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1 **An examination of microcystin-LR accumulation and toxicity using tethered bilayer lipid**
2 **membranes (tBLMs).**

3

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Abstract

Microcystin-LR (MC-LR) is a potent cyanobacterial toxin responsible for animal and human poisonings worldwide. MC-LR is found in organisms throughout the foodweb, however there is conjecture regarding whether it biomagnifies. Few studies have investigated how MC-LR interacts with lipid membranes, a determinant of biomagnification potential. We tested whether 1 μM MC-LR irreversibly associates with lipid bilayers or causes the creation of pore defects upon short and long-term exposure. Using tethered bilayer lipid membranes (tBLMs), we observed an increase in membrane conduction in tBLMs, representing an interaction of microcystin-LR with the lipid bilayer and a change in membrane packing properties. However, there were minimal changes in membrane capacitance upon short and long-term exposure, and MC-LR exhibited a rapid off-rate. Upon 24 h exposure to the toxin, no lipophilic multimeric complexes were detected capable of altering the toxin's off-rate. There was no evidence of the creation of new pores. This study demonstrates that MC-LR does not irreversibly imbed itself into lipids membranes after short or long-term exposure and suggests MC-LR does not biomagnify through the food web via lipid storage.

Keywords

Microcystin-LR; biomagnification; tethered bilayer lipid membrane; cyanobacteria.

43 ## 1. Introduction

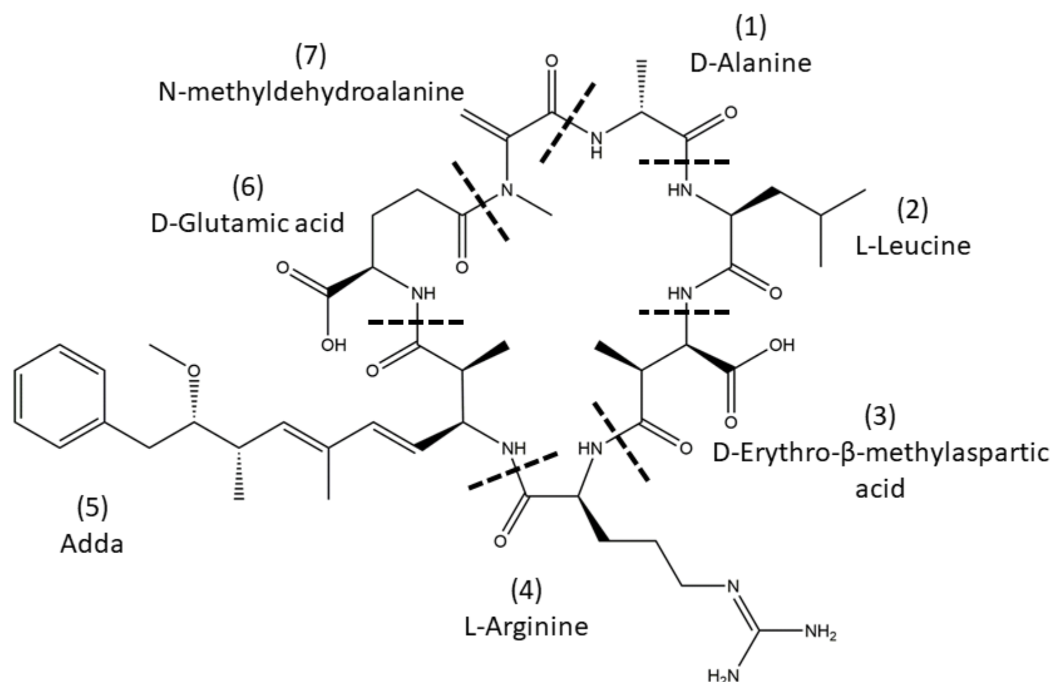
44 Cyanobacteria blooms are increasing in prevalence and severity, threatening both ecological
45 and anthropogenic water requirements (Paerl & Fulton, 2006; Paerl et al., 2011; Drobac et al.,
46 2013). Many bloom-forming cyanobacteria produce toxic secondary metabolites and
47 cyanotoxins that have been implicated in fish kills, deaths of waterbirds and other wildlife,
48 livestock poisonings and human fatalities (Azevedo et al., 1996; Carmichael, 2001; Ibelings et
49 al., 2005; White et al., 2005; Boopathi & Ki, 2014). Of all cyanotoxins, the hepatotoxic
50 microcystins (MC) are the most widespread and problematic (Quiblier et al., 2013; Mowe et
51 al., 2015).

