Universidade do Algarve

Toxicity Risks Linked to Cyanobacteria Blooms in Southern Portugal Reservoirs

Sandra Maria da Cruz Caetano

Tese para obtenção do Grau de Doutor em Ciências da Terra do Mar e do Ambiente
Ramo: Ciências e Tecnologias do Ambiente
Especialidade: Ambiente e Saúde

Trabalho efectuado sob a Orientação de
Maria Margarida Prazeres dos Reis

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Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

Sandra M. C. Caetano

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Resumo

A proliferação massiva de cianobactérias em ecossistemas de água doce representa actualmente a nível global mais do que um problema ambiental. Na verdade, tornou-se numa relevante preocupação no domínio da saúde pública. O principal objetivo deste trabalho foi fazer uma avaliação dos riscos de toxicidade ligados à proliferação de cianobactérias nas albufeiras do sul de Portugal. Pretendeu-se também alertar para a necessidade de ampliar e fortalecer a ligação entre o ambiente e a saúde humana, no contexto da monitorização da qualidade ecológica da água. Este trabalho constituiu ainda uma contribuição para a compreensão do papel da dinâmica do fitoplâncton no desenvolvimento de florescências de cianobactérias (CyanoHABs – "*Cyanobacteria Hazard Algal Blooms*"). Todas as albufeiras estudadas apresentaram dominância de cianobactérias, em termos de abundância, durante grande parte do ano, exibindo florescências, especialmente durante a estação seca. A presença constante de espécies toxigénicas constitui uma ameaça à saúde pública e muitas vezes condiciona a utilização da água. O sul de Portugal, dadas as suas condições climáticas e regime hidrológico deve ser considerado uma região de alta prioridade para o estudo do perigo associado a CyanoHABs. Além disso, numa perspectiva de futuras alterações climáticas, com eventual agravamento do regime hidrológico característico do sul será expectável um aumento da ocorrência de CyanoHABs. Há ainda a considerar a expansão biogeográfica de espécies exóticas toxigénicas de cianobactérias que já começaram a invadir estas albufeiras, nomeadamente *Cylindrospermopsis raciborskii*, com potenciais riscos para a saúde pública ainda mal conhecidos, uma vez que as toxinas produzidas por estas espécies não são regularmente analisadas, nem tidas em consideração pelos programas de monitorização nacionais. O presente trabalho reporta também a primeira notificação de *C. raciborskii* na albufeira de Alqueva, a maior da União Europeia, em abundâncias semelhantes às já detetadas por outros autores em outras albufeiras do sul de Portugal. Estes episódios confirmam que as espécies toxigénicas invasivas foram bem sucedidas nas nossas águas. Além disso, mesmo em albufeiras oligotróficas, como é o caso da albufeira do Beliche, as cianobactérias prevalecem, indiciando que os fatores "bottom-up" não são sempre tão importantes quanto assumido, devendo os fatores "top-down" ser alvo de investigação mais aprofundada. O desenvolvimento de índices ecológicos baseados na composição, abundância e biomassa de fitoplâncton como forma de medir a saúde dos ecossistemas, em especial das massas de água fortemente modificadas, como as albufeiras, deveria permitir inferir indiretamente sobre o potencial risco para a saúde pública. No entanto, das treze métricas ou índices multi-métricos abordados neste trabalho no contexto de cinco albufeiras do Algarve, só os que incluem informação sobre o biovolume de cianobactérias poderão ser indicativos de riscos ecotoxicológicos e de potencial toxicidade para humanos. Dada a simplicidade de obtenção e a qualidade da informação fornecida, os índices propostos, nomeadamente o índice de dominância de cianobactérias potencialmente tóxicas (PTCD) ou o índice de risco de biotoxicidade (BRI) poderão vir a constituir uma opção, logo que sejam aferidos através da aplicação a mais sistemas. Este trabalho constituiu portanto, uma contribuição para (i) a compreensão do papel das cianobactérias na dinâmica fitoplanctónica, (ii) o reconhecimento dos perigos associados à sua presença representa e (iii) a criação de uma base para a futura análise de potenciais impactos de alterações climáticas.

Palavras-chave: CyanoHABs, dinâmica fitoplânctonica, *Cylindrospermopsis raciborskii*, risco toxicológico, Beliche, Alqueva, albufeiras oligotróficas

Abstract

Cyanobacteria massive proliferation in freshwater ecosystems has become a relevant public health concern. The main objective of this study was to assess the toxicity risks linked to the proliferation of cyanobacteria in southern Portugal reservoirs, while alerting to the need to widen and strengthen the connection between environment and human health. This work also contributes to highlight the role of phytoplankton dynamics in the development of Cyanobacteria Hazard Algal Blooms (CyanoHABs). All addressed reservoirs revealed dominance of cyanobacteria in terms of abundance, exhibiting blooms, especially during the dry season. Following a global trend, these reservoirs exhibited CyanoHABs increase, with constant presence of toxigenic species which constitutes a threat to public health and often limits water use. From the perspective of future climate change, with expected worsening of the torrential hydrologic regime, increase in CyanoHABs occurrence is expected. Biogeographical expansion of exotic toxigenic cyanobacteria has begun in these reservoirs, also presenting public health risks, since their toxins are not regularly screened. This study includes the first notification of *Cylindrospermopsis raciborskii* in Alqueva reservoir, in similar abundances to those already detected by other authors in Southern Portugal reservoirs. Confirming invasive toxigenic species success in these waters. Even in oligotrophic reservoirs (Beliche), cyanobacteria prevail, indicating bottom-up factors are not always as important as assumed, whereby top-down factors must be further investigated. The development of ecological indices based on phytoplankton data should allow inderect inference about potential public health risks. Given its simplicity, the dominance index of potentially toxic cyanobacteria (PTCD) may constitute an option, when calibrated through its application to more systems. This work contributes to: (i) the understanding of cyanobacteria dynamics within phytoplankton, (ii) the acknowledgement of the hazards linked to their presence and (iii) constitutes a baseline for future studies on climate change impacts in Southern Portugal reservoirs.

Keywords: CyanoHABs, phytoplankton dynamics, *Cylindrospermopsis raciborskii*, toxicity risks, Beliche, Alqueva, oligotrophic reservoirs.

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CHAPTER I

INTRODUCTION

INTRODUCTION

Global aim

The main objective of this work was to assess toxicity risks linked to Cyanobacteria blooms in Southern Portugal reservoirs, while alerting and drawing attention to the necessity to expand and strengthen the connection between environment and human health, in the context of ecological water quality monitoring.

In spite of constituting a valuable tool to protect water quality and probably human health, ecological studies in aquatic environments are often overlooked and misunderstood by water managers. The European Directive 2000/60/EC (Water Framework Directive - WFD) while endorsing the use of ecological water quality as a criterion in aquatic ecosystem classification, addresses the importance of obtaining and preserving a classification of good for aquatic ecological status. However, the WFD does not clearly and unequivocally bridge ecological quality to public health.

Therefore this work aims to answer to an emerging need for studies researching and validating the links between aquatic ecology and public health.

1. Environment and human health links

Human populations have always been conditioned by the environment. Extreme weather events, such as droughts and floods, highlight it particularly well. Dependency of man's welfare on environment quality is intuitive and empirically recognized. The United Nations Conference on Environment and Development in 1992, in which the Agenda 21 was adopted, formalized the existence of a close relationship between human health and environment (Corvalán *et al.*, 1999; OECD, 2008). Among the raised issues, emerged questions such as: In what extent will the pollution of air and water shorten ours and our children lives? How does the environment affect human health? (OECD, 2008). According to the Organization for Economic Co-operation and Development (OECD) statistics (OECD, 2008), air and water pollution kill every year thousands of people, mostly children. Most of this pollution has directly or indirectly an anthropic origin.

Considering the rising requests of freshwater by human populations, the quality of water used for multiple purposes, is at present a growing concern, for water resource management authorities, which are struggling with the development of appropriate methodologies for freshwater sources protection.

The effects of environmental degradation are often overlooked as in the case of increased density, pathogenicity and/or toxicity of environmental microbial agents. The World Health Organization (WHO) states that from 1972 to 1999, thirty-five new pathogens have been discovered and many more re-emerged or expanded into areas where they had never been detected before (WHO, 2003). In this group, there are many waterborne biological agents (WHO, 2003). There is also a growing awareness of the possible effects of global change. Expected impacts on the hydrological regimes with increased frequency of extreme droughts alternating with violent floods shell revert into biological changes, which should be foreseen.

While assigning most water contaminations to poor, ineffective or absent effluent treatment or even to inadequate drinking water treatment methods (WHO, 2003), WHO recognizes the impossibility to detect all cases of contamination.

Aiming to protect populations from deterioration of potable water sources, water treatment systems are technological barriers designed to eliminate specific types of pollution. But increasing knowledge about the functioning of aquatic ecosystems and their microbial communities, calls for caution in the use of some commonly applied technologies and for their supplementation or adaptation to the detected specificities of the considered environment.

Water pollution can have a natural origin, such as algae growth in consequence of natural seasonal succession or dissolution of geological matrix components, or be anthropic in result of contamination by diffuse and point sources such as urban runoff or agricultural soils leaching.

The biological component of the ecosystem is structured into trophic levels, which operate on a network. This network or web of trophic relationships is organized in such way, that, what happens at one level will have impact on neighboring trophic levels and eventually on the most remote ones too. The better we apprehend the intricate chain of energy transfer, the faster, adequate and effective will be our response to emerging unbalances and the better will our models be. Human and animal populations health safeguarding actions, should be planed and taken, considering such models

2. Phytoplankton diversity

The word phytoplankton derives from two Greek words "phyton" meaning plant and "Planktos" which means errant wanderer or drifter (Langlois & Smith, 2001). Under this designation are gathered the main primary producers of some aquatic systems (Kyewalyanga, 2002). Broadly phytoplankton is the photosynthetic living mass suspended in the water column, drifting with the currents. Some of the species that are passively transported by currents are able, albeit with limitation, to control their movement. This living drifting mass is composed primarily of microalgae, a functional group including oxygenic and anoxygenic photosynthetic bacteria, autotrophic plastidic protists and mixotrophic fagotrophic protists, ranking in size from 0.2 to 4000 µm. The variety in sizes is linked to a huge taxonomic diversity, which is highlighted in **Figure I-1,** indicating on the "Tree of Life" (Delsuc et al., 2005) all the groups bearing mainly photosynthetic microorganisms (dark grey arrows) and the groups bearing only some photosynthetic species (light grey arrows).

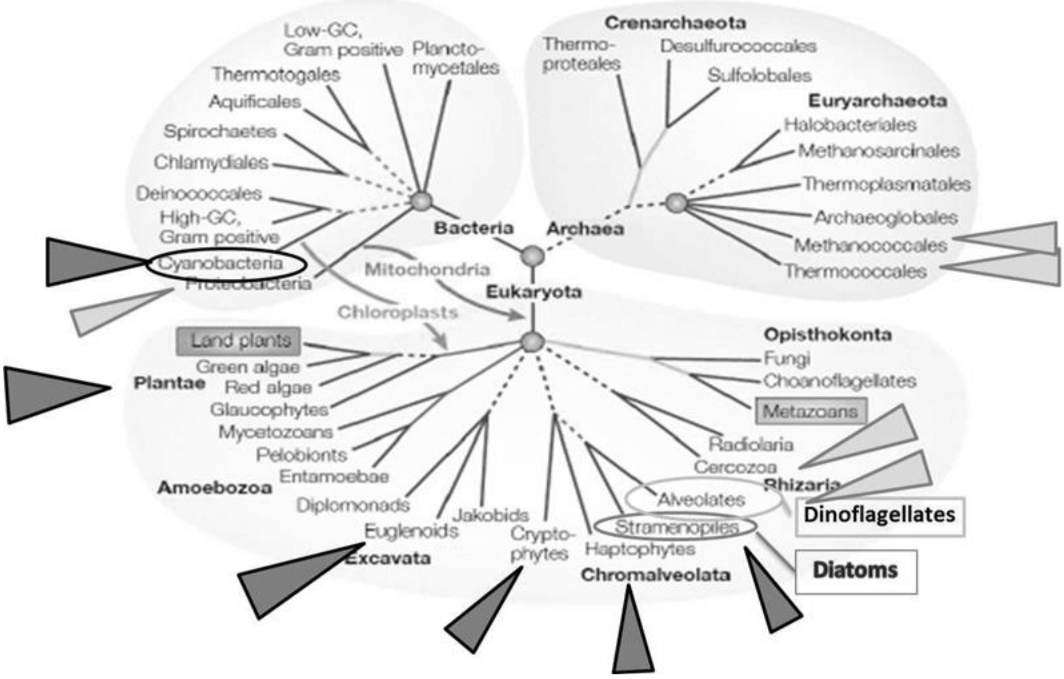
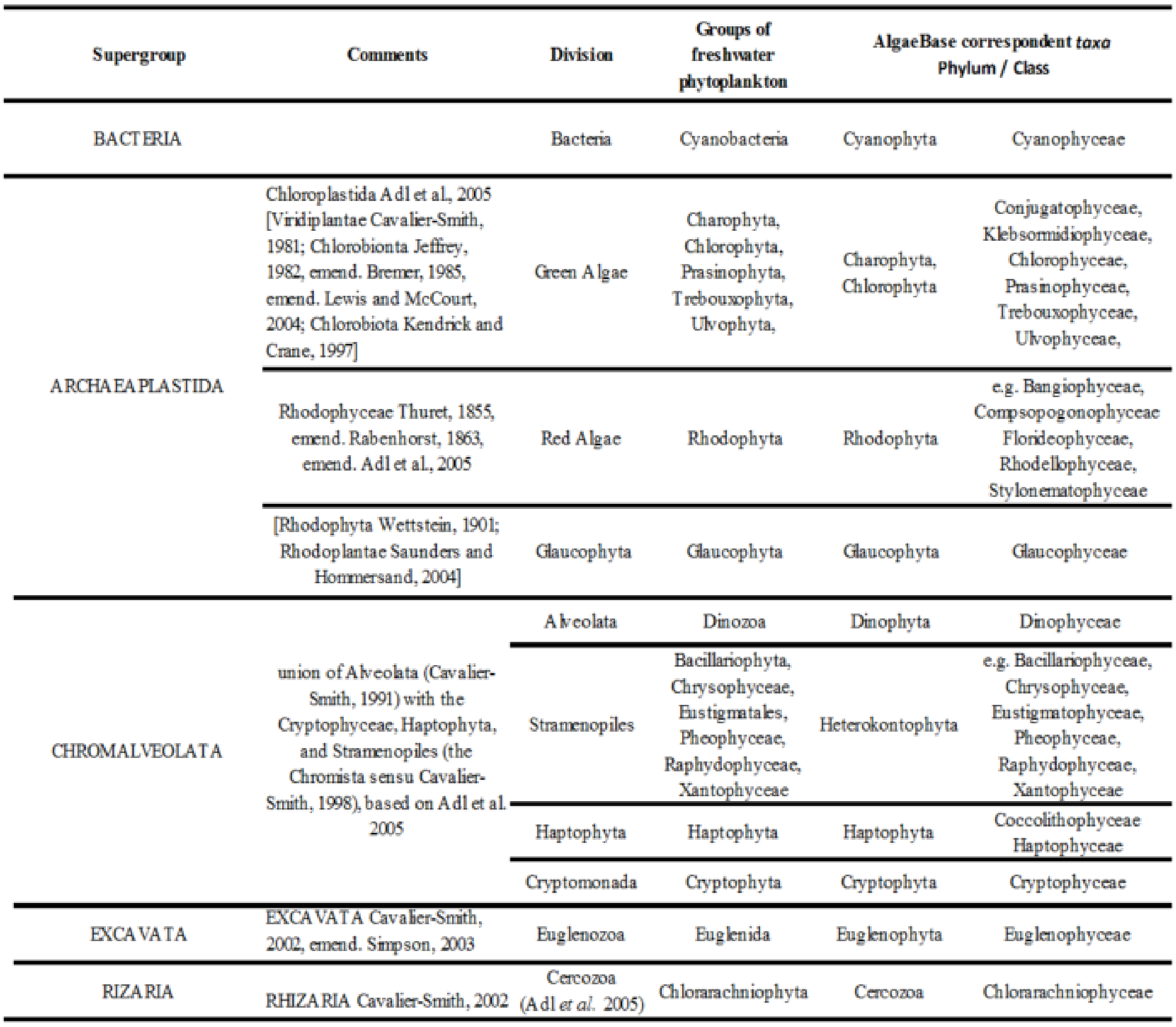


Figure I-1 – "Tree of Life" (Modified from Delsuc *et al.*, 2005) indicating main taxonomic groups bearing passively transported photoautotrophic aquatic (micro)organisms: dark grey arrows indicate groups of mainly photosynthetic microorganisms and light grey arrows the groups bearing some photosynthetic microbial species.

Most handbooks on morphological identification of freshwater phytoplankton still use, as classes and/or as *phyla,* the following groups: Cyanophyceae, Chlorophyceae, Prasinophyceae, Rhodophyceae, Glaucophyceae, Pyrrophyceae, Bacillariophyceae, Chrysophyceae, Cryptophyceae, Eustigmatophyceae, Haptophyceae, Phaeophyceae, Raphydophyceae, Xantophyceae and Euglenophyceae. Guiry, M.D. & Guiry, G.M. (2014), in their worldwide electronic database on algae (Algaebase) assemble these taxa in four kingdoms, namely Eubacteria, Plantae, Chromista and Protozoa. Comparing this nomenclature with the Taxonomy of Protists proposed by Adl *et al.* (2005), **Table I-I** indicates their relative taxonomic position and inclusion in higher ranks, in order to facilitate a much needed correspondence between different taxonomic approaches.

Table I-I - Comparison of main taxonomic groups bearing phytoplankton organisms based on Adl *et al.* (2005) and Guiry, M.D. & Guiry, G.M. (2014)



Supergroup	Comments	Division	Groups of freshwater phytoplankton	AlgaeBase correspondent *taxa*	
				Phylum	Class
BACTERIA		Bacteria	Cyanobacteria	Cyanophyta	Cyanophyceae
ARCHAEAPLASTIDA	Chloroplastida Adl et al., 2005 [Viridiplantae Cavalier-Smith, 1981; Chlorobionta Jeffrey, 1982, emend. Bremer, 1985, emend. Lewis and McCourt, 2004; Chlorobiota Kendrick and Crane, 1997]	Green Algae	Charophyta, Chlorophyta, Prasinophyta, Trebouxophyta, Ulvophyta,	Charophyta, Chlorophyta	Conjugatophyceae, Klebsormidiophyceae, Chlorophyceae, Prasinophyceae, Trebouxophyceae, Ulvophyceae,
	Rhodophyceae Thuret, 1855, emend. Rabenhorst, 1863, emend. Adl et al., 2005	Red Algae	Rhodophyta	Rhodophyta	e.g. Bangiophyceae, Compsopogonophyceae Florideophyceae, Rhodellophyceae, Stylonematophyceae
	[Rhodophyta Wettstein, 1901; Rhodoplantae Saunders and Hommersand, 2004]	Glaucophyta	Glaucophyta	Glaucophyta	Glaucophyceae
CHROMALVEOLATA	union of Alveolata (Cavalier-Smith, 1991) with the Cryptophyceae, Haptophyta, and Stramenopiles (the Chromista sensu Cavalier-Smith, 1998), based on Adl et al. 2005	Alveolata	Dinozoa	Dinophyta	Dinophyceae
		Stramenopiles	Bacillariophyta, Chrysophyceae, Eustigmatales, Pheophyceae, Raphydophyceae, Xantophyceae	Heterokontophyta	e.g. Bacillariophyceae, Chrysophyceae, Eustigmatophyceae, Pheophyceae, Raphydophyceae, Xantophyceae
		Haptophyta	Haptophyta	Haptophyta	Coccolithophyceae Haptophyceae
		Cryptomonada	Cryptophyta	Cryptophyta	Cryptophyceae
EXCAVATA	EXCAVATA Cavalier-Smith, 2002, emend. Simpson, 2003	Euglenozoa	Euglenida	Euglenophyta	Euglenophyceae
RIZARIA	RHIZARIA Cavalier-Smith, 2002	Cercozoa (Adl *et al.* 2005)	Chlorarachniophyta	Cercozoa	Chlorarachniophyceae

The exact number of phytoplankton species is uncertain, there have been mentioned numbers ranging from 30,000 to 1 million and the number 350 million has already emerged, about 20 times more than the total of all other known species in the planet (Guiry, 2012). On

AlgaeBase database 140,106 species and intraspecific algae names are listed (Guiry, M.D. & Guiry, G.M. 2015).

In some types of aquatic ecosystems, phytoplankton is the base of the food chain, *i.e.* the basic structural level, from whose balance everything else depends. Such is the case of ocean waters (Kyewalyanga, 2002), but even in lakes and coastal systems where there are other primary producers (*e.g.* macrophytes) phytoplankton plays an important role in nutrition dynamics and mineralization mechanisms, together with heterotrophic microorganisms.

Microalgae growth obeys to natural life cycles, regulated by the expression of their genetic potential. The regularity of these cycles has its own cadence, determined by adaptations of each species to the environmental conditions of each site. Intensive proliferation periods are part of the life cycles of many microalgae species. In these periods density increases exponentially, making those species dominant over others for a short period of time. These events are called blooms and tend to natural senescence, ending more or less quickly. The consequences for the ecosystem are in general reversible at a short-medium term. However, disturbances may occur in these spontaneous bloom cycles in response to environmental *stimuli*, which may be natural (*e.g.* abnormal weather phenomena) or anthropic (*e.g.* discharges of pollutants). These disturbances can result in alterations of frequency, intensity and duration of blooms, interfere with water quality and lead to increasing episodes of eutrophication.

3. How can phytoplankton blooms affect water quality?

Like the above mentioned blooming process, eutrophication can also occur naturally as part of the normal ecosystem succession (Zheng & Paul, 2007), or be promoted and/or enhanced by human activities. In this case the natural process is amplified, sometimes being referred as artificial eutrophication. Actually the word eutrophication derives from the Greek word "eutrophia" which means adequate and healthy nutrition or development. In an ecology context, the word eutrophication has been applied to aquatic systems, describing the process of enrichment in inorganic nutrients, particularly nitrogen and phosphorus, whenever this *stimulus* results in the massive development of plants and algae (Zheng & Paul, 2007), producing the mentioned artificial eutrophication.

In extreme cases eutrophication evolves into a state of anoxia due to excessive consumption of oxygen by the decomposition of organic matter, during the senescence of phytoplankton blooms. Oxygen depletion has serious consequences for aquatic life, killing oxygen breathing organisms. It can also have repercussions in terms of public health, since in anoxic environments the release of toxic gases and noxious odors may occur, as well as the production of hydrogen sulfide and the development of potentially pathogenic microorganisms (Reynolds & Walsby, 1975). This type of blooms is conventionally termed as Harmful Algal Blooms (HABs), even in the absence of special biotoxins.

Studies conducted in various environments prove the dependence between the concentration of nutrients in solution and the density and structure of phytoplankton populations (*e.g.* Duarte *et al.*, 2000; Örnólfsdóttir *et al.*, 2004). A regular input of nutrients over time will result in a biogeochemical balance, if they were consumed and exported in the same concentrations. According to Morel (1987), the dynamic between the processes of nutrient assimilation (uptake) and phytoplankton production (growth), commands the optimal nutritional average concentration for a specific organism in a specific environment and that depends on frequency and range of nutritional changes occurring in a particular environment. Thus in order to support healthy phytoplankton abundance, sustaining the natural life cycle of the species present, nutrient concentrations and their relative proportion must be kept within certain limits. Values for these limits vary for each aquatic ecosystem, according to its ecoregion. American and European environmental agencies aim to establish such limits for the water bodies of the several ecoregions.

Nutrient consumption is believed to depend on the stoichiometric balance between the concentrations of nitrogen and phosphorus, as mentioned by several authors for several aquatic and terrestrial environments, since in 1934, the oceanographers Redfield and Richards defined for organic matter production the average atomic ratio of C to N to P in marine phytoplankton to be 106:16:1 (Redfield-Richards Ratio), based on the molar proportions of the Photobiosyntheses process (Libes, 1992). Some authors even developed models that explain and simplify this relationship for different levels of aquatic microbial trophic web, that include phytoplankton (Mousseau *et al.*, 2001). According to Borchardt (1996) if the N: P ratio exceeds 20:1, it is considered that the phytoplankton growth is limited by phosphorus, and if it is less than 10:1, the system would be limited by nitrogen. This nutrient limitation affects the ecological phytoplankton succession, contributing to changes in phytoplankton composition over time in a regular cyclic way. Elser *et al.* (2007) provided a detailed review about the limitation of N and P to which primary producers are subject in several environments (freshwater, marine and terrestrial), concluding that the enrichment in N or P increases autotrophic production, but the simultaneous increase of both nutrients leads to uncontrolled primary production in ecosystems without other limitations. Excessive production may be so high, as to promote sensory changes in the water, such as color changes, smell, taste (Klapper, 1991) and sometimes even its viscosity. This excessive primary production is as stated above eutrophication.

In natural systems or where the human impact is smaller, nutritional balance, although fluctuating periodically according to seasonal changes, may remain healthy for several centuries. In this case periodic blooms and some sporadic light eutrophication events are integrated into the normal succession of the system being considered as part of the natural cycle. However, when the nutrients input rises, severe imbalances could occur and eventually evolve to extreme eutrophication situations.

In addition, eutrophication also promotes the loss of biodiversity in water bodies at all trophic levels (Codd, 2000), since during blooms few species are selected, sometimes only one or two, which temporarily dominate the ecosystem. In eutrophic waters those species are often cyanobacteria species. These constitute an additional concern because of their capacity to produce toxins (Codd, 1995; Codd *et al.*, 1989, 1997; Carmichael, 1992, 1997; Sivonen, 1996; Chorus and Bartram, 1999; Codd, 2000). HABs formed by cyanobacteria may be called CyanoHabs.

In freshwater reservoirs increased nutrient input can be of two types:

1 – Nutrient accumulation - due to agricultural or industrial activities in the drainage basin, as a result of runoff and soils leaching in consequence of excessive rainfall;

2 – Nutrient concentration - due to increased water evaporation in a frame of higher atmospheric temperatures and natural internal nutrient recycling.

In either case, the result is the same, *i.e.* the stimulation of phytoplankton growth, with the onset of blooms. These, whether natural or artificially stimulated by eutrophication, can by themselves become a serious problem, moreover in presence of potential production of toxic metabolites.

Most groups of microalgae include genera that can be considered as potential developers of HABs, and the group of cyanobacteria is by far the most problematic in freshwaters (Paerl *et al*. 2001). Of the 150 known genera of cyanobacteria, 40 have the ability to produce toxins (Carmichael, 2001) and are likely to form CyanoHABs, both in natural ecosystems and artificial reservoirs used as drinking water sources.

If the problem is limited only to a local increase in organic mass, it can be controlled with physical barriers and methods of physical restraint and removal of blooms. Such methods have been implemented in some situations, however, without great success (Granquist *et al.*, 2010). If the increase in organic matter is found in large parts of the water body or is due to a

CyanoHAB, it becomes more difficult to take any remedial or mitigation action. The difficulty is even greater if the affected waters are a source for drinking water supply, thus requiring specific water treatment measures (Teixeira and Rosa, 2006). In addition also some types of biological control, such as the introduction and manipulation of predators (from zooplankton to fish), lytic bacteria, cyanophages (virus), and even antibiotics have already been proposed, but these control methods are still experimental and have not yet demonstrated to be widely effective (Paerl, 2008; Paerl and Otten, 2013; Jacquet *et al.*, 2013; Wilken *et al.* 2014).

4. Explaining cyanobacteria success

Cyanobacteria are photosynthetic prokaryotic organisms, therefore considered functionally as microalgae, though taxonomically as Bacteria. In fact they are Gram-negative bacteria able to synthesize chlorophyll-*a*, along with light harvesting phycobiliproteins, like phycoerythrin, allophycocyanin and phycocyanin. High concentrations of the latter can in certain situations bluish these microorganisms (Whitton & Potts, 2000). Cyanobacteria are ancient, cosmopolitan and extremely resilient beings, present in almost all environments on the planet whether aquatic or terrestrial (Whitton, 1992; Whitton & Potts, 2000). These microorganisms play key roles in biogeochemical cycles and in maintaining and structuring microbial biodiversity as well as higher organism's communities (Codd *et al.,* 2005).

There is extensive literature on CyanoHABs and most studies mention that blooms result from complex interactions of high nutrient concentrations, warm temperatures, light availability, organic matter concentrations and slow-flowing or stagnant waters. Conductivity and salinity as well as pH were also considered as controlling variables. Generally, it is believed that the formation of cyanobacteria blooms is regulated by a combination of three primary environmental factors; namely (i) water temperature, (ii) light exposure and (iii) trophic dynamics of the aquatic system (Merel *et al.*, 2013):

(i) Water temperature – Most of cyanobacteria prefer warmer waters (25 °C or above). Consequently, global warming should increase the frequency and magnitude of CyanoHABs by favoring cyanobacteria over other phytoplankton species (Paul, 2008; El-Shehawy *et al.*, 2012; O'Neil *et al.*, 2012; Paerl and Paul, 2012). Also, cyanobacteria compete most effectively with eukaryotic primary producers, such as diatoms, chlorophytes, cryptophytes and dinoflagellates at elevated temperatures (Elliott *et al.*, 2006; De Senerpont Domis *et al.*, 2007;

Jöhnk *et al.*, 2008), meaning that cyanobacterial growth rates attain higher values as the growth rates of eukaryotic taxa decay. This is confirmed by the worldwide cyanobacteria blooms dependence upon temperature (Robarts and Zohary, 1987; Kanoshina *et al.*, 2003), and the spread of invasive species from the subtropics like *Cylindrospermopsis* sp. to North and South (Padisák, 1997; Wiedner *et al.*, 2007; Sukenik *et al.*, 2012). Additionally climatic oscillations due to global warming should affect patterns, intensities and duration of precipitation and droughts periods, promoting CyanoHAB dominance. Longer and intense precipitation events with higher runoff rates should contribute to nutrient leaching, increasing nutrient levels of receiving waters as well as adsorption to sediments, (Paerl and Fulton III, 2006; King *et al.*, 2007). Pearl and Paul (2012) stressed that freshwater discharges due to water excess, may at short-term prevent blooms by flushing them. However, as the water release diminishes, water residence time increases and loaded nutrients end up captured and cycled inside the water bodies, eventually promoting bloom occurrence. This will most likely occur if winter-spring precipitation increases and flushing events are followed by extended periods of drought (Pearl and Paul, 2012), as have occurred in Southern Portugal and predicted by Santos *et al.* eds. (2002) for climate changes scenarios.

(ii) Light exposure - Although several species of cyanobacteria can act as hetero- or chemotrophic, most species need a minimum of light availability for photosynthesis. Quality, intensity, and duration of light needed are species-specific (Merel *et al.*, 2013). Usually, accessory pigments of cyanobacteria protects the cell from high light intensities, preventing photo-inhibition and also allowing light absorption across a broader region of the visible spectrum (Mur *et al.*, 1999). So it seems that cyanobacteria are more efficient in overcoming deficiencies in light intensity or quality, when compared with other phytoplankton, which makes duration of light exposure an important growth parameter (Merel *et al.*, 2013).

iii) Trophic dynamics of the aquatic system - cyanobacterial blooms mainly occur in eutrophic reservoirs (Heisler *et al.*, 2008; El-Shehawy *et al.*, 2012) with N/P ratio ranging from10 to 15 (Mur *et al.*, 1999). However, Downing *et al.* (2001) found for 99 reservoirs a better correlation between concentration of total N (TN) and total P (TP) and the occurrence of cyanobacteria blooms, rather than with N/P ratio. It also should be stressed that different cyanobacteria species respond to nutrient loads in different ways and there is no simple relationship (Kaebernick and Neilan, 2001; Vézie *et al.*, 2002; Vuorio *et al.*, 2005). Reis *et al.* (2005), while studying CyanoHAB triggering conditions in Funcho reservoir (Southern Portugal), submitted a complex matrix of time series data with variables, as radiation, wind, air and water temperature, rain, conductivity, dissolved oxygen, pH, nutrients, photopigments and phytoplankton and zooplankton abundance and composition, to several multivariate analysis techniques (cluster analysis, distance based redundancy analysis, dynamic factor analysis and chronological clustering). Results indicated that (i) biological variables were indispensable to discriminate between bloom and non-bloom situations; (ii) dry season phosphorus limitation discriminated between type of seasonal dominant cyanobacteria; (iii) summer Chroococcales dominance was associated with elevated temperature; but (iv) almost 40% of the detected variance was not explainable by the measured variables, calling for research on microbial impact on phytoplankton, be it by viral infection, predation by microzooplankton, or competition with bacterioplankton. In other words, as stressed by Galvão *et al.* (2008) cyanobacteria success seems to be driven both by bottom up and top-down factors.

