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Analysis of cyanotoxins in water samples: method validation and application to monitoring and assessment of water quality

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Resumo

A literatura existente tem mostrado que cianotoxinas, como microcistina-LR (MC-LR) e cilindrospermopsina (CYN), são encontradas naturalmente em lagos, rios, lagoas e outras águas superficiais. Embora a ingestão de água contaminada seja a principal via de exposição humana às cianotoxinas, estudos têm revelado que estas podem efetivamente se acumular nos tecidos vegetais, o que pode aumentar a exposição humana e, consequentemente, ameaçar a saúde pública. No entanto, o uso de água contaminada com cianotoxinas para fins agrícolas está a aumentar em todo o mundo devido à eutrofização e escassez de água (estimulada pelas mudanças climáticas). Estes fatores podem trazer preocupações ao tema da segurança alimentar devido à possível absorção das cianotoxinas pelas plantas que podem levar à sua bioacumulação em tecidos comestíveis. Assim, o desenvolvimento de métodos rápidos e confiáveis para monitorização de cianotoxinas em águas superficiais pode ser uma vantagem importante para uma tomada de decisão baseada no risco. O objetivo deste trabalho foi identificar o principal grupo de cianotoxinas que possam existir em amostras de água do Alqueva, validar um método químico para estudar as cianotoxinas em amostras de água e posterior análise de risco deste reservatório com base em análises químicas.

A otimização da extração em fase sólida (SPE) foi realizada ao testar amostras de água ambiental enriquecidas com microcistina-LR (MC-LR) e cilindrospermospina (CYN) simultaneamente. Após a otimização do método, amostras ambientais das albufeiras do Alqueva foram submetidas ao mesmo procedimento e analisadas por cromatografia líquida e espectrometria de massa (LC-MS/MS).

Os resultados deste trabalho permitiram a validação do método de extração de várias toxinas em simultâneo, através da aplicação de SPE em amostras de água do ambiente, e a deteção de cianotoxinas no Alqueva, por LC-MS/MS, mesmo em baixas concentrações, sendo microcistinas (MCs) o grupo principal. Os resultados da monitorização da água obtidos pela aplicação do método implementado em amostras ambientais do Alqueva foram comparados com relatórios da literatura e orientações nacionais e internacionais podendo concluir-se que relativamente às cianotoxinas analisadas, o risco de utilização da água para diferentes fins humanos é negligenciável.

Abstract

The existing literature have shown that cyanotoxins, such as microcystin-LR (MC-LR) and cylindrospermopsin (CYN), are found naturally in lakes, rivers, ponds, and other surface waters. Although the ingestion of contaminated water is the main route of human exposure to cyanotoxins, studies have revealed that cyanotoxins can effectively accumulate in plant tissues, which might increase the human exposure and consequently threatening public health. Nevertheless, the use of cyanotoxins contaminated water for agricultural proposes is increasing worldwide due to eutrophication and water scarcity (stimulated by climate change). These factors can pose concerns on the topic of food safety due to the possible uptake of these cyanotoxins by plants that can lead to its high bioaccumulation in edible tissues. Thus, the development of fast and reliable methods for cyanotoxins monitoring in surface waters can be an important asset for a risk-based decision making. The aim of this work was to identify the main group of cyanotoxins that might exist in water reservoirs of Alqueva, to validate a method of extraction and analysis of multiple cyanotoxins simultaneously in water samples and based on monitoring data to proceed to the risk analysis of the water quality of Alqueva reservoirs monitored.

Optimization of solid phase extraction (SPE) was performed by testing spiked environmental water samples with both MC-LR and CYN simultaneously. After method optimization, environmental samples from Alqueva reservoirs were submitted to the same procedure and analyzed by liquid chromatography – tandem mass spectrometry (LC–MS/MS).

The results of this work allowed the validation of the extraction method of various toxins simultaneously, by applying SPE in environmental water samples, and the detection of cyanotoxins in Alqueva, by LC-MS/MS, even at low concentrations, being microcystins (MCs) the main group. The water monitoring results obtained by applying the implemented method in environmental samples from Alqueva were compared to literature reports and national and international guidelines and it can be concluded that regarding the analyzed cyanotoxins, the risk of using the water for different human purposes is negligible.

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Abbreviations

BW – Body Weight

CCMs - Carbon-Concentration Mechanisms

CIIMAR - Interdisciplinary Center for Marine and Environmental Research

CYN – Cylindrospermopsin

EDIA - Empresa de Desenvolvimento e Infra-estruturas do Alqueva, S.A.

ELISA - Enzyme-Linked Immunosorbent Assay

ESI - Electrospray Interface

ha - Hectare

HPLC - High Performance Liquid Chromatography

IZA - Intervention Zone of Alqueva

LC-MS/MS - Liquid Chromatography with tandem Mass Spectrometry

LD50 – Lethal Dose

LDO – Limit Detection Observed

LQO – Limit Quantification Observed

LPS's - Lipopolysaccharides

MC-LR - Microcystin-LR

MCs – Microcystins

MRM – Multiple Reaction Monitoring

N - Nitrogen

P - Phosphorus

PCR - Polymerase Chain Reaction

PDSI - Palmer Drought Severity Index

PGC – Porous Graphitic Carbon

Ppb – Parts per bilion

Ppm – Parts per million

SPE – Solid Phase Extraction

SD – Standard Deviation

TDI - Tolerable Daily Intake

US – United States

UV - Ultraviolet Light

WHO – World Health Organization

1. Introduction

In the second half of the 20th century, the waterbodies started to accelerate the eutrophication as a consequence of climate change and anthropogenic factors such as agriculture and urbanization. A vast number of studies and publications are asserting the concern of toxic cyanobacterial blooms drastically increasing, threatening the quality of water and consequentially human health, being this topic attracting the attention of the scientific community and public (Chorus et al., 2021). Cyanobacteria are an oxygen producing bacteria that utilizes sunlight as source of energy by converting carbon dioxide into biomass. These phototrophic bacteria originated about 3 billion years ago as they generated one giant event in the earth's history, which is the oxidation of the Earth's atmosphere (Huisman et al., 2018). Cyanobacteria is also known as blue-green algae although they are not algae, also they can display various colors, including several tones of red, brown, pink, yellow and green (Huisman et al., 2018). Cyanobacterial blooms are not considered a new environmental threat, in fact, these microorganisms are the oldest in the world with fossil evidence of 3.5-billion-year history. Among the Gram-negative photosynthetic bacteria, cyanobacteria are one of the most diverse groups when comes to physiology, morphology, and metabolism. Because of their photosynthetic capability under both anaerobic and aerobic state, these organisms occur in a range of distinct aquatic and terrestrial ecosystems (Lee et al., 2017)

Blue green algae, or Cyanobacteria, as mentioned above, are present in brackish, fresh, and marine waters but can also be found in terrestrial environments (Codd et al., 2005). These phylum is found literally in any illuminated environment and, surprisingly, in some dark subsurface ones (Bižić et al., 2020). The optimum temperature for several cyanobacteria species is higher than most eukaryotic algae, thus proliferating in warmer climates. The most common threat associated with the presence of cyanobacteria blooms is the production of toxins which could be very harmful for aquatic organisms, mammals and other type of living organisms (Whitton & Potts, 2012). The timing and extent of cyanobacteria bloom depend on the climate of the region, as well as other factors. On temperate areas, blooms are most noticeable in late summer and early autumn and can last for about 2 to 4 months (Van Apeldoorn et al., 2007). In aeras with Mediterranean or subtropical conditions, the bloom season could start sooner and can extend for a longer period of time (Van Apeldoorn et al., 2007). Also, the increase of industrial and agricultural activities in recent times are intensifying the eutrophication of

water bodies, and these events can lead to an intense growth in cyanobacterial blooms (Machado et al., 2016).

Cyanobacteria present in blooms have the ability to produce toxins (cyanotoxins) that are harmful to vertebrates, invertebrates, and plants. The toxins produced by cyanobacterial blooms can vary in space and time and is highly difficult to estimate the composition of the species and its abundance. These blooms frequently consist of combinations of strains that are toxic or non-toxic, if the strain composition changes can modify the whole composition of the blooms including the toxic profile in the water (Huisman et al., 2018). The main factors known to affect the cyanobacterial blooms are: (1) the increasing nutrients inputs, (2) the transport of cells via anthropogenic actions, (3) climate change, and (4) the increased aquaculture, also the overfishing that affects the food webs and permit harmful species to control algae communities can trigger a change to the blooms (O'Neil et al., 2012). Since the development of advance techniques of detection of cyanotoxins such as liquid chromatography tandem mass spectrometry (LC-MS/MS), the presence of these molecules in the water bodies have been reported more often in countries like China, United States, and in Europe. Therefore, methods based on LC-MS/MS have been used more regularly for monitoring water quality including drinking and recreational water (Kaushik & Balasubramanian, 2013; Lee et al., 2017).

The most common type of toxin produced by cyanobacteria appear to be liver toxic *microcystins* (MCs) that accumulate a 40 to 75% in cyanobacterial blooms (Corbel et al., 2013; McLellan & Manderville, 2017). Humans and animals are exposed to these toxins in numerous ways, for example, among the sources of toxins to humans are: drinking water, recreational waters, and food supplements made from cyanobacteria (Buratti et al., 2017; Huisman et al., 2018). The cyanotoxins are basically endotoxins that can be released to the environment, normally after cellular lysis. The toxins known to be involved in human incidents belong to the genera *Microcystis*, *Anabaena*, *Aphanizomenon*, *Planktothrix*, *Oscillatoria*, *Cylindrospermopsis* and less often *Gomphosphaeria*, *Coelosphaerium*, *Gloeotrichia*, *Nodularia* and *Nostoc* (Corbel et al., 2013).

The fresh and brackish water cyanotoxins fall into three broad chemical groups, the cyclic peptides (comprising of hepatotoxic *microcystins* and *nodularins*), the alkaloids (comprising the cytotoxic *cylindrospermopsin*, the neurotoxins *anatoxin-a*, *anatoxin-a(S)* in which "S" means salivation factor, and

saxitoxins) and the lipopolysaccharide (LPS's) which are possibly irritant. Additionally, there are two marine cyanotoxins belonging to the group of alkaloids (*aplysiatoxin* plus *debromoaplysiatoxin* and *lyngbyatoxin-a*) that can cause gastrointestinal and/or skin irritation (Van Apeldoorn et al., 2007).

Once cyanotoxins enter the soil and aquatic ecosystems, there are some processes that can remove them, such as, biodegradation, photochemical degradation by UV, adsorption in particles in suspension or onto sediments. Cyanotoxins have distinct chemical stability in the ecosystems they are present, being hepatotoxic cyclic peptide cyanotoxins very stable compounds that can persist in aquatic environments for weeks or, in natural conditions, even months after being released from the cells. Nevertheless, the processes mentioned above can speed up the removal of these toxins from the water. (Corbel et al., 2013).

