Fish metabolome from sub-urban lakes of the Paris area (France) and potential influence of noxious metabolites produced by cyanobacteria

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Abstract

The recent democratization of high-throughput molecular phenotyping allows the rapid expansion of promising untargeted multi-dimensional approaches (*e.g.* epigenomics, transcriptomics, proteomics, metabolomics, …). Indeed, these emerging omics tools, processed for ecologically relevant species, may present innovative perspectives for environmental assessments, that could provide early warning of eco(toxico)logical impairs**.**

In a previous pilot study (Sotton et al., Chemosphere 2019), we explore by ^{1}H NMR the bio-indicative potential of metabolomics analyses on the liver of 2 sentinel fish species (*Perca fluviatilis* and *Lepomis gibbosus*) collected in 8 water bodies of the peri-urban Paris' area (France). In the present study, we further investigate on the same samples the great potential of high-throughput UHPLC-HRMS/MS analyses. We show that the LC-MS metabolome remarkably allows clear separation of individuals according to the species, but also according to their respective sampling lakes. Interestingly, similar variations of *Perca* and *Lepomis* metabolomes occur locally indicating that site-specific environmental constraints drive the metabolome variations beyond the obvious genetic differences between the two species, and seem to be influenced by the production of noxious metabolites by cyanobacterial blooms in certain lakes. Thus, the development of such reliable environmental metabolomics approaches is constituting an innovative bio-indicative tool for ecological stress assessment, such as toxigenic cyanobacterial blooms, and aim at being further follow up.

Keywords: Untargeted metabolomics, environmental assessment, cyanobacteria, biomarkers, sentinel species

1. INTRODUCTION

In the past decades, the use of bio-indicator aquatic organisms has been widely adopted to monitor and to manage the quality of water bodies worldwide (Lorenz 2003), together with a growing recognition of the difficulties and the rational limits of direct chemical monitoring for providing sufficient information to adequately assess the risks from anthropogenic chemicals in the natural environment (Reyjol et al., 2014). More recently, the recent democratisation of high-throughput molecular phenotyping has allowed the rapid expansion of promising untargeted multi-dimensional approaches (*e.g.* epigenomics, transcriptomics, proteomics, metabolomics), constituting as much as responding biological traits, that now represent promising perspectives for environmental assessments (Bahamonde et al. 2016; Cordier et al., 2020). It is very likely that emerging omics-based analyses, such as metabolomics, developed for ecologically relevant aquatic species, may support the development of new ecological evaluation tools, that could provide even earlier warning of eco(toxico)logical impairs (Marie 2020).

The metabolome, defined as the set of primary metabolites synthesized at a given time and thus represents its global metabolic chemical picture, is assumed to represent a final endpoint of the phenotypic response of an organism that can potentially be modified when the ecological and environmental stress conditions change (Bundy et al., 2008). In this way, metabolomic studies have become a relevant approach to describe and analyse physiological processes involved in the homeostatic responses of the organisms encountering environmental stresses from potentially multiple origins (Capello et al., 2016; Good et al., 2020). However, despite its high potential to better understand the molecular mechanisms implicated in the ecotoxicological responses of aquatic organisms (Sardans et al., 2011; Viant, 2008), such environmental metabolomics investigations remains still rare.

Fishes, in particular, present unique features that make them especially relevant for bio-indication purposes, as they occupy almost any aquatic habitat, being capable of experiencing different and variable environmental conditions (Harris 1995). Fishes are also responsive to numerous abiotic (temperatures, water velocities, sediment loads, hypoxia, …) or biotic (famine, predation, parasitism, …) pressures, together with anthropogenic stressors, such as contaminants, that represent additional constraints that fish may experience in disturbed ecosystems (McClanahan 2019). Environmental stressors are known to induce biological variations, concerning the specific physiological, developmental and reproductive capabilities of the different fish species allowing them to colonize and occupy ecosystems (Hamilton et al., 2016).

As cyanobacterial blooms are one of the most common stress encountered by organisms living in modern freshwater lentic systems around the world (Harke et al., 2016; Burford et al., 2020), several recent efforts have been attempted to characterize the potential effect of cyanobacteria proliferation on various fish species according to experimentation performed in the lab, in mesocosms, or from field sampling (Amado et al., 2010; Ferrao-Filho et al., 2011; Sotton et al., 2017a, 2017b; Le Manach et al., 2018). To this end, we initially investigated the

liver metabolome of fish collected from different lakes encountering low or high cyanobacterial proliferation by ^{1}H NMR technics (Sotton et al., 2019). This pilot study leads to the description of putative metabolite concentrations, corresponding to specific NMR signals, that were significantly correlated with higher cyanobacterial occurrence.

The present study investigates the bio-indicative potential of high-throughput molecular phenotyping supported by LC-HRMS approaches on 2 sentinel fish specimens (*Perca fluviatilis* and *Lepomis gibbosus*) collected from a set of 8 water bodies of the peri-urban area of Paris (France), that were previously investigated by ^{1}H NMR (Sotton et al., 2019). We hypothesis that LC-MS-based metabolomics approach may present a higher potential for environmental diagnosis, as providing valuable multi-metric characters indicative of biological or ecological conditions, together with a prospective mechanistic under-standing that supports cause-effect relationship investigation. With regard to the cyanobacterial occurrence and the associated production of bioactive metabolites in the different lakes, we compare here the metabolome of two different sentinel species. Indeed, these organisms could present different or comparable sensitivity and responsiveness to local eco(toxico)logical constraints (*i.e.* potentially the production of noxious metabolites by cyanobacteria), supporting the general interest for omics-based environmental approach of cyanobacterial bloom threat.

