

In situ evaluation of oxidative stress and immunological parameters as ecotoxicological biomarkers in a novel sentinel species (*Mimachlamys varia*)



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ABSTRACT

Although the variegated scallop *Mimachlamys varia* seems to be a suitable sentinel species for contaminant monitoring, no study has identified biomarkers in this species. In order to fill this gap, this study conducted an *in situ* biomarker approach. *M. varia* were collected in contaminated and uncontaminated areas and responsiveness of oxidative stress and immunological biomarkers was evaluated in the digestive gland. In parallel, 14 trace element concentrations were evaluated in the same organ. Superoxide dismutase activity and malondialdehyde content responded efficiently to *in situ* contamination when a certain degree of contamination was reached. Laccase-type phenoloxidase showed a high sensitivity but saturation of the response was highlighted for the highest contaminations. Additionally, correlations were found between biomarkers and trace element concentrations. Taken together, results showed that biomarker approach conducted in the digestive gland of *M. varia* represents a sensitive analytical tool to highlight ecotoxicological issues in coastal marine ecosystems.

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1. Introduction

The European Union's Marine Strategy Framework Directive (MSFD Dir. 2008/56/EC) was adopted in June 2008 and requires member states to take the necessary measures to achieve or maintain Good Environmental Status (GES) of the marine environment by 2020. In the aim of highlighting ecotoxicological issues, the chemical assessment of contaminants is an interesting first step. However, only the biomonitoring approaches can reveal the impact of contaminants on the biota. In this context, the biomarker approach aims at measuring "biochemical, cellular, physiological or behavioral variations in tissue or body samples or at the level of whole organisms that provides evidence of exposure to and/or effects of, one or more chemical pollutants (and/or radiations)" (Depledge, 1993). Such an approach requires the use of a pertinent couple "biomarkers-species", meaning that biomarkers of value should be assessed

in an appropriate species. The value of a biomarker could be defined as a trade-off between its sensitivity, enabling an early warning of the environmental risk, and its significance, enabling to warn on biological impact upon high levels of biological organization (such as organisms or populations) (Amiard and Amiard-Triquet, 2008). Oxidative stress biomarkers have been much investigated to evaluate the sub-lethal effects of several contaminants such as trace elements, persistent organic pollutants, or polycyclic aromatic hydrocarbons (reviewed in Valavanidis et al., 2006). They have been claimed by several authors to be valuable biological indicators of environmental risks (Van der Oost et al., 2003; Valavanidis et al., 2006) due to their sensitivity as well as their significance as modulation of these parameters reflects a degradation of the functional integrity (Halliwell and Gutteridge, 1999). In the same way, immune parameters have also been investigated in several aquatic organisms exposed to contaminants (Galloway and Depledge, 2001; Hannam et al., 2010; Luna-Acosta et al., 2011; Milinkovitch et al., 2011) and they are considered valuable biomarkers due to their precocity and significance since altered immunity is commonly related to an impairment of the health status of an organism (Amiard and Amiard-Triquet, 2008). The appropriateness of species for biomonitoring (also called sentinel species) depends on several criteria such as how sedentary, how easily collected and how wide-spread

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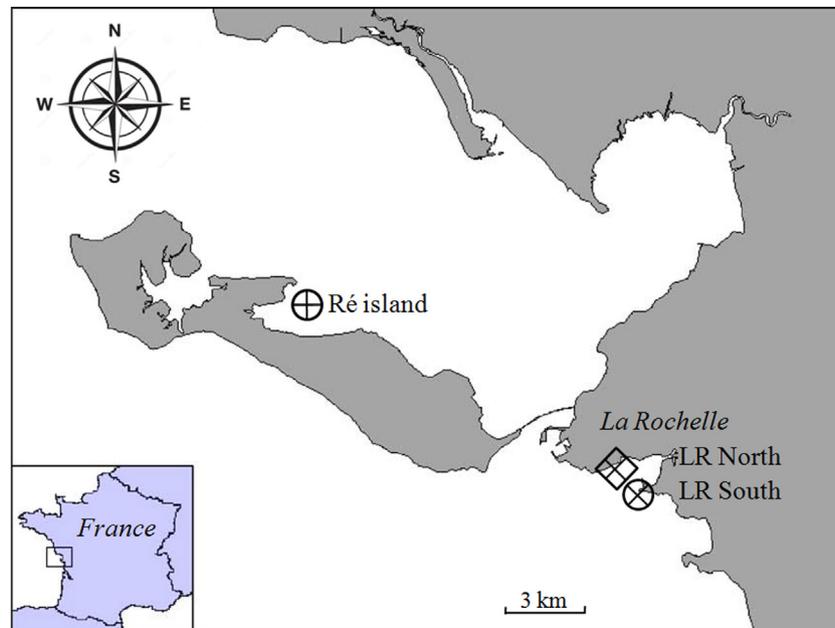