1.1 Cyanobacteria geographical distribution and dispersion on water bodies

Cyanobacterial Blooms (cyanobacterial harmful algal blooms) appear on a worldwide scale and have been documented in various countries. These harmful blooms are a global problem and exist in every continent, being present for instance in, Lake Erie in north America, several reservoirs in south America, the Baltic Sea in Europe, Lake Victoria in Africa, Lake Taihu in Asia, Murray River in Australia and even in some regions in Antarctica (Clark et al., 2017). In the U.S. numerous states had to shut down recreational areas or even issue health advisories due to the possible risk of exposure to cyanobacteria blooms (Clark et al., 2017). Data recorded and reported by Harke et al., (2016) describe *Microcystis* species appearing in 108 out of 257 countries and territories, most of the nations that didn't report any incident were small islands in the pacific region. The majority of the reports recorded on toxic cyanobacteria were from Europe, North America, and Australasian countries, with developing countries often not recording even single research.

The eutrophication of water bodies causing cyanobacterial blooms are increasing drastically due to a variety of factors, the increasing temperature and nutrients are considered to be the two main causes to this problem (Rigosi et al., 2014). Some studies also mention that cyanobacteria will thrive under the conditions expected for global climate change, and how this will affect the temperature/stratification as well as carbon dioxide (CO_2) and pH (Chittora et al., 2020).

Concerning water **temperature**, the warming of the waters can selectively stimulate cyanobacteria growth as they are prokaryotes their growth rate is enhanced at high temperatures (Paerl & Paul, 2012). The heating of surface waters strengthens vertical stratification in both freshwater and marine environments, and seasonal warming also lengthens the period of stratification (Paerl & Paul, 2012). Freshwater stratification starts around spring, is maintained during summer, and fall away in autumn (Domingues et al., 2011). Most toxic cyanobacteria possess gas vesicles which provide buoyancy, enabling them to shape dense surfaces blooms in stratified waters, where they can benefit of high-level irradiance to optimize photosynthesis (Zhang et al., 2021). As the burning of fossil fuel increases and the enormous quantities of CO_2 in the atmosphere continue to rise, the earth's surface temperature is expected to increase between 1.5˚C and 5˚C in this century causing changes in natural communities of phytoplankton and algae growth rate (O'Neil et al., 2012)

Regarding the **nutrients**, research indicates that eutrophication combined with an increased human population has triggered the occurrence of blooms. The freshwater bodies are becoming more supplemented with nutrients (Plaas & Paerl, 2021). Phosphorus (P) controls the proliferation of freshwater environments since most cyanobacteria in these ecosystems can fix nitrogen (N), so it is assumed that P and N are the regulators of cyanobacterial growth. Iron (Fe) has also been found to be an important nutrient for the growth of cyanobacteria (O'Neil et al., 2012). This growth of eutrophic activity is also related to the anthropogenic action over the last years, such as urban (increase human population density), agriculture, and industrial activities that load nutrients into many freshwater ecosystems (Davis et al., 2009)

Considering the changes in **CO_2** and **pH**, during the past two centuries the combustion of fossil fuels have boosted substantially the concentration levels of CO_2 in the atmosphere and its consequently worsening (O'Neil et al., 2012). The concentrations of atmospheric CO_2 that was increasing in the 20th century around 1% per year is now increasing at a rate of 3% per year even exceeding 800 ppm by the end of the century (O'Neil et al., 2012). Associating this event with climate change, the increase of atmospheric concentrations of CO_2 could have a more favorable impact on species that, unlike cyanobacteria, hold inferior carbon-concentration mechanisms (CCMs) which most eukaryotic algae and all cyanobacteria possess. It has been shown that CCMs in cyanobacteria are more

efficient than other algae or higher plants at low CO_2 concentrations and this may facilitate their dominance over low CO_2 concentration (O'Neil et al., 2012). It is hypothesized that surface-dwelling cyanobacteria could have the advantage over other phytoplankton because of the closer proximity to atmospheric CO_2 that can swiftly diffuse into surface waters and stimulate their growth (O'Neil et al., 2012). The pH of aquatic water bodies is deeply connected to the speciation of dissolved inorganic carbon, and the pH of most systems (between 7.5-8.1) maintains inorganic carbon principally in the structure of HCO_3^-. Several lakes are supersaturated with CO_2 due to terrestrial carbon inputs and sediment respiration, and the pH and speciation of inorganic carbon in lakes can vary from daily to episodic to seasonal (Paerl & Huisman, 2009). Various studies documented that cyanobacteria out-compete eukaryotic algae under high pH and low CO_2 conditions, also some cyanobacteria decrease cell division rates in response to lower pH conditions (O'Neil et al., 2012; Paerl & Huisman, 2009; Shapiro, 1990).

Climate change also affect the distribution of cyanobacteria on water bodies. Global warming and its associated changes in climatic oscillations affect patterns, intensities and duration of precipitation and droughts, are favoring cyanobacteria blooms. As an example, more intense precipitations can mobilize nutrients on land and rise the input of nutrients on water bodies (King et al., 2007). Concomitantly, the water-level reduction often results in higher nutrient concentrations, higher phytoplankton biomass, and lower water transparency in both shallow and deep lakes and reservoir. Drought may increase the water residence time in lakes, and several studies have shown that longer water residence times throughout dry years increase cyanobacteria biomass and dominance (Brasil et al., 2016). During the timeline of this project (July, August, and September), the drought levels according to the PDSI index (Palmer Drought Severity Index) were on the level H.2 – Alert (aggravation of the drought indicators that affect the normal level of hydric reservations) (APA, 2021).

1.2 Cyanotoxins (microcystin; cylindrospermopsin)

Among cyanobacteria, *Microcystis* is acknowledged to be the most prevalent and widely distributed bloom forming genus, and microcystin-LR the most predominant variant of microcystins produced by the species *Microcystis aeruginosa*. Another cyanotoxin, the tricyclic alkaloid cylindrospermopsin (CYN) is being recognized as a rising threat because of the invasive nature of its main producer, *Cylindrospermopsis raciborskii* (Freitas et al., 2015).

Microcystins (MCs) are cyclic heptapeptide molecules containing both L- and D-amino acids and an unusual hydrophobic C_{20} D-amino acid commonly known as ADDA (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid) (WHO, 2020). The ADDA residue is critical to the toxicity of MCs. There are over 250 distinct MCs, which vary mainly in the two L-amino acids at positions 2 and 4, being the most common and the most studied the MC-LR (position 2: Leucine, position 4: Arginine) (WHO, 2020). The majority of MCs are water soluble and stable in different conditions of pH and temperature, and light. WHO, (2020), established an order of MCs congeners, with a partition coefficients at pH 7 and pH 5 and the results were as following: MC-RR, MC-YR, MC-LR, MC-LW, MC-LF. The shift from intracellular to extracellular MCs pools is mostly due to the release of the toxins through cell lysis during the decline of blooms. Although other organs may be harmed, several acute and sub-chronic oral exposure studies conducted on animals and poisoning incidents linked to dialysis, suggest that the liver is the principal target of this toxin. There is also evidence that indicates that tumor promotion in a variety of tissues may be an outcome caused by long-term exposure of MC-LR (WHO, 2020; Gurbuz et al., 2009; Gutiérrez-Praena et al., 2013; IARC, 2010; Song et al., 2006).

Cylindrospermopsin (CYN) is an alkaloid consisting of a tricyclic guanidine moiety combined with hydroxymethyluracil. It is zwitterionic (dipolar ions with localized positive and negative charges), highly water soluble, and stable at extreme temperatures and pH. Humans are more vulnerable to exposure to CYN than other cyanotoxins because up to 90% of total CYN is released the extracellular medium (water). CYN also has an epimere called 7-epiCYN and another variant called deoxyCYN. Both structures were reported to be less toxic than CYN (Gutiérrez-Praena et al., 2013; Li et al., 2001; Rücker et al., 2007; Who, 2020).

Both toxins are known to be hazardous to various mammals, birds, fish, crustaceans, mollusks, and zooplankton (Machado et al., 2017). In table 1 is shown the Chemocomposition and toxicity of both toxins and the LD50 in mice. In Brazil, was reported the most severe case of acute toxicity caused by cyanobacterial toxins, during a single week following renal dialysis, 116 of 131 dialysis patients experienced disturbance of vision, vomiting, nausea, headache, muscle weakness and epigastric pain. Of these, 100 developed liver failure and 76 died. The deaths of 52 patients could be linked directly to liver failure (IARC, 2010; Machado et al., 2017). Cyanotoxins can also affect plants, either by reducing their germination

rates and growth, but also they can be taken up by plants and transmit the toxins to humans and other animals via food (Bavithra et al., 2019).

Table 1 - Chemocomposition and toxicity of MC-LR and CYN (Machado et al., 2016).

Toxin name	Chemical formula	Cyanotoxin variant	Toxin type	Toxicity (LD50)
Microcystins	$C_{49}H_{74}N_{10}O_{12}$	Microcystin-LR (MC-LR)	Hepatotoxin	50–100 μg/kg (mice)
Cylindrospermopsin	$C_{15}H_{21}N_5O_7S$	Cylindrospermopsin (CYN)	cytotoxin	2100 μg/kg (mice)

1.3 Water uses – effects of cyanotoxins on agricultural crops and monitoring of water resources

On most occasions cyanobacteria blooms occur in open aquatic systems, such as, oceans, rivers, lakes, and ponds, however it can also appear in water bodies that are destined to crop irrigation and agriculture. For this reason, it has been hypothesized that some toxins can accumulate in edible plants (crops and vegetables) and as such, be a risk for food safety and human health (Gutiérrez-Praena et al., 2014). Chen et al., (2012) refer that terrestrial plants could be exposed to MC-LR by using eutrophic water that could contain cyanobacterial blooms and respective toxins. Additionally, the author report that MC-LR was transferred from plant roots to shoots in the seedlings of eleven crop plants. (Chen et al., 2012). Citing Mohamed & Al Shehri, (2009), plants are able to take up considerable amount of cyanotoxins and undergo physiological and morphological changes. The concentration of MCs in surface waters used in crop irrigation range from 4 to 50 μg/L and up to 6500 μg/L, yet the higher concentrations would be observed in blooms and comprise intracellular and dissolved MCs (Machado et al., 2017). Although the studies documenting the concentrations of CYN in the environment are scarce, the concentration of total extracellular CYN in water appear to differ from imperceptible values up to 126 μg/L (Machado et al., 2017). In addition, because the MC-LR and CYN are chemically stable in irrigation water, these cyanotoxins may leak into the soil, compromising groundwater quality and lead to adverse public health consequences (Corbel et al., 2013; J. Machado et al., 2017). The World Health Organization (WHO), in 1999 proposed a tolerable daily

intake (TDI) of 0.04 μg MC-LR/kg body weight (BW). In some studies, MC-LR concentrations in food crops reached or even surpassed the WHO TDI, increasing the problematic concerning public health risks from consumption of vegetables irrigated with MC-LR contaminated water (Miller & Russell, 2017).

