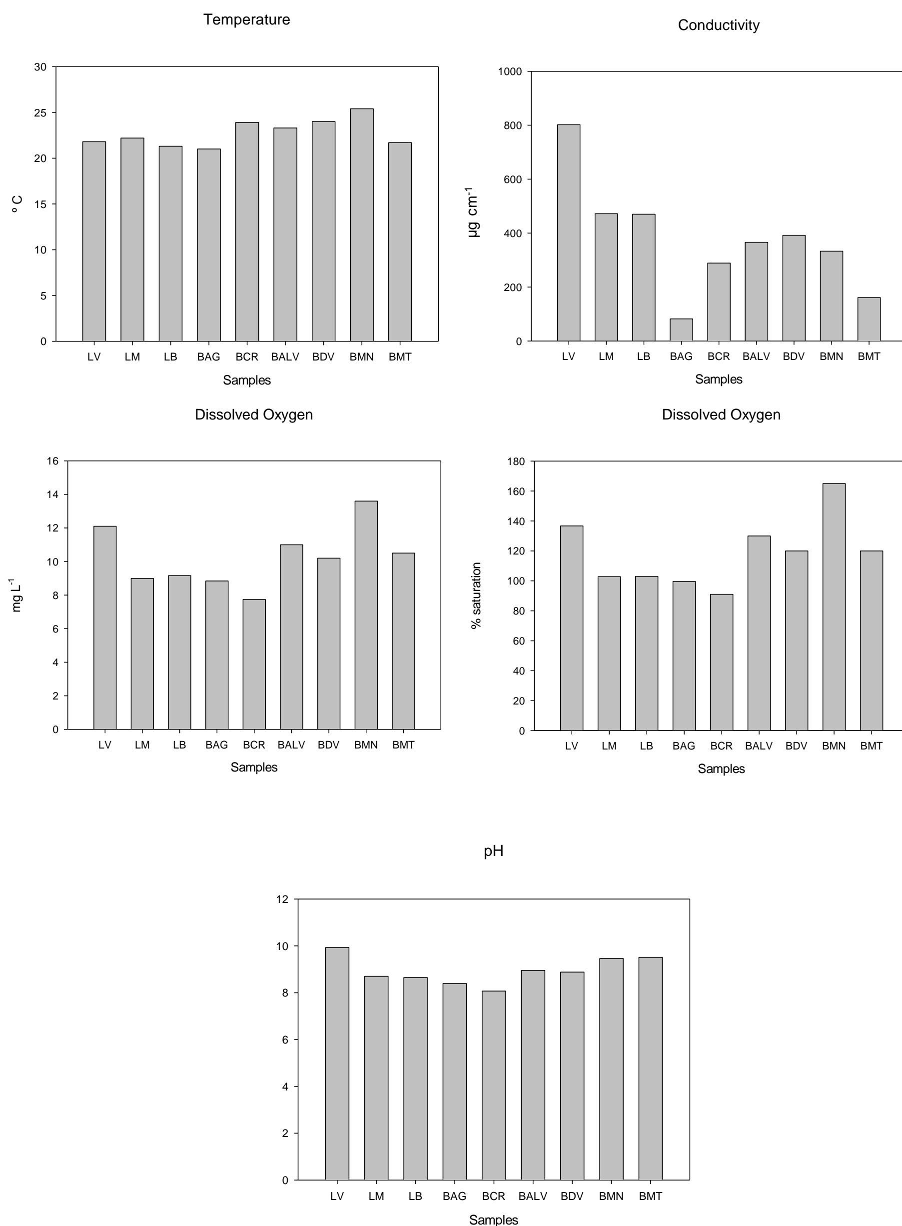



**Figure 4.1** Data for temperature, conductivity, OD (mg $L^{-1}$ and % of saturation) and pH from the field samples. Codes correspond to LV (Vela Lake); LM (Mira Lake); LB (Barrinha de Mira Lake); BAG (Aguieira Reservoir); BCR (Crestuma Reservoir); BALV (Alvito Reservoir); BDV (Odivelas Reservoir); BMN (Monte Novo Reservoir); and BMT (Montargil Reservoir).

Temperature varied between 21.0ºC at Aguieira Reservoir and 25.4ºC at Monte Novo Reservoir (Figure 4.1). For the studied reservoirs, the observed temperatures are between the minimum and the maximum registered by the Sistema Nacional de Informação de Recursos Hídricos (SNRIH) (http://snrih.pt) during early summer and late autumn (data register at stations of Aguieira from the year 2002-2011; 2007-2008 at Crestuma; 2001-2008 at Odivelas; and 1994-2008 at Monte Novo). These temperatures are related to blooms' development. Cyanobacterial blooms

normally prefer warmer waters with temperatures above 25ºC (WIEDNER et al., 2007). Temperature can exert great control over aquatic communities, if the overall water body temperature of a system is changed, an aquatic community shift can be expected (CKSB, July 2001). Higher values were found in the lakes, Vela, Mira and Barrinha de Mira (21.8 ºC, 22.2 ºC, 21.3 ºC, respectively).

The conductivity ranged between 82 µS $cm^{-1}$ at Aguieira Reservoir and 802 µS $cm^{-1}$ at Vela Lake (Figure 4.1). In natural waters, conductivity varies between 20 to 1500 µS $cm^{-1}$; this parameter provides a measure of mineralization, meaning that richer waters with higher nutrient concentration would have higher values of conductivity (FAO, 1987). Water conductivity depends on ionic concentrations and temperature, thus conductivity provides a good indication of water modifications, specially their mineral concentration, but gives no indication of the relative amounts of the various components (NISBET et al., 1970).

Data showed well oxigenated waters, with the minimum value at Crestuma Reservoir (7.74 mg $L^{-1}$) and a maximum at Monte Novo (13.60 mg $L^{-1}$) (Figure 4.1). For the reservoirs, only Alvito was within the historical data registered by SNIRH for the early summer and late autumn (data register at stations of Alvito from the year 2001-2008 and 2011); for the remaining reservoirs, registered data were higher than the historical values referents to the years of 2001-2008 at Odivelas; 1996-2008 at Monte Novo; and 1991-2008 and 2010-2011 at Montargil). In all samples, except Crestuma, the saturation percentage of DO was higher than 100%, which indicates an overproduction of oxygen, probably due to the presence of photosynthetic organisms. This may indicate the development of a bloom, since photosynthesis is one of the oxygen sources in water. Therefore, high values of DO are associated with high rates of photosynthesis. However, during blooms development there is an overproduction of oxygen during the day and consumption during the night, which can lead to small levels of oxygen causing death to many aquatic organisms (NCDENR). Although DO will not have a direct effect on users, it will influence microbial activity and the chemical oxidation state of various metals, such as iron (WHO, 2003). Low oxygen concentrations (less than 2 mg $L^{-1}$) is indicator of poor water quality (CKSB, July 2001) allowing the growth of nuisance organisms, causing taste and odour problems, including the formation of undesirable amounts of hydrogen sulphide (NHMRC, 2008). These factors are not a human health concern in recreational waters, but may give rise to aesthetic issues (WHO, 2003).

Data show a basic pH at all sites (Figure 4.1), with the minor value in Crestuma Reservoir (8.07) and the highest at Alvito Reservoir (9.95). The pH values registered stay between the values registered by SNIRH for the early summer and latte autumn period (data register at stations of Crestuma from the year 1995-1999 and 2003-2008; 2001-2008 and 2011 at Alvito; 2002 and 2004-2005 at Odivelas; 2001-2008 at Monte Novo; and 1989-1993 and 2002-2010 at Montargil), for the reservoirs, except for Monte Novo, which has a higher pH then the maximum registered (9.42). Cyanobacterial blooms cause high levels of pH, due to rapid consumption of inorganic carbon during the photosynthesis and pH raise can cause the release of SRP of the sediments, as well as dissolved inorganic nitrogen (DIN), which support the development of blooms (GAO et al., 2012).

pH levels only have a direct impact on the recreational users of water at very low or very high values. Under these circumstances, pH may have effects on the skin and eyes, exacerbating eyes irritation by chemicals (WHO, 2003). Both alkaline and acidic waters may cause eye and skin irritation and may affect the taste of water, and therefore, waters used for primary recreation should be in the pH range 6.5–8.5 (NHMRC, 2008).

