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

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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 the three MC variants most frequently reported in freshwaters worldwide (MC-LR, MC-RR and MC-YR) (Loftin et al., 2016; Mantzouki et al., 2018) .

One single sampling location was established per reservoir with the exception of the two largest reservoirs -San Juan and Alcántara- where samples were taken in 2 different sampling locations (Table S2). For each sampling location, sampling consisted in the collection of an integrated water sample from 5 different shore points (2 L per point) within the first meter of depth, covering the whole bathing area. Water samples were then transported cool (4 ˚C) to the laboratory for further analysis.

2.1.2 Biological characterization

Total chlorophyll *a*, and cyanobacterial chlorophyll *a* concentrations were determined using a benchtop BBE-Moldaenke Algae Analyser Fluorimeter, capable of discriminating among algal groups (green algae, diatoms, cryptophytes and cyanobacteria) within a water sample.

Cyanobacterial taxa identification of each sample was carried out microscopically using an Olympus BH2 microscope equipped with a Leica DF300 FX camera (Leica Microsystems, Germany) following the method described in (Cirés et al., 2013) . Species identification was based on diagnostic morphological traits according to (Anagnostidis, 1989; Komárek, 1999; Komárek and Anagnostidis, 2005) .

2.1.3 Extraction of cyanotoxins

Water samples were first filtered by GF/F glass fiber filters (Whatman, UK) and stored at -20˚C until extraction of intracellular cyanotoxins from the biomass retained in the filter.

2.1.3.1 Extraction of microcystins

Intracellular microcystins variants (LR, RR and YR) were extracted from the filters twice by sonication into 8 mL methanol 90% after Carrasco et al. (2007). The pooled extracts were concentrated under vacuum using a Heidolph Synthesis multiple evaporator (Heidolph

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

153 Beyond ~~microcystins~~ MCs, the eight samples were also analyzed for the presence of three
154 cyanotoxin groups (anatoxin-a, cylindrospermopsins and saxitoxins) considered as the most
155 widespread (Loftin et al., 2016; Mantzouki et al., 2018) .
156 Anatoxin-a was analyzed on a Waters Alliance 2695 high-pressure liquid chromatography
157 (HPLC) system equipped with a 996 photodiode array detector (PDA; Waters) (HPLC-PDA)
158 following Carrasco et al. (2007).
159 Cylindrospermopsin and saxitoxins were identified and quantified by electrospray ionization
160 liquid chromatography-tandem mass spectrometry (ESI LC-MS/MS) on a Varian 500 MS ion
161 trap mass spectrometer (Agilent Technologies) supported by two Varian 212 LC
162 chromatographic pumps and a 410 autosampler. Cylindrospermopsin was identified by ESI LC-
163 MS/MS as described by Cirés et al. (2011). Saxitoxins, the variants gonyautoxin 5 (GTX5),
164 neosaxitoxin (NEO), saxitoxin (STX), and decarbamoylsaxitoxin (dcSTX), were determined by
165 ESI LC-MS/MS following conditions detailed in Wörmer et al. (2011b).
166
167 *2.2 Toxicity of microcystins towards Anabaena sp. PCC7120 CPB4337*
168 *2.2.1 Strain and culture conditions*
169 The bioluminescent recombinant cyanobacterium *Anabaena* was routinely grown at 28°C under
170 continuous white light irradiance at approximately *ca*. 65 µmol photons m^{-2} s^{-1} on a rotary
171 shaker in 100 mL AA/8 medium (Allen and Arnon, 1955) supplemented with 5 mM nitrate
172 (hereinafter AA/8 + N) in 250 mL Erlenmeyer flasks and 10 µg/mL of neomycin sulfate for 3
173 days.
174
175 *2.2.2 Determination of toxicity by the bioluminescence assay*
176 The toxicity bioassays using *Anabaena* are based on the inhibition of constitutive luminescence
177 caused by the presence of a toxic substance (Rodea-Palomares et al., 2009b) . Acute
178 luminescence inhibition-based toxicity assays were performed as follows: cyanobacterial cells
179 grown as described, were centrifuged~~,~~ washed three times and re-suspended in fresh AA/8+N
180 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 the eight natural samples resuspended into 1 mL of Milli-Q water (see section 2.1.4.1 in the material and methods) were added to opaque white 96-well microtiter microplates, followed by 10 µL of tenfold concentrated AA/8+N and 20 µL of *Anabaena* to reach a final OD_{750nm} of 0.5. The bioassays were conducted during 1 h under the same conditions described before for cyanobacterial cells growth. Finally, luminescence was recorded in a Centro LB 960 luminometer during 10 min. Three independent experiments with triplicate samples were carried out for all *Anabaena* bioassays (Rodea-Palomares et al., 2009b) .