2. MATERIALS AND METHODS

2.1. Fish sampling

Field sampling campaigns was performed during late summer 2015 (7-10th September) in 8 peri-urban pounds around Paris' area (Île-de-France region, France), chosen for their respective eutrophication levels and the presence or the absence of recurrent cyanobacterial blooms: Cergy-Pontoise (Cer), Champs-sur-Marne (Cha), Maurepas (Mau), Rueil (Rue), Verneuil (Ver), Varennes-sur-Seine (Var), Fontenay-sur-Loing (Fon) and Triel (Tri) pounds (Supp. figure S1) (Maloufi et al., 2016; Sotton et al., 2019). These sites were sampled by the Hydrosphère company (www.hydrosphere.fr) with electric fishing device (FEG 8000, EFKO, Leutkirch, Germany) for capturing fish alive, that were then promptly sacrificed for liver collection. The investigation of the fish guild indicates that only the perch (*Perca fluviatilis*) and pumpkinseed sunfish (*Lepomis gibbosus*) were presents in all or almost all these pounds (supplementary table S1) and were further selected as sentinel species for further molecular phenotyping analyses by metabolomics.

Briefly, alive caught fishes (n=5-10 young-of-the-year per pounds and per species) were directly measured (12.0±4.8 cm), weighed (9.3±2.6 g), briefly euthanized by neck dislocation and then liver of each individual was shortly sampled, flash-frozen in liquid nitrogen and kept at -80°C until analyses, in accordance with European animal ethical concerns and regulations. In every lake, sub-surface chlorophyll-*a* equivalent concentrations attributed to the four-main phytoplankton groups (Chlorophyta, Diatoms, Cyanobacteria and Cryptophyta) were measured with an *in-situ* fluorometer (Fluoroprobe II, Bbe-Moldenke, Germany)

117 (Supplementary figure S2). Sub-surface water samples filtered on 20-μm mesh size were also
118 collected for phytoplanktonic community analysis and further metabolomics characterisation,
119 and then kept at -80°C until analysis as previously described (Sotton et al., 2019).
120
121 **2.2. Liver metabolite extraction and metabolomics analyses**
122 The liver extraction was performed on 132 individuals (comprising 78 *Perca* and 54 *Lepomis*)
123 with methanol/chloroform/water (ratio 2/2/1.8 – 22 mL.g^{-1} at 4°C) and the polar fraction was
124 analyzed on a 600-MHz NMR spectrometer equipped with a 5-mm cryoprobe (Advance III HD
125 Bruker, Germany) with a noesygppr1d pulse sequence as previously described (Sotton et al.,
126 2019). ^{1}H-NMR spectra were treated with Batman R-package for deconvolution, peak
127 assignment and quantification of 222 putative metabolites (Hoa et al., 2014).
128 The liver extracted polar phase was additionally injected (2 µL) on C_{18} column (Polar Advances
129 II 2.5 pore - Thermo), then eluted at a 300 µL.min^{-1} flow rate with a linear gradient of
130 acetonitrile in 0.1% formic acid (5 to 90 % in 21 min) with an ultra-high-performance liquid
131 chromatography (UHPLC) system (ELUTE, Bruker). Consecutively, the individual metabolite
132 contents were analysed using an electrospray ionization hybrid quadrupole time-of-flight (ESI-
133 Qq-TOF) high-resolution mass spectrometer (Compact, Bruker) at 2 Hz speed on positive MS
134 mode on the 50–1500 *m/z* range. The feature peak list was generated from recalibrated MS
135 spectra (< 0.5 ppm for each sample, as an internal calibrant of Na formate was injected at the
136 beginning of each sample analysis) within a 1-15 min window of the LC gradient, with a
137 filtering of 5,000 count of minimal intensity, a minimal occurrence in at least 50% of all
138 samples, and combining all charge states and related isotopic forms using MetaboScape 4.0
139 software (Bruker).
140 Additionally, five pools of six different individuals randomly selected for *Perca* and *Lepomis*
141 (quality check samples) and phytoplanktonic biomass extracts from the 8 lakes were similarly
142 eluted then analysed on positive autoMS/MS mode at 2-4 Hz on the 50-1500 *m/z* range for
143 further metabolite annotation. Molecular networks were performed with GNPS
144 (http://gnps.ucsd.edu) and/or MetGem (http://metgem.github.io) softwares, as previously
145 described (Kim Tiam et al., 2019; Le Manach et al., 2019) for cyanobacterial and fish
146 metabolite annotation was assayed using in-house (Le Manach et al., 2019) or CyanoMetDB
147 (Jones et al., 2021), and GNPS, Mona, HMDB and Massbank MS/MS spectral libraries. Water
148 samples of each lake, concentrated on a 20-μm mesh size, were also extracted with 75%
149 methanol (2 min sonication, 5 min centrifugation at 15,000 g - 4°C) and then similarly analysed
150 in triplicates on LC-HRMS on autoMS/MS positive mode, as earlier described, and metabolite
151 list annotated with same pipelines.
152
153 **2.4. Data matrix treatment**
154 The resulting the intensity data tables of the 222 metabolites (^{1}H NMR) and 1252 analytes (LC-
155 MS) were further treated for quantile normalization and Pareto's scaling, inspected data
156 representation by PCA and heatmap with hierarchical clustering (Euclidean distance) and then
157 analysed to investigate the influences of "Species" and "Lakes" parameters on the datasets by

