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## **1H-NMR metabolomics profiling of zebra mussel (*Dreissena polymorpha*): A field-scale monitoring tool in ecotoxicological studies**

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1 **<sup>1</sup>H-NMR metabolomics profiling of zebra mussel (*Dreissena polymorpha*): a field-scale**  
2 **monitoring tool in ecotoxicological studies**

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29 **Abstract**

30 Biomonitoring of aquatic environments requires new tools to characterize the effects of  
31 pollutants on living organisms. Zebra mussels (*Dreissena polymorpha*) from the same site in  
32 north-eastern France were caged for two months, upstream and downstream of three wastewater  
33 treatment plants (WWTPs) in the international watershed of the Meuse (Charleville-Mézières  
34 “CM” in France, Namur “Nam” and Charleroi “Cr” in Belgium). The aim was to test <sup>1</sup>H-NMR  
35 metabolomics for the assessment of water bodies’ quality. The metabolomic approach was  
36 combined with a more “classical” one, *i.e.*, the measurement of a range of energy biomarkers:  
37 lactate dehydrogenase (LDH), lipase, acid phosphatase (ACP) and amylase activities, condition  
38 index (CI), total reserves, electron transport system (ETS) activity and cellular energy  
39 allocation (CEA). Five of the eight energy biomarkers were significantly impacted (LDH, ACP,  
40 lipase, total reserves and ETS), without a clear pattern between sites (Up and Down) and  
41 stations (CM, Nam and Cr). The metabolomic approach revealed variations among the three  
42 stations, and also between the upstream and downstream of Nam and CM WWTPs. A total of  
43 28 known metabolites was detected, among which four (lactate, glycine, maltose and glutamate)  
44 explained the observed metabolome variations between sites and stations, in accordance with  
45 chemical exposure levels. Metabolome changes suggest that zebra mussel exposure to field  
46 contamination could alter their osmoregulation and anaerobic metabolism capacities. This  
47 study reveals that lactate is a potential biomarker of interest, and <sup>1</sup>H-NMR metabolomics can  
48 be an efficient approach to assess the health status of zebra mussels in the biomonitoring of  
49 aquatic environments.

50 Keywords: active biomonitoring, *Dreissena polymorpha*, energy metabolism, metabolomics,  
51 <sup>1</sup>H NMR

52 Caption: Results of this study showed the interest of <sup>1</sup>H-NMR metabolomics as an efficient  
53 approach in biomonitoring of aquatic environments, and lactate could be a potential biomarker.

## 54 **1. Introduction**

55 According to the European Environment Agency, point and diffuse source pollutions  
56 are the main pressures affecting over 55 % of the European surface water bodies (EEA, 2018).  
57 Among European water policies, the Water Framework Directive (WFD) (2000/60/EC) is  
58 considered as a pioneer framework for the monitoring and protection of aquatic environments,  
59 combining chemical and ecological approaches. These approaches are efficient but insufficient  
60 when it comes to relating water pollutant bioavailability to impacts on aquatic organisms and  
61 ecosystems, which underlines the need to develop new multidisciplinary tools to support and  
62 improve current approaches for biomonitoring aquatic environments (Wernersson et al., 2015).

63 In this context, several approaches promoted by scientific communities are largely used  
64 in ecotoxicology. For example, biomarkers (Amiard-Triquet et al., 2012; Arrighetti et al., 2019;  
65 Catteau et al., 2019) are indicators of several biological functions (immune system,  
66 reproduction, growth, metabolism, etc.). They have long been used in ecotoxicological studies,  
67 within a multi-parametric approach, and should ultimately be integrated as complementary  
68 tools into regulatory biomonitoring approaches such as the WFD (Adams et al., 2001; Arrighetti  
69 et al., 2019; Milinkovitch et al., 2019; Sanchez and Porcher, 2009). More recently, new  
70 biological approaches related to modern analytical techniques (*e.g.*, “-omics” approaches) have  
71 received special attention from ecotoxicology researchers because they can provide reliable  
72 information at very low levels of biological organization (Aggelen et al., 2010; Brockmeier et  
73 al., 2017).

74 Metabolomics is one of the recent “-omics” techniques with a large range of  
75 applications (Viant, 2007). It relies on the detection of small polar molecules called metabolites  
76 (molecular mass 50-2,000 Da) in different samples (biological fluids, cells, tissues, organs,  
77 organisms). This approach targets primary metabolites (sugars, amino acids) essential to

78 various biological functions of a living organism (*e.g.*, growth, reproduction), as well as  
79 specialized metabolites (alkaloids, flavonoids) involved in the interactions of organisms with  
80 their environment (Nicolè and Thomas, 2017). All these molecules form “the metabolome”  
81 which is the result of gene expression and the end-products of the metabolism. It characterizes  
82 the interactions of organisms with their environment, and makes metabolomics an emerging  
83 and promising approach in environmental science (Bundy et al., 2008; Nicolè and Thomas,  
84 2017).

85         Metabolomic studies can be performed using different analytical techniques, such as  
86 mass spectrometry (MS) (Courant et al., 2014) and nuclear magnetic resonance spectroscopy  
87 (NMR) (Watanabe et al., 2015). Proton nuclear magnetic resonance ( $^1\text{H-NMR}$ ) spectroscopy-  
88 based metabolomics is a commonly used tool for studying the effects of pollutants on the  
89 metabolic profiles of aquatic organisms, in laboratory or field-scale conditions (Cappello et al.,  
90 2017, 2016, 2013; Jones et al., 2008; Kwon et al., 2012; Maisano et al., 2017; Tuffnail et al.,  
91 2009; Xu et al., 2019). Because the spectral peak areas are quantitatively related to metabolite  
92 concentrations, this technique simultaneously provides unbiased and reproducible data about a  
93 wide range of metabolites. Furthermore,  $^1\text{H-NMR}$  has several other advantages, in that it is a  
94 rapid and cost-effective analytical technique that detects relative changes in the spectral pattern  
95 of a tissue / organ or even whole tissue extract, and can reflect biological effects of known and  
96 unknown environmental stressors on organisms (Tikunov et al., 2010). The potential of this  
97 technique to unveil the modes of action of environmental stressors and to identify possible  
98 biomarkers in ecotoxicology has been reported in a recent, detailed and comprehensive review  
99 covering NMR-based metabolomics research studies conducted to date on aquatic organisms  
100 (Cappello, 2020).

101           Despite the many upsides of biomarker and metabolomic approaches, downsides exist  
102 too. The main drawback is the possible interference of biological responses to stress conditions  
103 with other confounding factors, leading to false positive results or to background noises that  
104 can mask the real effect of chemical substances (false negative results) (Forbes et al., 2006).  
105 The variability of these biological parameters can be attributed to extrinsic abiotic factors (*e.g.*,  
106 temperature, salinity) or to intrinsic biotic factors (*e.g.* genotype, gender, age, reproduction)  
107 (Benito et al., 2019; Beyer et al., 2017; Freitas et al., 2019; Hines et al., 2007; Nam et al., 2017).  
108 All these factors can be species-, time-, and space-dependent; therefore, they are difficult or  
109 even impossible to control in the field, and their use may be limited in biomonitoring studies.  
110 To address this problem, some authors have proposed to work under semi-controlled field  
111 conditions (controlled provenance, sex, size, etc.) while keeping natural environmental  
112 complexity, by caging organisms in aquatic environments that they naturally inhabit or not  
113 (Catteau et al., 2019; Kerambrun et al., 2016; Le Guernic et al., 2016).