Cyanobacteria blooms are a persistent source of toxins in the aquatic environment that can be found in water supplies, recreational lakes, estuaries, etc. Since blooms are easily spotted, public health authorities can monitor their occurrence and dynamics. Many countries have implemented several programs in order to monitor cyanobacterial blooms (Backer, 2002). Cyanobacterial distributions as well as their spatial and temporal changes depend on the characteristics of the water body, (geographical, meteorological, hydrological, and morphological). Since the dissemination of cyanobacteria is crucial to hazard assessment, the monitoring programs should be specifically customized for each water body in order to optimize the relation of information - work. Knowledge of bloom history added to a good understanding of the environmental variables will significantly boost the ability to predict blooms (Chorus & Bartram, 1999). To guarantee the efficiency of the monitoring programs, these should be reviewed on a regular basis to provide the most cost-effective use of resources and to continue to satisfy the basic needs on which monitoring programs were established (Chorus & Bartram, 1999). In Portugal the departments of public health have the responsibility to regulate and secure the implementation of cyanobacteria monitoring program. This program must be implemented in locations that have an historic profile of occurrence of cyanobacteria blooms and toxins (Macário et al., 2018). The elements that are assessed are: aquatic flora, benthonic invertebrates, fish, temperature, oxygen balance, salinity, nutrients, acidic state, and other pollutants (APA, 2022). Even though there isn't legislation concerning the concentration of cyanotoxins in irrigation waters, on Decreto-Lei n.º 135/2009, of 3 of june are described diverse data of phytoplankton for the types of waters.

The primary methodologies used for identification and quantification of toxic cyanobacteria and its toxins are optic microscopy, real time and conventional PCR, ELISA essays, HPLC (High Performance Liquid Chromatography) and LC-MS (Liquid chromatography–mass spectrometry) (Churro & Valério, 2014; Moreira et al., 2011).

1.4 Characterization of study site - Alqueva

Alqueva is located in Alentejo that belongs to the Intervention Zone of Alqueva (IZA) that is situated between Elvas and Aljustrel. It has a population density of 22 habitants per km^2 and, occupies a region with approximately 900 000 ha distributed through 19 counties. Throughout this region, the Mediterranean climate and smooth landscape are responsible for the absence of precipitation in the warm season, which restricts the development of natural vegetation and agricultural activity. These extreme climate conditions are relevant since contribute to the scarce population that characterize the region and the distribution of urban nucleus far apart of each other. The IZA territory is predominantly used for agriculture in which 18,5% represents the weight of professional agriculture and signifies 31,0% of all utilized agricultural land (GPAa, 2004). The Alqueva dam, constructed in the Guadiana River is the main infrastructure of an irrigation system composed of, 69 dams and reservoirs, 382 km of primary network and 1620 km of extensions of conducts on the secondary network, 47 elevation platforms, 5 mini-hydric centrals and 1 photovoltaic central. The Alqueva subsystem covers an irrigated area of approximately 75000 ha (EDIA, 2019).

Water quality deteriorates during the spring-summer semester, in which the stratification of the water body is accentuated. The spring-summer semester, when the stratification of the water body is enhanced, sees a decline in water quality. When there is heavy, concentrated rain at the end of the summer, the reservoir's pollutant loads and sediment levels rise, making the problem worse (GPAa, 2004).

Currently, it is anticipated that the Alqueva reservoir's water quality will allow usage for agricultural activities, particularly for irrigation. If proper conduct is observed in agricultural activities throughout the basin region, protocols for quality in the emission of effluents will be followed, and the quality will improve along with the respect for the inflows agreed upon between Portugal and Spain (GPAa, 2004). The eutrophication levels of the reservoir, which may cause phytoplankton outbreaks or the development of atypical aquatic weeds, are what may cause problems for agriculture. In the latter scenario, irrigation system machinery can be used to lessen the consequences (GPAa, 2004).

1.5 Objectives

The general objective of this thesis is to enhance the capacity to perform the chemical analysis of cyanotoxins and assess the water quality in Alqueva. It was used an extraction method so there could be an efficient process for environmental samples and to ensure a rigorous monitorization. The following specific objectives were established: (1) to implement a Solid Phase Extraction (SPE) method for the extraction and analysis of multiple toxins and, (2) characterization of cyanotoxins present in Alqueva reservoirs.

2. Materials and Methods

2.1 Water Sample Collection

The sampling sites were located in Alqueva, which is situated in the border of Beja and Évora districts on south of Portugal. All samplings were made in the reservoirs that surround this area (figure 1). The samples were collected during July, August, and September of 2021. The reservoirs that were part of this sampling were "São Pedro", "Magra" and "Pisão". These reservoirs were chosen according to a risk prioritization, carried out by Silva et al., (2020) described as following: for the Risk analysis was established a risk prioritization based on the information provided by EDIA concerning the phytoplankton in Alqueva reservoirs. The risk matrix was made concerning the occurrence of toxic cyanobacteria (number of genera/species with toxic potential), and cellular density of toxic cyanobacteria (>20 000 cell/mL) from the data provided from EDIA (entity that monitories all of Alqueva reservoirs) concerning the months July and August of 2016 2017 and 2018. Both "São Pedro" and "Magra" reservoirs were classified as intermediate occurrence, as for "Pisão" reservoir, it was classified as high occurrence.

Table 2 - Risk prioritization index, based on the occurrence and density of toxic cyanobacteria.

	High occurrence of toxic cyanobacteria with cellular density ~ or >20 000 cell/mL
	Intermediate occurrence of toxic cyanobacteria with cellular density ~ or > 20 000 cell/mL
	Low occurrence of toxic cyanobacteria with cellular density ~ or > 20 000 cell/mL
	Low occurrence of toxic cyanobacteria and occurrence of toxic cyanobacteria with cellular density < 20 000 cell/mL

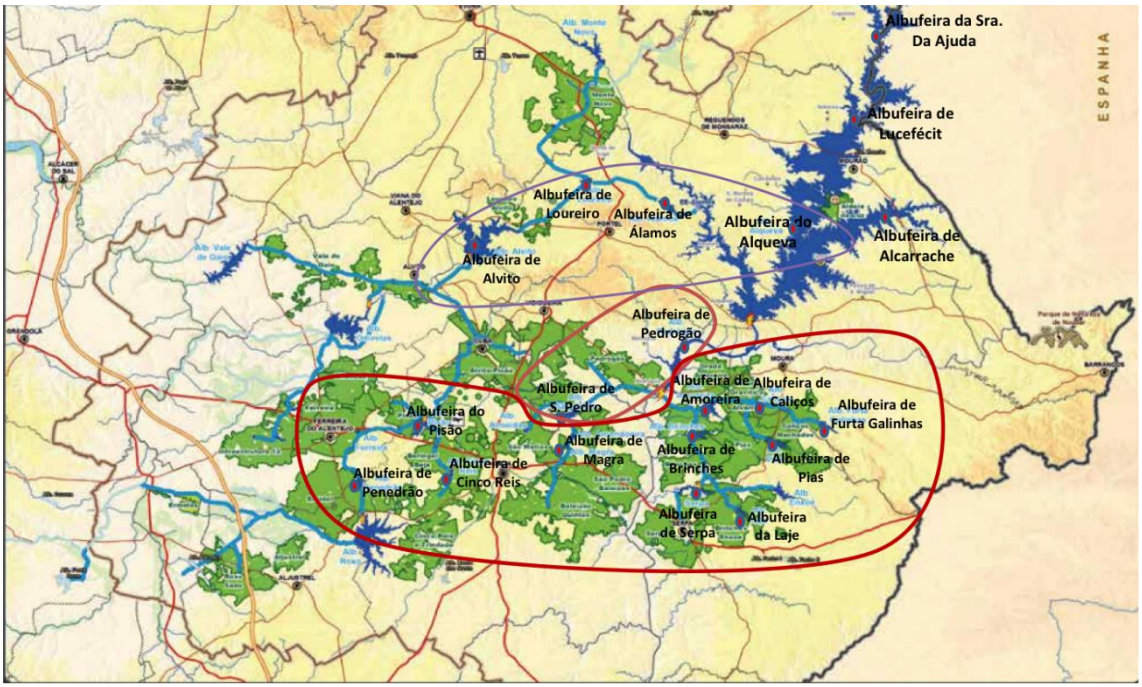

Figure 1 - Location of water reservoirs in Alqueva.

In all sites, except on "Magra", were performed 3 sample extractions, one at the water entrance, one at the water exit and one at the center of the reservoir (figures 2 and 3). When it came to the sampling in "Magra" it was collected one sample on the water entrance, and another at the water exit (figure 4). The water harvest consists of the collection of 5L/6L of water from the three specific sites. Depth-integrated samples that represent an overall reservoir condition are calculated with help of a Secchi disc (figure 5) which allow to estimate the photic zone (2,5 × depth of the Secchi disc) and a van-dorn bottle (figure 6). Furthermore, there are also other measurements that are recorded, such as, sampling time for each sample collected at a site, sample location coordinates using portable GPS that can provide with location information (latitude and longitude) and water quality parameters including temperature, conductivity, dissolved oxygen, and pH.

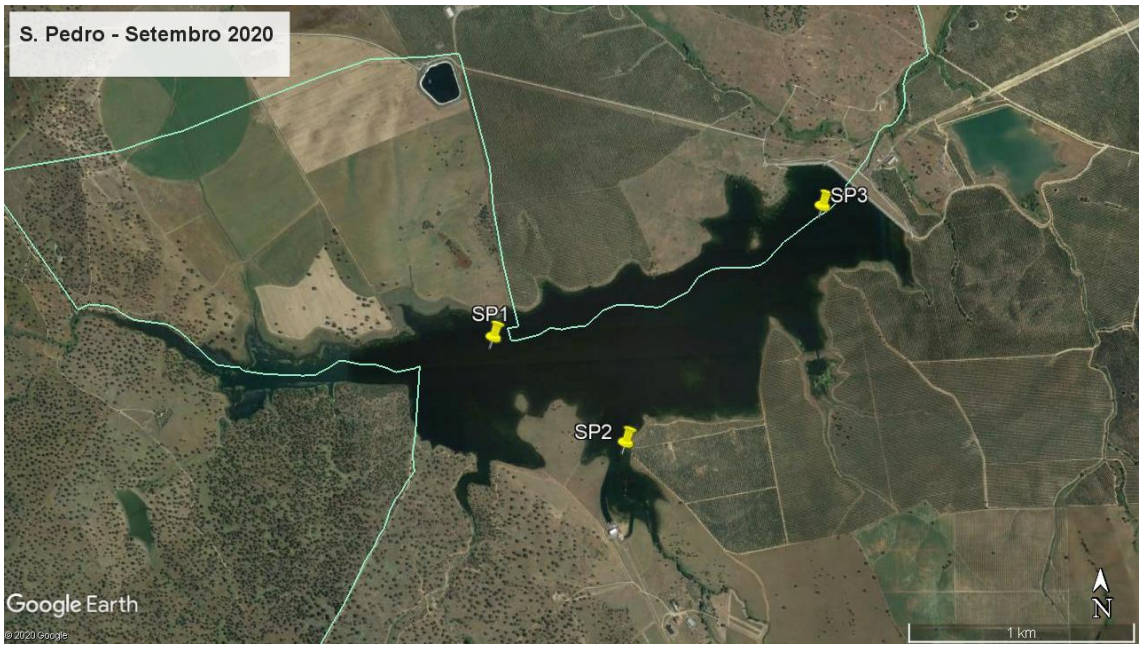

Figure 2 - S. Pedro sampling site.