52 MCs are a structurally diverse group of cyclic heptapeptides, with 90+ isomers (Boopathi &
53 Ki, 2014). They contain three D-amino acids (glutamic acid, *erythro*-β-methyl-aspartic acid
54 and alanine), two variable L-amino acids, and two unique residues (Mdha and Adda)
55 (Vesterkvist & Meriluoto, 2003). The toxicity of MC variants differs according to the
56 combination of variable amino acids in the peptide ring (de Figueiredo et al., 2004; Schmidt et
57 al., 2014). Microcystin-LR, a microcystin congener with a leucine and an arginine group, is
58 among the most toxic and common (Carmichael, 1997; de Figueiredo et al., 2004).

59 MC is frequently found in a variety of organisms, for example, aquatic plants (Mitrovic et al.,
60 2005), cladocerans (Ibelings et al., 2005), mussels (Barda et al., 2015), gastropods (Lance et
61 al., 2008; Barda et al., 2015), fish (Ibelings et al., 2005; Papadimitriou et al., 2012; Vasconcelos
62 et al., 2013) and mammals (Miller et al., 2010, van der Merwe et al., 2012). In higher
63 organisms, MCs are actively and preferentially accumulated in the liver through organic anion
64 transporting polypeptides (specifically, OATP1B1, OATP1B2, OATP1B3 and OATP1A2)
65 involved in bile-acid transport in hepatocytes (Chorus & Bartram, 1999; Ito et al., 2008;
66 McLellen & Manderville, 2017). They inhibit catalytic subunits of protein phosphatases-1 and
67 -2A, cause acute hepatotoxicosis and may promote cancer (Bagu et al., 1997; Carmichael,

68 2001; de Figeuiredo et al., 2004). Small but notable concentrations of MC are found in various
69 other tissues of rats and fish – for example the kidneys, heart, intestine, spleen, brain, gill,
70 muscle, gonad and stomach (Ito et al., 2000; Lei et al., 2008; Wang et al., 2008). The
71 identification of MCs in such varied organisms and tissues indicates a broad mechanism of
72 toxicity and accumulation beyond the well-known bile-acid transporters in hepatocytes. Petrov
73 et al. (1991) suggested that emplacement of MC's Adda residue in lipid bilayers may occur
74 due to its lipophilic and flexible tail that extends from the rigid cyclic backbone. This
75 potentially creates pores and compromises cell membrane integrity.

76 Lipophilic compounds, those that are soluble in lipids or oils, can impair the organisation of
77 lipids in a membrane and disrupt the functioning of the cell (Vesterkvist & Meriluoto, 2003).
78 Further, the capacity of a toxin to bioaccumulate and biomagnify can be influenced by
79 lipophilicity as lipophilic chemicals tend to accumulate in the lipids of organisms where they
80 are retained for extended periods and more readily transfer up trophic levels (Petersen &
81 Kristensen, 1998; Ibelings et al., 2005; Amiard & Amiard-Triquet, 2015). The water-octanol
82 partition coefficient (log K_{ow}) of MC-LR is -1 at pH 7 (Ibelings et al., 2005), suggesting that
83 the compound is relatively hydrophilic and may not strongly interact with lipids. However,
84 microcystins are amphipathic, containing both lipophilic and hydrophilic regions (**Figure 1**)
85 (Vesterkvist & Meriluoto, 2003). The lipophilic Adda residue and the variable amino acids in
86 the peptide ring of MCs may impair lipid membrane structure or facilitate MC-LR
87 accumulation within an organism (Orr & Jones, 1998; Zurawell et al., 2005; Schmidt et al.,
88 2014).



