^{1}H-NMR metabolomics profiling of zebra mussel (*Dreissena polymorpha*): a field-scale monitoring tool in ecotoxicological studies

Authors

Younes Mohamed Ismail HANI[1,2*], Sophie Martine PRUD'HOMME[1,3], Jean-Marc NUZILLARD[4], Isabelle BONNARD[1], Christelle ROBERT[5], Katherine NOTT[6], Sébastien RONKART[6], Odile DEDOURGE-GEFFARD[1], Alain GEFFARD[1]

[1] *Université de Reims Champagne-Ardenne (URCA), UMR-I 02 SEBIO (Stress Environnementaux et Biosurveillance des milieux aquatiques), Moulin de la Housse, Reims, France.*

[2] *Université de Bordeaux, UMR EPOC 5805, équipe Ecotoxicologie aquatique, Place du Dr Peyneau, 33120 Arcachon, France* (present address)

[3] *Université de Lorraine, CNRS, LIEC, F-57000, Metz, France* (present address)

[4] *Université de Reims Champagne Ardenne, CNRS, ICMR UMR 7312, 51097 Reims, France*

[5] *CERGroupe Marloie, Wallonia, Belgium*

[6] *La société wallonne des eaux, rue de la Concorde 41, 4800 Verviers, Belgium*

*Corresponding authors:

younes.hani@u-bordeaux.fr (permanent address: haniyounes@hotmail.fr)

Abstract

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

Keywords: active biomonitoring, *Dreissena polymorpha*, energy metabolism, metabolomics, ^{1}H NMR

Caption: Results of this study showed the interest of ^{1}H-NMR metabolomics as an efficient approach in biomonitoring of aquatic environments, and lactate could be a potential biomarker.

1. Introduction

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

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

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

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

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

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

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

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

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

2. Materials and methods

2.1. Experimental design

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

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

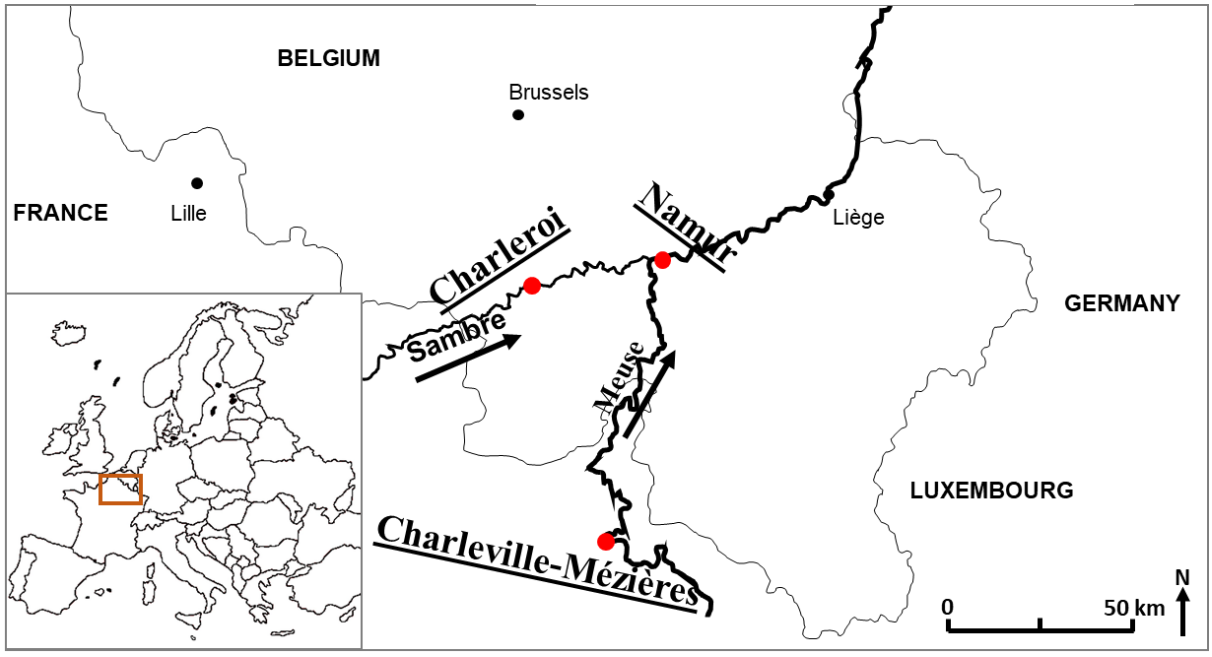


Figure 1: Geographical localizations of the experimental stations. Arrows: stream direction of the Meuse and Sambre rivers.