Fig. 1. Location of the three sampling areas: Ré island (⊕, geographical coordinates: 46.220, –1.404), LR South (⊕, geographical coordinates: 46.137, –1.175) and LR North (⊖, geographical coordinates: 46.149, –1.189). Adapted from Bustamante and Miramand, 2005b.

they are (Rand, 1985; White, 2004). On this basis, the variegated scallop *Mimachlamys varia* seems to be appropriate, since it is sessile, can easily be collected on the shore at low tide and has a large distribution (from the North Sea to the south east of the Atlantic Ocean and in Mediterranean sea, Gofas, 2014). Additionally, this species is of interest considering its very elevated incorporation and retention capacities for contaminants (Metian et al., 2009a,b,b) which seem to be higher than the ones of currently used sentinel species such as mussels or oysters: e.g., an *in situ* study of Bustamante et al. (2002) indicated a 2–4 times higher bioaccumulation of Polonium-210 in the soft parts of *M. varia* than in *Mytilus edulis*.

However, although *M. varia* can be considered interesting and suitable for *in situ* biomonitoring, to the best of our knowledge, no study has identified how possible biomarkers respond to contamination in this species.

Thus, the goal of this study was to identify, in *M. varia*, biomarkers that respond to *in situ* contamination. For this purpose, modulations of oxidative stress and immunological parameters were monitored in the digestive gland of *M. varia* collected in contaminated and uncontaminated areas on the Atlantic coast of France.

Oxidative stress has been evaluated through lipid peroxidation by quantifying malondialdehyde (MDA), a metabolite of cell lipid degradation. In addition, superoxide dismutase (SOD) and catalase (CAT) activities were measured considering their important role in antioxidant response (Valavanidis et al., 2006). Immune system alteration was evaluated by assessing a phenoloxidase (PO) activity (laccase-type) since this enzymatic activity already has been shown to be modulated by contaminants (Gagnaire et al., 2004; Bado-Nilles et al., 2008; Luna-Acosta et al., 2010) and plays an important role in the immune defense mechanism in invertebrates (Cerenius et al., 2008).

In parallel, the concentrations of 14 trace elements (Ag, As, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Se, Sn, V and Zn) have been assessed in the digestive gland of *M. varia* since these contaminants are considered as tracers of anthropogenic inputs in coastal waters (Sanudo-Wilhelmy and Flegal, 1992; Matthai et al., 2002) and

show a preferential bioaccumulation in this organ (Bustamante and Miramand, 2004, 2005a).

2. Materials and methods

2.1. *In situ* sampling and preparation of biochemical material

Sampling was conducted simultaneously on 3 sampling sites the 29th of March 2013. Sampling occurred at low tide in the infralittoral zone of the three areas (already studied in Bustamante and Miramand, 2005b) described in Fig. 1: Ré island, La Rochelle South and La Rochelle North. Fourteen scallops (with a height above 37 mm) were collected per area. The average height of the shells was 40.5 ± 0.7 mm (mean \pm SE) and the average weight of the soft parts was 1.61 ± 0.2 g (mean \pm SE) with no significant difference observed between areas. On the sampling site, the digestive gland was directly removed and stored at -60°C in dry ice then at -80°C back in the laboratory. The remaining soft parts of the organism were also stored at -80°C for further analysis.