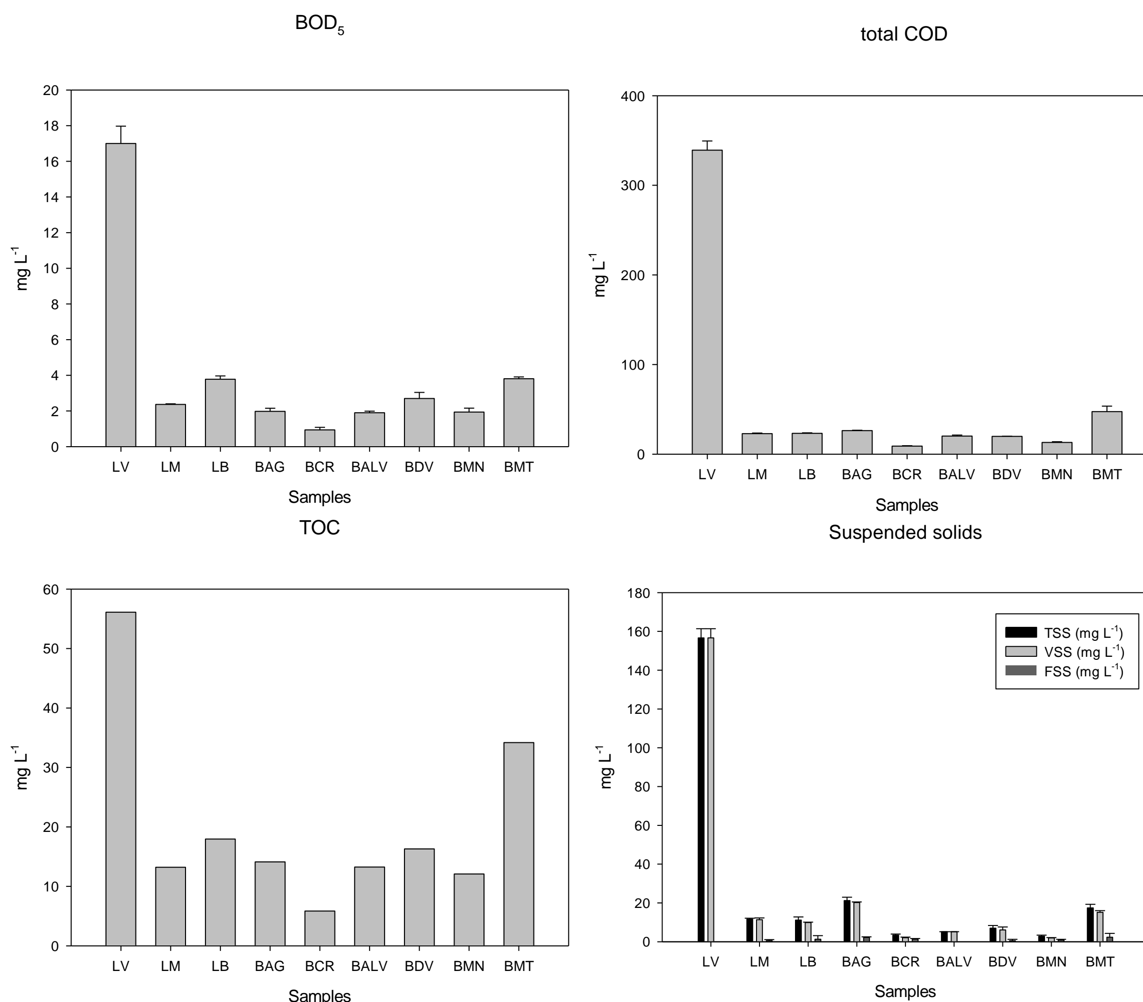



**Figure 4.2** Data for $BOD_5$, COD, TOC and suspend solids from the field samples. Codes correspond to LV (Vela Lake); LM (Mira Lake); LB (Barrinha de Mira Lake); BAG (Aguieira Reservoir); BCR (Crestuma Reservoir); BALV (Alvito Reservoir); BDV (Odivelas Reservoir); BMN (Monte Novo Reservoir); BMT (Montargil Reservoir); TSS (total suspended solids); VSS (Volatile suspended solids); and FSS (fixed suspended solids).

$BOD_5$ and COD (Figure 4.2) indicated organic contamination in Vela Lake (17.0 mg $L^{-1}$) where DO (136.7 % of saturation) and nutrients concentration were also high (4.88 mg N $L^{-1}$ and 0.3000 mg P $L^{-1}$) (see Table 4.3). These conditions could indicate the presence of a bloom, which could contribute to high concentration of organic material. All the values of the reservoirs remained between the SNIRH data for the early summer and late autumn (data registered at stations of Aguieira from the year 2001-2011; 1995-2011 at Crestuma; 2001-2008 and 2011 at Alvito; 2001-

2008 at Odivelas, 1994-2008 at Monte Novo; and 1986-2008 and 2010-2011 at Montargil). $BOD_5$ is an analytical parameter of water quality that indirectly measures the amount of biodegradable organic material present in the water. Thus, it is a measure of organic pollution to both waste and surface waters. High BOD is an indicatior of poor water quality (CKSB, July 2001), as recorded for Vela Lake.

COD data from the reservoirs are between the SNIRH records of this parameter for the period of the early summer to late autumn (data registered at stations of Aguieira from the year 2001-2011; 1995-2006 and 2008-2011 at Crestuma; 2001-2004, 2006-2008 and 2011 at Alvito; 2001-2008 at Odivelas; 1995-2008 at Monte Novo); for Montargil, COD register was higher than the historical values. COD is also an indicator of organics in the water, usually used with BOD. This parameter is often used as a measurement of pollutants in water (APHA, 1999). High organic inputs trigger the oxygenation and if excess organics are introduced into the system, there is potential for complete depletion of DO, threatening the aquatic community (CKSB, July 2001). High TOC levels can also contribute to oxygen depletion (DANET) and this parameter shows the same pattern than $BOD_5$ and COD (Figure 4.2). Data shows that Vela Lake had high values for $BOD_5$, COD and TOC, meaning that this water body is rich in organic matter although DO values were also high (Figure 4.1 and 4.2). Therefore, besides the high amount of organics in the system, the quantity was not high enough for depleting the system of oxygen, since part of the organic material correspond to phytoplankton, which produce oxygen during the photosynthesis process.

Vela Lake was the sample where the highest levels for TSS and VSS were found (Figure 4.2), and this is a place with high turbidity levels all over the year (VASCONCELOS et al., 1993). The values of TSS were within the SNIRH records taken for the reservoirs during the early summer to late autumn (data registered at stations of Aguieira from the year 2001-2011; 1995-2011 at Crestuma; 2001-2008 and 2011 at Alvito; 2001-2008 at Odivelas; and 1986-2011 at Montargil); for Monte Novo, the registered value was smaller than the historical values. These parameters also offer an approximation of the amount of organic matter present in the solid fraction (APHA, 1999) and corroborate the results, indicating high levels of organic material at Vela Lake.

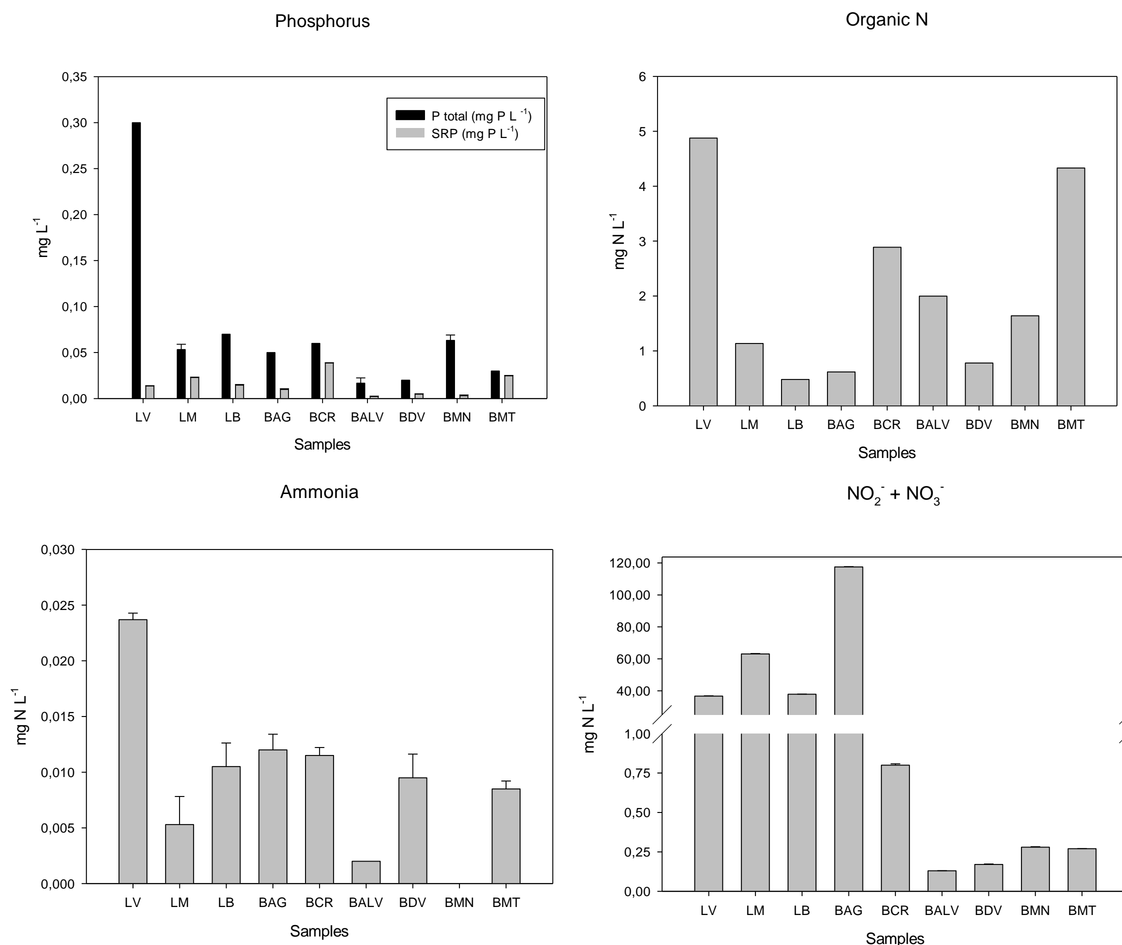



**Figure 4.3** Data for phosphorus, organic nitrogen, ammonia and nitrites+nitrates from the field samples. Codes corresponde to LV (Vela Lake); LM (Mira Lake); LB (Barrinha de Mira Lake); BAG (Aguieira Reservoir); BCR (Crestuma Reservoir); BALV (Alvito Reservoir); BDV (Odivelas Reservoir); BMN (Monte Novo Reservoir); BMT (Montargil Reservoir); P total (total phosphorus); and SRP (soluble reactive phosphorus).

