applied sciences

MDPI

Review

Review on Cyanobacterial Studies in Portugal: Current Impacts and Research Needs

Cristiana Moreira [1], Alexandre Campos [1], José Carlos Martins [1], Vitor Vasconcelos [1,2] and Agostinho Antunes [1,2,*]

[1] CIIMAR/CIMAR, Interdisciplinary Centre of Marine and Environmental Research, University of Porto, Terminal de Cruzeiros do Porto de Leixões, Av. General Norton de Matos s/n, 4050-208 Porto, Portugal; cmoreira@ciimar.up.pt (C.M.); amoclclix@gmail.com (A.C.); jmartins@ciimar.up.pt (J.C.M.); vmvascon@fc.up.pt (V.V.)

[2] Department of Biology, Faculty of Sciences, University of Porto, Rua do Campo Alegre, 4169-007 Porto, Portugal

* Correspondence: aantunes@ciimar.up.pt; Tel.: +351-223401813

Abstract: Cyanobacteria have long been associated with harmful effects on humans, animals and aquatic biota. Cyanotoxins are their most toxic metabolite. This review summarizes the current research, impacts and future needs in cyanobacterial studies undertaken in Portugal, the southernmost country of Europe, and with a recent multiplication of cyanotoxicity due to climate change events. Microcystins are still the most prevalent, studied and the only regulated cyanotoxins in Portuguese freshwater systems much like most European countries. With the development of some tools, particularly in molecular studies, the recent discovery of cylindrospermopsins, anatoxins and saxitoxins, both genes and toxins, in North and Center ecosystems of our country highlight current impacts that overall communities are facing with increased risks of exposure and uptake to cyanotoxins. Research needs encompass the expansion of studies at all aspects due to the uprising of these cyanotoxins and reinforces the urgent need of increasing the frequency of surveillance to achieve tangible effects of cyanotoxins in Portugal to ultimately implement regulations on cylindrospermopsins, anatoxins and saxitoxins worldwide.

Keywords: microcystins; cylindrospermopsins; anatoxins; saxitoxins; surveillance

Citation: Moreira, C.; Campos, A.; Martins, J.C.; Vasconcelos, V.; Antunes, A. Review on Cyanobacterial Studies in Portugal: Current Impacts and Research Needs. *Appl. Sci.* **2021**, *11*, 4355. https://doi.org/10.3390/app11104355

Academic Editors: Cristiana Moreira and Vitor Manuel Oliveira Vasconcelos

Received: 30 March 2021
Accepted: 5 May 2021
Published: 11 May 2021

1. Introduction

Cyanobacteria belong to an ancient group of prokaryotes where its toxic risks have not been completely evaluated. Eutrophication, anthropogenic pressure, rise of temperature are factors that contribute to the proliferation of these microorganisms (CyanoHAB's) in aquatic systems interfering with water quality and public health by the release of odor substances and toxic compounds (cyanotoxins). These latter possess negative effects such as hepatotoxic, cytotoxic, neurotoxic, genotoxic, immunologic and carcinogenic having resulted in episodes of human and animal mortality as well as morbidity in several countries worldwide [1–5]. Cyanobacteria occurrence possess its main impacts on freshwater ecosystems since activities such as drinking, irrigation and recreation can result in public health problems and derived economic losses. In Portugal, studies on cyanobacteria began in the 1930s mainly through taxonomic studies, followed by some reported toxic episodes associated with blooms and finally, in 1989 the first toxic cyanobacterium species was isolated and its cyanotoxin amount determined. Other strains were also isolated contributing to the knowledge on toxic cyanobacteria occurrence in Portuguese freshwater ecosystems. Bloom occurrence is not often accounted for by national and European governmental agencies where surveillance encompasses solely phytoplankton counting and enumeration of microcystins variant LR the only regulated cyanotoxin in Portugal and Europe [6]. Although other cyanotoxins such as cylindrospermopsins, anatoxins and

saxitoxins occurrence have been found in Portuguese freshwater systems [7] the lack of regulations on these cyanotoxins by Portuguese governmental authorities reflects the need for further research in order to fully assess cyanobacterial impacts, include in monitoring campaigns the evaluation of other cyanotoxins besides the legislated microcystins [6] to improve water management, water quality and provide with recommendations to national authorities in case of an episode of cyanotoxins intoxication occurring.

In toxic cyanobacteria and in cyanotoxins occurrence there can be applied quantitative and qualitative methods. The first (quantitative) allow the enumeration of the cyanotoxins applying techniques such as the High Performance Liquid Chromatography (HPLC), Liquid Chromatography–Mass Spectrometry (LC-MS) and Enzyme-Linked Immunosorbent Assay (ELISA) directly from isolates or environmental samples (water) that will permit after confronting the measured value with the adopted guideline value the evaluation of the toxic status of a given isolate or ecosystem. Matrix-Assisted Laser Desorption/Ionization (MALDI) is another used chemical method that aims to unravel the profile of cyanotoxicity and cyanobacteria metabolites in a given sample. The second (qualitative) allows the characterization and identification applying techniques such as the Polymerase Chain Reaction (PCR) and multiplex Polymerase Chain Reaction (mPCR) of toxic genotypes. These after DNA sequencing allow inferring of the phylogeny of given taxa. Other methods such as the quantitative PCR or Real-Time PCR permit the quantification of the toxic genotypes. Other used assays include the in vivo and in vitro assays based in experiments that englobe either living organisms (in vivo) or cell lines (in vitro) that after an incubation period to a given concentration of a cyanotoxin infer on the damage of the tested cyanotoxin. The present review summarizes the current research, impacts and future needs in cyanobacterial studies undertaken in Portugal (Table 1), contributing to redirect the continuous investigations globally by enumerating current impacts usually associated with cyanobacterial contamination and to foster the mitigation of toxic CyanoHABs, reinforce investigations in Portugal and worldwide by establishing research needs consequently turning the national and international communities and governments more resilient to this problem.

Table 1. Summary of the currents impacts and research needs per type of cyanotoxin in Portugal.

Cyanotoxin	Current Impacts	Research Needs
Microcystins (MC)	MCLR found in *M. aeruginosa*	Find MCLR in other genera besides *Microcystis* sp. Infer on the phylogeny of MCLR Portuguese DNA sequences
	Other MC variants found in *M. aeruginosa* isolates	Enumerate MC variants found in *M. aeruginosa* Find and enumerate other MC variants in bloom samples, other genera and in water samples In vivo and in vitro assays for other MC variants Infer on the phylogeny of MC variants Portuguese DNA sequences
Cylindrospermopsins (CYN)	Enumerated in Vela Lagoon	Expand enumeration studies to other ecosystems to assess possible risks
	Detected genes in North and Center regions	Expand surveillance to the South Region to assess possible risks Isolate the toxin-producing strain Infer on the phylogeny of CYN Portuguese DNA sequences
Anatoxin-a (ANA)	Found after laboratory cultivation of isolates of *Chrysosporum*, *Dolichospermum*, *Microcystis* and *Oscillatoria*	Find the environmental toxin-producing strain Study toxic effects through proteomic, in vivo and in vitro assays
	Enumerated in North and Center Regions	Expand surveillance to the South Region to assess possible risks Enumerate through chemical methods. Find other isoforms Infer on the phylogeny of Portuguese ANA DNA sequences
Saxitoxins (SXT)	Found in *C. gracile*	Study toxic effects through proteomic, in vivo and in vitro assays
	Enumerated in North and Center Regions	Expand surveillance to the South Region to assess possible risks Enumerate through chemical methods. Find other isoforms Infer on the phylogeny of Portuguese SXT DNA sequences
Others	Unidentified toxic metabolites in Portuguese *C. raciborskii*	Isolate *C. raciborskii* and perform chemical, in vivo and in vitro assays on strains to identify the toxicity

