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1 **The variegated scallop, *Mimachlamys varia*, undergoes alterations in several of its metabolic**
2 **pathways under short-term zinc exposure**

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6
7 **Abstract**

8 The variegated scallop (*Mimachlamys varia*) is a filter feeder bivalve encountered in marine regions of the
9 Atlantic coast. In particular, it is present in the La Rochelle marina (France), where it is used for the
10 biomonitoring of marine pollution, due to its ability to strongly bioaccumulate pollutants. In this semi-closed
11 environment, contamination generated by port activities leads to an accumulation of both organic and metal
12 pollutants. Zinc is one of these pollutants, present at a dose of up to 150 to $\mu\text{g}\cdot\text{L}^{-1}$. This study investigated the
13 effects of 48 h zinc exposure upon the metabolic profiles of *Mimachlamys varia* using UHPLC/QToF (ultra-
14 high performance liquid chromatography-quadrupole time-of-flight) tandem mass spectrometry
15 metabolomics. After acclimation in mesocosms recreating *in situ* conditions, both controls and exposed with
16 Zn^{2+} (150 $\mu\text{g}\cdot\text{L}^{-1}$) bivalves were dissected to recover the gills after 48 h and stored at -80°C before
17 metabolites extraction. UHPLC/QToF tandem mass spectrometry was performed to study metabolite
18 composition of samples. Statistical analysis of results using multivariate techniques showed a good
19 classification between control and exposed groups. Eleven identified metabolites were found to be down-
20 modulated in exposed scallops. These variations could reflect potential zinc effects on several of the
21 biological processes, such as energy metabolism, osmoregulation and defense against oxidative stress.
22 Among the eleven metabolites highlighted, four were reported for the first time in an aquatic organism
23 exposed to Zn. This study demonstrates once again the diversity of interactions between bivalves and metals
24 and the complexity of the physiological response of marine bivalves to pollutants.

25
26 **Keywords:** Zinc, UHPLC/QToF mass spectrometry, metabolomics, *Mimachlamys varia*.

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32

33 **Introduction**

34 Marine ecosystems are the final receptacle of contaminants released to the environment, especially in
35 seaports and other industrialized or strongly anthropised coastal areas. Boat harbours, for instance, may be
36 hotspots of contaminant accumulation (Bighiu et al., 2017; Johnston et al., 2011). Indeed, they are likely to
37 receive both a cumulative load of pollutants from upstream point and nonpoint sources from watershed, and
38 many pollutants generated at the marina itself. In addition, the construction of a marina generally creates a
39 condition of reduced water circulation and protection from wave action. The result is an area with limited
40 natural water circulation and exchange. The marina of La Rochelle, located in the south-west of France,
41 perfectly illustrates this phenomenon with its complex geometry and infrastructure, especially since its
42 expansion in 2014 (Huguet et al., 2019). Over time, this reduced circulation and increased pollutant
43 generation can increase pollutant concentrations in the water column, sediments, and aquatic organisms. This
44 often results in greater impacts in the area concerned on ecological communities (Sim et al., 2015). Because
45 of their environmental persistence, heavy metals are one of the major harmful toxic pollutants for the marine
46 environment (Stankovic et al., 2014) and especially in harbours and marina (Bighiu et al., 2017; Galkus et
47 al., 2012). The major proportion of these trace elements is trapped in coastal sediments where they can be
48 continuously released into the water column and incorporated into food webs. The availability of trace
49 elements to aquatic species is of prime concern both in terms of the prediction of the effects of metal
50 pollution on ecosystems and in terms of public health when bioaccumulated in edible animals.

51 In the following study, we focused attention on Zn pollution, at a concentration such as the maximum
52 concentration that can be reached in the marina of La Rochelle. Zinc is found at $157 \mu\text{g}\cdot\text{L}^{-1}$ in the water of
53 the Marillac area, which is one of the four docks that make up the marina of les Minimes (CREOCEAN,
54 2018). It may arise from nonpoint sources from watershed : rainwater runoff sources (outdoor zinc or
55 galvanised surfaces, tire wear debris, etc.), zinc-containing paints, anti-corrosion additives for water-
56 containing equipment, fireworks, motor oil/lubricating oils additives, industrial air emissions, industrial
57 runoff, zinc minerals or wastes in construction materials, fertiliser and agricultural micronutrient products
58 (California Stormwater Quality Association [CASQA] 2015). It also has its origin in the boat components
59 and their maintenance: zinc anodes used to deter corrosion of metal hulls and engine parts, zinc constituent
60 of motor oil and tires, antifouling paints (Eklund and Eklund, 2014).

61 Among the marine organisms most affected by metal pollution are bivalve mollusks, given their filter-
62 feeding attitude, which leads to a strong accumulation of the pollutants. In order to investigate the unknown
63 toxicological effects and mechanisms of metals on bivalves, environmental metabolomics is commonly
64 applied for several years now (for reviews see: Booth et al., 2011, Viant and Sommer, 2012, Lankadurai et
65 al., 2013). As examples of recent metabolomics application in the case of a metal contamination of bivalves,
66 Aru et al. (2016) investigated the effects of short-term lead and zinc exposure upon the metabolic profiles of
67 clams *Ruditapes decussatus* and *Ruditapes philippinarum*. Nguyen et al. (2018) applied an integrated
68 approach that combines flow cytometry and metabolomics to characterize cellular and molecular

69 mechanisms of copper immunotoxicity in mussel (*Perna canaliculus*). Wu et al. (2017) found that mussel
70 response to cadmium was dependent on life cycle stage. Many other current applications of
71 metabolomics to study the effects of metals or other water contaminants on marine organisms have been
72 reported in many studies (for a review see Pomfret et al., 2019).

