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1 **Response of Fish Gut Microbiota to Toxin-Containing Cyanobacterial Extracts: a**
2 **Microcosm Study on the Medaka (*Oryzias latipes*)**

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17

18

19 **Abstract**

20 The effect of toxin-producing cyanobacterial blooms on fishes health has been investigated
21 extensively, but no study to date has evaluated this effect on fish-associated microbiota. In this
22 study, we test the effect of pure microcystins and of crude extracts of metabolites from
23 *Microcystis aeruginosa* cultures on the composition of gut bacterial microbiota in Medaka
24 fishes (*Oryzias latipes*) exposed for 28 days in a microcosm experiment. A 16S rRNA-based
25 marker gene-based approach was used to investigate the composition of bacterial
26 communities. Results show that fish gut community compositions differ from those occurring
27 in the water, and among individual fishes. Exposure to extracts, rather than pure microcystin,
28 has a significant influence on gut community composition, with a marked increase in relative
29 abundances of pathogen-related bacteria (genera *Nocardia* and *Mycobacterium*) in the
30 presence of one extract, and of bacterial orders Sphingomonadales and Saprospirales in the
31 other. We suggest that compounds identified in the cyanobacterial extracts, but not
32 microcystin LR alone, alter the composition of bacterial communities, with possible
33 consequences for various biological functions in fishes. This pioneer microcosm experiment
34 indicates that cyanobacterial blooms probably have an effect on fish gut microbiota and
35 associated functions, including toxin degradation and feed efficiency, and should be further
36 explored.

37 **Introduction**

38

39 Cyanobacterial proliferations occur worldwide in freshwater, estuarine and marine
40 environments, when environmental conditions allow (*i.e.* nutrient inputs, light intensity, high
41 temperatures, pH and CO₂ concentration). In recent years, massive cyanobacterial blooms
42 increased in frequency and persistence around the world, causing serious threats to aquatic
43 ecosystems^{1,2}. Indeed, cyanobacteria produce numerous bio-active secondary metabolites,
44 including various cyanotoxins, that remain and bioaccumulate into the environment,
45 especially after bloom senescence³⁻⁵. The most widespread cyanotoxin is microcystin-leucine-
46 arginine (MC-LR) which can accumulate in the liver of aquatic vertebrates including fish^{6,7}.
47 Microcystins are produced, among others, by *Microcystis*, the most common cyanotoxin-
48 producing and bloom-forming genus in freshwater ecosystems⁸.

49 Over the last decade, MC-LR effects upon fish ecotoxicology have been documented in some
50 species⁹⁻¹². Among these, the Medaka fish (*Oryzias latipes*) is a model of choice for toxic
51 effects investigation thanks to its resistance to stress and diseases. Natural cyanobacterial
52 blooms, lab cultures, biomass extracts and pure microcystins (*e.g.* MC-LR) were shown to
53 adversely impact its development, reproduction, and to induce specific organ alterations,
54 notably hepatotoxicity and cardiotoxicity^{7,13-15}. However, despite the documented impact of
55 cyanobacterial blooms on fish health, no study has to our knowledge investigated the link
56 between these events and the composition of fish microbiota.

57 In ecology, the holobiont, *i.e.* the super-organism composed by one pluricellular host and its
58 associated microorganisms constituting its microbiota, is increasingly acknowledged as a
59 relevant level of investigation¹⁶. Indeed, microbiota play multiple fundamental roles in host
60 physiology, including in nutrition, immunity, protection and behavior¹⁷. In the context of
61 aquaculture and fisheries, many investigations have recently highlighted the importance of
62 fish microbiota for feed efficiency, and pathogen resistance¹⁸. Located at the interface between
63 an organism and its environment, the microbiota is exposed to the latter, and should thus be
64 accounted for in ecotoxicology studies. In this perspective, some cyanotoxins, known for their
65 antimicrobial effect, could directly impact the composition of fish microbiota. Such effects
66 have already been shown on the gut microbiota of the branchiopod *Daphnia*, for instance,
67 resulting in alteration of their tolerance to toxic cyanobacteria¹⁹. Cyanobacterial secondary
68 metabolites could also represent nutrient sources that select gut microbes able to use them.