4.1. Bottom-up driving factors

Compared to other algae, cyanobacteria have higher temperature optima (Paerl and Huisman, 2008), lower light requirements (Reynolds *et al.*, 1981) and possess carbon concentration mechanisms (Daley *et al.*, 2012) providing them a competitive advantage over eukaryotic algae especially at high pH. Basically at low pH more CO_2 is available in adequate supplies to all other algae outcompeting cyanobacteria (Paerl and Ustach, 1982), while these can flourish at low TN:TP ratios (Schindler, 1977). Some cyanobacteria (e.g. *Anabaena*, *Aphanizomenon*) can fix atmospheric nitrogen to match the phosphorus uptake when the supply in dissolved inorganic nitrogen is depleted. Non-nitrogen fixing cyanobacteria (e.g. *Microcystis*, *Lyngbya*) either have low nitrogen requirements or are able to migrate, within the water column using gas-vesicles to access benthic nitrogen sources (Smith, 1983; Ibelings *et al* 1991; Ferber *et al.*, 2004) surpassing, this way, the lack of a proper nitrogen fixation system, and also controlling sinking loss and enabling the rise to water surface where light and carbon dioxide are available. Dense algal blooms exhibit a strong demand for CO_2, in order to support high rates of photosynthesis needed to maintain the intense biomass production rate (Paerl and Ustach, 1982). This demand for CO_2 will crop the availability of free dissolved CO_2, therefore buoyancy regulation will constitute a huge advantage over subsurface phytoplankton populations, as surface-dwelling organisms can capture CO_2 directly from the atmosphere, minimizing carbon limitation of photosynthetic growth and taking advantage of rising atmospheric CO_2 levels (Paerl and Ustach, 1982). Also, Cyanobacteria able to migrate from the sediment to the water column can store internal phosphorus reserves as polyphosphates, which favor them when external phosphorus is low (Pettersson *et al.*, 1993). Non-nitrogen fixing cyanobacteria are favored by ammonium-nitrogen, while eukaryotic phytoplankton develops when nitrate-nitrogen is the main nitrogen source present. Nitrogen-fixing cyanobacteria are favored when nitrogen is depleted (Blomquist *et al.*, 1994; Glibert *et al.*, 2011). High dissolved inorganic nitrogen (e.g. urea) favors cyanobacteria growth over other algal groups (Glibert *et al.*, 2004). Presence of some trace metals also stimulates cyanobacteria growth, especially nitrogen-fixers which have higher requirements for these compounds, since nitrogen-fixation requires iron as a co-factor (Reuter and Pettersen, 1987).

Furthermore many cyanobacteria overwinter in the sediments and are re-suspended to the water column in spring, seeding the surface when conditions become favorable (Rengefors *et al.*, 2004). The increase of the anoxic zone during stratification promotes cyanobacteria recruitment (Trimbee and Prepas, 1988). Many lakes in the temperate zones present a trend to stratify earlier in spring, stratification is maintained throughout summer, and destratification is postponed to later fall (De Stasio *et al.*, 1996; Peeters *et al.*, 2007).

4.2. Top-down driving factors

Cyanobacteria losses through zooplankton grazing has been considered to be minimal due to large size of colonies or long trichomes of cyanobacteria, low content of essential nutrients (e.g. lipids) and toxicity (Lampert, 1987). Toxigenic cyanobacteria affect natural grazers and other aquatic biota, once they secrete organic compounds which suppress the growth of other algae (Murphy *et al.*, 1976; Keating, 1978; Suikkanen *et al.* 2004). Furthermore, if planktivorous fish are present in the ecosystem, they may remove large zooplankton reducing grazing pressure upon cyanobacteria (Fott *et al.*, 1980). Yet, bivalve mollusk (e.g. Corbula) grazing may provide competitive advantage to colonial and filamentous cyanobacteria (Glibert *et al.*, 2011). Increased concentrations in chemical products, such as pesticides and metals, due to runoff and other sources, may also disrupt the balance between cyanobacteria and their predators, and thus contribute to increase incidence of CyanoHABs, or lead to the expansion of more resilient strains of cyanobacteria through natural selection (Hudnell *et al.*, 2008). There are, also, recent works reporting lytic cyanophage activity controlling *Microcystis* spp. populations (Paerl, 2008; Yoshida *et al*., 2008; Mesquita *et. al.*, 2009; Li, *et al.*, 2013).

5. Cyanobacteria hazards

5.1. Ecological impacts

Heavy cyanobacteria blooms affect aquatic ecosystems by: (i) decreasing water transparency; (ii) promoting oxygen levels fluctuations; (iii) releasing toxins; and (iv) changing pH.

- (i) Decreased water transparency, which can drop till 1-2 cm, disturbs the whole ecology of the ecosystem by preventing light from reaching higher water depths (Vasconcelos, 2006). Increased turbidity suppresses the establishment and growth of aquatic macrophytes promoting changes in the underwater habitat for many invertebrates and fish species, affecting negatively their populations (Scheffer, 2007). Furthermore, sight is essential to many aquatic animals either to locate food, find partners to mate or simply to move (Vasconcelos, 2006).
- (ii) Heavy fluctuation of oxygen levels, since dense cyanobacterial blooms and the associated biomass will produce high amounts of oxygen during daytime leading to over saturation (Vasconcelos, 2006). However, high nocturnal respiration of all aquatic organisms (including cyanobacteria) may diminish dissolved oxygen concentration, to the point of causing the death of the more sensitive species such as some fish (Vasconcelos, 2006; Pearl and Huisman, 2009), especially in areas close to the bottom or the thermocline (Vasconcelos, 2006)
- (iii) Release of bioactive compounds with toxic properties by many cyanobacteria strains can cause death not only to aquatic organisms that come in direct contact with them, but also to livestock, domestic animals (such as canines see Backer *et al.,* 2013), aquatic birds, other wild animals (Codd *et al.,* 2005) and in some cases to humans (Carmichael, 2001; Caetano *et al*., 2013). Furthermore, the production of geosmin and other fetid chemicals during CyanoHABs are noxious to human and animals (Izaguirre and Taylor, 2004; Uwins *et al.*, 2007; Graham *et al.* 2010).
- (iv) Shifts in pH, with high values during daytime (>9 pH) and low levels at night may also stress the environment and cause biogeochemical changes that can increase the ecological effects stressed before.

5.2. Toxin production triggers

Cyanotoxins are generally accepted to be “secondary metabolites” (Carmichael, 1992a). Secondary metabolites production is subjected to general physiological control that responds to environmental factors (Holland and Kinnear, 2013). In the past, secondary metabolites such as alkaloids were regarded as metabolic waste, which the cell was unable to discard and therefore accumulated. Nowadays this is no longer generally accepted, and it is believed that such compounds have a beneficial role, such as control upon predation (*e.g.*, through poor palatability) (Vining, 1990). Some authors suggested a link between ecological disequilibria and microbial secondary metabolite production, which would have evolved during periods where these metabolites represented a competitive advantage (Lillehoj, 1982). It is conceivable that cyanobacteria secondary metabolites could have had multiple functions. In fact, a wide range of physiological roles have been speculated for cyanotoxins: cell signaling; nutrient uptake; iron scavenging; maintenance of homeostasis; and protection against oxidative stress (Utkilen and Gjolme, 1995; Pomati *et al*., 2004; Neilan *et al.,* 2012), thus blurring the labelling of cyanotoxins as “secondary metabolites”, since they would simultaneously offer functions in both primary and secondary metabolism. (Holland and Kinnear, 2013).

However, cyanobacteria blooms are not necessarily associated with the occurrence of toxins. Some toxigenic strains seem to be unable to synthesize the expected toxins (Sarazin *et al.*, 2002; Vasconcelos, 2006). The onset of genetic expression of the genes involved in cyanotoxin production is still not clearly understood.

Several leading laboratories around the world undertook efforts investigating what triggers toxin production. For instance, genes used in microcystin synthesis were identified and their operon intensively studied (Kaebernick and Neilan, 2001). In fact, this toxin is the most studied group of cyanotoxins, comprising at least 89 characterized analogs, isolated from several different cyanobacteria species (Fujiki *et al*., 2002; Welker and Van Döhren, 2006). Nevertheless, the way this operon is promoted is still unclear. Microcystin is the toxin produced by the highest number of cyanobacteria genera (see **Table II-I**), but the species most commonly associated with its production is *M. aeruginosa* (Chorus and Bartram, 1999). The synthesis of the microcystin peptides is non-ribosomal. Their production depends on a large enzymatic complex that incorporates a variety of different amino acids in the microcystin molecule, and thus produces variants with different carbon-nutrient stoichiometry and toxicity, all synthesized by the same enzymatic machinery, whose synthesis is regulated by the mentioned operon (Kaebernick and Neilan, 2001).

Other toxin-producing genes have been progressively identified in several strains (Tillett *et al*., 2000; Moffitt and Neilan, 2004; Kellmann *et al.,* 2006), allowing the development of PCR methods for the specific detection of potentially toxigenic cyanobacteria in environmental samples. Identification of strains bearing the appropriate genes, still remains the best way to know, whether a bloom may become toxic.

Considering that cyanotoxins are only produced by strains having the appropriate genes (Kurmayer and Christiansen, 2009), but that the presence of these genes does not automatically determine their production, only ongoing metagenomic and transcriptomic studies, done in the framework of adequate experimental design, will be able to elucidate triggers of cyanotoxin production.

Some environmental factors suspected to have a role in toxin production are radiation, water movement and flow, allelopathic influences and competition for resources, herbivory and grazing, nutrient concentrations and ratios, water temperature and salinity, cell division and growth rate (Orr and Jones, 1998; Tonk *et al*., 2005; Sevilla *et al*., 2008; Kosol *et al*., 2009). For instance, cylindrospermopsin production is highly affected by light intensity (Holland and Kinnear, 2013).

The multiplicity of environmental factors interacting with toxin production may be huge (Merel *et. al.*, 2013). While daunting, much more experimental work needs to be done regarding environmental factors promoting toxin production at the individual strain level.

6. CyanoHABs and cyanotoxin exposure

The potential effect of cyanobacteria on human health is an emerging issue in public health and has attracted a growing interest in the scientific community (Grivas, 2002). At least 40 of the known 150 Cyanobacteria *genera* are considered toxigenic (Skulberg *et al.*, 1993). These 40 *genera* include about 2000 species.

The production of cyanotoxins able to promote human pathologies has been scientifically proven. A lot of information has been produced on the subject, including books and review articles. The first major review on risks associated to cyanobacteria, was organized in 1999 by Chorus & Bertram for WHO. The book discusses in detail the risks of cyanotoxins contaminated water consumption (Chorus & Bartram, 1999). In 2005 OECD (2005) included cyanobacteria in the list of emerging pathogens, although these do not invade or colonize host organisms.

The main routes of human and animal exposure to cyanobacteria and cyanotoxins are outlined in **Figure I-2**.

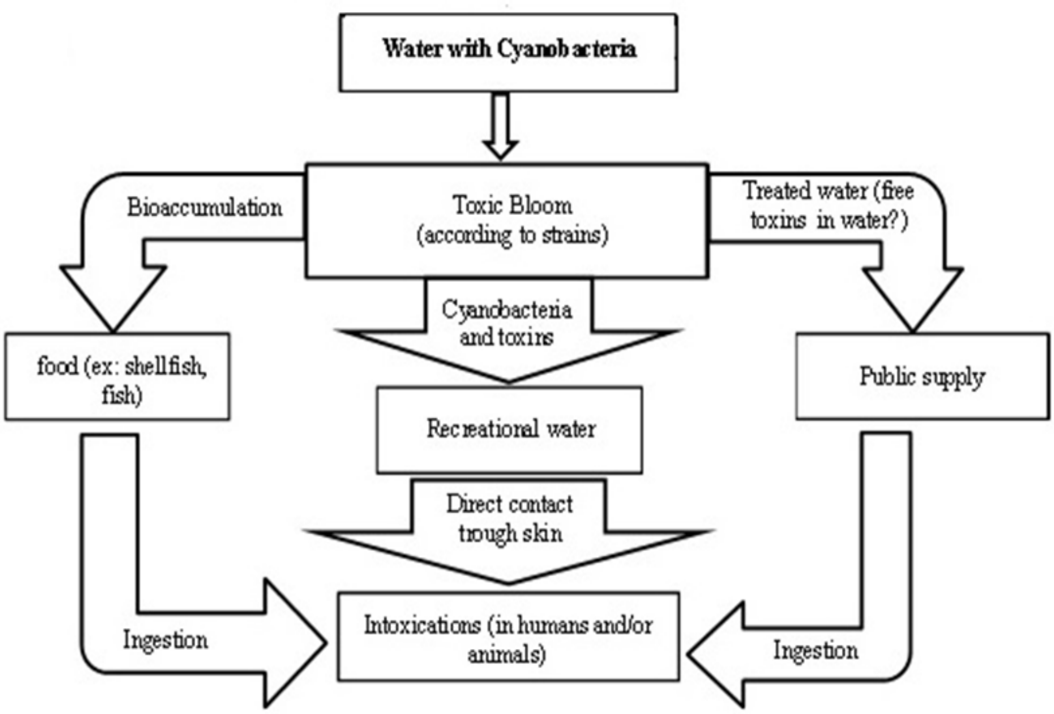


Figure I-2. – Cyanobacteria and cyanotoxins routes of exposure. (Based on Merel *et al.*, 2013).

There are many accounts in non-scientific literature describing the occurrence of scums and blooms in Europe over the last two millennia (Codd *et al*., 2005) and there is also evidence that native peoples of North America, Africa and Australia were aware of the toxicity present in waters with microalgae blooms (cf. Hayman, 1992 *in* Codd *et al*., 2005). Records of animal mortality episodes associated with the consumption of water blooms were common, some also referring episodes of human morbidity (Codd *et al*., 2005). However, the magnitude of the impact of cyanotoxins on human health with regard to mortality and morbidity remains probably underestimated due to notification failures, incorrect or inadequate diagnoses and lack of epidemiological studies (Skulberg *et al.*, 1994). The same reasoning applies to the identification and quantification of cyanobacteria and cyanotoxins in the environment, with many unanswered questions about the real risk to public health (Grivas, 2002).

According to several authors, the cyanobacterial *genera* most frequently associated with episodes of toxicity in freshwaters are *Microcystis*, *Planktothrix*, *Oscillatoria*, *Aphanizomenon*, *Anabaena* and *Lyngbia*. Each one of this genera is able to produce one or more cyanotoxins.

6.1. Cyanotoxins and human pathologies

Cyanotoxins are functionally classified into hepato-, neuro-, and cytotoxins (Hitzfeld *et al.,* 2000). Besides these, cyanobacteria also produce some lipopolysaccharides (LPS) and other secondary metabolites, some of them with pharmacological potential (Hitzfeld *et al.*, 2000). The consumption of water with high concentrations of cyanotoxins can cause gastrointestinal disorders, liver damage, neurological symptoms and potentially, although rarely verified, death (Falconer, 2008). Cyanotoxins modes of action are known (**Table I-II**), although much more work must be done, in order to investigate possible synergistic or antagonistic effects between different cyanotoxins or even with other chemicals present in water.

The impacts that can be generated in terms of public health due to exposure to long-term (chronic) to low doses of cyanotoxins, is not yet known (Backer, 2002, Hilborn *et al.*, 2008). Recently, it was hypothesized that there is a relationship between an amino acid produced by cyanobacteria (β-methylamino-L-alanine or BMAA), and neurological diseases such as Alzheimer's (Cox *et al.*, 2003; Murch *et al.*, 2004, Cox *et al.*, 2005), but much work still needs to be done in order to confirm this relationship.

Despite difficulties in proving the association between exposure to cyanotoxins and some human diseases, there is already a long list of morbidity cases and some mortality attributed to this relationship (**Table I-II**). An example was an incident at two hemodialysis centers in Caruaru city (Brazil), where at least 52 deaths were attributed to microcystin intoxication, after chemical and biological evidence obtained from the analysis of liver samples from 39 of the victims, which tested positive for this cyanotoxin. (Carmichael *et al.*, 2001).

In addition, various toxicological studies based on animals (rats, pigs, sheep, chickens, fish, etc.) have been carried out in the laboratory, proving cause-effect between exposure to cyanotoxins and observed pathologies. One example is the mitochondrial alterations and oxidative stress induced by microcystins, mechanisms involved in the initiation of tumors (Zanchett & Oliveira-Filho, 2013).

Table I-I - Main toxigenic genera/species, potentially produced cyanotoxins and their mode of action (Modified from Zanchett & Oliveira-Filho, 2013).

Main toxigenic genera/species)	Cyanotoxins	Mode of action	References
Hepatotoxic			
Anabaena, Planktothrix, Nostoc, Anabaenopsis	Microcystins (cyclic heptapeptides)	Inhibition of protein phosphatases type 1 and 2A	Pearson *et al.*, 2010 Mulvena *et al.*, 2012
Nodularia	Nodularins (cyclic pentapeptides)	Inhibition of protein phosphatases type 1 and 2A	Pearson *et al.*, 2010 Mulvena *et al.*, 2012
Cylindrospermopsis raciborskii, *Aphanizomenon ovalisporum,* *A. flos-aquae*	Cylindrospermopsins (guanidine alkaloids)	Glutathione and protein synthesis as well as cytochrome P450.	Kehr *et al.*, 2011
Neurotoxic			
Anabaena, *Aphanizomenon,* *Planktothrix*	Anatoxin-a (alkaloid)	Irreversible link to the nicotinic receiver S of acetylcholine	Ferrão-Filho, 2009 Falconer & Humpage, 2005 Falconer, 2008
Anabaena	Anatoxin-a(s) (organophosphate)	Irreversible inhibitor of acetylcholinesterase	Falconer, 2008 Rodriguez, *et al.*, 2006
Anabaena circinalis, *Aphanizomenon* sp., *A. gracile* *Cylindrospermopsis raciborskii,* *Lyngbya wollei*	Saxitoxins (carbamate alkaloids)	block sodium channels in nerve axons	Bláha *et al.*, 2009 Pearson *et al.*, 2010 Falconer, 2008
Dermatotoxic			
Lyngbya	Lyngbyatoxin-a alkaloid	potent tumor promoters, acting through potentiation of protein kinase C (PKC)	Chorus & Bartram, 1999 Funari & Testai, 2008
Lyngbya, Schizothrix, *Planktothrix* *(Oscillatoria)*	Aplysiatoxin alkaloids	potent tumor promoters, acting through potentiation of protein kinase C (PKC)	Chorus & Bartram, 1999 Funari & Testai, 2008
Cyanobacteria in general	Lipopolysaccharides (LPS)	Inflammatory agents, gastrointestinal irritants	Chorus & Bartram, 1999 Stewart *et al.*, 2006

Inadvertent drinking of water with high cyanobacteria density has been identified as the cause of acute human intoxication with symptoms of hepatogastroenteritis (Codd and Roberts, 1991) in many parts of the world. However in Portugal the occurrence of cyanobacteria blooms in water supplies had not been considered an environmental problem, with drinking water quality impacts and public health consequences, until the mid-90's of the last century (Araújo, 1995).

7. Southern Portugal Reservoirs

Southern Portugal has a Mediterranean climate, characterized by mild winters with heavy rainfall events and hot dry summers, where drought and flood episodes alternate irregularly. According to the National Water Institute (INAG) in southern reservoirs, maximum water levels are reached between January and June and minimum levels by the end of summer in September. Most of these reservoirs are located in lowland areas, with siliceous basins and substrate dominated by schists and sedimentary rocks. For all these reservoirs annual precipitation is inferior to 850 mm, mean water temperature is above 15 ºC and the water residence exceeds seven months (INAG, 2010). Their main uses are irrigation and drinking water production.

An exception to this typology is Alqueva reservoir, which is better framed as a main course reservoir. This typology includes water bodies with smaller hydraulic residence time (less than 10 days) and a drainage basin area higher than 20000 km^2 (INAG, 2010). Alqueva is located in the main course of the Guadiana River and is the biggest artificial lake of the European Union, draining a basin with almost 54000 km^2.

Data gathered on southern reservoirs reveal the regular occurrence of cyanobacteria blooms (Galvão *et al*., 2008).

While addressing in a review a total of 17 reservoirs in Southern Portugal, this work focuses specially on two of them, namely Beliche and Alqueva, as representatives of opposite situations in terms of nutrient enrichment and overall anthropic impacts. Beliche is a 27 years old, oligotrophic reservoir that should, therefore, have well-established phytoplanktonic assemblages. Alqueva is a much younger system (9 years old), subject to impacts of diverse human activities in its watershed, like intensive agriculture and livestock runoff, mining leaching, as well as domestic and industrial wastewater from the international part of the basin. Surveillance programs of Alqueva's waters also reveal the increased presence of cyanobacteria and CyanoHABs occurrence.

Specific goals

Within the general scope of this work and in order to consolidate and deepen the connection between ecological water quality and human health risks, the following specific goals were designed:

1) To review the available scientific literature on CyanoHABs occurrences in Southern Portugal listing/linking the impacts and risks detected;

2) To evaluate the effect of phytoplankton dynamics on the development of CyanoHABs linked to nutrient scarcity in an oligotrophic reservoir (case study: Beliche, Algarve, Portugal);

3) To stress the potential risks associated with the development of CyanoHABs dominated by new emergent species (case study: the emergence of *Cylindrospermopsis raciborskii* in Alqueva reservoir – Southern Portugal);

4) To assess how ecological quality indicators respond to the presence of CyanoHABs and how they relate to potential public health risks.

Thesis outline

As stated, the purpose of this study was to assess toxicity risks associated to Cyano HABs in Southern Portugal freshwater reservoirs. It addresses part of the biological component of these aquatic ecosystems, namely the phytoplankton, exploring the potentially toxic phytoplankton, especially cyanobacteria. While profiting from monitoring data accumulated during 10 years of environmental surveillance of reservoirs in Algarve, used as water sources for different human consumption purposes, it aims to contribute to a better understanding of the trinomial: Environment - Ecological Water Quality - Public Health.

Chapter I is a brief introduction to the topic Aquatic Environment vs Health, where the concepts of phytoplankton blooming and eutrophication are described in a context of linking water quality to public health risks, and explaining the specific objectives that will hereafter be addressed as independent studies (Chapter II to V) and presented as individual papers, according to the following list of manuscripts:

- Chapter II - Environmental and human health impacts of CyanoHABs in Southern Portugal reservoirs – a review;
- Chapter III - Toxicity risks linked to the biogeographic expansion of *Cylindrospermopsis raciborskii* in Southwestern Europe (Alqueva reservoir);
- Chapter IV - Phytoplankton dynamics and cyanobacteria related risks in Beliche reservoir (Portugal);
- Chapter V - Phytoplankton based indices applied to oligotrophic freshwater reservoirs classification.

Finally Chapter VI summarizes conclusions and future perspectives as final considerations.

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CHAPTER II

Environmental and human health impacts of CyanoHABs in Southern Portugal reservoirs – a review

Abstract

Reservoirs behave mostly as lentic aquatic ecosystems with high hydraulic residence and some carry relatively high concentrations of primary nutrients which will eventually lead to eutrophication, as a normal aging process. Massive proliferations of cyanobacteria, with relevant toxicity episodes, have been increasingly detected. Most of the toxic notifications in Portuguese freshwaters are attributed to *Microcystis aeruginosa*, although other toxigenic genera also occur, such as *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Gomphosphaeria*, and *Oscillatoria* among others. This work presents a review of studies conducted in Southern Portugal on cyanobacteria and cyanotoxins detection in reservoirs and the hazard that they represent. Mentioned reservoirs are Montargil, Maranhão and Divor on the Tagus river watershed, Alvito, Odivelas and Roxo on the Sado river watershed, Caia, Monte Novo, Vigia, Alqueva, Enxoé, Boavista, Odeleite and Beliche on the Guadiana watershed, Funcho and Arade on the Arade river and Bravura on the Odeáxere stream. Increasing notifications of cyanobacteria blooms are most probably a consequence of a mixture of factors acting synergistically, such as high radiation levels, longer periods of droughts and floods, wider periods of vertical stratification, high nutrient loadings during floods, increased turbidity, higher surface temperatures, mobilization of some chemicals from the bottom, during de-stratification and water scarcity periods. Cyanobacteria resilience is also due to their physiological surviving strategies like buoyancy regulation, nitrogen fixation, phosphorus storage, akinetes production and growth under poor light conditions. Furthermore exotic toxigenic species (*e.g. Cylindrospermopsis raciborskii*) have been gradually more detected representing increasing health risks, since toxins produced by exotic species aren't regularly screened. This biogeographical spreading suggests genetic plasticity, since these cyanobacteria have surpassed environmental constraints. This calls for a better strategic approach to the problem, involving water quality and public health authorities, as well as research institutes and scientists in order to: (i) assess cyanobacteria species composition and abundance rapidly and precisely, enhancing the current cyanobacteria monitoring efforts; (ii) investigate the potential relationship between cyanobacteria composition, toxicity and toxin concentrations across critical habitats; (iii) undertake efforts to develop molecular biology methods in order to overcome limitations of morphological identification, promoting accuracy and speeding up analytical procedures.

Keywords: CyanoHABs, cyanobacteria, cyanotoxins, Southern Portugal reservoirs

1. Introduction

Massive proliferation of cyanobacteria in freshwater ecosystems became more than just a worldwide environmental problem, turning out to be a major concern in the public health domain. Cyanobacteria constitute a primitive, highly adaptable, resilient and ubiquitous photosynthetic group of Prokaryota. This class comprises 150 genera and about 2000 species, but despite cyanobacteria ubiquity, each of their genera are not that omnipresent, at least till recently. Cyanobacteria species present a wide morphological variability (Mur *et al*., 1999) most probably achieved through evolutive adaptation to the different environments they came to colonize on Earth. They belonged to the firsts living organisms on Earth, seeming to be here since ever. In fact, fossils of these oxygen producing organisms were dated back to around 3.5 billion years ago (Schopf, 2002). So, what is the worry now?

In the last few decades cyanobacteria species considered to be exotic were found in unusual environments and bloom development events seem to have been increasing. Both, natural forces and human activities may be promoting Cyanobacteria Harmful Algal Blooms (CyanoHABs) through habitat alteration (Hudnell *et al*., 2008). In fact, several authors blame biogeographic expansion of tropical cyanobacteria species and growing frequency of CyanoHABs on global climate change and increased aquatic ecosystems eutrophication of anthropic origin (Messineo *et al.*, 2010; Sinha *et al*,. 2012).

The occurrence of massive concentrations of cyanobacteria that constitute a CyanoHAB creates a significant water quality problem, especially since many species may synthetize a wide range of odors, noxious compounds or potent toxins as secondary metabolites (Sivonen and Jones, 1999). The ability of around 40 genera of Cyanobacteria to produce cyanotoxins harmful to humans and animals constitutes a real worry, as accumulated literature data established a link between CyanoHAB occurrence and a higher probability of a toxicity event taking place. Ecotoxicological and human health impacts of these cyanotoxins have been documented worldwide (Merel *et al.*, 2013).

Cyanotoxins are a diverse group of natural toxins that may be peptidic, heterocyclic, lipidic or even organophosphatic (see Chapter III and IV). While some are hepatotoxic, namely microcystins, nodularins and cylindrospermopsins, others are neurotoxic like anatoxins, saxitoxins, or β-N-methylamino-L-alanine and some are dermatotoxic, for instance marine aplysiatoxins and lyngbyatoxins (Merel *et al.*, 2013). Some CyanoHAB species may simultaneously exhibit one or more of these types of toxicity and also be cytotoxic and/or genotoxic (De la Cruz *et al*., 2013).

Although, freshwater cyanobacteria blooms have been commonly reported in Portugal, since 1930 (Sampaio, 1933), toxicity only started to be detected in the last 25 years (Vasconcelos, 1993), after tragic events with hemodialysis patients in Évora (Portugal) as reported *a posteriori* by Araújo (1995).

In Portugal over the last decades many cyanobacteria blooms occurred throughout the whole country, getting authorities and the scientific community concerned. Freshwater monitoring programs started to focus on Cyanobacteria and data began to emerge despite usual budgetary difficulties. In fact in 1995/96 a proposal for a National Monitoring Program of Cyanobacteria in drinking and recreational water reservoirs was prepared by a working group coordinated by the Portuguese General Directorate of Health (Pereira *et al.*, 2000). Since then, various laboratories and scientists at Universities and Research Institutes have been carrying out regular screening of toxic freshwater cyanobacteria and conducting studies upon Portuguese water bodies, with special incidence on reservoirs, confirming suspicions and raising more questions about CyanoHABs impacts. Most of the toxic notifications in Portuguese freshwater environments have been attributed to *Microcystis aeruginosa*, although blooms of other potentially toxic genera such as *Anabaena*, *Aphanizomenon*, *Cylindrospermopsis*, *Gomphosphaeria*, and *Oscillatoria* have been detected (Vasconcelos, 1994; Mesquita *et al.*, 2002; Galvão *et al.*, 2012; Caetano *et al.*, 2013).

The present work reviews the main studies conducted in the Southern regions of Portugal, with the purpose of enlightening the potential hazard associated to cyanobacteria presence and their toxins in Southern Portugal reservoirs. The readers are invited to look up technical details of methodological protocols in the cited articles.

2. Cyanobacteria, cyanotoxins and human health

The risk of exposure to cyanotoxins through drinking water can be estimated to be high in regions where toxic cyanobacteria occur. Traditional water treatment methods of filtration, coagulation and disinfection were not efficient to remove cyanobacteria (Oliveira and Monteiro, 1993) and conventional treatment was considered ineffective for the removal of dissolved cyanotoxins (Campinas and Rosa, 2011). Bloom removal actions undertaken through the use of algicides (*e.g.* copper sulphate or hydrogen peroxide) in small drinking water reservoirs reverted into short-term human exposure to toxins, released in consequence of cyanobacteria cell wall and membranes rupture (Jones and Orr, 1994; Kuiper–Goodman *et al.*, 1999; Ross *et al*., 2006; Qian *et al*., 2010; Jančula and Marsalek, 2011).

The World Health Organization recommends the monitoring of microcystins when cyanobacteria density is higher than 2000 cells/ml (WHO, 2003), since microcystins are the most common cyanotoxins, comprising at least 89 analogs (Welker and Von Döhren, 2006) and are potentially produced by the highest number of cyanobacteria genera (see **Table II-I**). Furthermore 80% of microcystin exposure is estimated to occur through intake of drinking water (Kuiper-Goodman *et al.,* 1994). The mentioned guide value for alert level 1 of 2000 cells/ml, has been constantly exceeded whole year round in many South Portugal reservoirs.

Microcystins have different bioavailability and toxicity *in vivo* (Sivonen and Jones, 1999; Gupta *et al*., 2003; Chen *et al.*, 2007). WHO (2011) established the Tolerable Daily Intake (TDI) for microcystin-LR, as 0.04 μg/kg body weight (bw). The value was based on the NOAEL (non observed adverse effect level) value, that is 40 μg/kg/day in mice (Fawell *et al.*, 1994) and a security factor of 10^3 (10x for intra-specific variations, 10x for inter-specific variations and 10x for limitations in the database), assuming an adult body weight of 60 kg, a drinking water intake of 2 L per day, and an allocation factor for the exposure from drinking water of 0.8 (WHO, 2011). However, toxin exposure resulting from contaminated food (*e.g.* seafood) has not been considered in the NOAEL studies (Ibelings and Chorus, 2007). It is understood that humans can be exposed to combinations of toxins through food consumption (*e.g.* fish or shellfish) or by the intake of contaminated food and drinking water together. Little is known about synergistic effects of cyanobacterial toxins combinations, and this is a major knowledge gap for health risks, linked to cyanobacterial toxins in drinking water and food, proper assessment (Hilborn *et al.*, 2008). Furthermore, some studies by Falconer and Runnegar on microcystins physiopathology point to potential risks from continuous drinking of low-toxin doses (Carmichael and Falconer, 1993).

Using type of targeted organs most strongly affected as classification criterion (Codd *et al.*, 2005), **Table II-I** summarizes the known types of cyanotoxins, their potential producers, mode of toxicity, and the median lethal dose (LD_{50}) for intra peritoneal (i.p.) injection of pure toxin.