Digestive glands were then ground with a potter and divided into 2 samples used for trace element analysis and biochemical assessments of biomarkers. For trace element analysis samples were freeze-dried for 48 h. For biomarker assessment, samples were homogenised in ice-cold phosphate buffer (100 mM, pH 7.2, 1100 mOsm) containing ethylene diamine tetra acetic acid (EDTA 0.2 M) and free protease inhibitors (halt protease inhibitor cocktail 87785, Thermo Scientific). The homogenates were centrifuged at 12,500 g at 4°C for 15 min and the post-mitochondrial fractions (PMF) were used for biochemical assays.

2.2. Trace elements assessment

Analyses of Ag, As, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Se, Sn, V and Zn were performed with a Varian Vista-Pro ICP-OES and a ThermoFisher Scientific XSeries 2 ICP-MS. To this end, aliquots weighing between 0.1 and 0.2 g were digested using a 3:1 (v/v) 67–70% HNO₃/34–37% HCl mixture (Fisher, trace metal quality). Acidic digestion of the samples was carried out overnight at room temperature and then in a Milestone microwave oven (30 min with

constantly increasing temperature up to 120 °C, then 15 min at this maximal temperature). Each sample was made up to 50 mL with ultrapure quality water (Milli-Q). For samples with a weight less than 0.1 g, the mixture used was 3:1 (v/v) 67–70% HNO₃/34–37% HCl and the sample was completed with ultra pure water up to 25 mL. Two certified reference materials (CRMs) and one blank, treated and analyzed in the same way as the samples, were included in each analytical batch. CRMs were DOLT-4 (dogfish liver) and TORT-2 (lobster hepatopancreas) both from the National Research Council Canada (NRCC). Mean element concentrations are expressed in µg/g of dry weight. The detection limits were 0.02 (Ag), 0.25 (As), 1.24 (Cd), 0.02 (Co), 0.02(Cr), 1.24 (Cu), 4.97 (Fe), 1.24 (Mn), 0.05 (Ni), 0.02 (Pb), 0.12 (Se), 0.02 (Sn), 0.5 (V) and 4.97 (Zn) µg/g dry weight.

2.3. Biomarker assessment

Protein concentrations in the PMF were determined using Bradford (1976) with a BioRad assay kit (500-0002) that contained bovine serum albumin (BSA) as a standard and Brilliant Blue G 250 as a reactant. This reaction was measured at 570 nm using a spectrophotometer (SAFAS Flx-Xenius).

Lipid peroxidation was estimated by assessing the malondialdehyde (MDA) concentration in the PMF. For this purpose a commercially available MDA assay kit (Oxis International) was used. The method was based on the reaction of MDA with a chromogenic reagent (*n*-methyl-2-phenylindole). The blue product was quantified by measuring absorbance at 586 nm (Gérard-Monnier et al., 1998) using the SAFAS Flx-Xenius spectrophotometer. The results are presented in nmol of MDA/mg of protein.

The SOD activity was assessed in the PMF using the method developed by Paoletti et al. (1986). The assay, involving EDTA, MnCl₂ and mercaptoethanol, measured the decrease of nicotinamide adenine dinucleotide (NADH) oxidation. This inhibition of oxidation was monitored spectrophotometrically at 340 nm (using the SAFAS Flx-Xenius spectrophotometer) and is a function of SOD activity. Fifty percent inhibition of oxidation correspond to one unit of SOD. The results were presented in U of SOD/mg of protein.

The CAT activity was assessed according to the method of Babo and Vasseur (1992). Standard and PMF catalase reduced hydrogen peroxide into oxygen and water. Consequently, the degradation of hydrogen peroxide is a function of the enzyme activity. The degradation is assessed at 240 nm using the SAFAS Flx-Xenius spectrophotometer. The results were expressed in U of CAT/g of proteins.