69 The aim of the present study is to test whether changes occur in fish microbiota compositions
70 when hosts are exposed to cyanobacteria in a microcosm setup. As a natural cyanobacterial

71 bloom is difficult to stimulate in the laboratory, we investigated the effects of three
72 cyanobacterial extracts (two from distinct microcystin-producing *Microcystis aeruginosa*
73 strains and one pure MC-LR extract) on water-borne bacterial populations and on the
74 Medakas' gut microbiome composition. Cyanotoxins concentrations were chosen to mimic a
75 low-level intoxication, just above the norm for drinking water²⁰ to see how chronic exposure
76 may impact gut bacteria. Bacterial communities are investigated using an Illumina-based
77 metabarcoding approach on the V4-V5 region of the 16S rRNA-encoding gene. Potential
78 consequences for nutrition and pathogen resistance are discussed.

80 **Materials and methods**

82 ***Secondary metabolites in extracts of *Microcystis aeruginosa* strains***

83 Three different metabolite mixtures were used in exposure experiments. The first consisted in
84 pure microcystin-LR resuspended in 50% ethanol/water solution and then partially evaporated
85 (Novakit, France). The other two were extracted from cultures of *Microcystis aeruginosa*
86 strains Paris Museum Collection (PMC) 728.11 (Extract 1) and Pasteur Culture Collection
87 (PCC) 7820 (Extract 2) available upon request from the National Museum of Natural History
88 and Institut Pasteur collections, respectively. Both were cultured at 25°C in Z8 media (Rippka
89 1988, 16h: 8h light/dark cycle at 60 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). Cell cultures were sonicated in methanol,
90 centrifuged (10 min, 4°C, 3,500 g), methanol was evaporated, extracts were resuspended
91 (EtOH 50%), and ethanol was evaporated. Microcystin concentration was measured as MC-
92 LR equivalents (MC-LR eq.) using the Novakit Microcystin-ADDA ELISA kit (France).
93 Metabolites composition of the extracts were analyzed on an UHPLC (Ultimate 3000,
94 ThermoFisher Scientific) coupled with a high-resolution mass spectrometer (ESI-Qq-TOF
95 Maxis II ETD, Bruker). The analyte annotations were performed according to precise mass,
96 isotopic and fragmentation MS/MS patterns, as previously described¹⁴.

98 ***Exposure experiments and monitoring***

99 Adult male Medaka fishes were used in all experiments. Four treatments were applied: a
100 solvent control without any cyanobacterial metabolite; exposure to Extract 1 ($\sim 2.5 \mu\text{g MC-LR}$
101 $\text{eq}\cdot\text{L}^{-1}$); exposure to Extract 2 ($\sim 2.5 \mu\text{g MC-LR eq}\cdot\text{L}^{-1}$); and exposure to pure microcystin
102 ($\sim 2.5 \mu\text{g MC-LR eq}\cdot\text{L}^{-1}$). Each of the four treatments was applied on three replicate 10-liter
103 aquaria, each containing six fishes that were acclimatized 10 days prior to the beginning of
104 the exposure. Fishes were then exposed for 28 days at $25^\circ\text{C}\pm 1^\circ\text{C}$. They were fed twice a day

105 with T-0.3 Nutra HP (Skretting, Netherlands). Every two days, the aquaria were cleaned, half
106 of the water was replaced, and the extracts or MC-LR were renewed in totality in the
107 concentrations described above. Water temperature, conductivity, pH, nitrate and nitrite were
108 monitored every two days, and samples taken to further determine the total microcystin
109 content using the ELISA kit.

111 ***Fish dissection and tissue fixation***

112 At the end of the 28 days' exposure, three fishes (n=3) were sampled from each of the 12
113 aquaria, except for aquaria E (four fishes), F and I (two fishes each, see Table S1). Fishes
114 were anesthetized in 0.1% tricaine methane sulfonate (MS222; Sigma, St. Louis, MO),
115 sacrificed, dissected, and the whole intestine (including content because of small size) was
116 sampled and flash-frozen in liquid nitrogen. Aquarium water (50 mL) was filtered on a 0.22
117 µm nitrocellulose filter, then filters were flash frozen.

