Ecotoxicity assessment of microcystins from freshwater samples using a bioluminescent cyanobacterial bioassay

Miguel González-Pleiter [a, 1, *], Samuel Cirés [a, 1], Lars Wörmer [b], Ramsy Agha [c], Gerardo Pulido-Reyes [a], Keila Martín-Betancor [a], Andreu Rico [d], Francisco Leganés [a], Antonio Quesada [a], Francisca Fernández-Piñas [a]

[a] Departamento de Biología, C/Darwin, 2, Universidad Autónoma de Madrid, 28049, Madrid, Spain.

[b] Organic Geochemistry Group, MARUM—Center for Marine Environmental Sciences, University of Bremen, Leobener Str. 8, 28359 Bremen, Germany

[c] Department of Ecosystem Research, Leibniz-Institute of Freshwater Ecology and Inland Fisheries (IGB), Müggelseedamm 301, Berlin, 12587, Germany

[d] IMDEA Water Institute, Science and Technology Campus of the University of Alcalá, Avenida Punto Com 2, 28805, Alcalá de Henares, Madrid, Spain.

*Corresponding author: Miguel González-Pleiter, Departamento de Biología C/Darwin, 2, Universidad Autónoma de Madrid, ES-28049, Madrid, Spain

Telf. (+34) 914978198; Email: mig.gonzalez@uam.es

[1] These authors contributed equally to this work

Abstract

The hepatotoxic cyanotoxins microcystins (MCs) are emerging contaminants naturally produced by cyanobacteria. Yet their ecological role remains unsolved, previous research suggests that MCs have allelopathic effects on competing photosynthetic microorganisms, even eliciting toxic effects on other freshwater cyanobacteria. In this context, the bioluminescent recombinant cyanobacterium *Anabaena* sp. PCC7120 CPB4337 (hereinafter *Anabaena*) was exposed to extracts of MCs. These were obtained from eight natural samples from freshwater reservoirs that contained MCs with a concentration range of 0.04-11.9 µg MCs L^{-1}. MCs extracts included the three most common MCs variants (MC-LR, MC-RR, MC-YR) in different proportions (MC-LR: 100 – 0 %; MC-RR: 100 – 0 %; MC-YR: 14.2 – 0 %). The *Anabaena* bioassay based on bioluminescence inhibition has been successfully used to test the toxicity of many emerging contaminants (e.g., pharmaceuticals) but never for cyanotoxins prior to this study. Exposure of *Anabaena* to MCs extracts induced a decrease in its bioluminescence with ~~EC_{50} (~~effective concentration decreasing bioluminescence by 50 %~~)~~ ranging from 0.4 to 50.5 µg MC L^{-1} in the different samples. Bioluminescence responses suggested an interaction between MCs variants which was analysed via the Additive Index method (AI), indicating an antagonistic effect (AI < 0) of MC-LR and MC-RR present in the samples. Additionally, MC extracts exposure triggered an increase of intracellular free Ca^{2+} in *Anabaena*. In short, this study supports the use of the *Anabaena* bioassay as a sensitive tool to assess the presence of MCs at environmentally relevant concentrations and opens interesting avenues regarding the interactions between MCs variants and the possible implication of Ca^{2+} in the mode of action of MCs towards cyanobacteria.

Keywords: cyanotoxin, bioassay, bioluminescence, *Anabaena*, additive index, intracellular free Ca^{2+}

1.Introduction

Microcystins (MCs) are emerging pollutants of great concern for water managers (Sauvé and Desrosiers, 2014) since they are worldwide distributed and have been reported so far in freshwaters of at least 79 countries (Harke et al., 2016). MCs are cyclic heptapeptides comprising up to 248 chemical variants and are naturally biosynthesized by certain strains of the photosynthetic prokaryotes cyanobacteria (Spoof and Catherine, 2016). MCs are well known for their hepatotoxic effects in humans and other vertebrates and have also shown high toxicity potential for aquatic organisms including fish, zooplankton, plants and algae (Omidi et al., 2018) . Even though the ecological role of MCs remains unsolved, a number of studies indicate that they could have allelopathic effects, i.e., they may affect the growth of other photosynthetic microorganisms (microalgae and cyanobacteria) competing for resources in freshwater (Omidi et al., 2018) . Toxic effects of MCs on cyanobacteria have been evidenced on laboratory cultures for at least eight genera with varied responses including growth inhibition, reduction of photosynthetic performance and induction of oxidative stress, among others (Table S1). Despite these valuable evidences, there is a lack of studies evaluating the effects of MCs from an ecotoxicological point of view, but even more so using experimental conditions closer to those encountered in freshwater ecosystems. First, the exposure concentrations used in most laboratory studies (100-50,000 µg MCs L^{-1}) (Table S1) are about 1 to 3 orders of magnitude higher than the MC concentrations that have been measured in surface water ecosystems i.e., average concentrations of 1.2-3.0 µg L^{-1} in 1161 lakes from USA (Loftin et al., 2016) and 1.2-15 µg L^{-1} in 137 European lakes (Mantzouki et al., 2018) . Secondly, MC tests have been restricted to individual MCs variants, while MCs occur in complex mixtures in most freshwater ecosystems (Hercog et al., 2017) . Third, an essential condition towards a proper ecotoxicological assessment is the standardization of the exposure duration and the toxicological responses and endpoints to be investigated (e.g. EC_{50}, the effective concentration decreasing bioluminescence by 50 %), which has not been shown by previous works in cyanobacteria.

In this context, the present study aims at providing ecotoxicological insight into the effects of MCs extracts from eight natural samples from freshwater reservoirs on cyanobacteria via the use of a bioassay based on the recombinant bioluminescent cyanobacterium *Anabaena* sp. PCC7120 strain CPB4337 (hereinafter *Anabaena*). In this strain, the *Anabaena* chromosome bears a Tn5 derivative with *luxCDABE* from the luminescent terrestrial enterobacterium *Photorhabdus luminescens* (Fernández-Pinas and Wolk, 1994) . This bioassay, based on bioluminescence inhibition experienced by the strain after exposure to toxicants, has been successfully used to assess the toxicity of a number of emerging pollutants even at low concentrations naturally present in freshwaters (Rosal et al., 2010; González-Pleiter et al., 2013; Rodea-Palomares et al., 2016) .

