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1 **Specificity of the metabolic signatures of fish from cyanobacteria rich lakes**

2

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14

15

16

17 **Abstract**

18 With the increasing impact of the global warming, occurrences of cyanobacterial blooms in
19 aquatic ecosystems are becoming a main worldwide ecological concern. Due to their capacity
20 to produce potential toxic metabolites, interactions between the cyanobacteria, their
21 cyanotoxins and the surrounding freshwater organisms have been investigated during the last
22 past years. Non-targeted metabolomic analyses have the powerful capacity to study
23 simultaneously a high number of metabolites and thus to investigate in depth the molecular
24 signatures between various organisms encountering different environmental scenario, and
25 potentially facing cyanobacterial blooms.

26 In this way, the liver metabolomes of two fish species (*Perca fluviatilis* and *Lepomis gibbosus*)
27 colonizing various peri-urban lakes of the Île-de-France region displaying high biomass of
28 cyanobacteria, or not, were investigated. The fish metabolome hydrophilic fraction was
29 analyzed by ¹H-NMR analysis coupled with Batman peak treatment for the quantification and
30 the annotation attempt of the metabolites. The results suggest that similar metabolome profiles
31 occur in both fish species, for individuals collected from cyanobacterial blooming lakes
32 compared to organism from non-cyanobacterial dominant environments. Overall, such
33 environmental metabolomic pilot study provides new research perspectives in ecology and
34 ecotoxicology fields, and may notably provide new information concerning the
35 cyanobacteria/fish ecotoxicological interactions.

36

37 **Keywords:** fish; cyanobacteria; metabolomics; ecotoxicology; NMR

38

39 **1. Introduction**

40 Direct and indirect anthropogenic disturbances affect the global functioning and stability of
41 freshwater ecosystems. Global warming, physical alteration, habitat loss, water withdrawal,
42 pollutions, overexploitation and the introduction of non-native species constitute the main
43 threats affecting freshwater ecosystems and their biocenoses (Dudgeon et al., 2006; Bunn 2016;
44 Revenga et al., 2005). Over the past century, transformation of natural landscapes for industrial
45 and urban needs have led to a general increase of chemical nutrients concentrations in
46 freshwater ecosystems, and notably of phosphorus and nitrogen species that have caused the
47 massive eutrophication of freshwater bodies (Schwarzenbach et al., 2010). One of the main
48 consequences of this non-natural eutrophication process is a shift in the composition of the
49 primary producers due to the appearance of noxious autotrophic bacteria, such as cyanobacteria
50 (Chorus and Bartram 1999; O'Neil et al., 2012). In worldwide freshwater ecosystems,
51 cyanobacteria are mainly present during summers, forming large surface scums generally
52 accompanied by the presence of toxic compounds that have been pointed out to affect all trophic
53 food web compartments (Codd et al., 2005; Ferrao-Filho et al., 2011; Sotton et al., 2015).
54 *Microcystis*, *Aphanizomenon*, *Anabaena*, *Cylindrospermopsis* and *Planktothrix* are the main
55 bloom-forming freshwater cyanobacterial genera found during summers in lentic waterbodies,
56 and all have been often reported to produce potent toxic compounds, so-called cyanotoxins
57 (Codd et al., 2017). Among them, the microcystins (MCs), a family of hepatotoxins consisting
58 of more than 230 variants, are the most studied due to their high biological activity and their
59 wide occurrence during freshwater cyanobacterial blooms (Catherine et al., 2017). For exposed
60 macro-organisms, MCs may induce inhibition of the protein phosphatases 1 (PP-1) and 2A (PP-
61 2A) as well as occurrence of a cellular oxidative stress *via* the formation of reactive oxygen
62 species (ROS), with different physiological consequences depending on the organism and the
63 species studied (Amado et al., 2010; Malbrouck and Kestemont 2006). The effects of freshwater
64 cyanobacteria and their respective cyanotoxins, notably the MCs, have been widely studied on
65 fish maintained in microcosm or mesocosm conditions, and fish have been proposed as valuable
66 indicators of environmental disturbances associated to cyanobacteria proliferations (Malbrouck
67 and Kestemont 2006; Bols et al., 2001; Le Manach et al., 2018). However, the actual knowledge
68 on the genuine cyanobacteria bloom impacts for natural populations of fishes is mainly deduced
69 from short-time experimentation generally performed in micro- or mesocosms with high
70 concentrations of purified toxins. Furthermore, most of these studies were focused on the
71 mechanisms involved in the dynamic of the MC accumulation-detoxification. It appears that

72 there is still a lack in our understanding of the real ecotoxicological effects of cyanobacterial
73 biomass, particularly on the natural ichthyofauna population. Indeed, cyanobacterial blooms are
74 producing at the same time a “cocktail” of potentially bioactive compounds, and are also
75 potentially modifying other important ecological parameters of the water bodies.

76

77 The metabolome of an organism is constituted by the set of primary metabolites synthesized at
78 a given time. It represents its metabolic chemical picture, which can potentially be altered when
79 the ecological and environmental stress conditions change (Fiehn 2002; Bundy et al., 2008).
80 The observed metabolome regulations represent the chemical phenotypic response of an
81 organism that could potentially respond to counter-act the negative effects of stressors present
82 in the ecosystems, and thus to adapt to the variable conditions (Hamilton et al., 2016). In this
83 way, metabolomic studies have become a relevant approach to describe and analyze the
84 integrated response of the organisms under specific environmental context and scenario
85 (Franzosa et al., 2015; McLean 2013; Hultman et al., 2015). Then, the changes in the primary
86 metabolite concentrations help to provide valuable and useful information concerning the
87 physiological processes involved in the homeostatic or dys-regulated responses of the
88 organisms encountering environmental stresses from potentially multiple origins. Nuclear
89 magnetic resonance (NMR)-based metabolomics has been proved to be a powerful approach to
90 address hypotheses relating to fish physiology or pollutant induced toxicity or diseases
91 (Samuelsson and Larsson 2008; Viant 2008). However, despite its high potential for the
92 understanding of the molecular mechanisms implicated in the ecotoxicological responses of
93 organisms, the investigation of new qualitative and quantitative biomarkers characterizing the
94 interactions between populations and their biotopes in various ecological context remains still
95 rare and difficult to achieve (Sardans et al., 2013; Cappello et al., 2016; 2017; Qiao et al., 2016).