158 PERMANOVA or PLS-DA using MixOmics R Package (Rohart et al., 2017), MetaboAnalyst 5
159 (Chong et al., 2019) and MicrobiomeAnalyst tools (Chong et al., 2020).
160
161
162 ## 3. RESULTS AND DISCUSSION
163 ### 3.1. Distinction of *Perca* and *Lepomis* liver LC-MS metabolome
164 Figure 1 illustrates the global relative metabolites analyzed by LC-MS for each fish (comprising
165 *Perca* and *Lepomis*) on a heatmap with hierarchical classifications and individual plot principal
166 component analyses (PCA). A very strong and significant discrimination according to the
167 "species" was achieved for this LC-MS liver metabolomics dataset (PERMANOVA F-value =
168 94.92; R^2 = 0.43; P-value <0.001), when a slighter but still important discrimination can be also
169 retrieved for "lakes" (PERMANOVA F-value = 4.96; R^2 = 0.22; P-value <0.001).
170 In parallel, a molecular network performed with fragmentation data was obtained by LC-
171 MS/MS analyses of *Perca* and *Lepomis* metabolomes. This approach supports the global
172 annotation process of the fish liver metabolites considering both structural identity and
173 similarity (Nothias et al., 2020). The GNPS and the *t*-SNE representation of the molecular
174 network highlights that most, if not all, of the known metabolites (presenting structural
175 identity or analogy hits) are largely shared between the two species (*e.g.* nucleic acids,
176 carnitines, glutathiones, lipids, saccharides, …). Moreover, most of the species-specific cluster
177 metabolites remain uncharacterized, as corresponding molecules and clusters present no
178 match within the public chemical databases (supplementary figure S2) (da Silva et al., 2015).
179 Taken together, these analyses remarkably illustrate the various specificities of the
180 metabolome of these two species. It also shows the substantial portion of the specific liver
181 molecular metabolism still uncharacterized and that could be related to species-specific
182 nutritional, physiological or toxicological capabilities (Brusle et al., 2017).
183
184 ### 3.2. Comparison of *Perca* and *Lepomis* LC-MS metabolome between lakes
185 In addition, the LC-MS dataset of the two fish species considered separately clearly show even
186 more consistent discrimination of the metabolomes of fish originating from the different
187 sampling lakes. The un-supervised PCA and the heatmap with hierarchical classification
188 (Figure 2), performed with LC-MS dataset of liver metabolomes, present distinct local
189 signatures that support reliable discrimination of the lake of sampling for both *Perca* and
190 *Leptomis* (Fig. 2A-B and 2C-D, respectively). These observations strongly suggest that the
191 locality seems to globally influence the LC-MS metabolome composition for both species.
192 However, the liver metabolomes of individual *from* Fontenay-sur-Loing lake (Fon) exhibite a
193 quite species-specific relative positions on PCA, considering the relative position of individual
194 metabolomes from other lake in the two species (Figure 2A and 2C). This suggests that,
195 although for most localities the specific "lake" signature of the metabolome appears in good
196 agreement between the two species, in some specific environments, the metabolome
197 signatures are variable from one species to the other one. The species-specific signature of
198 the *Perca* and *Lepomis* metabolomes in Fon lake potentially traduce rather specific