73 Here, we used the marine bivalve *Mimachlamys varia*, in the family *Pectinidae*, commonly known as the
74 variegated scallop, as a model organism. Due to its relative abundance along the Atlantic coasts, its
75 sedentary nature and also because this species has been shown to have a very elevated pollutants
76 incorporation and retention capacity, it has been proposed as a potential indicator species for the marine
77 biomonitoring (Breitwieser et al., 2018a; Metian et al., 2009a, 2009b; Milinkovitch et al., 2015). In
78 particular, it is relatively easy to sample in the marina of La Rochelle, where it has been used for
79 ecotoxicological studies (Breitwieser et al., 2018b). Furthermore, it is a commercially harvested species also
80 captured for leisure activities, directly on the shore or by snorkeling and it represents therefore a definite
81 economic value.

82 Previously, we performed a first metabolomics study on *M. varia* that highlighted a complex metabolic
83 reprogramming that occurs during tidal cycles after an emersion period of 2 h and upon re-immersion (Ory et
84 al., 2019). It appeared that metabolites depend on the moment of the tidal cycle as well as on the ambient
85 environment (oxygenation, salinity and temperature conditions, seasonality, etc.). In order to minimise the
86 effects of all these important interplaying environmental factors and understand the underlying mechanisms
87 affecting *M. varia* exposed to zinc, we grew scallops in aquaria under controlled and continuously immersed
88 conditions and we exposed the animals to zinc concentration equal to that found in La Rochelle marina. A
89 comparative metabolomics study was performed in gills samples, using UHPLC/QToF mass spectrometry, in
90 order to provide an overview of the relationship between zinc exposure and metabolome changes. Gills were
91 preferred over other tissues, as they have been shown to be, along with the mantle and digestive gland, the
92 main storage organs for metals in oysters (Wang et al., 2018). In addition, gills are generally considered as
93 the main entry of dissolved metals in marine filter-feeder organisms (Marigómez et al., 2002).

94

95 **Materials and Methods**

96

97 *Animals and experimental design*

98 Scallops, *Mimachlamys varia*, (shell length from 3 to 5 cm) were collected from La Pointe du Grouin to Loix
99 en Ré (Ré island, France) and acclimated one month in the laboratory in filtered through a 50 µm nylon filter
100 and sterilised seawater. The physicochemical parameters of the water were controlled every two days and
101 were kept constant by a biweekly water renewal (pH 8.0, temperature 10°C, salinity 33 g.kg⁻¹ seawater,
102 nitrite, nitrate, and ammonia <0.025 mg.L⁻¹). Food as liquid algal culture (Shellfish Diet 1800®, Reed
103 Mariculture, Campbell, CA, USA) was distributed three times per week, at a rate of 0.02 mL per individual.
104 Mortality of less than 3% was observed in acclimation tanks.

105 For this experiment, 36 scallops were randomly distributed in six 18-litres aquaria. Three randomly selected
106 aquaria were contaminated with a ZnCl₂ solution (nominal zinc concentration 150 µg.L⁻¹). The other three
107 aquaria received the same amount of seawater.

108 After 48 hours of exposure, the six scallops remaining in each aquarium were sampled. The gill tissues of
109 each individual were dissected, drained on absorbent paper and put in cryovial on ice. Gill tissues from three
110 individuals were grouped, snap-frozen in liquid nitrogen and stored in a freezer at -80°C. From this
111 sampling, six exposed samples and six controls were obtained. These control samples were used as a
112 reference for the analyses.

113

114 *Sample preparation for metabolomics study*

115 Sample preparation was performed according to a previously described method (Ory et al., 2019), inspired
116 by a metabolomics study in Phycotoxines laboratory (Ifremer, Nantes, France) (Mondeguer et al., 2015). In
117 summary, samples were thawed, homogenized by manual grinding and then crushed with a homogenizer.
118 These three last operations were performed on ice. They were then adjusted to 1 g and stored at -80°C before
119 extraction procedure. This last protocol included a triple solvent extraction by successively using acetone
120 twice and methanol. The three pooled supernatants were recovered and dried with a stream of nitrogen,
121 before resuspension with 2 mL methanol/water 20/80 (v/v), storage at -80°C, filtration with 0.2 µm filters
122 and LC/MS analysis.

123

124 *UHPLC/QToF MS analysis of metabolites*

125 The analysis of metabolites in gills was performed by ultra-high-performance liquid chromatography
126 coupled to high resolution mass spectrometry, according to a previously described method (Ory et al., 2019),
127 with the same analytical equipment. 5 µL of the samples were injected in a column “Acquity UPLC HSST3”
128 (Waters) (2.1 × 150 mm, 1.7 µm), and the products were eluted at a flow rate of 300 µL.min⁻¹ using a
129 gradient composed of solvents A (water/formic acid 100/0.001 (v/v)) and B (acetonitrile/ formic acid
130 100/0.001 (v/v)). The gradient used was the same as described previously (Ory et al., 2019). The analyses
131 were performed in positive and negative ionisation mode with MS^E function in a centroid mode. The MS
132 parameters applied in the ESI source for the two ionisation modes were the same as described previously
133 (Ory et al., 2019), except that desolvation gas flow-rate was 300 L.h⁻¹ and capillary voltage was 3 kV (+) and
134 2.5 kV (-) and sampling cone 35 V. The instrument was adjusted for the acquisition on a 50–2100 m/z
135 interval, with a scan time of 0.5 s. A 20 to 40 V ramp was used as collision energy for the high energy mode
136 of MS^E.

137 The mass spectrometer was calibrated before analysis using 0.5 mM sodium formiate solution and the
138 Leucine Enkephalin (M = 555.62 Da, 1 ng.µL⁻¹) was used as a lock-mass.

139 Compound identification was performed as explained below.

140

141

142 *Quality Control*

143 A pool sample was prepared by combining 100 μ L of each gills tissue extract. This mixture was divided into
144 ten vials that were used as quality-control samples (QCs). They were regularly distributed and injected
145 throughout the sample list to ensure analytical repeatability. Samples of gill tissue extracts were measured in
146 randomised order. Blanks were prepared with the last extraction solvent and injected at the beginning and at
147 the end of the sample sequence, to allow the subsequent subtraction of contaminants or components coming
148 from the extraction solvent.