118 ***DNA extraction and 16S rRNA gene sequencing***

119 DNA was extracted from the intestine of 2 to 4 (mostly 3) replicate fishes from each
120 aquarium, from water-filters and from fish food using a ZymoBIOMICS™ DNA mini kit
121 (Zymo Research, CA), following mechanical lysis (bead-beater, 6 minutes, maximum speed).
122 A ~500 bp fragment of the rRNA-encoding gene corresponding to the V4-V5 variable region
123 of *Escherichia coli* was amplified using 515F and 926R primers²¹ and sequenced on an
124 Illumina MiSeq platform (2 X 250 bp, paired-end sequencing, Genoscreen, France). Raw
125 reads were deposited into the GENBANK Sequence Read Archive (SRA) database under
126 accession number PRJNA517613 (samples SAMN10839113 to SAMN10839169, see table
127 S2).

128 ***Sequence analysis***

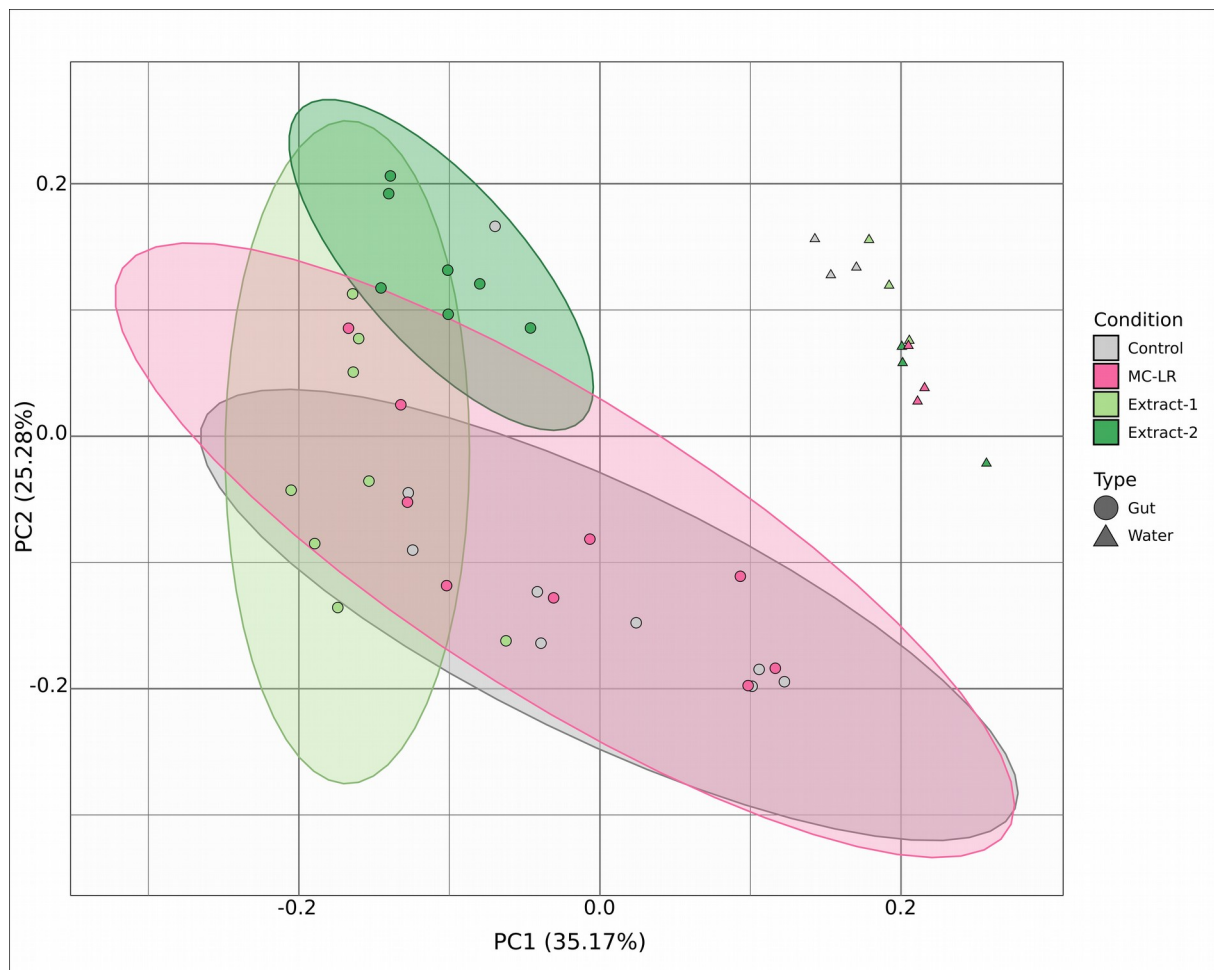
129
130 Raw reads were demultiplexed, quality checked and trimmed, and paired reads were
131 assembled in contigs using FLASH²². Sequence analysis was performed using QIIME2²³.
132 Amplicon Sequence Variants (ASVs²⁴) were identified using DEBLUR²⁵. Chimeric sequences
133 were identified using UCHIME (*de novo* chimera detection) and then removed²⁶. Taxonomic
134 affiliations were obtained by the sklearn-based classifier (GreenGenes 13-8-99 release).
135 Sequences matching “Chloroplast” and “Mitochondria” were discarded. Rarefaction curves,
136 alpha and beta diversity indices were generated using a sampling depth of 8,132
137 corresponding to the lowest number of quality-filtered reads obtained in a sample. Two gut
138