In all samples, total phosphorus was higher than 25 µg $L^{-1}$ (Figure 4.3) which means that all samples had high risk of bloom development (COALITION et al., 2009), except at Alvito and Odivelas reservoirs, that only represented a moderate risk. All data from the reservoirs are between the previously recorded data from SNIRH from the early summer to late autumn (data registered at stations of Aguieira from the year 2001-2002, 2007 and 2010-2011; 2008-2010 at Crestuma; 2001-2011 at Alvito; 2001-2010 at Odivelas; 1994-1995 and 2001-2010 at Monte Novo; and 1988-2011 at Montargil). In general, a total phosphorus levels of less than 10 µg $L^{-1}$ correspond to a low risk of cyanobacterial growth; concentrations between 10–25 µg $L^{-1}$ presents a moderate risk; and levels greater than 25 µg $L^{-1}$ provides high growth potential. However, growth can be maintained at low phosphorus concentrations allowing a rapid recycling of the nutrient (COALITION et al., 2009). Most of the phosphorus that enters into the aquatic environment comes from discharges from point sources, especially municipal sewage and industrial effluents

(CROUZET et al., 1999). Phosphorus does not present problems relating to health in drinking water. However, it is an essential element for the growth of phytoplankton and high concentrations in lakes and reservoirs can lead to a massive development of these organisms (NISBET et al., 1970). At Vela and Mira lakes, high phosphorus concentrations are common, since they receive substantial urban runoff or stormwater from agricultural areas. At Mira Lake an external loading from the bait used in sport fishing, may increase phosphorus levels temporarily and promote cyanobacterial blooms (GONÇALVES et al., 1996). Concerning SRP, the recorded values to reservoirs are in agreement with the previously recorded data from SNIRH for the period of the early summer to latte autumn (data register at stations of Aguieira from the year 2001-2011; 1995-2006 and 2009-2011 at Crestuma; and 2009 at Montargil); at Alvito, Odivelas and Monte Novo, the observed SRP were smaller than the historical values (2001-2008 and 2011 at Alvito; 2001-2008 at Odivelas; and 1994-2008 at Monte Novo). On the other hand, Vela Lake and Montargil Reservoir showed the highest values for nitrogen. Nitrogen is a key nutrient (as phosphorus), which can cause eutrophication of a water body. This may result in an accelerated growth of algae and higher forms of water plants, upsetting the biological balance and quality of the water. Among the major sources of nitrogen in aquatic environments are agricultural activities, especially due to the use of nitrogen fertilisers and manure, and discharges from municipal sewage (CROUZET et al., 1999).

In the sampled sites, concentrations of ammonia were very low (the highest value was found at Vela Lake, 0.0237 mg N $L^{-1}$) and below the toxic levels for aquatic animals (0.2 to 2 mg $L^{-1}$) (CROUZET et al., 1999) (Figure 4.3). Nitrites+nitrates levels were higher than ammonia levels; the highest levels were observed at the three lakes (Vela, Mira and Barrinha de Mira) and at Aguieira Reservoir (with a maximum of 117.5 mg N $L^{-1}$). In the remaining places, nitrites+nitrates levels were below 1 mg N $L^{-1}$. These values are in agreement with data from SNIRH for ammonia referring to the early summer to late autumn period (at stations of Crestuma from the year 1995-2006 and 2008-2011; 2001-2008 and 2011 at Alvito; 2001-2008 at Odivelas); at Monte Novo and Montargil, the registered values were higher than the historical records referent to the years of 1994-2008 at Monte Novo and 2010 at Montargil. Ammonia concentration is especially important for freshwater algae, because it is easier to incorporate into proteins. However, all the major forms of nitrogen (ammonium, nitrite, nitrate, urea, etc.) are ultimately bioavailable (CROUZET et al., 1999). The concentration of nitrate ($NO_3^-$) in aquatic environments may change over time due to the oxygen concentration and the intensity of primary productivity. When the oxygen concentration is low, normally nitrate is reduced to other forms of nitrogen (nitrite and ammonia). If the development of autotrophic organisms is high, the nitrate concentration tends to decrease. Nitrate is the most toxic form to aquatic organisms (CROUZET et al., 1999). The main nitrogen sources in the form of nitrates are from human and animal origins; therefore, nitrate concentrations above 5 mg $L^{-1}$ demonstrate inadequate sanitary conditions (NISBET et al., 1970). Nitrite ($NO_2^-$) is an oxidation intermediate state between nitrate and ammonia. The presence of nitrites in water indicates active biological processes influenced by organic pollution (NISBET et al., 1970). The

presence of ammonia and nitrates in natural waters are normally due to human activities, where agriculture is included (CROUZET et al., 1999).

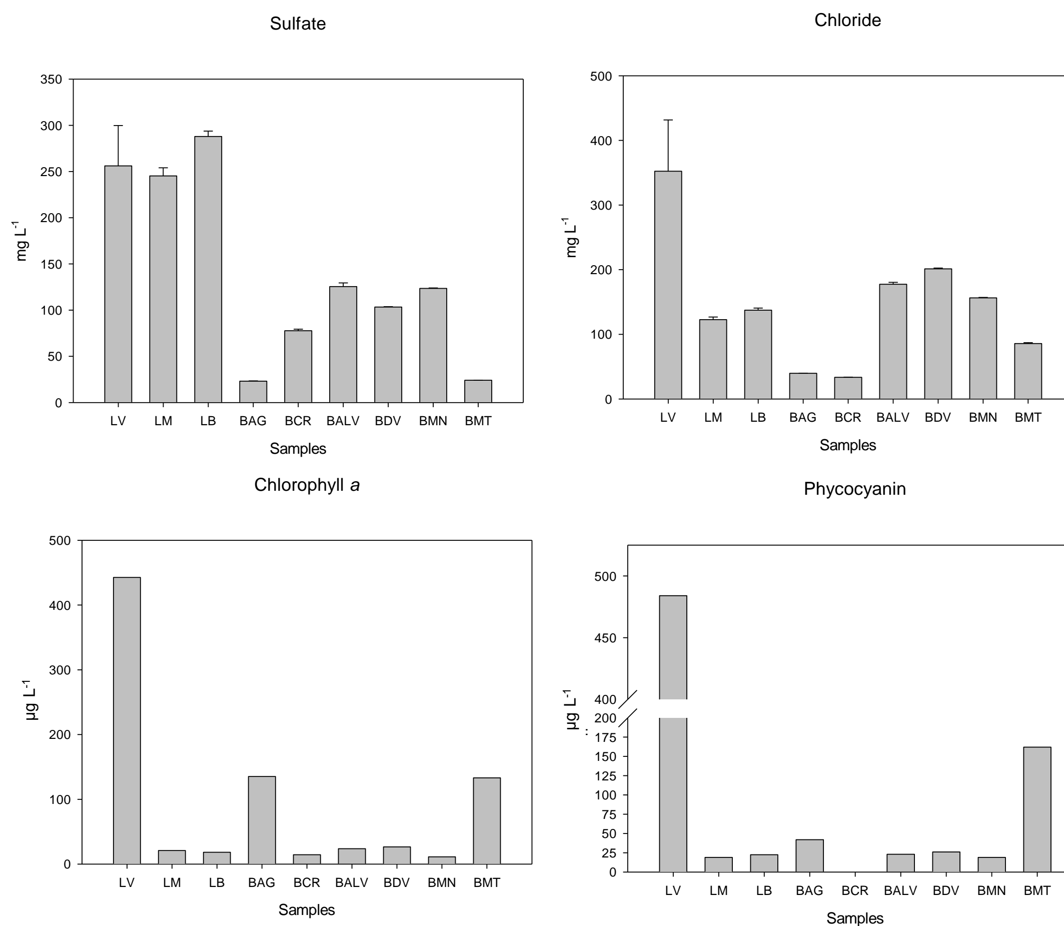



**Figure 4.4** Data for sulfate, chloride, chlorophyll *a* and phycocyanin from the field samples. Codes correspond to LV (Vela Lake); LM (Mira Lake); LB (Barrinha de Mira Lake); BAG (Aguieira Reservoir); BCR (Crestuma Reservoir); BALV (Alvito Reservoir); BDV (Odivelas Reservoir); BMN (Monte Novo Reservoir); and BMT (Montargil Reservoir).