149
150 *Chemicals*

151 Acetonitrile, Methanol, Acetone with HPLC grade purity were from Carlo Erba. Water was prepared using a
152 Milli-Q reagent water system. Succinate and L-phenylalanine were purchased from Sigma-Aldrich (now part
153 of Merck).

154
155 *Statistical analysis*

156 Metabolites data were obtained and treated as ion peak intensity as previously described (Ory et al., 2019).
157 Data were processed on the online and freely available Workflow4Metabolomics (W4M) platform
158 (<http://workflow4metabolomics.org>) for pretreatments and multivariate analyses processing as previously
159 described (Ory et al., 2019). Pretreatments included batch correction and removing metabolites with
160 coefficients of variation (CV) > 0.4 in the QC samples. Quality metrics tool (supplied by W4M Core
161 Development Team) provided visualising the data matrices, batch correction efficiency and supplied a rapid
162 highlight of potential sample outliers.
163 Log-transformation normalised the data and Pareto scaling was applied before the multivariate analysis
164 process. The data were analysed with unsupervised (Principal Component Analysis, PCA) and supervised
165 (Partial Least Square Discriminant Analysis, PLS-DA) methods. This last method is a multivariate regression
166 and prediction based on the separation between two classes: reference and Zn-exposed samples after a 48 h
167 exposure. It allowed the selection of metabolites involved in class separation in order to identify metabolites
168 affected by Zn exposure. Metabolites responsible for classification between groups were selected according
169 to their contribution as variable in the component prediction in PLS-DA model, described as Variable
170 Importance in Projection parameter (VIP), considering only variables with VIP values higher than 1
171 indicative of significant differences between groups.
172 Predictability performance was evaluated for each built PLS-DA model. To assess the statistical significance,
173 t-tests were performed on each metabolite intensities difference between reference and Zn exposed samples,
174 after normality and homoscedasticity were previously checked. Otherwise, Mann-Whitney tests were
175 performed.

176
177
178

179 *Identification and relative level of metabolites*

180 The identification of metabolites was performed by using the human metabolome database
181 (<http://www.hmdb.ca>) and the METLIN database
182 (https://metlin.scripps.edu/landing_page.php?pgcontent=mainPage) and also for lipids and lipid-derivatives
183 with the online lipid database: (http://www.lipidmaps.org/tools/ms/LMSD_search_mass_options.php). A
184 research was performed with the mass value of metabolites and a limit with a mass error lower 5 ppm.
185 Moreover, for succinate and L-phenylalanine, analytical standards were used with the LC/MS method to
186 verify this identification. Metabolites were finally expressed in relative abundance, which was calculated for
187 each metabolite by dividing the abundance in each sample by the median abundance across all the samples.

188

189

190 **Results**

191

192 *Data processing*

193 Mass spectrometry untreated analyses detected 20 946 *m/z* features for negative ionisation mode results in
194 LC/MS and 44 256 for positive ionisation mode. These large sets of data obtained for metabolomics profiling
195 were preprocessed applying a Lowess regression model fitting with the pool values as batch correction (Van
196 Der Kloet et al., 2009).

197 After preprocessing and removal of metabolites with $CV > 0.4$ in QC samples, the database of metabolites
198 counted for 1556 and 3581 compounds detected with negative and positive ionisation modes in LC/MS
199 respectively.

200

201 *PCA Analysis*

202 PCA was performed as a first method to confirm differences in metabolite levels between reference animals
203 and animals exposed to zinc and detect eventual outlier samples. The score plots of PCA analysis are shown
204 in Figure 1. Each symbol represented an individual sample, and the dot scattering indicated similarities or
205 differences in metabolic compositions between samples. PCA performed with data sets from both reference
206 and exposed individuals showed that two groups were well separated according to Zn exposure for the
207 negative mode but fairly well separated for the positive mode, due to a high dispersion of Zn-exposed
208 samples along the 2nd axis. Thus, the intensities of the compounds, considered as variables, contributed to the
209 separate structure observed between reference and exposed individuals. The two first axes accounted for
210 31%-12% and 24%-14% of the total variability among samples for negative and positive data sets
211 respectively. Reference samples and samples exposed to Zn were grouped and distinguished mainly along
212 the second axis (*t*₂) for both ionisation conditions. The model fitted the data well. However, too many
213 variables were considered to highlight a specific contribution to the total variability within both data sets.

214 Hotelling's T^2 statistic tested the presence of outliers measuring the variation within the PCA model.
215 Samples with large Hotelling T^2 had an unusual variation inside the model. In this case, they were not
216 representative of the modeled data and can be considered as outliers if p -value < 0.05 . The PCA results
217 showed one outlier sample in the negative mode of data sets (Zn48-2). This outlier sample was removed
218 from sample matrix data set to perform PLS-DA.

219
220 *PLS-DA analyses*
221 PLS-DA analyses were then performed, to identify metabolites whose abundance was related to exposure to
222 Zn (Figure 1). The first PLS-DA models were built to force the separation between the two populations in
223 the two datasets. (negative and positive modes). The performances of the model were proven, as the results
224 of both analyses in negative and positive modes showed a relative data consistency with R^2 (cumulative) =
225 0.99 and 0.99, respectively. Moreover, the prediction performance of the models reached Q^2 (cumulative) =
226 0.88 and 0.57 for negative and positive modes respectively, which corresponds to good prediction capability.
227 However, the permutation test was not significant (p -value > 0.05) for these first models due to the very
228 large number of variables. In order to improve the prediction performance of the model and to select the
229 variables with the largest contribution, successive PLS-DA models were built by selecting each time only the
230 variables with VIPs > 1 . This process was stopped when model predictive performance stopped to increase.
231 In the negative mode data set, a total of three successive models selecting 465, 155 and 59 metabolites with
232 VIPs > 1 were performed reaching $Q^2 = 0.931$. In the positive mode data set, three successive models
233 selecting 360, 137 and 40 metabolites with VIPs > 1 were performed reaching $Q^2 = 0.959$.
234 All these selected metabolites showed a significant difference between the reference samples and the
235 samples exposed to Zn (t-test or Mann-Whitney test, p -value < 0.05).