89

90 **Figure 1.** Chemical structure of microcystin-LR. (1) is D-alanine; (2) is the lipophilic
91 variable amino acid L-Leucine; (3) is D-erythro-β-methylaspartic acid; (4) is the
92 variable amino acid L-Arginine; (5) is the lipophilic residue Adda; (6) is D-glutamic
93 acid; and (7) is N-methyldehydroalanine.

94 Given that MC is regularly found in organisms across all trophic levels (Xie et al., 2005; Tokodi
95 et al., 2018), there are also concerns regarding whether MC can biomagnify to affect upper
96 trophic level organisms through the food web, such as fish, water birds, sea otters and humans
97 (Xie et al., 2005; Miller et al., 2010). Lipid membrane interactions are a common mechanism
98 of assimilation, accumulation and biomagnification of toxins; however this has not yet been
99 demonstrated with MC-LR (Ibelings et al., 2005; Kozlowsky-Suzuki et al., 2012; Schmidt et
100 al., 2014).

101 Despite the relatively low log K_{ow} of MC-LR, the lipophilic Adda and variable amino acid
102 regions of the compound, combined with suggestions of bioaccumulation and biomagnification

103 of MC-LR in the literature, may indicate some interaction between the cyanotoxin and lipid
104 membranes. To determine whether MC-LR irreversibly associates with lipid bilayers, we
105 investigated the interactions of the toxin with *tethered bilayer lipid membranes* (tBLMs), which
106 provide a model for natural biological membranes (Cranfield et al., 2014; Cranfield et al.,
107 2015a)

108

109 ## 2. Materials and Methods

110 ### 2.1 *Mass Spectroscopy*

111 Commercially available microcystin-LR was obtained and its purity tested prior to use using
112 mass spectroscopy. Matrix Assisted Laser Desorption Ionisation MassSpectrometry (MALDI-
113 MS) using a 5800 MALDI TOF/TOF (AB Sciex, Framingham USA) in positive ion reflector
114 mode was used. Its operation utilized a laser set to 4700, mass range set to 400-3000 Da with
115 focal mass of 995 Da. 500 spectra were accumulated on a spot containing 1 ng of microcystin-
116 LR standard (Abraxis) co-crystallised with α-Cyano-4-hydroxycinnamic acid (Sigma, USA)
117 to assist in ionization. The concentration of microcystin used for tBLM experiments was 1 μM
118 dissolved in phosphate buffered saline (PBS). This concentration was selected as it is on the
119 upper end of those found in natural systems, so is representative of a toxic bloom scenario
120 (Chorus, 2001).

121 ### 2.2 *Tethered Bilayer Lipid Membranes (tBLMs)*

122 Lipid bilayers were anchored to a pure gold substrate using a combination of 10% tethering
123 lipids and 90% spacer lipids as described previously (**Figure 2*A***) (Cranfield et al., 2015b). In
124 brief, pure 5N5 gold electrodes of 2.1 mm^2 were pre-prepared with a coating of tethered benzyl-
125 disulfide (tetra-ethyleneglycol)$_{n=2}$ C20-phytanyl tethers (DLP) and benzyl-disulfide-tetra-
126 ethyleneglycol-OH spacers (TEGOH) in the ratio of 1:9 (*SDx Tethered Membranes Pty Ltd,*

127 *Australia*). To these tethering chemistries were added 8 μL of a 3 mM solution of a mobile
128 lipid phase dissolved in ethanol. After a 2 min incubation, the mobile phase lipids were washed
129 three times with 3 × 400 μL of phosphate buffered saline (PBS). The lipids added consisted of
130 70% zwitterionic C20 Diphytanyl-Glycero-Phosphatidylcholine lipid and 30% C20
131 Diphytanyl-diglyceride-OH ether. To determine if the microcystin-LR might have an affinity
132 for other membrane components, the membranes were supplemented with either 20%
133 (mol/mol) 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE, Avanti Lipids,
134 USA) or 20% (mol/mol) cholesterol.