Using a method developed by Luna-Acosta et al. (2010), the phenoloxidase (PO) activity was measured in the PMF. The method is based on the fact that the oxidation of PPD (*p*-phenylenediamine) is catalyzed by laccase. Thus, the activity of laccase-type phenoloxidase is a function of PPD degradation. The reaction was determined at 420 nm using a SAFAS Flx-Xenius spectrophotometer. In parallel, the non-enzymatic auto-oxidation (oxidation in the absence of PMF) was subtracted. Laccase-type PO activity was expressed as U/mg of protein (one unit is defined as the amount of enzyme that catalyses the appearance of 1 µmol of product per min).

2.4. Statistical analysis

The statistical analysis was carried out using Statistica software. The homoscedasticity (using Levene test) was demonstrated for all variables and one-way analyses of variance (ANOVAs) were conducted to highlight significant differences between groups. If a significant difference was detected (for $P_{\text{value}} < 0.05$), pairwise comparisons between groups were conducted using an HSD Tukey *post-hoc* test. A multi-correlation Spearman test was used to highlight correlations between trace element concentrations and

Table 1

Trace element concentrations (µg/g of dry weight) in the digestive glands of *Mimachlamys varia* ($n = 14$ per sites) from the three sampling areas (Ré island, LR South, LR North).

	Sampling sites		
	Ré island	LR South	LR North
Ag	4.07 ± 0.83 ^a	9.06 ± 1.45 ^b	16.94 ± 1.16 ^c
As	10.17 ± 1.48 ^a	14.04 ± 0.14 ^b	14.99 ± 0.81 ^b
Cd	12.76 ± 1.72 ^a	25.74 ± 5.22 ^a	42.04 ± 3.58 ^b
Co	0.57 ± 0.08 ^a	0.92 ± 0.08 ^b	0.97 ± 0.06 ^b
Cr	0.93 ± 0.15 ^a	1.72 ± 0.19 ^{a,b}	1.63 ± 0.07 ^b
Cu	17.38 ± 1.77 ^a	54.77 ± 22.92 ^{a,b}	102.42 ± 12.84 ^b
Fe	677 ± 115 ^a	1115 ± 82 ^b	887 ± 54 ^{a,b}
Mn	17.27 ± 2.81 ^a	32.14 ± 1.52 ^a	23.97 ± 1.41 ^a
Ni	1.77 ± 0.27 ^a	2.61 ± 0.23 ^b	2.57 ± 0.13 ^b
Pb	1.36 ± 0.22 ^a	2.32 ± 0.17 ^b	2.03 ± 0.09 ^b
Se	5.23 ± 0.79 ^a	8.83 ± 0.25 ^b	8.55 ± 0.5 ^b
Sn	0.042 ± 0.0095 ^a	0.084 ± 0.0018 ^b	0.095 ± 0.0059 ^b
V	2.29 ± 0.41 ^a	4.07 ± 0.13 ^b	4.75 ± 0.31 ^b
Zn	59.5 ± 10.3 ^a	124 ± 11.5 ^b	90.63 ± 6.01 ^{a,b}

Different letters (^{a,b} and/or ^c) above values indicate a significant difference, where $P < 0.05$.

biomarkers values. The results were expressed as mean ± standard error of the mean.

3. Results

3.1. Trace element concentrations

Trace element concentrations are presented in Table 1. Ag concentrations were significantly different between all groups with the highest contamination recorded for LR North scallops, medium contamination for LR South ones and the lowest for those from Ré island. As, Co, Cr, Pb, V, Se and Sn concentrations showed significantly higher values in LR sites (North and South) but no significant difference between these sites were observed. For Cu and Fe, the only significant differences were found between LR North and Ré island whereas for Mn and Zn, significant differences were only found between LR South and Ré island). Cd concentrations were significantly higher in LR North scallops than in those from Ré island and LR South. Ni showed no significant variation of concentrations.

3.2. Lipid peroxidation (MDA content)

Malondialdehyde content in Ré island scallops showed an average value of 4.09 ± 0.50 nmol/mg of protein (Fig. 2A). Compared to this value, LR South scallops did not show significant difference but LR North had a 37% higher content. Pairwise comparison tests did not reveal significant difference between scallops from LR North and South.