114           Zebra mussels (*Dreissena polymorpha*) originate from the Ponto-Caspian region and  
115 are largely used as an animal model in aquatic ecotoxicology because (1) they are largely  
116 distributed in the freshwater ecosystems of the northern hemisphere, (2) are considered as one  
117 of the worst invasive species in the world, (3) occur at high densities, which facilitates their use  
118 for ecotoxicological tests without fear of causing damage to their sustainability (4) their high  
119 filtration capacities cause them to accumulate high levels of pollutants, and (5) they tolerate  
120 environmental stressors well (Binelli et al., 2015; Bourgeault et al., 2010; Karatayev et al.,  
121 2012; Louis et al., 2019). Except for a few studies, all scientific articles about the use of the  
122 zebra mussel in ecotoxicology have focused on classical biomarkers such as immunity,  
123 oxidative stress, energy metabolism, genotoxicity biomarkers, etc. (Binelli et al., 2009;  
124 Lafontaine et al., 2000; Minguez et al., 2012; Minier et al., 2006; Quinn et al., 2011). Although  
125 the zebra mussel is a valuable bioindicator species in aquatic biomonitoring, few researchers

126 (Leprêtre et al., 2019; Péden et al., 2019; Watanabe et al., 2015) have addressed the application  
127 of “-omics” approaches, especially metabolomics (Watanabe et al., 2015), to this model  
128 organism.

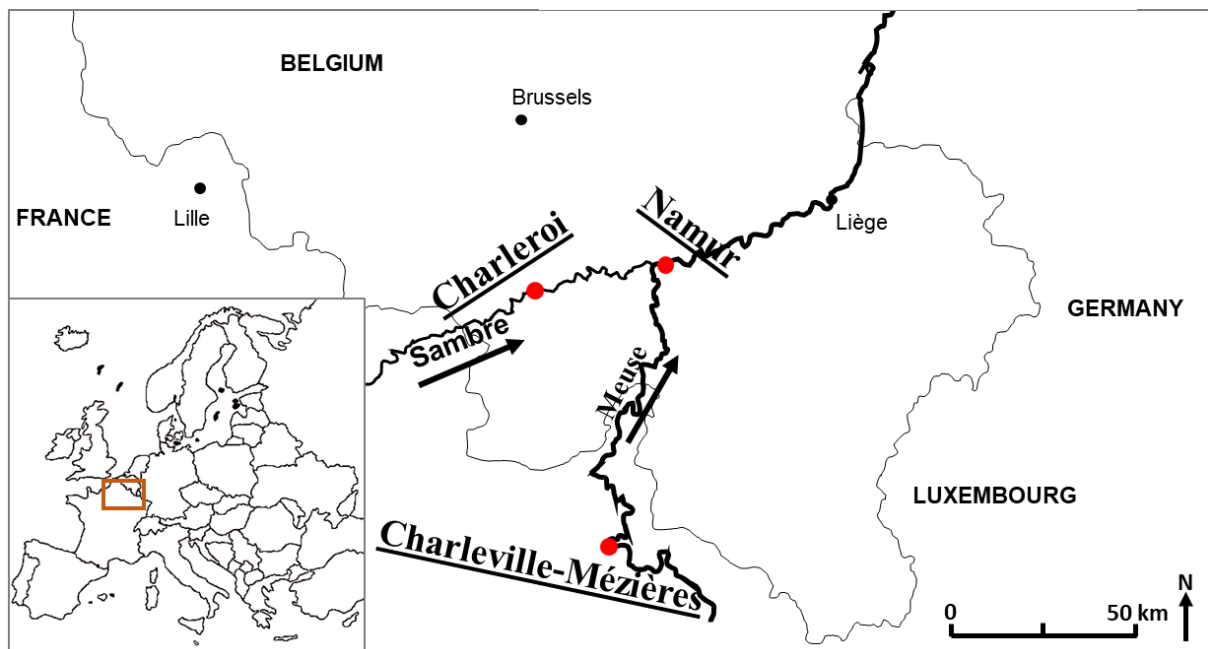
129 An <sup>1</sup>H-NMR metabolomic assay was developed in our laboratory for *D. polymorpha*  
130 (Prud’homme et al., 2020). The present study addresses the application of this approach to the  
131 analysis of the whole-body metabolome of *D. polymorpha* in an active field-scale  
132 biomonitoring study. Zebra mussels collected from the same site were caged for two months,  
133 upstream and downstream of three wastewater treatment plants (WWTPs) in the watershed of  
134 the Meuse river (Charleville-Mézières in France, Namur and Charleroi in Belgium). The aim  
135 was to determine whether we could identify metabolic profile differences among the three  
136 stations (interstation effects), and between the upstream/downstream locations of each  
137 experimental station (intrastation effects). Additionally, energy metabolism core biomarkers  
138 were measured, with a view to proposing the metabolomic approach as a complementary tool  
139 for diagnosing and monitoring the chemical quality of European water bodies.

## 140 **2. Materials and methods**

### 141 **2.1. Experimental design**

142 Zebra mussels ( $2 \pm 0.1$  cm shell length) were collected in October 2018 from the “Lac  
143 du Der-Chantecoq” (northeastern France, 4°45'00" E; 48°34'00" N). After a two-week  
144 acclimation period in aerated laboratory tanks, mussels were randomly placed in 2-mm-mesh  
145 polyethylene cages ( $7 \times 7 \times 14$  cm) (200 mussels *per* cage), that were installed for two months  
146 upstream (1 cage) and downstream (1 cage) of three WWTPs which treat the water of three  
147 municipalities: (1) Charleville-Mézières (France), (2) Charleroi and (3) Namur (Belgium)  
148 (Figure 1). In Namur, a third cage was installed in a supplementary upstream point, 5 km ahead

149 of the Meuse-Sambre confluence, and called Nam-Upstream1. For each station, cages were  
150 positioned 40 to 100 m apart, except in Charleroi where they were about 2 km away from each  
151 other. In total, 7 experimental cages were ballasted and placed in the same way at about 1.0 m  
152 depth on natural rocky structures of 7 experimental sites: 1- Charleville-Mézières upstream  
153 (CM-Upstream), 2- Charleville-Mézières downstream (CM-Downstream), 3- Charleroi  
154 upstream (Cr-Upstream), 4- Charleroi downstream (Cr-Downstream), 5- Namur upstream1  
155 (Nam-Upstream1), 6- Namur upstream2 (Nam-Upstream2) and 7- Namur downstream (Nam-  
156 Downstream). The GPS coordinates and water physico-chemical parameters of each site,  
157 recorded at the beginning and the end of the experiment, are reported in Table 1. Besides, water  
158 samples were collected at least 2 times per month and per site (at the beginning and at the end  
159 of the month) for chemical analysis.



160

161 **Figure 1: Geographical localizations of the experimental stations. Arrows: stream**  
162 **direction of the Meuse and Sambre rivers.**



**Table 1: GPS coordinates and water physico-chemical parameters (mean  $\pm$  S.D.) of each experimental site.**

Station	Site	GPS coordinates	Distance from downstream site (m)	Water parameters			
				Temperature (°C)	Dissolved Oxygen (mg/L)	pH	Conductivity ( $\mu$ S/cm)
Charleville- Mézières (CM)	Upstream	E 04°43'44.7" N 49°45'56.3"	100	11 $\pm$ 3	10 $\pm$ 0	8 $\pm$ 0	597 $\pm$ 13
	Downstream	E 04°43'46.9" N 49°45'59.6"	-	11 $\pm$ 3	10 $\pm$ 0	8 $\pm$ 0	598 $\pm$ 13
Charleroi (Cr)	Upstream	E 04°27'19.4" N 50°24'07.8"	2,000	12 $\pm$ 3	8 $\pm$ 1	8 $\pm$ 0	673 $\pm$ 16
	Downstream	E 04°29'27.4" N 50°23'44.7"	-	12 $\pm$ 3	6 $\pm$ 2	8 $\pm$ 0	1025 $\pm$ 172
Namur (Nam)	Upstream1	E 04°51'38.8" N 50°26'38.4"	8,000	13 $\pm$ 4	11 $\pm$ 0	8 $\pm$ 0	537 $\pm$ 39
	Upstream2	E 04°57'30.5" N 50°28'49.8"	40	13 $\pm$ 4	10 $\pm$ 0	8 $\pm$ 0	741 $\pm$ 147
	Downstream	E 04°57'32.9" N 50°28'49.4"	-	13 $\pm$ 3	10 $\pm$ 1	8 $\pm$ 0	763 $\pm$ 141