135 ## 2.3 *AC electrical impedance spectroscopy (EIS)*

136 Phase and impedance measures were performed using an SDx tethaPod™ operated with SDx
137 tethaQuick™ software (SDx Tethered Membranes Pty Ltd). Using a 50 mV peak-to-peak AC
138 excitation at frequencies between 0.1 and 10,000 Hz with four steps per decade. Swept
139 frequency EIS was employed to determine the change in membrane conduction (G_m) and
140 changes in membrane capacitance (C_m). The data were fitted to an equivalent circuit
141 comprising a *Constant Phase Element* (CPE) to represent the reservoir region (Krishna et al.,
142 2003) in series with a Resistor/Capacitor that described the tethered lipid bilayer (**Figure 2*B***).

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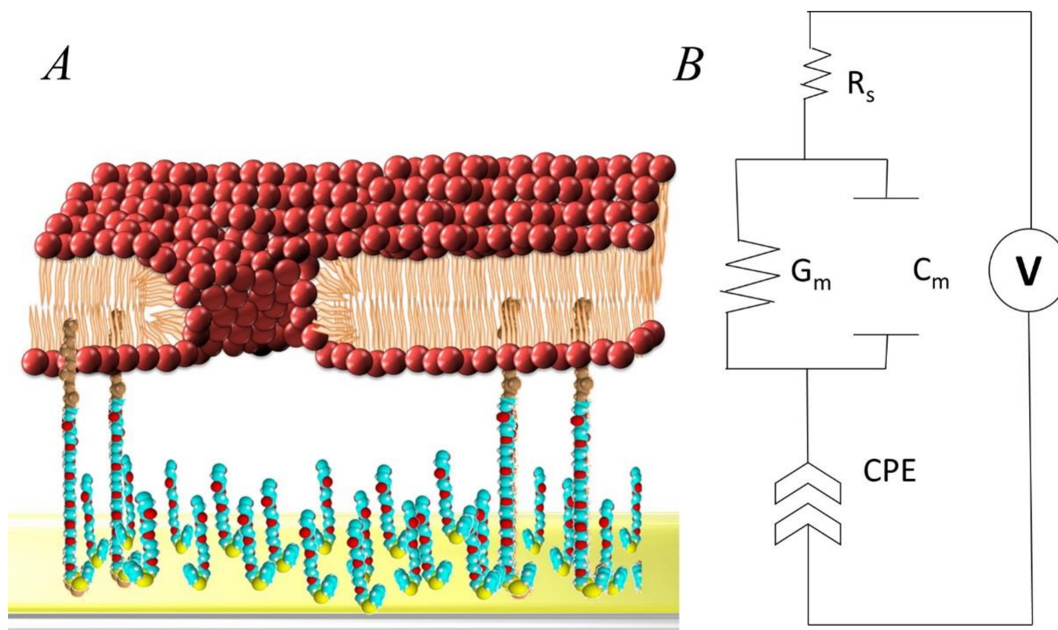


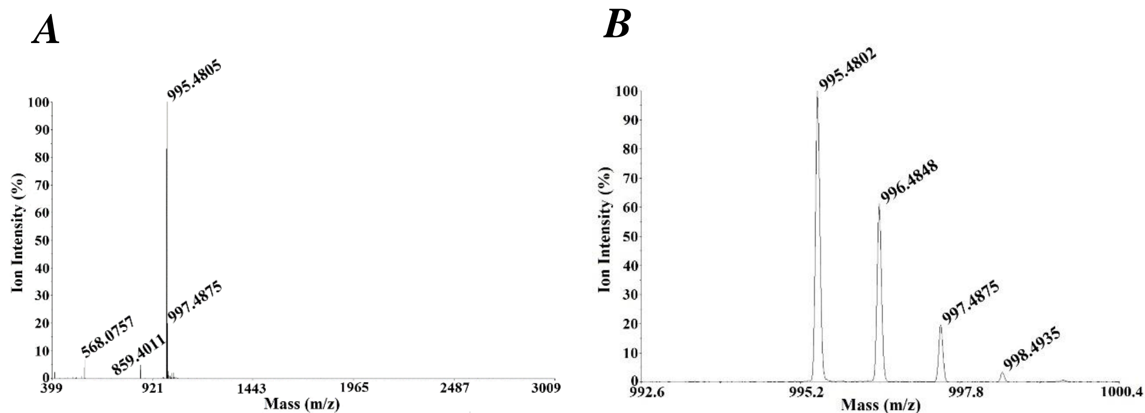
144 **Figure 2.** ***A***. Cartoon of a tethered lipid bilayer membrane (tBLM) with an
145 intrinsic membrane pore defect. Benzyl-disulfide (tetra-ethyleneglycol)$_{n=2}$ C20-
146 phytanyl tethers anchor a lipid bilayer to an underlying gold electrode. ***B*****.** The
147 *equivalent circuit* for modelling AC electrical impedance data. In this circuit, R_s
148 represents the resistance of the electrolyte solution (PBS), G_m represents the
149 conduction of the membrane, which is primarily determined by membrane pore
150 defects, *Cm* is the membrane capacitance and *CPE* is a *constant phase element*
151 used to model the tethering gold electrode and its surface chemistries.