In all sampled sites (Figure 4.4), sulfate concentrations were higher than 20 mg $L^{-1}$, which suggest pollution potential (NISBET et al., 1970). Alvito, Monte Novo and Montargil reservoirs showed values higher than those recorded by SNIRH data base for the period of the early summer to late autumn (data registered at stations of Alvito from the year 2001-2008 and 2011; 2001-2008 and Monte Novo; and 1988-1991at Montargil); for Aguieira, the observed values were within the historical records from the years 2002-2011. Sulfate can be related to the geologic nature or to the presence of industrial or urban effluents (NISBET et al., 1970).

Chloride values recorded ranged between 33.48 mg $L^{-1}$ at Crestuma Reservoir and 352.37 mg $L^{-1}$ at Vela Lake (Figure 4.4). All the reservoirs showed higher values than those recorded at SNIRH (at Aguieira from the year 2001-2011; 1995-2011 at Crestuma; 2001-2008 and

2011at Alvito; 1995 and 2001-2008 at Monte Novo; and 1988-1989, 1991, 2007 and 2009 at Montargil). The presence of chloride may be normal in natural waters, since it is originated from dissolved rocks and minerals (the mineralization process). However, high levels are due to anthropogenic pressure and water quality starts to degrade when chloride levels rise above 600 mg $L^{-1}$ (NAGPAL et al., 1981).

It is generally accepted that chlorophyll *a* levels persistently in excess cause problems for the water treatment. As concentrations increase further above 20 µg $L^{-1}$ problems with recreational waters become more relevant. WHO guidelines for the alert levels in recreational waters suggest a first alert level of 10 µg chl *a* $L^{-1}$ and a second alert level of 50 µg chl *a* $L^{-1}$, when cyanobacteria are the dominant phytoplanktonic group (CHORUS et al., 1999). All samples showed values above the first level (see Table 4.3), and Vela Lake and Aguieira and Montargil reservoirs had values above de second level. Comparing to SNIRH records taken from early summer to latte autumn, Aguieira, Crestuma, Odivelas and Monte Novo showed values within the historical records (data registered at stations of Aguieira from the year 2002-2003; 1995-2010 at Crestuma; 2001-2010 at Odivelas; and 2002-2010 at Monte Novo); in the case of Alvito and Montargil, the observed values were higher than the historical records from the years 2001-2011 at Alvito and 2001-2009 at Montargil. Chlorophyll *a* can be used to assess the biological response to nutrient concentrations in the medium, and it is a useful indicator of the degree of water eutrophication. The reference level of chlorophyll *a* is usually below 3 µg $L^{-1}$ (CROUZET et al., 1999), however, in this study, all chlorophyll *a* concentrations were above that value.

Microbiological results (Figure 4.5) showed the highest levels of fecal bacteria at Mira Lake. In fact, this is an urban lake with a significant urban pressure, particularly during summer months. A good microbiological water quality determined by these parameters may not correspond to safer water, namely concerning cyanobacterial blooms. However, it has been established a relationship between fecal contaminations during cyanobacterial blooms (SINGH et al., 2010). Since this analysis was performed once, it cannot be calculated percentiles from time-series data. However, these results suggest an excellent water quality at the sampled period, when compared to the values proposed by Directive 2006/7/CE of bathing waters use (Table 4.4).

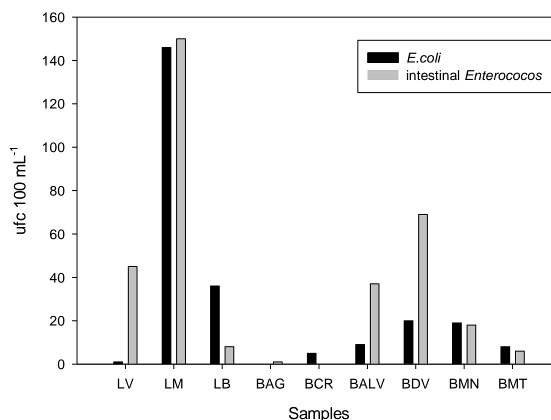



**Figure 4.5** Data from *Eschericia coli* and intestinal *Enterococos* from the field samples. Codes correspond to LV (Vela Lake); LM (Mira Lake); LB (Barrinha de Mira Lake); BAG (Aguieira Reservoir); BCR (Crestuma Reservoir); BALV (Alvito Reservoir); BDV (Odivelas Reservoir); BMN (Monte Novo Reservoir); and BMT (Montargil Reservoir).

For inland waters, this Directive establishes in Annex I, the following criteria:

**Table 4.4**: Quality standards for inland bathing water (taken from Directive 2006/7/CE, Annex I)

| **Parameter** | **Excellent quality** | **Good quality** | **Acceptable quality** |
|---|---|---|---|
| Intestinal *Enterococos* (*ufc* $100mL^{-1}$) | (*) 200 | (*) 400 | (**) 330 |
| *Escherichia coli* (*ufc* $100mL^{-1}$) | (*) 500 | (*) 1000 | (**) 900 |

(*) Based upon a 95-percentile evaluation; (**) Based upon a 90-percentile evaluation

### *Trophic state classification*

The development of blooms of some phytoplankton species is considered the most obvious signal of eutrophication and it is directly dependent on phosphorous and nitrogen overloads (SMITH et al., 1999). This increase of biomass causes turbidity in the water and that is generally seen as a deterioration of the recreational and aesthetic water value, because of the low transparency, odours and the possible presence of cyanobacteria and their toxins (CROUZET et al., 1999).

Eutrophication is the process by which water bodies are made more eutrophic through an increase in their nutrient supply. The susceptibility to eutrophication depends upon morphometrical, chemical and hydrological characteristics, and generally reservoirs, with higher residence times, are less susceptible to eutrophication than shallow lakes (CROUZET et al., 1999). According to criteria used in Portugal, for the assessment of eutrophication status in lentic systems, all places

were classified as eutrophic, being Vela Lake the place with higher concentrations of organic nitrogen (4.88 mg $L^{-1}$), phosphorus (0.3 mg $L^{-1}$) and chlorophyll *a* (442.7 µg $L^{-1}$). This place has already been considered highly eutrophic, mostly due to the regular nutrient inputs from adjacent agricultural fields, and Antunes *et al.* (2003) considered this place as more eutrophic than in the past (ANTUNES et al., 2003). The results of present work also showed high levels of TSS (156.67 mg $L^{-1}$), which seems to be normal, as turbidity is considered high and constant during the year (VASCONCELOS et al., 1993), and of $BOD_5$ (17.0 mg $L^{-1}$), COD (339.25 mg $L^{-1}$) and TOC levels (56.12 mg $L^{-1}$), suggesting organic contamination.

The supply rate of nitrogen and phosphorus strongly influences the growth of algae and vascular plants in freshwater and marine ecosystems (SMITH et al., 1999). In temperate lakes, phosphorus plays a major role in eutrophication and occurrence of phytoplankton blooms. However, high concentrations of phosphorus do not always ensure a bloom formation, since other nutrients may be limited or some inhibitors could also restrain the excessive growth of algae (AHN et al., 2002). Thus, although nutrients are important for assessment the trophic state, and eutrophic water showed to be related with algae blooms episodes, nutrients concentrations alone cannot be used as accurate indicators of bloom formation even though they are useful (AHN et al., 2002). Moreover, different ratios of phosphorus or nitrogen have differential influence among *taxa* (PEARL, 2008), and even with a constant N:P ratio in the water, the algal composition can still be changed by a temperature shift relative to the season, since the algal optimum N:P ratio changes with temperature (AHN et al., 2007). Other variables such as organic matter availability, light conditions, freshwater discharges, residence time and water stability also plays an important role in the cyanobacterial community (PEARL, 2008). The effects of nutrient loading on nuisance cyanobacteria can also depend on the structure of the local food web. Zooplankton is able to suppress cyanobacterial populations by grazing, which keeps the size of the bacterial filaments too small to form nitrogen-fixing heterocysts. When zooplankton abundances are reduced, filaments size increase, heterocysts are formed and nitrogen fixation begins. This implies that food web structure strongly interacts with phosphorus availability to control heterocystous cyanobacteria abundance (SMITH et al., 1999).

***Multivariate analyses***

Interestingly, according to all the environmental and biological parameters, samples from Central Portugal clustered together as well as samples from Southern Portugal. On the other hand, Crestuma Reservoir showed to be segregated from the remaining samples. Moreover, the samples from reservoirs at Alentejo region showed to be highly similar which suggests common features among the reservoirs for this region of Portugal. In fact, Alentejo region is strongly impacted from extreme climatic episodes such as drought, with high impacts on water quality and aquatic communities, as reported before (CAETANO et al., 2001, GALVÃO et al., 2008). The higher temperature values in summer are an important factor to consider for water quality monitoring and

management strategies for this region. Mira and Barrinha de Mira lakes also showed high similarity, probably due to their proximity. Vela Lake did not present high similarity with the remaining lakes, maybe due to the wider differences between the environmental variables in relation to other two lakes (see Table 4.3).