96

97 Thus, as toxic cyanobacterial blooms may represent important ecotoxicological and ecological
98 constraints in freshwater environments, it can be supposed that organisms exposed to
99 cyanobacterial blooms exhibit characteristic metabolome signatures, compared to others non-
100 exposed to cyanobacterial dominant conditions. Their metabolism may respond and/or counter-
101 act the potential negative effects of the cyanobacteria and thus adapt to the local environmental
102 pressures. To date, no studies have been carried out using a global metabolomic approach in
103 order to assess the specific metabolic changes that could be observed in fish exposed to
104 cyanobacterial blooms in contrasted aquatic natural ecosystems. In this way, during the summer
105 2015, young fish of two representative and common species of freshwater lakes from the

106 European temperate regions, the perch (*Perca fluviatilis*) and the pumpkinseed sunfish
107 (*Lepomis gibbosus*), have been sampled in eight peri-urban lakes of the Île-de-France region
108 contrasted by their phytoplanktonic community composition (“presence” or “absence” of
109 cyanobacterial blooms). ¹H-NMR metabolomic analyses were performed on the fish liver in
110 order to investigate the global metabolome local specificities of the two fish species collected
111 from a gradient of distinct ecological contexts (comprising the cyanobacteria dominance) and
112 to further identify the metabolic signatures related to these potential specific phenotypic
113 responses.

114

115 **2. Experimental section**

116 *2.1. Lakes and physico-chemical parameters measurements*

117 *In-situ* sampling campaigns were performed during summer 2015 in eight lakes of the Île-de-
118 France region (France), chosen for their different dominant phytoplankton communities already
119 described in previous studies and notably the presence or the absence of recurrent
120 cyanobacterial blooms (Catherine et al., 2008; Maloufi et al., 2016). Cergy “les Galets” lake
121 (C), Champs-sur-Marne “la Sablière” lake (CM), Maurepas “la Courance” lake (M), Rueil
122 “Saint Cucufa” lake (R), Verneuil “la grosse Pierre” lake (V), Varenne-sur-Seine “Le grand
123 Marais” lake (VS), Fontenay-sur-loing “le grand Fontenay” lake (F) and Triel lake (T)
124 (Supplementary figure S1) were thus studied and sampled with electric fishing device for
125 capturing fish alive. Previous investigation of the fish guild populating every one of these lakes
126 (using both electric device and fish net sampling) had indicated that only the perch (*Perca*
127 *fluviatilis*) and pumpkinseed sunfish (*Lepomis gibbosus*) were presents in all or almost all the
128 lakes and were further selected as bioindicative species for further metabolomic analyses.

129 In all lakes, dissolved oxygen (O₂) concentrations, pH, temperatures and conductivity were
130 measured in the water column using a multiparameter probe (YSI EXO2) (Supplementary table
131 S1).

132

133 *2.2. Phytoplankton sampling*

134 In every lake, sub-surface chlorophyll-*a* equivalent concentrations attributed to the four-main
135 phytoplankton groups (Chlorophyta, Diatoms, Cyanobacteria, Cryptophyta) were measured
136 with an *in-situ* fluorometer (Fluoroprobe II, Bbe-Moldenke, Germany) and samples of water
137 were filtered through 1.2 µm GF/C filters (Nucleopore, Whatman) and stored at -80°C until
138 total chlorophyll-*a* concentrations analyses using the ethanolic extraction as described by

139 Yepremian and co-workers (2017). Samples of water were fixed in Lugol iodine solution and
140 kept at 4°C until the identification. The estimation (%) of the abundance of the different
141 cyanobacterial genera was performed using an Utermohl's counting chamber and an inverted
142 microscope as described by Catherine et al. (2016). In parallel, phytoplankton biomass was
143 collected using an Apstein's type phytoplankton net (20- μ m mesh size) and kept at -80°C until
144 metabolite characterization by mass spectrometry (MS) and MCs content analyses.

145

146 *2.3. Metabolite characterization of phytoplanktonic biomass by mass spectrometry*

147 The collected biomass from the sampled lakes were freeze-dried and then sonicated in 75%
148 methanol, centrifuged at 4°C (15,000 g, 10 min). This step was performed twice, and the pooled
149 supernatants were collected with a Pasteur pipet and kept at -80°C prior to analysis. The
150 supernatant was transferred and acidified with formic acid and 5 μ L were analyzed in triplicats
151 on an HPLC (Ultimate 3000, ThermoFisher Scientific) coupled to an electrospray ionization
152 and quadrupole time-of-flight hybrid mass spectrometer (ESI-QqTOF, QStar® Pulsar i,
153 Applied Biosystems®, France).

154 High-performance liquid chromatography (HPLC) of 5 μ L of each of the metabolite extracts
155 was performed on a C₁₈ column (Discovery® Bio wide pore, 15cm x 1mm, 5 μ m, Sigma) at a
156 50 μ L.min⁻¹ flow rate with a gradient of acetonitrile in 0.1% formic acid (10 to 80% for 60 min).
157 The metabolite contents were then analyzed on positive mode using information dependent
158 acquisition (IDA), which allows switching between mass spectrometry (MS) and tandem mass
159 spectrometry (MS/MS) experiments, as previously described (Marie et al., 2012). The data was
160 acquired and analyzed with the Analyst QS software (Version 1.1). Peak lists were generated
161 from MS spectra acquired between 10 and 55 min, filtering noise threshold at 2% maximal
162 intensity and combining various charge states and related isotopic pattern. Metabolite
163 annotation was attempted according to the accurate mass of the molecules, then to their
164 respective MS/MS fragmentation pattern with regard to an in-house database of more than 700
165 cyanobacterial metabolites.