1.1. Toxic Cyanobacteria

Although cyanobacterial studies began in Portugal in the 1930s the first identification of the first toxic cyanobacterium species in our waters was attributed to Vasconcelos et al. [8] that with his surveillance and search for microcystins found that this was produced by the species *Microcystis aeruginosa* in several amounts and in a diverse range of freshwater systems of Portugal. In his survey 36 lakes, reservoirs and rivers were monitored. Then only *Microcystis* blooms were assessed and 60% of these were found to be toxic. The main species present in toxic blooms were *Microcystis aeruginosa* (72%) and *Anabaena flos-aquae* (28%) [9]. The main hepatotoxins in Portuguese freshwaters include MCYST-LR, MCYST-LA, MCYST-YR and [D-Aspl]MCYSTLR [8,10]. Later Pereira et al. [11] found evidence of saxitoxins production in Montargil Reservoir (South Region) through the chemical analysis of an isolate of *Chrysosporum flos-aquae*. Studies persisted and in 2009 Oswald et al. [12] found anatoxin-a production in strains of *Chrysosporum*, *Dolichospermum*, *Microcystis* and *Oscillatoria* with production values ranging from 0.06 μg/g to 24.62 μg/g of cyanobacterial dry weight [12]. In spite of this finding, the strains were only able to produce anatoxin-a under laboratory conditions failing so far its environmental detection through chemical methods. Cylindrospermopsins were found to occur in a lagoon located in the Center Region (Vela Lagoon) in amounts that reached 12 μg/L in the water [13]. In this study the cylindrospermopsin-producer identified belonged to *Chrysosporum* sp. and this was only achieved after sequencing and blast web search of a positive amplicon that belonged to the *cyrC* gene cluster since the isolation of the producing strain failed [13]. Since toxic cyanobacterium studies began in Portugal this was the first study to include molecular studies in the identification of a toxic cyanobacterium producer. More recently Moreira et al. [7] found evidence of cyanotoxins multiplication in Portugal since most of the previous studies were referred to the Center and South Regions of the country providing evidence that other cyanotoxins besides microcystins can be found in the colder North Region of Portugal. Other results from this study include that anatoxin-a and saxitoxins production were attributed to the *Chrysosporum* sp. genus [7]. The invasive and toxic *Cylindrospermopsis raciborskii* was found to occur for the first time in the colder North Region of Portugal without forming any blooms and with no cyanotoxicity being attributed so far [14]. Regarding bloom occurrence, the first intensive surveillance study that searched for all cyanotoxins in Portuguese freshwater systems belonged to Moreira et al. [14] where it was found that 50% of the ecosystems analyzed had blooms (Figure 1). Its composition was comprised of *Microcystis* alone or a mixture of *Microcystis* with *Chrysosphorum*/*Dolichospermum*. Though initial studies by Vasconcelos described that *Microcystis* was the dominant bloom forming genus in Portugal recently in a year (2017) with two heat waves during the sampling season lead to the rise of other types of cyanobacterium species which may have contributed to the multiplication of cyanotoxicity recommending the deepening of monitoring campaigns as well as the integration of other cyanotoxins besides the legislated microcystins-LR (MCLR). In this study only North and Center water systems were sampled; nonetheless, previously in 2005 in Algarve (South Region) at Beliche reservoir, it was found a bloom of *Planktothrix rubescens* that produced microcystins [15]. An extensive study on cyanobacterial blooms in the south regions of Alentejo and Algarve reservoirs highlighted the occurrence of blooms not only attributed to *M. aeruginosa* but to other species belonging to the Nostocales order in Algarve reservoirs and *Microcystis aeruginosa* and *Anabaena circinalis* in reservoirs from Alentejo region as the predominant bloom-forming species [16].

Figure 1. Bloom formation at Porto City Park Lake 1 (North Region) in 2012 (**A**) and at Marco de Canaveses (North Region) in 2012 (**B**).

1.2. Cyanotoxins Episodes

In worldwide cyanotoxins episodes they resulted in human and animal mortality and morbidity having a global impact and in Portugal, there is no exception. In fact, in 1993 the death of 20 patients in a hemodialysis unit in Évora Hospital (South Region) was attributed to cyanotoxins though none were measured [17]. More recently in 2017 the death of 25 cows in Alentejo (South Region) that ingested water from a nearby stream resulted in the detection of MCLR in the kidneys of one animal (0.13 µg/L). Phytoplankton examination revealed that *Microcystis* was the main genera found and total microcystins were detected in a concentration of 0.16 µg/L [18]. Though this is the first report of a cyanotoxins animal poisoning in Portugal the lack of epidemiological data through bloom occurrence in several ecosystems as described by Moreira et al. [7] may hinder other cyanotoxins outbreaks in national waters though no human fatality being identified to these metabolites similarly to the described mortality (Brazil) and morbidity (Australia) episodes [1,2]. Nonetheless continuous vigilance of these metabolites is essential along with cross-referencing with health and environmental agencies of Portugal.

2. Cyanobacterial Studies

2.1. Chemical Assays

Cyanotoxins based studies on chemical studies in Portuguese freshwater systems englobe the screening and enumeration of all the main cyanotoxins and a study on the peptide diversity of *M. aeruginosa* strains isolated from several Portuguese freshwater systems (Figure 2). Microcystins were the first to be chemically unraveled by HPLC where MCYST-LR, MCYST-LA, MCYST-YR and [D-Aspl] MCYSTLR were found to be present in both bloom samples and isolated strains of *M. aeruginosa* gathered from several freshwater systems with national representation [8,10]. Saxitoxins were the next cyanotoxins to be characterized this occurring through a HPLC-FLD method followed by LC/MS confirmation from a strain of *Chrysosporum flos-aquae* collected from a bloom of this cyanobacterium in Montargil reservoir (South Region) [11]. In this study, five PSP toxins, neoSTX, dcSTX, STX, GTX6, and GTX5 were found in the same isolate (LMECYA 31) [11]. Screening for anatoxins was followed later in the mid-2000s but only after laboratory cultivation of strains belonging to the genera *Chrysosporum*, *Dolichospermum*, *Microcystis* and *Oscillatoria* with anatoxin-a production values ranging 0.06 µg/g to 24.62 µg/g of cyanobacterial dry weight [12]. More recently cylindrospermopsins was found to occur in a lagoon in the Center Region of Portugal (Vela Lagoon) also through HPLC followed by confirmation of the mass spectrum through LC/MS in water samples and in the absence of blooms in concentrations that ranged a minimum of 1.4 µg/L to a maximum of 12 µg/L [13]. Apart from HPLC and LC/MS methods, a study by Martins et al. [19] used the MALDI-TOF

MS technique to determine the peptide diversity of Portuguese freshwater *M. aeruginosa* strains isolated from lakes, rivers and reservoirs. Results from their study include the finding of aeruginosins, microginins, anabaenopeptins, cyanopeptilins, microcystins, and microviridins [19]. In microcystins it was found the presence of the variants MCLR, -FR, -RR, -WR and -YR as the most commonly found [19].

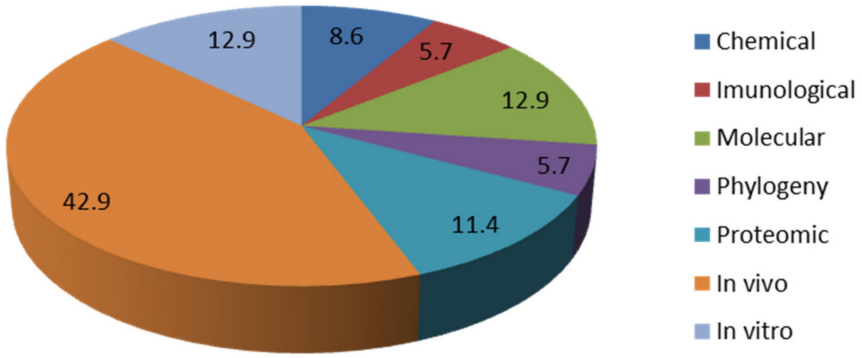

Figure 2. Graphical representation of the percentage of each assay published in Portugal.