3.3. Superoxide dismutase (SOD) activity

The SOD activity in the digestive gland of *M. varia* is presented in Fig. 2B. In Ré island, mean value was 29.40 ± 1.82 U/mg of protein. The activity of the enzyme is not significantly higher in LR South. However, scallops from LR North showed significantly higher (by 36% and 35%) activities when compared to Ré island and LR South ones, respectively.

3.4. Catalase (CAT) activity

The mean CAT activity in the digestive gland of *M. varia* was 426.8 ± 72.8 U/g of protein in Ré island scallops (Fig. 2C). No significant difference was observed between sampling sites. Enzyme activities showed a high intra-group variability.

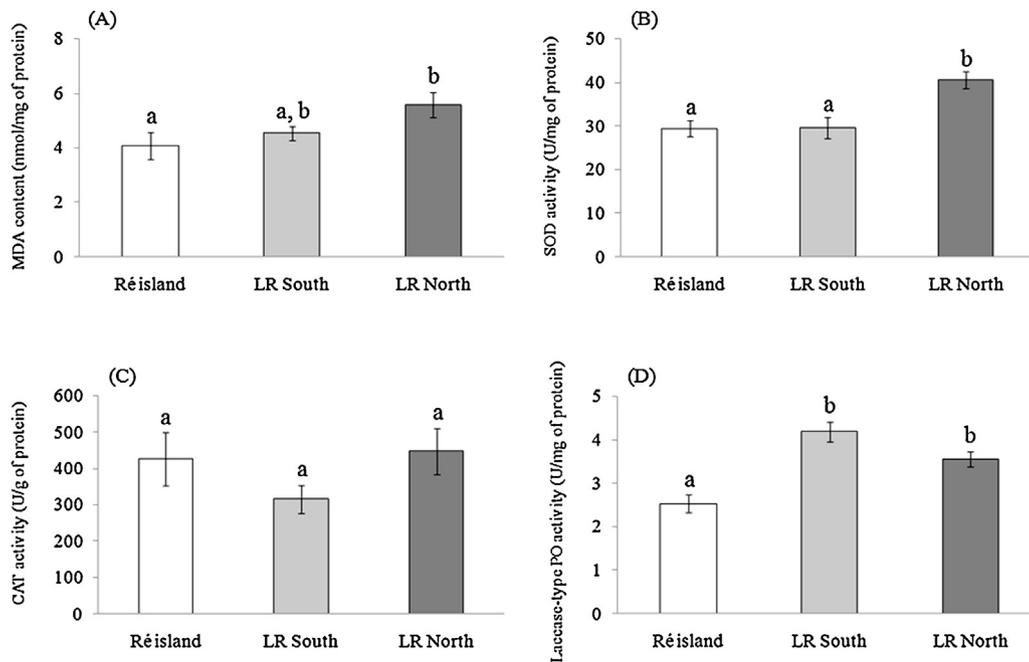


Fig. 2. Biomarkers assessed in the PMF of *Chlamys varia* digestive glands from Ré island, LR South and LR North. (A) MDA contents, (B) superoxide dismutase (SOD) activity, (C) catalase (CAT) activity, (D) laccase-type PO activity. Values represent mean \pm standard error ($n = 14$ per sites). Different letters above bars (a, b and/or c) indicate a significant difference, where $P < 0.05$.

3.5. Laccase-type PO activity

Laccase-type PO activity (Fig. 2D) was 2.53 ± 0.20 U/mg of protein in the digestive gland of organisms sampled in Ré island. When compared to this value, the activity in the digestive gland of South and North LR scallops is higher (64 and 28%, respectively). No significant difference was observed when conducting a statistical comparison between South and North LR groups.