166 A molecular network was created using the online workflow at GNPS (Wang et al., 2016). The
167 data was filtered by removing all MS/MS peaks within +/- 17 Da of the precursor m/z. The data
168 was then clustered with MS-Cluster with a parent mass tolerance of 1.0 Da and a MS/MS
169 fragment ion tolerance of 0.5 Da to create consensus spectra. Further, consensus spectra that
170 contained less than 2 spectra were discarded. A network was then created where edges were
171 filtered to have a cosine score above 0.6 and more than 5 matched peaks. Further edges between
172 two nodes were kept in the network if and only if each of the nodes appeared in each other's

173 respective top 10 most similar nodes. The spectra in the network were then searched against
174 GNPS' spectral libraries. The library spectra were filtered in the same manner as the input data.
175 All matches kept between network spectra and library spectra were required to have a score
176 above 0.6 and at least 5 matched peaks. All GNPS results are available at
177 <http://gnps.ucsd.edu/ProteoSAFe/status.jsp?task=a158701fa7894ab08c0df4c60994ccf1>

178

179 *2.4. Cyanotoxin quantification*

180 The 75% methanol extracts of the phytoplankton biomass were further analyzed by ELISA
181 (enzyme-linked immunosorbent assay), with the microcystins (Adda-specific) Kit (AD4G2
182 antibody, Abraxis), in order to globally measure the total MC content, and integrating the signal
183 obtained for all MC variants in presence. Prior to analysis, samples were dissolved with the
184 ELISA sample diluent to reach a methanol concentration below 5% to avoid any interactive
185 effect and to stay in the detection range of the kit (0.1-5 $\mu\text{g}\cdot\text{L}^{-1}$) for all samples. The results
186 were expressed in microcystin-LR equivalents ($\mu\text{g eq. MC-LR}\cdot\text{mg}^{-1}$ dry weight).

187 The free and bound β -Methylamino-L-alanine (BMAA) were also measured in the
188 phytoplankton biomass according to the hydrophobic interaction liquid chromatography
189 (HILIC)/MS-MS based methods described previously (Combes et al., 2014; Faassen et al.,
190 2016). This method used solid-phase extraction based on mixed mode sorbent to concentrate
191 and clean up the phytoplankton extract that contained free BMAA. After the acidic lysis of the
192 phytoplankton biomass, the bound fraction of BMAA was analyzed by LC/MS-MS. This
193 quantitative method has proved to be specific and reliable in a range of concentration level from
194 0.25 to 1.6 $\text{ng}\cdot\text{mg}^{-1}$ dry weight.

195

196 *2.5. Fish sampling and tissue extraction procedure for metabolomic analysis*

197 Immature individuals of perch (*Perca fluviatilis*) and pumpkinseed sunfish (*Lepomis gibbosus*),
198 two representative fish species of European freshwater lakes (that were selected according to
199 their presence in almost, if not, all investigated lakes), were targeted by electric fishing (FEG
200 8000, EFKO, Leutkirch, Germany) performed in the riparian area of every lake. Alive caught
201 fish ($n < 6-10$ per lake and per species) were directly measured, weighed (Table S2), briefly
202 euthanized by neck dislocation and then liver of each individual was shortly sampled, deep-
203 frozen in liquid nitrogen and kept at -80°C until metabolomics analyses, in accordance with
204 animal ethical concerns and regulations.

205 Individual liver metabolome extraction was carried out using the methanol/chloroform/water
206 (ratio 2/2/1.8) method, on the basis of existing literature (Lin et al., 2007; Wu et al., 2008).

207 Briefly, fresh frozen livers were weighed, homogenized in ice-cold methanol (8 mL per gram
208 of tissue, AnalaR Normapur, min. 99.8 %, VWR, Pennsylvania, USA) and ice cold milliQ water
209 (2.5 mL.g⁻¹), and then vortexed for 1 min. Subsequently, ice cold chloroform (4 mL.g⁻¹,
210 Normapur, 99.3 %, VWR, Pennsylvania, USA) and milliQ water (4 mL.g⁻¹) were added to
211 extract the hydrophobic metabolites. Then, the mixture was vortexed for 1 min and incubated
212 on ice for 10 min to obtain a complete solvent partition. The resulting supernatant was then
213 centrifuged at 4°C for 10 min at 2,000 g, resulting in a biphasic solution. The upper polar and
214 lower non-polar layers were carefully removed. The upper polar fraction was then transferred
215 to 2-mL Eppendorf tubes, dried under Speed-vac device (Speed-vac Plus SC110A, Savant) and
216 then kept at -80°C until NMR analysis. Prior to ¹H-NMR measurement, the polar tissue extracts
217 were dissolved in 550 µL of 0.1 M sodium phosphate buffer (pH 7.0) prepared in D₂O (10%
218 v/v) containing 0.25 mM sodium-3-tri-methylsilylpropionate (TMSP) as an internal standard.
219 Finally, the resulting samples were transferred to 5-mm NMR tubes (Norell, France) and
220 immediately analyzed by ¹H-NMR.

221

222 *2.6. ¹H-NMR spectroscopy and metabolite quantification.*

223 All NMR data were recorded at 298 K on a 600 MHz Bruker AVANCE III HD spectrometer
224 equipped with a 5-mm TCI CryoProbe (¹H-¹³C-¹⁵N) with Z-gradient. One-dimensional ¹H-
225 NMR spectra were acquired using a standard Bruker noesygppr1d pulse sequence to suppress
226 water resonance. Each spectrum consisted of 256 scans of 32,768 data points with a spectral
227 width of 7.2 kHz, a relaxation delay of 2.5 s and an acquisition time of 2.3 s.