2.2. Immunological Assays

Some studies have used the immunological assays (ELISA) marked by Abraxis in water samples collected from several lakes, rivers and reservoirs with national representation as well as in food supplements (Figure 2). This method was never used singularly but as complementary to other methods since it is an enumeration technique. Regarding water systems, initial studies were based on the search and enumeration of microcystins in southern reservoirs [20]. Valério et al. [20] in their study showed that microcystins were detected in 23% of the 53 samples tested and in some of them concentrations exceeded the WHO guideline of 1 μg/L [20]. Later, a cyanotoxins risk assessment conducted in eight Center Region reservoirs highlighted again the presence of microcystins in which two reservoirs resulted in high risk with values above 20 μg/L [21]. Until these studies, no ELISA immunoassays were conducted on other cyanotoxins besides microcystins. This situation was surpassed by the study of Moreira et al. [7] where all main cyanotoxins were enumerated by ELISA. In their study, North Region ecosystems were extensively studied and monitored for microcystins and resulted that in the five northern ecosystems analyzed 57% had values above 1 μg/L between April and September. Other cyanotoxins surveyed included in Vela Lagoon cylindrospermopsins with none of the six samples above the proposed guideline of 1 μg/L by Humpage and Falconer [22] and in the North and Center region ecosystems, the enumeration of anatoxin-a showed that 33% were above the proposed guideline value of 1 μg/L while in saxitoxins only one sample belonging to a North Region ecosystem exceeded the proposed guideline value of 3 μg/L [13]. Saker et al. [23] showed the presence of microcystins-LR equivalents with concentrations within the range of 0.1–4.72 μg/g in food supplements.

2.3. Molecular Studies

Molecular studies were initiated in the mid 2000s (Figure 2) with a first study applying *C. raciborskii* Portuguese isolated strains to determine their potential to produce either cylindrospermopsins or saxitoxins. Results from this analysis were negative for both cyanotoxins and englobe the application of peptide synthetase (PS) and polyketide synthase (PKS) genes through simple PCR analysis [24]. Recent studies have shown that environmental DNA belonging to *C. raciborskii* can be already found in the north and center regions of Portugal demonstrating its invasive nature but without forming any blooms and with no association with toxicity genes [14]. Other studies used the multiplex PCR technique in uncovering the toxigenic nature of dietary food supplements of cyanobacte-

rial origin. In fact, toxigenic *Microcystis* were found to be present in all analyzed dietary supplements produced from the nontoxic cyanobacterium *Chrysosporum flos-aquae* [25]. PCR was further considered an early warning technique in cyanobacterial investigations since it correlated well with ELISA assays when applying Portuguese water samples [26]. Valério et al. [27] developed a multiplex PCR that simultaneously amplified *mcyA*-cd, *mcyAB*, and *mcyB* amplicons of the microcystin gene cluster in 124 cyanobacterial isolates and in 37 environmental samples. The toxicological status of the isolates was assessed by high-performance liquid chromatography where the multiplex PCR developed showed a sensitivity of 92.3% and a specificity of 100%. In environmental samples, ELISA was used and results for the presence of microcystins in these samples gave a sensitivity of 80% and a specificity of 100% [27]. Studies applying Real-Time PCR analysis were initiated by Moreira et al. [28] with the variation of *C. raciborskii* and cylindrospermopsins in field samples gathered from Vela Lake with primers previously described characterizing total cyanobacteria (16S rRNA), *C. raciborskii* (*rpoC1*), and cylindrospermopsin synthetase gene (*cyrC*). The results report the high abundance of both cyanobacteria and *C. raciborskii* in Vela Lake, with *C. raciborskii* representing 0.4% to 58% of the total cyanobacteria population. Cylindrospermopsin synthetase gene was detected in one of the samples [28]. In the same year, Martins et al. [29] quantified *Microcystis* and MC-producing *Microcystis* to determine the genotypic composition of the natural *Microcystis* population. Results from his study further highlighted that a negative significant correlation was observed between toxic (with *mcy* genes) to non-toxic (without *mcy* genes) genotypes ratio and the overall *Microcystis* density [29]. More recently, Churro et al., [30] developed primers for the Real-Time PCR amplification of *Planktothrix agardhii* based on the *rpoC1* gene fragment. Cell concentration determined by the Real-Time PCR method showed a linear correlation with the cell concentration determined from direct microscopic counts and detection limit for cell quantification was 8 cells/µL. More recently Moreira et al. [7] carried an extensive surveillance study that included the application of representative genetic markers for the amplification in water samples of the four main cyanotoxins in the North and Center freshwater systems. Amplification results include that 79% of the samples had microcystins (*mcyA*), 40% had cylindrospermopsins (*cyrC*), 40% had anatoxin-a (*anaC*) and finally 50% had saxitoxins (*sxtI*) [13].

2.4. Phylogeny Studies

The first study to apply clustering analysis (Figure 2) belongs to Valério et al. [31], where in their study, M13 PCR fingerprinting, ERIC PCR fingerprinting and amplification of the internal transcribed spacer (ITS) region were used to characterize cultured strains of *C. raciborskii* obtained from several freshwater lakes and rivers of Portugal. Results showed that when including in this analysis other taxa such as *M. aeruginosa*, *Chrysosporum* spp., *Planktothrix agardhii* and *Oscillatoria neglecta* the potential of the fingerprinting method demonstrated only the ability to differentiate strains at an intra-specific level failing as an identification tool. Nonetheless, ITS amplification turned to be a good method for strain clustering [31]. In 2009 the same authors published a work on the applications of several molecular techniques (STRR and LTRR PCR fingerprinting, phylogenetic analysis with the 16S rRNA and *rpoC1* genes and M13 and ERIC PCR fingerprints) to evaluate the level of genetic diversity of 118 cyanobacterial isolates for taxonomic purposes. After the application of these methodologies in the 118 strains revealed good congruence, indicating the potential for use in cyanobacterial monitoring, as a quality management control. Later, Moreira et al. [32] when applying isolates of Portuguese *C. raciborskii* found that they cluster together with other European strains and were closely related to Asian and Australian strains. This study used for the first time the genetic information of four genetic markers that were concatenated in the analysis (16S rRNA, *rpoC1*, ITS-L and ITS-S) [32]. Later, the same authors studied the genetic diversity and population structure of Portuguese isolates of *M. aeruginosa* [33]. In their study, a phylogenetic tree was produced resulting from the concatenation of four distinct genetic markers (16S rRNA, 16S-23S ITS, DNA

gyrase subunit ß and cell division protein *ftsZ*) and showed that Portuguese *M. aeruginosa* population possesses 15 distinct genotypes with the southern strains clustering together. In this study, toxic strains were also analyzed and showed that they carry no cyanotoxicity significance appearing intermixed in the phylogenetic tree [33].

2.5. Proteomic Studies

Proteomics research methods have been providing complementary information concerning the toxicology of cyanotoxins and mechanisms of action of these molecules (Figure 2). It has been an essential research strategy to elucidate ecotoxicological outcomes in aquatic invertebrates (bivalves) and plants, and also cytotoxic and genotoxic effects of cyanotoxins in cell models such as *Saccharomyces cerevisiae*. Methods of global protein analysis, based on two-dimensional gel electrophoresis (2DE) and MALDI-TOF mass spectrometry (MS) were used, for example, to characterize the proteome of agricultural plant species, and describe the adverse effects related to the use of contaminated water in crop irrigation. This methodology enabled to reveal putative adverse alterations in the proteome of rice plants (12 and 20-day-old) exposed for 7 days to *M. aeruginosa* bloom extract with 0.26–78 µg/L MCLR [34]. Moreover, the same methodology revealed alterations in ATP synthase, Cytochrome b6-f complex iron-sulfur and oxygen-evolving enhancer protein markers, and other proteins related to carbohydrate metabolism in tomato plants, pointing to possible damages in ATP synthesis, energy metabolism and photosynthesis caused by the toxin MCLR [35]. Yet, a leaf proteome profile in lettuce confirmed some of the molecular disturbances previously observed and additional alterations in stress/defense response, protein synthesis and signal transduction, related to MCLR and CYN exposure. A concomitant decrease in plant growth was also reported [36].