152

153 ## 3. Results and Discussion

154 The microcystin-LR used in the experiment was determined to be of high purity with minimal
155 degradation by use of MALDI-MS, the highest abundant ions present in the sample being the
156 expected mass of the intact microcystin (**Figure 3**). Microcystin-LR was tested on tBLMs
157 containing Dyphytanyl PC lipids or mixtures of Diphytanyl lipids with 20% POPE or 20%
158 cholesterol. POPE and cholesterol are membrane constituents necessary for the interactions of

159 numerous other toxins and peptides with lipid bilayers (Palmer, 2001; Henriques et al., 2011;
160 Al Khamici et al., 2016; Cranfield et al., 2017). For each of the different membranes used there
161 was a consistent increase in membrane conduction due to the presence of 1 µM microcystin-
162 LR (**Figure 4*A*, *C* and *E***). This response is consistent with a change in membrane packing
163 brought about by the interaction of the microcystin-LR with the lipid bilayer. According to a
164 membrane packing model, this alteration of the membrane packing arrangement causes an
165 increase in the diameter of intrinsic toroidal pores within the membrane and is unlikely to be
166 due to the formation of new pores (Cranfield et al., 2016; Cranfield et al., 2017; Kuppusamy et
167 al., 2018). This data is supported by the fact that there is little change in membrane capacitance
168 as a result of adding 1 µM microcystin-LR (**Figure 4*B*, *D* and *F***). Membrane capacitance is
169 directly influenced by membrane thickness and/or the water content at the bilayer. As a positive
170 control, a pore-inducing cathelicidin peptide, *LL-37*, was later added (Turner et al., 1998;
171 Nizalapur et al., 2016). This addition of LL-37 induced a definite change in membrane
172 conduction and membrane capacitance.



173

174 **Figure 3.** ***A***, Mass-spectra of microcystin LR sample. The peak at 995.48 m/z
175 corresponds with microcystin LR at 97% total ion intensity, 859.40 m/z corresponds to
176 a fragmentation of the toxin releasing 136 Da corresponding to (2-
177 methoxyethyl)benzene. ***B***, spectra zoomed to display the carbon isotope distribution of
178 the molecule.

179

180 Following a PBS wash step microcystin-LR exhibited a rapid off-rate, which had little impact
181 on the capacitance of the membranes. This indicates that despite its amphipathic properties, its
182 lipophilicity is minimal as it can readily be removed by aqueous buffers. However, this does
183 not preclude the possibility microcystin-LR might form oligomers that are more lipophilic in
184 nature. To test this, we incubated tBLMs with microcystin-LR overnight before washing with
185 PBS. **Figure 5*A-D*** are these membrane conduction and membrane capacitance traces for
186 tBLMs exposed to 1 μM microcystin-LR in the presence of 20% POPE or 20% cholesterol
187 lipids, respectively. It is evident that the tBLMs exhibit an increase in membrane conduction,
188 with minimal changes in membrane capacitance. MC-LR is then readily washed out after the
189 allotted time-period. This suggests that no lipophilic multimeric complexes are being formed
190 by the toxin in membranes or in aqueous buffer.