Hepatotoxins cause death in animals either by liver failure or by hypovolemic shock. A group of these toxins, the cylindrospermopsins, may also cause kidney damage and may be cytotoxic and genotoxic. In natural waters microcystins were usually detected in concentrations between 0.1 – 10 µg/L (Sivonen and Jones, 1999), however higher concentrations have been sporadically detected. For instance, in Europe a maximum of 37 µg/L was found in the Tâmega River at Ponte de Canaveses in Northern Portugal (Ueno *et al.*, 1996). In the American continent higher concentrations have also been occasionally detected, e.g. at Lake Winnipeg in September 1996, during a bloom situation a microcystin-LR concentration of 300 µg/L (GCDWQ, 2002), another example occurred in the summer of 2010, when a sample collected from the Grand Lake St. Marys, in Ohio, revealed microcystin levels of at least 2,000 µg/L (Ohio EPA, 2010).

Results from bioassays carried by several laboratories established that all neurotoxins produce rapid lethal intoxications (mice injected with high doses die in a few minutes). The three major neurotoxin groups (see **Table II-I**), namely anatoxin-a, anatoxin-a(s) and saxitoxins, present diverse biochemical effects and different chemical structures. Anatoxin-a is an alkaloid but anatoxin-a(s) is a natural organophosphate, in fact the only naturally produced organophosphate, known to date. Anatoxin-a is a powerful agonist of muscular and neuronal nicotinic acetylcholine receptor. The bond receptor-toxin is irreversible, forcing sodium channels to stay open. In consequence, a constant inflow of sodium occurs, leading to a post-synaptic neuromuscular blocking, which may result in respiratory muscles dysfunction, brain deoxygenation, convulsions, asphyxia and death (Valério *et al.* 2010). Anatoxin-a(s) blocks acetylcholinesterase through irreversible inhibition, which brings about intensive salivation (Kuiper-Goodman *et al.*, 1999) and might also lead to paralysis and death by cardio-respiratory failure (Valério *et al.* 2010). Like anatoxin-a, saxitoxins are alkaloids, varying in composition according to changes in radicals (Sivonen and Jones, 1999). They block sodium inflow in sodium ion channels leading to the inhibition of impulses produced in the peripheral nerves and skeletal muscles, with consequent death by respiratory arrest (Kuiper-Goodman *et al*., 1999; Valério *et al.*, 2010).

Dermatoxins or irritants are the toxins that may be considered the least harmful to humans, except the ones produced by *Lyngbya majuscula* in tropical waters, as these may cause severe dermatitis and promote tumor (Moikeha and Chu, 1971; Osborne, et al. 2001).

Table II- I – Main known cyanotoxins and their potential producers, mode of toxicity and median lethal dose (LD_{50}) for intra peritoneal (i.p.) injection of pure toxin per kg of body-weight (bw) in mice. (adapted from WHO, 1998, Carmichael, 2001, and Churro *et al.*, 2012).

Cyanotoxins	**Known potential producers**	**Mode of action**	**LD_{50} (mouse i.p. injection of pure toxin)** µg/kg bw
Hepatotoxins			
Microcystins (Cyclic heptapeptides) 89 variants	*Microcystis, Anabaena, Oscillatoria /Planktothrix, Nostoc, Hapalosiphon, Anabaenopsis, Aphanocapsa, Woronichinia, Phormidium, Snowella.*	Protein phosphatase blocker. Bind covalently to protein phosphatases and cause hemorrhaging of the liver; chronic exposure may result in liver cancer	25 - ~1000
Nodularins (Cyclic pentapeptides) 9 variants	*Nodularia*	Same as microcystins	30 - 50
Cylindrospermopsins -Cytotoxin- (Guanidine alkaloid) 3 variants)	*Anabaena, Aphanizomenon, Lyngbya, Cylindrospermopsis, Umezakia, Raphidiopsis*	Blocks protein synthesis; inhibition of cytochrome P450; substantial cumulative toxicity	200 - 2100 /d; 200 /5-6 d
Neurotoxins			
Anatoxin-*a* (Tropane-related alkaloid) 5 variants	*Planktothrix (Oscillaria), Anabaena, Plectonema, Aphanizomenon, Rhaphidiopsis, Hyella*	Blocks post-synaptic depolarization - agonism of the muscular and neuronal nicotinic acetylcholine receptor	250
Anatoxin-a(s) (Guanidine methyl phosphate ester)	*Anabaena; Aphanizomenon*	Blocks acetylcholinesterase irreversible	40
Saxitoxins (Carbamate alkaloids) 20 variants	*Anabaena, Aphanizomenon, Lyngbya, Cylindrospermopsis, Planktothrix (Oscillatoria)*	Blocks sodium or calcium channels of nerve axon membranes	10 - 30
Dermatoxins			
Lyngbyatoxin-*a* (Alkaloid)	*Lyngbya, Schizothrix, Planktothrix (Oscillatoria***)**	Inflammatory agent, activates protein kinase C. Skin irritant and tumor promoter	250
Aplysiatoxins (Phenolic bislactone)	*Lyngbya, Schizothrix, Planktothrix (Oscillatoria), Microcoleus.*	Same as lyngbyatoxin-*a*	107 - 117
Lipopolysaccharides	All cyanobacteria		

Ibelings and Chorus (2007) refer animal studies, showing that crude cyanobacterial extracts can be far more toxic than purified toxins. This may indicate that there are important synergistic effects between ‘‘known’’ toxins that may co-occur in a bloom or between known toxins and other unidentified compounds produced by cyanobacteria (Ibelings and Chorus, 2007).

Thus, cyanotoxins might be life-threatening either through direct or indirect contact, inhalation or ingestion, and their presence in natural waters used for recreational, for irrigation or as drinking water sources is of great concern.

3. Studied Southern Portuguese reservoirs

Southern Portugal (**Figure II-1**) belongs to a region with a semi-arid Mediterranean climate, with mean annual precipitation < 800mm, a dry season usually prolonged from May to September and years of extended droughts alternating with years of intense rain and floods. This irregular torrential hydrologic regime would make the tourism and agriculture dominated economy unsustainable, were it not for the increasing regulation of freshwater flow patterns and watersheds, brought about by the construction of multiple dams (**Figure II-1**). These dams have been built since the 50’s of the last century, be it for irrigation, energy or drinking water production, in order to answer to growing water demands. The resulting reservoirs, while addressing freshwater scarcity for human activities, are managed in a way which promotes high hydraulic residence (>7 months), with a strong seasonality of stored water volumes driven by tourism enhanced demographic changes. This type of management seems to influence the development of cyanobacteria blooms and aggravate their harmful impacts (Galvão *et al.*, 2012).

The location of the reservoirs addressed in this study is indicated in **Figure II-1.**

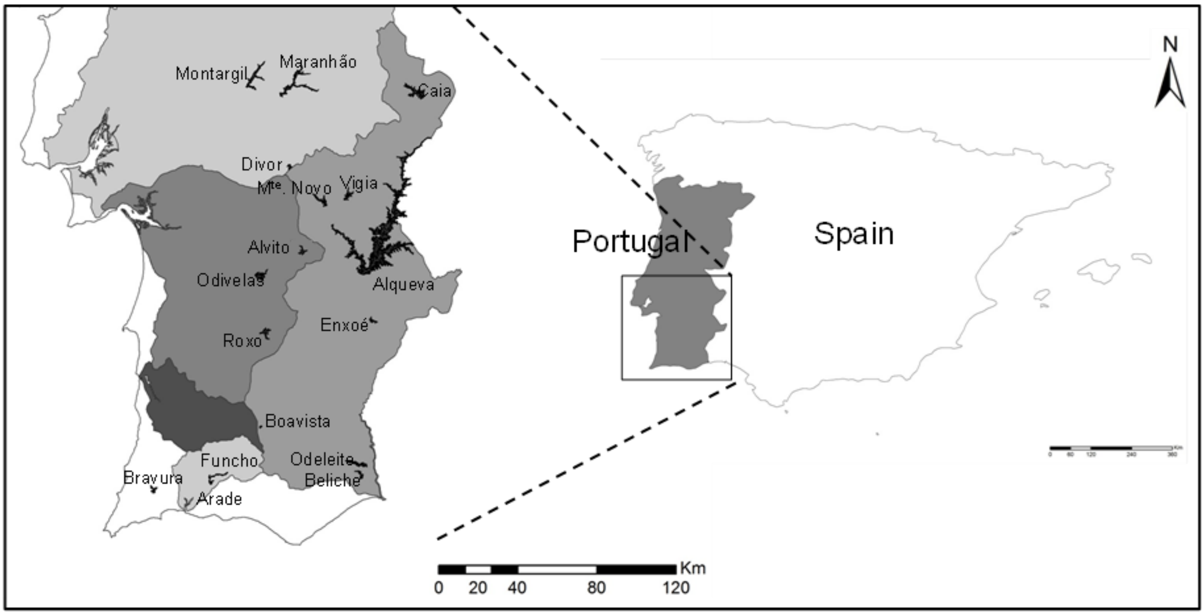


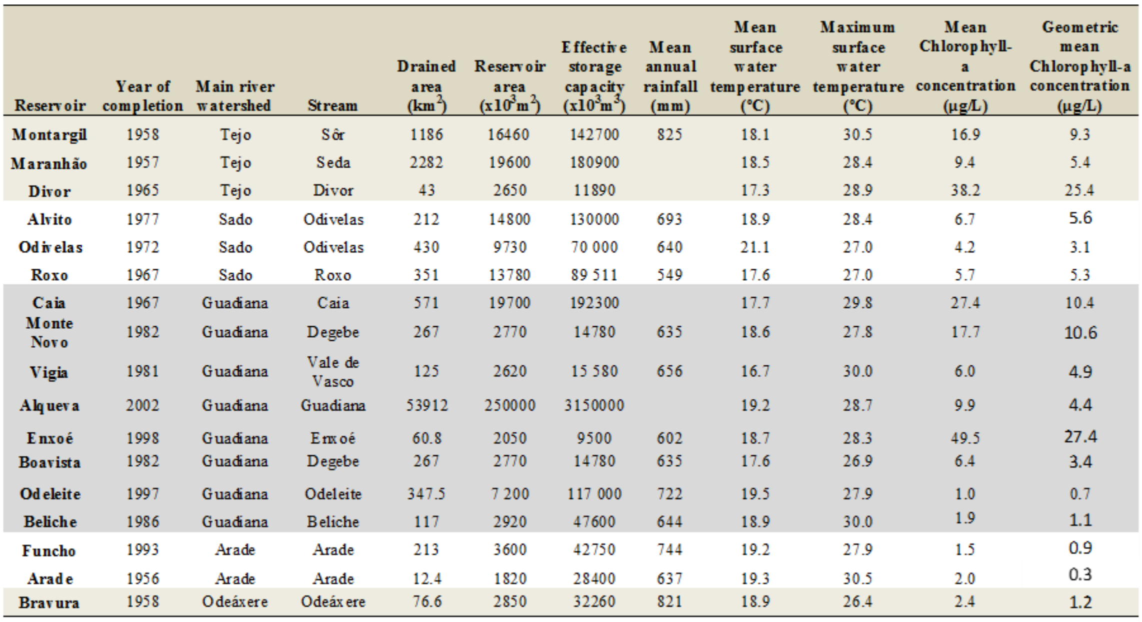
Figure II-1. Location of the studied Southern Portugal reservoirs (shading differentiates hydrographic basins).

Most of these reservoirs behave as warm monomictic lakes, with a mean annual water temperature >16.5 ºC (**Table II-II**), which favors CyanoHABs. Other characteristics of these reservoirs are also summarized in **Table II-II**, showing whenever data was available from the National Water Resources Service (SNIRH - http://snirh.pt/ and http://cnpgb.inag.pt) mean annual rainfall < 850mm. This apparent climatic homogeneity is contradicted by a wide range of drained basin sizes from 12.4 km^2 (Arade) to 53912 km^2 (Alqueva).

Geometric means of chlorophyll-*a* concentrations also reveal a great disparity in terms of trophic state. Applying the limits of the classification proposed by the Organisation for Economic Co-operation and Development (OECD), the Algarve reservoirs (Odeleite, Beliche, Funcho, Arade and Bravura) should be considered oligotrophic (<2.5 mg/m^3). Montargil, Maranhão, Alvito, Odivelas, Roxo, Vigia, Alqueva and Boavista would be classified as mesotrophic systems, Divor, Caia, Monte Novo and Enxoé as eutrophic (>10 mg/m^3).

Despite their trophic conditions diversity, all these freshwater reservoirs became dominated by cyanobacteria at least in summer, indicating that trigger conditions for CyanoHABs are not **always** bottom-up controlled factors.

Table II-II. Summary of attributes of the studied reservoirs.
Sources: http://snirh.pt/ and http://cnpgb.inag.pt/ and for Algarve reservoirs means of 10 year monthly data.



Reservoir	Year of completion	Main river watershed	Stream	Drained area (km^2)	Reservoir area (x10^{3}m^2)	Effective storage capacity (x10^{3}m^3)	Mean annual rainfall (mm)	Mean surface water temperature (°C)	Maximum surface water temperature (°C)	Mean Chlorophyll-a concentration (µg/L)	Geometric mean Chlorophyll-a concentration (µg/L)
Montargil	1958	Tejo	Sôr	1186	16460	142700	825	18.1	30.5	16.9	9.3
Maranhão	1957	Tejo	Seda	2282	19600	180900		18.5	28.4	9.4	5.4
Divor	1965	Tejo	Divor	43	2650	11890		17.3	28.9	38.2	25.4
Alvito	1977	Sado	Odivelas	212	14800	130000	693	18.9	28.4	6.7	5.6
Odivelas	1972	Sado	Odivelas	430	9730	70 000	640	21.1	27.0	4.2	3.1
Roxo	1967	Sado	Roxo	351	13780	89 511	549	17.6	27.0	5.7	5.3
Caia	1967	Guadiana	Caia	571	19700	192300		17.7	29.8	27.4	10.4
Monte Novo	1982	Guadiana	Degebe	267	2770	14780	635	18.6	27.8	17.7	10.6
Vigia	1981	Guadiana	Vale de Vasco	125	2620	15 580	656	16.7	30.0	6.0	4.9
Alqueva	2002	Guadiana	Guadiana	53912	250000	3150000		19.2	28.7	9.9	4.4
Enxoé	1998	Guadiana	Enxoé	60.8	2050	9500	602	18.7	28.3	49.5	27.4
Boavista	1982	Guadiana	Degebe	267	2770	14780	635	17.6	26.9	6.4	3.4
Odeleite	1997	Guadiana	Odeleite	347.5	7 200	117 000	722	19.5	27.9	1.0	0.7
Beliche	1986	Guadiana	Beliche	117	2920	47600	644	18.9	30.0	1.9	1.1
Funcho	1993	Arade	Arade	213	3600	42750	744	19.2	27.9	1.5	0.9
Arade	1956	Arade	Arade	12.4	1820	28400	637	19.3	30.5	2.0	0.3
Bravura	1958	Odeáxere	Odeáxere	76.6	2850	32260	821	18.9	26.4	2.4	1.2

In fact, Galvão *et al.* (2008, 2012) already suggested the importance of researching top-down variables impact on cyanobacteria proliferation, be it virio-, bacterio-, phyto- or zooplankton in oligotrophic reservoirs, where unfavorable conditions for other microalgae seem to be selecting cyanobacteria.

3.1. Reservoirs on the Tagus watershed (Divor, Montargil and Maranhão)

In Portugal, the first noticeable incident with cyanotoxins affecting humans occurred in Évora in 1993, after a severe drought 1991-1993, which left the freshwater reservoirs (**Figure II-1**) Divor (on the Tagus watershed) and Monte Novo (on the Guadiana watershed) at 19 % and 37 % of their maximum storage volumes, respectively. These two reservoirs were the main drinking water suppliers to Évora, a city located in Alentejo, which had around 50.000 inhabitants. In January 1993 changes in taste and color of drinking water began to be observed (Araújo, 1995). Meanwhile, the patients of the dialysis center of the hospital of Évora exhibited symptoms of encephalopathy, with high aluminum serum values. Twenty of them died. Though aluminum was not the only abnormal parameter at the water supply, and despite evidence of high phytoplankton density at both reservoirs with cyanobacteria blooms (Barros, 1994), no toxicity attributable to cyanobacteria was investigated. The dominant species found in those blooms was *Microcystis aeruginosa*, but species of the genera

Anabaena, *Oscillatoria* and *Aphanizomenon* were also present. All these genera are toxigenic and *Microcystis*, *Anabaena* and *Oscillatoria* species may produce hepatotoxins. Literature has plenty reports of microcystins causing intoxication to animals, resulting in liver damage, which can sometimes lead to death (*e.g.* Gorham, 1988). One of the considered potential signs for microcystin intoxication is alterations of the liver enzymes (Falconner, 1983). This was observed by Araújo (1995) in the epidemiological study undertaken at that time to evaluate the health effects resulting from cyanobacteria and the potential effects of hepatotoxins in the Évora drinking water supply. However insufficient data about cyanobacteria abundances and cyanotoxins analyses did not allow any inference of cause-effect relationship between the observed liver enzymes changes and the cyanobacteria blooms occurring in Monte Novo and Divor. Therefore, officially, blame was attributed to high aluminum levels (>0.2 mg/L), since it was excessively used at the water treatment plant, in the coagulation/flocculation process, in an attempt to eliminate the high densities of cyanobacteria.

As indicated in **Table II-II**, the geometric means of chlorophyll-*a* concentrations (>10 µg/L) for both these reservoirs continued to suggest high levels of eutrophication, especially in the case of the Divor reservoir (25.4 µg/L). Thus it is expectable that CyanoHABs occurrence in this system will persist.

The other two reservoirs in the south of the Tagus river watershed (Montargil and Maranhão, (**Figure II-1**) are believed to be mesotrofic, but are also mentioned in the scientific literature on account of CyanoHABs occurrences.

In May 1996, an intensive bloom of phytoplankton was detected at Montargil reservoir (Pereira *et al.*, 2000). This reservoir had already been biologically characterized and the phytoplankton studied by Oliveira (1984), more than a decade before, when phytoplankton was dominated by Chlorophyceae (Oliveira, 1984). Cyanophyceae, although present, were observed at concentrations not considered alarming and occasional low densities of *Microcystis aeruginosa* were not worrisome. The 1996 mentioned bloom was investigated and proven to be dominated by cyanobacteria (Pereira *et al.*, 2000). Analysis of periodically collected samples, from May to October, revealed the predominance of *Aphanizomenon flos-aquae* and *M. aeruginosa*, both potentially toxigenic species.

Mice bioassays performed using extracts of the collected samples revealed hepatotoxicity for the majority of the samples, whenever *M. aeruginosa* overcame (densities > 10^4 cells/ml) and neurotoxicity for the rest of the samples. In fact unusual toxicity associated with typical symptoms of paralytic shellfish poisoning (PSP) was observed. Only for two sampling dates no signs of toxicity where observed. Data also suggested that different types of toxins were being simultaneously produced during the bloom (Pereira *et al*., 2000). Strains of *A. flos-aquae* were isolated from the natural samples and cultured, for toxin production in order to eventually identify the toxins responsible for the observed effects on animals. Toxin analysis of culture extracts (Pereira *et al.*, 2000) by HPLC-FLD and confirmation by LC/MS detected five variants of PSP toxins, namely saxitoxin (STX), decarbamoyl-saxitoxin (dcSTX), neo-saxitoxin (neoSTX), gonyautoxin5 (GTX5) and gonyautoxin6 (GTX6).

Although Montargil reservoir was not used for drinking water supply, but to fulfill agricultural, electric and industrial requirements, the PSP toxins concentration found for the *A. flos-aquae* strain point out a potential risk for animal or/and human neuro-intoxication, *via* food consumption route *(e.g.* fish).

Still at the Tagus watershed, both Maranhão and Montargil belong to the firsts Southern Portuguese water bodies where *Cylindrospermopsis raciborskii* was detected (Saker *et al.*, 2003), in samples collected between July and October 1999, during a study that also addressed reservoirs in the Guadiana watershed.

3.2. Reservoirs on the Sado watershed (Alvito, Roxo and Odivelas)

In Alvito, Galvão *et al.* (2008) found different bloom-forming species in the reservoir, and microcystins were detected in several samples containing high abundances of cyanobacteria. Detected toxin concentrations were better correlated with *M. aeruginosa* biomass than with total cyanobacteria biomass. Bellém *et al.* (2013) results show a clear dominance by cyanobacteria, from the spring of 2003 till the fall of 2005, in the same reservoir. During this period the highest density (3.3 x 10^5cell/ml) was observed in March 2004, in a sample dominated by the potentially toxic *Snowella* sp. (Bellém *et al.*, 2013).

In the Roxo reservoir, high cyanobacteria densities were observed from July to December 2005. Again two cyanobacteria species dominated successively, *Microcystis incerta* in July and August (ca. 60% of the total cyanobacterial cell counts), followed by *Planktothrix* sp. from September to December. Microcystins analysis revealed high levels only at the beginning of the *Planktothrix* sp. bloom (September 2005). The highest abundance values were reached in the summer of 2005 with an *Oscillatoria* sp. peak of 3.2 x 10^5cell/ml (Bellém *et al.*, 2013). During 2006, there were no detectable cyanobacterial blooms until *Planktothrix sp*. attained abundances rounding 15.5 x 10^2cell/ml in July (Galvão *et al*., 2008).

In Odivelas, two distinct cyanobacteria blooms were also observed, but with a different species composition. Three different species of *Aphanizomenon spp*. predominated in August 2005, accounting for *ca*. 40% of the total cyanobacteria density. Other species, such as *Anabaena* spp. and *M. aeruginosa,* were also detected, but with much lower relative abundances (12 and 14% of total cyanobacteria counts respectively, about 6500 cells/ml). In September 2005, predominance drastically shifted towards *M. aeruginosa*, with abundances increasing up to 1.31 x 10^5cell/ml, representing *ca.* 50% of total cyanobacteria abundance. Toxin analysis performed revealed the presence of microcystin with concentrations reaching a maximum of 0.5 µg/L in samples collected, in September 2005, during maximal *M. aeruginosa* concentrations. In October an *Aphanizomenon* spp. bloom (also considered toxigenic) substituted the *M. aeruginosa* bloom. During winter, cyanobacteria decreased to negligible abundance values. They reemerged the following spring, dominating phytoplankton again, but with a different species composition and blooms occurring from April to May 2006, mainly composed of *Microcystis wesenbergii*. No detectable microcystins were found.

Odivelas reservoir is also mentioned as a location where *C. raciborskii* occurred, and used as an inoculum for laboratory cultures of this species. (Saker *et al*., 2004a).

In their study, Saker *et al*. (2004a) took samples from four sites: three reservoirs, used to supply drinking water, namely Odivelas, Caia (Guadiana watershed) and Maranhão (above mentioned in the Tagus watershed). The study, aimed the distribution of *C. raciborskii,* an “exotic” and highly toxic cyanobacteria, common inhabitant in tropical and subtropical freshwater ecosystems, but in geographical expansion to temperate regions of Europe (Padisak, 1997, Sinha *et al*., 2012, Caetano *et al*., 2013). In fact *C. raciborskii* was detected in Portugal not only in the four water bodies targeted by Saker *et al*. (2004) but in many others as referred by the same authors, namely the reservoirs of Montargil (referred above), Agolada, Bufo, Mértola and Patudos and Velas Lagoon.

Samples were taken from the mentioned 4 sites, between July and October 1999. Laboratory pure cultures were obtained and bioassays using mice were conducted. Results from the bioassays revealed high toxicity, but the toxin involved was not identified, since toxin HPLC analysis performed on the lyophilized biomass from all isolates failed to detect cylindrospermopsin (or deoxy-cylindrospermopsin), microcystins or saxitoxins. Nevertheless the histological examination executed revealed extensive liver damages, including necrosis and also enlargement of lymphatic follicles from intestinal mucosa. But no histological abnormalities were observed in the renal cortex, the medulla or any other organs, suggesting that the isolated *C. raciborskii* strain secreted one or more hepatotoxins, which were either not analyzed, or present in concentrations under the detection limit of the used methods, despite their huge toxic potential.

3.3. Reservoirs on the Alentejo area of the Guadiana watershed

3.3.1. Caia, Monte Novo, Vigia, Enxoé and Boavista

Caia reservoir belongs to the group of reservoirs where *C.raciborskii* was found (Saker *et al*., 2004a) and where saxitoxins were identified.

Monte Novo reservoir was also dominated by cyanobacteria from summer 2000 to Autumn 2007. Few exceptions were observed during winter in 2005 and 2007. A peak was reached in June 2006 with 1.1 x 10^5 cell/ml of *Microcystis* sp. (Bellém *et al.,* 2013).

Cyanobacteria dominance in Vigia reservoir increased since 2005, with a slight decrease in Cyanobacteria abundance during 2006/2007 autumn winter, but becoming dominant in May 2007, with 1.8 x 10^5 cells/ml of *Woronichinia* sp. (Bellém *et al.,* 2013).

In Enxoé reservoir, two *Planktothrix* spp. phytoplankton blooms were observed. However, microcystins were only detected in May 2006 during the second bloom (Galvão *et al.,* 2008). Bellém *et al.,* (2013) data shows a complete dominance by cyanobacteria during 2003-2004, with a peak for *Aphanizomenon* sp. (8.2 x 10^5 cells/ml) in the autumn of 2003.

From the summer 2006 till the autumn of 2007, cyanobacteria abundance dominated phytoplankton at Boavista reservoir, with exception of the 2006 fall, where Cryptophyceae took the lead. Cyanobacteria peaked in the fall 2007, with a bloom of 1.3 x 10^6 cells/ml, dominated by *M. aeruginosa* (Bellém *et al.,* 2013).

3.3.2. Alqueva

Alqueva Reservoir is the biggest artificial lake on the Iberian Peninsula and is located in Southern Portugal along 83 km of the main course of the Guadiana River (Serafim *et al.*, 2006). The Portuguese enterprise responsible for Alqueva management EDIA (Empresa de Desenvolvimento e Infra-Estruturas de Alqueva) implemented a monitoring programme in order to evaluate spatial and temporal variations in water parameters and thus document water quality changes during the early stages of reservoir filling in consequence of the Alqueva dam construction.

Morais *et al.* (2007) in their work, product of three years (2003 to 2006) of monitoring activities, found that algal successions were characterized by a highly marked contrast between the two main seasonal periods, a wet period (October to April) and a dry period (May to September). While cryptophytes-bacillariophytes-chlorophytes assemblages were characteristic of the wet period, cyanobacteria dominated the rest of the year, with surface blooms reaching over 10^4 cells/ml. Toxicity was not investigated, despite the fact that Alqueva reservoir was constantly under surveillance, being the most important water supply source in Southern Portugal (Morais *et al.*, 2007).

In their study (Caetano *et al.*, 2013) found massive and almost mono-specific, *C. raciborskii* blooms in September of both 2011 and 2012, throughout most of the reservoir (reaching 6.2 x 10^5cell/ml), much above the WHO second alert level (≥100 000 cells/ml). Also other toxigenic genera, such as *Raphidiopsis*, *Anabaena*, *Aphanizomenon*, *Oscillatoria/Planktothrix*, *Pseudanabaena*, *Microcystis*, *Phormidium* and *Woronichinia* were detected. Cyanotoxins were investigated and trace amounts of cylindrospermopsin (CYN) were detected by LC/ESI–MS/MS ion trap analyses. A battery of ecotoxicological tests was performed, using *Vibrio fischeri*, *Thamnocephalus platyurus*, *Daphnia magna*, *Pseudokirchneriella subcapitata* and *Brachionus calyciflorus* assays, but results were negative or inconclusive. In fact, there still is a need for experimental standardization of sampling and extracting cylindrospermopsin, as well as on analytical methods of detection and quantification. This was done for microcystin variants in a previous study also conducted on Alqueva samples, where a method using LC/ESI–MS/MS ion trap analyses was developed, allowing to minimize raw water matrix interferences. This study detected in several Alqueva samples three microcystin (MC) variants, namely MC-RR, LR and YR, although in amounts inferior to the WHO guide level of 1µg/L (Rodrigues *et al.*, 2013).

Worrisome is also the fact that mean chlorophyll-*a* concentrations, detected by our team along a two year monitoring period (2011 and 2012) on five sampling sites in Alqueva were superior to 25 µg/L, especially since means of official values given in **Table II-II** were lower than 10 µg/L. This suggests a recent increase in the eutrophication process, probably contributing to a shift in the blooming species. WHO guidance levels (Chorus and Bertram, 1999) suggest bathing inhibition in waters with chlorophyll-*a* concentrations above 10 µg/L (first guidance level).

3.3. Reservoirs on the Algarve area of the Guadiana watershed (Odeleite and Beliche)

Beliche and Odeleite are the main reservoirs used for drinking water production in Eastern Algarve. Both reservoirs are connected by an underground channel regulating the flow from Odeleite to Beliche. Water sluices are usually opened in spring and closed in autumn (Galvão *et al.*, 2008). Despite being oligotrophic reservoirs, Galvão *et al.* (2008, 2012) using accumulated data from 2003 to 2010, found that more than 80% of monthly water samples from both reservoirs, were dominated by cyanobacteria in terms of cell abundance. *Microcystis* was the dominant genus until spring 2008, with cell densities above WHO alert level 1 of 2000 cells/ml, occurring in 62-63% of all samples. It was also noticed that 13 to 17% of all samples presented concentrations above 2.0 x 10^4 cells/ml (Galvão *et al.*, 2008), with episodes of *Microcystis* blooms in June and July 2004 for Odeleite and Beliche respectively. During the summer of 2006, in a *Microcystis* spp. bloom, microcystin (MC) was detected both at the surface and at the bottom water samples of Beliche reservoir. MC concentrations had surprisingly a maximum of 3.5 µg/L in the bottom samples, but all other samples had MC concentrations below the WHO guide level of 1µg/L (Galvão *et al.*, 2012). Indeed in 2006 *Microcystis* blooms persisted during the whole summer with densities between 4.2 x 10^3 and 5.5 x 10^4 cells/ml, however the highest value were attained in March 2008, reaching 1.3 x 10^5 cells/ml (see chapter IV). Despite high cyanobacteria abundances, no significant levels of microcystins were detected under other bloom situations.

3.4. Reservoirs in Western Algarve (Bravura, Funcho and Arade)

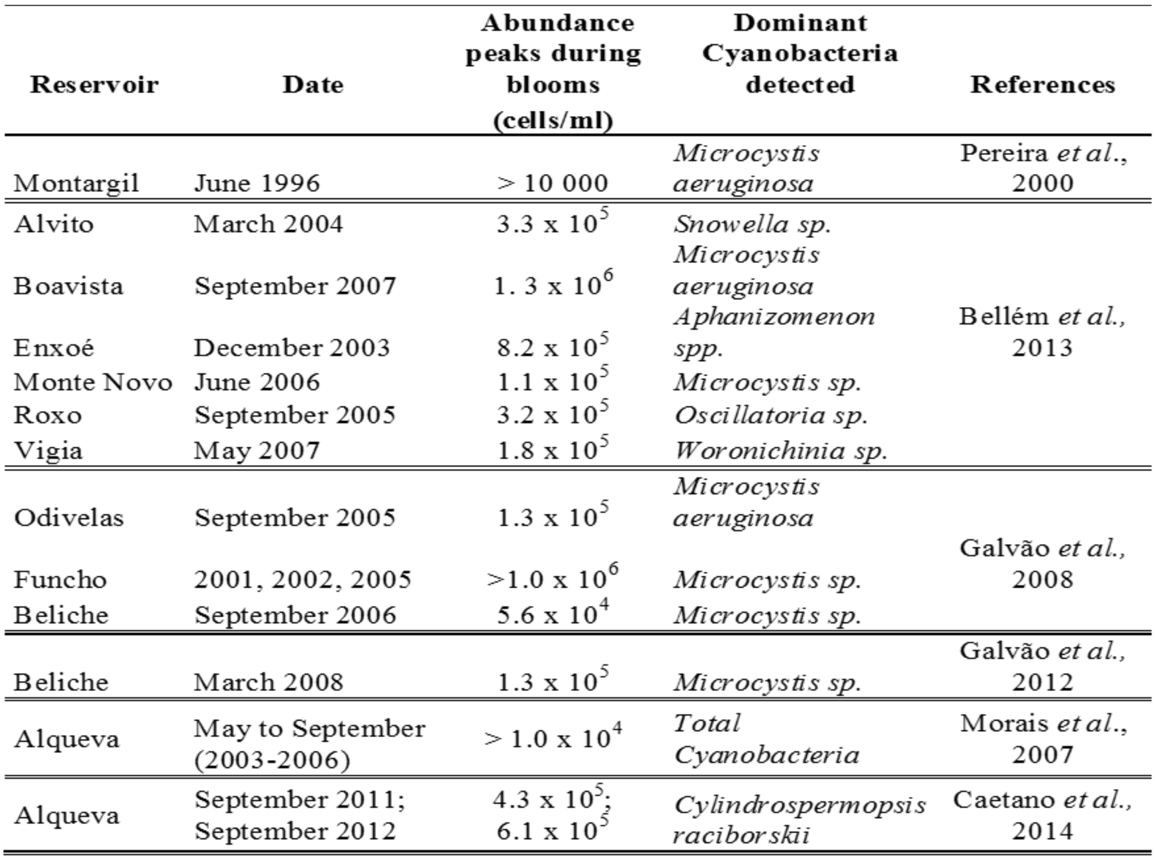
In Arade reservoir, a small irrigation reservoir, phytoplankton monitoring only started in May 2009. Since then cyanobacteria summer blooms with cell abundances above 10^4cell/ml occurred systematically, either dominated by *Microcystis* spp., or by *Aphanizomenon* spp. or *Anabaena* sp. Nevertheless no toxicity events have been recorded, as expected for oligotrophic water bodies.