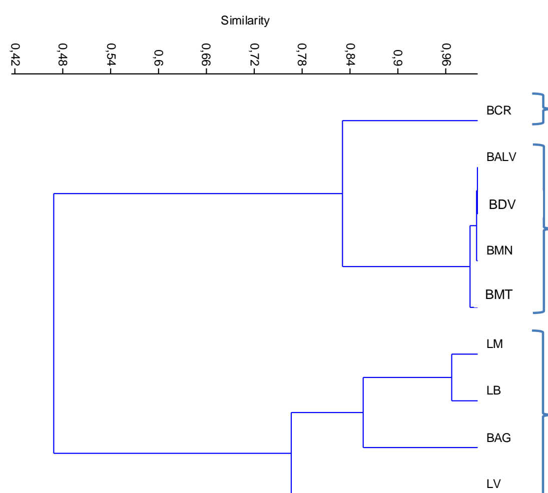



**Figure 4.6** Cluster analysis dendrogram of the samples using Pearson correlation. Codes correspond to LV (Vela Lake); LM (Mira Lake); LB (Barrinha de Mira Lake); BAG (Aguieira Reservoir); BCR (Crestuma Reservoir); BALV (Alvito Reservoir); BDV (Odivelas Reservoir); BMN (Monte Novo Reservoir); and BMT (Montargil Reservoir).

Correlation analysis between phycocyanin and chlorophyll *a* and the environmental parameters, using Pearson correlation coefficient (Table 4.5), showed that these two parameters correlated almost with the same environmental parameters.

**Table 4.5** Pearson correlation coefficient to assess relationships between the recorded environmental parameters and phycocyanin and chlorophyll a ($n$=9; $p < 0.05$)

| **Phycocyanin** | **Chlorophyll *a*** |
|---|---|
| $BOD_5$ ($R^2$=0.970) | $BOD_5$ ($R^2$=0.939) |
| COD total ($R^2$=0.976) | COD total ($R^2$=0.958) |
| SST ($R^2$=0.967) | SST ($R^2$=0.967) |
| SSV ($R^2$=0.964) | SSV ($R^2$=0.965) |
| Organic N ($R^2$=0.844) | Organic N ($R^2$=0.698) |
| Ammonia ($R^2$=0.826) | Ammonia ($R^2$=0.827) |
| P total ($R^2$=0.915) | P total ($R^2$=0.894) |
| Chlorophyll *a* ($R^2$=0.971) | Phycocyanin ($R^2$=0.971) |
| Chloride ($R^2$=0.724) | *P. aeruginosa* ($R^2$=0.794) |
| pH ($R^2$=0.758) | |

Phycocyanin and chlorophyll *a* presented a high correlation between them ($R^2$=0.971), as demonstrate in Chapter 2 and by other authors (AHN et al., 2002, ZAMYADI et al., 2012). These two variables were used as indicators of phytoplankton (chlorophyll *a*) (GREGOR et al., 2005) and of cyanobacteria (phycocyanin) (LEE et al., 1995), and they presented correlation with parameters that reflect organic matter ($BOD_5$, COD and suspended solids) and nutrients (P and N).

The variance explained by the CCA analysis was 83.5% (from which 52.6% were explained by the first two axes) (Figure 4.7, Table 4.6). Axis 1 was mainly defined by Aguieira Reservoir sample and *taxa Leptolyngbya* sp., *Fragilaria* sp. and Nostocales on the positive side and Vela Lake sample, short spiral cyanobacteria, centric diatoms and *Gloeocapsa* sp. on the negative side, with $Cl^-$ (-0.95), DO (-0.90) and DIN:SRP (0.71) levels as the main factors behind this distribution. The second axis was mainly related to DIN:SRP ratios (-0.68) and water temperature (0.39), with sample Montargil Reservoir and *Cylindrospermospis raciborskii* defining the positive side of the axis, and Aguieira Reservoir sample and *taxa Leptolyngbya* sp., *Fragilaria* sp. and Nostocales in the negative side of this same axis. The fact that DIN:SRP was a main modulator parameter is not surprising since the requirements for nutrients vary according to different species (HAVLIK et al., 2013).

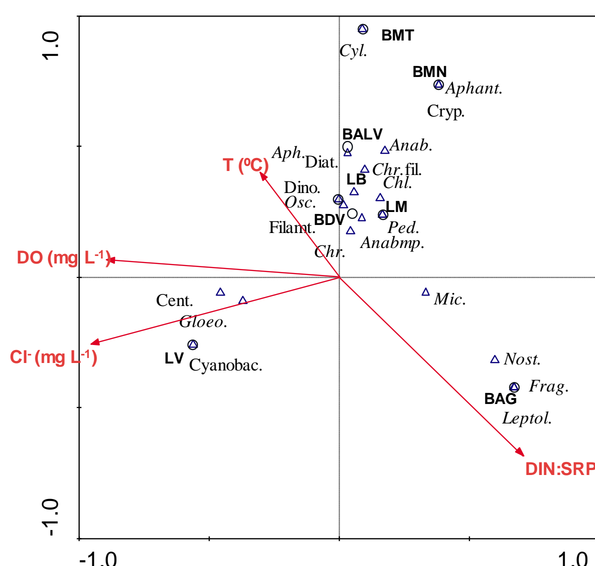



**Figure 4.7** Results from canonical correspondence analysis. Triplot for sampling sites, environmental variables and phytoplankton species. Codes correspond to LV (Vela Lake); LM (Mira Lake); LB (Barrinha de Mira Lake); BAG (Aguieira Reservoir); BALV (Alvito Reservoir); BDV (Odivelas Reservoir); BMN (Monte Novo Reservoir); BMT (Montargil Reservoir). *Aphanot.* (*Aphanothece* sp.); Chr. (Chroococcales); Chr. fil. (filamentous Chroococcales); Filamt. (filamentous cyanobacterial); *Mic* (*Microcystis* sp.); *Leptol*. (*Leptolyngbya* sp.); *Gloeo*. (*Gloeocapsa* sp.); Cyanobac. (cyanobacterial short spiral); *Nost.* (Nostocales); *Anab.* (*Anabaena* sp.); *Aph.* (*Aphanizomenon* sp.); Cent. (centric diatoms), Frag. (*Fragilaria* sp.); Diat. (Pennales diatoms); Cryp. (Cryptophyceae), Dino (Dinoflagellates); Osc. (Oscillatoriales sp.); *Ped.* (*Pediastrum* sp.); *Anabmp*. (*Anabaemopsis* sp.); *Cyl*. (*C. raciborskii*); and Chl. (Chlorophyceae).

**Table 4.6** Summary of canonical correspondence analysis between phytoplankton and environmental variables.

| | Axis 1 | Axis 2 | Axis 3 | Axis 4 | Total inertia |
|---|---|---|---|---|---|
| **Eigenvalues** | 0.847 | 0.760 | 0.571 | 0.375 | 3.058 |
| **Species-environmental correlations** | 0.999 | 0.996 | 0.871 | 0.950 | |
| **Cumulative percentage variance** | | | | | |
| **Of species data** | 27.7 | 52.6 | 71.2 | 83.5 | |
| **Of species-environment relation** | 33.2 | 63.0 | 85.3 | 100.0 | |
| **Sum of all eigenvalues** | 3.058 | | | | |
| **Sum of all canonical eigenvalues** | 2.553 | | | | |
| **Variance explained by the CCA** | 83.5 | | | | |
| **Variance explained by the first two axes** | 52.6 | | | | |

In the past, the ratio of total nitrogen to total phosphorous was thought to be a key parameter in the growth of cyanobacteria compared with other phytoplankton. However, more recent studies have refuted this factor and it is no longer considered a controlling factor (OLIVER et al., 2000). Cyanobacteria are known to develop blooms under low N:P ratios, since some species can store surplus phosphate in polyphosphate bodies and nitrogen in cyanophycin granules and phycobiliproteins (SRIVASTAVA et al., 2013). As cyanobacteria are better able to compete for

nitrogen than other phytoplankton when nitrogen is scarce, with a surplus supply of phosphorus, nitrogen becomes relatively scarce and these organisms are favoured (SMITH, 1983). Therefore, this group appears more from early spring to autumn when the nutrients levels are low and this group has advantage in relation to other organisms such as diatoms and chlorophytes (DE FIGUEIREDO et al., 2006). With low availability of ammonia or nitrate, nitrogen-fixing cyanobacteria (diazotrophic species) are enhanced, since their peak biomass is closely dependent on phosphorus loading into the system and not to nitrogen (SMITH et al., 1999). Under low N:P, these species are favoured. Therefore, in water bodies where nutrient concentrations are naturally low or have been decreased by remedial actions, cyanobacterial populations may still develop, with diazotrophyc species being favoured (SMITH et al., 1999). With global warning and nutrients input, it is expected that diazotrophic filamentous cyanobacteria, in particular, will be enhanced (MARKENSTEN et al., 2010). Some of these species, particularly, *Cylindrospermopsis raciborskii, Aphanizomenon* (*Cuspidothrix) issatschenkoi* and *Aphanizomenon aphanizomenoides* have already been considered invasive in Europe (WIEDNER et al., 2007). However, these N-fixing filamentous cyanobacteria do not have much success under phosphorus limitation or unavailability (DE FIGUEIREDO et al., 2011).