164 **2.2. Sample preparation**

165 To minimize the effects of field dissection on their metabolome as much as possible  
166 (Watanabe et al. 2015), a group of mussels (n=10 *per* site) was directly frozen in liquid nitrogen  
167 on-site, and then stored at -80 °C until metabolomic processing. The remaining mussels were  
168 transferred to the laboratory in aerated water recovered from each site and then acclimated for  
169 12 h before dissection. Then, they were weighed to the nearest 1 mg with a Sartorius ED224S  
170 balance, measured with vernier calipers to the nearest 0.1 mm (length x width x height), and  
171 dissected from their shells, and processed as follows: for the core biomarkers, soft samples were  
172 weighed and stored at -80°C as pools of three digestive glands (n= 9 pools *per* site) for digestive  
173 enzyme activities, and pools of three whole organisms (n= 9 pools *per* site) for the other energy  
174 biomarkers. For bioaccumulation assays, pools of 2 g of whole organisms were prepared: three  
175 pools were directly frozen at -80°C for inorganic bioaccumulation assays, and three other pools  
176 were frozen in liquid nitrogen, lyophilized and then stored at -80°C for organic bioaccumulation  
177 assays.

178 Water samples were collected at least 2 times per month and per site, at the beginning  
179 and at the end of the month, in amber 0.5-L glass bottles, and stabilized by adding sodium  
180 thiosulfate (2.5 mL of Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>·5H<sub>2</sub>O at 1.9 g/L). The samples were then stored at 5 ± 3 °C in  
181 the dark and extracted within 48 hours of sampling.

182 **2.3. Chemical analyses**

183 **2.3.1. Water analysis**

184 Water was filtered, and 0.25-L samples were processed after adjusting the pH at 7 ± 0.2.  
185 A solid-phase extraction was performed with an automatic extractor (Smartprep<sup>TM</sup>, Horizon  
186 Technologies). The samples were eluted with acetonitrile (LC-MS/MS grade), and analyzed

187 with an Agilent 1290 liquid chromatograph coupled to an Agilent 6490 QQQ tandem mass  
188 spectrometer. Extraction, quantification methods and the list of the targeted chemical  
189 compounds are detailed in Supplementary Data (*c.f.* S1.1, and tables S1, S2).

### 190 **2.3.2. Mussel bioaccumulation analysis**

191 For organic compounds (Polychlorinated biphenyls “PCBs”, Polycyclic aromatic  
192 hydrocarbons “PAHs”, Polybrominated diphenyl ethers “PBDEs”), freeze-dried homogenized  
193 mussels were treated with a modified QuEChERS extraction approach (Kalachova et al. 2011),  
194 and analyzed by atmospheric pressure gas chromatography/spectrometry (APGC), using an  
195 Agilent 7890B GC system (Agilent, Palo Alto, CA, USA). For heavy metals (Cadmium “Cd”,  
196 Copper “Cu”, Nickel “Ni”, and Zinc “Zn”), soft tissues were mineralized with Suprapur® nitric  
197 acid for 24 h at 80 °C. The resulting acidic solutions were adjusted to 10 mL with ultrapure  
198 water, and samples were analyzed using an inductively coupled plasma optical emission  
199 spectrometer (ICP-AOS, Thermo Scientific iCAP 6300 DUO). Results were reported in µg/kg  
200 wet weight for organic compounds, and µg/g wet weight for heavy metals.

### 201 **2.4. Core biomarker analyses**

202 Several energy biomarkers were addressed. Firstly, digestive enzyme (amylase, acid  
203 phosphatase (ACP), lipase and lactate dehydrogenase (LDH)) activities were assayed on the  
204 cytosolic fraction of digestive gland samples, after 30 minutes’ centrifugation (*c.f.* S1.2, for  
205 extraction), using Thermo-Scientific Gallery ready-to-use reagents on an automated  
206 photometric analyzer (Gallery™, Thermo Fisher Scientific Oy) following the manufacturer’s  
207 protocols. Each enzyme activity was expressed as U/g of protein.

208 Energy consumption (Ec) was evaluated by measuring the activity of the electron  
209 transport system (ETS) in the supernatant after 10 min of centrifugation at 3,000g (*c.f.* S1.2.),

210 according to De Coen and Janssen (1997a). ETS activity was then transformed into energy  
211 equivalents using an average oxyenthalpic equivalent of 480 kJ/mol O<sub>2</sub>, and results were  
212 expressed as mJ/mg wet wt/h (De Coen and Janssen, 1997a).

213 The condition index was calculated according to Lundebye et al. (1997), using biometric  
214 parameters recorded from individuals that were used for metabolomics: [CI= Soft tissue dry  
215 weight (g)/(length x width/height (mm))].

216 For energy reserves, protein contents were measured after extraction according to  
217 Bradford (1976), using bovine serum albumin (Sigma-Aldrich Chemicals, France) as a  
218 standard. Lipids and carbohydrates were measured according to protocols adapted from  
219 Plaistow et al. (2003), using olive oil (Sigma-Aldrich Chemicals, France) and glucose solutions  
220 (Sigma-Aldrich Chemicals, France) as standards. The whole-body energy reserves budget (E<sub>a</sub>)  
221 was calculated by summing the energetic values of the different reserves [E<sub>a</sub> = ∑ (total lipids,  
222 carbohydrates and proteins) (mJ/mg wet wt/h)], using an enthalpy of combustion of 17.5 kJ/g  
223 for carbohydrates, 39.5 kJ/g for lipids and 24 kJ/g for proteins.

224 Finally, an integrative biomarker (CEA: cellular energy allocation) was calculated after  
225 determining E<sub>a</sub> and E<sub>c</sub>, according to the following formula: [CEA = E<sub>a</sub>/E<sub>c</sub>] (Verslycke et al.,  
226 2004).

## 227 **2.5. <sup>1</sup>H-NMR metabolomics**

228 Frozen mussels were measured with vernier calipers to nearest 1mm (length x width x  
229 height), dissected on ice, and soft tissues were removed from the shell using a pre-cleaned  
230 surgical scalpel. Then, whole organisms were individually frozen in 2-mL Eppendorf tubes at  
231 -80 °C. Extraction was performed on 10 mg of ground whole organism lyophilizate, using the  
232 methanol:chloroform:water technique, as described in Supplementary Data S1.3.. Dried whole

233 tissue extracts were resuspended in 600  $\mu$ L of 0.1 M deuterated phosphate buffer (pH 7) (*c.f.*  
234 S1.4.), vortexed and then transferred into standard 5-mm NMR tubes (Norell<sup>®</sup>) for analysis  
235 with a Bruker AV III 600 NMR spectrometer (Bruker, Wissembourg, France) at the Institute  
236 of Molecular Chemistry of Reims (ICMR, Champagne Ardenne University, France). The  
237 detailed acquisition process is reported in Supplementary Data S1.4.