Toxicity response of the cyanobacterium was estimated as EC_{50} values~~, the median effective microcystins concentration that causes 50% of bioluminescence inhibition with respect to a non-treated control. EC_{50} values~~ and their standard deviation ~~were~~ calculated by the dose-response package (drc) using R Software, version 3.3.1.

2.2.3 Interactions of MCs in extracts from natural samples

Interactions between MCs presents in the MCs extracts from natural samples was evaluated using the additive index ~~(AI)~~ method (AI). The ~~additive index method (AI)~~ has been previously used to study chemical interactions in several bioassays (Coalova et al., 2014; Sultana Shaik et al., 2016; Xie et al., 2017; Wang et al., 2018) . In order to apply AI to our sample set, the following equation was used (Loewe and Muischnek, 1926; Loewe, 1928; Marking and Dawson, 1975) :

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

Where *Am* is the EC_{50} for MC-LR in mixture, *Ai* the EC_{50} for MC-LR individually (calculated using those extracts with only MC-LR). *Bm* the EC_{50} for MC-RR in mixture, *Bi* the EC_{50} for MC-RR individually (calculated using those extracts with only MC-RR). Regarding MC-YR, there was not any sample containing only this cyanotoxin (Table 1) and, as this method requires having at least one sample containing 100% of each of the single toxicant, MC-YR was

208 excluded from this study. S is the sum of the biological activity. S values were then used to
209 calculate AI using the following equation:
210 AI = (1/*S*) -1 for $S < 1$; AI = -*S* +1 for $S \geq 1$
211 To determine whether the range for AI overlapped zero (additive) the 95% confidence intervals
212 from EC_{50} were substituted into the AI formula to establish a range (Marking and Dawson,
213 1975) . The effects observed in the mixtures were then classified as additive (AI = 0; expected
214 action), synergistic (AI > 0; greater than additive effect), or antagonistic (AI < 0; less than
215 additive effect).

216

217 *2.2.4 Intracellular free Ca^{2+}*

218 *Anabaena* was exposed during 1 hour to both MC-LR a commercial standard of MC-LR (DHI
219 Water and Environment, Denmark) diluted with Milli-Q water up to a concentration equivalent
220 to the EC_{50} and to the samples diluted to reach EC_{50}, and the shifts in intracellular free Ca^{2+}
221 ($[Ca^{2+}]_c$) were analysed. $[Ca^{2+}]_c$ in *Anabaena* was analyzed by flow cytometry (FCM) staining
222 cells with the sensitive Ca^{2+} indicator Calcium Green-5N acetoxymethyl ester (Calcium Green
223 5N-AM) (Invitrogen Molecular Probes, USA) (Garcia-Pichel et al., 2010) and following the
224 protocol described by (Prado et al., (2012) with minor modifications. FCM analysis of
225 *Anabaena* cells was performed on a Cytomix FL500 MPL flow cytometer (Beckman Coulter
226 Inc., Fullerton, CA, USA) equipped with an argon-ion excitation laser (488 nm), detectors of
227 forward (FS) and side (SS) light scatter and four fluorescence detectors corresponding to
228 different wavelength intervals: 520 nm (FL1), 575 nm (FL2), 620 nm (FL3) and 675 nm (FL4).
229 The cell-permeant acetoxymethylester, non-fluorescent and Ca^{2+} insensitive, can be passively
230 loaded into cells, where it is cleaved by ubiquitous intracellular esterases to the cell-impermeant
231 fluorescent product Calcium Green 5N, which exhibits an increase in fluorescent emission
232 intensity (Ex/Em: 506/532 nm) upon binding Ca^{2+}. A Calcium Green 5N-AM stock solution was
233 prepared in DMSO. Cell suspensions were incubated with the fluorochrome (final
234 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*.