163 **Table 1: GPS coordinates and water physico-chemical parameters (mean ± 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 (µS/cm)
Charleville-Mézières (CM)	**Upstream**	E 04°43'44.7" N 49°45'56.3"	100	11 ± 3	10 ± 0	8 ± 0	597 ± 13
	Downstream	E 04°43'46.9" N 49°45'59.6"	-	11 ± 3	10 ± 0	8 ± 0	598 ± 13
Charleroi (Cr)	**Upstream**	E 04°27'19.4" N 50°24'07.8"	2,000	12 ± 3	8 ± 1	8 ± 0	673 ± 16
	Downstream	E 04°29'27.4" N 50°23'44.7"	-	12 ± 3	6 ± 2	8 ± 0	1025 ± 172
Namur (Nam)	**Upstream1**	E 04°51'38.8" N 50°26'38.4"	8,000	13 ± 4	11 ± 0	8 ± 0	537 ± 39
	Upstream2	E 04°57'30.5" N 50°28'49.8"	40	13 ± 4	10 ± 0	8 ± 0	741 ± 147
	Downstream	E 04°57'32.9" N 50°28'49.4"	-	13 ± 3	10 ± 1	8 ± 0	763 ± 141

2.2. Sample preparation

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

Water samples were collected at least 2 times per month and per site, at the beginning and at the end of the month, in amber 0.5-L glass bottles, and stabilized by adding sodium thiosulfate (2.5 mL of $Na_2S_2O_3.5H_2O$ at 1.9 g/L). The samples were then stored at 5 ± 3 °C in the dark and extracted within 48 hours of sampling.

2.3. Chemical analyses

2.3.1. Water analysis

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

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

2.3.2. Mussel bioaccumulation analysis

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

2.4. Core biomarker analyses

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

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

according to De Coen and Janssen (1997a). ETS activity was then transformed into energy equivalents using an average oxyenthalpic equivalent of 480 kJ/mol O_2, and results were expressed as mJ/mg wet wt/h (De Coen and Janssen, 1997a).

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

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

Finally, an integrative biomarker (CEA: cellular energy allocation) was calculated after determining Ea and Ec, according to the following formula: [CEA = Ea/Ec] (Verslycke et al., 2004).

2.5. ^{1}H-NMR metabolomics

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

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

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

2.6. Statistical analyses

2.6.1. Biological and chemical parameters

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

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

2.6.2. Metabolomics data

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

3. Results and discussion

3.1. Water quality and mussel exposure to field contamination

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

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

3.2. Energy metabolism biomarkers

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

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

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

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

Digestive enzyme activities (LDH, lipase and ACP) were dependent on station and site (in Namur, except for lipase). Energy acquisition from ingested food is ensured in the first place by enzymatic digestion. Digestive enzymes are admittedly potential biomarkers in 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-