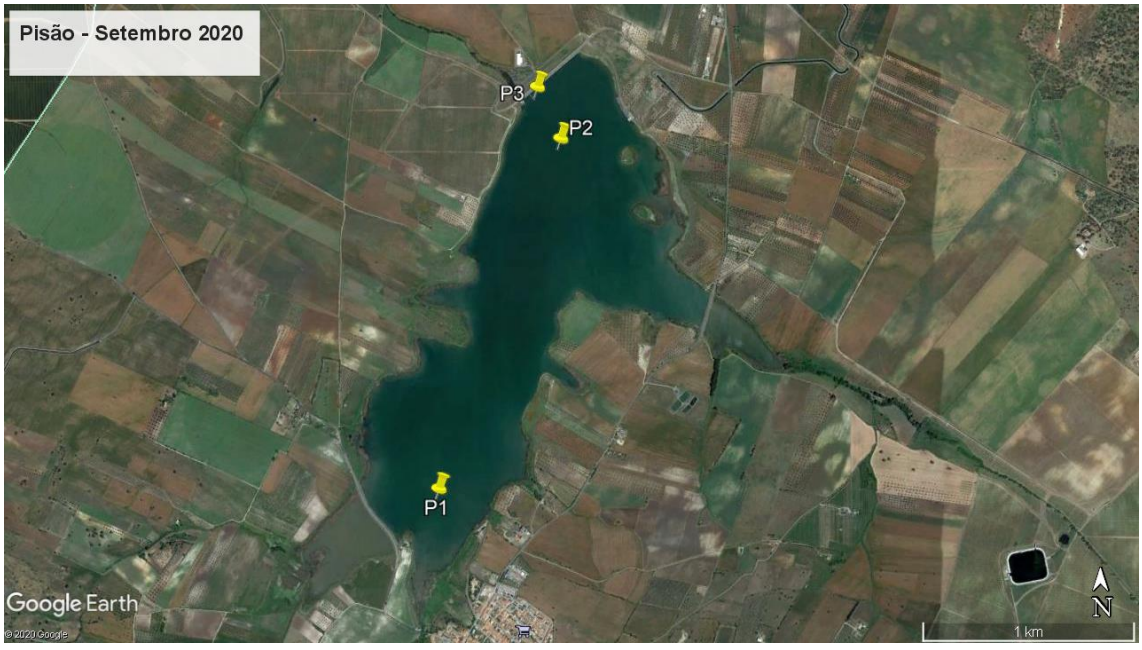

Figure 3 - Pisão sampling site.

Figure 4 - Magra sampling site.

Figure 5 - Secchi disc.

Figure 6 - Van-dorn bottle.

2.2 Water filtration and storage

After the harvest of the Alqueva samples, was performed a filtration using filters of 47mm (Fisherbrand™ MF 300 Microglass Fiber Filter Discs) of all the water samples collected, then, all the filters used in the filtration were identified and wrapped in aluminum foil and stored in -20˚C. After the filtration the filtered water was stored in 1.5L bottles properly identified and stored in -20˚C until it was used for the SPE protocol.

2.3 Validation of the SPE multi-toxin method and sample Preparation

For the analysis of multi-toxins, a pre-purification method described by Zervou et al., (2017), was implemented and validated regarding some parameters in the laboratory. This pre-purification method is based on SPE and the validation of the method was performed by using environmental water samples from "Parque da Cidade" located in Porto. Afterwards, the water samples were doped with a known concentration of 2.5µg/ml of a stock solution of a cyanobacterial extract. The volume used for the samples were 1ml; 2ml; 3ml, and 0.1, 1, and 5 µg/L of MC-LR and CYN respectively, that were prepared previously from cyanobacterial biomass. This volume was added to the 400 mL of water previously harvested. Afterwards an SPE protocol was performed.

2.4 Solid Phase Extraction (SPE) of environmental samples

SPE was carried out according to the method described by Zervou et al., (2017). It started by using an assembly of two cartridges, Oasis HLB (200 mg, 6 cc, 25–35 μm, Waters Corporation, USA) and HyperSep Hypercarb PGC (porous graphitic carbon, 200 mg, 3 cc,30–40 μm, Thermo Scientific, UK). The SPE was performed using a 12-port SPE vacuum manifold with large volume samplers and a diaphragm vacuum pump. Implementation of SPE was executed by analysing spiked water samples at the concentration level of 100 ng L^{-1} for each toxin. The SPE was preceded as follows: 5 mL of methanol were added to 400 mL of sample. Initial sample pH was adjusted to 11 with addition of NaOH 2 M (few drops). Then Oasis HLB and HyperSep PGC were connected in series (top Oasis HLB, bottom PGC) (Figure 7) and conditioned sequentially with 6 mL dichloromethane (DCM), 6 mL methanol and 6 mL water (pH11). Samples were passed through the SPE assembly and then the cartridges were dried for 15 min (air under vacuum). The sequence of the two cartridges in the SPE assembly was then reversed (top PGC, bottom Oasis HLB) and the analytes were eluted with a mixture of 10 mL DCM:MeOH (40:60, v/v), containing 0.5% formic acid (FA). After this the extract was dried under a gentle stream of nitrogen, the residue was re-dissolved with 400 μL MeOH: H2O (5:95, v/v) and sonicated in water bath for 5 min. The final extract was then transferred to an autosampler glass vial and analysed by LC–MS/MS.

Figure 7 - Assembly of cartridges in the SPE system.

The conditions for the LC-MS were as follow: The LC-MS system used to quantify MC-LR was a Liquid Phase Chromatograph Alliance e2695 HPLC system, coupled with a triple quadrupole spectrometry detector (Micromass® Quattro micro™ API), with electrospray interface (ESI). The program used for data acquisition and processing was MassLynx version 4.1. The mass spectrometer was operated in positive mode and quantification done with multiple reaction monitoring (MRM). The capillary voltage was maintained at 3.5 kV; cone at 20V; Extrator at 3V and Lens at 0.2V. The source temperature was held at 120 ˚C and Desolvation at 350 ˚C and 500 L/hr. Nitrogen was used as a sheath and auxiliary gas and Argon as a collision gas at a pressure of 0.5 bar.

Separation was achieved on C18 Hypersil Gold column (100 × 4.6 mm I.D., 5 μm, ThermoScientific, Waltham, MA, USA) kept at 45 ˚C, with a flow rate of 0.35 mL/min. and injected volume was 10 μL in loop partial mode. A gradient elution was used with mobile phase A, MeOH, and B ultrapure water both acidified with 0.1% formic acid (5 % A and 95 % B for 3 min, 40 % A and 60 % B at 4 min during 1

min, increasing to 60% A at 7 min for 2 min, increasing to 80% A for 1 min and returning to initial conditions at 20 min and equilibrating during 10 min).

Mass parameters were optimized with standard solutions an extracts of cyanobacteria toxin producers, all injected in positive polarity mode, in Full scan (30-1500 m/z) and MRM mode. Transitions, Cone, and collision energy voltages for each cyanotoxin are present in Table 2. The target compounds were: Cylindrospermopsin, Anatoxin-a, Saxitoxin, Nodularin-R and 7 Microcystin analogs (MC-RR, MC-YR, MC-LR, MC-LA, MC-LY, MC-LW and MC-LF).

The standards and samples were injected in duplicate and at each set of 10 samples was introduced a blank and two standards mix solutions of different concentration. Quantification was performed by external calibration curve.

Table 3 - Cyanotoxins addressed in this method and their MS operating parameters.

Cyanotoxin	Retention Time	Mass-to-charge ratio (*m/z*) transition	Cone (V)	Collision energy (V)
Microcystin-YR	12.90	1045.5>135.2 1045.5>599	20 20	60 55
Microcystin-LW	16.22	1047.3>579.5	50	20
Microcystin-LA	15.49	932.5>135 932.5>599.1	50 50	20 20
Microcystin-LR	13.25 15.00 (gradient 1 and 2)	995.5>135.05 995.5>599.2	50 50	30 30
Microcystin-RR	12.30	520.3>105.1 520.3>135.1	20 20	40 30
Microcystin-LF	16.16	1008.6>515.1	50	20
Microcystin-LY	15054	1024.5>677.2	50	20
Nodularin-R	12.98	825.3>135.5	50	20
Cilindrospermopsin	3.9016	415.9>176	20	80

		415.9>194 415.9>336.1	20 20	80 20
Saxitoxin	3.00	300>125 300>204 300>265	20 20 20	25 25 25
Anatoxin-a	5.05	166.1>107.2 166.1>131.1 166.1>149.1	20 20 20	25 15 10
Phenilalanine	2.94	166.1>120.0	20	10

Standards supplied by Cifga (Lugo, Spain). Microcystin-YR (CRM-00-MCYR, Lot 20-001, 99% purity), Microcystin-RR (CRM-03-MCRR, Lot 15-001, 99% purity) and Microcystin-LR (CRM-00-MC-LR, Lot 19-001, 96% purity). An Hepatotox Set™1 was supplied by ALEXIS Biochemicals (USA, Lot L26789, < 95% purity). All the standards were injected individually and then as a standard mixture with a concentration interval from 5 ppb to 500 ppb.

2.5 Extraction of cyanotoxins present in the biomass (intracellular fraction of water samples)

The extraction of the intracellular component from the biomass contained in the filters was performed as following: first the filters were macerated in a mortar with a slight stream of liquid nitrogen, just enough to cover all of the macerated filters. Afterwards was added 5 mL of solvent MeOH at 10% (v/v), to each filter. To complement the cell lysis step and release of intracellular content, was used the Sonopuls HD-2070.2 sonicator (20 kHz/25% amplitude), with the MS-72 probe and LS-40 isolator cabin (Bandelin, Germany) in 5x1 cycles. minutes at 20 kHz in an ice bath. For the separation of the dissolved and particulate components it was used the Heraeus Megafuge 16R centrifuge, at a speed of 13,000 g, for 20 min. The subsequent removal of solvents was carried out with an Acid-Resistant CentriVap Concentration system (Labconco®, USA) with a Coldtrap at -50 ˚C, coupled to an

RV5 Rotary Vane vacuum pump (Edwards, UK). The dehydrated samples were resuspended in an ultrasound bath in 1000 µL of 50% (v/v) MeOH, acidified with 0.1% (v/v) formic acid. After that they were filtered with SFPE-22E-050 into 2 ml vials for LC-MS.