228 The relative metabolite quantification was performed using the BATMAN (an acronym for
229 Bayesian AuTomatic Metabolite Analyser for NMR spectra) R-package (Hoa et al., 2014),
230 which deconvolutes peaks from 1-dimensional ¹H-NMR spectra to automatically assign
231 chemical shifts to specific metabolites from a target list and then estimate their respective
232 concentrations. This can be achieved thanks to an implementation of a Bayesian-based
233 modelling procedure. BATMAN uses, in a two-component joint model, resonances of every
234 assigned proton from a list of catalogued metabolites (this library is provided with the
235 BATMAN R-package, and includes the chemical shift information of various targeted
236 metabolites measured at pH 7.0) and noisy information to finally reconstruct the empirical
237 NMR spectrum. But, in absence of confirmatory analytical methods, they were further
238 mentioned as candidate metabolic biomarkers sharing the same ¹H-NMR parameters with the
239 catalogued metabolites. Therefore, 222 potential metabolites were quantified from Bruker
240 spectra files using the following parameters: i) the chemical shift regions belonging to the two

241 following regions: 0.5 to 4.60 ppm and 5.40 to 10.0 ppm, ii) 400 burn-in iterations, iii) 200
242 post-burn-in iterations and iv) 5000 iterations for batman rerun. Calculations were performed
243 on a HP Z820 workstation using two 3.30 GHz Intel Xeon® CPU E5 processors and 64 Go
244 RAM by activating 12-parallel threads processing.

245

246 2.7. Statistical exploration of data

247 The mixOmics library (Rohart et al., 2017) was used to carry out the multivariate analyses
248 (Tenenhaus et al., 2014). Regularized canonical correlation analysis (rCCA) is a multivariate
249 statistical method used to assess correlations between two multivariate datasets acquired on the
250 same individuals. Here, it was used as a factorial discriminant analysis that modeled the
251 relationships between the species (*Perca* and *Lepomis*), the quantitative chemical data measured
252 in the different lakes (total Chl-*a* concentrations, Chl-*a* estimated concentrations related to
253 cyanobacterial biomass (BBE_{cya}), MCs concentrations, O₂ concentrations, pH and
254 conductivity), together with the lake of sampling, constituting the Y matrix, and the semi-
255 quantitative levels of metabolites determined by the BATMAN algorithm with no
256 normalization of the data (X matrix) (supplementary data 1). The *rcc()* function was used to
257 define the canonical correlations and the canonical variates, the *network()* function was used to
258 produce the network of interactions, using default parameters. Cross-validation of MANOVA
259 results was obtained thanks to a bootstrap-based procedure with corrected *p*-value ($P < 0.05$) by
260 applying a random assignment of any statistical individual to a given group of treatments
261 (Krishnamoorthy and Lu, 2010). In order to evaluate the effects of the experimental factors
262 (species, chemical data and their interaction) on the relative concentrations of metabolites
263 highlighted by multivariate analyses and the subsequent relevant networks, simple two-way
264 ANOVAs followed by a Student-Newman-Keuls post-hoc test were performed.

265

266 3. Results

267 3.1. Phytoplankton, microcystins and chemical conditions of the studied lakes

268 During 2015 summer sampling campaign (Supplementary fig. S1), distinct phytoplanktonic
269 compositions and concentrations were observed in the eight lakes targeted (Fig. 1). Among
270 them, F, T, V and VS, exhibited remarkable high and/or dominance of cyanobacterial-specific
271 chlorophyll-*a* concentrations reaching 106, 14.8, 135 and 48.3 $\mu\text{g eq. Chl } a \text{ L}^{-1}$, respectively
272 (Fig. 1A). In the other lakes, chlorophyll-*a* concentrations of the total phytoplanktonic
273 community ranged between 7.2 and 241.5 $\mu\text{g. Chl } a \text{ L}^{-1}$ (supplementary table S1) but were

274 mainly dominated by chlorophytes, diatoms and/or cryptophytes (Fig. 1A), major
275 phytoplanktonic phyla, that are so far not known to produce any toxins nor other ecological
276 concerns as cyanobacteria does. In these lakes, only low concentrations of cyanobacterial-
277 specific chlorophyll-*a*, ranging from 2.9 to 10.9 $\mu\text{g eq. Chl } a \text{ L}^{-1}$, were measured (Fig. 1A).
278 Furthermore, in the cyanobacteria-dominated lakes, different cyanobacterial genera were
279 observed during samplings (Fig. 1B). F and T were dominated by *Planktothrix* (94% of the
280 cyanobacteria present in the sample) and *Pseudanabaena* (71%), respectively, whereas V and
281 VS, were both dominated by *Microcystis* (Fig 1B). Furthermore, we notice that the modeste
282 blue green signal observed in C corresponds to 100% *Spirulina* sp., a cyanobacteria genus that
283 is so far known not to produce any known toxic compounds, as it was further confirmed.
284 Microcystin concentration was investigated in order to estimate the amount of the
285 cyanobacterial bioactive compounds present in the various lake water (Fig. 2; supplementary
286 table S2). MC-LR equivalent concentrations were measured at concentrations ranging from
287 above the quantification limit ($> 1 \mu\text{g.g}^{-1} \text{ DW}$) to 3367 $\mu\text{g.g}^{-1} \text{ DW eq. MC-LR}$. The highest MCs
288 concentrations were found in lakes dominated by cyanobacteria and particularly in F, V and VS
289 where 2067 $\mu\text{g.g}^{-1}$, 166 $\mu\text{g.g}^{-1}$ and 3367 $\mu\text{g.g}^{-1} \text{ MC-LR eq.}$ were measured, respectively.
290 Interestingly, V that exhibited the highest cyanobacterial concentrations (dominated by
291 *Microcystis*) measured in this study, did not exhibit the highest MCs concentrations (3367 $\mu\text{g.g}^{-1}$
292 MC-LR eq. in VS) illustrating the fact that MCs concentrations are not linearly linked to the
293 cyanobacterial biomass measured in the lakes, as it is also dependent on the production of
294 metabolite by the dominant cyanobacterial clones, which may or not be producing MCs. On
295 one side, none of these samples presented detectable amount of the potential neurotoxin BMAA
296 (neither in free or bound form), saxitoxins nor anatoxins. On the other side, various classes of
297 cyanobacterial secondary metabolites comprising different variants of hepatotoxic
298 microcystins, together with various other potentially bioactive metabolite families, such as
299 anabaenopeptins, aeruginosins, microginins and cyanopeptolins, were detected in the
300 phytoplanktonic biomass of collected from the lakes where cyanotoxin-producing
301 cyanobacteria reached higher concentrations (Fig. 2; supplementary figure S2-S3).