199 responsiveness and/or sensitivity to a local factor.
200
201 Interestingly, the hierarchical classifications performed respectively on LC-MS metabolome
202 datasets of *Perca* and *Lepomis* show very similar lake relationships, grouping together the fish
203 from the lakes of Cer and Mau, from Cha, Fon and Rue, and from Tri, Var and Ver for *Perca* or
204 Tri and Ver for *Lepomis*, as this latter was not retrieved in Var (Figure 2B and 2D). This
205 observation suggests that these environments could present comparable, if not homogenous,
206 environmental conditions. Then, this hypothesis was further considered and potential
207 explicative factors investigated, considering the potential influence of cyanobacterial
208 proliferation.
209 Indeed, as previously shown, the Fon, Tri, Var and Ver lake encounter a remarkable high
210 amount of cyanobacteria belonging to the *Planktothix*, *Pseudo-Anabaena*, *Aphanizomenon*,
211 *Anabaena* or *Microcystis* genera, when others (Cer, Mau, Cha and Rue) exhibit mostly green
212 algae, diatoms or cryptophytes (Fig. 3A-B). Interestingly, a molecular network performed with
213 LC-MS/MS data obtained from the respective phytoplanktonic biomass of these lakes shows
214 that noticeable amounts of cyanopeptides are observed in the water column of the
215 cyanobacterial-rich lakes (Fon, Tri, Var and Ver), with the specific presence of most noxious
216 cyanopeptides (microcystins, cyanopeptolins and anbaenopeptins) being detected in Tri, Var
217 and Ver lakes, when Fon exhibits rather less toxic cyanopeptides (Janssen 2019), such as
218 microginins and aeruginosins (Fig. 3C). Taken together, these observations suggest that the
219 proliferation and the subsequent production of these potentially noxious cyanobacterial
220 metabolites could be considered as an explicative factor for the local metabolome singularity
221 of the liver metabolome of *Perca* and *Lepomis* from Tri, Var and Ver lakes, as previously
222 proposed (Sotton et al., 2019).
223
224 **3.3. Most discriminating metabolites between the different lake groups**
225 Thus, we re-explore the fish metabolome discrimination considering together fish from lake
226 presenting noxious cyanobacterial metabolites ("Tri/Var/Ver" group for *Perca* and "Tri/Ver"
227 group for *Lepomis*) or not ("Cer/Mau/Cha/Fon/Rue" group), by PLS-DA to identify metabolite
228 that the most discriminate between these two lake groups (Figure 4). Indeed, while
229 unsupervised PCA were first used to evaluate the global dispersion between species and
230 sampling groups, a supervised model such as partial least square differential analysis (PLS-DA)
231 allows to maximize the separation between sample classes (*e.g.* "lakes") and to extract
232 information on discriminating features (variable importance on projection - VIP). For both fish
233 species the metabolomes present a consistent discriminant analysis scoring (*Perca*: accuracy
234 = 1, performance R^2 = 0.78, predictability Q^2 = 0.72; *Lepomis*: accuracy = 0.93, R^2 = 0.59, Q^2 =
235 0.29) and large set of discriminating variables (*Perca* $VIP_{>1.5}$=109 and *Lepomis* $VIP_{>1.5}$=93)
236 (Figure 4A-B). Among these metabolites, 33 components have been successfully annotated
237 considering the molecular mass, the isotopic pattern, together with the fragmentation pattern
238 of all components with GNPS and MetGem algorithms and manual curation. Overall, the
239 observation of the metabolite semi-quantification of the annotated best VIP presents largely

240 similar variations between these two groups of lakes for *Perca* and *Lepomis* (Figure 4C; Supp.
241 fig. S3). Indeed, except for the reduced-glutathione that present higher concentrations in the
242 livers of *Perca* from "Tri/Var/Ver" lakes, when livers of *Lepomis* exhibits lower concentrations
243 than in the those of fish of the "Cer/Mau/Cha/Fon/Rue" group, all other metabolites present
244 similar pattern in the two species. This discrepancy suggests that glutathione-dependent
245 redox mechanisms could be more extensive in *Perca* than in *Lepomis* livers.
246 Globally, the main differences between the two lake groups of the different metabolite
247 concentration show that fishes collected from the "Cer/Mau/Cha/Fon/Rue" seem to present
248 greater energetic, anti-oxidant/detoxification and lipids reserves (*e.g.* various nucleic acids,
249 glutathiones, flavonoids and lipo-phosphocholines) than in lakes presenting more
250 cyanobacteria and related noxious metabolites. In addition, greater amounts of amino acids,
251 di-peptides and acylcarnitines could be related to higher protein catabolism and beta-
252 oxidation in the liver of fish originating from the "Tri/Var/Ver" lakes, hypothetically traducing
253 higher energetic requirements for those organisms. Taken together, these observations are in
254 agreement with the hypothesis of the occurrence of higher stress conditions locally occurring
255 in the Tri, Var and Ver lakes, in correspondence with the local production of potentially
256 noxious metabolites by cyanobacterial proliferation (Sotton et al., 2017a, 2017b; Le Manach
257 et al., 2018).
258
259 In general, various genetic or phenotypic factors (such as development stages, contamination
260 levels, predator/parasite pressures, food availability, …) could influence and explain lthe ocal
261 discrepancy of the metabolome of fishes collected from different environments (reviewed in
262 Marie 2020). However, in the present case, the fact that *Perca* and *Lepomis* metabolomes
263 analysed in parallel present similar metabolite variations clearly indicates that local
264 environmental constraints drive such phenotypic co-variations (beyond the obvious genetic
265 distinction between *Perca* and *Lepomis*). Then, these present results claim the great value of
266 LC-MS-based metabolomic imprints for molecular phenotyping on sentinel species and the
267 exploration of environmental stresses (Lohr et al., 2019).
268
269 **3.4. Comparison of ^{1}H NMR and LC-MS metabolomics for fish livers**
270 Considering the previous ^{1}H NMR metabolomics datasets of these two fish species retrieved
271 from the same samples (Sotton et al., 2019), the NMR liver metabolome of *Perca* and *Lepomis*
272 shows more limited specificity according to "species" or "lakes" factor than LC-MS dataset.
273 Although the 222 potential metabolites quantified by NMR on 132 fish (78 *Perca* and 54
274 *Lepomis*) present no obvious structuration of the individual dataset according to "species" or
275 "lakes" variables when observed on PCA or hierarchical classification, slight but significant
276 relations are however supported by PERMANOVA (F-values = 4.15 and 3.63; R^2 = 0.03 and
277 0.17; P-values < 0.01 and 0.01, for "species" and "lakes" respectively, Supp. fig. S3). Indeed,
278 according to the relatively lower performances of the hierarchical classifications and
279 PERMANOVA and the PLS-DA (Supp. fig. S4 and S5), the NMR liver metabolomes present