Hence, we hypothesized that if MCs are toxic to other non-toxin-producing cyanobacteria, *Anabaena* may also respond to MCs extracts from natural samples at environmentally relevant concentrations. Furthermore, we investigated whether intracellular free Ca^{2+} ($[Ca^{2+}]_c$) varies in response to MCs. The relevance of $[Ca^{2+}]_c$ relies on its suggested role as second messenger and early exposure biomarker for emerging pollutants in water (Barrán-Berdón et al., 2011; González-Pleiter et al., 2017) . In principle, MCs could behave as other freshwater pollutants and elicit changes in $[Ca^{2+}]_c$ in *Anabaena*, thereby providing insights on the still undescribed mode of action of MCs toward cyanobacteria. Therefore, this study provides novel information on cellular responses of non-toxin-producing cyanobacteria to MCs from natural samples at environmentally relevant concentrations.

2.Material and methods

2.1 Freshwater samples

2.1.1 Sampling

Eight natural samples containing MCs were obtained in four Spanish freshwater reservoirs: Alcántara (samples AL1 and AL2), San Juan (samples SJ1A-B, SJ2A-B), Cazalegas (sample CA) and Balsa de Morea (sample BM) (Table S2). The sampling locations were selected based on previous monitoring data (Wörmer et al., 2011a; Agha et al., 2012) confirming the presence of

96 the three MC variants most frequently reported in freshwaters worldwide (MC-LR, MC-RR and
97 MC-YR) (Loftin et al., 2016; Mantzouki et al., 2018) .
98 One single sampling location was established per reservoir with the exception of the two largest
99 reservoirs -San Juan and Alcántara- where samples were taken in 2 different sampling locations
100 (Table S2). For each sampling location, sampling consisted in the collection of an integrated water
101 sample from 5 different shore points (2 L per point) within the first meter of depth, covering the
102 whole bathing area. Water samples were then transported cool (4 ˚C) to the laboratory for further
103 analysis.
104
105 *2.1.2 Biological characterization*
106 Total chlorophyll *a*, and cyanobacterial chlorophyll *a* concentrations were determined using a
107 benchtop BBE-Moldaenke Algae Analyser Fluorimeter, capable of discriminating among algal
108 groups (green algae, diatoms, cryptophytes and cyanobacteria) within a water sample.
109 Cyanobacterial taxa identification of each sample was carried out microscopically using an
110 Olympus BH2 microscope equipped with a Leica DF300 FX camera (Leica Microsystems,
111 Germany) following the method described in (Cirés et al., 2013) . Species identification was
112 based on diagnostic morphological traits according to (Anagnostidis, 1989; Komárek, 1999;
113 Komárek and Anagnostidis, 2005) .
114
115 *2.1.3 Extraction of cyanotoxins*
116 Water samples were first filtered by GF/F glass fiber filters (Whatman, UK) and stored at -20˚C
117 until extraction of intracellular cyanotoxins from the biomass retained in the filter.
118
119 *2.1.3.1 Extraction of microcystins*
120 Intracellular microcystins variants (LR, RR and YR) were extracted from the filters twice by
121 sonication into 8 mL methanol 90% after Carrasco et al. (2007). The pooled extracts were
122 concentrated under vacuum using a Heidolph Synthesis multiple evaporator (Heidolph
123 Instruments GmbH, Germany), after which the dried extracts were resuspended into 1 mL of

Milli-Q water, filtered through 0.45 µm pore-size nylon filters (Teknokroma, Spain) and placed in chromatography vials for the subsequent analyses.

2.1.3.2 Extraction of anatoxin-a, cylindrospermopsin and saxitoxins

Anatoxin-a was extracted from the filters into 100% methanol following Carrasco et al. (2007). Cylindrospermopsin was extracted from the filters into Milli-Q water as described by Cirés et al. (2011). Saxitoxins were extracted from the filters into acetonitrile-water-formic acid (80:19.9:0.1) following Wörmer et al. (2011b). Pooled extracts were filtered through 0.45 µm pore-size nylon filters (Teknokroma, Spain) and placed in chromatography vials for the subsequent analyses.

2.1.4 Identification and quantification of cyanotoxins

Each sample was analyzed for three microcystins variants (LR, RR and YR), anatoxin-a, cylindrospermopsin and saxitoxins (gonyautoxin 5, neosaxitoxin, saxitoxin, and decarbamoylsaxitoxin).

2.1.4.1 Identification and quantification of microcystins (LR, RR and, YR)

MCs were identified and quantified by ESI LC-MS/MS using a Varian 500MS Ion Trap Mass Spectrometer coupled to two Varian 212 LC chromatographic pumps and a 410 autosampler, according to the procedures described in (Agha et al., 2012). Chromatographic separation of MC-LR, MC-RR and MC-YR was achieved using a Pursuit C18 3µm 2 x 150mm column and mobile phases MilliQ water (A) and methanol (B) both acidified with 0.2% formic acid and buffered with 2 mM ammonium formate. A chromatographic gradient (%A/%B) 60/40 to 0/100 in 18 minutes was applied. All quantifications were made by injecting commercial standards (Danish Hydraulic Institute, Denmark) and plotting calibration curves.

2.1.34.2 Identification and quantification of anatoxin-a, cylindrospermopsin and saxitoxins

Beyond ~~microcystins~~ MCs, the eight samples were also analyzed for the presence of three cyanotoxin groups (anatoxin-a, cylindrospermopsins and saxitoxins) considered as the most widespread (Loftin et al., 2016; Mantzouki et al., 2018) .

Anatoxin-a was analyzed on a Waters Alliance 2695 high-pressure liquid chromatography (HPLC) system equipped with a 996 photodiode array detector (PDA; Waters) (HPLC-PDA) following Carrasco et al. (2007).