302

303 3.2. Metabolomic analysis of sampled fish

304 $^1\text{H-NMR}$ raw files (supplementary fig. S4) were preprocessed thanks to the R BATMAN library
305 (Hao et al., 2014) to get a relative quantification of a preselected set of metabolites replacing
306 an analytical assignment based on different and complementary analyses. This approach allows
307 for each individual spectrum to determine a metabolite quantification, even for very low

308 concentrated metabolites when compared to the main ones, as the differences in their relative
309 concentrations could have been validated by both uni- and multivariate statistical tests (Hao et
310 al., 2014). A multivariate analysis was performed by using a rCCA analysis between the matrix
311 of the relative concentrations of the 222 metabolites (X) and the dummy matrix (Y)
312 corresponding to the following variables (supplementary data S1): *i.e.* fish species,
313 cyanobacterial-specific Chl-*a* and MCs concentrations, O₂, pH, conductivity and the total
314 Chlorophyll-*a* concentrations and their interaction using the mixOmics package in R and a
315 MANOVA bootstrap procedure applied to the dataset in order to highlight the significant effects
316 of the environmental variables on the observed metabotypes (*i.e.* the specific metabolic profiles
317 according to the environmental factors).

318 MANOVA bootstraps reveal significant effects of species ($p = 0$), cyanobacterial-specific chl-
319 *a* (BBE_{cy}) concentrations ($p < 10^{-43}$), MC concentrations ($p < 10^{-11}$), pH ($p < 10^{-28}$), O₂ ($p <$
320 10^{-28}), conductivity ($p < 10^{-47}$), total chlorophyll-*a* concentrations ($p < 0.011$) from the rCCA
321 model. However, a clear species effect is observable through the dimension 1 ($p < 10^{-16}$) with
322 *Perca* and *Lepomis* clearly separated by the first dimension whatever the lake considered (fig.
323 2A). On the dimension 2, a clear effect correlated with cyanobacterial biomass is observable (F
324 $= 1931$, $p < 10^{-16}$), as fish coming from cyanobacterial dominated lakes (in green) are clearly
325 separated by this dimension whatever the fish species considered (Fig. 3A). In addition, pH, O₂
326 concentrations and conductivity variables ($F = 599$ and $p < 10^{-16}$, $F = 867$ and $p < 10^{-16}$, and F
327 $= 419$ and $p < 10^{-16}$, respectively) are also significantly correlated to the different metabotypes
328 observed on this dimension but to a much lesser extent compared to cyanobacterial biomass
329 contents (Fig. 3A). In addition, no significant interactive effects between species and the
330 environmental factors could have been observed in this analysis suggesting that the
331 metabotypes of the two species seem to be similarly driven by the different environmental
332 factors.

333 The relevance network based on the second rCCA dimension specifically highlights metabolites
334 discriminating the fish from dominated and non-dominated cyanobacteria lakes and linking to
335 the correlated environmental factors (mainly BBE_{cy} concentrations but also pH) (Fig. 3B).
336 Overall, it appears that all these metabolites shown by this network exhibit negative correlation
337 with both cyanobacterial concentrations and pH values. In addition, two-way ANOVA (Table
338 1) were performed on all putative metabolites highlighted in the figure 3B. A significant effect
339 of the BBE_{cy} and pH is shown for putative metabolites annotated as 16 α -hydroxyestrone,
340 acetylcholine, androsterone, glycerophosphocholine, isovalerylcarnitine, L-glutamine and L-
341 palmitoylcarnitine whereas for glycerol, homo-L-arginine and L-arginine, a significant effect

342 of the BBECya concentrations, only, have been confirmed by two-way ANOVA analyses (Table
343 1). Furthermore, no interaction effect between the species and the BBECya concentrations or
344 pH has been observed for any metabolites suggesting the occurrence, for both fish species and
345 for these two discriminating environmental factors, the occurrence of a similar metabolic
346 responses. Negative correlations between relative concentrations of certain metabolites and the
347 BBECya concentrations and the pH conditions are corresponding to lowest relative
348 concentrations of these metabolites in the liver of fish captured in cyanobacterial dominated
349 lakes (Table 1; supplementary figure S5-S6). Globally, it appears that liver of fish sampled in
350 T, F and V lakes, and in VS lake in a lesser extent, exhibits the most significant differences in
351 the relative concentrations of the highlighted metabolites compared to fish coming from other
352 investigated lakes, where cyanobacteria are notably in low or not dominant proportions.

353

354 **Discussion**

355 *4.1. Diversity of ecosystems: environmental, chemical and biological conditions*

356 Cyanobacterial blooms are frequent phenoma in peri-urban lakes from the Île-de-France region
357 due to the eutrophic and hyper-eutrophic states of these environments. Interestingly, these
358 freshwater aquatic ecosystems are characterized by a wide range of environmental conditions
359 and anthropogenic pressures that influence the phytoplankton biomass and compositions
360 present in these ecosystems (Maloufi et al., 2016; Catherine et al., 2010; 2016). During summer
361 2015 sampling, in the lakes from Rueil (R), Cergy (C), Maurepas (M) and Champs-sur-Marne
362 (CM), diatoms or chlorophyta were dominant and cyanobacteria were present with chl-*a*
363 concentrations never exceeding 5 µg eq. chl-*a* L⁻¹ (supplementary table S1). In the other lakes,
364 the *Microcystis* genera appeared largely dominant, such as in Varenne-sur-Seine (VS) and in
365 Verneuil (V) lakes with cyanobacterial-specific chl-*a* (BBECya) concentrations reaching 135
366 and 48 µg eq. chl-*a* L⁻¹, respectively. In the Fontenay (F) lake, *Planktothrix* genera was found
367 to be dominant with BBECya concentration reaching 106 µg eq. chl-*a* L⁻¹. Interestingly, in Triel
368 (T) lake, the cyanobacteria belonging to the *Pseudanabaena* and *Anabaena* genera were present
369 at relatively lower but even substantial BBECya concentrations (around 15 µg eq. chl-*a* L⁻¹).
370 However, even though cyanobacterial amount in T appears maybe not as high as in other
371 cyanobacterial-dominated lakes of this study (F, V and VS), the presence in this T lake of
372 noticeable and recurrent amounts of other contaminants, such as heavy metals, polychlorinated
373 biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) was measured in both water
374 and fish samples (Teil et al., 2014; Gaspéri et al., 2010; Azimi and Rocher 2016), and would

375 indicate why the fish present in the T lake exhibit an even more divergent metabolic profile,
376 when compared to those of the non-cyanobacteria-dominated (R, C, M and CM) and the other
377 cyanobacteria-dominated (V, VS and C) lakes.