236
237 *Metabolite modulation*
238 Mass-matching analysis using the different online metabolome databases mentioned above or comparison
239 with standards allowed putative annotation of eleven metabolites showing a significant difference between
240 reference and Zn exposed samples (Table 1).
241 Interestingly, all of them were down-modulated when going to references to Zn exposed samples. The
242 relative abundance of all these metabolites decreased under zinc exposure by more than 30% (Figure 2) : L-
243 phenylalanine (-39.02%, $p = 0.05$), L-kynurenine (-70.31%, $p = 0.0038$), picolinic acid (-61.93%, $p = 0.013$),
244 neuroprostane 1 (-58.61%, $p = 0.027$), neuroprostane 2 (identified as 14-hydroperoxy-H4-neuroprostane or
245 11-hydroperoxy-H4-neuroprostane or 10-hydroperoxy-H4-neuroprostane) (-40.22%, $p = 0.05$),
246 neuroprostane 3 (256 candidates corresponding to neuroprostanes on 266 entries on lipid maps databank for
247 this m/z) (-52.28%, $p = 0.05$), succinate (-42%, $p = 0.05$), S-(PGA2)-glutathione or S-(PGJ2)-glutathione
248 (-64.07%, $p = 0.012$), xanthine (-46.65%, $p = 0.018$), hexanoylcarnitine (-36.81%, $p = 0.044$),
249 dodecenoylcarnitine (-49.12%, $p = 0.0005$).

250

251
252 **Discussion**
253 Scallops exposed for 48 h to a nominal zinc concentration of 150 $\mu\text{g}\cdot\text{L}^{-1}$ in laboratory conditions undergo
254 alterations of their metabolism. These alterations correspond to metabolism of two amino acids (e.g. L-
255 phenylalanine and L-tryptophan), bioactive lipid compounds (glutathione conjugated prostaglandin and
256 neuroprostanes), purine metabolism (e.g. xanthine), tricarboxylic acid (TCA) cycle intermediates (succinate)
257 and metabolism of fatty acids (hexanoylcarnitine and dodecenoylcarnitine). These variations could reflect
258 potential effects on several of the biological processes discussed below, such as energy metabolism,
259 including means to face increased energy demand and potential mitochondrial dysfunction, osmoregulation,
260 oxidative stress defense and purine and tryptophan metabolism as discussed below.
261 It should be mentioned that the decrease in phenylalanine could also result from disturbed food intake under
262 stress induction.
263
264 *Energy and fatty acid metabolism*
265 In the present study the decrease of four metabolites (L-phenylalanine, hexanoylcarnitine, trans-2-
266 dodecenoylcarnitine and succinate) for Zn exposed scallops, may be related with energy metabolism.
267 The decrease of phenylalanine may reflect its consumption as substrate for energy metabolism, in place of
268 carbohydrates or lipids, which are used in normal circumstances. Indeed, phenylalanine is an amino acid
269 being both glucogenic and ketogenic and it can be consumed under energy deficient conditions in aquatic
270 invertebrates (Sokolova et al., 2012). In case of Zn exposure, this energy deficiency may arise from an
271 elevated basal metabolic demand due to the need to develop protective mechanisms for the cells, such as
272 overproduction of metallothioneins, glutathione, molecular chaperones, antioxidants pathways (Cherkasov,
273 2006; Ivanina et al., 2008; Sokolova and Lannig, 2008). Moreover, toxic metals such as cadmium, copper,
274 zinc and mercury have been shown to reduce mitochondrial efficiency and coupling and elevated proton leak
275 in marine organisms (Sokolova et al., 2012). In the same vein, after 9 days of exposure with nominal Zn
276 concentration of 30 $\mu\text{g}\cdot\text{L}^{-1}$, oysters gills were analysed by proteomic analysis and it was concluded that Zn
277 affected the TCA cycle by influencing the expressions of Fe-containing proteins and disrupted the electron
278 transport chain by inhibiting complex I–IV related protein expressions (Meng et al., 2017). It should be
279 mentioned that other stress factors such as hypoxia, the presence of other pollutants or high temperature
280 could concomitantly modify the effect of zinc on the metabolism of bivalves. Recently Belivermis et al.
281 (2020) showed that hypoxia diminished the Ag and Zn uptake in mussels and induced significantly greater
282 abundance of several amino acids, amino sulfonic acids, dicarboxylic acids, carbohydrates and other
283 metabolites compared to Zn exposed mussels in normoxia. Sokolov et al. (2019) observed a decrease of
284 protein synthesis during hypoxia in intertidal oysters *Crassostrea gigas* and interpreted this as an energy-
285 saving mechanism.
286 Two different acylcarnitine, hexanoylcarnitine and trans-2-dodecenoylcarnitine, were found to be down-
287 regulated in presence of zinc. The first derivative of carnitine (hexanoylcarnitine) is a medium-chain

288 acylcarnitine, whereas the second one (trans-2-dodecenoylcarnitine) is a long-chain acylcarnitine. Long
289 chain fatty acids have to be combined with carnitine to enter the mitochondria, where they undergo beta-
290 oxidation for energy production. In contrast, the medium-chain fatty acids can enter the mitochondrial
291 membrane directly for beta-oxidation without the need of carnitine-mediated transport. Therefore, in our
292 case, the defect of trans-2-dodecenoylcarnitine mainly affects the oxidation of the corresponding long-chain
293 fatty acids. However, the metabolism of acylcarnitines is not only related to the transport of fatty acids, but
294 also plays a key role in maintaining the homeostasis of the mitochondrial acyl-CoA/CoA ratio (Connor and
295 Gracey, 2012; Gracey and Connor, 2016) and in other physiological processes such as peroxidation of fatty
296 acids. Therefore, the observed change in acylcarnitine profile in presence of zinc may be related more
297 generally with potential mitochondrial dysfunction and abnormal fatty acid metabolism.