3.6. Correlations between biomarkers responses and trace element concentrations (Table 2)

Multi-correlation test was conducted between trace element concentrations and biomarkers values. It revealed: (i) significant correlations of SOD activity with Ag, Co, Cd, Ni, and V, and a trend toward correlation with Cr ($P = 0.09$); (ii) no correlation of CAT activity with any of the considered trace elements; (iii) significant correlations of MDA content with Cr and V and trends toward correlation with Ag ($P = 0.09$), Cd ($P = 0.07$), Co ($P = 0.08$) and Pb ($P = 0.06$); (iv) significant correlations of laccase-type PO activity with all trace elements except with Ag and Sn.

4. Discussion

In order to identify biomarkers of contamination in *M. varia*, oxidative stress and immunological parameters were assessed in organisms collected in several areas potentially differentially contaminated: Ré island, previously identified as a reference site; LR South and LR North, subjected to important industrial and domestic contamination (Bustamante et al., 2002,b; Bustamante and Miramand 2004, 2005a,b,b). Levels of contamination of each area were estimated by assessing the trace element concentrations in the digestive glands of *M. varia* since these contaminants (and particularly Ag) are considered valuable tracers of sewage in coastal waters (Sanudo-Wilhelmy and Flegel, 1992).

Regarding all trace elements (except for Cd, Cr, Cu and Fe), results highlight a higher contamination of LR South, when compared to the reference site (Ré island). In the same way, regarding all

Table 2

Correlations between biomarkers responses and trace element concentrations in the digestive glands of *Mimachlamys varia* ($n = 14$ per sites) from the three sampling areas (Ré island, LR South, LR North).

	MDA	SOD	CAT	Lac-PO
Ag	<0.1	<0.05	–	–
As	–	–	–	<0.05
Cd	<0.1	<0.05	–	<0.05
Co	<0.1	<0.05	–	<0.05
Cr	<0.05	<0.1	–	<0.05
Cu	–	<0.05	–	<0.05
Fe	–	–	–	<0.05
Mn	–	–	–	<0.05
Ni	–	<0.05	–	<0.05
Pb	<0.1	–	–	<0.05
Se	–	–	–	<0.05
Sn	–	–	–	<0.1
V	<0.05	<0.05	–	<0.05
Zn	–	–	–	<0.05

<0.05 indicates a significant correlation where $P_{\text{value}} < 0.05$. <0.1 indicates a trend toward correlation where $P_{\text{value}} < 0.1$. – indicates no trend toward correlation neither significant correlation. MDA: malondialdehyde; SOD: superoxide dismutase; CAT: catalase; Lac-PO: laccase-type PO.

trace elements (except for Fe, Mn and Zn), the contamination is also higher in LR North than in the reference site. Taken together, these results confirmed that LR South and North remained highly contaminated decades after the previous studies of Bustamante et al. (2002) and Bustamante and Miramand (2004, 2005a,b),). Moreover, while most of the trace element concentrations did not show differences between the three sampling sites, Ag contamination was consistent with the release from the different activities of the city of La Rochelle (Fig. 1): contamination was highest in LR North, intermediate in LR South and lowest in Ré island. These results confirmed that Ag is a sensitive tracer of sewage in coastal waters (Sanudo-Wilhelmy and Flegel, 1992; Matthai et al., 2002) and suggested that anthropogenic inputs are more intense in LR North than in LR South.

While trace element measurements in the digestive gland of *M. varia* informed about the general anthropogenic sewage in coastal

waters and also validated the choice of the three sampling sites, this assessment revealed also, more particularly, the contamination and thus the potential biological effects (response, toxicity) of trace elements. Regarding this link between trace element bioaccumulation and biological effects, a specific attention could be given to non-essential metals (in the present study Ag, Cd and Pb) since these elements are not required by the organism and are thus likely to cause biological effects and then toxicity (Rainbow, 2002). Taken together, the concentrations of these 3 elements in the 3 sampling sites described a pattern showing the highest contamination in LR North, a medium one in LR South and the lowest for Ré island.

In summary, the trace element concentrations highlighted three sampling sites with different degrees of contamination: high, medium and low (or reference) contaminated sites.