A target proteomics approach, combining affinity chromatography and protein purification, 2DE and MALDI-TOF mass spectrometry, was used to investigate the role of glutathione s-transferases (GSTs) in the response of bivalves [37,38]. The studies carried out revealed protein profiles constituted by multiple GST isoforms in bivalve species. Alterations in individual isoforms were revealed by this methodology suggesting that the GST system may play a role in the detoxification processes of cyanotoxins in this group of aquatic invertebrates [37,38]. A large-scale proteomic profiling, combining liquid chromatography and high-throughput MS analysis, was used to depict other molecular processes related to the response of bivalves to cyanotoxins like MCs and CYN. Sub-toxic effects of cyanotoxins were related for instance to changes in protein folding and stabilization, cytoskeleton structure, and gene transcription/translation [39].

Proteomic and gene expression studies shed more light concerning the response of eukaryotic cells to MCs. Using the cell model *Saccharomyces cerevisiae*, several metabolic responses were revealed related to exposure to low MCLR concentrations. These studies revealed dose responses in Base Excision Repair (BER) DNA-repair system, suggesting genotoxic effects [40,41], and changes in a broad group of proteins with functions linked to gene translation and DNA replication, oxidative stress, cell cycle regulation and carbohydrate metabolism [41]. Alterations in protein markers such as translation initiation factor 1 (RLI1), thioredoxin reductase 1 (TRR1) and UV excision repair protein (RAD23) confirmed the link between oxidative stress and DNA damage mechanisms [41].

2.6. In Vivo Studies

Initial in vivo research findings on the effects of cyanobacteria and associated toxins focused mainly on the aquatic environment and their organisms (Figure 2). Zooplankton, bivalves and fish are among the most studied groups. These works comprehended various in vivo measurement approaches, using mostly laboratory-exposed organisms. In the early nineties, Vasconcelos [42] started by studying the impact of toxic and nontoxic cyanobacteria on some freshwater microcrustacean species. Results showed that a toxic *Microcystis aeruginosa* strain produced total lethality of three cladocerans (*Daphnia longispina*, *Ceriodaphnia pulchella* and *Simocephalus vetulus*) in two days. Furthermore, the non-toxic strain of

the same cyanobacteria species caused total mortality to *Daphnia* in four days while the other two species survived longer, suggesting that other compounds apart from MC may be involved in zooplankton dynamics [42,43]. In the following studies with this group of organisms, Nogueira et al. [44–46] assessed bioaccumulation and effects promoted by toxic and non-toxic cyanobacterial strains using *D. magna* as a biological model. Results suggested that the fitness and growth potential of natural *Daphnia* populations may be affected by cyanobacteria, especially when subjected to blooms of toxic strains. Furthermore, CYN and paralytic shellfish toxins (PSTs) were detected in the tissues of *D. magna* after exposure to the toxic strains of *Cylindrospermopsis raciborskii* and *Aphanizomenon issatschenkoi*, respectively, indicating that daphnids are potentially capable of passing cyanotoxins to higher trophic levels. In bivalve mollusks, the accumulation and depuration of cyanotoxins was reported in several studies where freshwater and intertidal bivalves were exposed to toxic cyanobacteria. Vasconcelos et al. [47] and Amorim and Vasconcelos [48] reported the accumulation of high levels of MC by *Mytillus galloprovincialis* when fed with toxic *M. aeruginosa* for several days. The majority of the toxins were contained in digestive tract, whereas the other organs were barely contaminated. Results also confirmed that mussels could retain detectable toxins after transfer to a non-toxic phytoplankton food source. Later, Pereira et al. [49] and Saker et al. [50] reported the accumulation of high levels of PSTs and CYN by *Anodonta cygnea* after 2 weeks exposure to the toxic cyanobacteria *A. issatschenkoi* and *C. raciborskii*, respectively. The pattern of PSTs accumulation in the various mussel organs was similar to the ones obtained for MC in *M. galloprovincialis*. In its turn, Saker et al. [50] were the first to report the concentrations of cyanobacterial toxins present in the haemolymph and found that this fluid contained the highest concentrations of CYN in *A. cygnea*, followed by viscera. Following a 2-week depuration period, trace to undetectable levels of PST were found in *A. cygnea*, whereas approximately 50% of the CYN remained in the tissues. Finally, Osswald et al. [51] reported no bioaccumulation of anatoxin-a by *M. galloprovincialis* fed with toxic *Anabaena* sp. (5×10^5 cell mL^{-1}) during 2 weeks. The lack of mortality during all these experiments associated to toxin persistence in the tissues supports the idea that bivalves are very resistant to cyanotoxins and good toxin vectors. Thus, it is proposed these mollusks possess detoxification mechanisms. Changes in activity and expression of GSTs in mussels and clams frequently found in Portuguese waters have already been linked to MC-producing microalgae or their purified toxins exposure [52–55]. Working with bivalves of several aquatic habitats, Carneiro et al. [38] showed that these mollusks presented specific adaptive biotransformation responses to MCs and other cyanobacteria compounds supported by the modulation of distinct cGST classes. Overall, results support the relative importance of the GST system as a protective mechanism to cope with MC-producing cyanobacterial blooms exposure which seems to be different between the bivalve species [56]. Despite the ability of cyanotoxins uptake and elimination, physiological alterations along with biochemical and genotoxic effects have been shown to be induced in bivalves by toxic cyanobacteria [39,57]. Studies with fish are in much lesser number when compared with bivalves. In one of the first, Vasconcelos [58] showed that several wild fish species, such as carp, barbel and grey mullet, which were collected from Portuguese freshwaters, could accumulate MC. However, the amounts of toxins detected in the edible parts were not very significant in terms of human health. The study of cyanobacterial effects in fish has been essentially related to the neurotoxin anatoxin-a (ATX). Results show that ATX producing cyanobacteria (*Anabaena* sp.) can promote the acute death of common carp juveniles (10^7 cell mL^{-1}), as well as induce adverse effects in the early development stages of this fish [59,60]. Furthermore, it has been shown that sublethal doses of ATX could induce bioaccumulation and alterations in relevant biochemical markers addressing energy metabolism and biotransformation mechanisms in rainbow trout [61,62].

In the last decade, the effects promoted by cyanobacterial toxins on crop plants attain growing research interest because of contamination via spray irrigation associated with agriculture activities. The effects of exposure of edible plants to MC and CYN (extracts and

purified) have been described for rice (*Oryza sativa*) [34,63], carrots (*Daucus carota*) [64,65], lettuce (*Lactuca sativa*) and spinach (*Spinacea oleracea*) [66,67]. Reported results include negative effects on plant growth, oxidative status and nutritional quality. Recently, Llana-Ruiz-Cabello et al. [68] reported increased sensitivity of spinach to both MC and CYN, individually and in mixture, in comparison with lettuce. Furthermore, plants exposed to CYN/MC mixture showed differential accumulation of both toxins, with CYN being assimilated in a greater amount than MC.