298 Lastly, succinate was found to decrease upon Zn exposure. It is a metabolite intermediate of the TCA cycle,
299 which is oxidized into fumarate by the iron-sulfur enzyme succinate dehydrogenase (SDH). Zn affects the
300 TCA cycle by influencing the expressions of Fe-containing proteins (Pagani et al., 2007) and it is therefore
301 so not surprising to observe that succinate is impacted by Zn in our study. What is more surprising is that the
302 succinate appears in the present study to be down-regulated after Zn exposure. Indeed, in several previous
303 studies, an accumulation of succinate was observed after Zn exposure : in the gills of Pacific oyster
304 (*Crassostrea gigas*) exposed to excess Zn solution (30 $\mu\text{g.L}^{-1}$) for 9 days (Meng et al., 2017), in the digestive
305 gland of *Ruditapes philippinarum* exposed to ZnCl_2 (nominal concentrations: 20, 50, 100 and 150 $\mu\text{g.L}^{-1}$) for
306 48 h (Aru et al., 2016) and in adductor muscles of the pedigree White clam of *Ruditapes philippinarum* after
307 exposure with Zn^{2+} (50 $\mu\text{g.L}^{-1}$) for 48 h (Wu et al., 2011). This accumulation of succinate was interpreted as
308 molecular sign of the shift in cellular metabolism to anaerobiosis. It was explained in details by proteomic
309 analysis, by a decrease in SDH expression and a simultaneously increase in enzyme succinyl-CoA synthetase
310 (SCS) expression, which allows succinate biosynthesis in the TCA cycle, under Zn exposure of oysters
311 (Meng et al., 2017). Nevertheless, in the same study, fumarase (FUM), another Fe-S enzyme that converts
312 fumarate to L-malate in the TCA cycle, showed significantly increased levels of expression at the same time.
313 This was interpreted as a compensation mechanism for ensuring that the TCA cycle remains active when
314 exposed to metals (Meng et al., 2017). This indicates that Fe-S enzymes of the TCA cycles can be either up-
315 or down-regulated upon Zn exposure and that defense strategy against Zn toxicity exists in bivalves, as it is
316 the case with bacteria exposed to metals such as Al that interfere with Fe metabolism (Chenier et al., 2008).

317 In another study, two pedigrees (White and Zebra) of clam *Ruditapes philippinarum* were exposed with
318 Zn^{2+} (50 $\mu\text{g.L}^{-1}$) during 48 h and the metabolomics results showed differences in digestive gland between
319 White and Zebra clam samples in succinate levels: in Zebra clam, succinate was increased, whereas it was
320 decreased in White clam after Zn exposure (Ji et al., 2015). In conclusion, it seems clear that Zn affects the
321 TCA cycle metabolites, through its effect on Fe-S enzymes involved in the cycle. Concerning the level of
322 succinate, it may be increased or decreased upon Zn exposure, depending on the species, with respect to
323 over- or under-expression of the different Fe-S enzymes of the TCA cycle.

324 However, succinate has multiple roles in cell metabolism and its decrease may be linked among others with
325 formation and elimination of reactive oxygen species, as discussed below.

326

327 *Osmoregulation*

328 Down-modulation of L-phenylalanine on the one hand and of prostaglandine-glutathione and neuroprostanes
329 on the other hand, in Zn exposed scallops may be related to osmoregulation phenomena.

330 Indeed, L-phenylalanine is a catecholamine precursor and its lower level may lead to a decrease in
331 dopamine. Dopamine and serotonin are neurotransmitters involved in the modulation of Na⁺ and K⁺ transport
332 in gills of crustaceans (Mo et al., 1998) and in lateral cilia in gills of bivalves (Carroll and Catapane, 2007).
333 The down-modulation of dopamine may thus have a repercussion on osmoregulation of cells submitted to Zn
334 exposure. A similar relationship between L-phenylalanine decrease and osmoregulation was previously
335 suggested (Aru et al., 2016). These authors observed a down-modulation of phenylalanine in presence of
336 ZnCl₂ (20, 50, 100 and 150 µg.L⁻¹) under laboratory conditions after 48 h exposure for the clam *Ruditapes*
337 *decussatus*, associated with a significant increment in organic osmolyte production (hypotaurine and
338 homarine) and a decrease of the content of free amino acid.

339 Prostaglandins (PGs) are an important group of bioactive lipid compounds in living organisms; they are
340 involved in mammals in a great variety of physiopathological processes like for instance inflammation,
341 signaling or reproduction. These molecules play similar roles in marine invertebrates, being involved in the
342 control of gametogenesis, ion transport, and defense. These molecules also represent very important lipid
343 mediators in marine invertebrates (Di Costanzo et al., 2019). PGs biosynthesis starts with arachidonic acid, a
344 polyunsaturated fatty acid (PUFA), that is oxidised by enzymes such as cyclooxygenase or lipoxygenase to
345 give precursors, which are then further transformed by a large number of possible enzymes, giving rise to the
346 formation of different families of PGs, like PG J₂ and PGA₂. These last PGs may undergo addition reactions
347 with glutathione (GSH) to give new reactive compounds in humans (Murphy and Zarini, 2002). In the
348 present study, among the metabolites showing a down regulation after 48 h Zn exposure, a compound
349 putatively identified as S-(PGA₂)-glutathione or S-(PGJ₂)-glutathione was obtained. This would indicate
350 that these types of conjugates are also present in bivalves and are impacted by exposure to Zn.

351 Isoprostanes (IsoPs) and the IsoP-like molecules are PG-like compounds formed *in vivo* via the
352 nonenzymatic free radical-initiated peroxidation of a number of different PUFAs. Among them,
353 neuroprostanes (MPs) are generated from the PUFA docosahexaenoic acid (DHA) (Milne et al., 2011). PGs,
354 IsoPs and IsoP-like molecules have been identified recently in molluscs (Bonnefille et al., 2018; Chakraborty
355 et al., 2014), which is not surprising given that PUFAs are essential molecules for the survival of bivalves
356 (Da Costa et al., 2015) and that they are highly susceptible to oxidation because of the large number of their
357 double bonds.