280 restricted discrimination on “lakes” for both species and could support here only a limited
281 discrimination potential, when compared to LC-MS metabolomics performances.
282 Overall, the dataset obtained from the LC-MS metabolome analysis presents a clearer
283 discriminant pattern between the two species or the 8 lakes, than the NMR dataset generated
284 on the same samples (with the same extraction procedure), regarding both hierarchical
285 classification, individual plots of PCA or PERMANOVA performances (Fig. 1 and 2; Supp. fig. S4
286 and S5). This observation is further confirmed by direct comparison of the NMR and LC-MS
287 metabolome dataset using MixOmics Diablo tool for multi-block PLS-DA (Supp. fig. S6 and S7).
288 Indeed, although the two datasets appear mostly to be congruent, the individual plot
289 distribution carries much more variance on the PLS-DA projection for the LC-MS analysis than
290 for the NMR, considering both fish species.
291
292 On one side, the ^{1}H NMR metabolomics allows the reliable quantification of main liver
293 metabolites (belonging mostly to amino acids, sugars, TCA metabolites, …), by detecting
294 characteristic chemical groups, with regards to their respective position within the molecules
295 (Martin et al., 2015). These primary metabolites are key contributors to the cellular
296 metabolism and are supposed to be responsive to variable physiological conditions of fish
297 encountering peculiar biological outcomes and/or environmental constraints (Roques et al.,
298 2020). However, this approach remains focused on few principal metabolites involved in main
299 cellular pathways which measured cellular contents (in *ppm*) can be involved in multiple
300 regulation processes, and not being specific of subtle molecular regulation processes. On the
301 other hand, ^{1}H NMR presents only low sensitivity and limited discrimination capability for
302 molecules presenting similar chemical structures, such as lipids, that exhibits an aggregated
303 and unspecific signal on NMR spectra (Emwas et al., 2019). For this reason, it is supposed to
304 present lower discriminating power than more selective and less qualitative, but more
305 sensitive, high-resolution mass spectrometry-based metabolomics approaches.
306 On the other side, although LC-MS-based metabolomic remains a selective analytical
307 approach (according to both LC separation and ionisation respective properties of each
308 molecule), it presents un-precedent analytical capabilities in terms of sensitivity, dynamic
309 range and number of characterized components and offer now unprecedented perspectives
310 for the investigation for molecular phenotyping applied to environmental sciences (Beyoglu
311 and Idle., 2020). We assume, that a single LC-MS fingerprint analyse does not embed the
312 whole metabolite picture of the biological compartment (the fish liver, in the present case),
313 especially because of its selectivity performances (Gika et al., 2019). However, it provided
314 specific and precise measurements of a large number of components that can endorse high-
315 throughput and in-depth phenotyping. For comparison, ^{1}H NMR metabolomics allows the
316 global quantification of the CH_2-CH_2 or CH_2-CH=CH fatty acid bounds (Marchand et al., 2018),
317 when LC-MS metabolomics can discriminate and characterize hundreds of different lipids
318 belonging to various sub-classes (Dreier et al., 2020), providing undoubtedly more informative
319 and discriminant features. In addition, multi-omics integration tools, such as MixOmic DIABLO
320 (Singh et al., 2019), provides innovative solutions for the comparison of heterogeneous

321 dataset matrices obtained from a single set of samples or individuals (supplementary figures
322 S6-S7).
323
324 **3.4. Use of LC-MS metabolomics fingerprint for environmental assessment?**
325 Although the value of LC-MS metabolomics for investigating the impacts of environmental
326 stressors or contaminants, and their respective mode of action has been well-explored in
327 medical sciences (Beyoğlu et al., 2020; Zhang et al., 2020) or in ecotoxicology laboratory-based
328 studies on aquatic models (Le Manach et al., 2018; Huang et al., 2017; Ekman et al., 2015; Gil-
329 Solsona et al., 2017), such methods have been used only faintly in field research so far (Bundy
330 et al., 2009; Lankadurai et al., 2013). Apart from a limited number of evidence of the utility of
331 NMR-based metabolomics in environmental fish studies (Capello et al., 2016; Sotton et al.,
332 2019; Wei et al., 2018), few other examples indicate that field-based LC-MS metabolomics
333 constitutes an emerging and powerful, but still underused, approach for increasing our
334 understanding of *in situ* biological, physiological, ecological or ecotoxicological processes
335 (Meador et al., 2020; Reverter et al., 2017; Goode et al., 2020).
336 Our analysis constitutes one of the first attempts to push forward the potential of high-
337 throughput molecular phenotyping, and especially through LC-MS-based metabolomics, for
338 environmental assessment. Indeed, this organism molecular phenotyping supported by multi-
339 variable chemometric investigation offers remarkably rich biological information that serves
340 at describing specific phenotypic plasticity. Moreover, this organism
341 variability/responsiveness can further be confronted to local environmental factors such as
342 noxious cyanobacterial metabolites in order to demonstrate correlation or causality
343 relationships.
344
345 As described by Pompfret and co-workers (2019), environmental metabolomics exhibit very
346 promising perspectives for operational bio-monitoring applications, according to its reliability,
347 its reproducibility, and its high predictive potential. However, these authors also point out
348 that the responsiveness and robustness of the bio-indicative object, which is characterized
349 through the analytical prism of the metabolomics, remains crucial and have to be carefully
350 evaluated and tested with an appropriate experimental design (Pompfret et al., 2019).
351 However, for ethical concerns, fish bio-monitoring would also gain at been less invasive and
352 deleterious for the organisms. To this end, non-lethal mucus sampling has been investigated
353 by LC-MS and have demonstrated the remarkable informativeness of this approach for
354 environmental studies (Reverter et al. 2017), but these efforts still remain explorative.
355
356
357 **4. Conclusions**
358 The main challenge facing us remains to be able to develop more integrative approaches that
359 aim at connecting chemical, biological and ecological assessment, in the context of
360 anthropized natural environments confronting multi-stressor pressures. A major caveat of the
361 use of fish environmental metabolomics remains maybe the lack of dedicated databases