Cylindrospermopsin and saxitoxins were identified and quantified by electrospray ionization liquid chromatography-tandem mass spectrometry (ESI LC-MS/MS) on a Varian 500 MS ion trap mass spectrometer (Agilent Technologies) supported by two Varian 212 LC chromatographic pumps and a 410 autosampler. Cylindrospermopsin was identified by ESI LC-MS/MS as described by Cirés et al. (2011). Saxitoxins, the variants gonyautoxin 5 (GTX5), neosaxitoxin (NEO), saxitoxin (STX), and decarbamoylsaxitoxin (dcSTX), were determined by ESI LC-MS/MS following conditions detailed in Wörmer et al. (2011b).

2.2 Toxicity of microcystins towards Anabaena sp. PCC7120 CPB4337

2.2.1 Strain and culture conditions

The bioluminescent recombinant cyanobacterium *Anabaena* was routinely grown at 28°C under continuous white light irradiance at approximately *ca*. 65 µmol photons m^{-2} s^{-1} on a rotary shaker in 100 mL AA/8 medium (Allen and Arnon, 1955) supplemented with 5 mM nitrate (hereinafter AA/8 + N) in 250 mL Erlenmeyer flasks and 10 µg/mL of neomycin sulfate for 3 days.

2.2.2 Determination of toxicity by the bioluminescence assay

The toxicity bioassays using *Anabaena* are based on the inhibition of constitutive luminescence caused by the presence of a toxic substance (Rodea-Palomares et al., 2009b) . Acute luminescence inhibition-based toxicity assays were performed as follows: cyanobacterial cells grown as described, were centrifuged~~,~~ washed three times and re-suspended in fresh AA/8+N medium at $OD_{750\,nm}$ of 2.5. 70 µL of commercial standard of MC~~icrocystin~~-LR (DHI Water and Environment, Denmark), as a representative cyanotoxin used in environmental studies, or MCs extracts from

178 the eight natural samples resuspended into 1 mL of Milli-Q water (see section 2.1.4.1 in the
179 material and methods) were added to opaque white 96-well microtiter microplates, followed by
180 10 µL of tenfold concentrated AA/8+N and 20 µL of *Anabaena* to reach a final OD_{750nm} of 0.5.
181 The bioassays were conducted during 1 h under the same conditions described before for
182 cyanobacterial cells growth. Finally, luminescence was recorded in a Centro LB 960 luminometer
183 during 10 min. Three independent experiments with triplicate samples were carried out for all
184 *Anabaena* bioassays (Rodea-Palomares et al., 2009b) .
185
186 Toxicity response of the cyanobacterium was estimated as EC_{50} values~~, the median effective~~
187 ~~microcystins concentration that causes 50% of bioluminescence inhibition with respect to a non-~~
188 ~~treated control. EC_{50} values~~ and their standard deviation ~~were~~ calculated by the dose-response
189 package (drc) using R Software, version 3.3.1.
190
191 *2.2.3 Interactions of MCs in extracts from natural samples*
192 Interactions between MCs presents in the MCs extracts from natural samples was evaluated using
193 the additive index ~~(AI)~~ method (AI). The ~~additive index method~~ ~~(~~AI~~)~~ has been previously used to
194 study chemical interactions in several bioassays (Coalova et al., 2014; Sultana Shaik et al., 2016;
195 Xie et al., 2017; Wang et al., 2018) . In order to apply AI to our sample set, the following equation
196 was used (Loewe and Muischnek, 1926; Loewe, 1928; Marking and Dawson, 1975) :

197 $$S = A_m/A_i + B_m/B_i$$

198 Where *Am* is the EC_{50} for MC-LR in mixture, *Ai* the EC_{50} for MC-LR individually (calculated
199 using those extracts with only MC-LR). *Bm* the EC_{50} for MC-RR in mixture, *Bi* the EC_{50} for MC-
200 RR individually (calculated using those extracts with only MC-RR). Regarding MC-YR, there
201 was not any sample containing only this cyanotoxin (Table 1) and, as this method requires having
202 at least one sample containing 100% of each of the single toxicant, MC-YR was excluded from
203 this study. S is the sum of the biological activity. S values were then used to calculate AI using
204 the following equation:

AI = (1/*S*) -1 for $S < 1$; AI = -*S* +1 for $S \geq 1$

To determine whether the range for AI overlapped zero (additive) the 95% confidence intervals from EC_{50} were substituted into the AI formula to establish a range (Marking and Dawson, 1975) . The effects observed in the mixtures were then classified as additive (AI = 0; expected action), synergistic (AI > 0; greater than additive effect), or antagonistic (AI < 0; less than additive effect).

2.2.4 Intracellular free Ca^{2+}

Anabaena was exposed during 1 hour to both MC-LR a commercial standard of MC-LR (DHI Water and Environment, Denmark) diluted with Milli-Q water up to a concentration equivalent to the EC_{50} and to the samples diluted to reach EC_{50}, and the shifts in intracellular free Ca^{2+} ($[Ca^{2+}]_c$) were analysed. $[Ca^{2+}]_c$ in *Anabaena* was analyzed by flow cytometry (FCM) staining cells with the sensitive Ca^{2+} indicator Calcium Green-5N acetoxymethyl ester (Calcium Green 5N-AM) (Invitrogen Molecular Probes, USA) (Garcia-Pichel et al., 2010) and following the protocol described by (Prado et al., (2012) with minor modifications. FCM analysis of *Anabaena* cells was performed on a Cytomix FL500 MPL flow cytometer (Beckman Coulter Inc., Fullerton, CA, USA) equipped with an argon-ion excitation laser (488 nm), detectors of forward (FS) and side (SS) light scatter and four fluorescence detectors corresponding to different wavelength intervals: 520 nm (FL1), 575 nm (FL2), 620 nm (FL3) and 675 nm (FL4). The cell-permeant acetoxymethylester, non-fluorescent and Ca^{2+} insensitive, can be passively loaded into cells, where it is cleaved by ubiquitous intracellular esterases to the cell-impermeant fluorescent product Calcium Green 5N, which exhibits an increase in fluorescent emission intensity (Ex/Em: 506/532 nm) upon binding Ca^{2+}. A Calcium Green 5N-AM stock solution was prepared in DMSO. Cell suspensions were incubated with the fluorochrome (final concentration: 8 mM) at 28 °C for 1h, and the green fluorescent emission was collected by the FL1 detector. In order to avoid the variability due to differences in cell size, fluorescence was corrected by cell size and estimated complexity using the FS and SS parameters.