139 samples (one from Extract 1 and one from Extract 2 experiments) were outliers in terms of
140 (very low) ASVs number compared to samples from the same conditions, and were discarded
141 from further analysis. A guide phylogenetic tree was produced to compute UniFrac
142 distances²⁷. Principal coordinates analysis (PCoA) plots based on Weighted and Unweighted
143 UniFrac distances (WU and UU) as well as Bray Curtis (BC) dissimilarities were generated
144 using ggplot2²⁸. Community richness were compared using ANOVA, and compositions were
145 compared using PERMANOVA. A Venn diagram was drawn using the web-based software
146 available at <http://bioinformatics.psb.ugent.be/webtools/Venn/>.

147 148 **Results and Discussion**

149
150 The analyzed parameters (temperature, conductivity, nitrites, pH and exposure levels
151 measured as MC-LR equivalents) were stable within a given aquarium during the 28-days
152 experiment (Table S1). Analysis of Extracts 1 and 2 from the *M. aeruginosa* strains revealed
153 very different compositions in secondary metabolites besides MC-LR, present at various
154 concentrations, including cyanopeptolins, aeruginosins, aerucyclamides, anabaenopeptins,
155 microviridins and/or microginins (Figure S1). Exposure levels ranged between mean 1.1 ± 0.4
156 $\mu\text{g MC-LR eq.L}^{-1}$ (Extract 1) and $5.6 \pm 1.1 \mu\text{g MC-LR eq.L}^{-1}$ (Extract 2), with $2.3 \pm 0.7 \mu\text{g.L}^{-1}$
157 in the pure MC-LR treatment. They were slightly above the norm for drinking water ($1.0 \mu\text{g}$
158 $\text{eq. MC-LR eq.L}^{-1}$), and represent chronic exposure levels rather than the acute levels
159 expected during or after a very intense bloom event²⁰. These conditions were previously
160 shown to lead to observable lesions on the liver of young and adult fish, but not to major
161 dysfunction^{14, 29-31}. The toxicity on hosts is thus limited, as are potential associated indirect
162 effects on microbiota linked for example to organ malfunction leading to changes in the
163 physico-chemical environment of microorganisms. Therefore, we expect to rather observe the
164 direct effect of the MC-LR and the extracts on the microbiota itself.