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

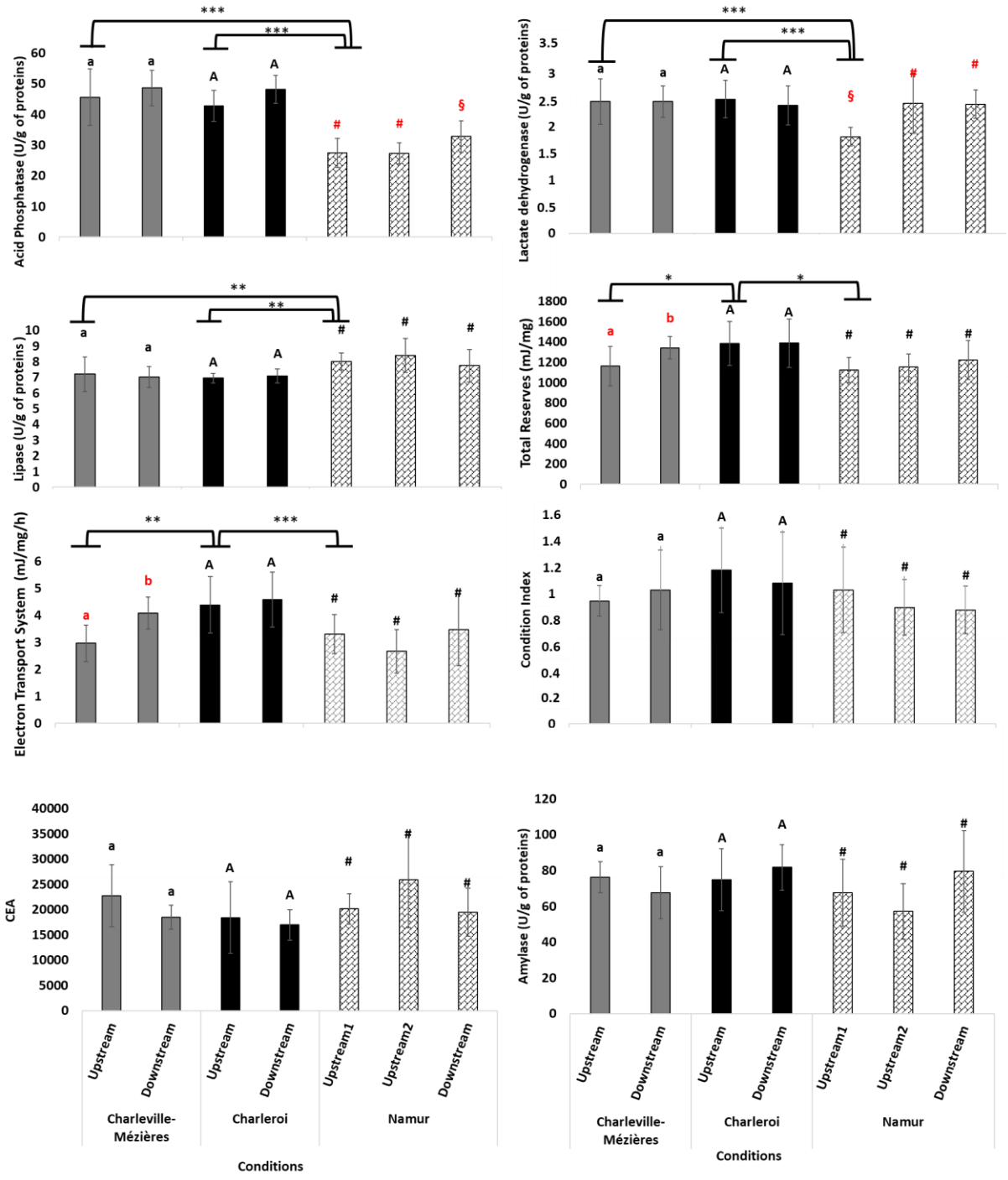


Figure 2: Energy metabolism biomarkers after 2 months of mussels caging upstream and downstream of three wastewater treatment stations (Charleroi, Charleville-Mézières, Namur). Results are presented as mean ± S.D. For the same station, different letters indicate significant differences between upstream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

3.3. ^{1}H-NMR metabolomics

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

To visualize the interstation effects on the zebra mussel metabolome, we compared the same data as we did for biomarkers, *i.e.*, CM-Upstream, Cr-Upstream and Nam-Upstream1. A non-supervised PCA was first performed. The score plot (Figure 3A) highlighted a clear separation between the three stations (especially for Charleroi), in line with energy biomarker results. The first two dimensions of the PCA expressed about 89 % of the total variance, mostly explained by PC1 (83 %, $p<0.01$). The metabolomic profiles clearly differed among stations, suggesting that the mussels' metabolic state was influenced by the local environmental conditions. A subsequent supervised approach was used to identify metabolites with a higher contribution to this interstation discrimination. The K-OPLS model confirmed the differences in metabolomic profiles among stations (Figure 3B). Our model was highly predictive (Q2=77 %), and explained 80 % of interstation variability, and the permutation test confirmed its validity ($p=0.02$). In K-OPLS, an important feature is the variable importance in projection (VIP) scores. High VIP-score variables strongly contribute to the model construction. In total, 80 variables (including assigned and non-assigned buckets) with a VIP>1.2 were observed; three of them were identified as being lactate (VIP= 1.32), glycine (VIP= 1.27) and maltose (VIP= 1.25). The others were mainly located in the 3-4 ppm region of the spectrum, corresponding to a highly complex region where resonance of carbohydrates, amino acids and