238 Prior to statistical processing, the spectra were pre-processed by NMRProcFlow, an  
239 open-source software program developed by Jacob et al. (2017) and dedicated to metabolomics,  
240 as described in Supplementary Data S2. Spectra were annotated following Prud'homme et al.  
241 (2020) and based on the combination of several 1D and 2D approaches to assign each  
242 metabolite as confidently as possible (*c.f.* S1.4.).

## 243 **2.6. Statistical analyses**

### 244 **2.6.1. Biological and chemical parameters**

245 Statistical analyses of all biomarkers and chemical analyses (bioaccumulation  
246 and water chemical results) were performed using R software (v3.3.1), and were considered  
247 significant when  $p < 0.05$ . One-way ANOVA and a Tukey HSD post-hoc test were performed  
248 after testing normality (Shapiro test) and homogeneity (Levene test). When normality or  
249 homoscedasticity were not met, data were transformed using a log or square-root method. A  
250 nonparametric test (Kruskal-Wallis) was used, followed by a non-parametric post-hoc test  
251 (Dunn's test with Bonferroni adjustment) for bioaccumulation and water chemistry results, and  
252 for biological parameters when data transformation was useless. Additionally, a multivariate  
253 statistical analysis (unsupervised PCA) was performed for energy biomarkers using Biostatflow  
254 platform (v.2.9.2; <http://biostatflow.org>). Statistical tests were performed on upstream results,  
255 using station (CM-Upstream, Cr-Upstream and Nam-Upstream1) as a factor to test the  
256 interstation effect. For bioaccumulation and water chemistry results, given the low number of

257 replicates and the absence of significant differences between upstream and downstream  
258 locations in the different stations for most chemicals, interstation effect was evaluated on the  
259 merged upstream and downstream data from each station. Intrastation effect was evaluated  
260 independently for each station using site (upstream/downstream) as a factor for all parameters.

## 261 **2.6.2. Metabolomics data**

262 NMRProcFlow pre-processing generated 418 buckets from 0.91 to 10 ppm after zeroing  
263 the water signal and blank contaminant peaks. The generated data matrix was imported into  
264 Biostatflow platform (v.2.9.2; <http://biostatflow.org>, a web application developed for the  
265 statistical analyses of “OMICS” Data, using R scripts). Hence, all multivariate statistical  
266 analyses (unsupervised principal component analysis “PCA” and supervised linear kernel-  
267 orthogonal projection to latent structures “K-OPLS”) were performed on this platform. Then, a  
268 univariate test (non-parametric Kruskal-Wallis test) was performed to check for significant  
269 abundance differences of the identified metabolites between the experimental groups (as  
270 described in Supplementary Data S1.5.). Interstation effect was tested on CM-Upstream, Cr-  
271 Upstream and Nam-Upstream1, and intrastation effect was tested on each station separately  
272 using site (upstream/downstream) as a factor. For the univariate and multivariate statistical  
273 tests, the dependent variables were the generated buckets.

## 274 **3. Results and discussion**

### 275 **3.1. Water quality and mussel exposure to field contamination**

276 The water physico-chemical parameters of the sites (Table 1) fell within the ecological  
277 requirements of the species found in the literature (McMahon, 1996; Navarro et al., 2006), and  
278 no mortality was observed at the end of the experiment. Although the oxygen concentration in  
279 Cr-Downstream (6 mg/L) was lower than in the other sites, it remained much higher than the  
280 growth-inhibiting concentration (2 mg/L) reported for *D. polymorpha* (Navarro et al., 2006).

281 Water chemical analysis and mussel bioaccumulation assay results are reported and  
282 discussed in detail in Supplementary Data S2. Briefly, mussels were exposed to different  
283 contaminants, more so in the Sambre than in the Meuse. Compared to other European rivers,  
284 the water quality of the Meuse watershed has improved over the last decades, with the  
285 implementation of WWTPs and the application of water policies (van Vliet and Zwolsman,  
286 2008). However, a spatial dichotomy still remains observable, with a less polluted upstream  
287 portion of the river (French Meuse) and a deteriorated water quality in the downstream portion  
288 beyond the Belgian border. This deteriorated quality of the Meuse is admittedly due to the  
289 inflow of the Sambre which remains greatly polluted compared to the Meuse (van Vliet and  
290 Zwolsman, 2008). Our results support this hypothesis.

### 291 **3.2. Energy metabolism biomarkers**

292 Our study addressed eight biomarkers of the energy metabolism in zebra mussels: ETS,  
293 digestive enzymes activities (ACP, lipase, LDH, amylase), energy reserves, CEA and condition  
294 index.

295 When addressing the interstation effects, we compared CM-Upstream, Cr-Upstream and  
296 Nam-Upstream1 data. Based on bioaccumulation and water chemistry results (*c.f.* S2.), water  
297 quality in Nam-Upstream2 tend to be impacted (though not significantly) by the Sambre river  
298 inflow, so we chose Nam-Upstream1 (located 5 km upstream of the Sambre-Meuse confluence)  
299 rather than Nam-Upstream2 for further analyses. Figure S1 shows graphical representations of  
300 PCA and KOPLS results according to stations and energy metabolism parameters. For PCA  
301 results, the first two principal components (PC1 and PC2) explained 64 59.9 % of total variance  
302 (figure S1 A and B). Namur individuals seem to have higher CEA and lipase activity. The other  
303 energy parameters (except CI) seemed to be higher in Charleville-Mézières and Charleroi  
304 mussels (figure S1 A and B). However, when applying the KOPLS analysis, no separation was

305 observed between groups, and the model was valid after permutation test ( $p=0.003$ ). Figure 2  
306 represents univariate statistical results of each biomarker. For condition index, amylase activity  
307 and CEA no significant differences were recorded. ACP, lipase and LDH activities, total  
308 reserves and ETS showed significant differences ( $p<0.05$ ) between Charleroi mussels and  
309 mussels from the other stations. Charleroi mussels notably displayed 1.2-fold higher energy  
310 reserves and a higher energy consumption rate (ETS activity 1.3 to 1.4-fold higher) compared  
311 to Charleville-Mézières and Namur mussels. In Namur individuals, ACP and LDH activities  
312 were 1.3 to 1.6 fold lower and lipase activity was 1.1 fold higher, compared to Charleville-  
313 Mézières (One-way ANOVA,  $df=2$ ,  $p<0.01$ ), which is in accordance with PCA results.

314         Considering intrastation effects, PCA results showed no separation between upstream  
315 and downstream of the three stations (Figure S2), while the univariate statistical test showed  
316 one in Charleville-Mézières and Namur but not in Charleroi (Figure 2). ACP and LDH activities  
317 were higher in Namur station (1.14 and 1.3-fold, respectively, Kruskal-Wallis,  $df=2$ ,  $p<0.03$ ),  
318 downstream of the discharge of the Sambre and Namur WWTP effluents into the Meuse (Nam-  
319 Upstream1 vs. Nam-downstream). However, although LDH activity was 1.3-fold lower in  
320 Nam-Upstream1 mussels than in Nam-Uptream2 mussels (Kruskal-Wallis,  $df=2$ ,  $p=0.01$ ), ACP  
321 activity did not differ, suggesting that only LDH activity was affected by the Sambre discharge  
322 into the Meuse. In Charleville-Mézières, mussels caged downstream of the WWTP had higher  
323 energy reserves (1.15-fold, Kruskal-Wallis,  $df=1$ ,  $p=0.02$ ) and displayed higher ETS activity  
324 (1.37-fold, One way ANOVA,  $df=1$ ,  $p<0.01$ ) than those caged upstream.