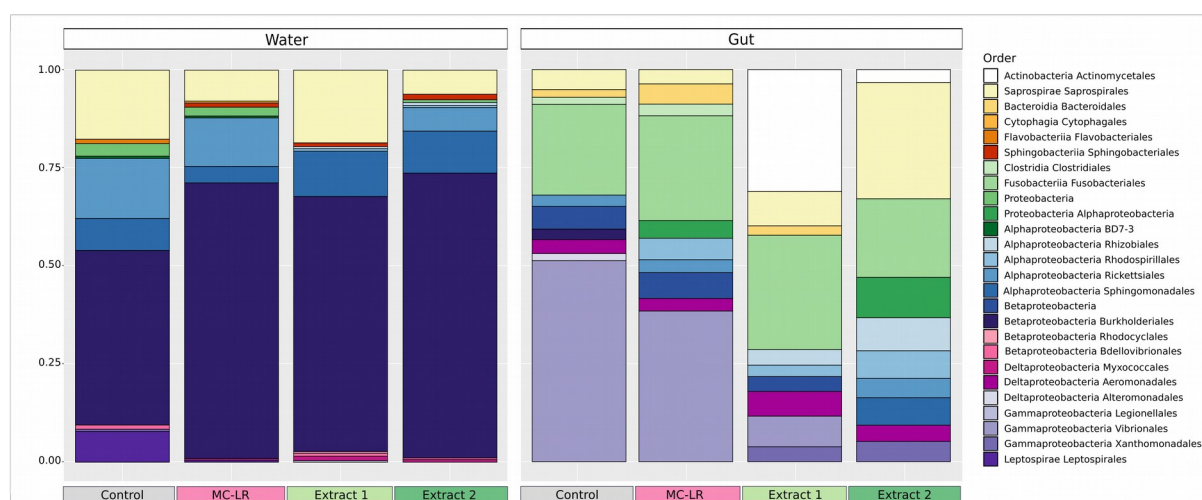
165 A total of 1,861,481 assembled paired-end reads were obtained from 12 water samples
166 (3 replicates per treatment) and 33 gut samples (7 to 9 per treatment, Table S1), of which
167 1,063,328 from bacterial origin passed quality filters. Deconvolution resulted in a total of 662
168 ASVs. Rarefaction curves reached saturation for all samples, indicating that the dataset
169 accurately represents the bacterial community (not shown). Depending on the sample,
170 between 8,132 and 37,511 assembled sequences were obtained, representing 18 to 178 ASVs
171 (Table S2). Comparisons indicate that the gut samples from specimens exposed to extracts 1
172 and 2 display overall higher diversity of ASVs and Shannon indices (Figure S2-3).



175 **Figure 1.** Principal Coordinates Analysis based on Weighted Unifrac dissimilarity metrics. Ellipses correspond
 176 to 95% confidence intervals for gut samples exposed to each of the four treatments.
 177