Using *M. varia* from these three sites, this study aims at identifying biomarkers that efficiently respond to *in situ* contamination. For this purpose, biomarkers of oxidative stress were assessed in *M. varia* from LR North, LR South and Ré island. Lipid peroxidation was evaluated through MDA content with values ranging between 4.1 and 5.6 nmol/mg of protein (in Ré island and LR North, respectively). Our results showed significantly higher contents of MDA in the digestive gland of scallops sampled in LR North than in the ones collected from Ré island. This result highlighted an increased lipid peroxidation in the scallops from the contaminated site. This is consistent with the results of Liu et al. (2012) who also showed this phenomenon studying *Chlamys farreri* from uncontaminated and contaminated sites. Although the contents of MDA in *M. varia* from LR South seem to be higher than those from Ré island and lower than those from LR North, no significant difference was observed. This shows that no modulation of lipid peroxidation occurred in organisms from LR South, when compared to the reference site. Taken together these results suggest that MDA, assessed in the digestive gland of *M. varia*, is a biomarker responding efficiently to *in situ* contamination, although a certain degree of contamination is required to induce this response.

Consequently, in order to identify the biomarkers that respond to an intermediate degree of contamination, this study also investigated the modulation of more sensitive oxidative stress biomarkers. For this purpose SOD and CAT activities were evaluated since these enzymes are considered as precocious biomarkers linked to oxidative stress, their induction (by reactive oxygen species, ROS) appearing before oxidative damages (Valavanidis et al., 2006).

Regarding SOD activity, values ranged between 29.4 and 40.7 U/mg of protein for scallops collected in Ré island and LR North, respectively.

The significantly higher SOD activity in LR North (when compared to Ré island) is in accordance with several studies conducted in *Pectinidae* that showed a modulation of this enzyme activity due to contaminants (Pan et al., 2005, 2006, 2009; Zhang et al., 2010). This higher activity revealed an antioxidant response (probably induced by ROS) in LR North organisms. However, in the same way as for MDA, SOD activity was not significantly different in LR South from that in Ré island suggesting that SOD responds efficiently to contamination although a degree of contamination is necessary.

Concerning CAT activities, no significant difference was found between the three groups of organisms (probably due to the high intra group variability) suggesting that this biomarker is not responsive enough to be used in future biomonitoring studies with *M. varia*.

In contrast, MDA contents and SOD activity can be considered responsive enough to be used for *in situ* biomonitoring studies with *M. varia* as a sentinel species. However, results also show that oxidative stress seems to not be induced by intermediate contaminations showing the need to consider biomarkers related to other key physiological functions. For this purpose, a biomarker

of immune system, laccase-type PO was assessed in the digestive gland.

Concerning laccase-type PO, our results revealed a significantly higher PO activity in the two contaminated sites (LR South and North) than in the reference site. This result is in accordance with those of Luna-Acosta et al. (2011, 2012), who found a modulation of the enzyme activity with different types of contaminants (hydrocarbons as well as herbicides and pharmaceuticals, respectively). Since both contaminated sites revealed a biomarker response, these results suggest that laccase-type PO activity is a sensitive biomarker responding to any degrees of contamination. However, no significant difference was found between contaminated sites. This could be due to a saturation of the response of laccase-type PO activity to contaminants: the higher contaminant stress did not induce a greater response of the enzyme.

Thus, results of this study showed several biomarkers responding to *in situ* contamination. Although identifying exactly the contaminants that are involved in this biomarker response is hardly possible, correlation tests were conducted to give information on this issue. Results revealed similarities in the correlation patterns of both responding biomarkers of oxidative stress (*i.e.*, SOD and MDA). Indeed, both biomarkers showed correlations or trends toward correlations with Ag, Co, Cd, Cr and V. These correlations suggest that oxidative stress could be partly due to (i) bioaccumulation of these trace elements or/and (ii) the contamination by sewage since trace elements (and more particularly Ag) are tracers of this type of contamination. Results also revealed discrepancies in the patterns of correlation between responding biomarkers and trace elements. Indeed, while Cu and Ni were correlated with SOD activity, no correlation was observed with MDA content. This could be due to the fact that, in this case, the SOD response was efficient enough to prevent the lipid peroxidation induced by these two trace elements. Consequently, the influx of Cu and Ni did not result in higher MDA contents. Regarding to the correlation between laccase type-PO and trace elements, results showed correlations for all trace elements except for Ag. This suggests that modulation of laccase-type PO activity is in part due to trace element bioaccumulation but seems not to be due to sewage contamination.