378

379 *4.2. Which cyanobacterial-related parameters may influence the fish metabolome?*

380 Cyanobacteria are well known to produce a wide variety of potentially toxic secondary
381 metabolites including MCs, that have been detected in remarkable amount especially in the VS
382 and F lakes, together with various other cyanobacterial bioactive compounds, such as those that
383 have been highlighted by the global network analysis, including cyanopeptolins, aeruginosins,
384 microginins and anabaenopeptins. Previous experimental studies have been able to show that
385 cyanobacterial exposure could induce significant physiological impairs in fish exhibiting
386 different metabolomic dysregulation, in relation with the various secondary metabolite content
387 of the investigated strains (Sotton et al., 2017a; 2017b). Nevertheless, the genuine ecological
388 consequences of the production of this chemical bioactive (including potent toxic) metabolites
389 by cyanobacteria remain largely uncovered.

390 Indeed, in addition to the production of such potentially toxic secondary metabolites, changes
391 in physico-chemical parameters of the water-bodies have also been reported during
392 cyanobacterial bloom episodes. Massive decrease of dissolved O₂ concentrations may occur
393 during important bloom senescence events, due to the bacterial degradation of cyanobacterial
394 cell biomass, inducing sometimes spectacular massive death of various fish species, also
395 described as fish-kill phenomenon (Pearl and Paul 2011). During the present study, no
396 noticeable depletion of the dissolved O₂ concentrations of neither surface nor bottom water or
397 any fish-kill have been observed, indicating that the high cyanobacterial biomass (observed in
398 V, VS, T and F lakes) were not in senescence. Furthermore, O₂ concentrations does not appear
399 to significantly drive the liver metabolic differences observed in the fish species studied,
400 suggesting that no metabolic disturbances linked to O₂ concentration variation were observed
401 here. Among the other environmental factors measured on the studied lakes that are noticeably
402 correlated with metabolic differences in fish livers based on rCCA analyses, the pH exhibits
403 large variations between sampled aquatic ecosystems. Indeed, it appears that lakes dominated
404 by cyanobacteria show higher pH values (above 9), excepted in Fontenay (F) lake where the
405 value is more similar (between 7.1 and 8.3) to those found in lakes where cyanobacteria are in
406 less abundance (supplementary table S1). Such high pH value phenomenon has been already
407 reported to be directly related with cyanobacteria photosynthesis process that removes carbon
408 dioxide from the water and increases hydroxide ion concentration (Lopez-Archilla et al., 2004).

409 However, in the present study, it is not possible to directly conclude whether the elevated pH
410 observed in cyanobacterial dominated lakes is a consequence of the photosynthetic activity of
411 cyanobacteria or is due to local geochemical conditions of the respective lake. To better
412 characterize the causality of these elevated pH in further investigation, it should be suitable to
413 monitor pH value before, during and at the end of the bloom. Further studies need now to be
414 performed in order to disentangle the specific and/or synergic effects of the various bioactive
415 metabolite production together with those of other physicochemical parameters associated with
416 cyanobacterial blooms.

417

418 *4.3. Metabolic changes correlated to perturbed lakes: cyanobacterial concentrations and pH* 419 *as environmental drivers of fish liver metabolome?*

420 Thanks to NMR and multivariate analyses, the present results show that comparable metabolic
421 changes are observable in both fish species exposed to higher cyanobacterial biomass (F, VS,
422 T and V lakes). In these analyses the most constraining factors correlated to the metabolic
423 changes in fish were the cyanobacterial concentrations and the pH values that exhibit the
424 strongest correlations with the changes of relative concentrations of various metabolites in the
425 two fish species sampled. In this study, it is also very likely that the combination of both high
426 pH values and high cyanobacterial biomass could lead to the more contrasted metabolome
427 changes in fish. This would be in agreement with the fact that fish from F lake, presenting high
428 cyanobacterial and MCs concentrations but moderate pH value around 7.5, exhibit intermediate
429 metabolomes compared to fish captured in T, V and VS (high cyanobacterial and MCs
430 concentrations and high pH) and fish captured in other lakes (C, CM, M, R), not dominated by
431 cyanobacteria.

432 The present results indicate that MCs may not be in this case the most constraining factor for
433 fish from those natural ecosystems. However, in addition to MC cyanobacteria produce a large
434 set of bioactive compounds that could also influence the fish health and the complexity of such
435 secondary metabolite mixtures aims at being more characterized in an ecotoxicological point
436 of view. One should take also into account that fish may not necessarily be exposed by direct
437 ingestion of cyanobacterial cells, and that trophic transfer may be another source of
438 cyanobacterial bioactive (including toxic) metabolites (Sotton et al., 2014). It would be now
439 interesting to consider other fish species feeding on phytoplankton in order to test whether they
440 are indeed exposed to higher cyanotoxin contents, by direct ingestion of cyanobacterial cells,
441 and whether they exhibit similar or even more drastic metabolome alterations than such
442 carnivorous ones.