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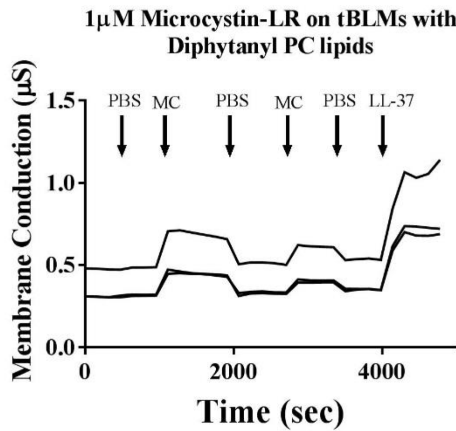

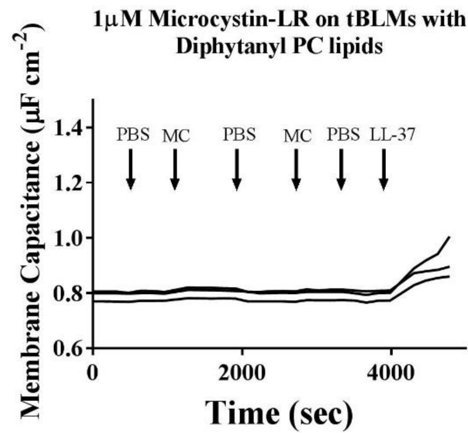

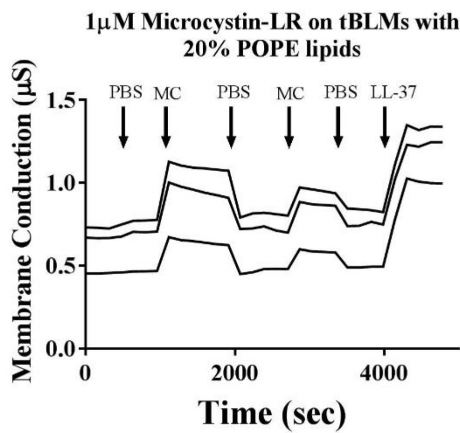

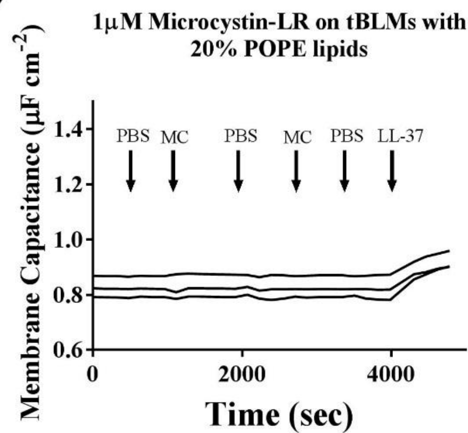

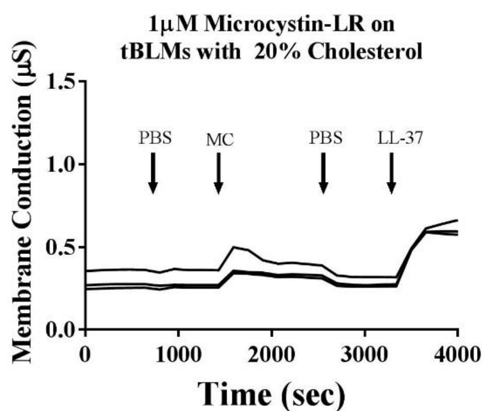

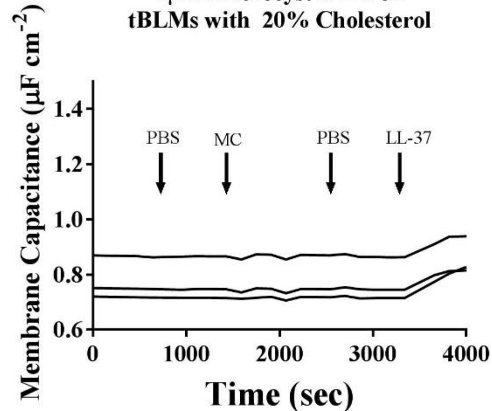

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193 **Figure 4.** ***A*** Membrane conduction changes over time as result of adding 1 μM
194 microcystin-LR to tBLMs made of Diphytanyl PC with 30% diphytanyl-
195 diglyceride-OH lipids. A small increase in membrane conduction can be seen,