(Viant et al., 2019) fulfilled by studies considering together different species, populations, development stages, seasons and environments. These data could be used to provide baseline reference values that would further support machine learning or artificial neural network tools for the training of decision-making models.

Author contributions

B.M. designed experiment, and performed sample analysis. B.M. and A.G. analysed data. B.M. wrote the manuscript. All authors have given approval to the final version of the manuscript.

Notes

The authors declare not conflict of interest

ACKNOWLEDGMENTS

We would like to thanks B. Sotton for his collaboration on NMR data acquisition and treatment. We are also greatful to A. Paris for its support and prolific discussions. This work was supported by grants from CNRS (Défi ENVIROMICS “Toxcyfish” project). The NMR and the MS spectra were respectively acquired at the Plateau technique de Résonance Magnétique Nucleaire and the Plateau technique de spectrométrie de masse bio-organique, Muséum National d’Histoire Naturelle, Paris, France. This work benefitted from the French GDR "Aquatic Ecotoxicology" framework which aims at fostering stimulating scientific discussions and collaborations for more integrative approaches.

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537 ## Legends of figures

538

539 **Figure 1.** Visualization of the dataset structuration for LC-MS metabolomics (comprising 132
540 fish and 1252 analytes) on PCA (A) and heatmap with hierarchical clustering (B) for *Perca*
541 (n=78) and *Lepomis* (n-54) fish collected during the 7-10th of September 2015 within 8 pounds
542 of the peri-urban Paris' area.

543

544 **Figure 2.** Individual plot of PCA (A and C) and heatmap with hierarchical classification (B and
545 D) of LC-MS liver metabolomes of *Perca* (n=78; A-B) and *Lepomis* (n=54; C-D) collected
546 during the 7-10th of September 2015 within 8 pounds of the peri-urban Paris' area.

547

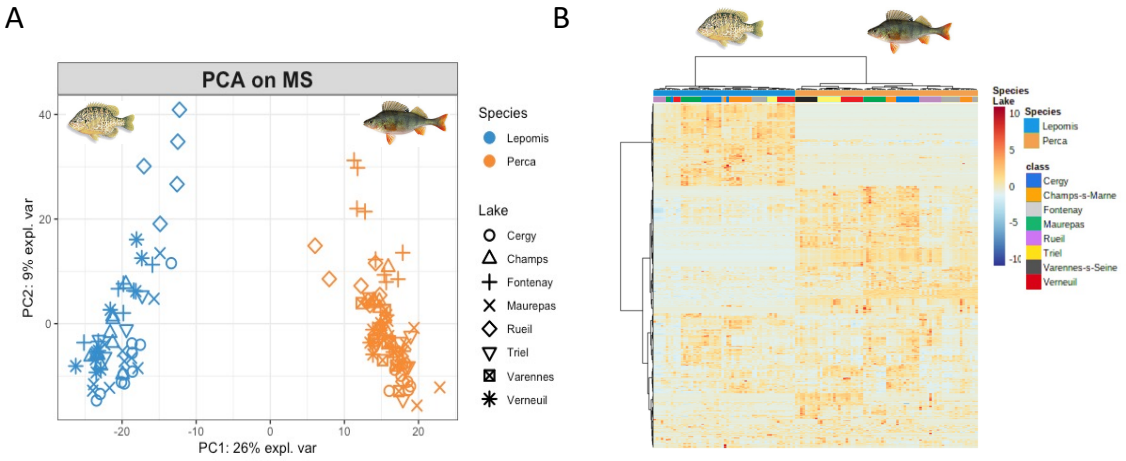
548 **Figure 3.** Phytoplankton composition estimated by BBE measurment of the 8 lake sub-
549 surface water (A), corresponding cyanobacteria relative composition for Fon, Tri, Var and
550 Ver (B), and molecular networking of metabolites extracted from the filtered biomass of the
551 respective water of the 8 lakes generated with *t*-SNE algorithm, with cyanobacteria peptide
552 clusters indicated in bold and potentially more noxious family clusters (microcystin,
553 cyanopeptolmin and anbaenopeptin) indicated in red (C).