3.4 Changes in intracellular free Ca^{2+} in Anabaena sp. PCC7120 CPB4337 after exposure to MCs extracts

Pure MC-LR (EC_{50} value) caused a significant increase (p-value < 0.001) of the intracellular free Ca^{2+} in *Anabaena* (226.7 ± 22.6 %) after 1 hour of exposure compared to the non-exposed control (not shown in Fig. 3). Besides bioluminescence, intracellular free Ca^{2+} was also altered in *Anabaena* after exposure to MC extracts at their EC_{50} values (Fig. 3). Indeed, 7 MCs extracts induced an increase in the intracellular free Ca^{2+} of *Anabaena* (Fig. 3). This novel report of an increase in intracellular free Ca^{2+} of cyanobacteria after exposure to MCs extracts from natural samples suggests that the MC-induced metabolic effects in cyanobacteria may be mediated by calcium. Intracellular free calcium could therefore be potentially used as an early biomarker of MC presence in freshwaters. Interestingly, our findings somewhat coincide with those of Cai et al., (2015) who proposed a critical role of calcium in the neurotoxicity of MCs toward vertebrates due to the $[Ca^{2+}]_c$ increase observed in primary hippocampal neurons from rats exposed to MC-LR.

4.Conclusion

Altogether, by using for the first time the bioluminescent bioassay of *Anabaena* sp. PCC7120 CPB4337 to MCs extracts from eight natural samples, the present study opens interesting avenues regarding: 1) a potential use of this bioassay as an early-warning detection tool of MCs in freshwaters; 2) study of toxicity interactions between MC in natural extracts; and 3) a possible involvement of intracellular free Ca^{2+} in the still unresolved mode of action of MCs towards cyanobacteria. This work puts us one step further towards a realistic risk assessment of MCs at environmental concentrations.

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524 **Figure captions**

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

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

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

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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

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562 **Table 2. Toxicity of pure MC-LR and MCs extracts from freshwater samples**
563 **towards *Anabaena* sp. PCC7120 CPB4337, expressed as median effective MCs**
564 **extracts concentrations causing 50% decrease in bioluminescence (EC_{50}).** Results
565 are 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

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Figure
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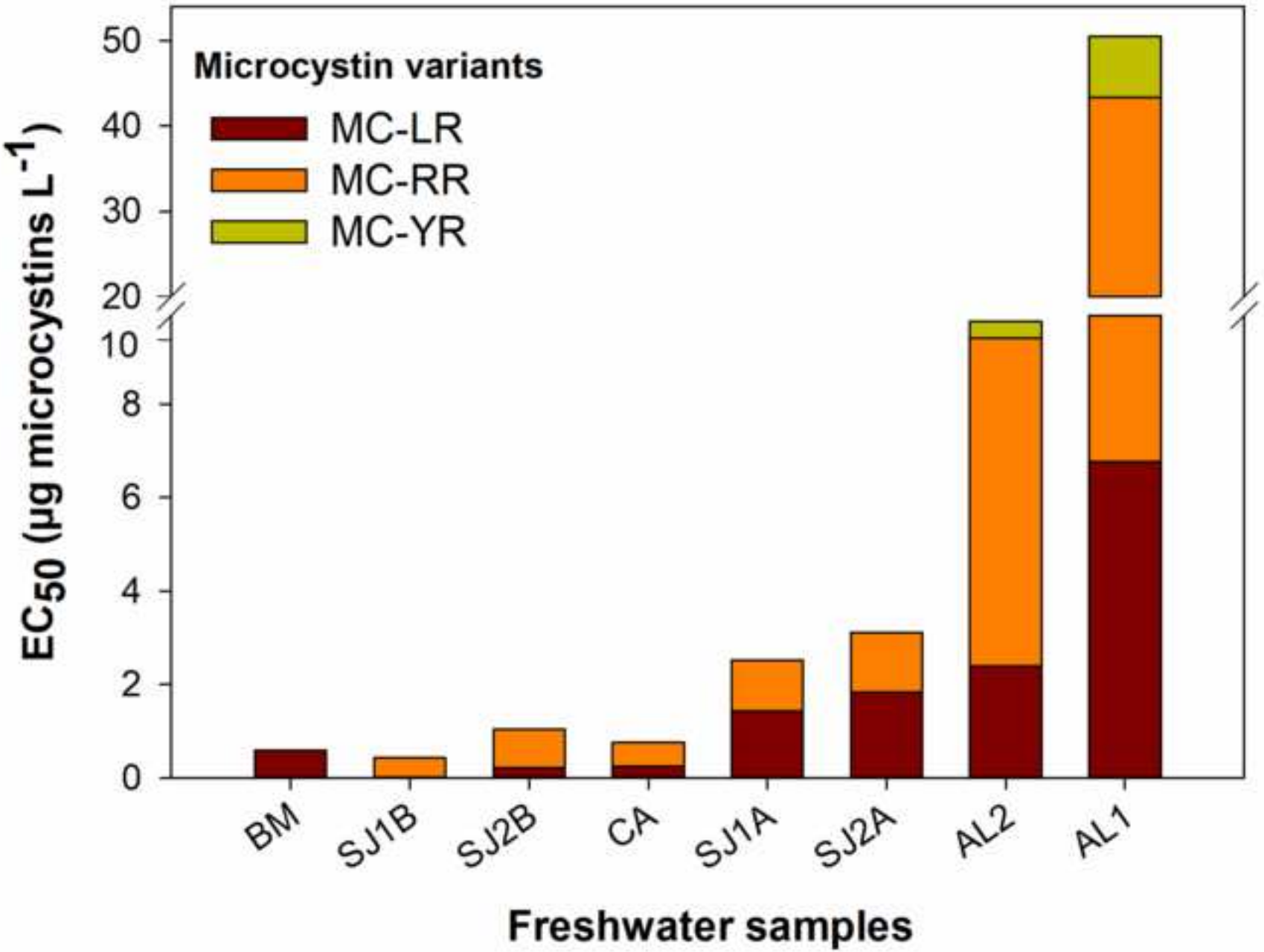