196 which is readily washed from the membrane subsequently by PBS buffer. LL-

197 37 peptide is added finally as a positive control. ***B,*** Capacitance measured over

198 the same period indicating minimal changes to membrane thickness and/or water

199 content as a result of the addition of microcystin-LR, which can be compared to

200 the significant capacitance change due to LL-37 peptide. ***C,*** similar conduction

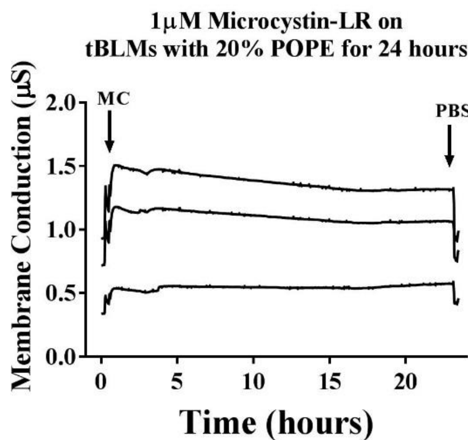
201 responses, and ***D***, capacitance responses, in membranes that include 20%

202 mol/mol POPE lipids. ***E,*** similar conduction responses, and ***F***, capacitance

203 responses, in membranes that include 20% cholesterol mol/mol. All samples are

204 n=3.

A



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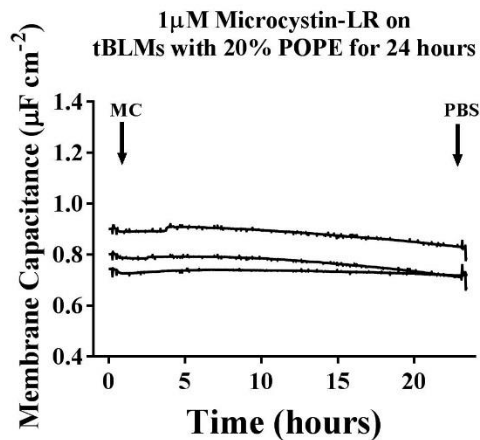

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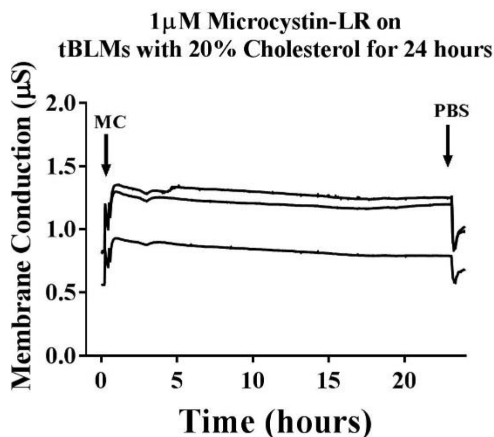

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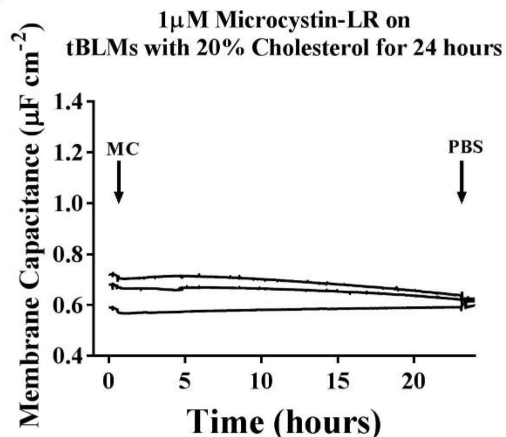

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206

207 **Figure 5.** ***A*****,** membrane conduction changes over a period of nearly 24 hours as
208 result of adding 1 µM microcystin-LR to tBLMs containing 20% POPE lipids**.**
209 A small increase in membrane conduction can be seen which is maintained a
210 relatively steady level until the microcystin-LR is subsequently washed from the
211 tBLM by PBS buffer. ***B,*** capacitance measures of the same period. ***C*** and ***D,*** are
212 similar conduction and capacitance measures, respectively, in tBLMs containing
213 20% cholesterol. All samples are n=3.