325         Digestive enzyme activities (LDH, lipase and ACP) were dependent on station and site  
326 (in Namur, except for lipase). Energy acquisition from ingested food is ensured in the first place  
327 by enzymatic digestion. Digestive enzymes are admittedly potential biomarkers in  
328 ecotoxicology to characterize the chemical contamination of aquatic environments (Dedourge-



329 Geffard et al., 2009; Hani et al., 2018; Palais et al., 2012; Wang et al., 2015). Decreased  
330 digestive enzyme activity has indeed been reported in various aquatic organisms (Chen et al.,  
331 2002; De Coen and Janssen, 1997b; Hani et al., 2018), although some authors reported no effect  
332 (Bourgeault et al., 2010). Our study reveals a high level of chemical contamination both in  
333 water and mussels in Charleroi station compared to the other stations (*c.f.* S2.). However,  
334 Charleroi mussels displayed higher, lower or similar digestive enzyme activities compared to  
335 mussels caged in the other stations. Besides, Nam-Downstream exhibited higher contamination  
336 by pharmaceuticals and iodinated contrast media (Table S4), which potentially caused the  
337 observed increase in ACP and LDH activities. Overall, our observations make it difficult to  
338 generalize links between digestive enzyme activity and site chemical pressures.

339 Charleroi mussels also displayed significantly higher ETS activity associated with  
340 greater energy reserves than the other stations. ETS activity is usually used to measure  
341 metabolic activity in the form of potential oxygen consumption, and has been proposed as an  
342 indicator of aquatic organism metabolism *in situ* (Cammen et al., 1990; Fanslow et al., 2001).  
343 We conducted our study in fall, when zebra mussels are at sexual rest and replenish their energy  
344 reserves (Palais et al., 2012). Our results suggest that Charleroi mussels had sufficient energy  
345 intake to restore their energy reserves, in parallel with high energy consumption, although the  
346 water in the area was the most contaminated (*c.f.* S2.). The higher ETS activity associated with  
347 higher energy reserves was also observed in CM-Downstream compared to CM-Upstream,  
348 although the effluents of this station did not seem to influence chemical water quality or the  
349 contamination state of mussels (*c.f.* S2.).

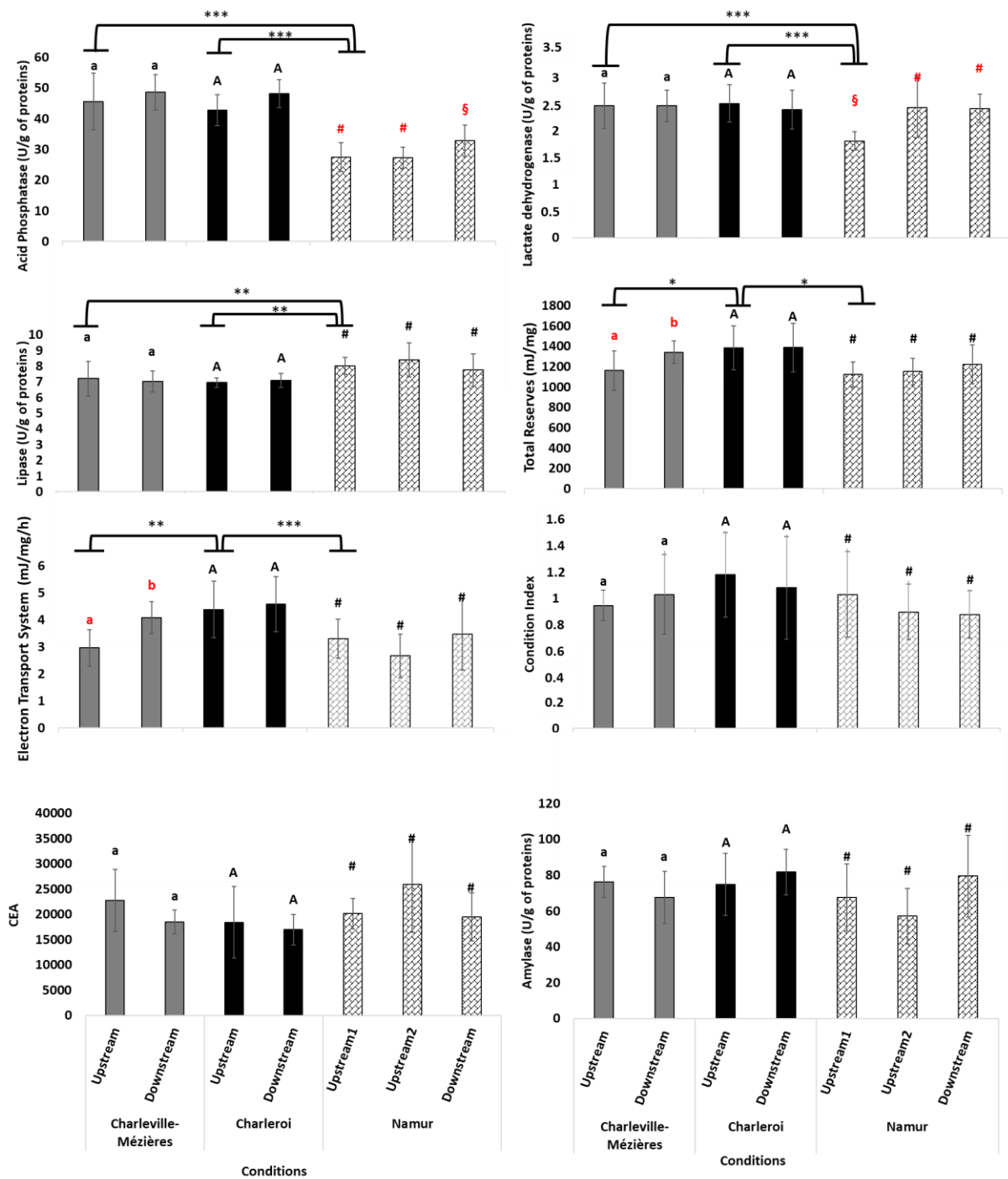
350 Energy is the most determining biological parameter involved in all the physiological  
351 processes of living beings; hence, energy metabolism parameters can be useful biomarkers of  
352 the effects of chemicals on different aquatic organisms (De Coen et al., 2000; Dedourge-

353 Geffard et al., 2012; Hani et al., 2018; Palais et al., 2012). In our study we addressed only 8  
354 biomarkers related to energy metabolism, and the multi- and univariate statistical analysis  
355 results evidenced an emerging trend that potentially differentiated the different experimental  
356 groups. However, highlighting a clear response pattern associated with chemical contamination  
357 levels remains difficult. This suggests that the response of energy-related biomarkers could not  
358 only be linked to chemical contamination but also to other environmental parameters  
359 representing confounding factors in *in situ* studies, even if we proceeded with a methodology  
360 which aimed to limit the influence of these factors. One solution could be to address many  
361 parameters at the same time and then to identify *a priori* a group of potential inter-related  
362 biomarkers whose impact could draw a response pattern clearly reflecting the health status of  
363 organisms and the chemical state of their environment. This is why a global metabolomics  
364 approach was applied to identify potential biomarkers and better understand mussel responses  
365 to water quality.

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370 **Figure 2: Energy metabolism biomarkers after 2 months of mussels caging upstream and**  
 371 **downstream of three wastewater treatment stations (Charleroi, Charleville-Mézières,**  
 372 **Namur). Results are presented as mean ± S.D.** For the same station, different letters indicate significant  
 373 differences between upstream and downstream samples (Intrastation effects,  $p < 0.05$ ). Asterisks indicate significant interstation  
 374 differences (Charleroi vs. Charleville-Mézières vs. Namur; \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ).