3.Results and discussion

3.1 Characteristics of freshwater samples

The eight natural samples from freshwater reservoirs contained MCs with a concentration range of 0.04-11.9 μg MCs L^{-1} (Table 1). These samples included different proportions of each of the ~~microcystins~~MCs variants (LR, RR and YR) (Table 1). Two of the samples contained only one MC variant each (sample BM with 100% MC-LR and sample SJ1B with 100% MC-RR); while there were four samples with binary mixtures of MC-LR and MC-RR in variable proportions (from 13.4% to 79.9% for each of the two variants) and two samples with ternary mixtures of MC-LR, MC-RR and MC-YR again in variable proportions of each individual MC variant from 3.6% to 72.5% (Table 1). Anatoxin-a, cylindrospermopsin, gonyautoxin 5, neosaxitoxin, saxitoxin and decarbamoylsaxitoxin were not detected in any of the eight freshwater samples analysed (data not shown). Taxonomic studies indicated the presence of toxin-producing cyanobacteria such as *Dolichospermum* and *Microcystis* (Table S2).

3.2 Toxicity of pure MC-LR and MCs extracts from freshwater samples towards Anabaena sp. PCC7120 CPB4337

Pure MC-LR caused a substantial decrease of the bioluminescence in *Anabaena* (EC_{50} = 45.5 ± 4.1 μg MC-LR L^{-1}) after 1 hour of exposure (Table 2). MC-LR has been previously used as a representative cyanotoxin in environmental studies inducing a toxic effect on growth (measured as increment in chlorophyll a content) of *Anabaena* PCC7120 wild type (Table S1). Therefore, bioluminescence appears to be more sensitive than growth as endpoint to evaluate the effect of MC-LR in this organism, at least, at short times of exposure.

The MCs extracts also induced a bioluminescence decrease in *Anabaena* after a short exposure of just 1 hour (Fig.1 and table 2). Table 2 shows EC_{50} values of the eight MCs extracts. The EC_{50} values ranged between 0.4 and 50.5 μg MCs L^{-1} (Table 2). These EC_{50} values and the EC_{50} value of the pure MC-LR in *Anabaena* are in the same order of magnitude (Table 2). These findings

suggest that *Anabaena* bioassay might be used as a sensitive early-warning tool responding to environmentally relevant concentrations of MCs in the range of µg/L and with short exposure time (1 hour). This fast and sensitive behaviour is likely attributable to the use of an endpoint (bioluminescence decrease) that can be recorded much earlier than growth inhibition, which requires several days to be evident in cyanobacteria (Table S1). Prior to this study, several authors have used the well stablished bioluminescence bioassay based on *Aliivibrio fischeri* (a naturally bioluminescent marine bacterium, formerly known as *Vibrio fischeri*) (Maršálek and Bláha, 2000; D'ors et al., 2012; Prasath et al., 2019). However, there are conflicting results regarding the suitability of *A. fischeri* to report on toxicity of cyanotoxins (Maršálek and Bláha, 2000), and also the use of marine organisms to test freshwater samples present some problems related to the high saline concentrations that are necessary in the analyte during the assay (Rodea-Palomares et al., 2009a; Hurtado-Gallego et al., 2019). Salinity may alter, among other parameters, the solubility of organic compounds. In this sense, the potential applications of *Anabaena* may be especially useful given that it is a bioassay based on a freshwater organism. Furthermore, ~~*Anabaena* showed very~~ the EC_{50} values of cyanotoxin towards *Anabaena* are much lower than those obtained in bioassays ~~the range of µg MC L^{-1}~~ based on aquatic invertebrates like *Daphnia magna* or *Thamnocephalus platyurus* (Tarczynska et al., 2001; Freitas et al., 2014). Therefore, based on our results (Fig.1 and table 2), *Anabaena* bioassay appears to be sensitive enough (EC_{50} = 0.4 - 50.5 µg MC L^{-1}) to assess water quality status and compliance with the standards set by the World Health Organization and other national institutions for recreational waters (6-20 µg MCs L^{-1}) and for drinking waters (1-1.5 µg MCs L^{-1}) in different countries (Ibelings et al., 2014).

3.3 Interactions of MCs in extracts from natural samples

The bioluminescence results in *Anabaena* evidenced that EC_{50} increased with the number of MC variants present in the sample, i.e., samples with a single variant were found to be more toxic (based on EC_{50} values) than those with two variants (MC-LR + MC-RR) while ternary mixtures (MC-LR + MC-RR + MC-YR) were the least toxic (higher EC_{50} values) (Fig. 1; Table 2). This suggested that the overall toxicity was influenced by interactions between the MC variants.

Two of the samples contained only one of each MC variant ~~each~~ (sample BM with 100% MC-LR and sample SJ1B with 100% MC-RR) (Table 1). In this context, AI can be used to evaluate the interactions of MCs extract from natural samples containing binary mixtures (MC-LR + MC-RR). AI analyses based on bioluminescence from the four samples containing MC-LR + MC-RR indicated an antagonistic interaction between these two MC variants (AI < 0; less than additive effect) (Fig. 2). One possible explanation is that a similar mode of action of MC-LR and MC-RR in cyanobacteria leads to a competition for the same receptor. Our analyses also indicated that AI turned out to be more negative (hence more antagonistic) with the increasing proportion of MC-LR, meaning that the greater the MC-LR/MC-RR ratio, the greater the antagonism between MC-LR and MC-RR (Fig. 2). A possible explanation of this trend would be that the toxicity of MC-LR towards cyanobacteria is lower than that of MC-RR and hence MC-LR partially counteracts the effect of the latter. This possibility is supported by the lower EC_{50} (i.e., higher toxicity) recorded for the sample containing only MC-RR (SJ1B, EC_{50} = 0.4 µg MC L^{-1}) compared to a slightly higher EC_{50} (i.e., lower toxicity) of the sample containing only MC-LR (SJ1B, EC_{50} = 0.6 µg MC L^{-1}). Babica et al. (2007) also found that the growth of the cyanobacterium *Microcystis aeruginosa* was more strongly inhibited by MC-RR than by MC-LR, in contrast with the opposite trend (greater toxicity of MC-LR than of MC-RR) observed in all studies with mice used as models for human toxicity (Bartram and Chorus, 1999) . This interesting paradox will require further generalization by additional interaction studies, considering mixtures of many more MC variants but also with other structurally different cyanotoxins (e.g., cylindrospermopsins, anatoxins, and saxitoxins). Although none of these other cyanotoxins (namely anatoxin-a, cylindrospermopsin and saxitoxin) was detected in the present samples according to our analyses (see supplementary material), they are increasingly found to co-occur with MCs in lakes worldwide (Pitois et al., 2018) hence offering very relevant targets to address by future studies with *Anabaena*.