Funcho is the main reservoir used for drinking water production in Western Algarve, while Bravura is seasonally used, from May to October (Galvão *et al.*, 2008). Funcho reservoir was dominated by cyanobacteria for the last 10 years during late summer and fall, but only exceeding the WHO alert level 2 (≥100 000 cells/ml) in 2001, 2002 and 2005, when *Microcystis* spp. reached >10^6 cells/ml. Since then, persistent summer blooms continued to occur but with densities below 10^5 cells/ml. Microcystin concentrations in this reservoir have always been negligible with respect to human health risk, only exceeding WHO guideline value of 1 µg/L, in scums, in January of 2002, during a mixed winter bloom of *A. flos-aquae* and *M. aeruginosa* (Mesquita *et al*., 2002).

For Bravura reservoir which is more exposed to anthropic influence, the WHO alert level 2 was well surpassed in 2005, 2006 and 2009 during summer blooms of non toxigenic Chroococcales.

Data available for the addressed South Portugal reservoirs is summarized in **Table II-III.**

Table II-III – Most significant documented toxigenic blooms observed in Southern reservoirs, displaying abundances, dominant species and dates.



Reservoir	Date	Abundance peaks during blooms (cells/ml)	Dominant Cyanobacteria detected	References
Montargil	June 1996	> 10 000	*Microcystis aeruginosa*	Pereira *et al.*, 2000
Alvito	March 2004	3.3×10^5	*Snowella sp.*	Bellém *et al.*, 2013
Boavista	September 2007	1.3×10^6	*Microcystis aeruginosa*	
Enxoé	December 2003	8.2×10^5	*Aphanizomenon spp.*	
Monte Novo	June 2006	1.1×10^5	*Microcystis sp.*	
Roxo	September 2005	3.2×10^5	*Oscillatoria sp.*	
Vigia	May 2007	1.8×10^5	*Woronichinia sp.*	
Odivelas	September 2005	1.3×10^5	*Microcystis aeruginosa*	Galvão *et al.*, 2008
Funcho	2001, 2002, 2005	$>1.0 \times 10^6$	*Microcystis sp.*	
Beliche	September 2006	5.6×10^4	*Microcystis sp.*	
Beliche	March 2008	1.3×10^5	*Microcystis sp.*	Galvão *et al.*, 2012
Alqueva	May to September (2003-2006)	$> 1.0 \times 10^4$	*Total Cyanobacteria*	Morais *et al.*, 2007
Alqueva	September 2011; September 2012	4.3×10^5; 6.1×10^5	*Cylindrospermopsis raciborskii*	Caetano *et al.*, 2014

In summary, published data (**Table II-III**) only refer, for the 17 studied reservoirs, two CyanoHABs with densities over 1 million cells/ml, both dominated by *Microcystis* species, potentially hepatotoxigenic.

Highest densities for filamentous cyanobacteria species were found for *Aphanizomenon* spp. and *Cylindrospermopsis raciborskii*, which are potential producers of both cylindrospermopsins and saxitoxins.

4. Unpublished monitoring data for studied reservoirs

Unpublished data, obtained from the Portuguese Environmental Agency (APA) with monitoring information on phytoplankton abundances and biovolume, from 2009 to 2014, for all the 17 studied reservoirs, relates to a total of 367 samples. Analysis of this data revealed that five cyanobacteria blooms reached densities above one million cells per milliliter (see **Table II-IV**). Of these, one sample surpassed 3.9 million cells per milliliter (see **Table II-IV**).

Table II-IV – Extreme bloom occurrences (> 1 million cells per milliliter) from 2009 to 2014, indicating cell abundance of dominant species, and proportion of phytoplankton biovolume represented by dominant cyanobacteria species.

Bloom Density	Number of occurrences	Reservoir	Date	Peaks (cells/ml)	Dominant cyanobacteria	Dominant cyanobacteria contribution to the total phytoplankton biovolume (%)
> 1 million cells/ml	5	Caia	28-06-2011	3.96×10^6	*Microcystis aeruginosa*	82.67
		Caia	26-07-2011	1.87×10^6	*Aphanocapsa incerta*	31.86
		Enxoé	24-08-2010	1.41×10^6	*Limnothrix* sp.	28.35
		Enxoé	10-09-2012	1.28×10^6	*Aphanizomenon flos-aquae*	97.11
		Enxoé	11-09-2014	1.55×10^6	*Aphanizomenon flos-aquae*	98.94

Two of these CyanoHABs, in Caia reservoir, were dominated by Chroococcales, namely potentially microcystin producers, like *Microcystis aeruginosa* and *Aphanocapsa incerta*. But the other three were dominated by filamentous genera *Limnothrix* and *Aphanizomenon*. It must also be mentioned, that the dominant species in three of these extreme blooms surpassed 80% of the total phytoplankton biovolume (**Table II-IV**) and therefore, dominant cyanobacteria not only overtook the other cyanobacteria species but also all the remaining phytoplankton.

Furthermore about 64.6 % of these 367 samples, presented cyanobacteria densities above 2000 cells per milliliter, from which about 70.9% had more than 10.000 cells per milliliter and 30.4% of these had densities higher than 100.000 cells per milliliter.

5. Discussion

South Portugal reservoirs behave mostly as lentic aquatic ecosystems, since due to water scarcity and in view of strong agriculture and tourism water demands, they are managed in a way where hydraulic residence is very high. Most of them carry relatively high concentrations of primary nutrients (nitrogen, phosphorous and carbon), which already lead some of them to have evident eutrophication symptoms, as is the case of Divor, Caia, Monte Novo, Enxoé and even Alqueva. Eutrophication, either natural or accelerated by man, has been proven to favor CyanoHABs over other types of HABs, since cyanobacteria possess some eco-strategies that allow them to overcome other organisms and become dominant, especially in eutrophic environments (Reynolds, 2006; Paerl and Fulton, 2006; Churro *et al.*, 2012). *Ergo* cyanobacteria are the most common phytoplankton organisms associated with eutrophication in freshwater ecosystems, and are expected to produce massive blooms causing severe changes in water quality, as well as economic losses (Vasconcelos, 2006). This association of cyanobacteria with eutrophication forecasts high toxicity risks for populations using water from Caia and Monte Novo, but especially from Divor and Enxoé since high mean concentrations of chlorophyll-*a* (see **Table II-II**) denunciate high eutrophication levels and episodes. The same applies to Alqueva reservoir, where despite official mesotrophy, our team found mean chlorophyll-*a* values superior to 25 µg/L.

However, not only cyanobacteria, appreciate nutrient enrichment, but most phytoplankton organisms also do so, developing massive phytoplankton blooms and impairing water transparency. This decrease in light availability favors, again, cyanobacteria, capable to photosynthesize under low light intensity circumstances. Furthermore, climate change, has been discussed to be linked with cyanobacteria ecology and growth (Paerl and Huisman, 2008, 2009; Paul, 2008; Paerl *et al.*, 2011; Paerl and Paul, 2012), since cyanobacteria are fond of warm weather (Falconer and Humpage, 2005), increased atmospheric concentrations of carbon dioxide and UV fluxes alteration (Beardall and Raven, 2004). Therefore, it is expected that cyanobacteria populations benefit from global warming

(Falconer and Humpage, 2005; Merel *et al.,* 2013). Such cyanobacteria growth stimulation may lead to very high toxin concentrations within CyanoHABs, often several orders of magnitude higher than the toxin concentrations in the surrounding waters (Pearl and Huisman, 2009). Moreover there are forecasts on changes in the hydrological regime to happen in Portugal, in the scope of a global change, exacerbating further the cycle of droughts and floods and prolonging springs and falls (Santos, *et al.* 2002). Such changes also impact phytoplankton dynamics, favoring cyanobacteria.

All the addressed reservoirs, in Southern Portugal, were dominated by cyanobacteria during part of the dry season, exhibiting blooms, especially during summer and fall, when the higher abundance peaks occurred. According to Falconer and Humpage (2005) a reservoir or lake with a consistent cyanobacteria summer bloom occurring during several years, should be considered to have established cell populations, creating a phenomenon very difficult to reverse.

In this framework where exotic species have already been detected, and allowed to "sow" these water bodies with resilient akinetes, high toxicity risks must be anticipated.

An example of an exotic species already at home in South Portugal reservoirs waters is *C.raciborskii*, a tropical and subtropical toxigenic species increasingly detected in Portuguese reservoirs. How did this species reach our waters? Processes of invasion and proliferation are under examination (Paerl and Huisman, 2009). However, it is known that *C. raciborskii* is usually distributed along the water column, and is adapted to low-light conditions found in many turbid, eutrophic waters (Padisák, 1997). Therefore eutrophication is a favoring factor. It also prefers water temperatures above 20ºC, and survives adverse conditions using specialized resting cells, the akinetes (Paerl and Huisman, 2009). Birds during migration, may carry on their paws these akinetes that might germinate where adequate environment conditions are found, such as reservoirs with eutrophic situations. Summer water temperatures in Southern Portugal reservoirs are well above 20 ºC (**Table II-II**) and indeed Portuguese *C.raciborskii* strains present high concentrations of akinetes in cultured isolates, while cultures from the same species from other places of the world (e.g. Northern Australia) rarely present akinetes. It was suggested by Vasconcelos (2006) that *C. raciborskii* from temperate regions such as Portugal are better adapted to lower growth temperatures, where akinetes might be of greater importance for survival during the winter. One could speculate that this morphological difference could be an adaptation to the new environment. And if so, this means that some invasive cyanobacteria species possess enough genetic plasticity to be successful in our reservoirs, even if environmental conditions were not exactly the most propitious. Although considering that till recently no cylindrospermopsin or saxitoxin production had been detected in *C. raciborskii* strains isolated from European waters, high toxicity risks must be taken into account, since in some cases toxicity still was detected.

Nevertheless, current scientific knowledge on cyanotoxins still doesn't allow a precise assessment of public health risks from exposure to toxigenic cyanobacteria (Churro *et al.,* 2012) and available epidemiological data is also insufficient for an accurate approach to the many toxicological aspects that need to be clarified (Churro *et al.,* 2012). This precludes a more accurate forecast of eventual risks in these reservoirs, requiring continuous monitoring not only of phytoplankton, but also of toxins and their ecotoxicological effects, especially since newly introduced species might produce still unknown toxins as detected by Pereira *et al.* (2000).

In this perspective South Portugal, due to its geographical situation, climatic conditions and hydrological regime should be considered a high-priority study area in relation to CyanoHABs, and rehabilitation measures for impaired water bodies should be urgently prepared.

The strong presence of *Microcystis aeruginosa* in some water supplying reservoirs (Vasconcelos *et al.,* 1996) anticipates acute and long-term adverse effects on human health due to exposure to hepatotoxins. This has centered the diagnostic of toxic occurrences mainly on microcystins, which are already regularly monitored in some water treatment plants.

As mentioned before, different cyanobacterial species respond to nutrient concentrations in different ways. Nevertheless increased temperatures and phosphorous concentrations seemed to favor all, but as stressed by Davis *et al*., (2009) especially promote the growth of toxigenic strains. This could explain the prolonged absence of toxicity in oligotrophic reservoirs, though sometimes dominated by *Microcystis* in summer, as has been the case of the reservoirs in Algarve.

Blooms with co-dominant species and also with switchovers or succession of flourishing cyanobacteria species were also observed in our reservoirs, as in Odeleite and Beliche, where mixed cyanobacterial blooms of Nostocales and Oscillatoriales in late spring, were gradually replaced by Chroococcales, including *Microcystis* sp. (Galvão *et al.,* 2008), which is the most known Chroococcale cyanobacteria.

The possible association between the toxic episode at the dialysis center of Évora (1993) and *Microcystis* sp. blooms occurring in the water supply reservoirs (Divor and Monte Novo) was pointed out (Barros *et al.,* 1994; Araújo, 1995). The patients exhibited encephalopathy symptoms and changes in their liver enzymes, which are considered as a possible consequence of the microcystins intoxication (Falconner, 1983). As mentioned, casualties were diagnosed as aluminum intoxication due to high amounts of aluminum in the water supply. Hepatotoxins or neurotoxins analysis were never done, despite the presence of *M. aeruginosa* and *A. flos-aquae* in the source water.

Mentioned animal studies, showing that crude cyanobacterial extracts can be far more toxic than purified toxins, (Ibelings and Chorus, 2007) may indicate that there are important synergistic effects between co-occurring ‘‘known’’ and unknown toxins and even other unidentified compounds produced by cyanobacteria (Ibelings and Chorus, 2007). This assumption is of great concern since data from Montargil reservoir suggests that different types of toxins were being simultaneously produced during the mentioned bloom (Pereira *et al.,* 2000).

The brief summary about new unpublished data (cf. section **4.**) reinforces previous evidence of the presence of potential producers of multiple hepato- and meurotoxins, like cylindrospermopsins, microcystins and saxitoxins. Such findings should be enough to acknowledge the need for improving actual monitoring programs, by introducing toxicity screening and subsequent toxin detection and quantification in order to assess environmental and public health risks associated with CyanoHABs. These will not disappear, but instead probably increase in occurrence and intensity.

6. Conclusions

Data from the several discussed studies suggests that cyanobacteria blooms detection is rising in South Portugal reservoirs. These phenomena, which are not exclusive to our water bodies, are most probably a consequence of a mixture of factors that synergistically affect phytoplankton dynamics. South Portugal reservoirs have high hydraulic residence, high sun exposure levels, increasing periods of droughts and floods, with high nutrient loadings during floods, which promote phytoplankton growth and increase turbidity favoring cyanobacteria, since some prefer to grow under shadow. Furthermore rising water surface temperatures, in a context of global change, and prolonged vertical stratification periods could also promote cyanobacteria, especially the growth of species that can regulate their position in the water column, such as *Microcystis* spp.. Also re-suspension of sediments, during the periods of water scarcity mobilizes nutrients and other compounds, such as metals that can be used by cyanoacteria.

While cyanobacteria are able to drive both in eutrophic and in oligotrophic systems, blooms in oligotrophic reservoirs only rarely displayed any toxicity. Such oligotrophic reservoirs should be the object of long term monitoring programs, since their study will best allow the differentiation between climate change and eutrophication impacts. In any case, precaution measures should include toxicity tests for any CyanoHAB in any type of reservoir.

Additionally exotic and toxigenic species have invaded our reservoirs (*e.g. C. raciborskii*), increasing the health risks, since toxins produced by such species aren't regularly screened. Suggested genetic plasticity, is also a concern, as it means that cyanobacteria can adapt and surpass environmental difficulties, indicating that they may also come to surpass solutions advanced by man.

This well succeeded invasion calls for a better strategic approach to the problem, involving water quality responsible authorities and public health authorities, as well as research institutes and scientists in order to: (i) assess cyanobacteria species composition and abundance rapidly and precisely, enhancing the current cyanobacteria monitoring efforts (identification and quantification), (ii) investigate the potential relationship between cyanobacteria composition, toxigenicity and toxin concentrations across critical habitats; (iii) undertake efforts to use molecular biology methods to overcome morphological identification limits, thus promoting accuracy and speeding up analytical procedures.

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CHAPTER III

Toxicity risks linked to the biogeographic expansion of *Cylindrospermopsis raciborskii* in Southwestern Europe (Alqueva reservoir)

ABSTRACT

Cylindrospermopsis raciborskii, a potentially highly toxic cyanobacterium has been increasingly detected in temperate regions, gaining an almost cosmopolitan distribution. In the Iberian Peninsula *C. raciborskii* has been identified in several small reservoirs, exhibiting some toxicity of unknown origin, but no production of cylindrospermopsin (CYN) was hereto attested. The Alqueva reservoir is the largest man-made lake in the European Union, in a Mediterranean climate ecoregion that favors summer cyanobacteria blooms. While monitoring spatial and temporal phytoplankton dynamics, this highly potentially toxic species was recorded for the first time in Alqueva, forming massive blooms in September 2011 and 2012 (cell ml^{-1} ranging from $9.6x10^4$ to $6.0x10^5$ in 80% of the sampling sites). Inversion microscopy identification of *C. raciborskii* was confirmed through Polymerase Chain Reaction amplification of extracted DNA with specific primers. Trace amounts (< 250 ng L^{-1}) of CYN were also detected through LC-MS/MS, at two sampling sites during these almost mono-specific blooms, suggesting the presence of genes for CYN production. An extensive battery of ecotoxicological assays representing different taxonomic and functional groups revealed no significant toxicity, except for September 2011 samples of three sampling sites. These findings call for toxicity monitoring in Iberian reservoirs as an emergent priority.

Keywords: *Cylindrospermopsis*, Alqueva, cylindrospermopsin, Cyanobacteria blooms, ecotoxicological assays, molecular identification, invasive species.

INTRODUCTION

Despite poor public awareness, health risks associated to planktonic blooms seem to have been acknowledged for millennia (Codd et al., 2005), sometimes through anecdotal reports of actual events resulting in animal mortalities (Falconer and Humpage, 2006; Stewart et al., 2006). Nevertheless, scientific reports of toxicity linked to blue-green algae (cyanobacteria) only appeared during the last quarter of the 19th century (Vasconcelos, 2006). Since then, cyanobacteria harmful algae blooms (CyanoHAB) have been blamed both for animal and human health endangering episodes (Metcalf and Codd, 2009), with reports of human fatalities in at least three countries, namely Brazil, Portugal and USA (Wisconsin) (Araújo, 1995; Jochimsen et al., 1998; Stewart et al., 2006). In fact, these phytoplankton microorganisms are able to produce several toxic metabolites (cyanotoxins) interfering with vital human functions. Routes of exposure to these compounds include direct skin contact, inhalation of aerosols and/or ingestion through water or food consumption, making toxic CyanoHAB a key concern in drinking-water production.

Chemically, cyanotoxins may be peptidic (microcystins and nodularin), alkaloids (anatoxins, saxitoxins, cylindrospermopsin, aplysiatoxins, and lyngbyatoxin), organophosphatidic (anatoxin-a(s)) or lipopolysaccharidic (irritants) (Fristachi et al., 2008). Microcystins (MCT), nodularin and cylindrospermopsins (CYN) act as hepatotoxins, while anatoxins, saxitoxins (STX), aplysiatoxins, and lyngbyatoxin are neurotoxic. Furthermore, CYN was shown to be cytotoxic, dermatotoxic, genotoxic and developmental toxic (De la Cruz et al., 2013), by inducing irreversible protein synthesis inhibition (Žegura et al., 2011) and DNA fragmentation (Štraser et al., 2013). The first report of an human poisoning episode, caused by CYN-contaminated drinking water, was recorded in Palm Island (Australia) in November 1979, when an aboriginal community was affected by an outbreak of hepatoenteritis (Griffiths and Saker, 2003), which was linked to a *Cylindrospermopsis raciborskii* bloom in a domestic water supply reservoir (Hawkins et al., 1985).

Cylindrospermopsis raciborskii is a potentially highly toxic species, a diazotrophic, filamentous cyanobacterium of the order Nostocales, bearing no sheaths, with terminal heterocysts as shown in **Fig. III-1** and subterminal akinetes, featuring efficient buoyance regulation (Padisák, 1997) and opportunistic up-take of phosphate with storage adaptation (Padisák, 1997; Isvánovics et al., 2000). This species is commonly found in tropical to subtropical freshwater ecosystems, but has been increasingly detected in temperate regions of Europe (Padisák, 1997; Briand et al., 2002) and Northern America (Hill, 1970; Hamilton et al., 2005; Hong et al., 2006). For the latter regions Sinha et al. (2012) reported an increase from 8 occurrences in 40 years (1950 to 1990), to 18 occurrences in 20 years (1990 to 2010). The expansion of *C. raciborskii* geographical distribution has been attributed to global warming phenomena (Messineo et al., 2010; Sinha et al., 2012); but might also be linked to increased eutrophication, as well as synergistic effects with other bottom-up and top-down control variables that need to be investigated (Sinha et al., 2012). Yamamoto and Shiah (2012) point out high shade tolerance, during little water transparency and low dissolved inorganic nitrogen concentrations in summer, leading to enhanced heterocyst production, as key factors in promoting *C. raciborskii* growth. In contrast, autumnal rainfall may be a critical factor leading to *C. raciborskii* blooms collapse (Yamamoto and Shiah, 2012). Albeit all the information gathered over four decades on *C. raciborskii* there still is a need for integrated ecological research in order to understand triggering factors of toxic events (Sinha et al., 2012).

Figure III-1. Microphotography of *Cylindrospermopsis raciborskii* under differential interference contrast phase (DIC) microscopy (Zeiss Axio Imager Z2 Fluorescence microscope with Apotome) Sample from Mourão, September 2011.

Following the Palm Island episode in 1979, CYN was purified from *C. raciborskii* cultures, characterized, and named by Ohtani et al. (1992). Since then, CYN production by *C. raciborskii* blooms was detected in Australia (Hawkins et al., 1997) and New Zealand (Wood and Stirling, 2003); however South American *C. raciborskii* strains produced STX instead of CYN (Lagos et al., 1999). Also surprisingly, European and North American strains have thus far been considered non-producers of either CYN or STX (Sinha et al., 2012), despite toxicity detected through mouse bioassays (Bernard et al., 2003; Fastner et al., 2003; Saker et al., 2003) suggesting the presence of other toxic metabolite(s), that need to be investigated (Saker et al., 2003). Thus not all strains of *C. raciborskii* produce CYN. It is now generally accepted that some species of the genera *Anabaena, Aphanizomenon, Lyngbya, Raphidiopsis* and *Umezakia* can produce CYN or one of its variants (Kinnear, 2010), although it remains unclear whether the known variants, 7-epicylindrospermopsin (7-epi-CYN) and 7-deoxycylindrospermopsin (7-deoxy-CYN), are CYN precursors, or CYN degradation products (De la Cruz et al., 2013). In a recent review on the importance, detection, and toxicity of CYN as well as on its chemical and biological degradation and removal by natural and artificial processes, De la Cruz et al. (2013) pointed out that different CYN structures can have different lipophilicities and polarities which can affect their reactivity and toxicity. As acknowledged for STX isomers and structurally similar compounds, some may be reactive or toxic while others possess much less activity, no activity at all, or completely different mechanisms of interaction (Wiese et al., 2010; De la Cruz et al., 2013). In relation with the multiplicity of potential producers, CYN detection has been recorded for all continents except the Antarctic (Padisák, 2003) gaining an almost cosmopolitan distribution (Kinnear, 2010).

CyanoHAB occurrence in Portugal was documented since 1991 (Vasconcelos, 1993a; Araújo, 1995) but CYN had so far not been recorded, even though species of the genera *Aphanizomenon* (Pereira et al., 2000), *Lyngbya and Raphidiopsis* are commonly found, *and C. raciborskii* was detected at Caia, Maranhão and Odivelas reservoirs as well as at the Ardila River (Saker et al., 2003). Accordingly, microcystin (MCT), and STX were detected in Portuguese reservoirs (Vasconcelos, 1993b; Pereira et al., 2000; Rodrigues et al., 2013) along with several cyanobacteria genera known to be potential MCT or STX producers. Furthermore, CyanoHAB have been documented throughout the Guadiana watershed before and after the construction of the Alqueva dam, with high densities of one or more potentially toxic species from the genera *Pseudanabaena*, *Anabaena*, *Oscillatoria/Planktothrix*, *Geitlerinema*, *Aphanizomenon*, *Merismopedia*, *Microcystis*, *Woronichinia*, *Synechocystis*, and *Aphanocapsa* (Galvão et al., 2012).

Alqueva reservoir is the largest artificial water body in the European Union, draining a 55,000 km^2 watershed into the Guadiana River. It is located in Alentejo (southeast Portugal), a semi-arid region with high levels of water scarcity and agriculture as main economic activity. The Alentejo region is included in the Mediterranean basin, one of the 25 biodiversity hotspots, occupying only 1.4% of the earth's surface, but bearing high species richness both of vascular plants and of vertebrate animals, mainly mammals, birds, reptiles and amphibians (Myers et al., 2000). The great biodiversity of this region makes this reservoir an environmentally important area not only for Portugal but also for the rest of Europe. Recent environmental risk assessment on this reservoir, revealed growing evidence of eutrophication and water quality impairment, due to: (i) high nutrient and organic matter input, from intensive agriculture and cattle farming (Palma et al., 2010a); (ii) extensive contamination by untreated wastewater discharges, upstream of the reservoir (Palma et al., 2010a); (iii) presence of pesticides such as. atrazine, simazine, diuron and terbuthylazin (Palma et al., 2009); (iv) high levels of As, Fe and Mn in the water column and As, Cd and Pb in sediments (Palma et al., 2010a, 2014a); (v) mean chlorophyll *a* concentrations > 25 mg L^{-1} (Rodrigues et al., 2013); (vi) presence of potential cyanotoxin producers (Galvão et al., 2008); (vii) detection of microcystin-RR and microcystin–LR (Rodrigues et al., 2013); (viii) ecotoxicological chronic responses detection during the dry season (Palma et al., 2010b; Palma et al., 2014b).

The present work spanning a two year period, reports on the presence of *C. raciborskii* (with trace amounts of CYN) in the Alqueva reservoir, together with the results of a wide range of ecotoxicological assays using aquatic organisms from different taxonomic and functional groups. It also applies molecular methods to the identification of *C. raciborskii*.

MATERIALS AND METHODS

Sampling sites

Alqueva reservoir is located in Alentejo region (Southeast Portugal) within the Guadiana watershed (**Fig. III-2**). The hydrologic regime of Alqueva reservoir reflects the Mediterranean climate, which is characterized by dry and hot summers and mild and wet winters with concentrated rains; the annual average of atmospheric temperature ranges from 24 to 28 °C, in the warm months (July/August), and from 8 to 11 °C in the cold months (December/January).

Based on a previous established program for the EcotoxTools project (Palma et al., 2014a), five sampling sites were used, as being representative of contamination levels at upstream, middle and downstream areas (Palma et al., 2014a). Of the five, three were along the main Guadiana channel, Senhora da Ajuda (38°46'28.56''N, 7°10'47.00''W), Lucefécit (38°33'6.32''N, 7°17'52.86''W) and Mourão (38°23'60.00''N, 7°23'25.80''W); and two were at the channels of each of the main tributaries Alcarrache (38°19'1.53''N, 7°19'51.10''W) on Alcarrache stream and Álamos (38°20'30.00''N, 7°34'40.00''W) on the Dejebe River (**Fig. III-2**). At Senhora da Ajuda the reservoir displays lotic conditions due to the influx of the Guadiana

River coming from Spain, while at the other four sites, the system behaves like a lentic water body with a warm monomictic regime corresponding to strong summer stratification and winter water-column mixing.

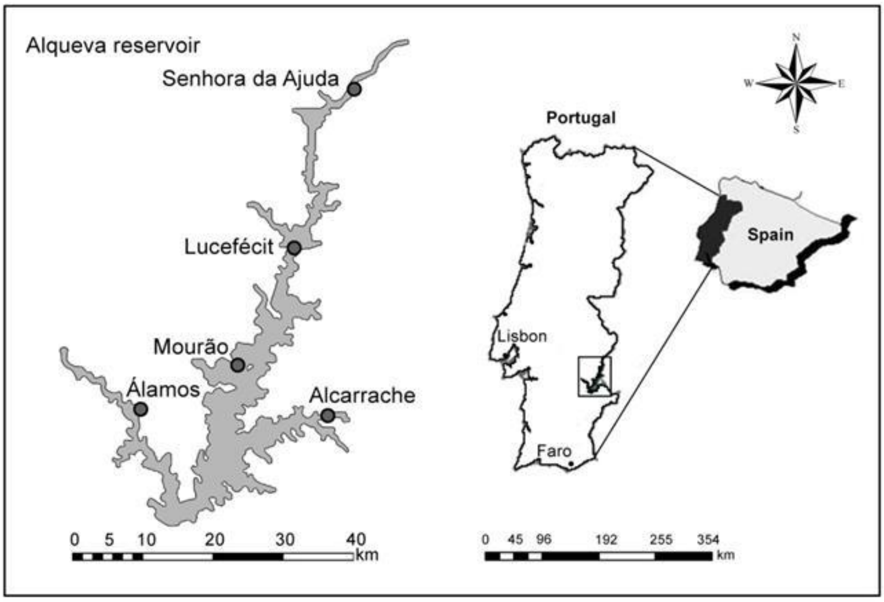

Figure III-2. Study area and sampling sites in Alqueva reservoir

Sampling

Water samples were collected during 2011-2012 at each of the five selected sampling sites, even though the present study only integrates data obtained for samples collected in September of both years when *C. raciborskii* presence was detected. For phytoplankton analysis, samples taken in July and November of both years were also included.

For determination of general water quality parameters and to perform ecotoxicological assays with aquatic organisms, surface water samples were collected using a 5 L Van Dorn bottle at the depth of 0.5 m (Palma et al. 2014b), while for phytoplankton and toxin analysis, vertically integrated samples of the euphotic zone (assumed as 2.5-fold the measured Secchi disk distance) were produced by mixing of discrete samples collected at increasing 1 m depths, as recommended by European standardization initiatives. Phytoplankton samples for cyanobacteria abundance and biovolume determination were preserved with acid Lugol´s iodine solution (0.5%), while for samples for toxin analysis, 1 L of each integrated water sample was divided into 3 replicate volumes of 200 mL, kept in amber borosilicate bottles and frozen at -20 ºC until analytical analysis.

Phytoplankton analysis

Total phytoplankton and cyanobacteria abundance and composition were determined under inversion microscopy according to the methodology described by Utermöhl (1958) as recommended by CEN TC 230/WG 2/TG 3/N83 (2004). As recommended, specific biovolumes were calculated using the median values obtained by the measurement of not less than 20 cells, for each *taxon*, and calculations done choosing the geometric solid, which resembled the most, the observed shape as foreseen by Hillbrand et al. (1999). Chlorophyll *a* was determined by spectrophotometry, after filtration through glass fiber filters GF/F of 0.7 µm pore size (Whatman, GE Healthcare) and acetone extraction according to ISO 10260 (1992) and Clesceri et al. (1999). The calculations were carried out using the monochromatic equation of Lorenzen (1967).

Cylindrospermopsis raciborskii molecular differentiation

DNA was extracted both from cultures of *C. raciborskii* isolates and from Lugol fixed samples. In the latter case, a prewash step was introduced as recommended by Bertozzini et al. (2005). Extraction followed the E.Z.N.A.® Plant DNA Kit (Omega Bio-Tek Inc.) protocol.

The primers used in this study are described in **Table III-I**.

Table III-I. Primers used in *Cylindrospermopsis raciborskii* molecular differentiation.

Primer	Target	Sequence	Specificity	Reference
CYA108F	16S	5’-ACGGGTGAGTAACRCGTRA-3’	*Cyanobacteria*	Urbach *et al*., 1992
CYAN 16S R	16S	5’-CTTCAYGYAGGCGAGTTGCAGC-3’	*Cyanobacteria*	Urbach *et al.,* 1992 (modified by Rinta-Kanto *et al*., 2005)
CYA377R	16S	5’-CCATGGCGGAAAATTCCCC-3’	*Cyanobacteria*	Nübel *et al.,* 1997
CYLnif F	nifH	5'-TAARGCTCAAACTACCGTAT-3’	*C. raciborskii*	Dyble *et al.,* 2002
CYLnif R	nifH	5'-ATTTAGACTTCGTTTCCTAC-3’	*C. raciborskii*	Dyble *et al.,* 2002
CYLcpc F	cpcBA-IGS	5'-GGCTTACGCGAAACCTATATA-3’	*C. raciborskii*	Dyble *et al.,* 2002
PCα R	cpcBA-IGS	5'-CCAGTACCACCAGCAACTAA-3’	*C. raciborskii*	Neilan *et al.,* 1995

For total cyanobacteria polymerase chain reaction (PCR) 16S rDNA amplification, each 25 µL reaction was composed of 400 nM of each primer, 200 µM of dNTPs, 1 x Mg-free PCR buffer (Promega), 2 mM $MgCl_2$, 300 ng μL^{-1} BSA and 0.4 U/µL Taq DNA polymerase (Promega). PCR was performed in a DOPPIO thermal cycle (VWR Collection), with an initial denaturation step at 95 ºC for 5 min, followed by 50 cycles of 94 ºC for 30 s, 56 ºC for 60 s and 72 ºC for 30 s, and a final elongation step at 72 ºC for 15 min.