554

555 **Figure 4.** Individual plots of PLS-DA, testing the difference between the different lake groups
556 presenting, or not, high content of potentially noxious cyanobactérial metabolites
557 (Cha/Cer/Fon/Mau/Rue and Tri/Var/Ver, respectively) according to the LC-MS metabolomes of *Perca*
558 (A) and *Lepomis* (B), and corresponding list of annotated best VIP list (metabolite score > 1.5) of
559 *Perca* and *Lepomis* metabolomes (C). Accuraty, predictability and quality performances and number
560 of VIP metabolite score > 1.5) for sampling lake discrimination are indicated for each species. Dots
561 between brackets indicate when the metabolite concentration is more important in the
562 Cha/Cer/Fon/Mau/Rue (green) or Tri/Var/Ver (red) lake group. * indicates that this metabolite was
563 also observed as being negatively correlated with cyanobacteria occurrence within the different lake
564 according to NMR analysis (Sotton et al., 2019).

565

566 **Figure 1.**



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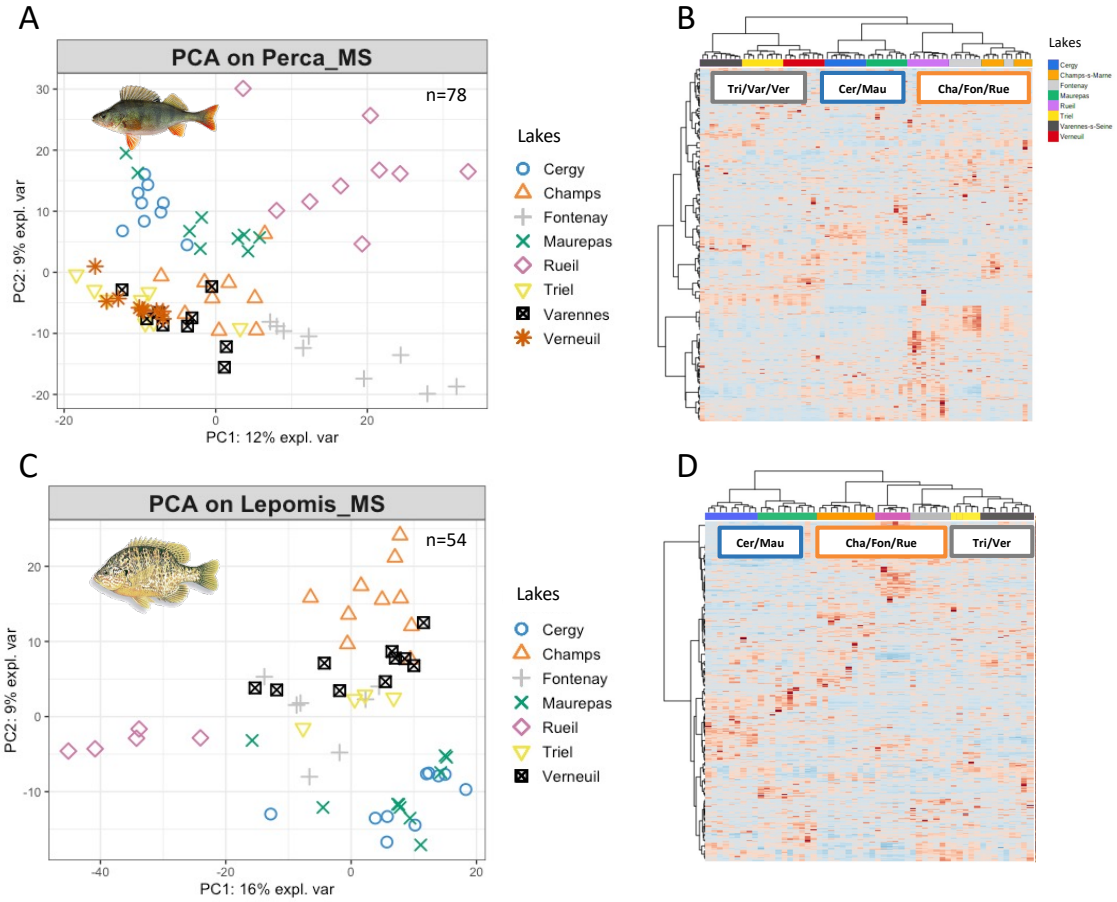
568 **Figure 2.**