358 The role of PGs, IsoPs and Iso-P like molecules in invertebrates has not yet been fully elucidated. Some
359 studies show that they have a role in osmoregulation : PGE₂ was shown to be involved in Na transport
360 regulation in gills of the freshwater mussel *Ligumia subrostrata* and also in clams (Saintsing and Dietz, 1983)

361 and it was shown that hyposmotic stress significantly increased prostaglandin synthesis in the marine bivalve
362 *Modiolus demissus* (Freas and Grollman, 1980). In the present case, a down-modulation of three compounds
363 identified as neuroprostanes was obtained after Zn exposure and this may be linked also with
364 osmoregulation. In fish, prostaglandins were shown to be involved in Cl⁻ secretion (Avella et al., 1999) and
365 effluent exposure resulted also in widespread reduction (between 50% and 90%) in PG profiles obtained
366 through metabolomics in fish tissues and plasma (David et al., 2017).

367

368 *Oxidative stress defense*

369 Besides their possible role in the above mentioned osmoregulation, IsoPs and Iso-P like molecules have
370 become biomarkers of choice in human for assessing endogeneous oxidative stress linked with many
371 pathological disorders, because they are chemically stable (Milne et al., 2008). Oxidative stress is
372 characterised by an imbalance between cellular antioxidant defenses and overproduction of free radicals,
373 particularly reactive species of oxygen (ROS). Lipids are readily attacked by free radicals, resulting in the
374 formation of a number of peroxidation products. In bivalves, like in other animals, there is a close
375 relationship between environmental stress and the rate of cellular ROS generation and consequently lipid
376 peroxidation reactions (Regoli and Giuliani, 2014). In our case, the modification of the Iso-P like molecules
377 profile could therefore be linked to an increased oxidative stress in the presence of Zn.

378 Another down-modulated metabolite under Zn exposure that may be linked with oxidative stress defense is
379 succinate. The roles of succinate in mitochondrial ROS formation and elimination are numerous and
380 complex (Tretter et al., 2016). It was shown that succinate exerted an inhibitory effect on lipid peroxidation
381 in mitochondria. This protective role of succinate against oxidation was attributed to the maintenance of a
382 pool of reduced coenzyme Q. Succinate was oxidised by the membrane-bound succinate dehydrogenase and
383 the enzyme activity was only moderately sensitive to oxidants, therefore it was more efficient in maintaining
384 reduced coenzyme Q, thus maintaining membrane integrity.

385 Other metals and in particular copper are able to induce oxidative stress in bivalves. Cu²⁺-exposed
386 haemocytes showed a remarkable increase in ROS production, which induced oxidative stress in mussel
387 (*Perna canaliculus*) (Nguyen et al., 2018). This was accompanied by a decrease in GSH. This compound is
388 an important antioxidant, able to react with electrophilic oxidants (e.g., H₂O₂) resulting in conversion of two
389 GSH molecules into its oxidized form (GSSG). Two other molecules decrease simultaneously upon Cu²⁺
390 exposure: cysteine, which is involved in the GSH metabolic pathway and methionine, which takes place in
391 the transsulfuration pathway, a source of cysteine for GSH. These concomitant phenomena were identified as
392 important signatures of oxidative stress (Nguyen et al., 2018).

393

394 *Purine metabolism*

395 A lower level of xanthine in presence of Zn suggests a disordered nucleotide metabolism. Indeed, in the
396 catabolism of purine nucleotides, adenosine monophosphate (AMP) and guanosine monophosphate (GMP)
397 both lead to xanthine (Van Waarde, 1988). AMP is degraded to inosine and then by phosphorolysis to

398 hypoxanthine. In presence of xanthine oxidase (XOD), xanthine is formed from oxidation of hypoxanthine.
399 GMP is degraded to guanosine and then by phosphorolysis to guanine, which is transformed to xanthine by
400 guanine deaminase. This makes the metabolic interrelationships between nucleotides and purine base
401 nucleosides, and finally xanthine, very strong (Harkness, 1988). Therefore, we can consider that xanthine is
402 at the center of the interplay between ATP and GTP metabolism. Since the nucleotides ATP and GTP are the
403 energy carriers molecules in cells, it can be hypothesised that zinc would induce an increased demand for
404 energy by the cells.

405 Previously, a decrease of inosine (between -30 and -80%) and hypoxanthine (between -40 and -50%) was
406 observed in whole body of zebrafish, after exposure to ZnCl₂ at different concentrations (10, 100, 500 and
407 1000 µg.L⁻¹ for 72 h). It was interpreted as an impairment of the purine metabolism caused by exposure to
408 Zn. As it was combined with an ATP level increased, it was hypothesised that Zn would induce an increased
409 energy demand to fulfil essential maintenance costs and ensure organism survival (e.g. cellular protective
410 mechanisms, detoxification) (Kim et al., 2016; Sokolova et al., 2012).

411 412 *Tryptophan metabolism*

413 In humans, L-tryptophan is metabolised by two main pathways : the serotonin pathway (around 1% of L-
414 tryptophan metabolism) and the kynurenine pathway (around 95% of L-tryptophan metabolism). In the
415 kynurenine pathway, L-tryptophan gives one of three main end products: kynurenic acid, picolinic acid and
416 NAD. This pathway begins with the oxidative cleavage of L-tryptophan by either one of the two enzymes
417 indoleamine 2,3-dioxygenase (IDO) or tryptophan 2,3-dioxygenase also called tryptophan pyrrolase (TDO) to
418 produce L-formylkynurenine, which is then converted to L-kynurenine by a formamidase. L-kynurenine is a
419 branch point and can be converted to the following: 1) kynurenic acid by kynurenine aminotransferases; 2)
420 3-hydroxykynurenine by kynurenine 3-monooxygenase; or 3) anthranilic acid. 3-hydroxykynurenine further
421 undergoes enzymatic or chemical transformations to give either picolinic acid or NAD (Grant et al., 2009).
422 The kynurenine metabolism was shown to be highly conserved throughout evolution and the enzyme IDO
423 was discovered in mammals, lower vertebrates, several invertebrates, fungi and a number of bacterial species
424 (Cervenka et al., 2017).