2.7. In Vitro Studies

The first in vitro study (Figure 2) belongs to Dias et al., [69] that evaluated the cytotoxicity of MCLR on a kidney cell line (Vero-E6). Results showed that the lowest cytotoxic MCLR concentration varied between 11 and 100 µM and that cytotoxic effects observed were attributed to MCLR and not to other bioactive compounds. Results further suggest that Vero-E6 cell line may constitute a cell model to evaluate the nephrotoxicity of microcystins [69]. In the following year the same authors, Dias et al., [70] studied the impact of MCLR on tumor promotion at kidney level when applying the same cell line Vero-E6 since MCLR is considered a potent tumor promoter. Results showed that at nanomolar concentrations MCLR stimulates cell cycle progression in Vero-E6 kidney cell line and that the analysis of mitogen-activated protein kinase ERK1/2 activity revealed that MCLR is associated with the activation of this same pathway. Menezes et al. [71] studied the involvement of the endoplasmic reticulum and autophagy in MCLR toxicity in Vero-E6 and HepG2 cell lines. Results showed that HepG2 cells had an increased sensitivity to MCLR than Vero cells. Autophagy is triggered in both cell lines as a survival response to low MCLR concentrations. This study further suggests the involvement of the endoplasmic reticulum in HepG2 apoptosis triggered by MCLR, while in Vero cells endoplasmic reticulum destructuration could be a consequence of cytoskeleton inflicted damages [71]. Dias et al. [72] used the comet and/or the micronucleus (MN) assays to study the genotoxicity of MCLR in kidney- (Vero-E6) and liver-derived (HepG2) cell lines and in blood cells from MCLR-exposed mice. MCLR treatment caused a significant induction in the MN frequency in both cell lines. Moreover, they showed that in HepG2 cells, MCLR induces both chromosome breaks and loss. Alternatively, the comet assay results were negative in Vero-E6 cells and in mouse leukocytes. In this study Dias et al. [72] evidence MCLR genotoxicity. In the same year, a study from Valério et al. [73] evaluated the effect of MCLR on the growth, ROS levels, antioxidant system response and apoptosis induction in *Saccharomyces cerevisiae*. After exposure to several concentrations of this cyanotoxin, results showed that *Saccharomyces cerevisiae* possesses MCLR toxicity effects known to occur in higher eukaryotes [73]. Miguens and Valério [74] studied the impacts of microcystins in the growth of heterotrophic bacteria. In their study MCLR, MCRR and MCYR can reduce the growth of some heterotrophic bacteria that were initially isolated from freshwater sources. In the same year, Dias et al. [75] in their study evaluated the susceptibility of four cyanobacterial isolates (*M. aeruginosa*, *C. gracile*, *C. bergii*, *P. agardhii*) to distinct antibiotics (amoxicillin, ceftazidime, ceftriaxone, kanamycine, gentamicine, tetracycline, trimethoprim, nalidixic acid and norfloxacin). The overall reduced susceptibility suggests that the cyanobacteria tested might be naturally non-susceptible to these compounds [75]. Finally, Salvador et al. [76] studied the effects of light intensity on the levels of expression of *mcyA* in two cyanobacterium species: *M. aeruginosa* and *P. agardhii*. Results showed that there were differences in the expression of *mcyA* between the two species. In *M. aeruginosa*, the highest levels of expression occurred at 4 µmol photons/m/s in the adaptation phase, whereas for *P. agardhii* it was at 4 µmol photons/m/s in the exponential growth phase [76].

2.8. Culture Collections

Portugal currently harbors three culture collections that possess isolates of cyanobacteria both with marine and freshwater origin. Located in the National Institute of Health Dr. Ricardo Jorge (INSA) the Estela Sousa e Silva Algal Culture Collection (ESSACC) has nearly

50 years of existence. The living isolates maintained in the ESSACC contain more than 170 isolates including freshwater cyanobacteria strains isolated from bloom occurrences in Portugal [77]. Another culture collection is the Coimbra Culture Collection of microalgae (ACOI) with over 3000 isolates including freshwater cyanobacteria isolates established over 40 years ago (http://acoi.ci.uc.pt/index.php, accessed on 20 February of 2021). The last and most recently established culture collection in Portugal belongs to the Blue Biotechnology and Ecotoxicology Culture Collection (LEGE CC) (http://lege.ciimar.up.pt/, accessed on 20 February of 2021). LEGE CC holds 386 strains, mainly collected in coastal (48%), estuarine (11%), and fresh (34%) water bodies, the most part from Portugal (84%) [78].

3. Conclusions

With less than 100 publications since studies began in the 1930s, Portugal is still at its infancy in comparison to some European countries regarding cyanobacterial risks in freshwater systems. Much like other European countries studies are mainly circumscribed to microcystins LR though other variants have been reported and studied. National regulations demand since 2007 the surveillance of MCLR in drinking water failing its environmental surveillance in lakes and lagoons with recreational impact according to national authorities. With other cyanotoxins been recently reported such as cylindrospermopsins, anatoxin-a and saxitoxins, the risks to the national population and the local communities that inhabit near the sampling sites and use the water for several daily purposes have increased exponentially endangering their life and health. Given the current impacts, it is demanded the increase of investigations mainly in the surveillance of the recently reported cyanotoxins (CYN, ANA and SXT) without neglecting MCLR due to regulations. Investigations at all aspects with toxin-producing strains (known and unknown metabolites) isolated from Portuguese freshwater ecosystems and incorporate them into in vivo and in vitro assays allows to establish the amount of toxicity and permit to determine the level of exposure to humans and animals. Also retrieve environmental data (continuous surveillance) to support in case of any intoxication by any of the new existing cyanotoxins is a need since globally there is still a lack of epidemiological studies. Given that cyanotoxins contamination is a threat to freshwater ecosystems and public health this review summarizes the current impacts and research needs of Portugal, a southern European country, due to the recent discovery of three harmful cyanotoxins in our waters (CYN, ANA and SXT) and the existence of an unknown toxic metabolite. This review permits, under the global scenario of toxic cyanobacteria, the summary of relevant research needs to other countries that are faced with the uprise of new toxic forms simultaneously redirecting investigations globally. Finally, it is demanded to expand and increase the frequency of surveillance of cylindrospermopsins, anatoxins and saxitoxins in Portuguese freshwater systems towards implementation of national and global future regulations simultaneously contributing to the knowledge and mitigation on toxic cyanobacteria globally and specifically in the European continent.