### 375           **3.3.   <sup>1</sup>H-NMR metabolomics**

376           Among the metabolites identified in Prud'homme et al. (2020), we detected and  
377 assigned 28 compounds, including 11 proteinogenic amino acids (alanine, aspartate, glutamate,  
378 glutamine, glycine, isoleucine, leucine, lysine, phenylalanine, tyrosine, valine), 5 amine  
379 compounds (betaine, cadaverine, choline, putrescine, trimethylamine), 3 nucleotides (ATP,  
380 ADP, AMP), 3 nucleosides (adenosine, uridine, inosine), 2 organic acids (succinate, lactate), 2  
381 carbohydrates (glucose, maltose), 1 alkaloid (nicotinic acid), and 1 coenzyme (NAD).

382           To visualize the interstation effects on the zebra mussel metabolome, we compared the  
383 same data as we did for biomarkers, *i.e.*, CM-Upstream, Cr-Upstream and Nam-Upstream1. A  
384 non-supervised PCA was first performed. The score plot (Figure 3A) highlighted a clear  
385 separation between the three stations (especially for Charleroi), in line with energy biomarker  
386 results. The first two dimensions of the PCA expressed about 89 % of the total variance, mostly  
387 explained by PC1 (83 %,  $p<0.01$ ). The metabolomic profiles clearly differed among stations,  
388 suggesting that the mussels' metabolic state was influenced by the local environmental  
389 conditions. A subsequent supervised approach was used to identify metabolites with a higher  
390 contribution to this interstation discrimination. The K-OPLS model confirmed the differences  
391 in metabolomic profiles among stations (Figure 3B). Our model was highly predictive ( $Q^2=77$   
392 %), and explained 80 % of interstation variability, and the permutation test confirmed its  
393 validity ( $p=0.02$ ). In K-OPLS, an important feature is the variable importance in projection  
394 (VIP) scores. High VIP-score variables strongly contribute to the model construction. In total,  
395 80 variables (including assigned and non-assigned buckets) with a  $VIP>1.2$  were observed;  
396 three of them were identified as being lactate ( $VIP= 1.32$ ), glycine ( $VIP= 1.27$ ) and maltose  
397 ( $VIP= 1.25$ ). The others were mainly located in the 3-4 ppm region of the spectrum,  
398 corresponding to a highly complex region where resonance of carbohydrates, amino acids and

399 nucleotides overlapped, making their identification difficult, as explained by Prud'homme et  
400 al. (2020). Then, we used univariate analysis (Kruskal-Wallis nonparametric test) to verify if  
401 metabolite abundance differed significantly among mussels from the different stations. Lactate  
402 was more abundant in mussels from Charleroi, followed by Charleville-Mézières and then  
403 Namur (Kruskal-Wallis,  $df=2$ ,  $p<0.01$ , Figure S3 A). A significant difference was also observed  
404 for glycine (Kruskal-Wallis,  $df=2$ ,  $p=0.01$ ) and maltose (Kruskal-Wallis,  $df=2$ ,  $p=0.04$ ), in line  
405 with lactate (higher in Charleroi mussels than in the other groups) (Figure S3 B and C,  
406 respectively). Those three metabolite abundances seemed to be particularly impacted by  
407 station-specific environmental conditions.

408         Concerning intrastation (upstream/downstream) effects, PCA results (Figure 4 A, C, E)  
409 showed no significant separations between upstream and downstream in Charleroi and  
410 Charleville-Mézières. However, Nam-Downstream mussels were slightly distinguished from  
411 Nam-Upstream1 and Nam-Upstream2 mussels, with variability mostly explained by PC1 (85  
412 %,  $p=0.02$ ). Supervised K-OPLS was then applied to search for a significant separation between  
413 the upstream and downstream sites of each station (Figure 4 B, D, F). K-OPLS showed a great  
414 upstream vs. downstream separation in Namur and Charleville-Mézières, but not in Charleroi.  
415 The absence of effects in Charleroi could be explained by the high contamination level of the  
416 Sambre both upstream and downstream of the WWTP. Interestingly, although Charleroi cages  
417 were 2 km apart (Table 1), the mussels' metabolome was highly modulated compared to the  
418 other stations, and in the same way upstream and downstream. The generated statistical models  
419 were valid ( $p<0.05$ ), predictive ( $Q^2= 56\%$  and  $46\%$ ), and explained  $33\%$  and  $82\%$  of  
420 variability in Charleville-Mézières (Figure 4B) and Namur (Figure 4F), respectively. K-OPLS  
421 satisfactorily explained and predicted metabolome variation. This variation was explained by  
422 49 and 60 variables (including assigned and non-assigned buckets) with a VIP score  $>1.20$  for  
423 Charleville-Mézières and Namur, respectively. Among these, 8 metabolites were identified to

424 be glutamine (VIP=2.07), putrescine (VIP=1.71), ADP/ATP (VIP=1.53), trimethylamine  
425 (VIP=1.51), cadaverine (VIP=1.40), AMP (VIP=1.32), lysine (VIP=1.22) and UDP/UTP  
426 (VIP=1.20) in Charleville-Mézières sites. Kruskal-Wallis tests showed no significant difference  
427 between CM-Upstream and CM-Downstream ( $p>0.05$ ) for these metabolites; moreover,  
428 unsupervised multivariate PCA failed to clearly discriminate upstream mussels from  
429 downstream mussels in Charleville-Mézières, suggesting that intrastation changes identified by  
430 supervised K-OPLS (which explained only 33 % of data variability) may have been masked by  
431 high interindividual variability, leading to inconclusive univariate approaches. High  
432 interindividual metabolome variability could be due to a gender effect, as previously evidenced  
433 in bivalves (Hines et al., 2007). Our study was conducted when zebra mussels were at sexual  
434 rest, a period where gender identification is difficult since the gonad are not differentiated. In  
435 Namur, only 2 metabolites were identified: glutamate (VIP=1.29) and lactate (VIP=1.24). Both  
436 of them were lower in Nam-Upstream1 than in the other two sites, but only significantly  
437 (Dunn's test,  $p<0.05$ ) when compared to Nam-Downstream (Figure S4). Therefore, lactate was  
438 higher when the pollution was higher, as in Nam-Downstream and Charleroi compared to the  
439 other stations. Other variables (non-identified buckets) highly contributed to site separation but  
440 were mainly located in the 3-4 ppm region of the NMR spectrum and could not be associated  
441 with a specific metabolite.

442 Both metabolomics and classical biomarker approaches distinguished Charleroi from  
443 the other stations, and the upstream/downstream of Namur and Charleville-Mézières stations.  
444 In our study, metabolomics was more efficient than the chosen biomarkers, perhaps because  
445 with the classical approach we addressed only 8 biomarkers, whereas with metabolomics we  
446 addressed several parameters at the same time without a priori, which broadens the potential to  
447 identify a clear response pattern related to our experimental conditions. Metabolomics probably  
448 identified other markers more informative about the effect of water quality than the

449 conventional biomarkers addressed in this study. This underlines the potential and  
450 complementarity of the two approaches in the biomonitoring of aquatic environments.

451 Metabolomics is a novel branch in ecotoxicology (Lin et al., 2006), and can be efficient  
452 to assess the health status of organisms by measuring small metabolites involved in different  
453 aspects of physiological processes (Brew et al., 2020; Nguyen and Alfaro, 2020). The  
454 production and levels of these metabolites can be modulated by physiological and/or  
455 environmental factors (Campillo et al., 2019; Cappello et al., 2013; Dumas et al., 2020; Lin et  
456 al., 2006). We identified 4 metabolites (glycine, glutamate, maltose and lactate) involved in  
457 different metabolic pathways and significantly impacted by the experimental conditions. As all  
458 mussels originated from the same population, metabolite modulation is attributable to changes  
459 in environmental conditions in each station and site.