425 In the present study, a down-regulation of both picolinic acid and kynurenine was observed after Zn
426 exposure, showing an impact of this metal on tryptophan metabolism. This trend was also observed in
427 another study with the marine bivalve *Mytilus galloprovincialis*, when exposed to a wastewater treatment
428 plants effluent extract: both L-formylkynurenine and L-kynurenine were found to be down-regulated
429 (respectively -55%, $p \leq 0.05$ and -63%, $p \leq 0.05$) compared to non-exposed animals thanks to a non-targeted
430 metabolomics study (Dumas et al., 2020).

431 In another study using proteomic analysis with the pacific oyster *Crassostrea gigas* the enzyme kynurenine
432 formamidase was found to be down-regulated under aerial exposure (Zhang et al., 2015), which again
433 corresponds to an impairment of the tryptophan metabolism. This was accompanied by the suppression of

434 many physiological activities and this was interpreted as a means of redistributing available resources to fuel
435 stress responses (Zhang et al., 2015).

436

437 **Conclusion**

438 In the present study, we chose the variegated scallop *Mimachlamys varia*, for assessing zinc contamination
439 effects on their metabolism, at a concentration such as the one the metal can reach in La Rochelle marina. An
440 untargeted UHPLC/QToF tandem mass spectrometry metabolomics approach was chosen, as it offers a high
441 level of sensitivity to highlight impacts due to short-time metal exposure. Metabolite profiling of gills tissues
442 could clearly separate scallops with no metal inputs and those exposed to 48 h zinc exposure, under
443 laboratory conditions. Among the high quantity of modulated metabolites, only a few of them were
444 identified, because of the current lack of knowledge on scallop metabolism. They include amino acids or
445 their metabolites, bioactive lipid compounds, including four metabolites never before identified in bivalves,
446 metabolites of purine or the TCA cycle and carnitine conjugates of fatty acids. These molecules reflect
447 potential zinc effects on several of the biological processes, such as energy metabolism, osmoregulation,
448 defense against oxidative stress, metabolism of purine and tryptophan.

449 This study demonstrates once again that metabolomics approach, when used in untargeted mode is able to
450 reveal novel and unanticipated physiological perturbations, without *a priori* knowledge of the metabolome.
451 This is valuable in the present case where a poorly studied animal model is used. The limitations of the
452 technique come from the fact that there are a large number of unknown metabolites that remain unannotated
453 in the databases, so that some metabolite features cannot be attributed to a molecular structure. The
454 development of new methods dedicated to the identification of metabolites on the one hand and to the search
455 for the connectivity of metabolic pathways on the other hand, combined with the enrichment of databases,
456 will make it possible in the future to carry out more and more advanced impact analyses.

457 Moreover, further study is needed to assess the effect of other trace elements present in the marine
458 environment and better understand the combined effects of pollutants. La Rochelle marina sets up multiple
459 actions to ensure a sustainable development of coastal activities while preserving the aquatic environment.
460 These actions, framed by the "Ports Propres" AFAQ certification (AFNOR : Association française de
461 normalisation), are based on criteria approved at the European level by the CWA 16987 agreement of the
462 "Clean Harbour Guidelines". Among them, some aim to develop approaches to highlight the physiological
463 modifications of marine organisms impacted by environmental variation. Our results show that
464 metabolomics is a powerful method that can brings out small environmental variations, such as zinc
465 concentration, through metabolic changes in scallops.

466

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479

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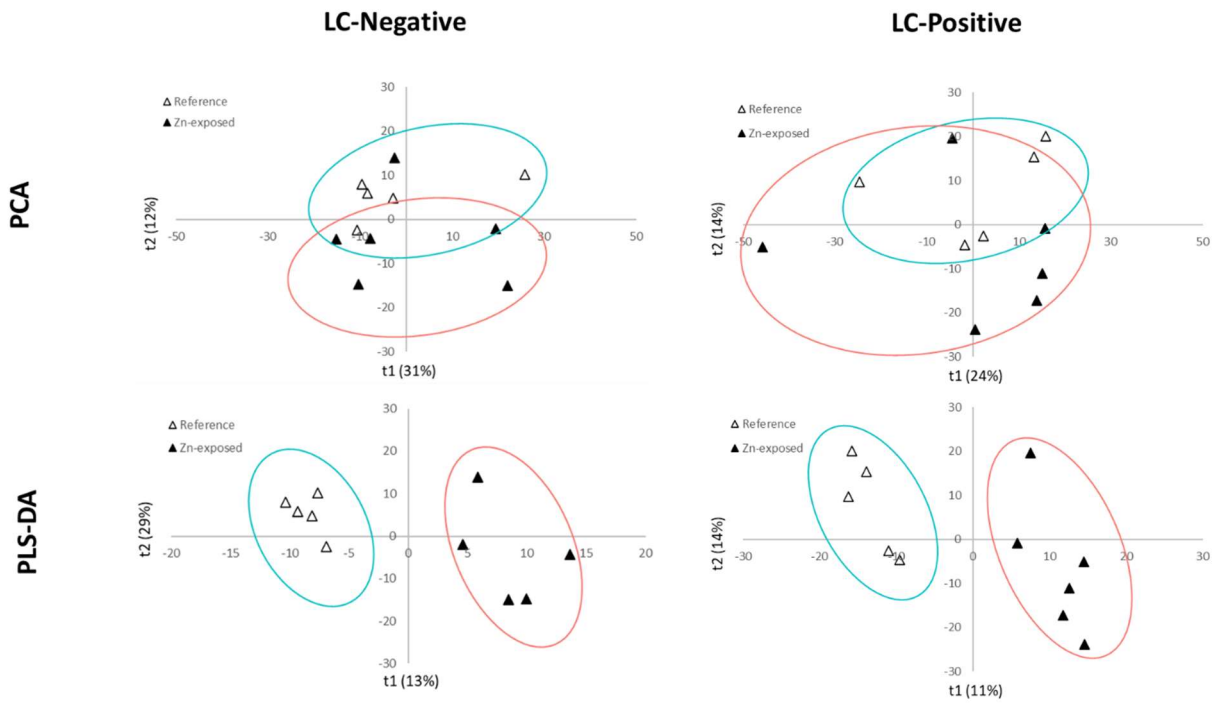
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653 FIGURE CAPTIONS

