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

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

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Abstract

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

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

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Introduction

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

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

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

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

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

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

Materials and Methods

Animals and experimental design

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

For this experiment, 36 scallops were randomly distributed in six 18-litres aquaria. Three randomly selected aquaria were contaminated with a $ZnCl_2$ solution (nominal zinc concentration 150 µg.L^{-1}). The other three aquaria received the same amount of seawater.

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

Sample preparation for metabolomics study

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

UHPLC/QToF MS analysis of metabolites

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

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

Compound identification was performed as explained below.

Quality Control

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

Chemicals

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

Statistical analysis

Metabolites data were obtained and treated as ion peak intensity as previously described (Ory et al., 2019). Data were processed on the online and freely available Workflow4Metabolomics (W4M) platform (http://workflow4metabolomics.org) for pretreatments and multivariate analyses processing as previously described (Ory et al., 2019). Pretreatments included batch correction and removing metabolites with coefficients of variation (CV) > 0.4 in the QC samples. Quality metrics tool (supplied by W4M Core Development Team) provided visualising the data matrices, batch correction efficiency and supplied a rapid highlight of potential sample outliers.

Log-transformation normalised the data and Pareto scaling was applied before the multivariate analysis process. The data were analysed with unsupervised (Principal Component Analysis, PCA) and supervised (Partial Least Square Discriminant Analysis, PLS-DA) methods. This last method is a multivariate regression and prediction based on the separation between two classes: reference and Zn-exposed samples after a 48 h exposure. It allowed the selection of metabolites involved in class separation in order to identify metabolites affected by Zn exposure. Metabolites responsible for classification between groups were selected according to their contribution as variable in the component prediction in PLS-DA model, described as Variable Importance in Projection parameter (VIP), considering only variables with VIP values higher than 1 indicative of significant differences between groups.

Predictability performance was evaluated for each built PLS-DA model. To assess the statistical significance, t-tests were performed on each metabolite intensities difference between reference and Zn exposed samples, after normality and homoscedasticity were previously checked. Otherwise, Mann-Whitney tests were performed.

Identification and relative level of metabolites

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

Results

Data processing

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

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

PCA Analysis

PCA was performed as a first method to confirm differences in metabolite levels between reference animals and animals exposed to zinc and detect eventual outlier samples. The score plots of PCA analysis are shown in Figure 1. Each symbol represented an individual sample, and the dot scattering indicated similarities or differences in metabolic compositions between samples. PCA performed with data sets from both reference and exposed individuals showed that two groups were well separated according to Zn exposure for the negative mode but fairly well separated for the positive mode, due to a high dispersion of Zn-exposed samples along the 2nd axis. Thus, the intensities of the compounds, considered as variables, contributed to the separate structure observed between reference and exposed individuals. The two first axes accounted for 31%-12% and 24%-14% of the total variability among samples for negative and positive data sets respectively. Reference samples and samples exposed to Zn were grouped and distinguished mainly along the second axis (t2) for both ionisation conditions. The model fitted the data well. However, too many variables were considered to highlight a specific contribution to the total variability within both data sets.

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

PLS-DA analyses

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

Metabolite modulation

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

Discussion

Scallops exposed for 48 h to a nominal zinc concentration of 150 µg.L^{-1} in laboratory conditions undergo alterations of their metabolism. These alterations correspond to metabolism of two amino acids (e.g. L-phenylalanine and L-tryptophan), bioactive lipid compounds (glutathione conjugated prostaglandin and neuroprostanes), purine metabolism (e.g. xanthine), tricarboxylic acid (TCA) cycle intermediates (succinate) and metabolism of fatty acids (hexanoylcarnitine and dodecenoylcarnitine). These variations could reflect potential effects on several of the biological processes discussed below, such as energy metabolism, including means to face increased energy demand and potential mitochondrial dysfunction, osmoregulation, oxidative stress defense and purine and tryptophan metabolism as discussed below.

It should be mentioned that the decrease in phenylalanine could also result from disturbed food intake under stress induction.

Energy and fatty acid metabolism

In the present study the decrease of four metabolites (L-phenylalanine, hexanoylcarnitine, trans-2-dodecenoylcarnitine and succinate) for Zn exposed scallops, may be related with energy metabolism.