Author Contributions: Conceptualization, C.M. and A.A.; methodology, C.M.; software, C.M.; validation, C.M.; formal analysis, C.M.; investigation, C.M., A.C. and J.C.M.; resources, C.M.; data curation, C.M.; writing—original draft preparation, C.M., A.C. and J.C.M.; writing—review and editing, V.V. and A.A.; visualization, C.M.; supervision, V.V. and A.A.; project administration, C.M.; funding acquisition, C.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by national funds through FCT-Foundation for Science and Technology within the scope of UIDB/04423/2020 and UIDP/04423/2020 and the FCT projects PTDC/AAG-GLO/2317/2014 (POCI-01-0145-FEDER-016799) and PTDC/CTA-AMB/31774/2017 (POCI-01-0145-FEDER/031774/2017) and to the Postdoctoral fellowship attributed to Cristiana Moreira (SFRH/BPD/122909/2016) by the FCT.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Bourke, A.; Hawes, R.; Neilson, A.; Stallman, N. An outbreak of hepato-enteritis (the Palm Island mystery disease) possibly caused by algal intoxication. *Toxicon* **1983**, *21*, 45–48. [CrossRef]
2. Jochimsen, E.M.; Carmichael, W.W.; An, J.; Cardo, D.M.; Cookson, S.T.; Holmes, C.E.M.; Antunes, M.B.; Filho, D.A.D.M.; Lyra, T.M.; Barreto, V.S.T.; et al. Liver Failure and Death after Exposure to Microcystins at a Hemodialysis Center in Brazil. *N. Engl. J. Med.* **1998**, *338*, 873–878. [CrossRef]
3. Gugger, M.; Lenoir, S.; Berger, C.; Ledreux, A.; Druart, J.-C.; Humbert, J.-F.; Guette, C.; Bernard, C. First report in a river in France of the benthic cyanobacterium Phormidium favosum producing anatoxin-a associated with dog neurotoxicosis. *Toxicon* **2005**, *45*, 919–928. [CrossRef] [PubMed]
4. van Apeldoorn, M.E.; van Egmond, H.P.; Speijers, G.J.A.; Bakker, G.J.I. Toxins of cyanobacteria. *Mol. Nutr. Food Res.* **2007**, *51*, 7–60. [CrossRef] [PubMed]
5. Sieroslawska, A. Immunotoxic, genotoxic and carcinogenic effects of cyanotoxins. *Centr. Eur. J. Immunol.* **2010**, *35*, 105–110.
6. Decreto-Lei, n.º 306/2007 de 27 de Agosto, Diário da República, I Série, n.º 164, 27 de Agosto de 2007. Available online: https://dre.pt/application/dir/pdf1sdip/2007/08/16400/0574705765.pdf (accessed on 20 February 2021).
7. Moreira, C.; Gomes, C.; Vasconcelos, V.; Antunes, A. Cyanotoxins Occurrence in Portugal: A New Report on Their Recent Multiplication. *Toxins* **2020**, *12*, 154. [CrossRef]
8. Vasconcelos, V.; Sivonen, K.; Evans, W.R.; Carmichael, W.W.; Namikoshi, M. Isolation and characterization of microcystins (heptapeptide hepatotoxins) from Portuguese strains of Microcystis aeruginosa Kutz. emed Elekin. *Arch. Hydrobiol.* **1995**, *134*, 295–305. [CrossRef]
9. Vasconcelos, V. Toxic Cyanobacteria (Blue-green Algae) in Portuguese Freshwaters. *Detect. Methods Cynobacterial Toxins* **1994**, 133–135. [CrossRef]
10. Vasconcelos, V.M.; Sivonen, K.; Evans, W.R.; Carmichael, W.W.; Namikoshi, M. Hepatotoxic microcystin diversity in cyanobacterial blooms collected in Portuguese freshwaters. *Water Res.* **1996**, *30*, 2377–2384. [CrossRef]
11. Pereira, P.; Onodera, H.; Andrinolo, D.; Franca, S.; Araújo, F.; Lagos, N.; Oshima, Y. Paralytic shellfish poisoning toxins in the freshwater cyanobacterium Aphanizomenon flos-aquae, isolated from Montargil reservoir, Portugal. *Toxicon* **2000**, *38*, 1689–1702. [CrossRef]
12. Osswald, J.; Rellán, S.; Gago-Martinez, A.; Vasconcelos, V. Production of anatoxin-a by cyanobacterial strains isolated from Portuguese fresh water systems. *Ecotoxicology* **2009**, *18*, 1110–1115. [CrossRef] [PubMed]
13. Moreira, C.; Mendes, R.; Azevedo, J.; Vasconcelos, V.; Antunes, A. First occurrence of cylindrospermopsin in Portugal: A contribution to its continuous global dispersal. *Toxicon* **2017**, *130*, 87–90. [CrossRef] [PubMed]
14. Moreira, C.; Gomes, C.; Vasconcelos, V.; Antunes, A. Monitoring the invasive cyanobacterium Cylindrospermopsis raciborskii —A case of dispersion into northern Portuguese freshwater systems. In: Harmful Algae 2018—From ecosystems to socioecosystems. Nantes. International Society for the Study of Harmful Algae 2020. In Proceedings of the 18th Intl. Conf. on Harmful Algae, Nantes, France, 21–26 October; 2018; pp. 130–134.
15. Paulino, S.; Valério, E.; Faria, N.; Fastner, J.; Welker, M.; Tenreiro, R.; Pereira, P. Detection of Planktothrix rubescens (Cyanobacteria) associated with microcystin production in a freshwater reservoir. *Hydrobiologia* **2009**, *621*, 207–211. [CrossRef]
16. Galvão, H.M.; Reis, M.P.; Valério, E.; Domingues, R.B.; Costa, C.; Lourenço, D.; Condinho, S.; Miguel, R.; Barbosa, A.B.; Gago, C.; et al. Cyanobacteria blooms in natural waters in Southern Portugal—A water management perspective. *Aquat. Microb. Ecol.* **2008**, *53*, 129–140. [CrossRef]
17. CyanoNews. Toxin Suspect in Mass Killing. Cyanonews 1996, 12, 4. Available online: http://wwwcyanosite.bio.purdue.edu/cyanonews/cn122/cn122.pdf (accessed on 20 February 2021).
18. Menezes, C.; Nova, R.; Vale, M.; Azevedo, J.; Vasconcelos, V.; Pinto, C. First description of an outbreak of cattle intoxication by cyanobacteria (blue-green algae) in the South of Portugal. *Bov. Pract.* **2019**, *53*, 66–70.
19. Martins, J.; Saker, M.L.; Moreira, C.; Welker, M.; Fastner, J.; Vasconcelos, V.M. Peptide diversity in strains of the cyanobacte-rium Microcystis aeruginosa isolated from Portuguese water supplies. *Appl. Microbiol. Biotechnol.* **2009**, *82*, 951–961. [CrossRef]
20. Valério, E.; Faria, N.; Paulino, S.; Pereira, P. Seasonal variation of phytoplankton and cyanobacteria composition and associated microcystins in six Portuguese freshwater reservoirs. *Ann. De Limnol. Int. J. Limnol.* **2008**, *44*, 189–196. [CrossRef]
21. Menezes, C.; Churro, C.; Dias, E. Risk Levels of Toxic Cyanobacteria in Portuguese Recreational Freshwaters. *Toxins* **2017**, *9*, 327. [CrossRef]
22. Humpage, A.R.; Falconer, I.R. Oral toxicity of the cyanobacterial toxin cylindrospermopsin in male Swiss albino mice: Determination of no observed adverse effect level for deriving a drinking water guideline value. *Environ. Toxicol.* **2003**, *8*, 94–103. [CrossRef]
23. Saker, M.; Jungblut, A.-D.; Neilan, B.; Rawn, D.; Vasconcelos, V. Detection of microcystin synthetase genes in health food supplements containing the freshwater cyanobacterium Aphanizomenon flos-aquae. *Toxicon* **2005**, *46*, 555–562. [CrossRef] [PubMed]