Protocols employing two different pairs of primers (*Nif* H and *cpc* BA-IGS) were used for PCR amplification (Dyble et al., 2002). The reaction mixture contained 10 pmol of each primer, 200 µM of dNTPs, 1 x PCR buffer (Invitrogen), 2.5 mM $MgCl_2$, 1 U Platinum®Taq DNA Polymerase (Invitrogen) and 1 µL (10 ng) DNA template.

PCR amplification parameters for *nifH* primers were 94 ºC for 5 min, followed by 30 cycles of 94 ºC for 10 s, 55 ºC for 20 s and 72 ºC for 60 s, and a final elongation step at 72 ºC for 7 min. For *cpcBA-IGS* primers PCR parameters were 94 ºC for 5 min, followed by 30 cycles of 94 ºC for 10 s, 52 ºC for 20 s and 72 ºC for 60 s, and a final elongation step at 72 ºC for 7 min.

All PCR products were confirmed by electrophoresis on a 1% (m/v) agarose gel, and visualized through GelStar™ Nucleic Acid Gel staining, under ultraviolet (UV) illumination.

Ecotoxicological assays

Ecotoxicological analyses of reservoir water samples (Palma *et al.*, 2010b; 2014b; Rosa *et al.*, 2010) were carried out using representative test organisms as follows:

30 min. luminescence of the marine bacterium *Vibrio fischeri* (NRRL B-11177) using Luminotox protocol (ISO 11348-2, 1998);

24 h lethality of the crustacean *Thamnocephalus platyurus* in accordance to the protocol provided with the Thamnotoxkit F assay (Persoone, 1999a). (MicroBioTests, Nazareth, Belgium);

48 h lethality of the cladoceran *Daphnia magna* assays were performed following the ISO 6341 protocol (1996);

21 d *Daphnia magna* reproduction assay was conducted according to the OECD guidelines (1998);

72 h *Pseudokirchinella subcapitata* growth assay followed the OECD (1984) and EC (1992) guidelines with minor modifications and stock culturing conditions as described in detail by Rosa *et al.* (2010);

48 h *Brachionus calyciflorus* feeding and population growth assays were carried out following the Rotoxkit F Chronic protocol (Persoone, 1999b);.

30 min luminescence of the marine bacterium *Vibrio fischeri*, the 24 h lethality of the crustacean *Thamnocephalus platyurus* and the 48 h lethality of the cladoceran *Daphnia magna* were evaluated both with the 2011 and 2012 samples and on a range of dilutions of each collected water sample, using reconstituted hard water (ASTM, 2002) as dilution and control medium. All remaining assays (sublethal) were only performed with the 2011 samples and with the non-diluted water samples from each site. During all assays, temperature, pH, dissolved oxygen, and conductivity were measured and were in accordance with the standard protocol used.

Statistical analysis

For assays in which a dilution range of the water samples was tested, and whenever possible, the median effective concentration (EC_{50}, in %) and respective 95% confidence limits (CL) was estimated; for the *T. platyurus* and *D. magna* lethal assays using the Probit analysis (Finney, 1971), whereas for the *V. fischeri* bioluminescence assay using LUMISsoft 4 Software (Lange). For all remaining assays, potential water toxixity at the Alqueva was evaluated by comparing organism responses among the standard assay control and the waters from the five sites of the Alqueva reservoir by one-way analysis of variance; when significant differences were detected the Dunnett's test was performed to compare each water sample with the control. For both feeding assays a paired Student's *t*-test comparing algal cell densities in the standard control at the start and end of the assay confirmed that no algal growth took place during the exposure period. The assumptions of normality and homoscedasticity were verified using Shapiro–Wilk's and Bartlett's tests, respectively. All statements of significant difference were set at the 0.05 level. All statistical analyses on sub-lethal assays were conducted using Statistica 7.0 software (StatSoft, Tulsa, OK, USA).

Cylindrospermopsin ion trap LC/ESI–MS/MS analysis

For CYN analysis, certified standard solutions (10 µg mL^{-1} in methanol) of CYN (> 95%), were supplied by ABRAXIS Cylindrostd-S (Pennsylvania, USA). Membrane filters RC55 of 0.45 µm pore size were provided by Whatman (GE Healthcare). All reagents used were analytical reagent grade. Acetic acid was purchased from Sigma Aldrich (Taufkirchen, Germany). Acetonitrile and methanol were high-performance liquid chromatography (HPLC) grade and supplied by Merck (Darmstadt, Germany). Ultra-Pure water was obtained from Simplicity 185 water system. A CHROMABOND vacuum manifold solid phase extraction (SPE) system, used for concentration and clean-up of the water samples, was purchased from Macherey–Nagel (Germany), and Bond Elut Carbon cartridges, 500 mg, 6 mL, were supplied by Agilent Technologies (Varian, Inc., Europe).

The extraction procedure involved intentional cell disruption through a thaw freeze procedure in order to also extract intracellular toxin and sample filtration through 0.45 µm membrane filters (Whatman RC55),. The filtered water was stored at 4 ºC and submitted to CYN extraction within 24 h and the filters stored at -20 ºC until required. The extraction method was similar to the reported previously by Wörmer et al. (2009) and Guzmán-Guillén et al. (2012). Sample cleanup was carried out using Bond Elut Carbon, 500 mg, SPE cartridges, and elution with 10 mL of solvent mixture of dichlorometane:methanol (1:4, v/v). The SPE eluates, after drying under a nitrogen stream at 40 ºC, were dissolved in 1 mL water, acidified with 0.05% acetic acid, and stored at -20 ºC before quantitative analysis through liquid chromatography coupled to ion trap tandem mass spectroscopy with electrospray ionization (LC/ESI-MS/MS). Collision-induced dissociation (CID) experiments were performed on a Bruker Daltonics HCT ultra mass spectrometer (ion trap detector) equipped with an electrospray ion source (ESI – operated in positive and negative mode) and coupled to a Agilent Technologies 1200 Series liquid chromatograph.

The CYN LC separation was achieved on a GraceSmart RP-18 analytical column (150 mm x 2.1 mm, 3 µm, Grace Davison Discovery Sciences) maintained a 30 ºC. Binary gradient was used employing a Milli-Q water and acetonitrile (both contained 0.05% formic acid [v/v]) mobile phase at a flow rate of 0.250 mL min^{-1}. The acidified acetonitrile was firstly run at a 5 % for 2 min, increased to 85% over 4 min and held for 4 min before returning to conditions in 2 min and the column equilibrated until 8 min before injection. The injection volume was 10 µL.

Standard solution of CYN (500 mg L^{-1}) was firstly infused into the mass spectrometer for mass spectrometry studies. Full-scan mass spectra were acquired both in the positive and negative modes, by tuning for CYN on the $[M+H]^+$ at *m/z* 416.0 and the $[M-H]^-$ at *m/z* 414.0, respectively. MS/MS was performed by extracted ion chromatographic (EIC) with isolation of the parent ion and a fragmentation cut-off of at *m/z* 112.

RESULTS

Phytoplankton abundance and biovolume

There were massive, almost mono-specific blooms of *C. raciborskii* blooms in September of 2011 and 2012, throughout most of the reservoir (**Figure III-3**).

Figure III-3. Inversion microscopy photograph of a September 2011 sample taken from water collected at the Alqueva reservoir (Mourão site) illustrating *Cylindrospermopsis raciborskii* dominance (observed with a 10x objective on a Zeiss Axio Observer A1 microscope).

In fact, this species was identified in 13 samples collected at Alqueva reservoir, from which 5 were collected in July and September 2011 and 8 in July, September and November 2012 (see **Figure III-4**). High densities and biovolumes of *C. raciborskii* cells (10^5 cells ml^{-1}) were only registered for September samples of both years (see **Figures III-4** and **III-5**). One interesting feature about the detected *C. raciborskii* strains is their morphology, In fact, Komàrec and Kormáková (2003) mention that in this species akinetes rarely are adjacent to the terminal heterocyte, but many of the trichomes observed in Alqueva samples showed this characteristic. (**Figures III-1** and **III-3**).

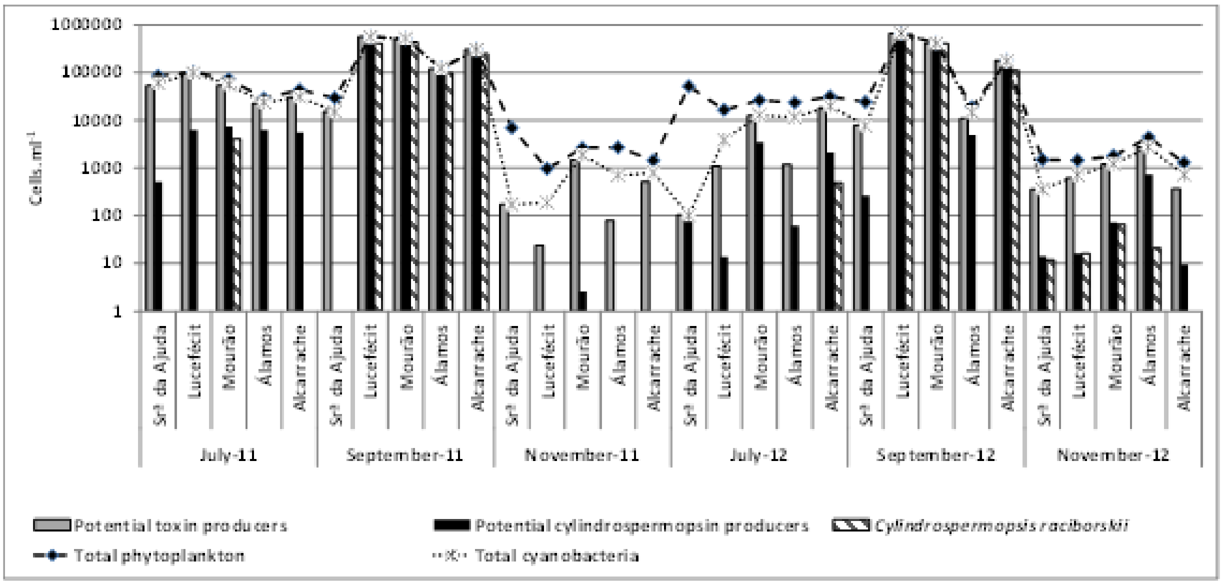

Figure III-4. Phytoplankton abundance dynamics in 2011 and 2012, from July to November at five sampling sites, compared with cyanobacteria abundance, and the fractions of cyanobacteria that are *Cylindrospermopsis raciborskii*, potential toxin producers or potential cylindrospermopsin producers.

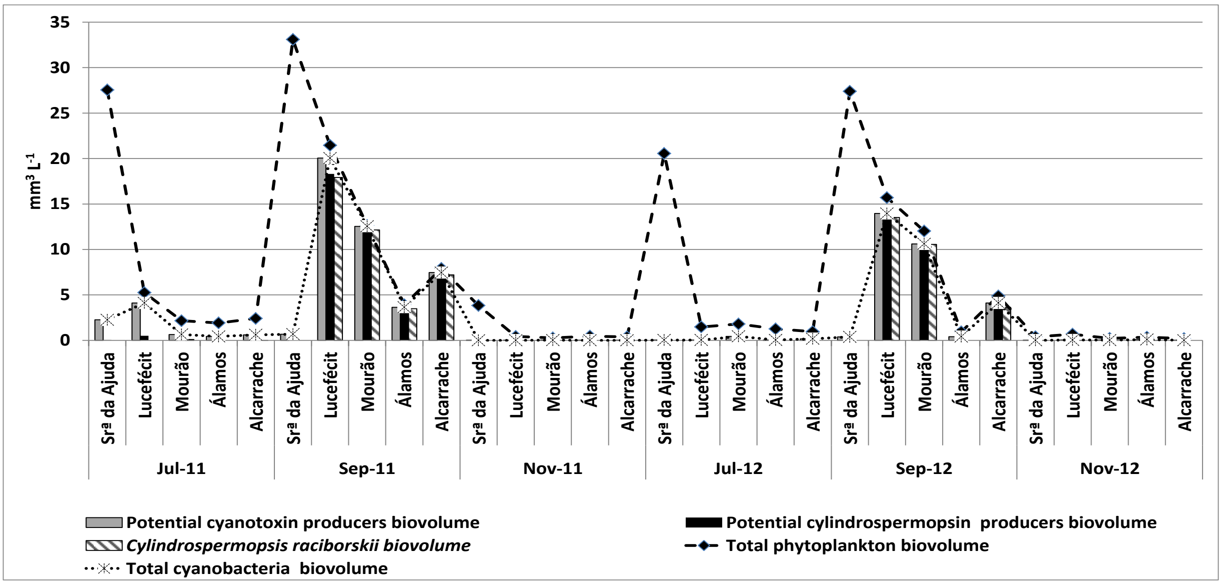

Figure III-5. Phytoplankton biovolume dynamics in 2011 and 2012, from July to November, compared with cyanobacteria biovolume, and the biovolume of the fractions of cyanobacteria that are *Cylindrospermopsis raciborskii*, potential toxin producers or potential cylindrospermopsin producers.

Data summarized in Table 2 considered the following potentially toxic genera found in Alqueva reservoir with different distributions: (i) as potential CYN producers *Cylindrospermopsis*, *Raphidiopsis*, *Anabaena* and *Aphanizomenon*, (ii) while potential toxin producers includes potential CYN producers and *Oscillatoria/Planktothrix, Pseudanabaena*, *Microcystis*, *Phormidium* and *Woronichinia*.

While no *C. raciborskii* cells were detected at Senhora da Ajuda, located at the influx of Guadiana River, this species dominated the 2011 CyanoHAB in 4 out of 5 sampling sites, namely Lucefécit, Mourão, Álamos and Alcarrache (**Table III-II**), and the 2012 bloom at 3 sites (Lucefécit, Mourão and Alcarrache). At these sampling stations in September 2011 and 2012, 100% of the biovolume of cyanobacteria present corresponded to potentially toxic species, cyanobacteria abundance constituted 99.2% to 99.9% of total phytoplankton abundance, and *C. raciborskii* abundance ranged from 92.7% to 100% of the abundance of potential CYN producers (**Table III-II**).

Table III-II. Relative abundance and biovolume proportions of cyanobacteria over total phytoplankton (Cyano/Total Phyto), potential toxin producers over cyanobacteria (Pot. Tox./Cyano) and *Cylindrospermopsis raciborskii* over potential cylindrospermopsin producers (*C.raciborskii*/ Pot CYN) in samples taken in September 2011 and 2012.

Sampling site	**Sampling month**	**Cyano/Tot.Phyto abundance (%)**	**Cyano/Tot.Phyto biovolume (%)**	**Pot. Tox. /Cyano abundance (%)**	**Pot. Tox. /Cyano biovolume (%)**	***C. raciborskii/ Pot. CYN*** **abundance (%)**	***C. raciborskii/ Pot. CYN*** **biovolume (%)**
Srª da Ajuda	Sep-11	51.2	2.1	100.0	100.0	0.0	0.0
	Sep-12	32.0	1.4	100.0	100.0	0.0	0.0
Lucefécit	Sep-11	99.7	93.5	100.0	100.0	97.3	97.8
	Sep-12	99.9	88.9	100.0	100.0	99.6	98.1
Mourão	Sep-11	99.8	99.3	100.0	100.0	100.0	100.0
	Sep-12	99.5	88.3	100.0	100.0	100.0	100.0
Álamos	Sep-11	99.3	93.0	98.7	100.0	99.7	99.5
	Sep-12	78.9	43.3	72.6	99.0	0.0	0.0
Alcarrache	Sep-11	99.2	94.4	100.0	100.0	99.6	99.9
	Sep-12	97.7	84.2	100.0	100.0	92.7	94.0

Figure III-4 shows *C. raciborskii* abundance in samples taken in July, September and November of 2011 and 2012. *C. raciborskii* was present in Mourão samples as early as July 2011 and in Alcarrache samples taken in the same month in 2012. It was also present, albeit in low densities (<100 cells mL^{-1}) in November 2012. Abundance values recorded in September 2012 ranged from 9.6 x 10^{4} cells.mL^{-1} in Alcarrache to 6.0 x 10^{5} cells.mL^{-1} in Lucefécit. During September 2011 abundances were generally greater than 1.2 x 10^{5} cells.mL^{-1} (and as high as 5.5 x 10^{5} cells mL^{-1} in Lucefécit).

Figure III-5 which shows abundances expressed as total cell volume, points to the dominance of summer blooms by chlorophytes at Senhora da Ajuda, despite the high cell number values for *Microcystis* (as shown in **Fig III-4**).

Molecular confirmation of *Cylindrospermopsis raciborskii* identification

Positive PCR amplification of specific *C. raciborskii* gene sequences were obtained for the nitrogen fixing gene *nifH*, a highly conserved gene encoding for dinitrogenase reductase (Dyble *et al.*, 2002). Illustrating amplification of specific sequences of this gene, obtained for cultured isolates and environmental water samples. Product bands are shown in **Fig III-6.**

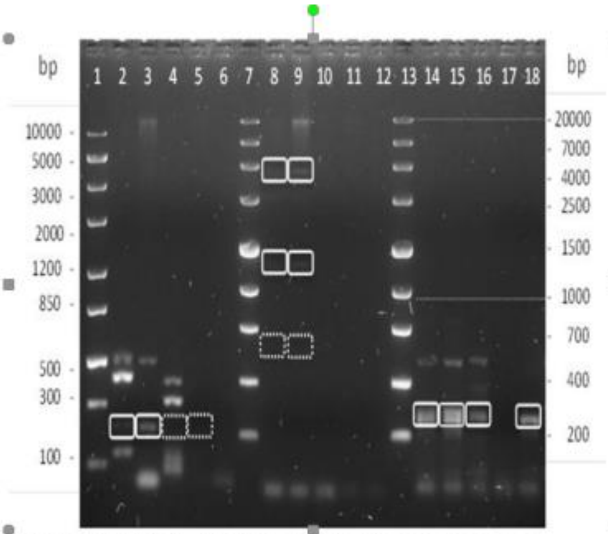

Figure III-6. Electrophoresis agarose gel showing positive results encircled with a continuous line and negative results with a dashed line. PCR products were obtained with *nifH cpcBA-IGS* and general cyanobacteria (*cya)* primers for cultured isolates of *Cylindrospermopsis raciborskii* (lanes 2, 8 and 14) and natural samples DNA (lanes 3, 4, 9, 10, 15 and 19). Negative controls in lanes 5, 6, 11, 12 and 17; and positive control for PCR in lane 16. Lane sequence loaded with:

1) ZipRuler™ Express DNA Ladder 1
2) *Cylindrospermopsis raciborskii* isolate *pcr nifH* product
3) Lucefécit Sep/2012 (lugol fixed) *nifH* product
4) Mourão Sep/2011 (frozen water sample) *nifH* product
5) *Microcystis aeruginosa* PCC 7005 *nifH* product
6) *nifH* PCR negative control
7) ZipRuler™ Express DNA Ladder 2
8) *Cylindrospermopsis raciborskii* isolate *pcr cpcBA-IGS* product
9) Lucefécit Sep/2012 (lugol fixed) *cpcBA-IGS* product
10) Mourão Sep/2011 (frozen water sample) *cpcBA-IGS* product
11) *Microcystis aeruginosa* PCC 7005 *cpcBA-IGS* product
12) *cpcBA-IGS* PCR negative control
13) ZipRuler™ Express DNA Ladder 2
14) *Cylindrospermopsis raciborskii* isolate *pcr 16S cya* product
15) Lucefécit Sep/2012 (lugol fixed) *16S cya* product
16) *Microcystis aeruginosa* PCC 7005 *16S cya 16S cya* product
17) 16s Cya PCR negative control
18) Mourão Sep/2011 (frozen water sample) *16S cya* product.

As negative controls, amplification products for *Microcystis aeruginosa* (PCC7005) DNA (Lane 5) and for no DNA (Lane 6) were used. Positive amplification of a fragment of 225 bp of the *nifH* gene is shown for a culture of an isolate recovered from a Mourão 2011 sample (Lane 2) as well as for a Lugol fixed 2012 sample from Lucefécit (Lane 3), but was absent for a frozen September 2011 sample from Mourão (Lane 4), which instead presented larger amplification products, some also present in previous adjacent lanes. Positive samples for the *nifH* 225 bp fragment were also amplified by the *cpcBA-IGS* gene primers (lanes 8 - 9), but generated much larger fragments than the expected 638 bp. Positive controls for the whole procedure were provided by the amplification of *C. raciborskii* DNA samples as well as *M. aeruginosa* (PCC7005) DNA with general *cya* 16S primers (lanes 14-18).

Toxicity sublethal assays and Toxicity assays

Toxicity assays results are summarized in **Table III-III**. Most of the tests conducted in 2011 revealed no toxicity, justifying why the battery of assays used was strongly reduced for 2012. Toxicity was only detected for 2011 water samples from Mourão and Alcarrache (*Vibrio fischeri* - 30 min EC_{50} respectively of 48.7% and 44.3%) and for Senhora da Ajuda sample (*Thamnocephalus platyurus* – 24 h EC_{50} 63.0% and *Daphnia magna* feeding inhibition of 40% after 24 h).

Table III-III. Lethal and sublethal effects (expressed either as median effective concentration in % – EC_{50} or as percentage inhibition relatively to the standard control) of water samples collected in September 2011 and 2012 at five sites of the Alqueva reservoir.

Sampling site	Sampling month	*Vibrio fischeri*	*Thamnocephalus platyurus*	*Daphnia magna*			*Pseudokirchneriella subcapitata*	*Brachionus calyciflorus*	
		Bioluminescence 30 min - EC_{50} (%);(v/v)	Mortality 24 h -EC_{50} (%);(v/v)	Immobilization/ mortality 48 h EC_{50} (%);(v/v)	Feeding after 24 h EC50 (%);(v/v)	Reproduction after 21 days	Growth after 72 h	Feeding after 48 h	Population growth after 48 h
Sr^a da Ajuda	Sep-11	n.t.	63.0	n.t.	40.0	n.t.	n.t.	n.t.	n.t.
	Sep-12	n.t.	n.t.	n.t.	n.d.	n.d.	n.d.	n.d.	n.d.
Lucefécit	Sep-11	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
	Sep-12	n.t.	n.t.	n.t.	n.d.	n.d.	n.d.	n.d.	n.d.
Mourão	Sep-11	48.7	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
	Sep-12	n.t.	n.t.	n.t.	n.d.	n.d.	n.d.	n.d.	n.d.
Álamos	Sep-11	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
	Sep-12	n.t.	n.t.	n.t.	n.d.	n.d.	n.d.	n.d.	n.d.
Alcarrache	Sep-11	44.3	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
	Sep-12	n.t.	n.t.	n.t.	n.d.	n.d.	n.d.	n.d.	n.d.

n.d.- not determined
n.t. - no toxicity detected

Detection of cylindrospermopsin

Traces of CYN (< 0.25 µg l^{-1}) were observed in September of 2011 and 2012 at Mourão and also at Alcarrache in September 2012, since as shown in **Figure III-7**, the parent peak (*m/z* 414.0) and the 3 main fragmentation peaks (*m/z* 271.9, 301.9 and 371.0) of the MS^2 standard are present in the sample MS^2 mass spectra for the same LC peak retention time (2.7 min).

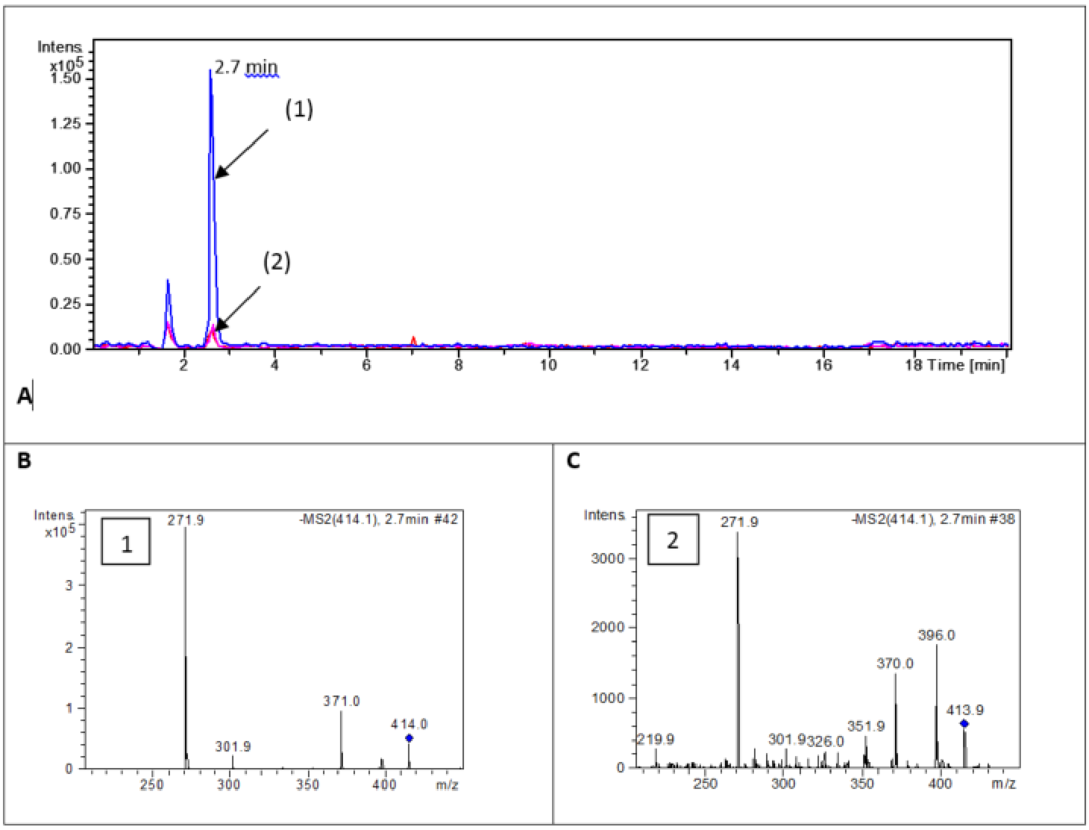

Figure III-7. Cylindrospermopsin ion trap LC/ESI–MS/MS analysis. (A) - Extracted ion MS^2 chromatograms, using the ESI negative mode, of a standard solution of CYN, peak (1), and of a water sample from Mourão (2012), peak (2). (B) - CYN standard solution MS^2 mass spectrum (RT – 2.7 min). (C) - Mourão sample MS^2 mass spectrum (RT – 2.7 min).

DISCUSSION

Though the present work constitutes the first report of *C raciborskii* CyanoHABs in Alqueva reservoir, data from a previous monitoring program have reported very high summer CyanoHABs density (Galvão *et al.*, 2008), without indicating the species composition of the blooms. It would be interesting to re-examine preserved samples for molecular confirmation of *C. raciborskii* presence or absence.

Molecular identification of cyanobacterial samples is important since morphological characteristics can vary depending on environmental conditions. In fact, there have been some studies using genetic identification to discriminate cyanobacteria species of the genera *Microcystis*, *Anabena*, *Nodularia*, and *Cylindrospermopsis* (Beltran and Neilan, 2000; Bolch *et* *al.,* 1996, Neilan *et al*., 1995; Otsuka *et al*., 1999; Zehr *et al.*, 1997; Dyble *et al*., 2002; Wilson *et al*., 2000) but most studies differentiating between cyanobacteria filotypes were done on axenic cultures, while environmental samples pose a vaster challenge (Wilson *et al.*, 2000, Dyble *et al.*, 2002). One of their major setbacks is the presence of PCR inhibitors, which require special treatment during extraction and present a challenge in every reaction (Wilson *et al.*, 2000). Prewashing of cyanobacterial samples before DNA extraction was used in this study in an attempt to counter possible PCR inhibitors. The same approach could be applied to DNA extracted from other Alqueva preserved samples in order to establish *C. raciborskii* expansion in this watershed. Nevertheless a better understanding of *C. raciborskii* genomic diversity is still needed in order to explain detected variability in amplification products. Unlike results presented by Dyble *et al.* (2002), not all fragments obtained with *nifH* gene primers had the expected 225 bp size, indicating some variability in gene sequences amplified by these primers, which should be investigated. Likewise amplification for the *cpcBA-IGS* gene primers (lanes 8 – 9 in **Figure III-6**) generated much larger fragments than the expected 638 bp in agreement with high diversity also found by Dyble *et al.* (2002). Other sets of specific primers with less variability have been used by other authors (Wilson *et al.*, 2000), but fail to discriminate between *C. raciborskii* isolates. In view of detected morphological specificities (akinetes adjacent to terminal heterocyte), characteristics also shared with the CYN producing strains from New Zealand (Wood and Stirling, 2003), more thorough genomic studies of the present isolates should be performed.

Detected *C. raciborskii* dominated, with precise regularity in 2011 and 1012, late summer CyanoHABS in Alqueva reservoir, indicating the potential for new occurrences each year, probably linked to akinete accumulation and synchronized germination. According to Moore *et al.* (2003) akinete formation is promoted by high concentrations of soluble reactive phosphorous and akinetes may rest for long periods in the sediments, meaning that preexisting *C. raciborskii* blooms in lentic systems favor the development of future blooms, once conditions become favorable. Furthermore, Portuguese *C. raciborskii* strains from other nearby water bodies have also been found to produce high concentrations of akinetes (Saker *et al.*, 2003). Akinete germination in *C. raciborskii* seems to be temperature dependent, requiring water temperatures above 22–24 °C. Such temperatures are common in Alqueva waters probably favoring akinete germination. In fact, summer average water temperatures, registered for June, July and September at Lucefécit (25.5 ºC), Mourão (25.3 ºC), Álamos (25.1 ºC) and Alcarrache (25.6 ºC) were all above 25 ºC, which might help explain the detected periodicity for this type of CyanoHAB development in Alqueva waters. Present trophic conditions recorded for all sampling sites are evidenced by mean chlorophyll *a* content determined for June, July and September samples overpassing 33 µg l^{-1} in 2011 and 2012. Corresponding high phytoplankton abundances seem also propitious, both for akinete germination and vegetative cell growth, as Wiedner *et al.* (2007) concluded that excessive light might be a limiting factor. This species seems to prefer to grow in the shade, below the euphotic zone, and might use water clarity decrease as an opportunity to increase density. Noticeably Alqueva *C. raciborskii* CyanoHABs had similar abundances to blooms of the same species found for other areas in Portugal (Saker *et al.*, 2003), but the hereby reported blooms were almost mono-specific, absolutely dominated by *C. raciborskii*, while other detected blooms occurred in co-dominance with other cyanobacteria (Saker *et al.*, 2003).

While establishing *C. raciborskii* presence in Alqueva waters, concomitantly with trace amounts of CYN and absence of other potential CYN producers, data suggests the presence of *C.raciborskii* CYN producing strains, which should also be investigated to elucidate carriers of cylindrospermopsin production genes.

In spite of CYN production detection in laboratory cultures of strains isolated from Hungarian lakes (Antal *et al.*, 2011) and a significant correlation allegedly found in Italy between *C. raciborskii* and the presence of CYN (Manti *et al.*, 2005), *C. raciborskii* strains from Northern America and Europe are believed not to produce CYN (Sinha *et al*., 2012). Thus, present findings assume special relevance, pointing out for the need of more research on European *C. raciborskii* strains. Furthermore other potential CYN producers (*Anabaena* spp., and *Aphanizomenon* spp.) are regularly detected in Southern Portugal reservoirs (Galvão *et al*., 2008), where hereto cyanotoxin detection has been limited to microcystin variants (Galvão *et al*., 2012, Rodrigues *et al*., 2013).

CYN is known to undergo photolysis (Senogles *et al.,* 2001) and adsorption to some metals (Senogles *et al.,* 2001). It is also known that pH, but not temperature can interfere with these processes (Senogles *et al.,* 2001). Such effects might explain the apparently low levels of CYN detection recorded in the present study. Since CYN degradation kinetics is still not totally understood, an interrogation about a potential masking effect on detected CYN concentrations, due to high UV levels as well as high metal concentrations (e.g. As^+, Mn^+, Fe^{2+}) as detected by Palma *et al.* (2014), remains. In fact Barón-Sola *et al.* (2013) also found that some cations, like Mn^+, interfere with enzymes involved in CYN production. Furthermore, since CYN analysis was not included in the initial monitoring protocol, samples used for CYN detection were all types of frozen samples that remained after all other analysis were performed, meaning that they were kept frozen for long periods. Studies on the type of molecular damage generated by prolonged freezing and the effect of the extraction thaw-freeze procedure on toxin loss are still needed, since they might help explain the low amounts of CYN found.