315 *3.4 Changes in intracellular free Ca^{2+} in Anabaena sp. PCC7120 CPB4337 after exposure to*
316 *MCs extracts*
317 Pure MC-LR (EC_{50} value) caused a significant increase (*p*-value < 0.001) of the intracellular free
318 Ca^{2+} in *Anabaena* (226.7 ± 22.6 %) after 1 hour of exposure compared to the non-exposed control
319 (not shown in Fig. 3). Besides bioluminescence, intracellular free Ca^{2+} was also altered in
320 *Anabaena* after exposure to MC extracts at their EC_{50} values (Fig. 3). Indeed, 7 MCs extracts
321 induced an increase in the intracellular free Ca^{2+} of *Anabaena* (Fig. 3). This novel report of an
322 increase in intracellular free Ca^{2+} of cyanobacteria after exposure to MCs extracts from natural
323 samples suggests that the MC-induced metabolic effects in cyanobacteria may be mediated by
324 calcium. Intracellular free calcium could therefore be potentially used as an early biomarker of
325 MC presence in freshwaters. Interestingly, our findings somewhat coincide with those of Cai et
326 al., (2015) who proposed a critical role of calcium in the neurotoxicity of MCs toward vertebrates
327 due to the $[Ca^{2+}]_c$ increase observed in primary hippocampal neurons from rats exposed to MC-
328 LR.
329
330 **4.Conclusion**
331 Altogether, by using for the first time the bioluminescent bioassay of *Anabaena* sp. PCC7120
332 CPB4337 to MCs extracts from eight natural samples, the present study opens interesting avenues
333 regarding: 1) a potential use of this bioassay as an early-warning detection tool of MCs in
334 freshwaters; 2) study of toxicity interactions between MC in natural extracts; and 3) a possible
335 involvement of intracellular free Ca^{2+} in the still unresolved mode of action of MCs towards
336 cyanobacteria. This work puts us one step further towards a realistic risk assessment of MCs at
337 environmental concentrations.
338
339 **Acknowledgments**
340 This research was supported by CTM2016-74927-C2-1/2-R grant from Spanish Ministry of
341 Economy and Competitiveness (MINECO). Miguel González-Pleiter holds a postdoctoral
342 contract (PEJD-2017-POST/AMB-3520) from Comunidad de Madrid – European Union (EU).

343 Samuel Cirés was recipient of a Juan de la Cierva-Incorporación Postdoctoral Fellowship
344 (IJCI2014-19151) and Andreu Rico holds a Juan de la Cierva-Formación Postdoctoral
345 Fellowship (FJCI 2015-27190), both from the Spanish Ministry of Economy and
346 Competitiveness (MINECO).
347

References

Agha, R., Cirés, S., Wörmer, L., Domínguez, J.A., Quesada, A., 2012. Multi-scale strategies for the monitoring of freshwater cyanobacteria: Reducing the sources of uncertainty. water research 46, 3043-3053.

Allen, M.B., Arnon, D.I., 1955. Studies on nitrogen-fixing blue-green algae. I. Growth and nitrogen fixation by Anabaena cylindrica Lemm. Plant Physiology 30, 366.

Anagnostidis, K., 1989. Modern approach to the classification system of Cyanophytes 4-Nostocales. Algological Studies/Archiv f¸ r Hydrobiologie, Supplement Volumes, 247-345.

Babica, P., Hilscherová, K., Bártová, K., Bláha, L., Maršálek, B., 2007. Effects of dissolved microcystins on growth of planktonic photoautotrophs. Phycologia 46, 137-142.

Barrán-Berdón, A.L., Rodea-Palomares, I., Leganés, F., Fernández-Piñas, F., 2011. Free Ca 2+ as an early intracellular biomarker of exposure of cyanobacteria to environmental pollution. Analytical and bioanalytical chemistry 400, 1015-1029.

Bartram, J., Chorus, I., 1999. Toxic cyanobacteria in water: a guide to their public health consequences, monitoring and management. CRC Press.

Cai, F., Liu, J., Li, C., Wang, J., 2015. Intracellular calcium plays a critical role in the microcystin-LR-elicited neurotoxicity through PLC/IP3 pathway. International journal of toxicology 34, 551-558.

Carrasco, D., Moreno, E., Paniagua, T., Hoyos, C.d., Wormer, L., Sanchis, D., Cires, S., Martín-del- Pozo, D., Codd, G.A., Quesada, A., 2007. Anatoxin- a occurrence and potential

cyanobacterial anatoxin- a producers in Spanish reservoirs 1. Journal of Phycology 43, 1120-1125.

Cirés, S., Wörmer, L., Agha, R., Quesada, A., 2013. Overwintering populations of Anabaena, Aphanizomenon and Microcystis as potential inocula for summer blooms. Journal of Plankton Research 35, 1254-1266.

Cirés, S., Wörmer, L., Timón, J., Wiedner, C., Quesada, A., 2011. Cylindrospermopsin production and release by the potentially invasive cyanobacterium Aphanizomenon ovalisporum under temperature and light gradients. Harmful Algae 10, 668-675.