The decrease of phenylalanine may reflect its consumption as substrate for energy metabolism, in place of carbohydrates or lipids, which are used in normal circumstances. Indeed, phenylalanine is an amino acid being both glucogenic and ketogenic and it can be consumed under energy deficient conditions in aquatic invertebrates (Sokolova et al., 2012). In case of Zn exposure, this energy deficiency may arise from an elevated basal metabolic demand due to the need to develop protective mechanisms for the cells, such as overproduction of metallothioneins, glutathione, molecular chaperones, antioxidants pathways (Cherkasov, 2006; Ivanina et al., 2008; Sokolova and Lannig, 2008). Moreover, toxic metals such as cadmium, copper, zinc and mercury have been shown to reduce mitochondrial efficiency and coupling and elevated proton leak in marine organisms (Sokolova et al., 2012). In the same vein, after 9 days of exposure with nominal Zn concentration of 30 µg.L^{-1}, oysters gills were analysed by proteomic analysis and it was concluded that Zn affected the TCA cycle by influencing the expressions of Fe-containing proteins and disrupted the electron transport chain by inhibiting complex I–IV related protein expressions (Meng et al., 2017). It should be mentioned that other stress factors such as hypoxia, the presence of other pollutants or high temperature could concomitantly modify the effect of zinc on the metabolism of bivalves. Recently Belivermis et al. (2020) showed that hypoxia diminished the Ag and Zn uptake in mussels and induced significantly greater abundance of several amino acids, amino sulfonic acids, dicarboxylic acids, carbohydrates and other metabolites compared to Zn exposed mussels in normoxia. Sokolov et al. (2019) observed a decrease of protein synthesis during hypoxia in intertidal oysters *Crassostrea gigas* and interpreted this as an energy-saving mechanism.

Two different acylcarnitine, hexanoylcarnitine and trans-2-dodecenoylcarnitine, were found to be down-regulated in presence of zinc. The first derivative of carnitine (hexanoylcarnitine) is a medium-chain

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

Lastly, succinate was found to decrease upon Zn exposure. It is a metabolite intermediate of the TCA cycle, which is oxidized into fumarate by the iron-sulfur enzyme succinate dehydrogenase (SDH). Zn affects the TCA cycle by influencing the expressions of Fe-containing proteins (Pagani et al., 2007) and it is therefore so not surprising to observe that succinate is impacted by Zn in our study. What is more surprising is that the succinate appears in the present study to be down-regulated after Zn exposure. Indeed, in several previous studies, an accumulation of succinate was observed after Zn exposure : in the gills of Pacific oyster (*Crassostrea gigas*) exposed to excess Zn solution (30 $\mu g.L^{-1}$) for 9 days (Meng et al., 2017), in the digestive gland of *Ruditapes philippinarum* exposed to $ZnCl_2$ (nominal concentrations: 20, 50, 100 and 150 $\mu g.L^{-1}$) for 48 h (Aru et al., 2016) and in adductor muscles of the pedigree White clam of *Ruditapes philippinarum* after exposure with Zn^{2+} (50 $\mu g.L^{-1}$) for 48 h (Wu et al., 2011). This accumulation of succinate was interpreted as molecular sign of the shift in cellular metabolism to anaerobiosis. It was explained in details by proteomic analysis, by a decrease in SDH expression and a simultaneously increase in enzyme succinyl-CoA synthetase (SCS) expression, which allows succinate biosynthesis in the TCA cycle, under Zn exposure of oysters (Meng et al., 2017). Nevertheless, in the same study, fumarase (FUM), another Fe-S enzyme that converts fumarate to L-malate in the TCA cycle, showed significantly increased levels of expression at the same time. This was interpreted as a compensation mechanism for ensuring that the TCA cycle remains active when exposed to metals (Meng et al., 2017). This indicates that Fe-S enzymes of the TCA cycles can be either up- or down-regulated upon Zn exposure and that defense strategy against Zn toxicity exists in bivalves, as it is the case with bacteria exposed to metals such as Al that interfere with Fe metabolism (Chenier et al., 2008). In another study, two pedigrees (White and Zebra) of clam *Ruditapes philippinarum* were exposed with Zn^{2+}(50 $\mu g.L^{-1}$) during 48 h and the metabolomics results showed differences in digestive gland between White and Zebra clam samples in succinate levels: in Zebra clam, succinate was increased, whereas it was decreased in White clam after Zn exposure (Ji et al., 2015). In conclusion, it seems clear that Zn affects the TCA cycle metabolites, through its effect on Fe-S enzymes involved in the cycle. Concerning the level of succinate, it may be increased or decreased upon Zn exposure, depending on the species, with respect to over- or under-expression of the different Fe-S enzymes of the TCA cycle.

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

Osmoregulation

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

Indeed, L-phenylalanine is a catecholamine precursor and its lower level may lead to a decrease in dopamine. Dopamine and serotonin are neurotransmitters involved in the modulation of Na^+ and K^+ transport in gills of crustaceans (Mo et al., 1998) and in lateral cilia in gills of bivalves (Carroll and Catapane, 2007). The down-modulation of dopamine may thus have a repercussion on osmoregulation of cells submitted to Zn exposure. A similar relationship between L-phenylalanine decrease and osmoregulation was previously suggested (Aru et al., 2016). These authors observed a down-modulation of phenylalanine in presence of $ZnCl_2$ (20, 50, 100 and 150 $\mu g.L^{-1}$) under laboratory conditions after 48 h exposure for the clam *Ruditapes decussatus*, associated with a significant increment in organic osmolyte production (hypotaurine and homarine) and a decrease of the content of free amino acid.