460 Lactate (2-hydroxypropanoate), is a hydroxycarboxylic acid, and one of the end-  
461 products of anaerobic pyruvate metabolism (De Zwaan and Dando, 1984). The significant  
462 increase in lactate in Charleroi mussels compared to the other stations (interstation effect) and  
463 in those of Nam-downstream compared to the other Namur sites (intrastation effect) suggests a  
464 disturbance of the mussels' energy metabolism inducing higher anaerobic energy production.  
465 This can presumably be explained by pollution-induced closing of mussels valves, which  
466 obviously stimulated the anaerobic metabolism of our experimental animals. Closure of valves  
467 is one of the behavioral adaptations of mussels to limits the harmful effects of pollutants which  
468 leads to a switch to anaerobic pathways (Isani et al., 1995; Madon et al., 1998; Rist et al., 2016;  
469 Slooff et al., 1983; Wright et al., 2013). In green mussels (*Perna viridis*), higher lactate was  
470 observed after one and two weeks' exposure to cadmium, copper and their combination, and  
471 the authors supposed that the mussels' energy metabolism was impacted by this exposure (Wu  
472 and Wang, 2010, 2011). Lactate production has been reported as an energy modulation strategy

473 in aquatic organisms under different stress conditions. For example, in *Perna canaliculus*  
474 mussels, lactate was one of 38 identified metabolites detected 24 h post injection with *Vibrio*  
475 *sp.* Lactate accumulation suggested that *Vibrio sp.* impacted the mussels' energy metabolism  
476 by limiting pyruvate conversion into citrate (Thao V. Nguyen et al., 2018). In olive flounder  
477 fish (*Paralichthys olivaceus*), lactate was one of the metabolites identified by Kim et al. (2020)  
478 after thermal stress. Additionally, when NMR metabolomics was used to evaluate the freshness  
479 of commercial mussels (*Mytilus galloprovincialis*), lactate was one of the metabolites that  
480 discriminated fresh mussels from stored ones (Aru et al., 2016). Hence, lactate is a potential  
481 biomarker of the health status of zebra mussels in ecotoxicological studies. Lactate is produced  
482 by a reversible redox reaction catalyzed by the enzyme lactate dehydrogenase (LDH) (Adeva-  
483 Andany et al., 2014; Le et al., 2010). The significant decrease in lactate in Nam-Upstream1 and  
484 increase in Charleroi were corroborated by our LDH assay results, which revealed a low activity  
485 in Nam-Upstream1 compared to the other stations and other Namur sites (Figure 2). This  
486 interesting result supports the complementary aspect of metabolomics, which identified new  
487 parameters likely to complete the set of energy biomarkers currently used to evaluate the health  
488 status of aquatic organisms.

489 Glycine and glutamate are free amino acids that were higher in Charleroi mussels  
490 (compared to the other stations) and in Nam-Upstream2 and Nam-Downstream mussels  
491 (compared to Nam-Upstream1). This could suggest changes in zebra mussel osmotic regulation  
492 and anaerobic energy metabolism under our experimental conditions. In bivalves, free amino  
493 acids are important in osmoregulation and considered as end-products, substrates or  
494 intermediates of anaerobic energy metabolism (Zurbug and De Zwaan, 1981). Zebra mussels  
495 are hyperosmotic regulators (Dietz et al., 1996), and hyperosmotic regulation in mollusks  
496 appears to depend on the synthesis of amino acids involving anaerobic pathways (Zurbug and  
497 De Zwaan, 1981). Four anaerobic pathways are known in invertebrates: the aspartate-succinate,



498 glucose-succinate, opine, and lactate pathways (Lee and Lee, 2011). In bivalves, the anaerobic  
499 metabolism is dominated by the opine pathway involving opine dehydrogenases (*e.g.*,  
500 strombine dehydrogenase (SDH), octopine dehydrogenase (ODH)), whereas in vertebrates the  
501 lactate pathway (involving LDH) is the main route for anaerobic energy production (De Zwaan  
502 and Dando, 1984; Lee and Lee, 2011). Gäde and Zebe (1973) found that ODH played a more  
503 important role than LDH in zebra mussels, which supports the predominance of the opine  
504 pathway in their anaerobic metabolism. We observed glycine accumulation in Charleroi (the  
505 most contaminated station) mussels. Glycine is known to be catalyzed anaerobically (with  
506 pyruvate and NADH) by SDH to produce energy (one of the opine pathways) (Lee and Lee,  
507 2011). Glycine and lactate accumulation could suggest that under our experimental conditions,  
508 the mussels' opine pathway (represented by SDH activity) was inhibited and anaerobic energy  
509 production was ensured by the lactate pathway. This corroborates the energy metabolism  
510 disturbance hypothesis. This also supports the relevance of lactate as a potential biomarker in  
511 zebra mussels. In addition, glycine and glutamate with cysteine are also involved in the  
512 glutathione metabolic pathway (production of glutathione "GSH") (Thao V Nguyen et al.,  
513 2018). GSH is an important cellular thiol, involved in different biological processes such as the  
514 protection of cells against the toxic effects of a variety of endogenous and exogenous  
515 compounds, including oxygen reactive species and pollutants (antioxidant role) (Canesi et al.,  
516 1999). The accumulation of glycine and glutamate could represent a signature of a possible  
517 oxidative stress due to the disruption of the anaerobic metabolism of zebra mussels under our  
518 experimental conditions.

519 Maltose was higher in mussels of Charleroi and Charleville-Mézières compared to  
520 Namur individuals, and could suggest a disturbance of the energy metabolism of zebra mussels  
521 under our experimental conditions.

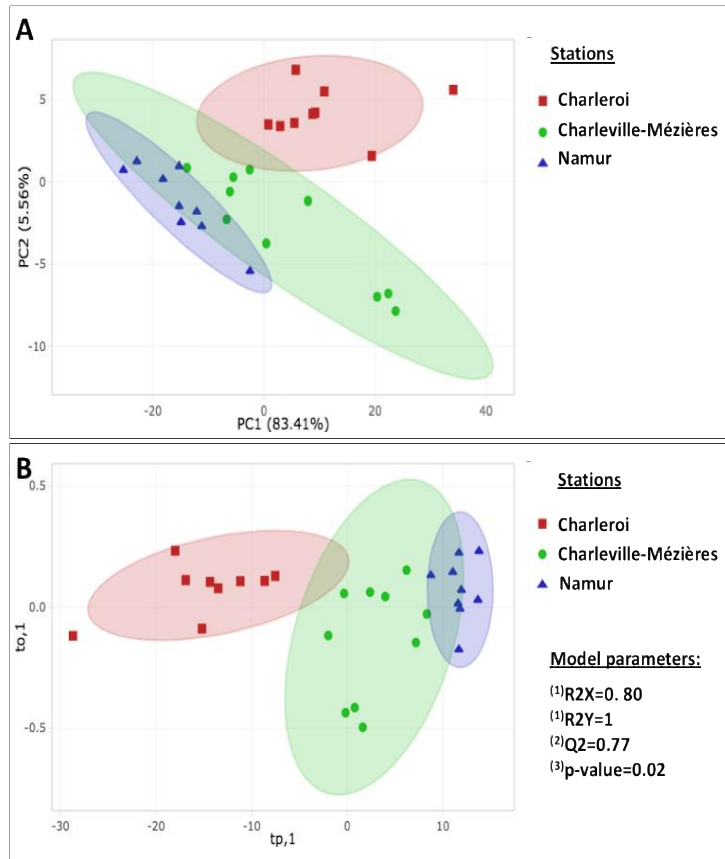
522 <sup>1</sup>H-NMR metabolomics emerges as a strong and sensitive approach that could be used  
523 to assess the global health status of zebra mussels in biomonitoring of aquatic environments.  
524 Using this global approach, we identified different metabolic profiles among mussels from the  
525 same provenance and the same population, caged no more than 100 m apart, upstream and  
526 downstream of two WWTPS (Namur and Charleville-Mézières). <sup>1</sup>H-NMR metabolomic results  
527 were consistent with the contamination states of the different stations/sites, and allowed us to  
528 identify 4 metabolites (lactate, maltose, glutamate and glycine). One of them (lactate) is a  
529 potential future biomarker of the health status of zebra mussels in ecotoxicological studies.  
530 Interestingly, one of the “classical approach” energy biomarkers – LDH – was modulated  
531 together with its substrate – lactate – identified by the metabolomic approach; this underlines  
532 the potential of metabolomics, especially if combined with a classical biomarker approach. One  
533 advantage of metabolomics is that it focuses on several metabolites at a time, with significant  
534 time saving and the possible identification of potential biomarkers targetable individually  
535 thereafter. Therefore, we suggest using metabolomics without *a priori* in the first place to  
536 identify metabolites and subsequently evaluate and validate their potential as biomarkers.