178 ***Bacterial communities in the water are similar***

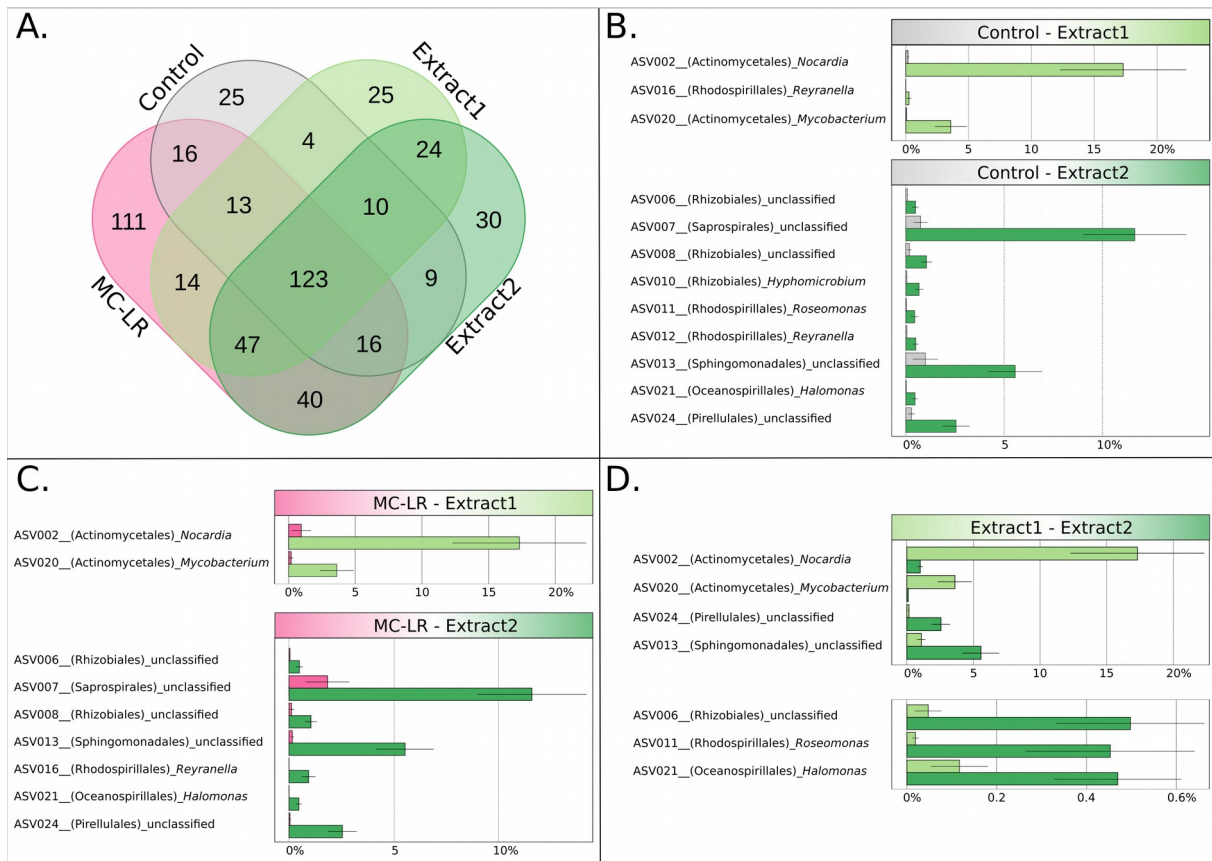
179 Bacterial communities compositions were not significantly different among water
 180 samples whatever the treatment (WU distances, PERMANOVA $p > 0.09$), and clustered
 181 together in the principal coordinates analysis (PCoA, Figure 1). Similar results were observed
 182 in PcoA plots obtained using UU and BC dissimilarities (figure S4). These communities were
 183 dominated by Burkholderiales (Betaproteobacteria, mean $60.1 \pm 13.5\%$ of reads), Saprospirales
 184 (Bacteroidetes, $11.8 \pm 6.3\%$), Sphingomonadales ($8.1 \pm 4.6\%$) and Rickettsiales ($8.1 \pm 8.8\%$)
 185 (Alphaproteobacteria, Figure 2). Interestingly, abundances of Leptospirales (Spirochaetes),
 186 some of which are known to be animal pathogens, were significantly lower in the presence of
 187 cyanobacterial extracts or MC-LR compared to control ($p\text{-value} < 0.01$), suggesting selective
 188 antibacterial effects of these compounds. In total, the water samples displayed 390 ASVs, of
 189 which 145 also occurred in gut samples.



191 **Figure 2.** Relative abundances of major bacterial orders (mean values) present in water and gut samples in each
 192 treatment.
 193

194 *Gut bacterial community composition is influenced by treatment*

195 Communities compositions from gut samples were different from those in the water
 196 (p -value <0.001) and well separated on the PCoA plots (Figure 1 and S4). Differences between
 197 fish gut and water communities have previously been reported in various studies^{18,32–34}. In
 198 contrast to water, gut bacterial communities were much more spread on the PCoA plot (Figure
 199 1), emphasizing high inter-individual variability within a given experimental condition. This
 200 variability is commonly observed among vertebrate hosts^{34–37} and is not explained by the
 201 different aquaria used in this study (factor “aquarium” on gut community composition, p -
 202 value >0.4). Bacterial community compositions were not significantly different between
 203 control and MC-LR-exposed fish guts ($p>0.7$, Figure 1). These on the other hand strongly
 204 differed from compositions in guts of fish exposed to Extract 1 and Extract 2 (p -values <0.01),
 205 which also differed between them (p -value <0.01). The same results were obtained in UU and
 206 BC analyses (figure S4).