24. Valério, E.; Pereira, P.; Saker, M.; Franca, S.; Tenreiro, R. Molecular characterization of Cylindrospermopsis raciborskii strains isolated from Portuguese freshwaters. *Harmful Algae* **2005**, *4*, 1044–1052. [CrossRef]
25. Saker, M.L.; Welker, M.; Vasconcelos, V.M. Multiplex PCR for the detection of toxigenic cyanobacteria in dietary supplements produced for human consumption. *Appl. Microbiol. Biotechnol.* **2007**, *73*, 1136–1142. [CrossRef] [PubMed]
26. Saker, M.L.; Vale, M.; Kramer, D.; Vasconcelos, V.M. Molecular techniques for the early warning of toxic cyanobacteria blooms in freshwater lakes and rivers. *Appl. Microbiol. Biotechnol.* **2007**, *75*, 441–449. [CrossRef] [PubMed]
27. Valério, E.; Chambel, L.; Paulino, S.; Faria, N.; Pereira, P.; Tenreiro, R. Multiplex PCR for detection of microcystins-producing cyanobacteria from freshwater samples. *Environ. Toxicol.* **2009**, *25*, 251–260. [CrossRef]
28. Moreira, C.; Martins, A.; Azevedo, J.; Freitas, M.; Regueiras, A.; Vale, M.; Antunes, A.; Vasconcelos, V. Application of real-time PCR in the assessment of the toxic cyanobacterium Cylindrospermopsis raciborskii abundance and toxicological potential. *Appl. Microbiol. Biotechnol.* **2011**, *92*, 189–197. [CrossRef] [PubMed]
29. Martins, A.; Moreira, C.; Vale, M.; Freitas, M.; Regueiras, A.; Antunes, A.; Vasconcelos, V. Seasonal Dynamics of Microcystis spp. and Their Toxigenicity as Assessed by qPCR in a Temperate Reservoir. *Mar. Drugs* **2011**, *9*, 1715. [CrossRef]
30. Churro, C.; Pereira, P.; Vasconcelos, V.; Valério, E. Species-specific real-time PCR cell number quantification of the bloom-forming cyanobacterium Planktothrix agardhii. *Arch. Microbiol.* **2012**, *194*, 749–757. [CrossRef] [PubMed]
31. Valério, E.; Chambel, L.; Paulino, S.; Faria, N.; Pereira, P.; Tenreiro, R. Molecular identification, typing and traceability of cyanobacteria from freshwater reservoirs. *Microbiology* **2009**, *155*, 642–656. [CrossRef] [PubMed]
32. Moreira, C.; Fathalli, A.; Vasconcelos, V.; Antunes, A. Genetic Diversity and Structure of the Invasive Toxic Cyanobacterium Cylindrospermopsis raciborskii. *Curr. Microbiol.* **2011**, *62*, 1590–1595. [CrossRef]
33. Moreira, C.; Vasconcelos, V.; Antunes, A. Genetic characterization of Microcystis aeruginosa isolates from Portuguese freshwater systems. *World J. Microbiol. Biotechnol.* **2016**, *32*, 118. [CrossRef] [PubMed]
34. Azevedo, C.C.; Azevedo, J.; Osório, H.; Vasconcelos, V.; Campos, A. Early physiological and biochemical responses of rice seedlings to low concentration of microcystin-LR. *Ecotoxicology* **2014**, *23*, 107–121. [CrossRef]
35. Gutiérrez-Praena, D.; Campos, A.; Azevedo, J.; Neves, J.; Freitas, M.; Guzmán-Guillén, R.; Cameán, A.M.; Renaut, J.; Vasconcelos, V. Exposure of Lycopersicon Esculentum to Microcystin-LR: Effects in the Leaf Proteome and Toxin Translocation from Water to Leaves and Fruits. *Toxins* **2014**, *6*, 1837. [CrossRef]
36. Freitas, M.; Campos, A.; Azevedo, J.; Barreiro, A.; Planchon, S.; Renaut, J.; Vasconcelos, V. Lettuce (Lactuca sativa L.) leaf-proteome profiles after exposure to cylindrospermopsin and a microcystin-LR/cylindrospermopsin mixture: A concentra-tion-dependent response. *Phytochemistry* **2015**, *110*, 91–103. [CrossRef] [PubMed]
37. Martins, J.C.; Campos, A.; Osorio, H.; Da Fonseca, R.; Vasconcelos, V. Proteomic Profiling of Cytosolic Glutathione Transferases from Three Bivalve Species: Corbicula fluminea, Mytilus galloprovincialis and Anodonta cygnea. *Int. J. Mol. Sci.* **2014**, *15*, 1887. [CrossRef] [PubMed]
38. Carneiro, M.; Antas, P.; Reis, B.; Azevedo, J.; Osório, H.; Campos, A.; Vasconcelos, V.; Martins, J. Modulation of hepatic glutathione transferases isoenzymes in three bivalve species exposed to purified microcystin-LR and Microcystis extracts. *Toxicon* **2017**, *137*, 150–157. [CrossRef] [PubMed]
39. Oliveira, F.; Diez-Quijada, L.; Turkina, M.V.; Morais, J.; Felpeto, A.B.; Azevedo, J.; Jos, A.; Camean, A.M.; Vasconcelos, V.; Martins, J.C.; et al. Physiological and Metabolic Responses of Marine Mussels Exposed to Toxic Cyanobacteria Microcystis aeruginosa and Chrysosporum ovalisporum. *Toxins* **2020**, *12*, 196. [CrossRef] [PubMed]
40. Valério, E.; Campos, A.; Osório, H.; Vasconcelos, V. Proteomic and Real-Time PCR analyses of Saccharomyces cerevisiae VL3 exposed to microcystin-LR reveals a set of protein alterations transversal to several eukaryotic models. *Toxicon* **2016**, *112*, 22–28. [CrossRef]
41. Valério, E.; Barreiros, S.; Rodrigues, S.; Turkina, M.V.; Vasconcelos, V.M.; Campos, A. New Insights in Saccharomyces cerevisiae Response to the Cyanotoxin Microcystin-LR, Revealed by Proteomics and Gene Expression. *Toxins* **2020**, *12*, 667. [CrossRef] [PubMed]
42. Vasconcelos, V. Preliminary results of a study on the impact of toxic and nontoxic cyanobacteria on some freshwater micro-crustacean species. *Crustaceana* **1990**, *59*, 316–318. [CrossRef]
43. Vasconcelos, V. Cyanobacteria toxins: Diversity and ecological effects. *Limnetica* **2001**, *20*, 45–58.
44. Nogueira, I.C.; Pereira, P.; Dias, E.; Pflugmacher, S.; Wiegand, C.; Franca, S.; Vasconcelos, V.M. Accumulation of paralytic shellfish toxins (PST) from the cyanobacterium Aphanizomenon issatschenkoi by the cladoceran Daphnia magna. *Toxicon* **2004**, *44*, 773–780. [CrossRef]
45. Nogueira, I.C.G.; Saker, M.L.; Pflugmacher, S.; Wiegand, C. Toxicity of the cyanobacteriumCylindrospermopsis raciborskii toDaphnia magna. *Environ. Toxicol.* **2004**, *19*, 453–459. [CrossRef] [PubMed]
46. Nogueira, I.C.; Lobo-Da-Cunha, A.; Vasconcelos, V.M. Effects of Cylindrospermopsis raciborskii and Aphanizomenon ovalispo-rum (cyanobacteria) ingestion on Daphnia magna midgut and associated diverticula epithelium. *Aquat. Toxicol.* **2006**, *80*, 194–203. [CrossRef] [PubMed]
47. Vasconcelos, V.M. Uptake and depuration of the heptapeptide toxin microcystin-LR in Mytilus galloprovincialis. *Aquat. Toxicol.* **1995**, *32*, 227–237. [CrossRef]
48. Amorim, Á.; Vasconcelos, V. Dynamics of microcystins in the mussel Mytilus galloprovincialis. *Toxicon* **1999**, *37*, 1041–1052. [CrossRef]