3. Results

3.1 Validation of the SPE method for multiple toxins

In order to improve the capacity to analyse multiple class toxins in water samples, an SPE method was tested and validated in the laboratory, to enable to extract, purify and concentrate several types of cyanotoxins from an individual environmental sample at the same time. During this phase of the work, as was mentioned above, the validation was performed using spiked water from "Parque da Cidade, Porto". Relatively to the MC-LR validation the method linearity and range of measurement were studied by analysing solutions at 3 different concentrations in the range of 2.5 – 7.5 µg/L in triplicates. Linear regression analysis was done giving a coefficient of determination r^2>0.9959 (figure 8). Regarding the CYN validation the method linearity and range of measurement were studied by analysing solutions at 3 different concentrations: 0.1, 1, and 5 µg/L in triplicate. Linear regression analysis was done giving a coefficient of determination r^2>0.9846 (figure 9).

Since there isn't any particular guidelines for performance criteria for the analysis of cyanotoxins in water, the performance of the method was assessed based on available directives and guidelines referring to the analysis of other organic pollutants in water (Conselho da União Europeia, 1998; European Union, 2015; Parlamento Europeu e do Conselho, 2009; Zervou et al., 2017). The obtained mean recoveries and precision parameters for the proposed analytical method are, in general, in agreement to the above guidelines for all common and frequently found toxins.

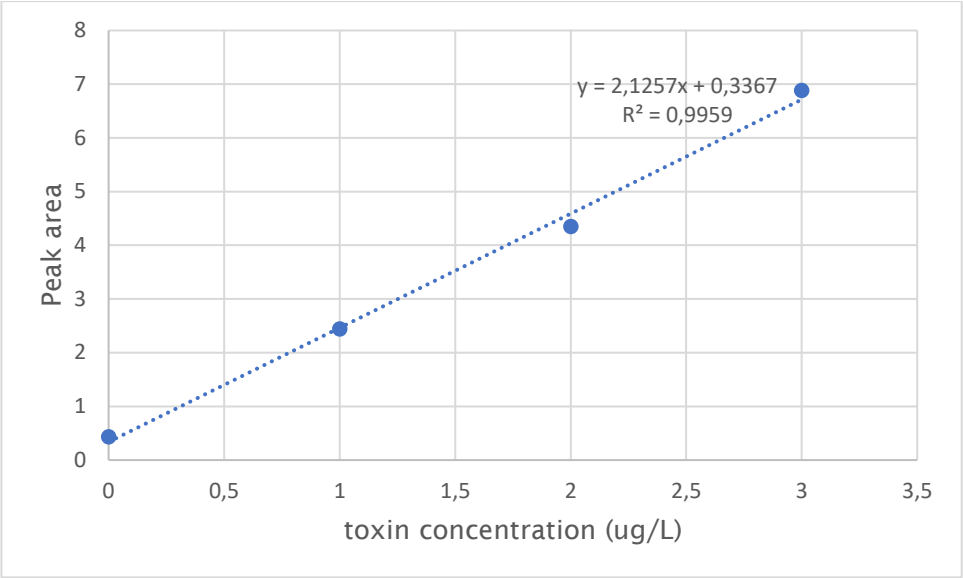

Figure 8 – Linear regression analysis regarding MC- LR measurements.

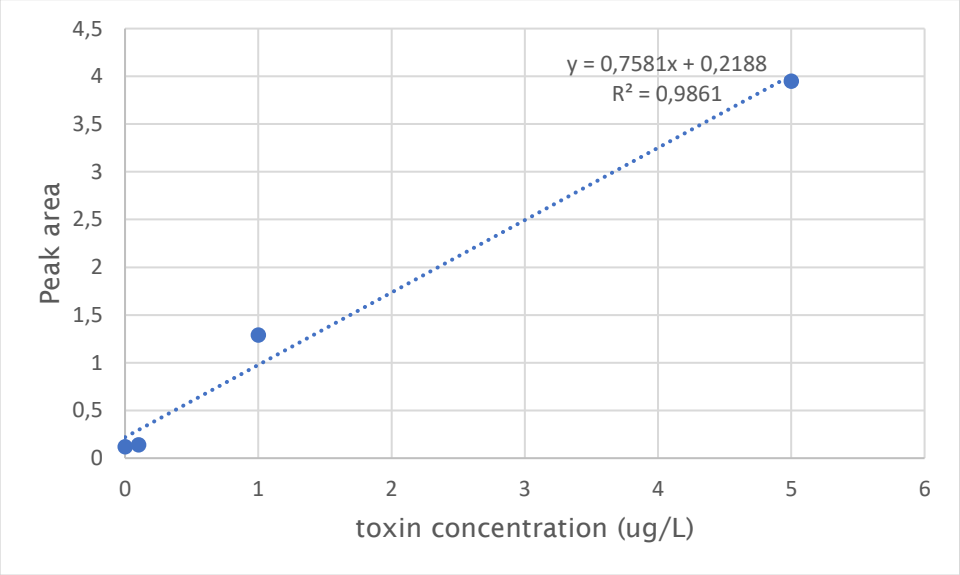

Figure 9 – Linear regression analysis regarding CYN measurements.

Method trueness, as % recovery, was evaluated by analysing water samples. Three spiked waters with MC-LR, with respective triplicates, were analysed, and mean recoveries were in the range of 87.1% to 97.7% (table 4). Method precision, as % standard deviation (SD) was also assessed by repeated measurements of spiked samples. The SD values ranged from 7.25 to 13.71 for all analytes. Three spiked waters with CYN, with respective triplicates, were analysed, and mean recoveries varied between 79% and 139% (table 4). Method precision, as SD was also assessed by repeated measurements of spiked samples. The SD values ranged from 0.01 to 0.4 for all analytes.

Table 4 – Mean recovery (%) of MC-LR and CYN after SPE method.

Cyanotoxin	Expected µg	µg obtained	% Mean recovery	SD
MC-LR	2.5	2.44	97.72%	13.71
	5.0	4.35	87.06%	8.82
	7.5	6.88	91.73%	7.25
CYN	0.1	0.14	139.00%	0.01
	1.0	1.29	129.00%	0.06
	5.0	3.95	79.00%	0.40

3.2 Alqueva reservoirs

First, LC-MS/MS analysis was carried out to check the presence of several groups of toxins, e.g., MCs and CYN. CYN was not detected on any of the water samples analysed. With these results we could establish that all reported values were below the limit values established by the Portuguese government for MC-LR (Decreto-Lei Nº 306/2007, de 27 de Agosto, Do Ministério Do Ambiente, Do Ordenamento Do Território e Do Desenvolvimento Regional, alterado pelo Decreto-Lei nº 152/2017). Following this analysis, a quantitative analysis was carried out to the different MC variants (MC-LR, MC-RR, MC-YR, MC-LA, MC-LY) both the dissolved fractions (water) and intracellular fractions (biomass). No toxins were detected in the dissolved fractions of the Alqueva samples. Positive detection was verified in the intracellular fractions. The results are presented in table 5 to 9.

Table 5 – MC-LR in dissolved fractions from Alqueva reservoirs, assessed through LC-MS/MS.

Reservoir	Month	Mean (ng/L)
São Pedro	July	<LOD
Magra		<LOD
Pisão		<LOD
São Pedro	August	<LOD
Magra		<LOD
Pisão		<LOD
São Pedro	September	<LOD
Magra		<LOD
Pisão		<LOD

LOD – Limit of Detection

Table 6 – MC-RR in dissolved fractions from Alqueva reservoirs, assessed through LC-MS/MS.

Reservoir	Month	Mean (ng/L)
São Pedro	July	<LOD
Magra		<LOD
Pisão		<LOD
São Pedro	August	<LOD
Magra		<LOD
Pisão		<LOD
São Pedro	September	**<LOQ**
Magra		<LOD
Pisão		**<LOQ**

LOD – Limit of Detection; LOQ – Limit of Quantification

Table 7 - MC-YR in dissolved fractions from Alqueva reservoirs, assessed through LC-MS/MS.

Reservoir	Month	Mean (ng/L)
São Pedro	July	<LOD
Magra		<LOD
Pisão		<LOD
São Pedro	August	<LOD
Magra		<LOD
Pisão		<LOD
São Pedro	September	<LOD
Magra		<LOD
Pisão		<LOD

LOD – Limit of Detection

Table 8 - MC-LA in dissolved fractions from Alqueva reservoirs, assessed through LC-MS/MS.

Reservoir	Month	Mean (ng/L)
São Pedro	July	<LOD
Magra		<LOD
Pisão		<LOD
São Pedro	August	<LOD
Magra		<LOD
Pisão		**<LOQ**
São Pedro	September	<LOD
Magra		<LOD
Pisão		<LOD

LOD – Limit of Detection; LOQ – Limit of Quantification

Table 9 - MC-LY in dissolved fractions from Alqueva reservoirs, assessed through LC-MS/MS.

Reservoir	Month	Mean (ng/L)
São Pedro	July	<LOD
Magra		<LOD
Pisão		<LOD
São Pedro	August	<LOD
Magra		<LOD
Pisão		<LOD
São Pedro	September	<LOD
Magra		<LOD
Pisão		<LOD

LOD – Limit of Detection

Analysing these results, we can conclude that the results obtained were all bellow the limit of detection except in 3 points where there was presence of toxins but below the limit of quantification. For MC-RR in September (São Pedro and Magra) and for MC-LA in August (Pisão). Even though the results are far below the guidelines limits, further study should be conducted to provide more accurate conclusions. Other studies have reported toxic *M. aeruginosa* and MC concentrations of 0.5 to 2.58 μg l−1, in other reservoirs from Alqueva and Alentejo region (Valério et al., 2010).