N:P ratios do not only influence bloom development, but also play a role in allelopathy and toxin production (GRANÉLI et al., 2008). In the present study, nitrogen concentrations were higher than phosphorus, however, phosphorus concentrations, despite of low were high enough to allow the development of cyanobacterial blooms, as stated before. Higher levels of nitrogen in relation to phosphorus are normal, since over the years, nitrogen fixation from the atmosphere and return from sediments allowed the total mass of nitrogen in the lake to increase relative to phosphorus (SMITH et al., 1999). *Microcystis* spp. is a species that could form blooms under low concentrations of phosphorus, due to its ability to store phosphorus (DOKULIL et al., 2000). Blooms of this species were recorded in Vela Lake and in Aguieira Reservoir (see Chapter 3, Figures 3.5 and 3.6), where relatively low concentration of nitrogen (0.0237 mg N $L^{-1}$ of ammonia and 36.66 mg N $L^{-1}$ of nitrite+nitrates, for Vela Lake; 0.0120 mg N $L^{-1}$ of ammonia and 117.5 mg N $L^{-1}$ of nitrite+nitrates, for Aguieira Reservoir) and phosphorus (0.0137 mg P $L^{-1}$ and 0.0099 mg P $L^{-1}$ of SRP, respectively) were recorded. *Aphanizomenon* requires low amounts of nitrogen (OLIVER et al., 2000) but is phosphorus dependent (DOKULIL et al., 2000), and appeared with higher densities at Alvito Reservoir (see Chapter 3, Figure 3.6), with (0.0020 mg N $L^{-1}$ of ammonia and 0.13 mg N $L^{-1}$ of nitrite+nitrates; 0.0167 mg P $L^{-1}$ of phosphorus), Odivelas Reservoir (see Chapter 3, Figure 3.6), with (0.0095 mg N $L^{-1}$ of ammonia and 0.17 mg N $L^{-1}$ of nitrite+nitrates; 0.0200 mg P $L^{-1}$ of phosphorus) and in smaller densities in Monte Novo Reservoir (see Chapter 3, Figure 3.6), (no ammonia levels was detected, but 0.28 mg N $L^{-1}$ of nitrite+nitrates was registered; 0.0633 mg P $L^{-1}$); *Cylindrospermopsis* also require small amounts of nitrogen, since is a diazotrophyc species, but it have also the capacity to scavange and store phosphorus (BURFORD et al., 2006). This species was found in Montargil Reservoir (see Chapter 3, Figure 3.6), where low levels of ammonia (0.0085 mg N $L^{-1}$), nitrite+nitrates (0.27 mg N $L^{-1}$) and phosphorus

(0.300 mg P $L^{-1}$) were registered. Although these diazotrophic species could develop in environments with low nitrogen levels, they prefer to uptake DIN rather than consuming energy for nitrogen-fixation. Co-occurrence of diazotrophic and non-diazotrophic species can occur, typically with the trio *Anabaena, Aphanizomenon* and *Microcystis* (PEARL, 2008), this trio appears in Odivelas Reservoir (see Chapter 3, Figure 3.6), which had higher levels of nitrogen than phosphorus. At Mira Lake, higher densities of Chlorophyceae (*Pediastrum* sp.) were recorded, instead of cyanobacteria (see Chapter 3, Figure 3.7).

Although several empirical models based on lake survey data have shown that total phosphorus and total nitrogen concentrations are the best predictors of cyanobacterial dominance and biomass, much of the variance associated with these nutrient models remains unexplained. This happens because cyanobacteria are dynamic organisms and other variables influence their development (TARANU et al., 2012), such as high water temperature, low light intensity, high pH and low $CO_2$ (SRIVASTAVA et al., 2013). However, a combination of three primary environmental factors has high influence in the development of a bloom: temperatures above 25ºC; intensity and duration of light exposure and the trophic status (MEREL et al., 2013). Therefore, global changes and increased temperature could enhance bloom development, namely of harmful genera (PAERL et al., 2011). Beside this factor, cyanobacteria are also able to regulate buoyancy, which provides them with a competitive advantage in stratified water column, compared with other phytoplankton groups. However, literature suggests that the importance of climate-related variables *versus* nutrients is ecotype specific (TARANU et al., 2012).

Chloride also showed to have major importance for the samples and phytoplankton distribution. However, there is a lack on studies relating chloride to phycocyanin levels, and therefore, with cyanobacterial dominance (CCA from Figure 4.7). On the other hand, water temperature and DO showed to have a strong influence in phytoplankton species distributions, and several studies have already described the strong relationship of high temperatures (WIEDNER et al., 2007) and high DO levels (NCDENR) during blooms' development.

Toxin analysis revel the presence of MC in Vela Lake and Aguieira Reservoir (> 12.3 µg $L^{-1}$ and 1.4 µg $L^{-1}$, respectively), where strong *Microcystis* sp. blooms was found (see Chapter 3, Table 3.3). At these places, toxic *Microcystis* spp. blooms have already been recorded (VASCONCELOS et al., 1993, VASCONCELOS et al., 2011), meaning there is persistence in the occurrence of these toxic blooms. This is of major importance for cyanobacterial blooms prediction and to the creation of the management plans for these water bodies.

## 4.4 Conclusions

Management strategies require a previous knowledge about the environmental variables that most affect the communities and the interactions between them. These kinds of studies are of increasing importance, since global warming is expected to potentiate cyanobacterial blooms

development. Multivariate analysis represents a good technique to test associations between these variables. Several studies determined the variables that most affect phytoplankton; for cyanobacteria, in particular, temperature, light intensity (intensity and duration) and nutrient levels are the main ones. Environmental parameters for trophic status assessment revealed that all sites could be considered eutrophic; being Vela Lake the most eutrophic sample, with high levels for nutrients, chlorophyll *a*, phycocyanin and organic material. Moreover, at Vela Lake and Aguieira Reservoir, toxic *Microcystis* spp. blooms were recorded meaning there is a persistent formation of toxic blooms – this can have major importance for cyanobacterial blooms prediction and management for these water bodies. Alentejo reservoirs showed a high similarity in environmental parameters, possibly due to common features in reservoirs of this region. From multivariate analyses, the most influent variables in phytoplankton distribution were temperature, $Cl^-$, DO and DIN:SRP ratios. This highlights the importance to control and manage nutrient inputs for lentic water bodies, particularly under the rising global temperature.

## 4.5 References


AHN, C. Y.; OH, H. M. - Rainfall, phycocyanin and N:P ratios related to cyanobacterial blooms in a Korean large reservoir. Hydrobiologia. Vol. 474 (1-3) (2002), p. 117-124.

AHN, C.Y. [et al.] - Alternative Alert System for Cyanobacterial Bloom, Using Phycocyanin as a Level Determinant. The Journal of Microbiology. Vol. 45(2) (2007), p. 98-104.

ANTUNES, S. C.; ABRANTES, N.; GONÇALVES, F. - Seasonal variation of the abiotic parameters and the cladoceran assemblage of Lake Vela: Comparison with previous studies. Annales de Limnologie. Vol. 39, n.º 3 (2003), p. 255-264.

APHA - Standard Methods for the Examination of Water and Wastewater. 20th. Washington, DC: American Public Health Association, 1999.

BELLINGER, G.E.; SIGEE, D.C. - Freshwater Algae: Identification and Use as Bioindicators. 2º. Spain, 2010. ISBN 978-0-470-05814-5.

BURFORD, M. A.; MCNEALE, K. L.; MCKENZIE-SMITH, F. J. - The role of nitrogen in promoting the toxic cyanophyte *Cylindrospermopsis raciborskii* in a subtropical water reservoir. Freshwater Biology. Vol. 51, n.º 11 (2006), p. 2143-2153.

CAETANO, S. [et al.] - Cyanobacteria blooms and cyanotoxin occurrence in the Guadiana (SE Portugal) - preliminary results. Ecotoxicology and Environmental Restoration. Vol. 4 (2001), p. 53-59.

CHORUS, I.; BARTRAM, J. - Toxic Cyanobacteria in Water: A guide to their public health consequences, monitoring and management London: E & FN Spon, 1999. ISBN 0-419-23930-8.

CKSB - Special environmental impact assessment (SEIA) for the proposed Sino-Malaysia JV forest plantation at Kalabakan and Gunung Rara Forest Reserves, Tawau Distric, Sabah July 2001.

COALITION, GLOBAL WATER RESEARCH; AUSTRALIA, WATER QUALITY RESEARCH - Guidance manual for managemement of toxic cyanobacteria. Gayle Newcombe, 2009. ISBN 978–90–77622–21–6

CODD, G.A.; METCALF, J.S.; BEATTIE, K.A. - Retention of *Microcystis aeruginosa* and microcystins by salad lettuce (*Lactuca sativa*) after spray irrigation with water containing cyanobacteria. Toxicon. Vol. 37, n.º 8 (1999), p. 1181–1185.