Coalova, I., de Molina, M.d.C.R., Chaufan, G., 2014. Influence of the spray adjuvant on the toxicity effects of a glyphosate formulation. Toxicology in Vitro 28, 1306-1311.

D'ors, A., Bartolomé, M., Sánchez-Fortún, S., 2012. Importance of strain type to predict the toxicological risk associated with Microcystis aeruginosa blooms: comparison of Microtox® analysis and immunoassay. Journal of water and health 10, 256-261.

Fernández-Pinas, F., Wolk, C.P., 1994. Expression of luxCD-E in Anabaena sp. can replace the use of exogenous aldehyde for in vivo localization of transcription by luxAB. Gene 150, 169-174.

Freitas, E.C., Pinheiro, C., Rocha, O., Loureiro, S., 2014. Can mixtures of cyanotoxins represent a risk to the zooplankton? The case study of Daphnia magna Straus exposed to hepatotoxic and neurotoxic cyanobacterial extracts. Harmful Algae 31, 143-152.

Garcia-Pichel, F., Ramírez-Reinat, E., Gao, Q., 2010. Microbial excavation of solid carbonates powered by P-type ATPase-mediated transcellular Ca2+ transport. Proceedings of the National Academy of Sciences 107, 21749-21754.

González-Pleiter, M., Gonzalo, S., Rodea-Palomares, I., Leganés, F., Rosal, R., Boltes, K., Marco, E., Fernández-Piñas, F., 2013. Toxicity of five antibiotics and their mixtures towards photosynthetic aquatic organisms: implications for environmental risk assessment. Water research 47, 2050-2064.

González-Pleiter, M., Leganés, F., Fernández-Piñas, F., 2017. Intracellular free Ca 2+ signals antibiotic exposure in cyanobacteria. RSC advances 7, 35385-35393.

Harke, M.J., Steffen, M.M., Gobler, C.J., Otten, T.G., Wilhelm, S.W., Wood, S.A., Paerl, H.W., 2016. A review of the global ecology, genomics, and biogeography of the toxic cyanobacterium, Microcystis spp. Harmful Algae 54, 4-20.

Hercog, K., Maisanaba, S., Filipič, M., Jos, Á., Cameán, A.M., Žegura, B., 2017. Genotoxic potential of the binary mixture of cyanotoxins microcystin-LR and cylindrospermopsin. Chemosphere 189, 319-329.

Hurtado-Gallego, J., Pulido-Reyes, G., González-Pleiter, M., Fernández-Piñas, F., 2019. Luminescent microbial bioassays and microalgal biosensors as tools for environmental toxicity evaluation. Handbook of Cell Biosensors, 1-58.

Ibelings, B.W., Backer, L.C., Kardinaal, W.E.A., Chorus, I., 2014. Current approaches to cyanotoxin risk assessment and risk management around the globe. Harmful Algae 40, 63-74.

Komárek, J., 1999. Cyanoprokaryota 1. Teil: Chroococcales. Subwasserflora von Mitteleuropa 19, 1-548.

Komárek, J., Anagnostidis, K., 2005. Süßwasserflora von Mitteleuropa, bd. 19/2: Cyanoprokaryota: Oscillatoriales. Spektrum Akademischer Verlag.

Loewe, S., 1928. Die quantitativen probleme der pharmakologie. Ergebnisse der Physiologie 27, 47-187.

Loewe, S.t., Muischnek, H., 1926. Über kombinationswirkungen. Naunyn-Schmiedeberg's Archives of Pharmacology 114, 313-326.

Loftin, K.A., Graham, J.L., Hilborn, E.D., Lehmann, S.C., Meyer, M.T., Dietze, J.E., Griffith, C.B., 2016. Cyanotoxins in inland lakes of the United States: Occurrence and potential recreational health risks in the EPA National Lakes Assessment 2007. Harmful Algae 56, 77-90.

Mantzouki, E., Lürling, M., Fastner, J., de Senerpont Domis, L., Wilk-Woźniak, E., Koreivienė, J., Seelen, L., Teurlincx, S., Verstijnen, Y., Krztoń, W., 2018. Temperature effects explain continental scale distribution of cyanobacterial toxins. Toxins 10, 156.

Marking, L.L., Dawson, V.K., 1975. Method for assessment of toxicity or efficacy of mixtures of chemicals. US Fish and Wildlife Service.

Maršálek, B., Bláha, L., 2000. Microbiotests for cyanobacterial toxins screening. New microbiotests for routine toxicity screening and biomonitoring. Springer, pp. 519-525.

Omidi, A., Esterhuizen-Londt, M., Pflugmacher, S., 2018. Still challenging: the ecological function of the cyanobacterial toxin microcystin–What we know so far. Toxin Reviews 37, 87-105.

Pitois, F., Fastner, J., Pagotto, C., Dechesne, M., 2018. Multi-Toxin Occurrences in Ten French Water Resource Reservoirs. Toxins 10, 283.

458 Prado, R., Rioboo, C., Herrero, C., Cid, Á., 2012. Screening acute cytotoxicity biomarkers using
459 a microalga as test organism. Ecotoxicology and environmental safety 86, 219-226.
460
461 Prasath, B.B., Santhanam, P., Nandakumar, R., Jayalakshmi, T., 2019. Detection of Cyanotoxins
462 of Cyanobacterial (Microcystis aeruginosa) Strain Using Microtox® Bioluminescence Bioassay.
463 Basic and Applied Phytoplankton Biology. Springer, pp. 211-219.
464
465 Rodea-Palomares, I., Fernández-Piñas, F., González-García, C., Leganés, F., 2009a. Use of lux-
466 marked cyanobacterial bioreporters for assessment of individual and combined toxicities of
467 metals in aqueous samples. Handbook on Cyanobacteria: Biochemistry, Biotechnology and
468 Applications, 283-304.
469
470 Rodea-Palomares, I., Gonzalez-Garcia, C., Leganes, F., Fernandez-Pinas, F., 2009b. Effect of pH,
471 EDTA, and anions on heavy metal toxicity toward a bioluminescent cyanobacterial bioreporter.
472 Archives of environmental contamination and toxicology 57, 477.
473
474 Rodea-Palomares, I., Gonzalez-Pleiter, M., Gonzalo, S., Rosal, R., Leganes, F., Sabater, S.,
475 Casellas, M., Muñoz-Carpena, R., Fernández-Piñas, F., 2016. Hidden drivers of low-dose
476 pharmaceutical pollutant mixtures revealed by the novel GSA-QHTS screening method. Science
477 advances 2, e1601272.
478
479 Rosal, R., Rodea-Palomares, I., Boltes, K., Fernández-Piñas, F., Leganés, F., Gonzalo, S., Petre,
480 A., 2010. Ecotoxicity assessment of lipid regulators in water and biologically treated wastewater
481 using three aquatic organisms. Environmental Science and Pollution Research 17, 135-144.
482
483 Sauvé, S., Desrosiers, M., 2014. A review of what is an emerging contaminant. Chemistry Central
484 Journal 8, 15.
485