4. Discussion

The increased occurrence of MCs and CYN in water bodies explains the necessity of reliable and fast analytical techniques, enabling a consistent and data-based risk analysis of use of water for different human purposes. Among the different parameters commonly considered for analytical methods validation, the linearity as well as the percentage of recovery are crucial. The results obtained in this work demonstrated to be consistent with the requirements regarding the above-mentioned parameters. Concerning the linearity of the method validation, comparing to Haddad et al., (2019), the authors reported a coefficient correlation of r^2>0.998 for MC-LR, which is similar to our findings of the coefficient correlation of r^2>0.9959 for MC-LR, however these authors didn't report any linearity for CYN. However according to Díez-Quijada et al., (2018), the authors stated they observed a coefficient correlation of r^2>0.999 for CYN, which is an improvement of the results obtained in this work, r^2>0.9846. These authors also reported the coefficient correlation for MC-LR, r^2>0.9996, which is also similar to our findings. Concerning the mean recovery (%) of the method, Díez-Quijada et al., (2018), reported a recovery of 62–84% for MC-LR, and 45–69% for CYN, which, comparing to the results obtained in this work, its showed a range of recovery of 87.1–97.7% for MC-LR, and 79–129% for CYN, which makes the results obtained in this thesis better. Although, Haddad et al., (2019), only reported values for MC-LR, we can also compare their values (94%) to ours (87.1–97.7%) and state that they are similar.

According to WHO, (2013) there are three levels of risk associated with the presence of cyanobacteria, which are: Relatively low probability of adverse health effects (20 000 cyanobacteria/ml), Moderate probability of adverse health effects (100 000 cyanobacteria/ml) and, High probability of adverse health effects (Cyanobacterial scum formation), where scum is often termed bloom. Comparing the guidelines mentioned above and the data previously reported by EDIA, these reservoirs presented a moderate probability of adverse health effects. With this knowledge, it was performed a monitoring program to oversee these locations.

Comparing the results from this thesis and the values provided by different countries (table 10 and 11), it can be seen that the toxin concentrations found in Alqueva samples are below the limits set in different countries considering different uses of water. The low levels registered for MCs are in accordance with the mesotrophic state attributed to the reservoirs investigated.

Table 10 - Guidelines and standards for cyanobacterial toxins in water used for recreation or bathing water in various countries (Burch et al., 2005; Macário et al., 2018).

Australia	Two Level Guideline Level 1: 10 μg L-1 total MCs or >50,000 cells mL-1 toxic *M.aeruginosa* or biovolume equivalent of >4 mm3 L-1 for the combined total of all cyanobacteria where a known toxin producer is dominant in the total biovolume. Level 2: either the total biovolume of all cyanobacterial material exceeds 10 mm3 L-1 or scums are consistently present
Germany	Three Level Guideline Level 1: <10 μg L-1 MCs Level 2: >10 - <100 μg L-1 MCs Level 3: >100 μg L-1 MCs
Netherlands	20 μg MC-LR L-1
France	Three Level Guideline Level 1: 20,000 cyanobacterial cells mL -1 Level 2: >20,000 -<100,000 cyanobacterial cells mL-1 Level 3: Presence of scums
Portugal	2 - 10 μg/L MCs

Table 11 - Guidelines or standards for cyanobacterial toxins in drinking water in various countries (Burch et al., 2005; Decreto-Lei Nº 306/2007, de 27 de Agosto, Do Ministério Do Ambiente, Do Ordenamento Do Território e Do Desenvolvimento Regional, alterado pelo Decreto-Lei nº 152/2017).

Australia	1.3 μg L-1 Total MCs, expressed as toxicity equivalents of MC-LR
Brazil	1.0 μg L-1 MCs 3.0 μg L-1 saxitoxins (equivalents) 15 μg L-1 CYN
Canada	1.5 μg L-1 cyanobacterial toxins as MC-LR
Czech Republic	1 μg L-1 MC-LR
China	1 μg L-1 MC-LR
France	1 μg L-1 MC-LR
Italy	0.84 μg L-1 total MCs
Japan	1 μg L-1 MC-LR
Korea	1 μg L-1 MC-LR
New Zealand	For cyanobacteria: <1 potentially toxic cyanobacterium present in 10 mL of sample. PMAV for cyanobacterial toxins: Anatoxin: 6.0 μg L-1 Anatoxin-a (S): 1.0 μg L-1 CYN: 1.0 μg L -1 Homoanatoxin-a: 2.0 μg L-1 MC-LR Toxicity Eq: 1.0 μg L-1 Nodularin: 1.0 μg L-1 Saxitoxins (as STX-eq):3.0 μg L
Norway	1 μg L-1 MC-LR
Poland	1 μg L-1 MC-LR
Portugal	1 μg L-1 MC-LR
Spain	1 μg L-1 MC-LR

Gkelis & Zaoutsos, (2014), after various harvests of water on several water sources, found out that the dominant species were from de *Microcystis* genera, specifically, *M. wesenbergii*, *M. novacekii* and *M. viridis*. MCs were detected by ELISA

in all samples. MC concentration ranged from 3.9 mg L^{-1} to 108 mg L^{-1}. They also recorded three blooms of *C. raciborskii* which concentrations ranged from 0.3 mg L^{-1} to 2.8 mg L^{-1}. The results of this work can relate to the results found by the author mentioned above since *Microcystis* is a predominant genus in Alqueva and we were able to detect microcystin analogs such as, MC - RR, MC - LA. However, due to the low concentration of these toxins (bellow LOQ), their quantification was not possible.

Regarding the results described by Kaloudis et al., (2013), the authors showed that the compounds investigated in their work were partially present, missing any concentration of nodularin. However, various types of MCs were found (i.e., MC-LR, -RR, -YR, -LA), with several concentration levels, MC-LR 2 – 451 ng L^{-1}, MC-RR 2 - 174 ng L^{-1}, MC-YR 2 – 717 ng L^{-1}, and MC-LA 5 – 8 ng L^{-1}. They also state that their results are similar to additional reports concerning other Greek lakes and in good correlation with those for other Mediterranean countries, including Portugal, Spain, and Italy. Also, concerning Mchau et al., (2021), the authors observed the presence of CYN, MCs, (-RR, -LR and -YR) on lakeshores in Tanzania. They reported that CYN was present in 89% of the lakeshores collection sites with concentrations ranging from 0.004 to 0.01μg/L. The study shows the presence of MC congeners, -RR, -LR and -YR with concentrations ranging from 0.003 - 0.009 μg /L for MC-RR, 0.010 – 0.012 μg /L for MC-LR and 0.012 – 0.013 μg /L for MC-YR. Comparing to results showed in this thesis, our results are below any observed values reported by the authors (Kaloudis et al., 2013; Mchau et al., 2021).

According to Baralla et al., (2017), their experiment was performed in June, July and August in two places. In Cabras lagoon it was obtained a peak of MC-LR in July, 0.75 ± 0.07 ng/L and August, 0.63 ± 0.18 ng/L. As for Calich lagoon there was a constant trend between all months and with a mean MC-LR concentration of 0.20 ± 0.06 ng/L. The results reported above are optimal for the water quality since the concentrations were very low. Moreover the authors linked the calm wind and drought with the high temperatures of summer which makes this results more impressive, since these parameters can cause cyanotoxins release (Baralla et al., 2017). The results presented by Baralla et al., (2017), also are in accordance with the results found in this thesis. Both studies were performed during, at least, 2 common months, in summer in North Hemisphere and have peaks for MC-LA in August, in Pisão. In September the peaks for MC-RR were in São Pedro and Pisão

although they were not quantifiable. All these results are ideal for water use purposes due to their very low (not harmful) concentrations of cyanotoxins.

A study in Canada recorded the presence of MCs in samples collected from 16 lakes from Quebec and Ontario. MC-LR was the most frequently detected microcystin (12/16 samples) and it was also found at the highest concentrations (range in positive samples: 29–3476 ng L^{-1}). The authors also reported the presence of MC-RR (4/16 samples), and MC-LA (3/16 samples), however was less recurrent and not in high concentrations (Roy-Lachapelle et al., 2019). In comparison, this work found all MC congeners in all reservoirs, although nearly all the results found were not detected.

Overall, the results obtained were surprising, since samples were collected during the summer, which in other water sources in the country, have higher values of MCs. However, one possible explanation for these results is the fact that most reservoirs are often monitored by the respective authority (EDIA), which makes it easier for taking any measures in case of high concentrations. The water of these reservoirs is being frequently renovated (transferred from the main reservoir) due to the intense use of the water in agriculture, which do not favor the development of blooms. Since there aren't values established for cyanotoxins for irrigation water some studies in the literature have been suggesting values, such as, Campos et al., (2021) that set values to MCs in irrigation water to 10 μg/L, so that would have a minor impact on plant growth, yield and quality. However, considering the values determined for recreational and drinking water, the Alqueva water supply doesn't represent any risk. Further studies should be applied to the monitoring of reservoirs, such as Alqueva, due to the likelihood of containing perilous values that can harm human and animal health.

5. Conclusion

Cyanobacteria in environment is a topic that is becoming more relevant in the environmental monitoring field since eutrophication is a serious problem that affects both the environment and human health. Furthermore, climate change is strengthening eutrophication due to global warming and its associated changes in climatic oscillations such as increase in nutrients, higher phytoplankton biomass and lower water transparency. Even though many countries are making various efforts to establish cyanobacterial monitoring programs, there is still much work that can be done, such as the one carried in this thesis. Moreover, it was possible to implement a SPE method in Interdisciplinary Center for Marine and Environmental Research (CIIMAR), for the analysis of multiple toxins. This will facilitate the monitoring of environmental water quality and risk evaluation of toxins in water sources, such as Alqueva. There is still much work to do in relation of guidelines and directives, both in Portugal and in the European Union, especially on irrigation water. However, we can succeed in making the monitoring of cyanobacteria/cyanotoxins in water a more routine procedure to assess water quality. Relatively to the monitoring of water quality of Alqueva, it can be observed that MCs concentrations were below the regulatory limits (1 ug/L in drinking water) which is a positive situation for this water source. However, the monitoring performed in Alqueva should be maintained and even established for other months due to global warming and anthropological consequences that tend to worse in the future.