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

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

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

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

Oxidative stress defense

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

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

Other metals and in particular copper are able to induce oxidative stress in bivalves. Cu^{2+}-exposed haemocytes showed a remarkable increase in ROS production, which induced oxidative stress in mussel (*Perna canaliculus*) (Nguyen et al., 2018). This was accompanied by a decrease in GSH. This compound is an important antioxidant, able to react with electrophilic oxidants (e.g., H_2O_2) resulting in conversion of two GSH molecules into its oxidized form (GSSG). Two other molecules decrease simultaneously upon Cu^{2+} exposure: cysteine, which is involved in the GSH metabolic pathway and methionine, which takes place in the transsulfuration pathway, a source of cysteine for GSH. These concomitant phenomena were identified as important signatures of oxidative stress (Nguyen et al., 2018).

Purine metabolism

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

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

Previously, a decrease of inosine (between -30 and -80%) and hypoxanthine (between -40 and -50%) was observed in whole body of zebrafish, after exposure to $ZnCl_2$ at different concentrations (10, 100, 500 and 1000 $\mu g.L^{-1}$ for 72 h). It was interpreted as an impairment of the purine metabolism caused by exposure to Zn. As it was combined with an ATP level increased, it was hypothesised that Zn would induce an increased energy demand to fulfil essential maintenance costs and ensure organism survival (e.g. cellular protective mechanisms, detoxification) (Kim et al., 2016; Sokolova et al., 2012).

Tryptophan metabolism

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

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

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

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

Conclusion

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

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

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

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FIGURE CAPTIONS

Figure 1: Score plots of PCA and PLS-DA for negative mode (LC-Negative), positive mode (LC-Positive) of liquid chromatography. Ellipses score plots represented the confidence limit (95%) of Hotelling's T2 statistic (Green ellipse for reference samples and red ellipse for Zn-Exposed samples).

Figure 2: Box plots of the relative abundance of the 11 identified metabolites in *Mimachlamys varia* gills showing a significant difference between reference and Zn-exposed samples. Green plots represent the reference samples, red plots represent the zinc exposed samples. The mean value is given by the dark diamond-shaped symbol.