49. Pereira, P.; Dias, E.; Franca, S.; Carolino, M.; Vasconcelos, V. Accumulation and depuration of cyanobacterial paralytic shellfish toxins by the freshwater mussel Anodonta cygnea. *Aquat. Toxicol.* **2004**, *68*, 339. [CrossRef]
50. Saker, M.L.; Metcalf, J.S.; Codd, G.A.; Vasconcelos, V.M. Accumulation and depuration of the cyanobacterial toxin cylindrospermopsin in the freshwater mussel Anodonta cygnea. *Toxicon* **2004**, *43*, 185–194. [CrossRef]
51. Osswald, J.; Rellán, S.; Gago, A.; Vasconcelos, V. Uptake and depuration of anatoxin-a by the mussel Mytilus galloprovincialis (Lamarck, 1819) under laboratory conditions. *Chemosphere* **2008**, *72*, 1235–1241. [CrossRef]
52. Vasconcelos, V.M.; Wiegand, C.; Pflugmacher, S. Dynamics of glutathione-Stransferases in Mytilus galloprovincialis exposed to toxic Microcystis aeruginosa cells, extracts and pure toxins. *Toxicon* **2007**, *50*, 740–745. [CrossRef]
53. Fernandes, S.; Welker, M.; Vasconcelos, V.M. Changes in the GST activity of the mussel Mytilus galloprovincialis during exposure and depuration of microcystins. *J. Exp. Zool. A Ecol. Genet. Physiol.* **2009**, *311*, 226–230. [CrossRef]
54. Carneiro, M.; Reis, B.; Azevedo, J.; Campos, A.; Osorio, H.; Vasconcelos, V.; Martins, J.C. Glutathione Transferases Responses Induced by Microcystin-LR in the Gills and Hepatopancreas of the Clam Venerupis philippinarum. *Toxins* **2015**, *7*, 2096. [CrossRef] [PubMed]
55. Reis, B.; Carneiro, M.; Machado, J.; Azevedo, J.; Vasconcelos, V.; Martins, J.C. Transcriptional responses of glutathione trans-ferase genes in Ruditapes philippinarum exposed to microcystin-LR. *Int. J. Mol. Sci.* **2015**, *16*, 8397. [CrossRef] [PubMed]
56. Antas, P.; Carneiro, M.; Reis, B.; Castelo-Branco, R.; Azevedo, J.; Urbatzka, R.; Campos, A.; Vasconcelos, V.; Martins, J.C. GST transcriptional changes induced by a toxic Microcystis aeruginosa strain in two bivalve species during exposure and recovery phases. *Ecotoxicology* **2018**, *27*, 1272–1280. [CrossRef]
57. Martins, J.C.; Machado, J.P.; Martins, A.; Azevedo, J.; Teles, L.O.; Vasconcelos, V. Dynamics of Protein Phosphatase Gene Expression in Corbicula fluminea Exposed to Microcystin-LR and to Toxic Microcystis aeruginosa Cells. *Int. J. Mol. Sci.* **2011**, *12*, 9172. [CrossRef] [PubMed]
58. Vasconcelos, V.M. Cyanobacteria toxins in Portugal: Effects on aquatic animals and risk for human health. *Braz. J. Med. Biol. Res.* **1999**, *32*, 249–254. [CrossRef] [PubMed]
59. Osswald, J.; Rellán, S.; Carvalho, A.P.; Gago, A.; Vasconcelos, V. Acute effects of an anatoxin-a producing cyanobacterium on juvenile fish—Cyprinus carpio L. *Toxicon* **2007**, *49*, 693–698. [CrossRef] [PubMed]
60. Osswald, J.; Carvalho, A.; Claro, J.; Vasconcelos, V. Effects of cyanobacterial extracts containing anatoxin-a and of pure anatoxin-a on early developmental stages of carp. *Ecotoxicol. Environ. Saf.* **2009**, *72*, 473–478. [CrossRef]
61. Osswald, J.; Azevedo, J.; Vasconcelos, V.; Guilhermino, L. Experimental determination of the bioconcentration factors for anatoxin-a in juvenile rainbow trout (Oncorhynchus mykiss). *Proc. Int. Acad. Ecol. Environ. Sci.* **2011**, *1*, 77–86.
62. Osswald, J.; Carvalho, A.P.; Guimarães, L.; Guilhermino, L. Toxic effects of pure anatoxin-a on biomarkers of rainbow trout, Oncorhynchus mykiss. *Toxicon* **2013**, *70*, 162–169. [CrossRef]
63. Prieto, A.; Campos, A.; Cameán, A.; Vasconcelos, V. Effects on growth and oxidative stress status of rice plants (Oryza sativa) exposed to two extracts of toxin-producing cyanobacteria (Aphanizomenon ovalisporum and Microcystis aeruginosa). *Ecotoxicol. Environ. Saf.* **2011**, *74*, 1973–1980. [CrossRef]
64. Machado, J.; Azevedo, J.; Freitas, M.; Pinto, E.; Almeida, A.; Vasconcelos, V.; Campos, A. Analysis of the use of microcys-tin-contaminated water in the growth and nutritional quality of the root-vegetable, Daucus carota. *Environ. Sci. Pollut. Res.* **2017**, *24*, 752–764. [CrossRef]
65. Guzmán-Guillén, R.; Campos, A.; Machado, J.; Freitas, M.; Azevedo, J.; Pinto, E.; Almeida, A.; Cameán, A.M.; Vasconcelos, V. Effects of Chrysosporum (Aphanizomenon) ovalisporum extracts containing cylindrospermopsin on growth, photosynthetic capacity, and mineral content of carrots (Daucus carota). *Ecotoxicology* **2016**, *26*, 22–31. [CrossRef]
66. Pereira, S.; Saker, M.L.; Vale, M.; Vasconcelos, V.M. Comparison of Sensitivity of Grasses (Lolium perenne L. and Festuca rubra L.) and Lettuce (Lactuca sativa L.) Exposed to Water Contaminated with Microcystins. *Bull. Environ. Contam. Toxicol.* **2009**, *83*, 81–84. [CrossRef]
67. Freitas, M.; Azevedo, J.; Pinto, E.; Neves, J.; Campos, A.; Vasconcelos, V. Effects of microcystin-LR, cylindrospermopsin and a microcystin-LR/cylindrospermopsin mixture on growth, oxidative stress and mineral content in lettuce plants (*Lactuca sativa* L.). *Ecotoxicol. Environ. Saf.* **2015**, *116*, 59–67. [CrossRef] [PubMed]
68. Llana-Ruiz-Cabello, M.; Jos, A.; Cameán, A.; Oliveira, F.; Barreiro, A.; Machado, J.; Azevedo, J.; Pinto, E.; Almeida, A.; Campos, A.; et al. Analysis of the Use of Cylindrospermopsin and/or Microcystin-Contaminated Water in the Growth, Mineral Content, and Contamination of *Spinacia oleracea* and *Lactuca sativa*. *Toxins* **2019**, *11*, 624. [CrossRef] [PubMed]
69. Dias, E.; Andrade, M.; Alverca, E.; Pereira, P.; Batoreu, M.C.C.; Jordan, P.; Silva, M.J. Comparative study of the cytotoxic effect of microcistin-LR and purified extracts from Microcystis aeruginosa on a kidney cell line. *Toxicon* **2009**, *53*, 487–495. [CrossRef] [PubMed]
70. Dias, E.; Matos, P.; Pereira, P.; Batoréu, M.C.; Silva, M.J.; Jordan, P. Microcystin-LR activates the ERK1/2 kinases and stimulates the proliferation of the monkey kidney-derived cell line Vero-E6. *Toxicol. In Vitro* **2010**, *24*, 1689–1695. [CrossRef]
71. Menezes, C.; Alverca, E.; Dias, E.; Sam-Bento, F.; Pereira, P. Involvement of endoplasmic reticulum and autophagy in microcystin-LR toxicity in Vero-E6 and HepG2 cell lines. *Toxicol. In Vitro* **2013**, *27*, 138–148. [CrossRef]
72. Dias, E.; Louro, H.; Pinto, M.; Santos, T.; Antunes, S.; Pereira, P.; Silva, M.J. Genotoxicity of microcystin-LR in in vitro and in vivo experimental models. *Biomed. Res. Int.* **2014**, *2014*, 949521. [CrossRef]

73. Valério, E.; Vilares, A.; Campos, A.; Pereira, P.; Vasconcelos, V. Effects of microcystin-LR on Saccharomyces cerevisiae growth, oxidative stress and apoptosis. *Toxicon* **2014**, *90*, 191–198. [CrossRef]
74. Miguéns, D.; Valério, E. The impact of some microcystins on the growth of heterotrophic bacteria from Portuguese freshwater reservoir. *Limnetica* **2015**, *34*, 215–226.
75. Dias, E.; Oliveira, M.; Jones-Dias, D.; Vasconcelos, V.; Ferreira, E.; Manageiro, V.; Caniça, M. Assessing the antibiotic suscep-tibility of freshwater Cyanobacteria spp. *Front. Microbiol.* **2015**, *11*, 799.
76. Salvador, D.; Churro, C.; Valério, E. Evaluating the influence of light intensity in mcyA gene expression and microcystin production in toxic strains of Planktothrix agardhii and Microcystis aeruginosa. *J. Microbiol. Methods* **2016**, *123*, 4–12. [CrossRef]
77. Paulino, S.; Sam-Bento, F.; Churro, C.; Alverca, E.; Dias, E.; Valério, E.; Pereira, P. The Estela Sousa e Silva Algal Culture Collection: A resource of biological and toxicological interest. *Hydrobiologia* **2009**, *636*, 489–492. [CrossRef]
78. Ramos, V.; Morais, J.; Castelo-Branco, R.; Pinheiro, A.; Martins, J.; Regueiras, A.; Pereira, A.L.; Lopes, V.R.; Frazão, B.; Gomes, D.; et al. Cya-nobacterial diversity held in microbial biological resource centers as a biotechnological asset: The case study of the newly es-tablished LEGE culture collection. *J. Appl. Phycol.* **2018**, *30*, 1437–1451. [CrossRef] [PubMed]