208 **Figure 3. A.** Venn Diagram displaying the number of gut bacterial ASVs shared among treatments. **B-D:** Major
 209 gut ASVs displaying significant differences in relative abundance between two conditions (pairwise t-tests with
 210 Bonferroni correction, p -values < 0.01). The bar plots display the mean proportion of ASV in gut samples from
 211 each of the two treatments, named by their ID and their affiliated genus, when available. Among gut ASVs, 26
 212 displayed significantly different relative abundances between at least two treatments, of which only the 12 that
 213 represented at least 1% of reads in at least one gut sample are listed.
 214

215 *Identity of stable and variable taxa and possible link with treatments*

216 Overall, 507 distinct ASVs occurred in gut samples, of which 123 were shared among
 217 all treatments and 25 to 111 were unique to one treatment (Figure 3A). The 20 most abundant
 218 ASVs represented between 53.7 and 97.8% of total reads in a given individual. In each
 219 bacterial order, a single or two ASVs represented most of the reads. Significant variations in
 220 taxa relative abundances were observed among treatments (summarized at the Class, Order,
 221 Family and Genus levels in figure S5, and for ASVs in figure 3). Despite variation among
 222 treatments, order Fusobacteriales (Fusobacteria), dominated by a single ASV affiliated to
 223 *Cetobacterium somerae* (ASV253), was abundant in fish gut microbiota in all conditions
 224 (mean reads per treatment between 12.9% and 26.2%, Figure 2). *C. somerae* notably produces
 225 B12 vitamins beneficial to various hosts and plays an essential role in healthy host physiology
 226 in several species^{32,38-40}. Vibrionales (Gammaproteobacteria) represented by two main ASVs,

227 namely ASV220 and ASV378, was the second most abundant bacterial order in three
228 treatments (mean reads per treatment between 7.9% and 46.3%), but was almost absent in
229 guts from fishes exposed to Extract 2 (mean=0.17%, Figure 2, Table S3, Figure S5). This
230 order includes commensal and animal pathogenic genera which are common in fish guts^{36,39,40}.
231 Both taxa, *C. somerae* and Vibrionales, have already been reported in congeneric *Oryzias*
232 *melastigma* (marine Medaka), and in a small 16S rRNA clone library obtained from *O. latipes*
233 gut^{39,40}.

234 Various other taxa were related to common fish pathogens, for example *Aeromonas*
235 *salmonicida* (ASV237, mean 2.9 % of reads over all treatments³⁴). Among these, two ASVs
236 within Class Actinobacteria were significantly more abundant in guts exposed to *Microcystis*
237 *aeruginosa* Extract 1 compared to Extract 2, Control and MC-LR treatments (Figure 3B-D,
238 Figure S5). The first ASV was related to genus *Nocardia*, namely ASV002 (mean 17.2% of
239 reads in Extract 1 samples, occurring in 7 of the 8 specimens), and the second to genus
240 *Mycobacterium* (ASV020, 3.6% of reads in Extract 1 samples, present in 7 out of 8
241 specimens). *Nocardia* and *Mycobacterium* genera, both belonging to the family
242 Mycobacteriaceae, are known as pathogens of animals, including fish⁴¹. Interestingly,
243 Mycobacteria are frequently reported to be more abundant in guts of animals suffering
244 inflammatory bowel diseases even though their role in these pathologies remains unclear^{42,43}.
245 Nevertheless, dysbioses (*i.e.* lasting changes of the microbiota), as those occurring in
246 inflammatory bowel diseases, are known to favor the increase of bacterial pathogens
247 abundances by disrupting the protective barrier provided by the healthy gut microbiota^{44, 45}.
248 Therefore, the change in bacterial community and the increase of Mycobacteria abundances
249 may reflect a dysbiosis induced by chronic exposure to Extract 1 (Figure S5).