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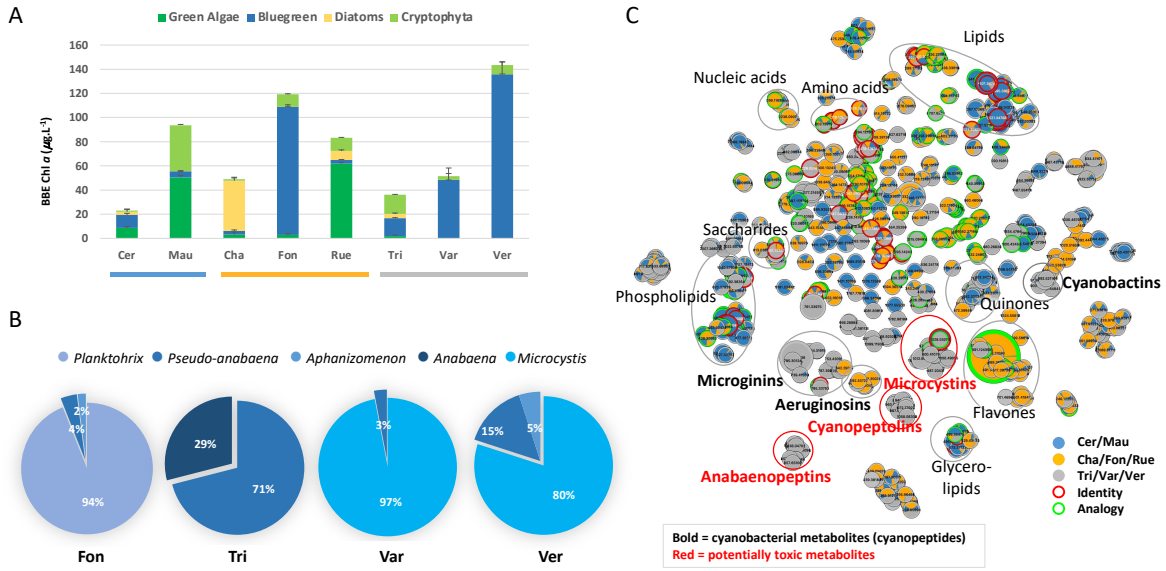
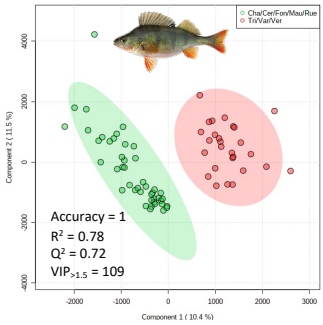
570 **Figure 3.**



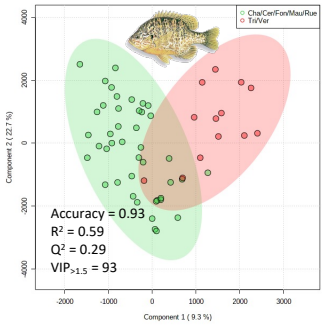
571

572 **Figure 4.**

A



B



C

MW	Annotation	Comp. 1_*Perca*	Comp. 1_*Lepomis*
267.09601	Adenosine	9.33 (●<●)	14.3 (●<●)
347.06212	Adenosine 5'-mono-phosphate	2.02 (●<●)	7.64 (●<●)
307.08355	Glutathione (reduced)	7.49 (●<●)	5.28 (●<●)
335.11488	Trp-Met	5.64 (●<●)	0.17
203.11734	Acetyl-carnitine	5.27 (●<●)	2.25 (●<●)
284.13698	Analog: Isoflavonoid	4.84 (●<●)	1.53 (●<●)
463.07428	Adenylosuccinic acid	4.06 (●<●)	0.07
612.15076	Glutathione (oxidized)	3.82 (●<●)	0.05
245.16244	Valeryl-carnitine*	3.76 (●<●)	1.64 (●<●)
463.07419	Adenylosuccinic acid	3.46 (●<●)	1.99 (●<●)
383.10736	Analog: Cyanidin arabonosine	3.41 (●<●)	0.41
567.33206	Lysophosphatidylcholine 22:6	3.34 (●<●)	1.60 (●<●)
626.16765	Analog: N-acetylglutamic acid	1.51 (●<●)	3.08 (●<●)
181.07481	Tyr	1.55 (●<●)	2.67 (●<●)
250.02948	Analog: N-acetylglutamic acid	2.57 (●<●)	0.03
300.13247	Analog: Hydroxyflavanone	2.50 (●<●)	1.03
559.07138	Adenosine 5'-diphosphoribose	2.49 (●<●)	0.59
404.00264	Uridine 5'-diphosphate	2.40 (●<●)	0.55
135.05505	Adenine	1.77 (●<●)	2.38 (●<●)
607.08199	Uridine-5-diphosphoacetylgalactosamine	2.37 (●<●)	0.14
363.05757	Guanosine 5-monophosphate	2.33 (●<●)	1.59 (●<●)
324.10545	Analog: 6-O-α-D-glucopyranosyl-D-fructose	1.89 (●<●)	2.30 (●<●)
392.16974	Trp-Trp	0.02	2.29 (●<●)
321.06967	Cytidine triphosphate	2.28 (●<●)	0.19
278.15223	Tyr-Pro	2.21 (●<●)	0.02
521.19585	*sn*-glycero-3-phosphocholine	1.69 (●<●)	2.21 (●<●)
665.12496	1,4-Dihydronicotinamide adenine dinucleotide	1.54 (●<●)	2.16 (●<●)
204.07959	Trp	2.13 (●<●)	0.10
242.06799	Thymidine	2.12 (●<●)	0.36
283.09143	Guanosine	0.44	2.08 (●<●)
429.14971	Analog: Adenosine 5'-monophosphate	1.54 (●<●)	2.06 (●<●)
257.10277	*sn*-glycero-3-phosphocholine	2.04 (●<●)	0.60
325.37028	N-Oleoylethanolamine	2.03 (●<●)	0.77

573