Spoof, L., Catherine, A., 2016. Appendix 3: tables of microcystins and nodularins. Handbook of cyanobacterial monitoring and cyanotoxin analysis, 526-537.

Sultana Shaik, A., Shaik, A.P., Jamil, K., Alsaeed, A.H., 2016. Evaluation of cytotoxicity and genotoxicity of pesticide mixtures on lymphocytes. Toxicology mechanisms and methods 26, 588-594.

Tarczynska, M., Nalecz- Jawecki, G., Romanowska- Duda, Z., Sawicki, J., Beattie, K., Codd, G., Zalewski, M., 2001. Tests for the toxicity assessment of cyanobacterial bloom samples. Environmental Toxicology: An International Journal 16, 383-390.

Wang, Y., Wu, S., Chen, J., Zhang, C., Xu, Z., Li, G., Cai, L., Shen, W., Wang, Q., 2018. Single and joint toxicity assessment of four currently used pesticides to zebrafish (Danio rerio) using traditional and molecular endpoints. Chemosphere 192, 14-23.

Wörmer, L., Agha, R., Cirés, S., Galán, E., Ratón, C., Al-Ismail, S., Quesada, A., 2011a. Informe de los análisis realizados en las zonas de baño continentales durante las temporadas 2008 y 2009. Cianobacterias. Edited by Ministerio de Medio Ambiente, Medio Rural y Marino (MMAMRM).

Wörmer, L., Cirés, S., Agha, R., Verdugo, M., de Hoyos, C., Quesada, A., 2011. First detection of cyanobacterial PSP (paralytic shellfish poisoning) toxins in Spanish freshwaters. Toxicon 57, 918-921.

Xie, J., Yang, D., Sun, X., Cao, R., Chen, L., Wang, Q., Li, F., Ji, C., Wu, H., Cong, M., 2017. Combined toxicity of cadmium and lead on early life stages of the Pacific oyster, Crassostrea gigas. Invertebrate Survival Journal 14, 210-220.

513

514

515 **Figure captions**

516 Figure 1. Toxicity of MCs extracts from freshwater samples on bioluminescent *Anabaena* sp.
517 PCC7120 CPB4337 after 1 hour of exposure. Vertical bars stand for EC_{50} values~~, the median~~
518 ~~effective MCs concentration that causes 50% of bioluminescence inhibition with respect to a~~
519 ~~control not exposed to MCs extracts~~. Freshwater samples on X axis are classified according to the
520 number of MCs variants naturally present.

521 Figure 2. Interactions of MCs extract from freshwater samples containing MC-LR + MC-RR in
522 *Anabaena* sp. PCC7120 CPB4337. Vertical bars stand for ~~A~~additive ~~I~~index (AI), which classifies
523 the effects in mixtures as additive (AI = 0), synergistic (AI > 0), or antagonistic (AI < 0). Error
524 bars represent 95% confidence intervals for AI. Letters mark groups with significant differences
525 for AI indexes ($p < 0.05$, Dunnett´s test). The line and scatter plot represents the ratio between
526 concentrations of MC-LR and MC-RR in each freshwater sample.

527 Figure 3. Changes in intracellular free Ca^{2+} concentration in *Anabaena* sp. PCC7120 CPB4337
528 after exposure to MCs extract from freshwater samples. MCs extracts exposure concentrations
529 were the EC_{50} values recorded for each sample (Table 2). Results are expressed as relative
530 fluorescence (%) compared to a control not exposed to MCs extracts. Error bars represent standard
531 deviation (n = 3). Asterisks mark significant differences with control (*p*-value <0.05*; *p*-value
532 <0.01**; *p*-value < 0.001 ***) after Dunnett´s test.

533

534

535

536

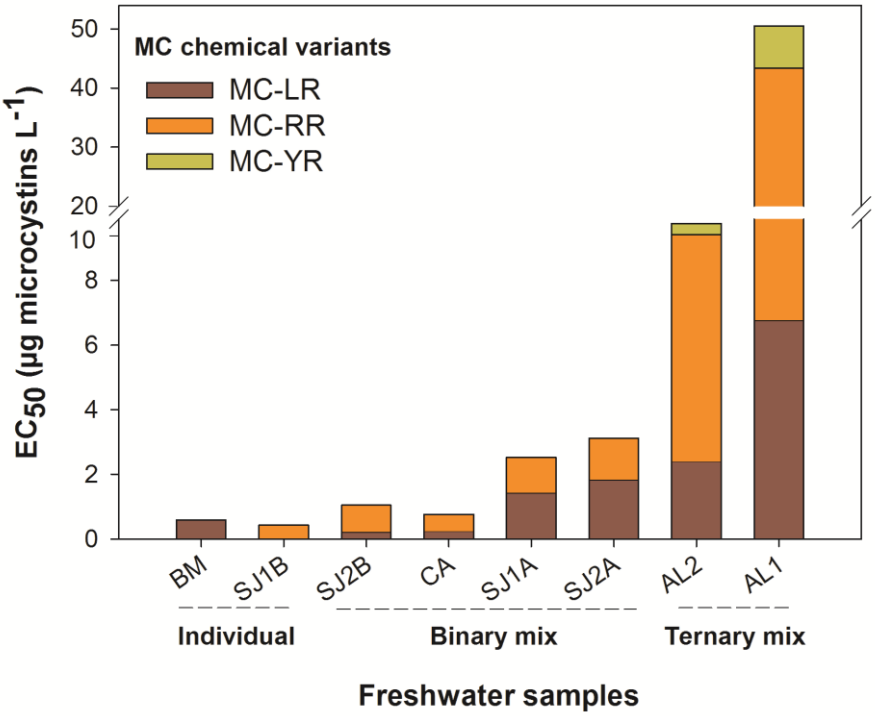
Table 1. Microcystin concentrations and proportion of each variant in the freshwater samples tested in the present study. Abbreviations: MCs: microcystin; MC-LR: microcystin LR; MC-RR: microcystin RR; MC-YR: microcystin YR.