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

Concerning intrastation (upstream/downstream) effects, PCA results (Figure 4 A, C, E) showed no significant separations between upstream and downstream in Charleroi and Charleville-Mézières. However, Nam-Downstream mussels were slightly distinguished from Nam-Upstream1 and Nam-Upstream2 mussels, with variability mostly explained by PC1 (85 %, $p=0.02$). Supervised K-OPLS was then applied to search for a significant separation between the upstream and downstream sites of each station (Figure 4 B, D, F). K-OPLS showed a great upstream *vs*. downstream separation in Namur and Charleville-Mézières, but not in Charleroi. The absence of effects in Charleroi could be explained by the high contamination level of the Sambre both upstream and downstream of the WWTP. Interestingly, although Charleroi cages were 2 km apart (Table 1), the mussels' metabolome was highly modulated compared to the other stations, and in the same way upstream and downstream. The generated statistical models were valid ($p<0.05$), predictive (Q2= 56 % and 46 %), and explained 33 % and 82 % of variability in Charleville-Mézières (Figure 4B) and Namur (Figure 4F), respectively. K-OPLS satisfactorily explained and predicted metabolome variation. This variation was explained by 49 and 60 variables (including assigned and non-assigned buckets) with a VIP score >1.20 for 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

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

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

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

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

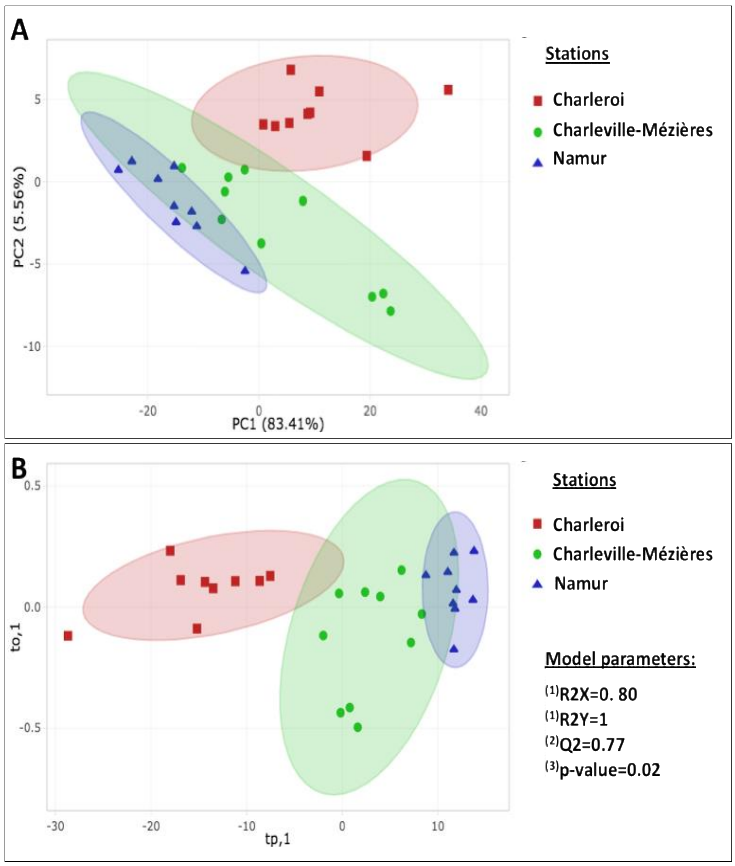
Glycine and glutamate are free amino acids that were higher in Charleroi mussels (compared to the other stations) and in Nam-Upstream2 and Nam-Downstream mussels (compared to Nam-Upstream1). This could suggest changes in zebra mussel osmotic regulation and anaerobic energy metabolism under our experimental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