CODD, GEOFFREY A. - Cyanobacterial toxins, the perception of water quality, and the prioritisation of eutrophication control. Ecological Engineering. Vol. 16, n.º 1 (2000), p. 51-60.

CRONBERG, G.; ANNADOTTER, H. - Manual on aquatic cyanobacteria. A photo guide and a synopsis of their toxicology. Denmark: International Society for the Study of Harmful Algae United Nations Educational, Scientific and Cultural Organisation, 2006. ISBN 87-990827-0-5.

CROUZET, P. [et al.] - Nutrients in European ecossystems. Environmental assessment report no.4. Copenhagen: European Environment Agency, 1999.

DANET, A. - Pollution Database [em linha]. [Consult. Disponível WWW:<URL:http://pollution.unibuc.ro/?substance=48>.

DE FIGUEIREDO, D. R. [et al.] - Microcystin-producing blooms--a serious global public health issue. Ecotoxicology and Environmental Safety. Vol. 59, n.º 2 (2004), p. 151-63.

DE FIGUEIREDO, D. R. [et al.] - Differential inter- and intra-specific responses of Aphanizomenon strains to nutrient limitation and algal growth inhibition. Journal of Plankton Research. Vol. 33, n.º 10 (2011), p. 1606-1616.

DE FIGUEIREDO, D. R. [et al.] - The effect of environmental parameters and cyanobacterial blooms on phytoplankton dynamics of a Portuguese temperate lake. Hydrobiologia. Vol. 568, n.º 1 (2006), p. 145-157.

DIAZ, R.J. ; SOLOW, A. - Ecological and Economic Consequences of Hypoxia. Topic 2 Report for the Integrated Assessment of Hypoxia in the Gulf of Mex-ico. NOAA Coastal Ocean Program Deci sion Analysis Series No. 16. (1999).

DOKULIL, M. ; TEUBNER, K. - Cyanobacterial dominance in lakes. Hydrobiologia. Vol. 438 (2000), p. 1-12.

FAO - Chemical Features of Water [em linha]. Nigeria: N.M. Kutty. [Consult. Disponível em WWW:<URL:http://www.fao.org/docrep/field/003/ac183e/AC183E00.htm#TOC>.

FOSS- Application Note 5201 - Determination of the sum of nitrate and nitrite in water by FIAstar 5000.

GALVÃO, H. M. [et al.] - Cyanobacterial blooms in natural waters in southern Portugal: A water management perspective. Aquatic Microbial Ecology. Vol. 53, n.º 1 (2008), p. 129-140.

GAO, Y. [et al.] - Effects of cyanobacterial-driven pH increases on sediment nutrient fluxes and coupled nitrification-denitrification in a shallow fresh water estuary. Biogeosciences. Vol. 9, n.º 7 (2012), p. 2697-2710.

GONÇALVES, F. [et al.] - Anthropogenic influences on seasonal chages of nutrients, physical and chemical factors in three coastal freshwaters shallow lakes (Portugal). Limnetica. Vol. 12(2) (1996), p. 47-52.

GRANÉLI, E.; WEBERG, M.; SALOMON, P. S. - Harmful algal blooms of allelopathic microalgal species: The role of eutrophication. Harmful Algae. Vol. 8, n.º 1 (2008), p. 94-102.

GREGOR, JAKUB; MARŠÁLEK, BLAHOSLAV - A Simple In Vivo Fluorescence Method for the Selective Detection and Quantification of Freshwater Cyanobacteria and Eukaryotic Algae. Acta hydrochimica et hydrobiologica. Vol. 33, n.º 2 (2005), p. 142-148.

HAMMER, Ø. ; HARPER, D.A.T.; RYAN, P.D. - PAST: Paleontological Statistics Software Package for Education and Data Analysis Palaeontologia Electronica Vol. 4, n.º 1 (2001), p. 4-9.

HAVLIK, I. [et al.] - On-line monitoring of large cultivations of microalgae and cyanobacteria. Trends in Biotechnology Vol. 31, n.º 7 (2013), p. 406-414.

INAG, I.P. - Manual para a avaliação da qualidade biológica da água em lagos e albufeiras segundo a Directiva Quadro da Água. Protocolo de amostragem e análise para o Fitoplâncton. Ministerio do Ambiente, do Ordenamento do Território e do Desenvolvimento Regional. Instituto da Água, I.P., 2009.

LEE, T.Y. [et al.] - Quantitative determination of cyanobacteria in mixed phytoplankton assemblages by an *in vivo* fluorimetric method. Analytica Chimica Acta. Vol. 302 (1995), p. 81-87

LORENZEN, C.J. - Determination of chlorophyll and pheo-pigments: spectrophotometric equations. Limnology and Oceanography. Vol. 12 (1967), p. 343-346.

MARKENSTEN, H.; MOORE, K.; PEARSON, I. - Simulated lake phytoplankton composition shifts toward cyanobacteria dominance in future warmer climate. In: de Figueiredo, D. R., A. M. M. Goncalves, et al. (2011). "Differential inter-and intra-specific responses of *Aphanizomenon* strains to nutrient limitation and algal growth inhibition." Journal of Plankton Research 33(10): 1606-1616. (2010).

MEREL, S. [et al.] - State of knowledge and concerns on cyanobacterial blooms and cyanotoxins. Environment International. Vol. 59 (2013), p. 303-27.

MUR, L. R.; SKULBERG, R.; UTKILEN, H. - Cyanobacteria in the environment. In: Coalition, G. W. R. and W. Q. R. Australia (2009). Guidance manual for managemement of toxic cyanobacteria, Gayle Newcombe. (1999).

NAGPAL, N.K.; LEVY, D.A.; MACDONALD, D.D. - Ambient Water Quality Guidelines for Chloride - Overview Report. Canada: Ministery of Environment, 1981.

NCDENR - Algal Blooms [em linha]. Division of water resources - water quality programs. [Consult. Disponível em WWW:<URL:http://www.ncdenr.gov/web/wq/ess/eco/blooms>.

NHMRC - Guidelines for managing Risks in Recreational Water. . Australian Government, National Health and Medical Research Council 2008.

NISBET, M.; VERNEAUX, J. - Composantes chimiques des eaux courantes. Discussion et proposition de classes en tant que bases d'interprétation des analyses chimiques. Annales de Limnologie. ISSN 0003-4088. Vol. 6, n.º 2 (1970), p. 161-190.

OLIVER, R.L.; GANF, G.G. - Freshwater Blooms. Chapter 6. In: Coalition, G. W. R. and W. Q. R. Australia (2009). Guidance manual for managemement of toxic cyanobacteria, Gayle Newcombe. (2000).

PAERL, H. W.; HALL, N. S.; CALANDRINO, E. S. - Controlling harmful cyanobacterial blooms in a world experiencing anthropogenic and climatic-induced change. Science of the Total Environment. Vol. 409, n.º 10 (2011), p. 1739-45.

PAERL, H. W.; HUISMAN, J. - Climate change: a catalyst for global expansion of harmful cyanobacterial blooms. Environmental Microbiology Reports. Vol. 1, n.º 1 (2009), p. 27-37.

PEARL, H. - Nutrient and other environmental controls of harmful cyanobacterial blooms along the freshwater–marine continuum In: HUDNELL, H. K. - Cyanobacterial Harmfull Algal Blooms: state of the science and research needs. Springer, (2008), p. 217-237.

PEREIRA, L.A.M.; RODRIGUES, M.A.R. - Avaliação do estado trófico das águas nas albufeiras de Lisboa e Vale do Tejo Lisboa: Comissão de Coordenação e Desenvolvimento Regional de Lisboa e Vale do Tejo Direção de Serviços de Monitorização Ambiental Divisão de Monitorização Ambiental-Divisão de Laboratórios, 2005.

SAKER, M. L. [et al.] - Molecular techniques for the early warning of toxic cyanobacteria blooms in freshwater lakes and rivers. Applied Microbiology and Biotechnology. Vol. 75, n.º 2 (2007), p. 441-449.

SINGH, R.K. [et al.] - Microbial evaluation of water bodies from Jaunpur, UP, India. Pollution Research. n.º 2 (2010), p. 365-370.

SMITH, V.H. - Low nitrogen to phosphorus ratios favor dominance by blue-green algae in lake phytoplankton. In: Havens, K. E., R. T. James, et al. (2003). "N:P ratios, light limitation, and

cyanobacterial dominance in a subtropical lake impacted by non-point source nutrient pollution." Environmental Pollution 122. (1983).

SMITH, V.H.; TILMAN, G.D.; NEKOLA, J.C. - Eutrophication: impacts of excess nutrient on freshwater, marine and terrestrial ecosystems. Environmental Pollution. Vol. 100 (1999), p. 179-196.

SNRIH - Sistema Nacional de Informação de Recursos Hídricos [em linha]. [Consult. Disponível em WWW:<URL:http://snirh.pt/>.

SRIVASTAVA, A. [et al.] - Monitoring approaches for a toxic cyanobacterial bloom. Environ Sci Technol. Vol. 47, n.º 16 (2013), p. 8999-9013.