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Appendix

Appendix

Table i - Results of MC-LR analysis in the several water samples collected from Alqueva reservoirs by LC-MS

Sample ID	Type	Std. Conc	RT	Area	IS Area	Response	tection Fla	ppb	%Dev	S/N	LOD	LOQ
SP _J_ 1 FA2	Analyte		14,17	2,084		2,084	bb			0,375	<	
SP _J_ 1 FB2	Analyte		14,7	5,461		5,461	bb			0,534	<	
SP _J_ 2 FA2	Analyte			0								
SP _J_ 2 FB2	Analyte		14,38	4,808		4,808	bb			0,069	<	
SP _J_ 3 FA2	Analyte		14,28	3,312		3,312	bb			1,813	<	
SP _J_ 3FB2	Analyte		14,17	2,244		2,244	bb			0	<	
M _J_ 1 FA2	Analyte											
M _J_ 1FB2	Analyte		14,7	2,992		2,992	bb			0,194	<	
M _J_ 2FA2	Analyte											
M _J_ 2 FB2	Analyte											
P _J_ 1 FA2	Analyte											
P _J_ 1 FB2	Analyte		14,49	1,336		1,336	bb			0,484	<	
P _J_ 2 FA2	Analyte											
P _J_ 2 FB2	Analyte		14,38	1,656		1,656	bb			0,405	<	
P _J_ 3 FA2	Analyte											
P _J_ 3 FB2	Analyte		14,81	1,015		1,015	bb			0,233	<	
SP _A_ 1 Fa2	Analyte		14,17	3,312		3,312	bb			0	<	
SP _A_ 1 Fb2	Analyte		14,6	4,701		4,701	bb			0,205	<	
SP _A_ 2 Fa2	Analyte		14,6	4,381		4,381	bb			0,634	<	
SP _A_ 2 Fb2	Analyte											
SP _A_ 3 Fa2	Analyte		14,28	1,977		1,977	bb			0,327	<	
SP _A_ 3 Fb2	Analyte		14,49	1,389		1,389	bb			0,128	<	
M _A_ 1 Fa2	Analyte		14,38	1,175		1,175	bb			0,057	<	
M _A_ 1 Fb2	Analyte											
M _A_ 2 Fa2	Analyte		14,38	5,556		5,556	bb			2,32	<	
M _A_ 2 Fb2	Analyte											
P _A_ 1 Fa2	Analyte		14,28	1,923		1,923	bb			0	<	
P _A_ 1 Fb2	Analyte		14,28	5,823		5,823	bb			2,856	<	
P _A_ 2 Fa2	Analyte		14,38	1,336		1,336	bb			0,546	<	
P _A_ 2 Fb2	Analyte		14,92	1,194		1,194	bb			0,957	<	
P _A_ 3 Fa2	Analyte		14,17	0,748		0,748	bb			0	<	
P _A_ 3 Fb2	Analyte											
SP _S_ 1 Fa2	Analyte											
SP _S_ 1 Fb2	Analyte		14,81	9,3		9,3	bb			2,369	<	
SP _S_ 2 Fa2	Analyte											
SP _S_ 2 Fb2	Analyte		14,92	2,506		2,506	bb			0,789	<	
SP _S_ 3 Fa2	Analyte		14,7	4,775		4,775	bb			0	<	
SP _S_ 3 Fb2	Analyte											
M _S_ 1 Fa2	Analyte											
M _S_ 1 Fb2	Analyte		14,28	2,137		2,137	bb			0,259	<	
M _S_ 2 Fa2	Analyte		14,38	1,442		1,442	bb			0,471	<	
M _S_ 2 Fb2	Analyte		14,28	1,816		1,816	bb			0,669	<	
P _S_ 1 Fa2	Analyte		14,28	4,06		4,06	bb			1,925	<	
P _S_ 1 Fb2	Analyte		14,28	0,641		0,641	bb			0,426	<	
P _S_ 2 Fa2	Analyte		14,7	2,03		2,03	bb			0,443	<	
P _S_ 2Fb2	Analyte											
P _S_ 3 Fa2	Analyte		14,28	0,534		0,534	bb			0,328	<	
P _S_ 3 Fb2	Analyte		14,81	1,496		1,496	bb			0,509	<	

Table ii - Results of MC-RR analysis in the several water samples collected from Alqueva reservoirs by LC-MS

Name	Type	Std. Conc	RT	Area	IS Area	Response	Detection	ppb	%Dev	S/N	LOD	LOQ
SP _J_ 1 FA2	Analyte		12,28	6,372		6,372	bb			0,862	<	
SP _J_ 1 FB2	Analyte		12,21	9,897		9,897	bb			1,43	<	
SP _J_ 2 FA2	Analyte		12,71	7,761		7,761	bb			1,516	<	
SP _J_ 2 FB2	Analyte		12,64	20,007		20,007	bb			1,575	<	
SP _J_ 3 FA2	Analyte		12,85	1,246		1,246	bb			0,995	<	
SP _J_ 3FB2	Analyte		13,28	15,758		15,758	bb			2,577	<	
M _J_ 1 FA2	Analyte		13,06	4,541		4,541	bb			1,219	<	
M _J_ 1FB2	Analyte		12,5	16,091		16,091	bb			1,769	<	
M _J_ 2FA2	Analyte		11,96	3,916		3,916	bb			1,198	<	
M _J_ 2 FB2	Analyte		12,89	13,334		13,334	bb			1,61	<	
P _J_ 1 FA2	Analyte		12,85	13,085		13,085	bb			0,963	<	
P _J_ 1 FB2	Analyte		12,5	1,816		1,816	bb			1,743	<	
P _J_ 2 FA2	Analyte		12,57	4,557		4,557	bb			1,963	<	
P _J_ 2 FB2	Analyte		12,6	17,836		17,836	bb			1,213	<	
P _J_ 3 FA2	Analyte		12,71	17,232		17,232	bb			1,289	<	
P _J_ 3 FB2	Analyte		12,78	5,981		5,981	bb			1,305	<	
SP _A_ 1 Fa2	Analyte		12,71	8,366		8,366	bb			1,887	<	
SP _A_ 1 Fb2	Analyte		13,06	5,413		5,413	bb			1,51	<	
SP _A_ 2 Fa2	Analyte		12,17	10,075		10,075	bb			2,01	<	
SP _A_ 2 Fb2	Analyte		12,78	1,602		1,602	bb			1,069	<	
SP _A_ 3 Fa2	Analyte		12,67	1,869		1,869	bb			1,593	<	
SP _A_ 3 Fb2	Analyte		12,74	3,934		3,934	bb			1,376	<	
M _A_ 1 Fa2	Analyte		12,57	5,999		5,999	bb			1,497	<	
M _A_ 1 Fb2	Analyte		12,64	4,45		4,45	bb			1,008	<	
M _A_ 2 Fa2	Analyte		13,14	20,779		20,779	bb			2,136	<	
M _A_ 2 Fb2	Analyte		12,74	25,39		25,39	bb			2,827	<	
P _A_ 1 Fa2	Analyte		11,96	2,065		2,065	bb			1,902	<	
P _A_ 1 Fb2	Analyte		12,74	5,767		5,767	bb			1,169	<	
P _A_ 2 Fa2	Analyte		12,74	14,084		14,084	bb			1,69	<	
P _A_ 2 Fb2	Analyte		12,85	2,083		2,083	bb			1,949	<	
P _A_ 3 Fa2	Analyte		12,6	2,456		2,456	bb			0,724	<	
P _A_ 3 Fb2	Analyte		12,89	2,67		2,67	bb			1,419	<	
SP _S_ 1 Fa2	Analyte		13,28	7,836		7,836	bb			3,15	>	<
SP _S_ 1 Fb2	Analyte		12,78	4,272		4,272	bb			1,147	<	
SP _S_ 2 Fa2	Analyte		12,92	6		6	bb			1,527	<	
SP _S_ 2 Fb2	Analyte		12,89	2,261		2,261	bb			1,741	<	
SP _S_ 3 Fa2	Analyte		12,71	5,162		5,162	bb			1,69	<	
SP _S_ 3 Fb2	Analyte		12,64	6,177		6,177	bb			1,581	<	
M _S_ 1 Fa2	Analyte		12,67	6,728		6,728	bb			1,704	<	
M _S_ 1 Fb2	Analyte		13,14	9,954		9,954	bb			1,099	<	
M _S_ 2 Fa2	Analyte		13,14	24,712		24,712	bb			2,144	<	
M _S_ 2 Fb2	Analyte		12,5	26,382		26,382	bb			1,922	<	
P _S_ 1 Fa2	Analyte		12,64	7,761		7,761	bb			2	<	
P _S_ 1 Fb2	Analyte		13,06	6,802		6,802	bb			2,031	<	
P _S_ 2 Fa2	Analyte		12,53	8,313		8,313	bb			5,092	>	<
P _S_ 2Fb2	Analyte		12,28	5,892		5,892	bb			1,378	<	
P _S_ 3 Fa2	Analyte		12,78	6,676		6,676	bb			2,039	<	
P _S_ 3 Fb2	Analyte		12,57	3,097		3,097	bb			0,979	<	

Table iii - Results of MC-YR analysis in the several water samples collected from Alqueva reservoirs by LC-MS

SP _J_ 1 FA2	Analyte		13,67	5,075		5,075	bb			0,145	<	
SP _J_ 1 FB2	Analyte		13,53	1,781		1,781	bb			0,112	<	
SP _J_ 2 FA2	Analyte		13,88	4,684		4,684	bb			0,314	<	
SP _J_ 2 FB2	Analyte		13,31	1,496		1,496	bb			0,142	<	
SP _J_ 3 FA2	Analyte		14,35	1,923		1,923	bb			0,215	<	
SP _J_ 3FB2	Analyte											
M _J_ 1 FA2	Analyte		13,53	4,559		4,559	bb			0,762	<	
M _J_ 1FB2	Analyte		13,96	3,562		3,562	bb			0,258	<	
M _J_ 2FA2	Analyte		14,67	3,312		3,312	bb			0,16	<	
M _J_ 2 FB2	Analyte		14,13	12,947		12,947	bb			1,823	<	
P _J_ 1 FA2	Analyte		13,1	6,401		6,401	bb			1,789	<	
P _J_ 1 FB2	Analyte		14,13	5,147		5,147	bb			0,995	<	
P _J_ 2 FA2	Analyte											
P _J_ 2 FB2	Analyte											
P _J_ 3 FA2	Analyte		14,24	6,126		6,126	bb			1,018	<	
P _J_ 3 FB2	Analyte		13,17	3,562		3,562	bb			2,622	<	
SP _A_ 1 Fa2	Analyte		13,81	4,416		4,416	bb			0,604	<	
SP _A_ 1 Fb2	Analyte		13,81	1,923		1,923	bb			0,216	<	
SP _A_ 2 Fa2	Analyte		13,53	2,707		2,707	bb			0,364	<	
SP _A_ 2 Fb2	Analyte		14,03	2,493		2,493	bb			0,366	<	
SP _A_ 3 Fa2	Analyte		13,31	2,386		2,386	bb			0,813	<	
SP _A_ 3 Fb2	Analyte		14,35	2,564		2,564	bb			0,674	<	
M _A_ 1 Fa2	Analyte		13,39	4,274		4,274	bb			0,247	<	
M _A_ 1 Fb2	Analyte		13,88	2,867		2,867	bb			0,593	<	
M _A_ 2 Fa2	Analyte		13,17	3,063		3,063	bb			0,303	<	
M _A_ 2 Fb2	Analyte		13,74	2,956		2,956	bb			1,454	<	
P _A_ 1 Fa2	Analyte		13,81	1,14		1,14	bb			1,142	<	
P _A_ 1 Fb2	Analyte		14,35	4,167		4,167	bb			3,302	>	<
P _A_ 2 Fa2	Analyte											
P _A_ 2 Fb2	Analyte											
P _A_ 3 Fa2	Analyte		14,24	8,352		8,352	bb			1,507	<	
P _A_ 3 Fb2	Analyte		13,81	4,399		4,399	bb			1,302	<	
SP _S_ 1 Fa2	Analyte		14,35	1,603		1,603	bb			1,234	<	
SP _S_ 1 Fb2	Analyte											
SP _S_ 2 Fa2	Analyte											
SP _S_ 2 Fb2	Analyte		13,74	3,953		3,953	bb			1,156	<	
SP _S_ 3 Fa2	Analyte		13,31	8,049		8,049	bb			1,753	<	
SP _S_ 3 Fb2	Analyte		14,13	4,612		4,612	bb			0,792	<	
M _S_ 1 Fa2	Analyte											
M _S_ 1 Fb2	Analyte											
M _S_ 2 Fa2	Analyte		13,81	3,864		3,864	bb			0,848	<	
M _S_ 2 Fb2	Analyte		13,88	7,408		7,408	bb			0	<	
P _S_ 1 Fa2	Analyte		13,17	2,208		2,208	bb			0,944	<	
P _S_ 1 Fb2	Analyte		13,74	1,282		1,282	bb			1,122	<	
P _S_ 2 Fa2	Analyte		13,74	3,74		3,74	bb			0,6	<	
P _S_ 2Fb2	Analyte		13,39	11,825		11,825	bb			2,353	<	
P _S_ 3 Fa2	Analyte		13,53	1,71		1,71	bb			0,367	<	
P _S_ 3 Fb2	Analyte		13,53	5,271		5,271	bb			1,228	<	