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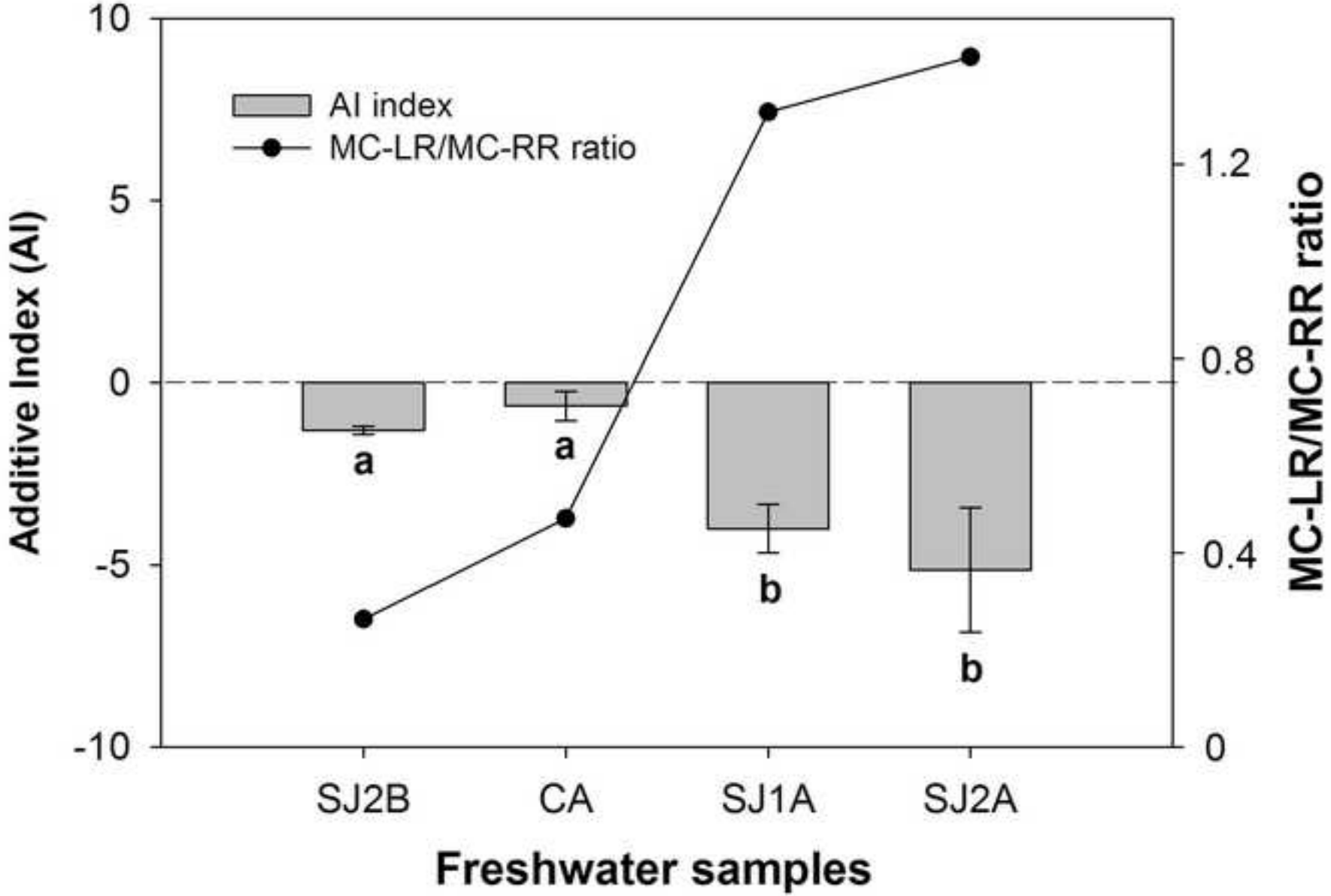