250 In Extract 2 treatment, two other bacterial orders displayed significantly higher
251 abundances compared to other treatments, namely Saprospirales (ASV007, 11.6% of reads in
252 Extract 2 samples) and Sphingomonadales (ASV013, 5.5% of reads, Figures 2, 3B and 3C),
253 and ASVs were present in all specimens. These orders are not reported to include animal
254 pathogens, but some are able to degrade a wide variety of metabolites including complex
255 carbohydrates, proteins and aromatic compounds⁴⁶. For instance, plant-based diets promote
256 Saprospirales population in fish guts⁴⁷. Growth of Sphingomonadales members has been
257 shown to correlate with that of cyanobacteria including *Microcystis*, and the former were
258 suspected to benefit from organic matter produced by cyanobacteria^{48,49}. Furthermore, several
259 members of Sphingomonadales can use microcystins as nutrient sources⁴⁸. Overall, observed
260 increase of Saprospirales and Sphingomonadales abundances thus suggests that Extract 2

261 exposure selected microbiota members able to metabolize cyanobacterial secondary
262 metabolites. By modulating gut microbiota compositions, relatively low cyanobacterial
263 metabolites abundances could thus have serious effects on host feed efficiency.

264
265 Overall, results from this study first show that MC-LR alone had no noticeable effect
266 on the composition of gut-associated bacterial communities as recently shown on pooled
267 individuals of zebrafish⁵⁰, at the levels and duration applied here, while full extracts of
268 *Microcystis aeruginosa* both had a significant effect. Full extracts contained various
269 secondary metabolites besides MCs, among which some probably directly affect animal-
270 associated microbial communities, either alone or together. For instance, aerucyclamides,
271 some of which were identified here (Figure S1), are cyclic peptides that may present
272 antimicrobial and cytotoxic activities. Cyanobacterial secondary metabolites may also include
273 compounds interfering with bacterial quorum sensing, or exhibiting allelopathic or
274 antibacterial properties⁵¹. These compounds may lead to dysbioses and their consequences,
275 such as the increase in abundances of potential pathogens observed in Extract 1, or the
276 increase in bacterial taxa that may use cyanobacterial metabolites as hypothesized in Extract
277 2. Possible functional consequences for fish health need to be further explored, but these
278 changes in microbiota composition could alter feed efficiency, pathogen susceptibility or
279 protective functions of the gut. Experiments from this study mimic what may happen in a lake
280 after the end of a bloom, when compounds are released in the water following cyanobacteria
281 senescence. Given the reported consequences of blooms for fish health in the wild and in
282 aquaculture settings where they may greatly affect yields, this topic appears as a promising
283 line of research in animal-bacteria interactions and a microcosm-based approach using
284 extracts is an appropriate first step to tackle the issue. The next step will be to investigate the
285 effect of direct exposure to cyanobacterial blooms, ideally in mesocosm setups, to test
286 whether the actual presence of bacteria modifies these effects.

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291 **Supporting Information Available**

294 Supporting information contains additional data and analyses on the composition of
295 cyanobacterial extracts, alpha diversity (observed ASVs and Shannon indices), beta diversity
296 (PcoA plots and tests based on UU and BC metrics), and community composition at the Class,
297 Order, Family and Genus levels. Three supporting tables are provided that summarize water
298 parameters, samples ID and characteristics, and relative abundances, taxonomic affiliation and
299 sequences of all ASVs. This information is available free of charge on the ACS Publications
300 website.

301

302 **Authors contribution**

303 SD and BM conceived the study

304 MH, CD, BM and SD conducted the exposure experiments

305 SD, SH, AG, BM and MH performed molecular analyses, and analyzed the data

306 HH performed the analyses on liver.

307 SD, SH and AG drafted the manuscript, all authors contributed and agreed on the contents

308

309 ***Ethics statement***

310 Experiments on fishes were conducted according to good practice, validated by comité Cuvier
311 (Author. APAFiS#19316-2019032913284201) and under the supervision of accredited
312 personnel (CD, BM).

313

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316

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