Among all toxicity tests used, only *V. fischeri* bioluminescence assay, *T. platyurus* lethal assay and *D. magna* feeding assay had any response and only to the 2011 September water samples. The most sensitive species was *V. fischeri* inhibited by two different water samples (Mourão and Alcarrache). Nevertheless, this test failed to detect toxicity with Senhora da Ajuda 2011 water sample, which exhibited moderate toxicity for other assays. This toxicity cannot be related to CYN, since *C. raciborskii* and CYN were not detected in this upstream site. Responses detected for Mourão and Alcarrache 2011 September samples might also not be linked to the low concentrations of CYN detected, which were well below the LC_{50} of 574µg ml^{-1} mentioned for *T. platyurus* by De la Cruz *et al.* (2013).

CONCLUSIONS

Presented data calls for better understanding of *C. raciborskii* ecology in temperate regions and calls for genomic research on European filotypes to elucidate their toxicity potential. It also leads to the conclusion that phytoplankton monitoring programs in surface waters prone to the development of CyanoHABs should include ecotoxicological assays in order to screen for the need of specific toxin detection. Furthermore issues as: “1) how long does CYN and CyanoHABs take to sink to the bottom? 2) How long does CYN persist at different levels of the water column? 3) Does toxicity persist beyond initial CYN degradation?” - call for proper answers.

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CHAPTER IV

Phytoplankton dynamics and cyanobacteria related risks in Beliche reservoir (Portugal)

Abstract

Beliche is an oligotrophic, 27 years old reservoir that supplies water to half of the population of Algarve and is included on the national water quality monitoring program. In this study cyanobacteria related risks were investigated trough phytoplankton dynamics, and Cyanobacteria Harmful Algae Blooms (CyanoHABs) detection. Abundance proportions between phytoplankton groups as well as their fluctuations and shifts in *taxa* density and biovolume were studied in an attempt to shed light on relationships established between organisms, one of the most structuring features, in ecosystems. These links either synergistic or antagonistic could eventually affect water quality. Richness expressed as *taxa* number varied throughout time as expected and proportions between taxonomical groups were reasonably constant or suffered minor fluctuations. Chlorophyceae and Cyanobacteria diversities are inversely related but Diatoms seem to trend with Cyanobacteria indicating some kind of link. Clear detection of synergisms or antagonisms between taxonomical groups should be investigated with multivariate numerical analysis. The constant presence of potentially toxic cyanobacteria indicates that Beliche reservoir bears the strains needed to inoculate a highly toxic bloom, however densities of toxigenic species only surpassed the 2000 cells/ml guide level in 20 occurrences during the 9 years of this study and only one occurrence surpassed the second alert level of 10^5 cells/ml. Given the low levels of toxins and toxicity detected, even though Beliche reservoir is intensively used for drinking water production, and dominated by CyanoHABs producing species, human health toxicity risks don’t seem to be of great concern. Nevertheless the following questions remain to be answered in future works: - Which phytoplankton species are present when toxins are secreted? - Which environmental conditions trigger the expression of toxin producing genes? What promotes the productions of akinetes? What promotes akinetes germination?

Keywords: Phytoplankton dynamics, oligotrophic, cyanobacteria, Beliche reservoir.

1. Introduction

It has been acknowledged that phytoplankton surveillance gives more information on water quality changes than simple nutrient or chlorophyll-*a* concentrations (Vuorio *et al.* 2007). Cyanobacteria are common in freshwater lakes and reservoirs throughout the world. Under favourable conditions, some cyanobacteria strains can dominate phytoplankton within a water body and form nuisance blooms (Stewart *et al.* 2006). These microalgae are of great concern due to their potential production of noxious compounds and dangerous toxins.

In this study, abundance proportions between phytoplankton groups as well as their fluctuations and shifts in relative *taxa* abundance were analysed, exploring the concept that, relationships established between organisms, either synergistic or antagonistic, are one of the most structuring features in ecosystems, since established links define communities and affect biodiversity, which will eventually come to affect water quality.

There are several studies on relationships within plankton communities, most of them conducted in marine waters (eg. Decelle *et al*. 2012; Duarte *et al.* 2006). However there are also, some studies on freshwater species (e.g. Descamps-Julien & Gonzalez, 2005).

Most studies on the phytoplankton of freshwater ecosystems focus on bottom-up regulation of seasonal succession, neglecting other kinds of plankton dynamics. Nevertheless, relationships within phytoplankton populations, including cyanobacteria, should interfere with the dynamics of this prokaryotic plankton group and affect toxic bloom development.

This study addresses phytoplankton dynamics and toxicity risks in an oligotrophic, 27 years old Portuguese water supply reservoir, namely Beliche (**Figure IV-I**). As mentioned elsewhere (Galvão *et al.*, 2012), in Beliche stream hydrographic basin, deforestation and farming mal-practices, enforced for historical reasons, lead, decades ago, to agriculture desertion and human desertification. Farming activities are now limited to forestry, mainly by replanting pine woods, and extensive cropping of sparse almond trees. Human population density is below 20 habitants /Km^2 forecasting no significant human impacts in this catchment area, since no sewages, no pig style farms, no intensive or extensive farming and no industry are to be found.

This study is not based on a specifically designed experiment to analyse growth rates or toxicity development, but uses long time series of monthly monitoring data to infer eventual relationships between different *taxa*. The intention was to carry out an exploratory study that could enlighten eventual impacts on water quality, resulting from the mentioned relationships in order to study them in future works. An oligotrophic reservoir was chosen to minimise the “background noise” due to the complex nutritional dynamics that occurs in systems with nutrient enrichment. Therefore, the objective of this work was to get some insight into the following questions:

Are there detectable relationships between phytoplankton populations affecting phytoplankton composition and abundance? How and when do these relations contribute to the promotion of Cyanobacterial Hazard Algae Blooms (CyanoHABs)?

For a better approach, some assumptions have been presumed throughout this work:

1) predator populations dynamics were stable throughout the years;

2) and as stated by the Hutchinson paradox multiple species might dominate simultaneously this ecosystem.

Summarizing, the present study focused on temporal shifts in phytoplankton abundance and composition in an attempt to elucidate how ecological phytoplankton succession affects the dynamics of CyanoHABs.

2. Methods

Study area

Beliche dam is a 54 m high earth and rockfill wall (completed in March 1986), located in South Portugal in the Algarve region (Alonzo *et al.* 2005). It was built on the Beliche stream (Guadiana river basin) to supply the population from Eastern Algarve with urban and irrigation water (**Figure IV-1**)

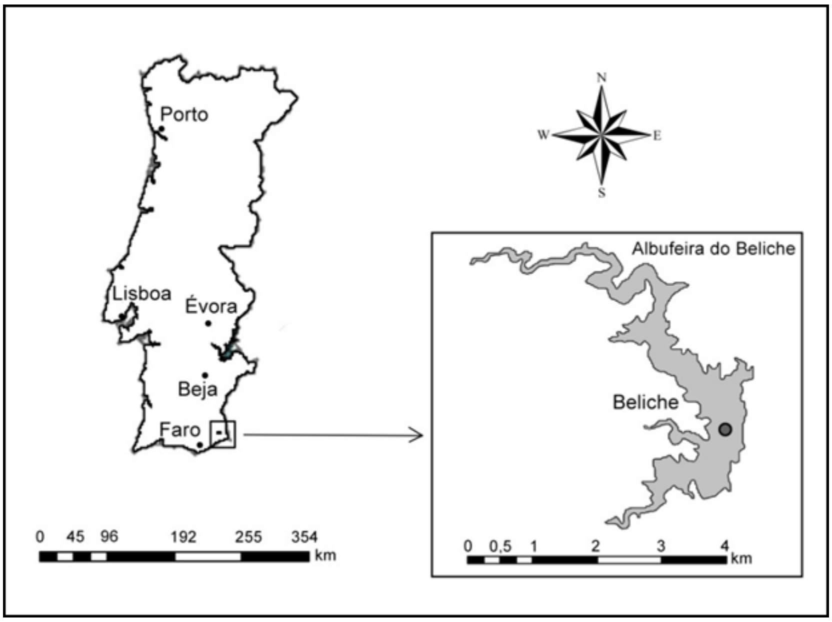


Figure IV-1 –Location of Beliche reservoir

Representing the Mediterranean climate irregularity, the region has been subjected to extreme periods of intensive rainfall and severe droughts. In fact, the hydrographic regime has repercussions on the reservoir hydraulic levels, and consequently on the nutrient cycling and aquatic life. Seasonal temperature variations are less wide-ranging, being mild along dry and wet seasons, and thus probably having less ecological impact than the hydrological regime. The reservoir drains a 98 km^2 watershed (**Table IV-I**) in the poorest and most deserted counties in Portugal.

Table IV-I –-Summary of hydrometric characteristics of Beliche reservoir

Reservoir	**Beliche**
Stream	Ribeira de Beliche
Watershed	Guadiana
Catchment area (km^2)	98.47
Latitude (mean)	37º 16' 35"
Longitude (mean)	-7º 30' 33"
Year of clompletion	1986
Max. water column height (m)	30
Total volume (x 10^6 m^3)	48
Flooded surface (ha)	292
Mean anual precipitation (mm)	644

Apart from cyanobacteria dominance in terms of phytoplankton abundance, water quality monitoring reveals a high quality status for this oligotrophic reservoir (Galvão *et al.* 2012).

Sampling and monitoring

Water samples were collected since July 2003 until April 2010 on a monthly basis, although in some months, more than one sample was collected. In these cases monthly data resulted from the arithmetic mean of data obtained for that month. Nevertheless, for some purposes, all the raw data were considered. Sampling campaigns were funded by several successive research projects CIANOALERTA I, II and III, CIANOTOOLS and ECOCRITERIO in the scope of INTERREG IIIA Programme and ECOTOXTOOLS funded by the National Science and Technology Foundation (FCT). These projects gathered for the same objectives several institutions in Algarve (Portugal) and Andalusia (Spain).

The sampling and analytical methods used in this study have been described elsewhere (Caetano *et al.*, 2014, see Chapter III). The phytoplankton monitoring program included, beyond composition and abundance analysis, the study of physical and chemical variables such as temperature, precipitation, nutrients and pigment concentrations, which were monitored simultaneously, through standard European methods.

Cellular volume and linked parameters were also calculated since May 2009. All the determinations were done as recommended (INAG, 2009) by the Portuguese Environmental Agency (APA) and European Union Standards (CEN).

3. Results and discussion

Throughout the sampling period (2003-2012) mean water temperatures (MWT) of 19 ºC, and maximum precipitation in the10 days preceding sample collection (Prec10d) of 84 mm (see **Table IV-II**) for a mean annual rainfall of 644 mm (see **Table IV-I**) confirmed the expected Mediterranean climate conditions. In **Table IV-II** are also given maximal, minimal, median and mean mass concentration values for dissolved inorganic nitrogen (DIN), soluble reactive phosphorus (SRP) and chlorophyll-*a* (Chlo-*a*) as well as the molar ratio DIN:SRP.

Table IV-II - Maximal, minimal, median and mean values for water temperature (ºC), mass concentration of dissolved inorganic nitrogen (DIN), soluble reactive phosphorus (SRP) and chlorophyll-a (Chlo-a), the molar ratio DIN:SRP and precipitation in the10 days preceding sample collection (Prec10d).

Variable Unit	MWT ºC	DIN mg/L	SRP mg/L	Chlo-a µg/L	DIN:SRP *	Prec10d mm
Maximum	27	1,24	0,20	40	93	84
Minimum	11	0,06	0,01	0,0	0,8	0
Median	19	0,27	0,02	1,2	24	1
Mean	19	0,35	0,03	1,9	32	11
Date of maximum	**11-08-2003**	**12-12-2005**	**09-08-2004**	**10-03-2004**	**15-01-2007**	**09-12-2008**

Nutrient concentrations confirmed oligotrophy and chlorophyll-*a* geometric mean of 1.1 µg/L (see Chapter II) was also well below 2.5 µg/L, considered to be the upper limit of oligotrophy (Vollenweider and Kerekes, 1982). High molar DIN:SRP ratios, well above the Redfield–Richards ratio of 16:1, also contradict the existence of favoring conditions for diazotrophic cyanobacteria, which are believed to outcompete other groups when nitrogen is limiting, profiting from their ability of fixing atmospheric N_2 (Chislock, *et al.*, 2014)

And yet, Cyanobacteria (CYA) dominated in terms of phytoplankton abundance for the whole sampling period, which is illustrated in **Figure IV-2 A**. Nevertheless, in terms of cellular biovolume proportions of the main taxonomic phytoplankton groups, Bacillariophyceae (Diatoms – DIA) dominated the system as illustrated by **Figure IV-2 B**. Biovolume data was only obtained since 2009, but the comparison holds when using data for exactly the same years.

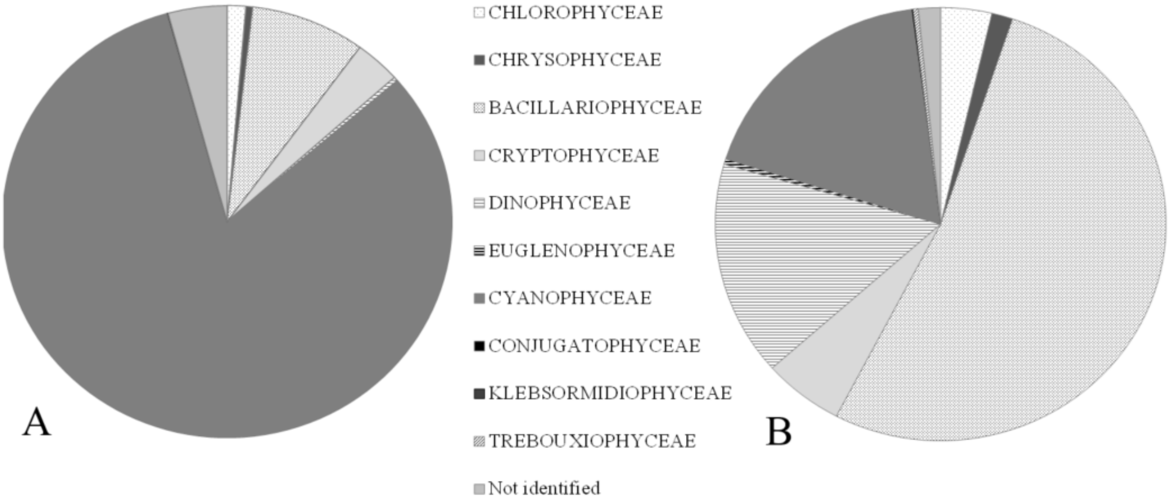


Figure IV-2: – **A** -Total phytoplankton abundance distribution by taxonomical group (data since 2003); - **B** - Total phytoplankton biovolume distribution by taxonomical group (data since 2009).

Cyanobacteria biovolume exceeded the proportion of 9.2 %, considered to be the limit of good ecological potential for Northern Portuguese reservoirs (European Union, Decision 2008/915/CE, 2008)

Chlorophyceae (CLO) presented the highest diversity in terms of species/genera richness, Diatoms (DIA) the second highest and Cyanophyceae (CYA) only the third, as indicated in **Table IV-III**, where acronyms for the main phytoplankton taxonomic groups are also given. Density distribution by these groups was very different (**Figure IV-3**). Conjugatophyceae and Klebsormidiophyceae were extremely scarce in total cell numbers and also in number of *taxa* present, throughout the all sampling period and were therefore disregarded for the purpose of the present study.

Table IV-III – Number of *taxa* observed per group, during the sampling period (2003-2010) with indication of acronyms.

Groups	Acronym	*Taxa* richness
Chlorophyceae	CLO	47
Crysophyceae	CRYS	8
Bacillariophyceae	DIA	35
Criptophyceae	CRIP	4
Dinophyceae	DINO	6
Euglenophyceae	EUG	4
Cyanophyceae	CYA	21
Conjugatophyceae	CONJ	2
Klebsormidiophyceae	KLEB	2
Total *taxa* observed		**129**

In order to better weigh the contribution of Cyanobacteria abundance to total phytoplankton abundance, comparison with other *taxa*, year by year (2003-2010), of proportions of accumulated densities are shown in **Figure IV-3**. CYA alone contributed more than all other *taxa* together. For the rest of the groups, abundances of Diatoms, Criptophyceae and Chlorophyceae (%DIA+CRIP+CLO) together account for higher abundances than all the remaining *taxa* together. Biovolume proportions for samples taken since May 2009 are presented in **Figure IV-4**, confirming diatom dominance with exception of 2009, where CYA alone account for almost 50% of the biovolume of the considered groups.

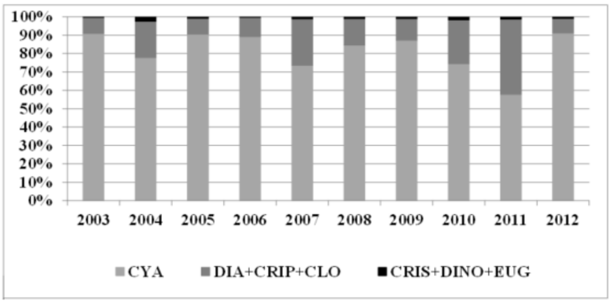


Figure IV-3 – Distribution of abundance proportions (% cell/ml) by group throughout sampling period.

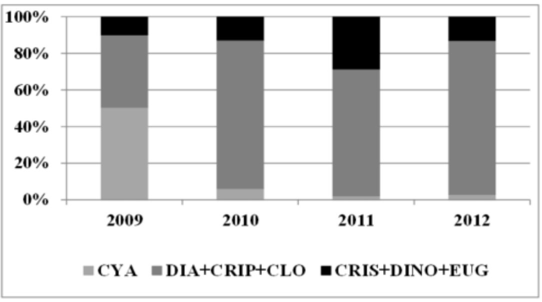


Figure IV-4 – Distribution of biovolume proportions (% mm^3/L) by group since 2009.

In fact a bloom of *Oscillatoria/Planktothrix* occurred in October 2009 (cf. **Table IV-IV**), diminishing the relative presence of all other species, especially since this filamentous cyanobacteria has a high specific biovolume, thus contributing to the high proportion of CYA biovolume in that specific sample, and to the total CYA biovolume, of that year, in general, while other groups account for less than 12 % in abundance, as shown in **Figure IV-3**.

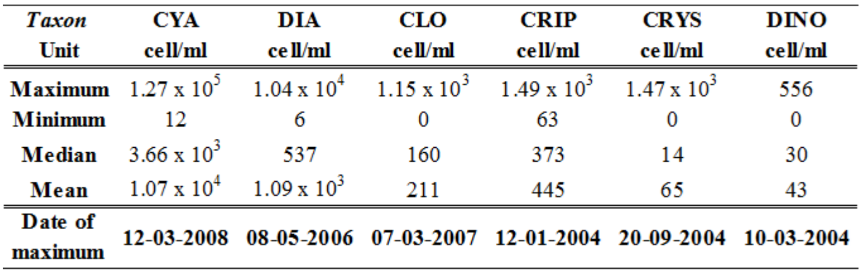
In **Table IV-IV** *maximum* abundance values for the four toxigenic genera/species that have surpassed the World Health Organization (WHO) alert level 1 of 2000 cells/ml are highlighted in dark grey. These are only the 4 *maxima* of a total of 20 such occurrences, from which only one was higher than the second alert level of 10^5 cells/ml. Other maxima for potentially toxigenic genotypes are highlighted in lighter grey. Nevertheless mean abundance values for several groups including the toxigenic genus *Microcystis* are well above 2000 cells/ml, contributing for the high values for Cyanobacteria in **Table IV-V**.

Table IV-IV– Cyanobacteria Genera – Total concentrations, *maxima*, *minima*, mean values, and dates of abundance *maximum*, with toxigenic genera highlighted in grey.

Identified genera / groups	Sum of total abundances (cell.mL^{-1})	Maximum (cell.mL^{-1})	Minimum (cell.mL^{-1})	Mean (cell.mL^{-1})	Date of the maximum
Anabaena	2.55×10^4	3.39×10^3	24	749	**21-08-2006**
Aphanizomenon	3.08×10^3	2.51×10^3	85	1.03×10^3	**09-08-2004**
Aphanothece	9.51×10^3	4.29×10^3	28	1.19×10^3	**02-11-2004**
Chroococcus	35	12	12	12	**12-07-2004**
Coelosphaerium	4.87×10^3	3.2×10^3	1.6×10^3	2.4×10^3	**10-07-2006**
Gomphosphaeria	2.13×10^3	592	189	355	**28-09-2006**
Lyngbya	177	177	177	177	**17-06-2004**
Merismopedia	297	142	12	49	**14-09-2005**
Microcystis	3.12×10^5	1.26×10^5	2	5.47×10^3	**12-03-2008**
Oscillatoria/Planktothrix	6.67×10^4	2.80×10^4	4	1.08×10^3	**19-10-2009**
Pseudanabaena	156	77	8	39	**13-09-2006**
Spirulina	379	379	379	379	**23-09-2009**
Woronichinia	272	272	272	272	**21-07-2004**
Other Chroococcales	4.95×10^5	7.12×10^4	12	5.10×10^3	**10-07-2006**
Other filamentous	2.18×10^4	4.99×10^3	2	303	**16-10-2006**
Other Cyanophycea	9.12×10^3	7.36×10^3	379	3.04×10^3	**14-07-2003**

Highest and lowest densities attained for the different groups in the reservoir, and dates for these occurrences are indicated in **Table IV-V** together with median and mean values. Median values for Cyanobacteria abundance are also well above the WHO alert level 1 of 2000 cells/ml, since 62.1% of the samples had CYA abundances above this limit.

Table IV-V – *Maxima*, *minima*, median and mean abundances for the main groups observed in Beliche reservoir throughout the sampling period with indication of date for each maximum value. CLO – Chlorophycea, CRYS - Chrysophycea, DIA – (Diatoms) Bacillariophyceae, CRIP – Criptophycea, DINO – Dinophycea, CYA – Cyanobacteria



Taxon **Unit**	**CYA** **cell/ml**	**DIA** **cell/ml**	**CLO** **cell/ml**	**CRIP** **cell/ml**	**CRYS** **cell/ml**	**DINO** **cell/ml**
Maximum	1.27×10^5	1.04×10^4	1.15×10^3	1.49×10^3	1.47×10^3	556
Minimum	12	6	0	63	0	0
Median	3.66×10^3	537	160	373	14	30
Mean	1.07×10^4	1.09×10^3	211	445	65	43
Date of maximum	**12-03-2008**	**08-05-2006**	**07-03-2007**	**12-01-2004**	**20-09-2004**	**10-03-2004**

Cumulative abundances by year and group are presented in **Figure IV-5**, where the logarithmic scale allows visualization of all groups, since the range of densities was extremely high. **Figure IV-5** illustrates once more the dominance of cyanobacteria in cell numbers, along the whole sampling period. Diatoms and Criptophyceae maintained proportions throughout the sampling period. Chlorophyceae were probably the most consistent, since the lower densities observed in 2003 are due to the fact that in 2003 only the last 6 months were sampled. It's also visible that Chrysophyceae were absent in 2003, probably because the sampled months were not the seasonally adequate for this group. However in 2006 their abundance was also very low. 2003 and 2006 were also the years, where the Cyanobacteria cumulative abundances were higher. It could be that the presence of high numbers of Cyanobacteria affects negatively Chrysophyceae.

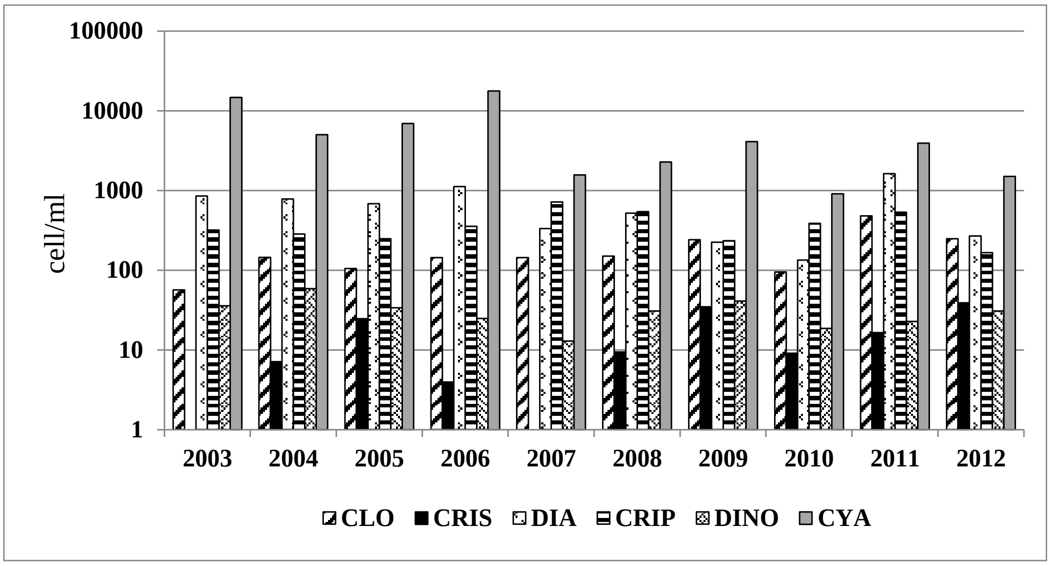


Figure IV-5 – Accumulated abundances of the main groups of phytoplankton by year. CLO – Chlorophyceae, CRIS - Chrysophyceae, DIA – Diatoms, CRIP – Criptophyceae, DINO – Dinophyceae, CYA – Cyanobacteria.

As shown in **Figure IV-6**, although the maximal abundance of cyanobacteria occurred in March 2008 (see Tables above), surpassing the WHO second alert level of 10^5 cells/ml, the year when this potentially toxic group, maintained its higher concentration was 2006, since from June to October their numbers were between WHO alert level 1 and 2, and almost reached the level 2 twice. It's also visible that Chlorophyceae were highly regular trough time and that Diatoms seem to trend with Cyanobacteria. In fact there are some studies that refer symbiotic relationships between Diatoms and Cyanobacteria (Foster *et al*., 2011; Prema and Anand, 2012). It's also mentioned in literature (Bucka, 1989) that some Chlorophyceae benefit or are stimulated in their growth after Cyanophyceae bloom decomposition, not just because of the decrease in water turbidity but also because toxins seem to act as growth stimulators for some Chlorophyceae species (e.g. *Scenedesmus quadricauda* commonly present in cyanobacteria blooms and appearing in large numbers specifically after hepatotoxic blooms). Therefore it could be expected that at the end of cyanobacteria blooms, especially the ones that were dominated by *Microcystis* spp., Chlorophyceae abundance increased, or at least the abundance of some Chlorophyceae species. However this relationship was not detected in this study, reinforcing the absence of toxin production.

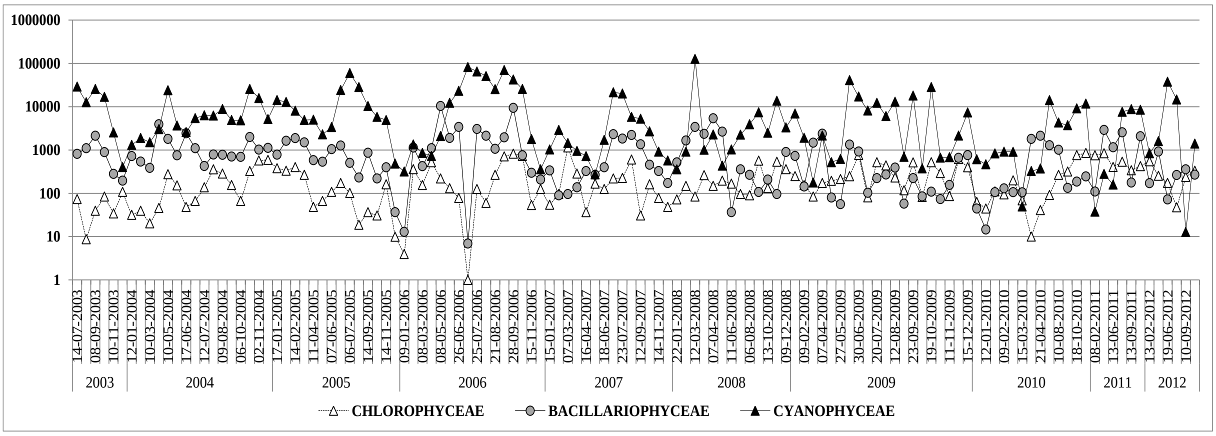


Figure IV-6 – Temporal evolution of abundance (cell/ml) distribution of the most relevant groups - Cyanobacteria, Bacillariophyceae (Diatoms) and Chlorophyceae throughout the sampling period.

To evaluate diversity trends throughout the sampling period the genera/species richness per group was also analysed by year (**Figure IV-7**).

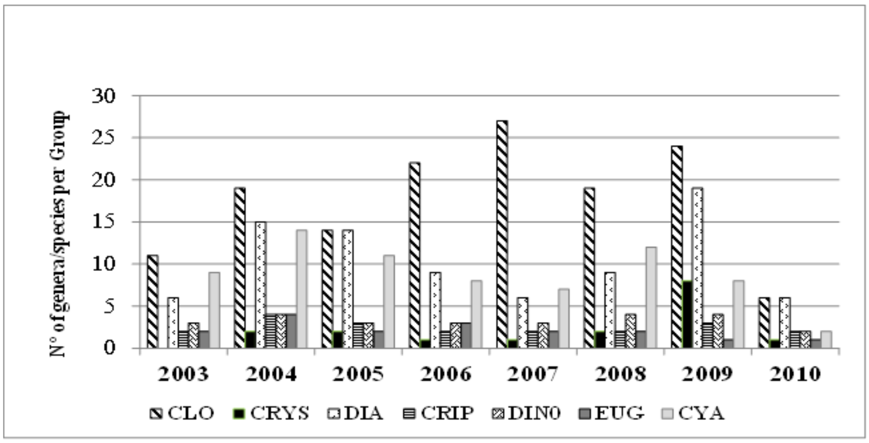


Figure IV-7 – Evolution of the number of *taxa* per group through the sampling period. CLO – Chlorophycea, CRYS - Chrysophycea, DIA – Diatoms, CRIP – Criptophycea, DIN – Dinophycea, EUG - Euglenophycea, , CYA – Cyanophycea.

It seems that Chrysophyceae (CRYS), Euglenophyceae (EUG), Criptophyceae (CRIP) and Dinophyceae (DINO) had during these years a low and stable genera/species richness. Richness for Cyanobacteria (CYA) and Diatoms (DIA) seem again to trend together, but DIA presented a higher global diversity than CYA (see **Table IV-II**) for the whole period, with a richness maximum for 2009 samples. Excluding data from 2010, Chlorophyceae species richness was always above 10, and even above 20 in 2006, 2007 and 2009, years that followed an extreme drought in 2004/2005. Interesting is also the fact that in these years of higher CLO diversity, cyanobacteria diversity seems to have diminished. Highest CYA genera/species richness in 2004, happened during the mentioned severe drought. Both CYA and DIA suffered drastic reduction of species richness in the years following this drought (2006/2007), recovering CYA richness in 2008 and DIA richness in 2009.

Figure IV-8 presents the abundance distribution of main CYA genera, presenting also the mean, maximum and minimum values attained. As observed the highest concentrations were obtained by *Microcystis*, a toxigenic genus, and for Other Chroococales. This last *taxon* is very broad, and the genera/species included in this group were not identified, meaning that it may also contain toxigenic cyanobacteria. The same occurred with the groups, designated as Other filamentous and Other Cyanophyceae. *Microcystis* was also the genus presenting the wider range of abundance.