443 Interestingly, the fish collected from the T lake exhibits one of the most divergent global
444 metabolotypes, suggesting that it could potentially be influenced by the occurrence of multi-stress
445 conditions, comprising cyanobacteria proliferations together with the other heavy metal, PCB
446 and PAH contaminants, that have been observed in this specific environments (Catherine et al.,
447 2016). Indeed, T lake was not characterized by the highest concentration of cyanobacteria
448 observed during this study. However, due to the presence of other pollutants already monitored
449 in past studies in this pound and not in the other one (Teil et al., 2014; Gaspéri et al., 2010;
450 Azimi and Rocher 2016), this observation suggests that additive and/or synergistic effects of
451 multi-pollutants together with cyanobacterial bloom seem to be involved in similar metabolic
452 variations than those of fish from pounds which are the most stressed by cyanobacterial blooms.
453 But such assumption now need further investigations before been fully validated. On order to
454 fully validate potential metabolite biomarkers of cyanobacterial exposure pressure on fish liver
455 metabolome, further investigation would now aim at investigating a larger set of lake from a
456 wider geographic area, and also to firmly confirm the metabolite identification by orthogonal
457 approaches such as 2-D NMR, spiking of standard, and or to develop in parallel mass
458 spectrometry based metabolomic investigations.

459

460 **5. Conclusion**

461 The present study demonstrates that cyanobacterial blooms occurrence can locally be correlated
462 with metabolic divergences in relation to the fish response to environmental stress. Such
463 metabolomic analyses support experimentally observed processes but also give us innovative
464 perspectives for the characterization of the environmental and genuine interactions between fish
465 and cyanobacteria proliferations.

466 In this way, further studies should be performed in order to investigate longitudinal variations
467 along seasonal variations, fish life history, populational genetic distance and the specific
468 changes of fish metabolomes during bloom episodes, which could help us to better understand
469 and characterize the main environmental factors potentially disturbing fish physiology. Also,
470 the understanding of the ecosystem functioning under ecotoxicological pressure induced by
471 cyanobacterial blooms would gain at investigating other fish species feeding directly on
472 phytoplankton organisms. It should highlight whether higher metabolome and toxicological
473 changes would be driven by directly feeding on cyanobacterial cells and higher contact with
474 with the various potentially toxic secondary metabolites, or by other ecological parameters
475 impacted by cyanobacterial proliferations. Furthermore, complementary omic analyses, such as

476 proteomics and transcriptomics, would provide additional support to generate system biology
477 understanding of the global organism response to environmental stress, and help at
478 characterizing potential biomarkers useable at the fish population levels in various aquatic
479 environments.

480

481 **Conflicts of interest**

482 None

483

484 **Declaration of interest**

485 None

486

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497

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650

651 **Table**

652

Putative metabolites	BBECya		pH	
	<i>F-value</i>	<i>p-value</i>	<i>F-value</i>	<i>p-value</i>
16a-Hydroxyestrone	26.604	1.02e⁻⁰⁶	8.295	0.00472
Acetylcholine	43.903	1.08e⁻⁰⁹	5.892	0.01672
Androsterone	19.932	1.85e⁻⁰⁵	10.776	0.00135
Glycerophosphocholine	38.040	1e⁻⁰⁸	8.892	0.00348
Glycerol	29.455	3.1e⁻⁰⁷	3.908	0.0504
Homo-L-arginine	32.501	8.95e⁻⁰⁸	1.795	0.18287
Isovalerylcarnitine	32.028	1.08e⁻⁰⁷	9.392	0.0027
L-Arginine	30.269	2.22e⁻⁰⁷	2.228	0.13820
L-Glutamine	30.004	2.47e⁻⁰⁷	14.025	0.000280
L-Palmitoylcarnitine	24.965	2.05e⁻⁰⁶	12.888	0.000483

653

654 **Table 1:** Effects of significantly-related environmental factors on the putative metabolite
655 expressions revealed by two-ways ANOVA analyses performed on the environmental
656 parameter and metabolite matrix for all individuals (supplementary data 1). Bold p-values
657 correspond to p-values < 0.05.

658

659

660 **Legends of figures**

661 **Figure 1. Phytoplanktonic chlorophyll-a concentrations ($\mu\text{g.L}^{-1}$ eq. Chl-a) in the sampled**
662 **lakes (A) and the cyanobacterial genus composition observed in the cyanobacteria-**
663 **dominated lakes (B).** ND = non-detected. Cergy (C), Champs-sur-Marne (CM), Maurepas lake
664 (M), Rueil lake (R), Verneuil lake (V), Varenne-sur-Seine lake (VS), Fontenay lake (F) and
665 Triel lake (T).

666

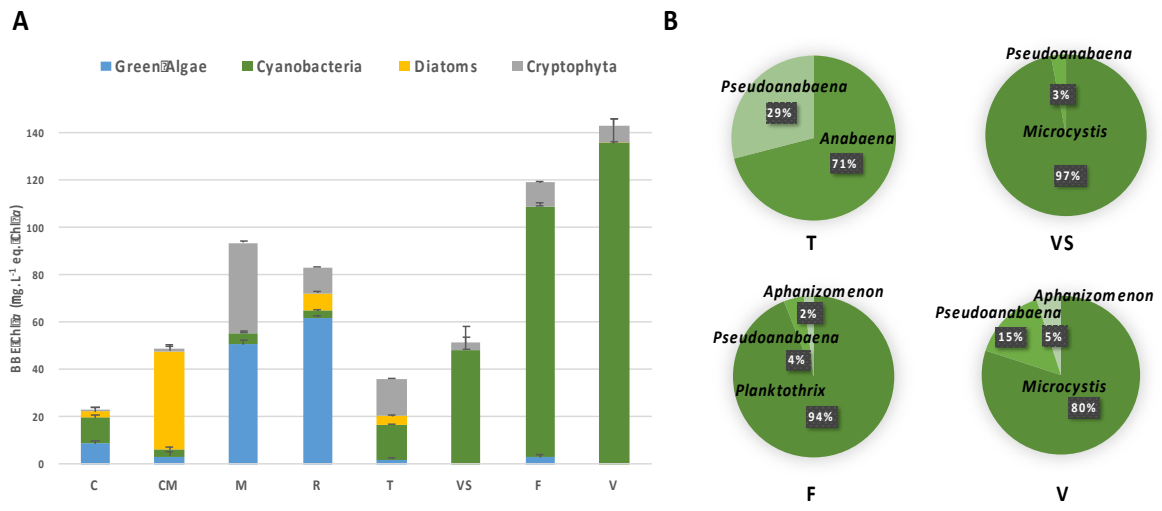
667 **Figure 2. Diversity of metabolites observed in the lakes, dominated or not by**
668 **cyanobacterial proliferations revealed by molecular networking based on MS/MS**
669 **fragmentation data.** When various phospholipids are observed in all lakes, potentially bio-
670 active metabolites known to be produced by certain cyanobacteria, such as microcystins
671 (hepatotoxin), aeruginosins, cyanopeptolins, anabaenopeptins, microginins, are only observed
672 in cyanobacterial dominated lakes.