654 Figure 1: Score plots of PCA and PLS-DA for negative mode (LC-Negative), positive mode (LC-Positive)
655 of liquid chromatography. Ellipses score plots represented the confidence limit (95%) of Hotelling's T²
656 statistic (Green ellipse for reference samples and red ellipse for Zn-Exposed samples).
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658 Figure 2: Box plots of the relative abundance of the 11 identified metabolites in *Mimachlamys varia* gills showing
659 a significant difference between reference and Zn-exposed samples. Green plots represent the reference samples,
660 red plots represent the zinc exposed samples. The mean value is given by the dark diamond-shaped symbol.
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662 Figure 1



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665 Figure 2

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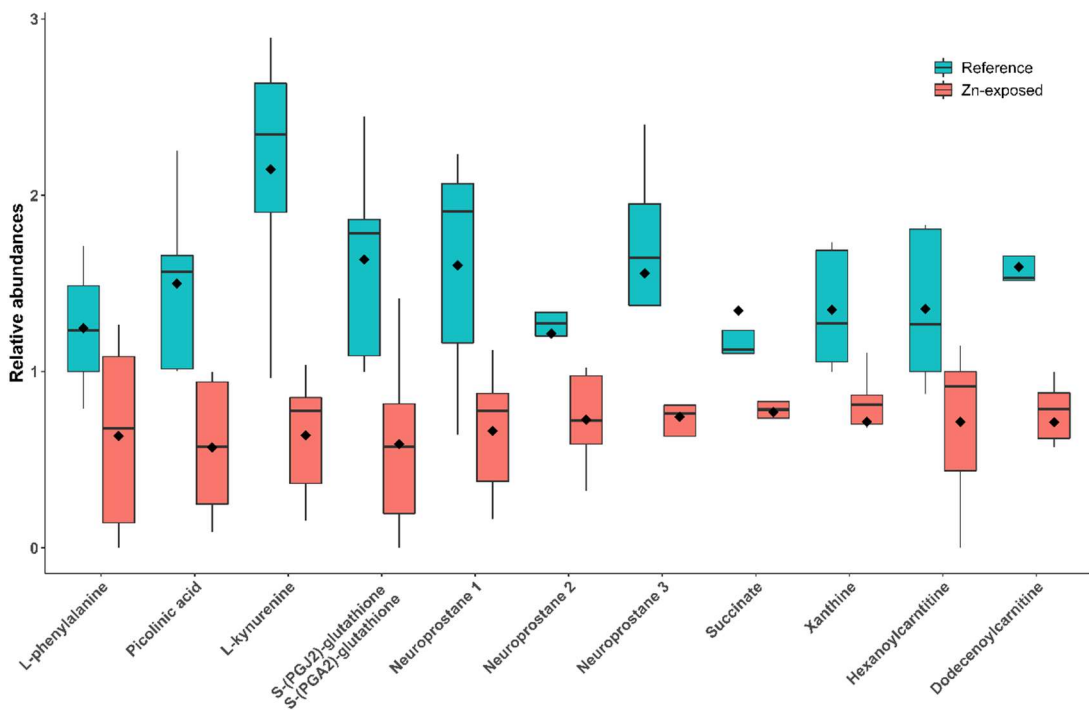
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Table 1: Identified metabolites varying after 48h-zinc exposure

Metabolite	Platform	Retention time (s)	Formula	Monoisotopic mass (Da)	Adduct	Observed mass (m/z)	Theoretical mass (m/z)	Mass error (ppm)	Ratio between reference and P-value exposed to Zn	
Picolinic acid	LC/MS Neg	198	C ₅ H ₄ N	123.032	[M-CO ₂ -H] ⁻	78.0346	78.0349	3.8	2.62	0.013
L-kynurenine	LC/MS Neg	417	C ₁₀ H ₁₁ N ₂ O ₃	208.0848	[M-H] ⁻	207.0770	207.0775	2.4	3.37	0.004
Neuroprostane 1	LC/MS Neg	678	C ₂₂ H ₂₉ O ₄	358.2144	[M-H] ⁻	357.2066	357.2071	1.4	2.42	0.027
Neuroprostane 2	LC/MS Neg	601	C ₂₂ H ₃₁ O ₆	392.2199	[M-2H ₂ O-H] ⁻	391.2123	391.2126	0.8	1.67	0.050
Neuroprostane 3	LC/MS Neg	981	C ₂₂ H ₃₁ O ₅	376.225	[M-H ₂ O-H] ⁻	375.2167	375.2177	2.7	2.09	0.050
Succinate	LC/MS Neg	247	C ₄ H ₅ O ₄	118.0266	[M-H] ⁻	117.0188	117.0193	4.3	1.75	0.050
L-phenylalanine	LC/MS Pos	417	C ₉ H ₁₂ NO ₂	165.079	[M+H] ⁺	166.0869	166.0863	3.6	1.96	0.050
S-(PGA2)-glutathione S-(PGJ2)-glutathione	LC/MS Pos	658	C ₃₀ H ₄₇ N ₃ O ₁₀ SNa	641.2982	[M+Na] ⁺	664.289	664.2874	2.4	2.78	0.001
Xanthine	LC/MS Pos	367	C ₅ H ₅ N ₄ O ₂	152.0334	[M+H] ⁺	153.0412	153.0407	3.3	1.89	0.018
Hexanoylcarnitine	LC/MS Pos	486	C ₁₃ H ₂₆ NO ₄	259.1784	[M+H] ⁺	260.186	260.1856	1.5	1.90	0.044
Dodecenoylcarnitine	LC/MS Pos	562	C ₁₉ H ₃₆ NO ₄	341.2566	[M+H] ⁺	342.2637	342.2639	0.6	2.23	<0.001

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