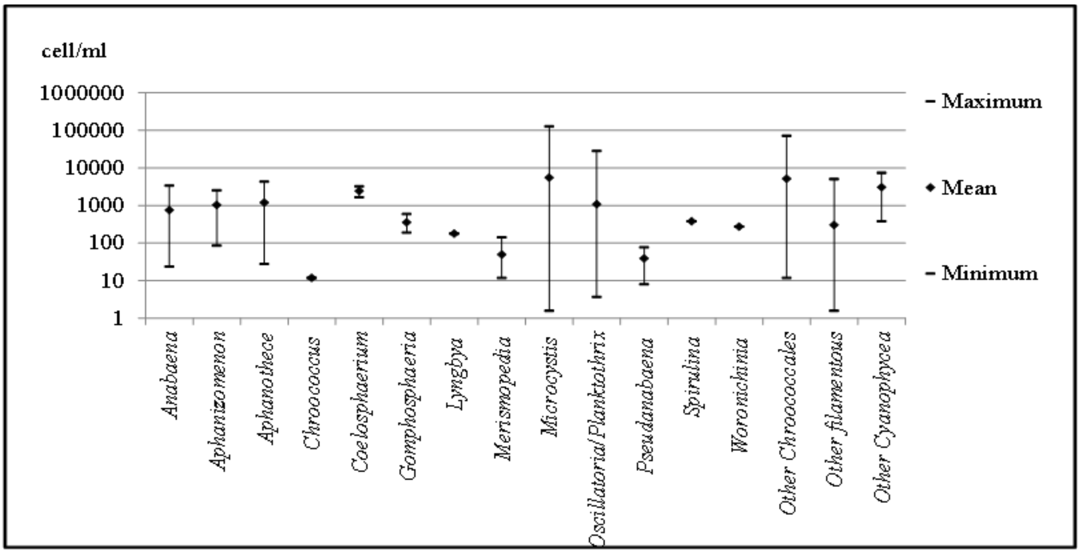


Figure IV-8 – Cyanobacteria abundance distribution by genera, observed throughout the sampling period. Mean, maximum and minimum are indicated on the line giving the range of abundances where zero abundances were disregarded..

The most significant CyanoHAB detected presented a *maximum* for *Microcystis*, in March 2008 (see **Table IV-IV** and **Figure IV-9)**. *Oscillatoria/Planktothrix*, also toxigenic, was another CYA genus that reached a very high abundance. Indeed *Mycrocistis* spp. and *Oscillatoria/Planktothrix* spp. were consistently present along the sampling period, though in low densities. During the summer of 2004, toxigenic species were present in very low densities, namely *Lyngbya* sp. (177 cells/ml in June), *Woronichinia* sp. (272 cells/ml in July) and *Aphanizomenon flos-aquae* (2514 cells/ml in August, 85 cells/ml in September and 471 cells/ml in October). Only *A. flos-aquae* abundance surpassing 2000 cells/ml is shown in **Figure IV-9**. *Anabaena* spp. were frequently detected but only formed mixed blooms in the summers of 2004 and 2006.

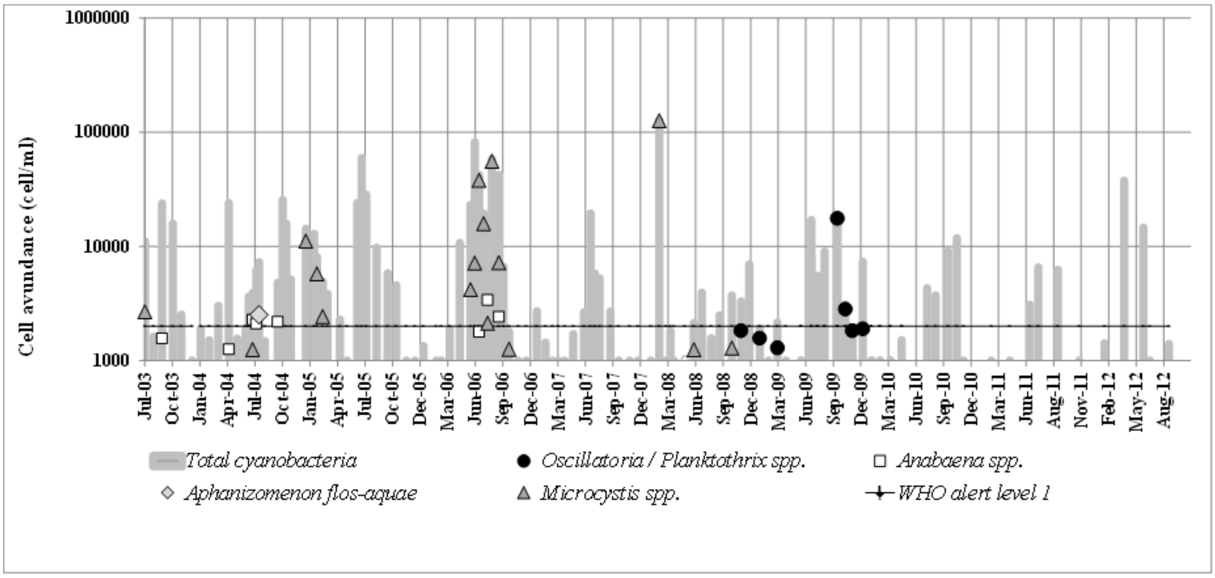


Figure IV-9 – Temporal abundance evolution of total cyanobacteria and main toxigenic *taxa* detected, indicating WHO alert level 1

Beliche reservoir probably bears the strains needed to inoculate a highly toxic bloom, but as shown in **Figure IV-9**, densities of these potentially toxigenic species only surpassed the 2000 cells/ml guide level in 20 occurrences in 9 years. Microcystin concentrations, for these events, were always below the WHO guide level of 1µg/L, except for one sample taken at the bottom of the reservoir (summer 2006) in which surprisingly 3.5 µg/L of microcystin were detected. Ecotoxicological assays were only performed since 2011, and were always negative for water samples (Reis *et al.*, 2013), though some bottom sediments with high metal concentrations tested positive. In fact, 2010 and 2011 were very rainy years, leading to surface discharges in this reservoir. This type of water management might have contributed to a decrease in cyanobacteria species densities.

In brief, though Beliche reservoir is intensively used for drinking water production, and dominated by CyanoHABs producing species, human health toxicity risks don't seem to be of great concern, given the absence of worrisome toxin concentrations. This confirms findings by other authors for cyanobacteria dominated oligotrophic systems, which also reported lower levels of toxin production than in eutrophic systems (Mur *et al.*, 1999).

4. Conclusions and final considerations

For the present study heavy statistical treatment was postponed, in order to graphically grasp the most evident relationships in 9 year monitoring data. This approach prevented the loss of information due to data normalizations, interpolations, outlier extractions or data ranking. However future application of multivariate techniques to treatment of the hereby produced database, that includes long time series for phytoplankton composition and abundance, shell probably elucidate phytoplankton succession (chronological clustering) or differentiate bottom-up from top-down driven CyanoHabs events.

Despite the fact that cyanobacteria have lower maximal growth rates than the majority of other phytoplankton species, in certain conditions they are able to overtake all other species, resulting in cyanobacteria blooms (Sedmak and Kosi, 1998), even in oligotrophic systems. This study revealed the consistent presence of toxigenic Cyanobacteria in Beliche reservoir, an oligotrophic environment, thus calling for the inclusion of toxicity monitoring in the Official Monitoring Program, through at least ecotoxicological assays.

However the performed data analysis did not allow a clear observation of synergisms between taxonomical groups or antagonisms unfavorable to cyanobacteria. The only mutually positive relationship observed was found between Cyanobacteria and Diatoms that trended together, both in abundances, biovolumes and diversity measured as genera richness. Controled mesocosmal experiments could be designed to better understand a possible negative trend between Cyanobacteria and Criptophyceae and/or Chrysophyceae. The same type of experiments could also help to understand Chlorophyceae stimulation by CyanoHAbs scenescence, as well as CyanoHABs promotion by Chlorophyceae bloom shadowing.

This study also lead to the perception that monthly sampling was insufficient for some purposes, such as detecting bloom beginnings, or the time lag needed to correlate physical and chemical variables with phytoplankton shifts. Such analysis would need higher sampling frequencies, even though the study site was an oligotrophic reservoir. While for precipitation, the accumulated rainfall from the last 10 days before samplings was considered, DIN and SRP concentrations were the determined for the same water collected for phytoplankton analysis. It seems obvious that, during a CyanoHAB in an oligotrophic environment, nutrients were already depleted, since they were consumed during bloom development, thus precluding correlations between high phytoplankton densities and nutrient enrichment. In fact, in a nutrient limited system, all the new inputs of nutrients are welcome and used for primary production.

According to Mur *et al.* (1999) cyanobacteria slow growth rates are compensated by high prevalence of populations once they have been established. Therefore it became clear that eco-strategic mechanisms developed by cyanobacteria have to be accounted for in phytoplankton dynamics studies. Questions like: -what promotes the productions of akinetes? What promotes akinete germination? – Which other phytoplankton species are present when toxins are secreted? – What triggers the expression of toxin production genes? Should be addressed.

Furthermore, interactions with other biological variables like zooplankton abundance and composition, as well as with virio- and bacterioplankton are still not well understood, and deserve future data treatment as (António, 2011) started to do for this reservoir.

Nevertheless, presented data consists in a much required baseline for this reservoir, recently classified as having a good ecological potential, specially, since the analyzed period includes one 18 month long extreme drought and two exceptionally rainy years. Additionally, intensive monitoring for another ten years will provide data adequate for time series analysis and eventual prediction of future climate change impacts.

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CHAPTER V

Phytoplankton based indices applied to oligotrophic freshwater reservoirs classification

ABSTRACT

Phytoplankton is used in the scope of the Water Frame Directive (WFD) as a biological indicator to estimate ecological potential in artificial and heavily modified systems, like reservoirs resulting from river dams. Present work compares several ecological indices, used to evaluate lentic system’s state or ecological potential, through their application to 5 oligotrophic reservoirs from the Algarve region (Bravura, Arade, Funcho, Beliche e Odeleite), profiting from long phytoplankton data series collected throughout 11 years. Therefore based on a detailed characterization of Algarve’s reservoirs, the goal was to assess and verify metrics in order to infer the ecological potential of these reservoirs in way to optimize the monitoring effort without loss of relevant information. Beyond the metrics recommended in the frame of European deliberations, others easier to obtain but with significantly special relevance to Southern Portugal reservoirs, were tested. The comparison allowed to recommend metrics simple and specifically adapted to the ecological potential evaluation based on phytoplankton, aiming to adequately stablish the boundary conditions between the ecological potential classes of Good and Moderate in reservoirs.

Key-words: phytoplankton based indices; ecological potential; trophic status; freshwater reservoirs

1- INTRODUCTION

Quality water resources are essential to all forms of life on Earth, but they tend to scarcity and thus attained high economic values. Their integrated management gained an indisputable relevance, no matter the intended use, be it for industrial or agriculture purposes, public supply or ecosystems and biodiversity conservation, for stream flow regulation or renewable energy production. These multiple water uses introduce a number of constraints to the quantity and quality of available water. The conjugation of these different water uses requires, as a common goal, the preservation and/or improvement of water quality, as foreseen in the Water Framework Directive (European Union, 2000) and resulting national laws.

In heavily modified water bodies, such as reservoirs resultant from the damming of waters, the main environmental and anthropic pressures on ecological water quality are: (i) accelerated siltation, (ii) decline of water level, (iii) toxic contamination, (iv) eutrophication and (v) acidification (Kira, 1997). Measurement of phytoplankton abundance, composition and biomass can easily be related to one of the three last mentioned pressures, namely toxic contamination, eutrophication and acidification. Phytoplankton biomass and composition has been often identified as linked to abiotic variables triggering eutrophication and consequent biological alterations. In fact, changes in the good ecological potential of a reservoir arise, in many cases, from disturbances in this type of variables, affecting the development of phytoplankton (Rodrigues *et al.*, 2013). Spatial distribution and seasonal succession of phytoplankton result from a multiplicity of ecophysiological responses of various phytoplankton species coexisting in a water body, and their continuous interaction with environmental variables, which justifies the use of phytoplankton composition and abundance as a biological indicator of water quality. In fact, phytoplankton was first used in water quality evaluation, in lentic water bodies by Naumann, in Sweden in the early XX century (Naumann, 1919). Since then, thousands of studies on phytoplankton were done contributing to clarify the relationship between biomass and phytoplankton composition and the trophic status of lakes and reservoirs. With the implementation in the European Union of the Water Framework Directive (WFD), the composition, abundance and biomass of phytoplankton reached the status of key component in the ecological characterization of reservoirs, assuming that changes in composition and abundance of many different *taxa* also reveal biomass increments and increases in frequency and intensity of phytoplankton blooms, which in turn reflect water quality degradation in lentic systems (European Union, 2000). European Union (EU) Member States sharing the same ecoregion organized Geographical Inter-calibration Groups (GIG), which worked to establish the upper and lower limits of the scales used in the ecological status classification of the various water bodies in Europe. With regard to lentic systems, the European Union (EU) funded several international projects EUROLAKES (Eurolakes, 2004), WISER (Wiser, 2012) and CIANOALERTA (Forján *et al*., 2008), that evaluated and compared data on abundance, composition and biovolume of phytoplankton in lakes and reservoirs of different EU ecoregions. The GIG from northern and central Europe ecoregions could benefit from data acquired in those projects, but the GIG from the Mediterranean region (MedGIG) did not, even though the CIANOALERTA projects had long term series of phytoplankton abundance and composition data for the main reservoirs in Algarve. Therefore even recognizing limitations in number and typology of reservoirs subjected to inter-calibration exercises and also to the biological data used to establish metrics for lakes in the Mediterranean region, provisional adoption of several indices according to the sub-regions were recommended. For the geographic area where Portuguese reservoirs are included, metrics listed on 2008/915/CE from October 30th (European Union, 2008), are chlorophyll-a (Chlo-a), total phytoplanktonic biovolume (TPB), cyanobacteria biovolume proportion (CBP) and Catalàn algal group index (IGA). Some of these metrics aren't at all applicable to Algarve reservoirs (Galvão *et al*., 2012), since these do not fall within the definition provided for lakes and reservoirs in Portugal. The repeal of that decision resulted in its replacement by a new European Commission Decision 2013/480/UE September 20 (European Union, 2013), which in what concerns the southern Portugal reservoirs remains in fault. Metrics for this region have been postponed to December 2016, in order to integrate the outcome of pending inter-calibration exercises for biological data. It is foreseen that the new Decision will recommend the adoption of a new system for assessing the Mediterranean phytoplankton (NMASRP) in reservoirs. Thus, due to the lack of inter-calibration with data from southern Portugal reservoirs, and the absence of EC law transposition into national legislation of the boundary values for their classification based on other phytoplankton metrics, the actual classification of the ecological potential of these reservoirs continues to be based only on the concentration of chlorophyll-a (INAG, 2009b).

Indeed, results from the research projects CIANOALERTA I, II and III that along with Chlo-*a* also evaluated the composition and abundance of phytoplankton, were not taken into account by MedGIG during the first phase of the calibration exercise. However, according to these projects all of these reservoirs are warm monomictic systems, with strong water column stratification from spring until autumn, with high hydraulic retention and high temperature. Summer surface water temperatures above 26 ºC systematically contribute to positively select cyanobacteria populations, many of them potentially toxic, during the dry season and sometimes also during the wet season. This dominance of cyanobacteria even in oligotrophic reservoirs (Galvão *et al.*, 2012), raises important questions in terms of ecological and ecotoxicological quality, not foreseen by the proposed indices.

Taking advantage from phytoplankton long term data series, collected since 2003, during the research projects CIANOALERTA I, II and III, CIANOTOOLS and ECOCRITERIO, (funded under the INTERREG IIIA support program), the present study compares 13 ecological indices to evaluate the ecological potential of lentic systems through the application to five reservoirs in the Algarve region.

Thus the objective was to evaluate, based on the characterization data from Algarve reservoirs, different metrics that allow to optimize the monitoring effort without loss of relevant information and which will also allow in the future to establish accurately the boundary conditions between the ecological potential classes - Good and Moderate - for freshwater reservoirs of southern Portugal.

2 – METHODS

2.1 – Study Area

Southern Portugal reservoirs are mainly used for drinking water production and agriculture. In this type of reservoir, the residence time is usually above seven months, with a high annual fluctuation in storage, reaching maximum values from January to June and minimum in September. They have an annual average temperature above 15 °C and less than 800 mm annual rainfall. Most watersheds are siliceous, dominated by shale and sedimentary rocks, the water hardness exceeding 50 µg $CaCO_3$/L (Reis *et al.*, 2004). This study considered the main Algarve reservoirs identified in **Figure V-1** through the location of their sub-basins, as Bravura, Arade, Funcho, Odeleite and Beliche respectively.

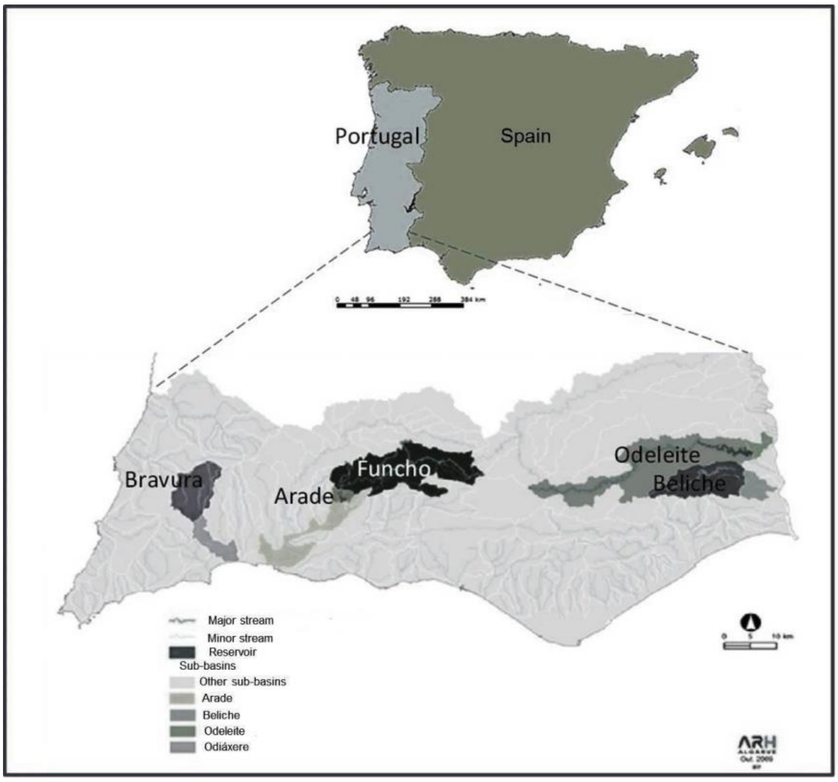


Figure V-1. Geographic location of the sub-basins that drain into the reservoirs of Bravura, Arade, Funcho, Odeleite and Beliche.

In **Table V-1**, some characteristics of the reservoirs under study are displayed. Its construction had as main objective the use of stored water for agriculture and/or public supply.

Table V-1. Some watershed data of the studied systems (http://www.snirh.pt/ "accessed in 02/05/2014").

Reservoir	Municipality	Year of completion	Stream	Drained basin (km^2)	Total capacity (hm^3)	Mean altitude (m)	Average annual precipitation (mm)
Bravura	Lagos	1958	Odiáxere	76.6	34.8	202	821
Arade	Silves	1956	Arade	12.39	28.4	121	637
Funcho	Silves	1993	Arade	212.6	47.7	223	744
Odeleite	Castro Marim	1996	Odeleite	347.5	130	293	722
Beliche	Castro Marim	1986	Beliche	98.5	48	198	644

2.2 – Sampling

Sampling campaigns were always performed by the same team, currently integrated in the Portuguese Environmental Agency (APA), subsamples of each recollection were sent to the laboratories of all research projects partners including “Águas do Algarve, SA (AdA)”; Algarve University (UAlg) and Huelva University (UHU). Initially adopted sampling methodology consisted in collecting discrete surface and bottom samples from the catchment towers. However, starting May 2009, methodology was altered to obtain integrated samples from the euphotic water column, in order to perform the phytoplankton analysis as recommended by the Sampling Protocol and Analysis of Phytoplankton Manual prepared by INAG (INAG, 2009a) according to European standards.

2.3 - Physical, chemical, biological and biochemical variables studied

The physical, chemical and biological data used in this study reported to 11 years of water quality monitoring in Algarve reservoirs. Entities involved used standard methods in all the performed analyzes. The physical, chemical and biological data were obtained under CIANOALERTA I, II and III, and CIANOTOOLS and ECOCRITERIO research projects since: 2001 for the Funcho reservoir, 2003 for Bravura, Beliche and Odeleite reservoirs and 2009 for Arade reservoir. Among the multiple physicochemical parameters determined in this study only data that could be integrated as nutrient pressure indicator was considered. Biological parameters, including composition and abundance of phytoplankton were determined by AdA until 2009, and afterwards by UAlg and APA, which were also responsible for the determination of specific biovolumes. Teams involved in these determinations joined international inter-calibration tests, on a regular basis. Phytoplankton analyzes, identification and quantification, were always performed in harmony with European Committee for Standardization (CEN, 2004) standards, according to the above mentioned Sampling and Analysis of Phytoplankton Protocol prepared by INAG Manual (2009a). As recommended specific biovolumes were calculated using the median obtained by the measurement of not less than 20 cells, for each *taxon*. For chlorophyll-a (Chlo-a) determination, phytoplankton was concentrated through filtration on GF/F Whatman membranes, which were cryopreserved and properly transported to the Biochemistry Laboratory of UHU, where pigment extraction and HPLC analysis was performed, according to Young's (1997) methods.

2.4 - Calculation of ecological indexes

The various trophic indices used were calculated either for annual mean values, for wet season (November to April) mean values and for dry season (May to October) mean values. All biovolumes based indices took into consideration data from May 2009 until April 2011 of each reservoir, except for Beliche in which data up to November 2012, was included.

2.4.1 - Trophic state indices (TSI (Chlo-a) Chlo to DIN and SRP)

Carlson's Trophic State Index (TSI) (Carlson, 1977), uses algal biomass duplication as a criterion to establish trophic state classes. TSI was determined based on the Chlo-a as indicated in Equation 1, since TSI based on Secchi disk transparency or total phosphorus are not appropriate for Algarve reservoirs (Galvão *et al*., 2012).

Equation 1 – Calculation of Carlson's Trophic State Index - TSI (Chlo-*a*).

$$TSI(Chlo\,\mathrm{a}) = 10 \times \left(6 - \frac{2{,}04 - 0{,}68\, ln(Chlo\,\mathrm{a})}{ln\,(2)}\right) = 9{,}81 \times\ ln\,(Chlo\,\mathrm{a})\ + 30{,}6$$

For each reservoir median and mean concentrations of Chlo-a, dissolved inorganic nitrogen (DIN) and soluble reactive phosphorus (SRP) were also calculated.

2.4.2 - Total phytoplankton biovolume (TPB)

Along with Chlo-a as a measure of phytoplankton biomass, total phytoplankton biovolume (TPB) of each sample, obtained through the sum of total cell volume of phytoplankton species present in each sample was also calculated.

2.4.3 - Cyanobacteria biovolume of (CB) and their proportion (CBP)

Cyanobacteria biovolume (CB) was determined for each sample as the sum of all biovolumes of detected cyanobacteria species. The proportion of cyanobacteria biovolume (CBP) of each sample corresponded to the proportion (%) of the total phytoplankton biovolume represented by cyanobacteria biovolume. However, for calculation of IGA (see 2.4.4) Cyanobacteria biovolume excluded Chroococcales species, with exception of *Microcystis* and *Woronichinia* genera. This metric, therefore, includes a weighing of potentially more toxic Chroococcales role.

2.4.4 - Index of algal groups (IGA)

Index of algal groups (IGA), proposed by Catalàn *et al.* (2003), being one of the currently recommended metrics to evaluate nutrient pressure on composition and phytoplankton abundance is based on 10 different proportions of algal groups biovolumes, weighing their potential contribution to the development of algal blooms, according to authors criteria. For IGA calculation biovolume proportions of different phytoplankton groups were used as indicated in Equation 2.

Equation 2: IGA index

$$IGA = \frac{1 + 0{,}1 \times Cr + Cc + 2 \times (Dc + Chc) + 3 \times Vc + 4 \times Cya}{1 + 2 \times (D + Cnc) + Chnc + Dnc}$$

Where:

D	Dinophyceae	Cc	Chrysophyceae colonial
Cnc	Chrysophyceae non colonial	Dc	Bacillariophyceae colonial
Chnc	Chlorococcales non colonial	Chc	Chlorococcales colonial
DnC	Bacillariophyceae non colonial	Vc	Volvocales colonial
Cr	Cryptophyceae	Cia	Cyanobacteria

2.4.5 - Phytoplankton Trophic status Index (PTI)

The trophic state index for phytoplankton (PTI) was developed under the WISER (2012) project in order to compare the results of the national classification systems. This metric weighs the total biovolume of each taxon present, in the sample, by the indicator value of its trophic optimum (Phillips et al., 2013), according to Equation 3.

Equation 3 - Phytoplankton trophic index (PTI).

$$PTI = \frac{\sum_{j=1}^{n} a_j \cdot s_j}{\sum_{i=1}^{n} a_i}$$

were $\boldsymbol{a}$ represents the total biovolume of species $\boldsymbol{j}$ to $\boldsymbol{n}$ and $\boldsymbol{s}$ the indicative value of its trophic optimum.

2.4.6 - Mediterranean Phytoplanktonic Trophic state Index (MedPTI)

The index of the trophic status for Mediterranean phytoplankton, MedPTI, was proposed by CNR - Istituto per lo Studio degli ECOSISTEMI - Italian (Marchetto et al., 2009), to assess the impact of eutrophication in Mediterranean reservoirs. This index weighs the biovolume of 44 selected *taxa* throuh the value of the trophic indicator assigned to each *taxon*, as indicated in Equation 4..

Equation 4 - MedPTI

$$MedPTI_i = \frac{\sum_{k=1}^{m} B_{j,k} \cdot v_k \cdot i_k}{\sum_{k=1}^{m} B_{j,k} \cdot i_k}$$

Where:

B_j represents the total biovolume of species k to m, v the trophic value of species k and i the indicative trophic value of k , obtained by squaring the inverse value of its tolerance determined by Marchetto *et al.* (2009), where v and i values for 44 species are given.

MedPTI determination is based on annual averages, according to authors guidance, and requires that listed species represent at least 70% of total annual phytoplankton biovolume within the studied reservoir.

2.4.7 - Berger-Parker dominance index (BP)

This simple dominance index is just the proportion of the most abundant phytoplankton species within the total phytoplankton. In this case it was decided to calculate it as a biovolume proportion.

2.4.8 - Potentially toxic cyanobacteria dominance Index (PTCD)

In Algarve reservoirs and in most other Southern Portugal reservoirs the three most abundant cyanobacteria species correspond to >90% of the total cyanobacteria biovolume. Thus, the hereby proposed index weighs the presence of potentially toxic cyanobacteria, among the three most abundant cyanobacteria species in each sample. For this calculation the following cyanobacteria genera detected in southern Portugal reservoirs were considered as potentially toxic: *Anabaena, Aphanizomenon, Cylindrospermopsis, Geitlerinema, Lyngbia, Microcystis, Oscillatoria / Planktothrix, Phormidium, Pseudoanabaena, Rhaphidiopsis* and *Woronichinia* (Caetano *et al.,* 2013). If the most abundant species belongs to one of these genera, the sum of its biovolume is multiplied by 4, the biovolume of the second most abundant species is multiplied by 3 if also potentially toxic, and should the third species also belong to this group of toxigenic genera, its biovolume is multiplied by 2. The multiplying factor for any non-toxic species is 1. The weighed sum thus obtained is divided by the non-weighed sum of their biovolume and multiplied by an indicator of the trophic status of the reservoir, represented by 1 for oligotrophy, 2 for mesotrophy and 3 for eutrophy.

The ponderation by the trophic status is essential, since several authors proved that toxigenic species produce higher concentrations of toxins in eutrophic systems, than in mesotrophic, the oligotrophic systems having the lowest loads of cyanotoxins. The index thus reflects the conditions where toxigenic species exhibit higher toxicity, assuming a minimum value of 1 for the presence of only non-toxigenic species in an oligotrophic reservoir, and a maximum value of 12 for toxigenic species in eutrophic reservoirs.

2.4.9 Phytoplankton Biotoxicity Risk Index (BRI)

Considering that the highest biovolume for a toxigenic species is sometimes the species biovolume of a dinoflagellate, and that these have been responsible for ichthyotoxicity episodes also in freshwaters of other countries (e.g. Angelo, 1991; Rengefors and Legrand, 2001) a phytoplankton biotoxicity risk índex is also proposed. This is in practice a very simplified index of dominance of potentially toxic cyanobacteria and dinoflagellates, obtained by the sum of the biovolume of the toxigenic species among the 3 most abundant cyanobacteria species and the 2 most abundant dinoflagellates. For this purpose the following genera detected in Southern Portugal reservoirs were considered as potentially toxigenic: (i) the dinoflagellates *Gymnodinium* e *Peridinium*; (ii) and the cyanobacteria *Anabaena, Aphanizomenon, Cylindrospermopsis, Geitlerinema, Lyngbia, Microcystis, Oscillatoria/Planktothrix, Phormidium, Pseudanabaena, Rhaphidiopsis e Woronichinia.*

The absence of any ratio in the calculation of this index allows the immediate differentiation of samples in terms of toxicity risks.

2.5 - Analysis of metrics suitability

The suitability of these metrics was based on the comparison of each index results with the results obtained for trophic classification of reservoirs over 11 years of evaluation of their nutritional regime, integrating the project reports (CIANOALERTA CIANOTOOLS and ECOCRITERIO) findings. Each index was classified according to the following criteria: (i) conceptual relevance as pressure indicator for nutrients, toxic contamination and / or acidification, (ii) suitability to specific goals as ecotoxicity and public health, (iii) feasibility and analytical reliability, (iv) integration of seasonal variability, (v) consistency, (vi) discriminant capacity, (vii) interpretability and (viii) usefulness for aquatic resources management.

3 - RESULTS AND DISCUSSION

Table 2 shows median and mean values of Chlo-a, DIN and SRP concentrations, obtained since the beginning of the study, including values of an 18 month long extreme drought (2005-2006), as well as particularly rainy years (2010/2011). From the comparison of these values with limits of the OECD indices or, in their absence, with the proposed values for xeric regions in the United States (EPA-822-B-01-008, USA), resulted the trophic classification also shown in **Table V-2**.

Table V-2. Median and mean values obtained for Chlo-a, DIN and SRP for the five reservoirs since the beginning of study.

		Bravura	**Arade**	**Funcho**	**Odeleite**	**Beliche**
Chlo-*a* *(mg/m^{3})*	**median**	0.99	0.84	0.97	0.72	1.16
	mean	2.35	2.04	1.50	1.04	1.85
	geometric mean	1.20	0.30	0.90	0.70	1.10
	dry season geo. mean	1.19	0.20	1.00	0.74	1.03
	result [a]	oligotrophy	oligotrophy	oligotrophy	oligotrophy	oligotrophy
DIN *(mg/m^{3})*	**median**	359	266	266	266	266
	mean	419	302	402	273	341
	result [b]	oligotrophy	oligotrophy	oligotrophy	oligotrophy	oligotrophy
SRP *(mg/m^{3})*	**median**	* 24.9	* 24.9	* 24.9	* 24.9	* 24.9
	mean	* 26.3	* 25.1	* 29.2	* 26.2	* 25.9
	result [c]	* mesotrophy	* mesotrophy	* mesotrophy	* mesotrophy	* mesotrophy

* values strongly biased by the high quantification limit of the used method

[a] <2.5 upper limit of oligotrophy according to OECD indices

[b] by comparison with limits for xeric regions in USA (EPA-822-B-01-008, USA).

[c] >10 lower limit of mesotrophy according to OECD indices

Methods used for determination of both total phosphorus and inorganic phosphate, have quantification limits above the lower limit of mesotrophy, impairing the assessment of the trophic status of these reservoirs using the obtained SRP data. Therefore the mesotrophy classification should be considered false. The remaining data points towards oligotrophy of these systems, although, over the years, specific situations of mesotrophy have occurred as well as exceptionally eutrophic events, particularly in Bravura, Arade and Funcho reservoirs.

TSI (Chlo-a), TPB, CBP, PTI and IGA indices calculation results are plotted as box-plots in **Figures V-2** to **7**, showing for each of the five reservoirs global data and also data corresponding to both the wet and the dry seasons fractions.

TSI data obtained using Chlo-a are compiled in **Figure V-2**. Upper limit for oligotrophy is also indicated, corresponding to a TSI (Chlo-a) = 30. According to the median of this index, Bravura, Funcho and Beliche reservoirs should be considered as mesotrophic and Arade and Odeleite reservoirs as oligotrophic, which contradicts the available nutrient data. According to nutrient data, only Bravura and Funcho might, be considered mesotrophic systems if a limit of DIN< 400 mg/m^3 is used. Beliche reservoir is clearly oligotrophic, according to nutrient data, a fact consistent with the low population density in its catchment area (<20 inhab./km^2) and the absence of potential impacts of human activities.