537 The combination of metabolomics and classical biomarkers was already reported in the  
538 literature in several marine organisms including fish and bivalves (Brandão et al., 2015; Caricato  
539 et al., 2019; Digilio et al., 2016; Ji et al., 2015), however, to our knowledge this is the first study  
540 that combines the use of biomarkers and metabolomics to assess the health status of zebra  
541 mussels.

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546 **Figure 3: PCA score plots (A) and Kernel-Orthogonal Projections to Latent Structures**

547 **(K-OPLS) model (B) of mussels after 2 months of mussel caging in Charleville-Mézières**

548 **upstream, Charleroi upstream and Namur upstream 1 (data of interstation effect).** Ellipses

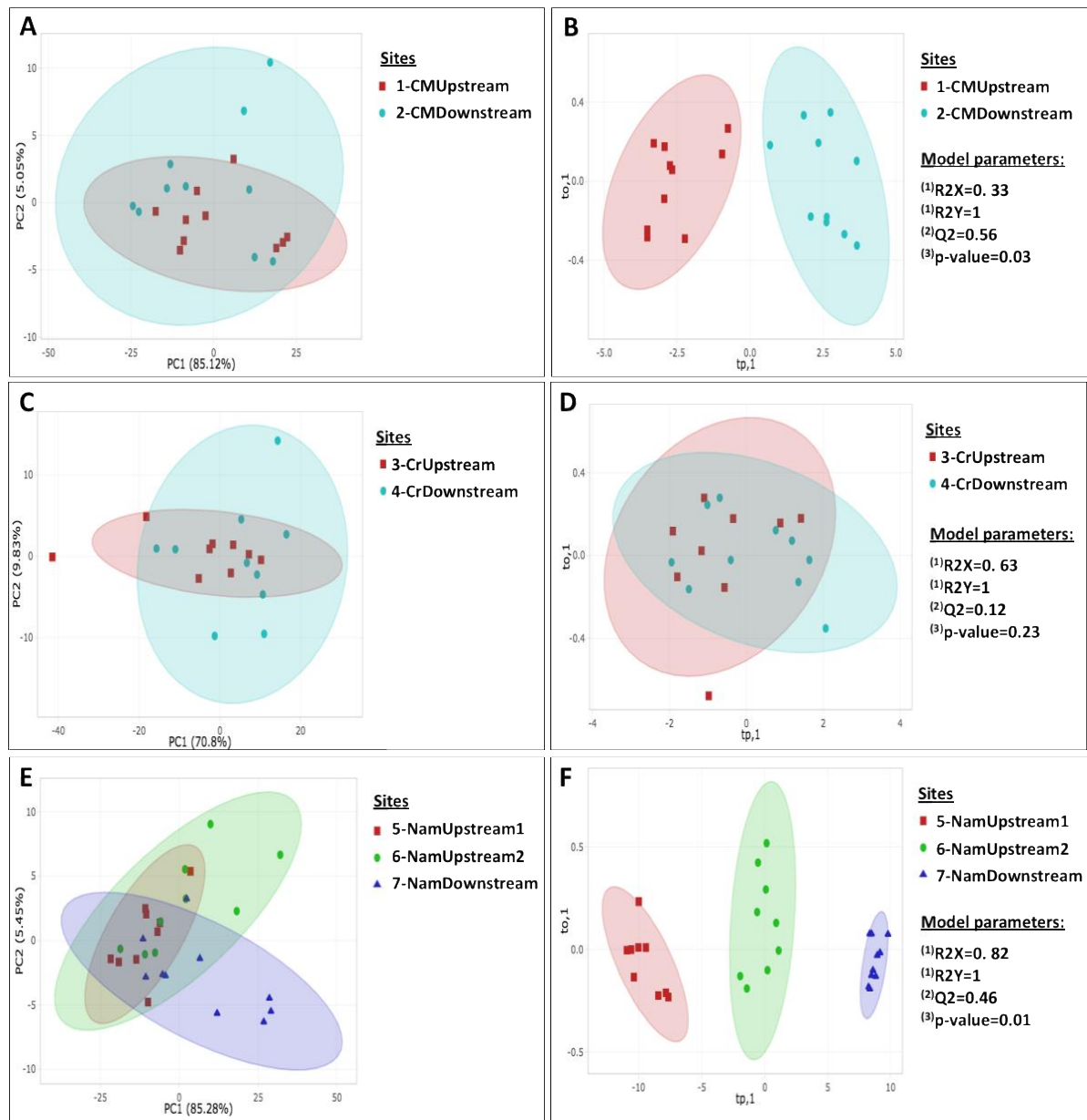
549 were drawn with a 95 % confidence interval. In (B), K-OPLS model validation parameters were: (1) R2X and R2Y, which

550 represent the cumulative explained variation for all model components; (2) Q2, the predictive power of the model; and (3) the

551 *p*-value of the permutation test (model valid when  $p < 0.05$ ).

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555 **Figure 4: PCA score plots (A, C, E) and Kernel-Orthogonal Projections to Latent**

556 **Structures (K-OPLS) models (B, D, F) showing mussel separation upstream and**

557 **downstream of Charleville-Mézières (A and B), Charleroi (C and D) and Namur (E and**

558 **F) stations (data of intrastation effect). Ellipses were drawn with a 95 % confident interval. For (B), (D) and**

559 (F), K-OPLS model validation parameters were: (1) R2X and R2Y, which represent the cumulative explained variation for all

560 model components; (2) Q2, the predictive power of the model; and (3) the *p*-value of the permutation test (model valid when

561 *p*<0.05).

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#### 4. Conclusion

564 Our study demonstrates the relevance of <sup>1</sup>H-NMR metabolomics as an efficient  
565 approach to assess the health status of zebra mussels in the biomonitoring of aquatic  
566 environments. <sup>1</sup>H-NMR metabolomics showed a clear separation of the mussels' profiles  
567 according to experimental conditions. This separation was also observed by the classical  
568 approach (energy biomarkers), but was not very marked. Our results have nevertheless  
569 demonstrated the potential and the corroboration of these two approaches by identifying a  
570 metabolite (*i.e.* lactate) whose abundance was impacted by experimental conditions; in parallel,  
571 this impact was observed by the conventional approach, on the enzyme (LDH) which catalyzes  
572 the reaction responsible for the production of this metabolite. In addition to lactate, the  
573 metabolomic approach made it possible to identify 3 other metabolites (glutamate, maltose and  
574 glycine), which were also significantly impacted by the experimental conditions of our study,  
575 which suggests that local pollution alters the osmoregulation and energy metabolism of zebra  
576 mussels. To our knowledge, our study is the first to combine metabolomics and classical  
577 biomarkers to evaluate the effect of field contamination on the health status of zebra mussels.

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