Figure 1

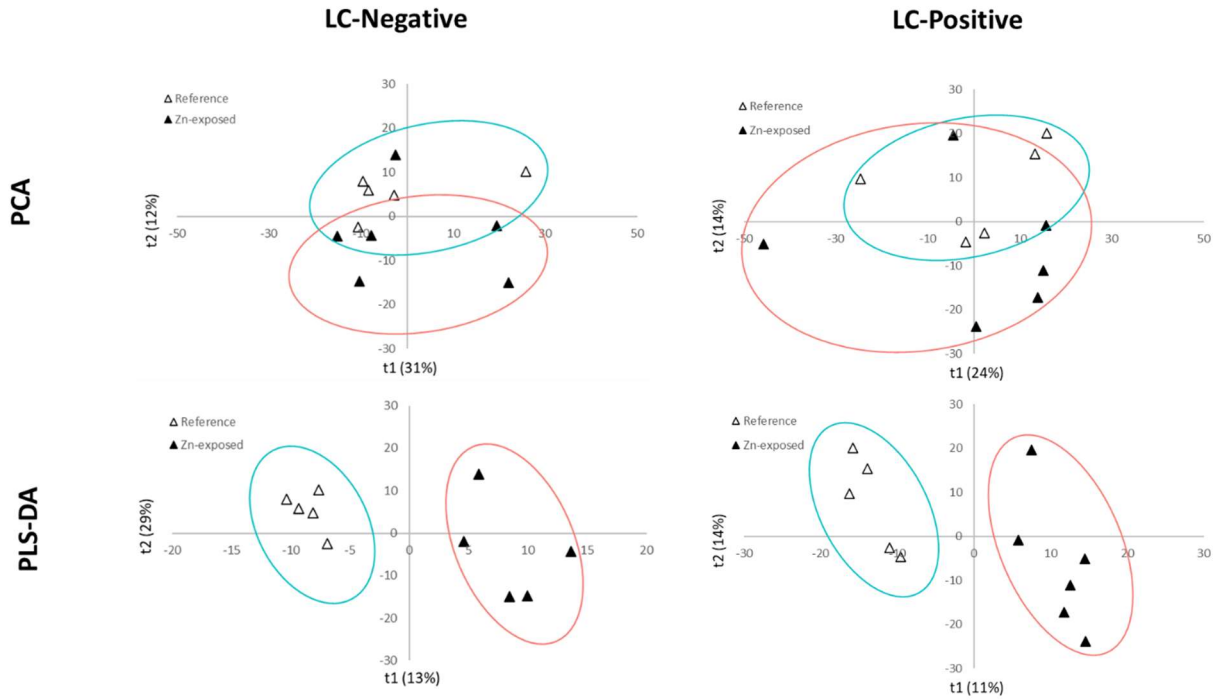

Figure 2

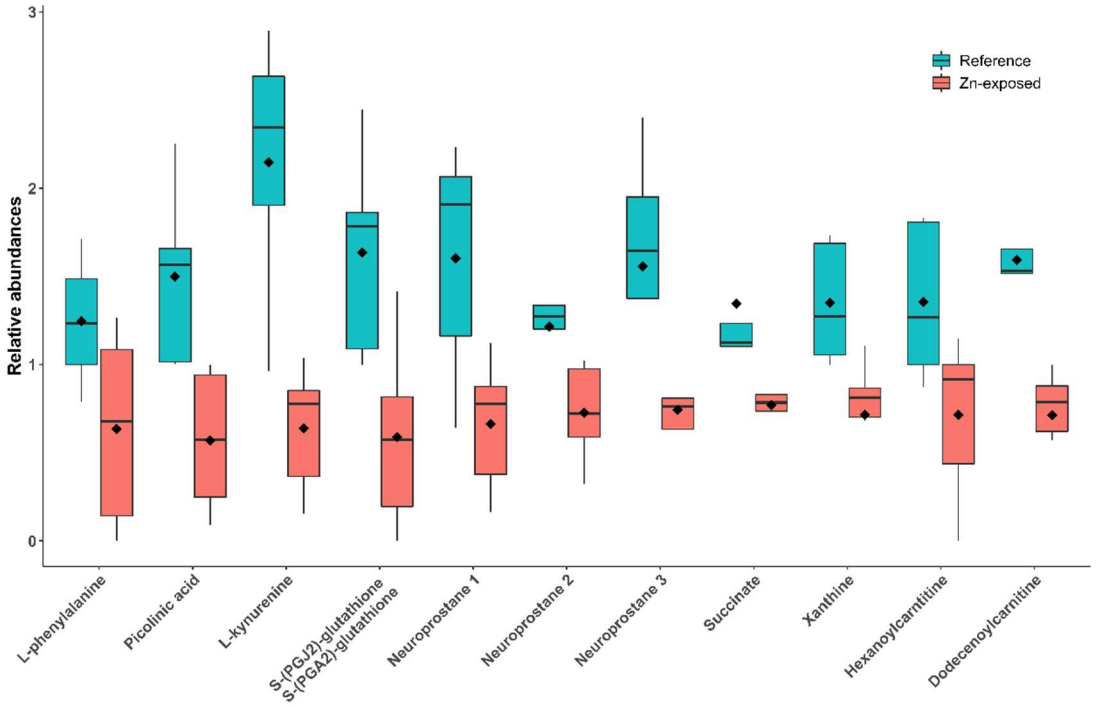

Table 1: Identified metabolites varying after 48h-zinc exposure

Metabolite	Plateform	Retention time (s)	Formula	Monoisotopic mass (Da)	Adduct	Observed mass (m/z)	Theorical mass (m/z)	Mass error (ppm)	Ratio between reference and exposed to Zn	P-value
Picolinic acid	LC/MS Neg	198	C_5H_4N	123.032	$[M-CO_2-H]^-$	78.0346	78.0349	3.8	2.62	0.013
L-kynurenine	LC/MS Neg	417	$C_{10}H_{11}N_2O_3$	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	$[M-2H_2O-H]^-$	391.2123	391.2126	0.8	1.67	0.050
Neuroprostane 3	LC/MS Neg	981	$C_{22}H_{31}O_5$	376.225	$[M-H_2O-H]^-$	375.2167	375.2177	2.7	2.09	0.050
Succinate	LC/MS Neg	247	$C_4H_5O_4$	118.0266	$[M-H]^-$	117.0188	117.0193	4.3	1.75	0.050
L-phenylalanine	LC/MS Pos	417	$C_9H_{12}NO_2$	165.079	$[M+H]^+$	166.0869	166.0863	3.6	1.96	0.050
S-(PGA2)-glutathione S-(PGJ2)-glutathione	LC/MS Pos	658	$C_{30}H_{47}N_3O_{10}SNa$	641.2982	$[M+Na]^+$	664.289	664.2874	2.4	2.78	0.001
Xanthine	LC/MS Pos	367	$C_5H_5N_4O_2$	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