Water body	Sample code	Microcystins			
		Total MCs ($\mu g\ L^{-1}$)	MC-LR (%)	MC-RR (%)	MC-YR (%)
Balsa Morea	BM	0.04	100	0	0
Alcántara	AL1	11.9	13.4	72.5	14.2
	AL2	1.7	24.6	71.9	3.6
San Juan	SJ1A	0.3	56.7	43.3	0
	SJ1B	0.1	0	100	0
	SJ2A	0.5	58.7	41.3	0
	SJ2B	0.06	20.8	79.9	0
Cazalegas	CA	0.2	32	68	0

553 **Table 2. Toxicity of pure MC-LR and MCs extracts from freshwater samples**
554 **towards *Anabaena* sp. PCC7120 CPB4337, expressed as median effective MCs**
555 **extracts concentrations causing 50% decrease in bioluminescence (EC_{50}).** Results are
556 presented as median ± 95% confidence intervals. MCs: microcystins.

Water body	Sample code	EC_{50} (µg MCs L^{-1})
-	Pure MC-LR	45.5 ± 4.1
Balsa Morea	BM	0.6 ± 0.1
Alcántara	AL1	50.5 ± 10.2
	AL2	9.8 ± 1.1
San Juan	SJ1A	2.5 ± 0.2
	SJ1B	0.4 ± 0.07
	SJ2A	3.1 ± 0.6
	SJ2B	1.0 ± 0.04
Cazalegas	CA	0.8 ± 0.1

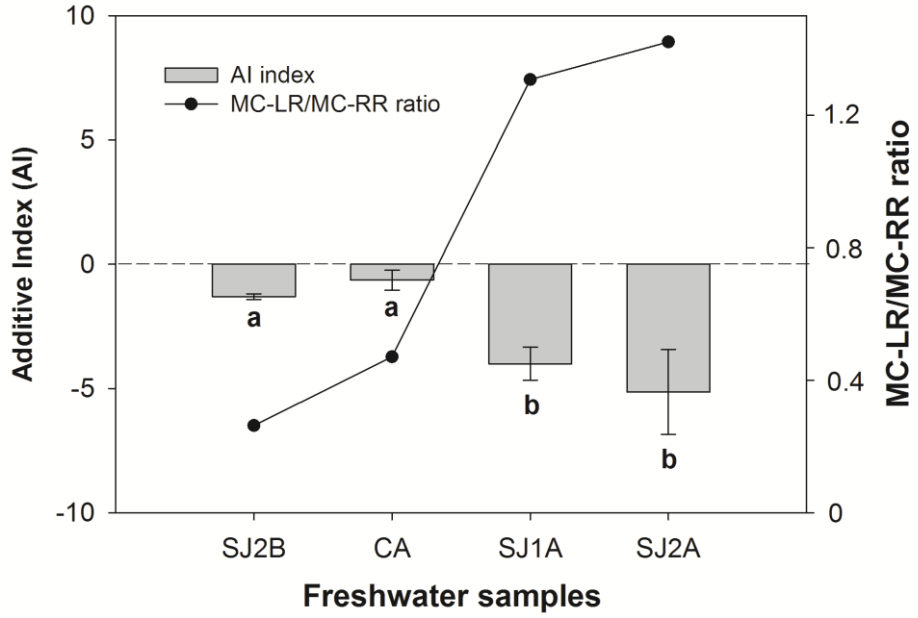
557
558
559
560
561



562

563 **Figure 1.**

564



565

566 **Figure 2**.

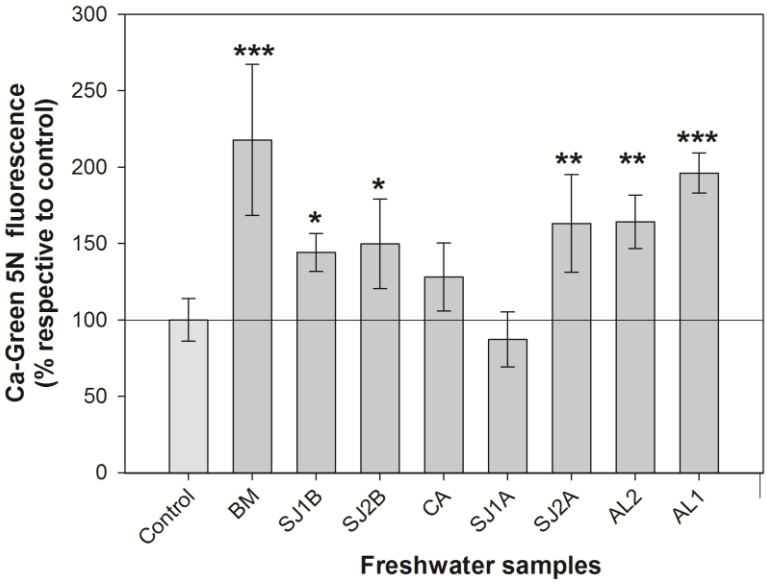

Ca-Green 5N fluorescence
(% respective to control)
300
250
200
150
100
50
0
***
*
*
**
**
***
Control
BM
SJ1B
SJ2B
CA
SJ1A
SJ2A
AL2
AL1
Freshwater samples

Figure 3.