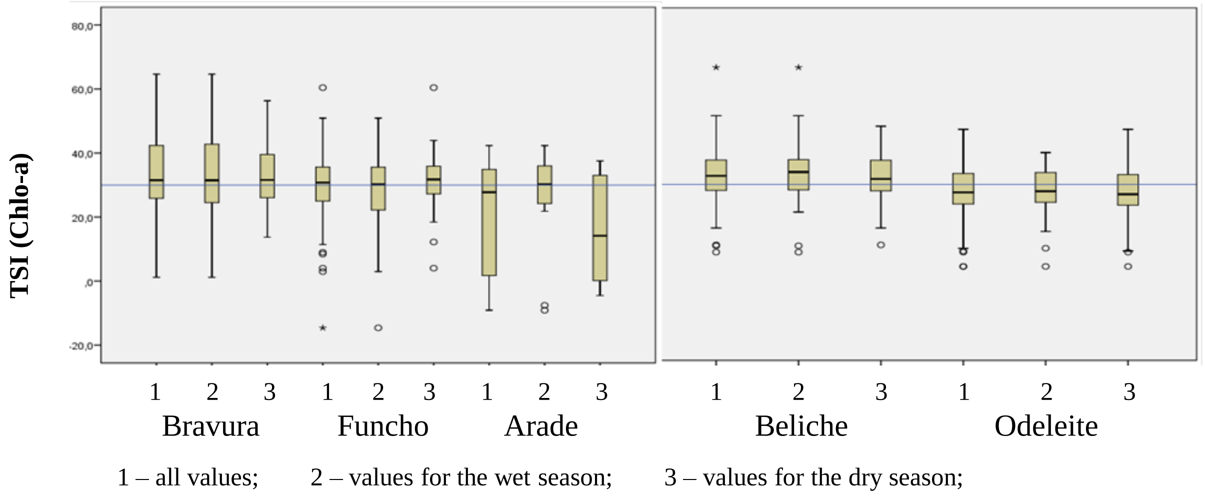


Figure V-2. Boxplots of the values of trophic state index TSI – Chlo-a, for the five studied reservoirs (—— oligo-mesotrophic limit).

Plotting TPB values in the same type of diagrams, median determined for all reservoirs appear well below the Good-Moderate boundary (**Figure V-3**). Only a few samples collected on Arade and Beliche reservoirs present singular values above the upper limit of class GOOD (1.9 mm^3/L). So according to this index the five reservoirs have good ecological potential.

Nevertheless, the extreme values detected in Arade (4.61 and 4.68 mm^3/L) match potentially toxic blooms. The first value spotted in September 2009 is from a cyanobacteria bloom of the genus *Anabaena*, which contributed to 85% of the TPB. This bloom lasted till October 2009, but with the presence of *Gymnodinium* spp,, with large cells of these dinoflagellates also potentially toxic.

Two TPB extreme values detected in Beliche are also related with two natural phytoplankton blooms, occurred in 2011, in the context of a healthy ecological succession triggered by massive precipitation, promoting diatom development. Only a singular value of 2.74 mm^3/L, which occurred in October 2009, matched to a potentially toxic cyanobacteria bloom from the genus *Oscillatoria,* contributing with 89 % to TPB.

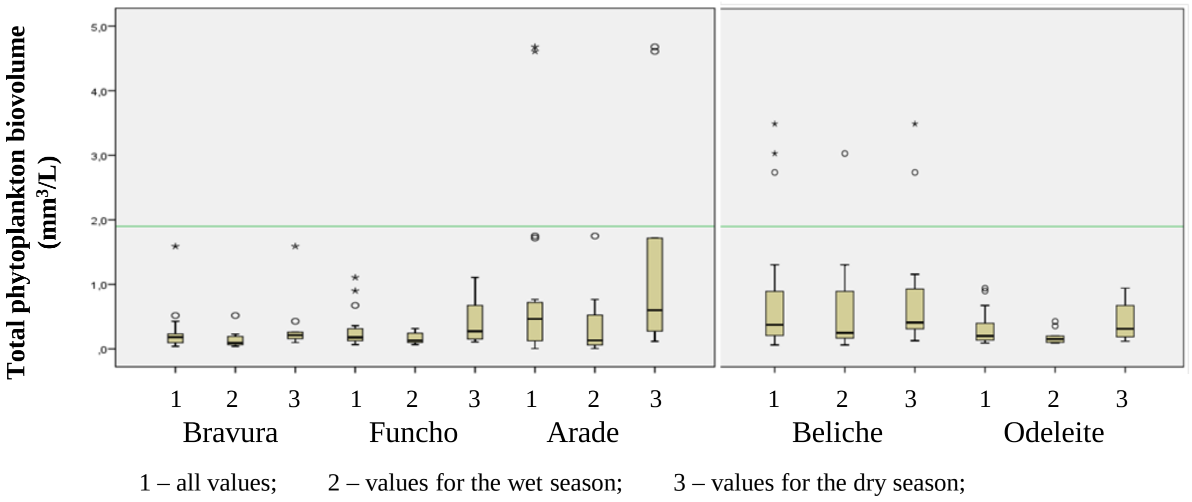
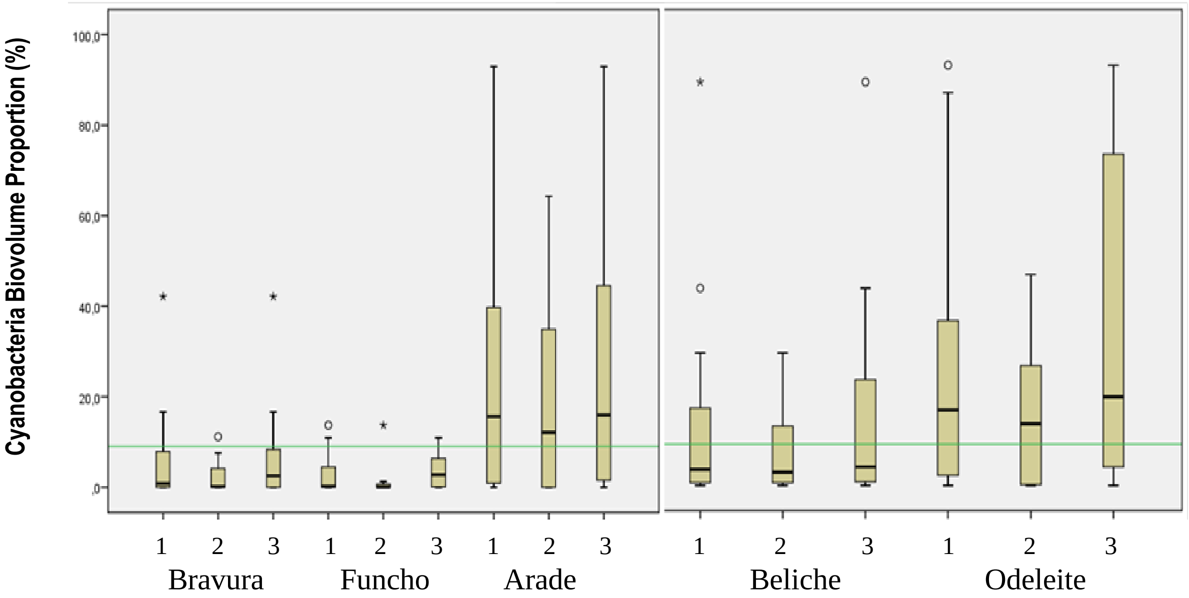


Figure V-3. Boxplots with the total phytoplankton biovolume values (TPB), indicating the GOOD class limit (<1.9 mm^3/L).

Using the cyanobacteria biovolume proportion (CBP), median values were inside the GOOD boundaries (below the GOOD-MODERATE limit) except for Arade and Odeleite reservoirs (**Figure V-4**). The few values that exceeded the limit for this proportion in the Bravura and Funcho reservoirs, in June 2009 (42.1%); July 2010 (16.6%) and February 2011 (11.2%), are negligible because they were related to very low TPB values, all lower than 0.2 mm^3/L, which do not represent any bloom. Beliche reservoir, although presenting 34% of the values above the limit, could be considered inside the limits of good potential, once the values of medians of all sites were well below the adopted limit for North Portugal reservoirs. On the other hand Arade reservoir with 58% of the values above the limit, reached CBPs of 93% in September 2009 for a total biovolume of 4.6 mm^3/L, reinforcing potential toxicity risks. Surprisingly Odeleite reservoir, considered a reference reservoir, obtained the worse results for this index, with 63% of the values classified as Moderate, sometimes reaching a CBP of 93%, as in October 2009.

Therefore applying TSI (Chlo-a) and CBP indices, and evaluating the result based on the index with worse median value, scored by each one of the five reservoir, all of them would be classified as Moderate. Although all are in compliance and well inside the Good potential according to TPB. The artificiality generated by the classifications obtained for these two indices is created by the fact that any slight increase in the numerator of the calculated equation, not followed by a similar increase in the denominator, will produce high values, without a real observed alteration in the trophic conditions. Very high values of CBP might be obtained when cyanobacteria are present in conditions of very low TPB.

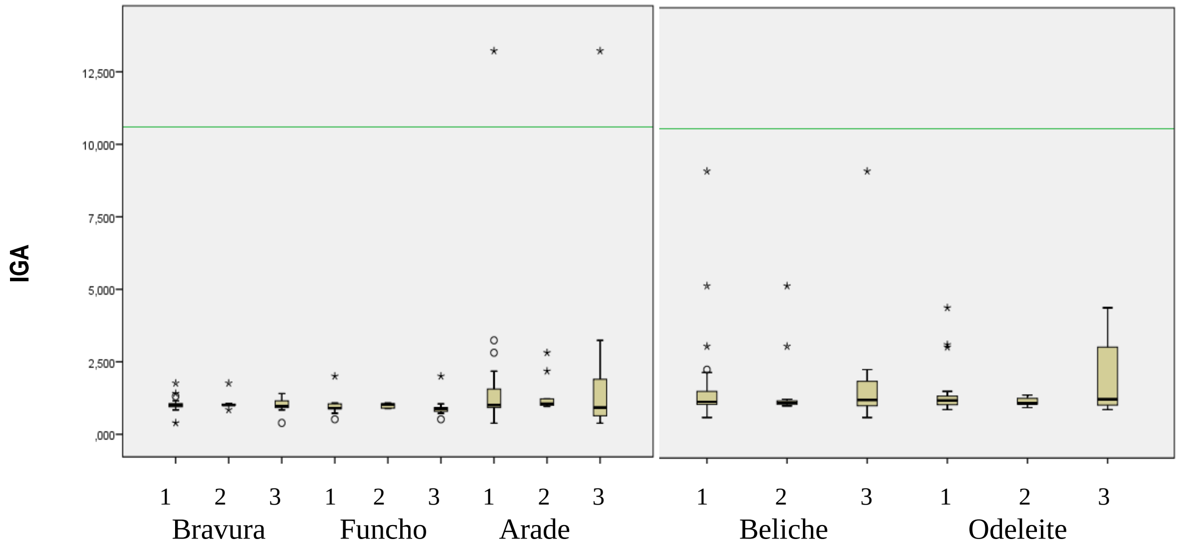


1 – all values; 2 – values for the wet season; 3 – values for the dry season;

Figure V-4. Box plots with CBP values and Good-Moderate limit (> 9.2 % Moderate and < 9.2 % Good).

CBP was considered an important indicator because it covers the presence of potentially toxic microalgae. But, in view of its dependence of TPB it should be always weighed by TPB classes, or instead the direct use of cyanobacteria biovolume (CB) values should be implemented.

Distribution of calculated IGA values are presented in **Figure V-5**, along with the line indicating the level above which the potential is no longer classified as Good. The figure clearly illustrates that all the studied reservoirs are inside the quality standards proposed by this index, revealing coherence between data from wet and dry seasons in all the sites. Only IGA from September 2009 sampling in Arade reservoir (IGA = 13.2), was higher than the boundary value (10.6). This extreme value could be explained by high cyanobacteria biovolume found in this sampling, since IGA uses a multiplying factor of 4 for cyanobacteria biovolume. In spite of the good results obtained for this index its interpretation is far from being simple, since its application to other clearly eutrophic systems, also produces good classifications (Reis *et al.*, 2013). In fact, IGA relevance seems to be controversial, once it weighs phytoplankton groups by taxonomy instead of function. IGA differentiates only colonial species from the non-colonial, does not distinguish *taxa* at the species or the genus level, and does not take their role in the ecosystem in consideration, as do for example PTI and MedPTI. Weighing all cyanobacteria as indicators of eutrophication IGA ignores the fact that they can be dominant in oligotrophic systems. On the other hand, other potentially toxic phytoplankton but non colonial, such as dinoflagellates, are not considered. But worse than that, to calculate IGA, it is necessary to obtain an enormous amount of information, whose relevance is masked when summarized in a multimetric index difficult to interpret. It seems to be possible to get better indicators with less information.

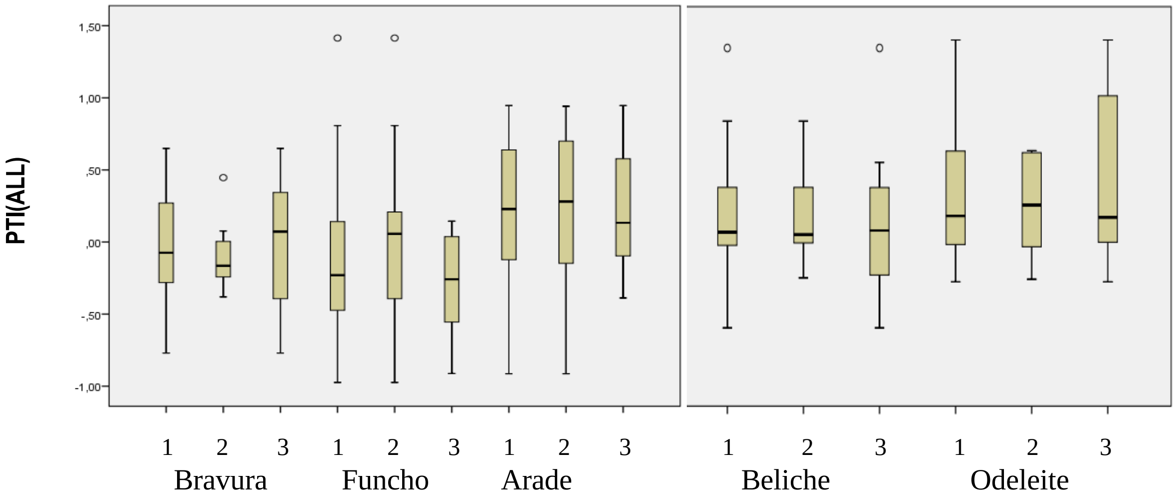


1 – all values; 2 – values for the wet season; 3 – values for the dry season;

Figure V-5. IGA values box plots (Good-Moderate limit line) found to the five studied reservoirs in booth wet and dry season.

Figure V-6 gathers data obtained for the PTI index gauged by the listed optimal trophic values of ALL list (Phillips *et al.*, 2013). This index weighs biovolume of each present *genus* by their optimal trophic value in terms of phosphorus. PTI development in the scope of EU FP7-WISER project, involved the analysis of data of 1795 lakes from twenty European countries. In addition to the list of optimal trophic values designated as ALL, other lists where produced only with data from lakes of different sub-regions. In the present work, results obtained using the Med-GIG optimum values were omitted, since they are not very different from the ones shown in **Figure V-6**, and the Med-GIG species list (Phillips *et al.*, 2013) is more incomplete in relation to species detected in Algarve reservoirs. As in previous figures, box-plot diagrams represent results using all the samples of each system, and the same data spread for samples taken during the wet season and the dry season. In the absence of reference values for this index, the relative homogeneity of medians obtained for any season in any reservoir should be noticed, although WISER project used only summer data for the PTI development.

PTI obtained data interpretation seems to still be too complex, but phytoplankton weighing at genus level, and not at large taxonomic groups level, should give this index a higher relevance and reliability than IGA. This should be studied by applying it to a different set of reservoirs, including mesotrophic and eutrophic ones.



1 – all values; 2 – values for the wet season; 3 – values for the dry season;

Figure V-6. Box plots with PTI (*ALL*) values to all the studied reservoirs.

In fact, considering that phytoplankton blooms of different species can be triggered by diverse environmental factors, since they depend on the specificity of the ecophysiological response (Galvão *et al.*, 2012), it will be necessary to develop indexes such as the PTI, that try to integrate this information.

Regarding the MedPTI index, the fact that it has been built based upon 44 species of Sardenha lakes limits its application to other lentic systems, since as shown in **Table V-3**, only occasionally, and especially in the year 2010, the percentage of biovolume of those species (> 70%) was compatible with its use (values shaded) in accordance to the authors recommendations (Marchetto *et al.*, 2009).

Table V-3. MedPTI and percentage of MedPTI species used for the five water systems in the years 2009, 2010, 2011 and 2012.Valid values are shaded.

		Bravura	**Arade**	**Funcho**	**Odeleite**	**Beliche**
2009	**% of MedPTI species**	65.0	52.0	55.8	65.5	55.7
	MedPTI	3.1	3.1	3.0	3.1	3.1
2010	**% of MedPTI species**	83.7	74.1	80.8	76.3	64.6
	MedPTI	3.0	3.1	3.1	3.1	3.1
2011	**% of MedPTI species**	95.2	25.1	32.2	49.6	93.1
	MedPTI	2.6	3.0	3.0	2.7	3.1
2012	**% of MedPTI species**	-	-	-	-	25.4
	MedPTI	-	-	-	-	3.1

Considering only the years where a percentage above 70%, was attained, all index values could be included in the Excellent class (values above 2.77), except the value for Bravura in 2011, which falls under the Good class (values above 2.45). The inapplicability of this index to the studied reservoirs suggests, however, the possibility of adopting a new list of indicator species, based on determinations of CIANOALERTA projects, in order to build a reliable and relevant index for these reservoirs.

Given the interpretation difficulties experienced with the European Commission Decision 2013/480 / EU of 20 September (European Union, 2013) proposed indexes, other metrics simpler to obtain and producing particularly relevant information in the present study were also determined. The results for Berger-Parker dominance index (BP) are summarized in **Figure V-7.** Since this is a particularly simple metric, BP has an easy interpretation.

Immediately it is noticed an increase in median values during the dry season as a result of systematic phytoplankton blooms in late spring and late summer. However, this index suffers from the same limitation that the CBP. BP is a ratio, whose denominator is the TPB, In case of low TPB values its value increases without corresponding ecological significance. Furthermore, without specific notes, it does not give any information about toxicity or taxonomic identity of the dominant species. Anyway, given that it is very easy to obtain, the setting of limits for this index it's recommended.

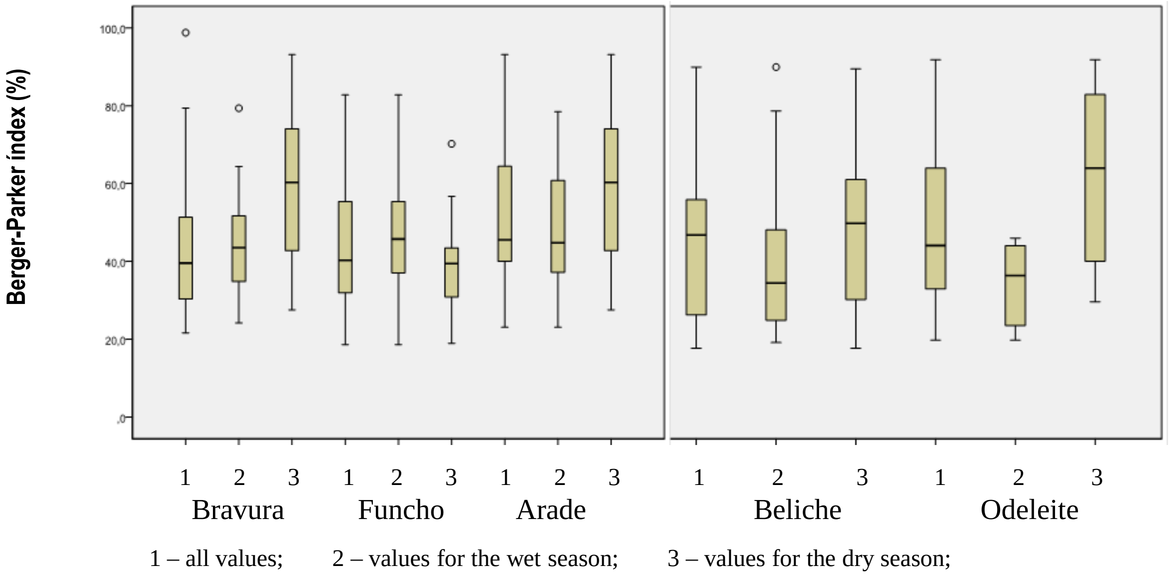


Figure V-7. Dispersion of Berger-Parker index values for total data of each studied reservoir and discriminated for dry and wet seasons.

In an attempt to overcome the difficulties discussed above with the addressed indices, two new indices are hereby suggested, taking in account the dominance of potential toxigenic species. **Table V-4** shows values used in the calculation of the potentially toxic cyanobacteria dominance index (PTCD). This index uses the biovolumes of the 3 most abundant cyanobacteria species in terms of biovolume, weighing biovolume of the ones that are considered potentially toxic (cf. genus listed in 2.4.8.) by multiplicative factors of 4 for the more abundant, 3 for the 2nd most abundant and 2 for the 3rd most abundant, if also potentially toxic. The ratio for this weighed biovolumes sum to the sum of unweighted biovolumes, is an indicator of potential toxicity associated with the system, which will range between 1 and 4. This metric can also be weighted by the trophic state of the reservoir (eg. Chlo-a data classification), in order to better explain, conditions under which cyanobacteria exhibit more toxicity, using a multiplying factor of 3 for eutrophy, 2 for mesotrophy and 1 for oligotrophy.

Table V-5 presents values used in the calculation of the biotoxicity risk index (BRI). The maximum value observed is < 5 mm^3/L, and thus compatible with oligotrophy. For mesotrophic reservoirs in Alentejo values up to 50 mm^3/L have been detected, and for eutrophic reservoirs in the same region up to 100 mm^3/L, but a thorough calibration is still needed.

Table V-4. Metrics based on the biovolume of the 3 most abundant cyanobacteria species in each sample

		Bravura	Arade	Funcho	Odeleite	Beliche
(A)Potential toxicity weighed biovolume sum ***(mm^3/L)***	**median**	0.017	0.073	0.010	0.192	0.073
	mean	0.051	1.808	0.043	0.531	0.515
	maximum	0.335	16.718	0.389	3.503	9.804
(B) Unweighed biovolume sum ***(mm^3/L)***	**median**	0.008	0.020	0.005	0.050	0.021
	mean	0.020	0.477	0.013	0.135	0.132
	maximum	0.106	4.286	0.099	0.879	2.451
$\mathbf{PTCD} = \frac{(A)}{(B)} \times \mathbf{1^*}$	**median**	1.4	3.6	2.9	3.9	3.6
	mean	2.1	3.1	2.5	3.2	3.3
	maximum	3.8	4.0	4.0	4.0	4.0

* 1 stands for oligotrophy

Table V-5. Biotoxicity Risk Index (BRI) based on the biovolume of potentially toxic phytoplankton in each sample (mm^3/L)

		Bravura	Arade	Funcho	Odeleite	Beliche
Sum of biovolumes of the 2 most abundant potentially toxic dinoflagellates ***(mm^3/L)***	**median**	0.002	0.003	0.014	0.005	0.010
	mean	0.016	0.078	0.047	0.011	0.103
	maximum	0.153	1.290	0.386	0.076	1.820
Sum of biovolumes of the 3 most abundant potentially toxic cyanobacteria ***(mm^3/L)***	**median**	0.003	0.015	0.001	0.039	0.018
	mean	0.011	0.483	0.010	0.126	0.129
	maximum	0.077	4.286	0.097	0.879	2.451
BRI (mm^3/L)	**median**	0.007	0.027	0.018	0.045	0.044
	mean	0.027	0.536	0.057	0.137	0.232
	maximum	0.154	4.286	0.387	0.879	2.451

These toxicity indicating indices, properly calibrated and together with TPB or Chlo-a, offer the reservoirs managers easily obtained information and probably more relevant, than other metrics based on phytoplankton, since they allow to foresee eco-toxicological and public health risks, due to cyanobacteria and/or dinoflagellate potential toxicity.

Their calibration will require statistical analysis of data from other reservoirs in the South of Portugal, applying indices also to eutrophic or mesotrophic systems, in order to define boundaries for the Good-Moderate classification. Despite the relevance of possible toxicity information, this may be present in clearly oligotrophic reservoirs, as in this work. Therefore it will be necessary that these indices allow the discrimination of such cases.

4 - CONCLUSION

Application of different indices to the five studied water systems allows to compare the obtained results and analyze their relevance and applicability. The evaluation of each of these indices in accordance with criteria set by the Environmental Protection Agency (USA) and/or the EU, such as: (i) conceptual relevance in terms of nutrients pressure indicator, toxic contamination and/or acidification (ii) suitability to specific goals as ecotoxicity and public health, (iii) feasability and analytical reliability, (iv) seasonal variability integration (v) response consistency, (vi) discriminant capacity, (vii) easiness of interpretation and (viii) usefulness for water resources managers was summarized in **Table V-6**, scoring each index for each criterion on a scale of 1 to 5, the latter corresponding to the maximum score.

Table V-6. Studied ecological indices comparative evaluation

	Conceptual relevance	Suitability to specific goals	Feasibility and analytical reliability	Seasonal variability integration	Consistency	Discriminant capacity	Interpreta-bility	Usefulness for aquatic resources managers	Mean
Chlo-*a* *(mg/m^3)*	3	4	5	4	4	3	5	5	4.1
DIN *(mg/m^3)*	2	3	5	4	4	3	5	5	3.9
SRP *(mg/m^3)*	2	3	4	3	4	3	5	5	3.6
TSI (Chlo-*a*)	4	3	5	4	3	3	4	3	3.6
TPB *(mm^3/L)*	5	5	3	3	2	5	4	5	4.0
CB *(mm^3/L)*	5	5	3	3	2	5	4	5	4.0
CBP *(%)*	4	4	3	3	2	4	4	4	3.5
IGA	1	2	2	3	2	1	1	2	1.8
PTI	5	5	3	4	4	5	2	4	4.0
MedPTI	5	5	1	3	2	1	3	4	3.0
BP *(%)*	3	4	4	4	4	2	4	3	3.5
PTCD	5	5	4	3	4	4	5	5	4.4
BRI	5	5	3	3	4	3	4	5	4.0

Considering only the indices scored with an average of 4 or more, the use of a combination of one of the phytoplankton biomass measurements (Chlo-a or TPB) with at least one of phytoplankton composition measurements (CB, PTCD, BRI or PTI) is sugested, in order to obtain the information required by WFD for the ecological potential classification of these reservoirs.

Given the simplicity and the quality of the provided information, PTCD or preferably BRI should be implemented as soon as calibration occurs through its application to more systems. PTI is more demanding in terms of data acquisition, but promising in terms of ecologic analysis, as soon as a list of specific trophic optimal for the species found in southern Portugal reservoirs exists.

In any case data from the present study constitutes a well-informed baseline for future studies on the impacts of Climate Change on oligotrophic reservoirs in the Mediterranean region.

5 – REFERENCES

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CHAPTER VI

General Conclusions and Future Perspectives

Discussion and conclusions

Detailed conclusions regarding the specific goals of this work were drawn in chapters II to V. The present chapter summarizes and integrates the most relevant findings.

To undertake the general purpose of assessing toxicity risks linked to cyanobacteria hazard algal blooms (CyanoHABs) in Southern Portugal, this work started by reviewing available literature reporting toxicity events in eighteen reservoirs of this region. The presence of invasive species and their toxicological potential was also investigated and ecotoxicological risks assessed while profiting from unexpected massive blooms of *Cylindrospermopsis raciborskii*, occurring in September 2011 and 2012 in Alqueva reservoir. These allowed a better insight into exotic emerging species in Southern Portugal. Phytoplankton dynamics in an oligotrophic environment, dominated by cyanobacteria was studied in an attempt to establish the influence that other microalgae could have upon CyanoHaBs development, since previous studies indicated that biological variables play an important role in specifically controlling/promoting CyanoHABs. Since in the scope of the European Water Framework Directive several criteria for classifying ecological potential of these reservoirs were adopted, this work also looked into the links between ecological quality indicators and water safety in terms of CyanoHABs.

Available historical data as well as the provided by the monitoring studies done in the scope of this work made it evident that CyanoHABs occurrence is increasing in Southern Portugal freshwater reservoirs, probably due to a combination of factors that synergistically interact to favor CyanoHaBs development.

Indeed Southern Portugal reservoirs assemble a set of characteristics known to promote cyanobacteria growth, such as high hydraulic residence linked to poor annual rainfall, high radiation levels, mean temperatures above 15 ºC with maxima above 25 ºC. The torrential hydrological regime and consequent high turbidity increases, during and after floods, also favor cyanobacteria, since some prefer to grow under shadow. Furthermore vertical stratification periods are becoming longer in consequence of water extractions from the meta- and hipolimnion, contributing to the rising of water surface temperatures. Moreover many cyanobacteria species are strongly favored by stratified conditions (*e.g. Microcystis* spp.). This situation is worsened in reservoirs with high nutrient loadings, and also in reservoirs with wide ranges of water levels, where besides the winter mixing of the water column, also during periods of water scarcity in the summer sediments may be re-suspended and nutrients and other compounds (*e.g.* metals) mobilized, becoming available for cyanobacteria. Additionally re-suspension is also a tool for the recruitment of akinetes, (resting spores), seeding the waters and renewing cyanobacteria populations.

Thus abiotic factors also contributed to favor exotic toxigenic species to invade Southern reservoirs (*e.g. Cylindrospermopsis raciborskii*), increasing the health risks, since some of the toxins produced by such species aren’t regularly screened.

Eventhough, only trace amounts of cylindrospermopsin were detected in samples of the *C.raciborskii* blooms reported in Alqueva reservoir, which also did not reveal significant toxicity. The fact that tests for this unexpected toxin were performed on water kept frozen for long periods, may be responsible for eventual false negative results. This means that readiness to deal with eventual invasion by allochtonous species must rise.

This study also revealed the consistent presence of toxigenic cyanobacteria in Beliche reservoir, an oligotrophic environment. Even though toxicity events were occasional, with low levels of measured toxins (microcystin), the presence of toxigenic species, calls for the inclusion of toxicity monitoring in the Official Monitoring Program, through at least ecotoxicological assays.

In relation to the ecological indexes based on phytoplankton to infer about the ecosystem health and indirectly about public health risk, more work should be done, and is already in progress although not included in this thesis. Most of the calculated indexes seem to be inadequate to Southern Portugal reservoirs, according to the purpose they are intended. Some of the studied metrics seem promising if adequately calibrated, as explained in chapter V and could be suitable to assess freshwaters ecological status as well as potential toxicity risks for public health. The index of dominance of potentially toxic cyanobacteria (PTDC) and the Biotoxicity Risk Index (BRI), which also includes dinoflagellates borne risks will now be applied to other southern reservoirs in order to link them to public health risk assessment. In the absence of cost effective monitoring methods to differentiate between toxigenic and non-toxigenic strains of the so called potentially toxic species, nor to detect actual expression of toxin producing genes in environmental samples the use of such indices should constitute a precautious measure in order to protect public health.

In fact, public health risks must be assumed with a precautious criterion attributing more relevance to risks in mesotrophic and eutrophic reservoirs, since toxicity risks are linked to toxigenic cyanobacteria biovolume, and not to the relative dominance of phytoplankton groups.

The several studies hereby presented, indeed alert and highlight the need to expand and reinforce the connection between environment and human health.

Future perspectives

The presence of CyanoHABs in Southern Portugal reservoirs is a constant and there is no safe method to get rid of them without causing damage to the cells, which makes the use of chemicals inappropriate. In fact the only safe and effective way of avoiding contact between human populations and cyanobacteria and/or their toxins is prevention, or rather the anticipation of blooms. This could be achieved with enhanced surveillance programs, allowing for proper mitigation measures to be taken. In the framework of accounting for expected global changes, special attention should be given to exotic or unusual detected cyanobacteria species.

The use of molecular biology methods to complement and overcome morphological identification limitations, will in the future improve accuracy and speed up analytical procedures.

CyanoHABs, must be considered not only in view of their environmental risks and contribution to eutrophication, but mainly because of potential impacts of toxigenic species on public human health. An ecological index that might be useful in assessing both types of risks, impairment of ecological status and human intoxication should be implemented.