Table iv - Results of MC-LF analysis in the several water samples collected from Alqueva reservoirs by LC-MS

Name	Type	Std. Conc	RT	Area	IS Area	Response	Detection	ppb	%Dev	S/N	LOD	LOQ
SP _J_ 1 FA2	Analyte											
SP _J_ 1 FB2	Analyte											
SP _J_ 2 FA2	Analyte											
SP _J_ 2 FB2	Analyte											
SP _J_ 3 FA2	Analyte											
SP _J_ 3FB2	Analyte											
M _J_ 1 FA2	Analyte											
M _J_ 1FB2	Analyte											
M _J_ 2FA2	Analyte											
M _J_ 2 FB2	Analyte											
P _J_ 1 FA2	Analyte											
P _J_ 1 FB2	Analyte											
P _J_ 2 FA2	Analyte											
P _J_ 2 FB2	Analyte											
P _J_ 3 FA2	Analyte											
P _J_ 3 FB2	Analyte		16,97	6,91		6,91	bb			0,523	<	
SP _A_ 1 Fa2	Analyte											
SP _A_ 1 Fb2	Analyte											
SP _A_ 2 Fa2	Analyte											
SP _A_ 2 Fb2	Analyte											
SP _A_ 3 Fa2	Analyte											
SP _A_ 3 Fb2	Analyte											
M _A_ 1 Fa2	Analyte											
M _A_ 1 Fb2	Analyte											
M _A_ 2 Fa2	Analyte											
M _A_ 2 Fb2	Analyte											
P _A_ 1 Fa2	Analyte											
P _A_ 1 Fb2	Analyte											
P _A_ 2 Fa2	Analyte											
P _A_ 2 Fb2	Analyte											
P _A_ 3 Fa2	Analyte											
P _A_ 3 Fb2	Analyte											
SP _S_ 1 Fa2	Analyte											
SP _S_ 1 Fb2	Analyte											
SP _S_ 2 Fa2	Analyte											
SP _S_ 2 Fb2	Analyte											
SP _S_ 3 Fa2	Analyte											
SP _S_ 3 Fb2	Analyte											
M _S_ 1 Fa2	Analyte											
M _S_ 1 Fb2	Analyte											
M _S_ 2 Fa2	Analyte											
M _S_ 2 Fb2	Analyte											
P _S_ 1 Fa2	Analyte											
P _S_ 1 Fb2	Analyte											
P _S_ 2 Fa2	Analyte											
P _S_ 2Fb2	Analyte											
P _S_ 3 Fa2	Analyte											
P _S_ 3 Fb2	Analyte		17,29	10,961		10,961	bb			1,551	<	

Table v - Results of MC-LA analysis in the several water samples collected from Alqueva reservoirs by LC-MS

Name	Type	Std. Conc	RT	Area	IS Area	Response	Detection	ppb	%Dev	S/N	LOD	LOQ
SP _J_ 1 FA2	Analyte		17,35	18,025		18,025	bb			1,162	<	
SP _J_ 1 FB2	Analyte		17,51	19,267		19,267	bb			1,246	<	
SP _J_ 2 FA2	Analyte		16,83	7,264		7,264	bb			1,07	<	
SP _J_ 2 FB2	Analyte		17,19	13,304		13,304	bb			1,039	<	
SP _J_ 3 FA2	Analyte											
SP _J_ 3FB2	Analyte											
M _J_ 1 FA2	Analyte											
M _J_ 1FB2	Analyte		17,19	2,897		2,897	bb			0,403	<	
M _J_ 2FA2	Analyte		17,51	19,731		19,731	bb			0,859	<	
M _J_ 2 FB2	Analyte											
P _J_ 1 FA2	Analyte											
P _J_ 1 FB2	Analyte		17,51	88,119		88,119	bb			4,149	>	
P _J_ 2 FA2	Analyte		17,51	81,58		81,58	bb			3,674	<	
P _J_ 2 FB2	Analyte		17,19	31,408		31,408	bb			1,281	<	
P _J_ 3 FA2	Analyte		17,35	14,5		14,5	bb			0,734	<	
P _J_ 3 FB2	Analyte											
SP _A_ 1 Fa2	Analyte											
SP _A_ 1 Fb2	Analyte											
SP _A_ 2 Fa2	Analyte		17,35	16,013		16,013	bb			1,264	<	
SP _A_ 2 Fb2	Analyte											
SP _A_ 3 Fa2	Analyte											
SP _A_ 3 Fb2	Analyte		17,35	7,868		7,868	bb			2,267	<	
M _A_ 1 Fa2	Analyte		17,51	10,969		10,969	bb			0,92	<	
M _A_ 1 Fb2	Analyte											
M _A_ 2 Fa2	Analyte											
M _A_ 2 Fb2	Analyte		17,02	6,132		6,132	bb			0,505	<	
P _A_ 1 Fa2	Analyte											
P _A_ 1 Fb2	Analyte											
P _A_ 2 Fa2	Analyte											
P _A_ 2 Fb2	Analyte											
P _A_ 3 Fa2	Analyte		17,19	13,84		13,84	bb			1,436	<	
P _A_ 3 Fb2	Analyte											
SP _S_ 1 Fa2	Analyte											
SP _S_ 1 Fb2	Analyte											
SP _S_ 2 Fa2	Analyte											
SP _S_ 2 Fb2	Analyte		17,19	1,252		1,252	bb			0,222	<	
SP _S_ 3 Fa2	Analyte		17,19	7,644		7,644	bb			0,863	<	
SP _S_ 3 Fb2	Analyte											
M _S_ 1 Fa2	Analyte											
M _S_ 1 Fb2	Analyte											
M _S_ 2 Fa2	Analyte		17,35	2,092		2,092	bb			0,345	<	
M _S_ 2 Fb2	Analyte											
P _S_ 1 Fa2	Analyte											
P _S_ 1 Fb2	Analyte											
P _S_ 2 Fa2	Analyte											
P _S_ 2Fb2	Analyte											
P _S_ 3 Fa2	Analyte											
P _S_ 3 Fb2	Analyte											

Table vi - Results of MC-LY analysis in the several water samples collected from Alqueva reservoirs by LC-MS

Name	Type	Std. Conc	RT	Area	IS Area	Response	Detection	ppb	%Dev	S/N	LOD	LOQ
SP _J_ 1 FA2	Analyte											
SP _J_ 1 FB2	Analyte											
SP _J_ 2 FA2	Analyte											
SP _J_ 2 FB2	Analyte											
SP _J_ 3 FA2	Analyte											
SP _J_ 3FB2	Analyte		16,86	5,995		5,995	bb			0,67	<	
M _J_ 1 FA2	Analyte											
M _J_ 1FB2	Analyte											
M _J_ 2FA2	Analyte											
M _J_ 2 FB2	Analyte											
P _J_ 1 FA2	Analyte		16,67	3,341		3,341	bb			0,474	<	
P _J_ 1 FB2	Analyte											
P _J_ 2 FA2	Analyte		17,54	4,184		4,184	bb			0,573	<	
P _J_ 2 FB2	Analyte		17,38	2,039		2,039	bb			0,102	<	
P _J_ 3 FA2	Analyte											
P _J_ 3 FB2	Analyte											
SP _A_ 1 Fa2	Analyte											
SP _A_ 1 Fb2	Analyte		16,86	3,27		3,27	bb			0,602	<	
SP _A_ 2 Fa2	Analyte											
SP _A_ 2 Fb2	Analyte		17,54	12,391		12,391	bb			1,351	<	
SP _A_ 3 Fa2	Analyte											
SP _A_ 3 Fb2	Analyte											
M _A_ 1 Fa2	Analyte											
M _A_ 1 Fb2	Analyte											
M _A_ 2 Fa2	Analyte		17,38	6,983		6,983	bb			0,555	<	
M _A_ 2 Fb2	Analyte		16,67	23,84		23,84	bb			2,435	<	
P _A_ 1 Fa2	Analyte		16,67	3,931		3,931	bb			1,08	<	
P _A_ 1 Fb2	Analyte											
P _A_ 2 Fa2	Analyte		17,38	5,48		5,48	bb			1,153	<	
P _A_ 2 Fb2	Analyte											
P _A_ 3 Fa2	Analyte		17,38	11,346		11,346	bb			0,882	<	
P _A_ 3 Fb2	Analyte											
SP _S_ 1 Fa2	Analyte		16,86	2,948		2,948	bb			0,699	<	
SP _S_ 1 Fb2	Analyte											
SP _S_ 2 Fa2	Analyte											
SP _S_ 2 Fb2	Analyte											
SP _S_ 3 Fa2	Analyte											
SP _S_ 3 Fb2	Analyte											
M _S_ 1 Fa2	Analyte		17,06	4,674		4,674	bb			0,78	<	
M _S_ 1 Fb2	Analyte											
M _S_ 2 Fa2	Analyte		16,67	6,147		6,147	bb			0,596	<	
M _S_ 2 Fb2	Analyte											
P _S_ 1 Fa2	Analyte											
P _S_ 1 Fb2	Analyte											
P _S_ 2 Fa2	Analyte		17,22	3,12		3,12	bb			0,518	<	
P _S_ 2Fb2	Analyte											
P _S_ 3 Fa2	Analyte		17,06	5,487		5,487	bb			0,552	<	
P _S_ 3 Fb2	Analyte		17,22	7,982		7,982	bb			1,198	<	