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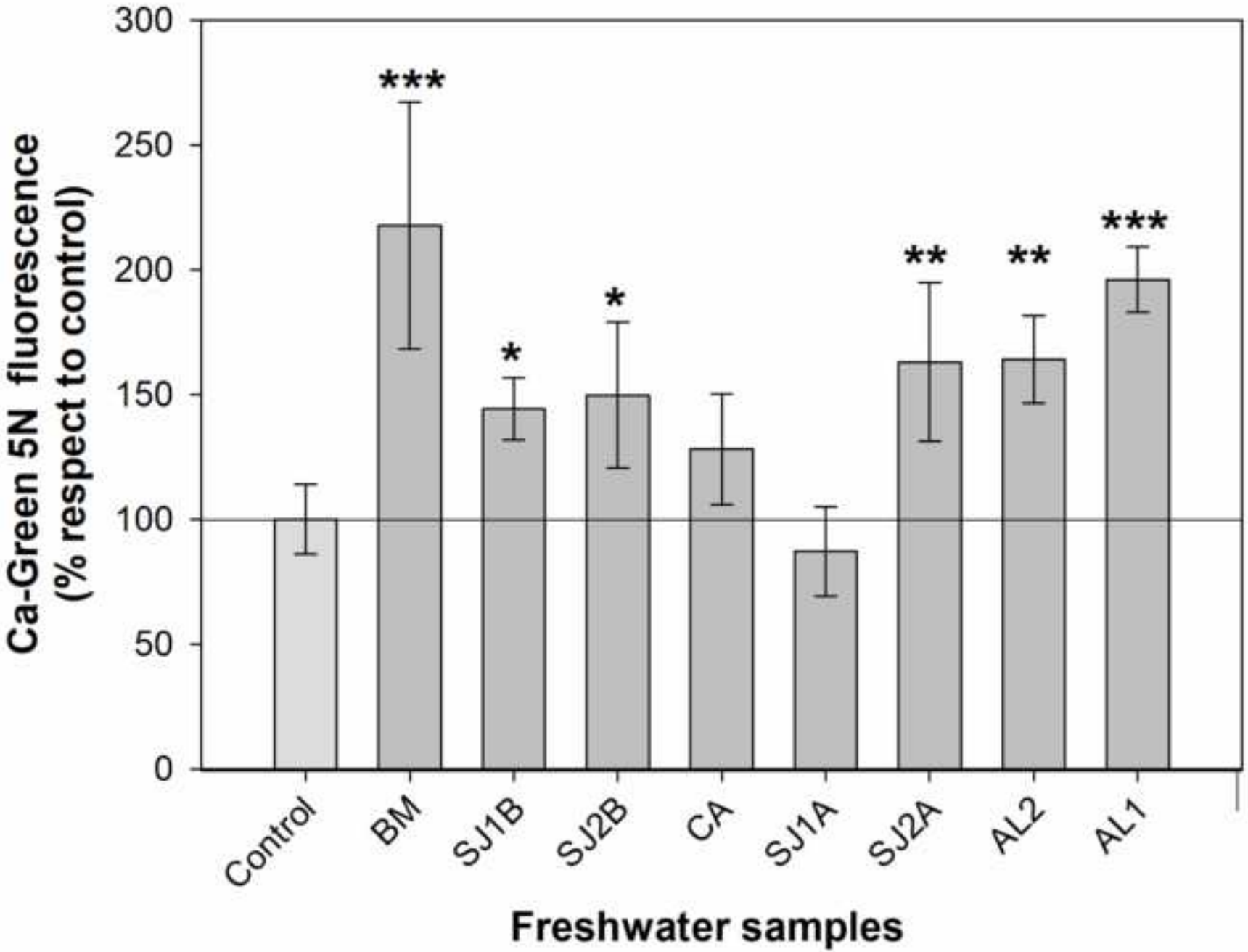

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