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

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



545

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 mussels 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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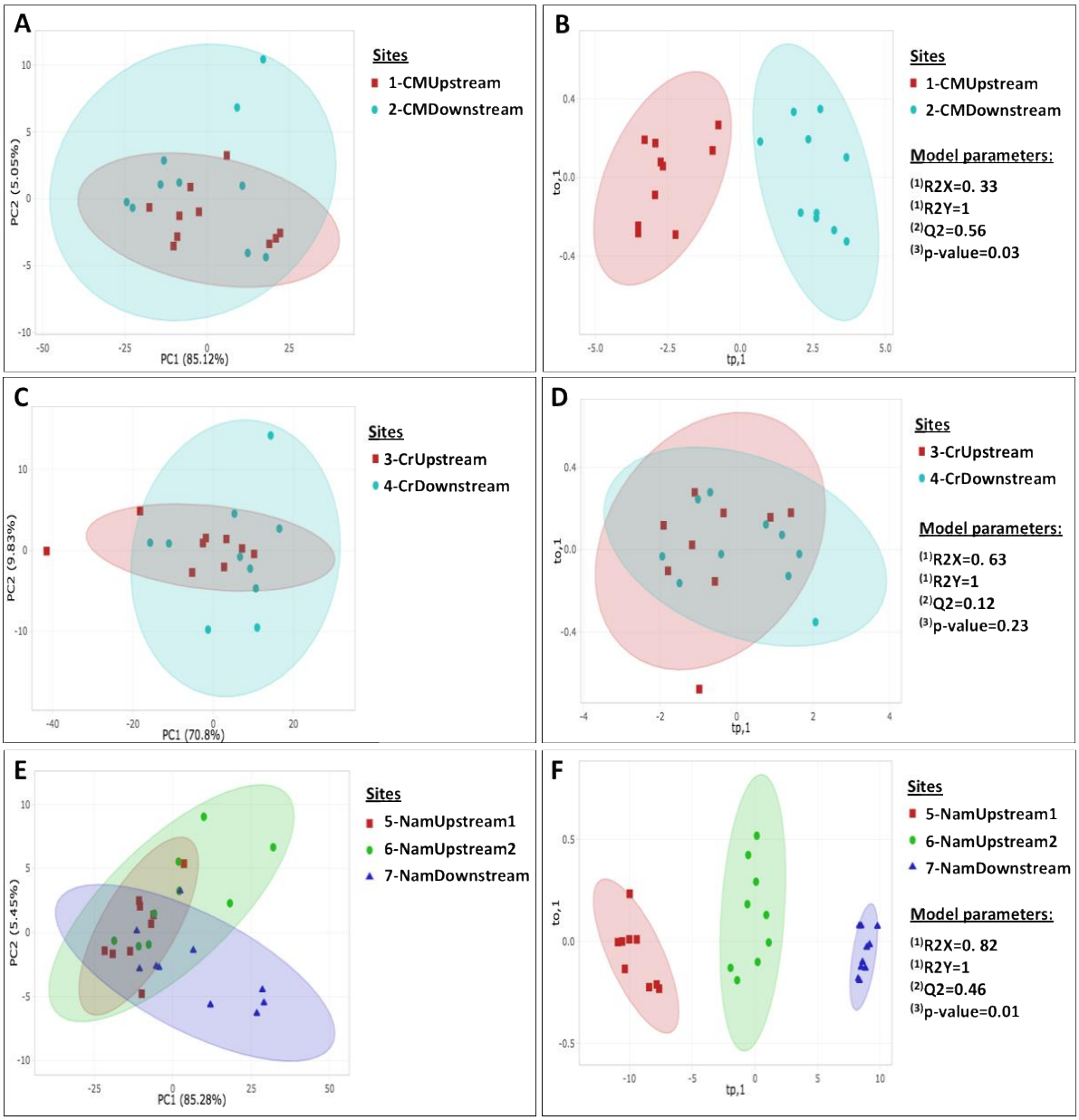

Figure 4: PCA score plots (A, C, E) and Kernel-Orthogonal Projections to Latent Structures (K-OPLS) models (B, D, F) showing mussel separation upstream and downstream of Charleville-Mézières (A and B), Charleroi (C and D) and Namur (E and F) stations (data of intrastation effect). Ellipses were drawn with a 95 % confident interval. For (B), (D) and (F), K-OPLS model validation parameters were: (1) R2X and R2Y, which represent the cumulative explained variation for all model components; (2) Q2, the predictive power of the model; and (3) the *p*-value of the permutation test (model valid when $p<0.05$).

4. Conclusion

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

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