673

674 **Figure 3. $^1\text{H-NMR}$ liver metabolomes and relevance network of fish sampled in the different**
675 **lakes.** The individual plots of regularized canonical correlations analysis (rCCA) for
676 dimensions 1–2 (A). Perch individuals are on the left side and pumpkinseed individuals on the
677 right side of the graphic. Lakes are represented by their respective letters that in blue correspond
678 to control lakes and in green to perturbed lakes. Cergy (C), Champs-sur-Marne (CM),
679 Maurepas lake (M), Rueil lake (R), Verneuil lake (V), Varenne-sur-Seine lake (VS), Fontenay
680 lake (F) and Triel lake (T). Relevance network providing from rCCA analysis on the dimension
681 2 (B). Putative metabolites above a correlation threshold of $|0.3|$ were kept, revealing that the 2
682 most correlated variables are pH and BBECya (cyanobacterial specific content of chlorophyll a
683 measured by BBE fluorescence probe). Green edges correspond to negative correlations with
684 the discriminant ecological factors.

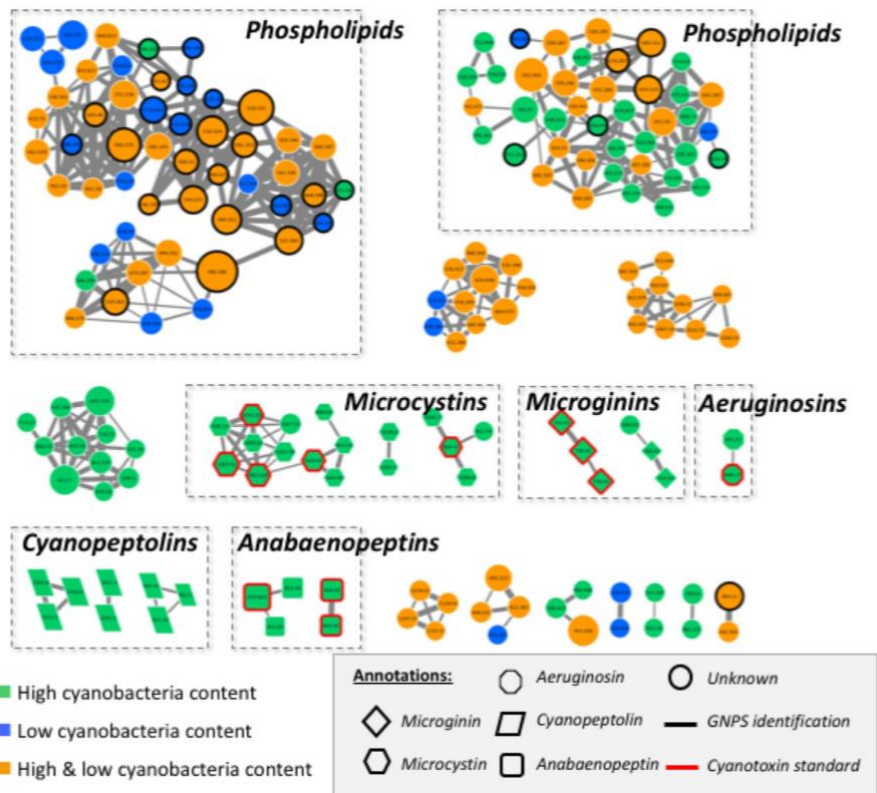
685

686 **Fig. 1.**



687

688 Fig. 2.

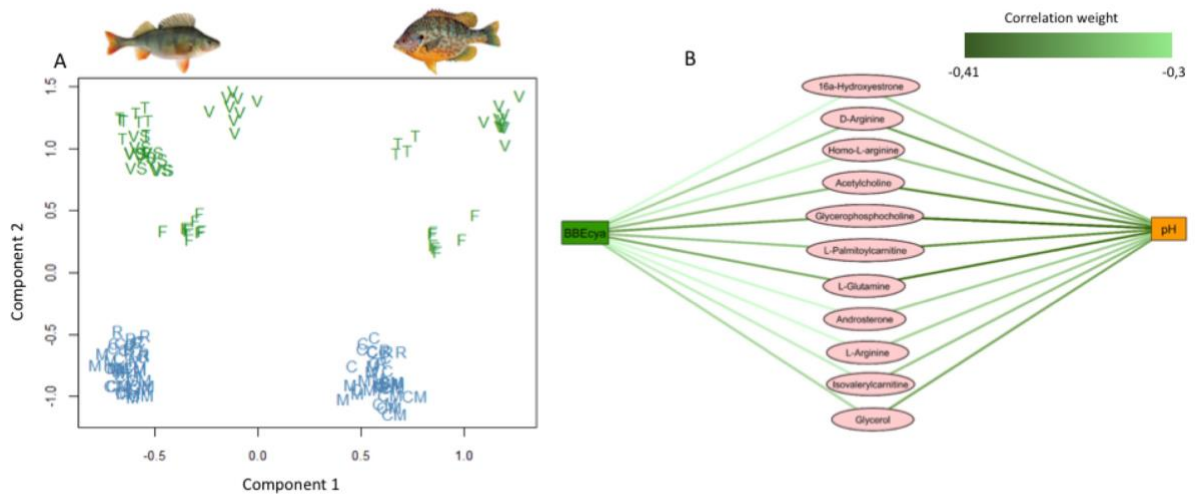


689

690

691

692 **Fig. 3.**



693