214 The minimal effect of MC-LR on membrane capacitance after short and long-term exposure,
215 and the rapid off-rate following the PBS wash, indicates that the toxin has minimal potential to
216 imbed itself in or disrupt biological membranes. Therefore, the creation of pores, and/or the
217 deterioration of membrane integrity, as was suggested by Petrov et al. (1991), is unlikely to be
218 a contributing factor to MC-LR's toxicity. Biomagnification of MC-LR is unlikely to occur
219 due to the low residence time in lipids, and biodilution is likely to be the prevailing process
220 (Ibelings, 2005; Kozlowsky-Suzuki, 2012). This is supported by Dyble et al. (2011) who found
221 that orally administered MC-LR was eliminated rapidly (within 24 h) from the tissue of
222 juvenile yellow perch.

223 The present study showed some minor, temporary interaction of MC-LR with lipid membranes,
224 most likely caused by the insertion of the lipophilic Adda residue. This interaction may explain
225 the detection of the toxin in low quantities in a diverse range of species and tissue types (Ito et
226 al., 2000; Wang et al., 2008; Lei et al., 2008). The introduction of cholesterol and POPE lipid
227 to membranes indicated that the presence of lipid micro-domains has little influence on the
228 toxin's membrane insertion and retention time. Localised accumulation of MC-LR in

229 hepatocytes through the OATP transporters remains the likely route of exposure to toxic levels
230 of MC-LR (Chorus & Bartram, 1999).

231 While this study suggests that MC-LR does not biomagnify through the food web through lipid
232 storage, this does not discount the possibility of other MC variants doing so. MC-LR is
233 frequently cited as the most toxic and widespread of the microcystin family (Schmidt et al.,
234 2014; Rastogi et al., 2015), and many water quality guidelines often focus solely on MC-LR,
235 such as those by the World Health Organisation (WHO) (Guidelines for drinking-water quality:
236 fourth edition incorporating the first addendum, 2017). It should also be noted that the majority
237 of cyanotoxin research focuses on MC-LR (de Figueiredo et al., 2004). Some microcystin
238 isomers, such as MC-LF and MC-LW, are more lipophilic than the MC-LR variant (Vesterkvist
239 et al., 2012) and Xie et al. (2004) noted that depuration of MC-RR in silver carp occurs far
240 more slowly than MC-LR. These microcystins may represent a greater biomagnification
241 potential than MC-LR. Further research should seek to clarify the differences in
242 biomagnification potential between the microcystin congeners and establish water quality
243 guidelines that incorporate other MC congeners that may threaten higher trophic level
244 organisms.

245

246 **4. Conclusion**

247 Using tethered bilayer lipid membranes, we demonstrated that MC-LR does not irreversibly
248 imbed itself into lipid membranes after short or long-term exposure. We observed an increase
249 in membrane conduction in tBLMs, most likely caused by insertion of the lipophilic Adda
250 residue, altering the overall membrane packing properties. However, there was minimal
251 changes in membrane capacitance and MC-LR exhibited a rapid off-rate. When left to incubate
252 with the membrane over 24 hours, the data suggests that no lipophilic multimeric complexes

were formed capable of altering the toxin's off-rate, and no new pores were created. The temporary insertion of the Adda residue may explain the identification of the toxin in low quantities in a diverse range of species and tissue types (Ito et al., 2000; Wang et al., 2008; Lei et al., 2008), however MC-LR appears incapable of accumulating to high levels as a result of lipid insertion in these tissues. These findings indicate that biodilution, as opposed to biomagnification, is the prevailing process when MC-LR enters the foodweb.

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