TARANU, ZOFIA E. [et al.] - Predicting cyanobacterial dynamics in the face of global change: the importance of scale and environmental context. Global Change Biology. Vol. 18, n.º 12 (2012), p. 3477-3490.

TECATOR- Application Note 86/87 - Determination of Kjeldahl Nitrogen content with Kjeltec System 1026.

TER BRAAK, CAJO J. F. - Canonical correspondence analysis: a new eigenvector technique for multivariate direct gradient analysis. Ecology. Vol. 67, n.º 5 (1986), p. 1167-1179.

VASCONCELOS, V. M. - Cyanobacterial toxins in Portugal: Effects on aquatic animals and risk for human health. Brazilian Journal of Medical and Biological Research. Vol. 32, n.º 3 (1999), p. 249-254.

VASCONCELOS, V. M. [et al.] - Toxicidade de estirpes de cianobactérias isoladas a partir das lagoas das Braça, Vela e Mira. Boletim UCA, Universidade do Algarve, UCTRA Vol. 1 (1993), p. 193-201.

VASCONCELOS, V.; MORAIS, J.; VALE, M. - Microcystins and cyanobacteria trends in a 14 year monitoring of a temperate eutrophic reservoir (Aguieira, Portugal). Journal of Environmental Monitoring. Vol. 13, n.º 3 (2011), p. 668-72.

WHO - Algae and cyanobacteria in fresh water. In: Guidelines for safe recreational water environments. Geneva, Switzerland: World Health Organization, 2003. p. 136-158.

WIEDNER, C. [et al.] - Climate change affects timing and size of populations of an invasive cyanobacterium in temperate regions. Oecologia. Vol. 152, n.º 3 (2007), p. 473-84.

WILLIAMS, D.E. [et al.] - Bioaccumulation and clearance of micro-cystinsfrom salt water mussels, *Mytilus edulis*, and *in vivo* evidence for covalently bound microcystins in mussel tissues. Toxicon. Vol. 35, n.º 11 (1997), p. 1617–1625.

ZAMYADI, A. [et al.] - Monitoring of potentially toxic cyanobacteria using an online multi-probe in drinking water sources. Journal of Environmental Monitoring. Vol. 14, n.º 2 (2012), p. 579-88.

ZAR, J.H. - Biostatistical analysis. 3º edition. 1996.

# Chapter V

General conclusions

Cyanobacterial blooms are becoming a global issue and countries are showing more and more concern about this problem. At the moment, several monitoring programs are in place. However, measures are only taken when blooms are already established, with potential high risks for the health of the water users. In Portugal, like in many countries, Cyanobacterial Monitoring Programme is based mainly on chlorophyll *a* concentration and cell counts. However, for cyanobacterial assessment, these parameters present some limitations. Therefore, the main goals of this work were to develop a stepwise strategy for a faster, easier and more cost-effective monitoring of cyanobacterial blooms in Portuguese lentic freshwater bodies, particularly those used for recreational purposes, where massive blooms occur. For this strategy, the use of fluorometry for determining phycocyanin (a cyanobacterial specific pigment) as the first monitoring step showed effective results with immediate exclusion of samples for further and potentially costly procedures. However, the establishment of phycocyanin thresholds is very controversial and this work proves it, by demonstrating the high inter-specific variation on the phycocyanin content between the tested cyanobacteria (*C. raciborkii* showed the highest phycocyanin content and *M. aeruginosa* the lowest). Therefore, different thresholds, according to the dominant bloom forming cyanobacteria, should be considered. This also highlights the necessity of using several complementary approaches to identify the dominant species (to choose the proper threshold) and to assess the toxicity. Nevertheless, due to the facility and fastness of fluorometry, on the cases where the phycocyanin levels recorded rise up to the defined thresholds, immediate actions can be taken, in order to initiate a chain of responses to deal with the cyanobacterial blooms' development.

Therefore, before determining the dominant cyanobacterial species, the minimum phycocyanin threshold value should be considered as a reference value, above which further analysis should be performed. In our study, this value would be 0.8 µg $L^{-1}$ determined for *M. aeruginosa*, which is one of the most common species found in freshwater lakes and reservoirs, with several reports of toxicity all over the world, including in Portugal.

The fact that algal presence did not show interference in the phycocyanin signal and the mixtures of two cyanobacteria showed high correlation coefficients between the phycocyanin measured and the phycocyanin expected, suggests that this may be a reliable technique for using in environmental samples for assessing cyanobacterial concentration. However, before the implementation of PC thresholds in monitoring programmes, further laboratory assays should be perform to quantify the interference of other compounds and to assess differences in pigment content for more species. An intercalibration study should be performed from north to southern Portugal, using isolated toxic bloom-forming species to determine several PC thresholds. Validation of these data in environmental samples and standardization of fluorometric techniques are essential, in order to create robust thresholds, which could be safely implemented in cyanobacterial monitoring programs.

However, *per se*, phycocyanin determination is limited for assessing cyanobacterial risk, when cyanobacteria are present above the thresholds. Therefore, a stepwise monitoring approach was developed, incorporating several techniques (phycocyanin assessment through fluorometry, chlorophyll *a* determination, cyanobacterial identification and cell counts, toxic potential evaluation and cyanotoxins analysis). However, in the present economic context, cyanobacterial blooms are not considered a priority, and therefore, management strategies need to be efficient in assessing risk assessment, but also need to allocate resources (human and financial). This justifies the creation of a stepwise strategy that allows the exclusion of samples which do not represent a risk for the public health, for further analysis. This approach proved to improve the effectiveness of the risk assessment and reduction of sites within alert levels, allowing a substantial saving of resources in intervention measures. And this saving of resources compensates the investment in extra equipment and analyses proposed by this strategy. Therefore, this approach is a valuable tool and a faster, easier and a long-term more cost-effective alternative to be used in management and monitoring strategies for recreational waters with records of cyanobacterial blooms. Moreover, if the effort would be distributed by several stakeholders such as universities, environmental agencies, government and local authorities, more the costs would be dissipated, and more involved and alert these important actors would be for this problematic and global issue.

Not only reactive measures should be taken. Instead, proactive measures could help to deal and avoid the development of cyanobacterial blooms. The implementation of these measures requires a previous knowledge of the environmental and biological associations that are behind and promote the development of these blooms. Multivariate analysis represents a good technique to test these associations, to understand how environmental parameters influence species distributions, in order to develop management strategies. In this study, all sites have been considered eutrophic, being Vela Lake the most eutrophic sample, with high levels for nutrients, chlorophyll *a*, phycocyanin and organic material. Multivariate analyses indicated that the most influent variables in phytoplankton distribution were temperature, chloride, dissolved oxygen and DIN:SRP ratios, which highlights the importance to control and manage nutrient inputs for lentic water bodies, particularly under the rising global temperature.

More studies in the field of fluorometry and phycocyanin determination in several bloom-forming species should be conducted, and one of the major challenges would be to improve fluorometry techniques to minimize environmental interference. Also, the use of continuous real-time *in situ* monitoring or though remote satellite and airborne sensors should be improved, in order to create an organized network for continuous real-time phycocyanin determination in lakes and reservoirs, allowing a more accurate monitoring of this phenomenon.

# Annex

## List of publications from the present work

*Conference proceedings*

MACÁRIO, I., NUNES, M.I., GONÇALVES, F., CASTRO, B.B., DE FIGUEIREDO, D.R. Proposta de uma metodologia de monitorização faseada para deteção de blooms de cianobactérias. *10ª Conferência Nacional do Ambiente (10CNA). November, 6 to 8, Aveiro, Portugal* (2013)

*Oral communications*

MACÁRIO, I., NUNES,M.I., GONÇALVES, F., CASTRO, B.B., DE FIGUEIREDO, D.R. Proposta de uma metodologia de monitorização faseada para deteção de blooms de cianobactérias. *10ª Conferência Nacional do Ambiente (10CNA). November, 6 to 8, Aveiro, Portugal* (2013)

*Poster communications*

MACÁRIO, I., NUNES, M.I., FONSECA, D., GONÇALVES, F., CASTRO B.B., DE FIGUEIREDO, D.R. *Pedagógicas de Educação Ambiental - Aprender fora de portas: redes, recursos e potencialidades. January, 17 to 19, Leiria, Portugal.* (2013)

FONSECA, D., CASTRO, B.B., MACÁRIO, I., GONÇAVES, F., DE FIGUEIREDO, D.R. Quando as Cianobactérias crescem demais... *XX Jornadas Pedagógicas de Educação Ambiental - Aprender fora de portas: redes, recursos e potencialidades. January, 17 to 19, Leiria, Portugal.* (2013)

DE FIGUEIREDO, D.R., MACÁRIO, I., NUNES, M.I., CORREIA, A., CASTRO, B.B., GONÇALVES, F. A multilevel approach to monitor and help manage the development of cyanobacterial blooms in lakes and reservoirs. *TWAM2013: Transboundary water management across borders and interfaces: present and future challenges. March 20-26, Aveiro, Portugal.* (2013)