Although trace element concentrations have been shown to be correlated with biomarker responses, other *in situ* parameters could also have effects on the interrelationship. Among the several natural abiotic factors, oxygen level, temperature and salinity are the most likely to modulate oxidative stress (Lushchak, 2011) as well as immunological parameters in aquatic animals (*e.g.*, Wang et al., 2012; Munari et al., 2011). In this study, variation of these parameters could be partly involved in the biomarker modulation observed. However, monitoring conducted 5 days before and after the sampling date in two sites close to the sampling areas (46.278, –1.377 and 46.055, –1.131), revealed only slight variability of these parameters: the average water surface temperature was 8.6 and 9.2 °C (in both sites respectively), salinity was 32.3 and 30.3‰, and oxygen concentration was 9.4 and 9.9 mgO₂/L (data from IFREMER database via personal communication of Yann Coupeau).

5. Conclusion

By assessing the modulation of oxidative stress and immunological parameters in the digestive gland of *M. varia*, collected in contaminated and uncontaminated areas, this study highlighted biomarkers responding to *in situ* contamination. Both SOD and MDA, as biomarkers of oxidative stress, responded to *in situ* contamination, although a certain degree of contamination was necessary to trigger this response. Laccase-type PO was higher in sites with intermediate and high levels of contamination than in the reference site but no difference between these two

different degrees of contamination was observed. Thus, each biomarker cannot independently differentiate the different degrees of contamination, but, considering the three responding biomarkers, three levels of biological effects, corresponding to the three levels of contamination, could be discerned. Such results showed that a biomarker approach in *M. varia* could be a sensitive and valuable tool to highlight ecotoxicological issues. However, the responsiveness of several other biomarkers (linked to several organism functions) should be investigated in order to support these conclusions. Additionally, complementary studies, comparing the responsiveness of common biomarkers in the variegated scallop and in conventional species used for biomonitoring (such as mussels or oysters), could be of great interest in order to assess the value of *M. varia* as a sentinel species. Indeed, little information is available on this topic: to the best of our knowledge only one biomonitoring study conducted by Cotou et al. (1998) found a higher concentration of methallothionein in the soft parts of *M. varia* when compared to the blue mussel *Mytilus galloprovincialis*.

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References

- Amiard, J.C., Amiard-Triquet, C., 2008. *Les Biomarqueurs Dans L'évaluation De L'état Écologique Des Milieux Aquatiques*. Tec & Doc Lavoisier, Paris, pp. 372.
- Babo, S., Vasseur, P., 1992. In vitro effects of thiram on liver antioxidant enzyme activities of rainbow trout (*Oncorhynchus mykiss*). *Aquat. Toxicol.* 22, 61–68.
- Bado-Nilles, A., Gagnaire, B., Thomas-Guyon, H., Le Floch, S., Renault, T., 2008. Effects of 16 pure hydrocarbons and two oils on haemocyte and haemolymphatic parameters in the Pacific oyster, *Crassostrea gigas* (Thunberg). *Toxicol. In Vitro* 22, 1610–1617.
- Bradford, M.M., 1976. A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254.
- Bustamante, P., Germain, P., Leclerc, G., Miramand, P., 2002. Concentration and distribution of ^{210}Po in the tissues of the scallop *Chlamys varia* and the mussel *Mytilus edulis* from the coasts of Charente-Maritime (France). *Mar. Pollut. Bull.* 44, 997–1002.
- Bustamante, P., Miramand, P., 2004. Interspecific and geographical variations of trace element concentrations in *Pectinidae* from European waters. *Chemosphere* 57, 1355–1362.
- Bustamante, P., Miramand, P., 2005a. Evaluation of the variegated scallop *Chlamys varia* as a biomonitor of temporal trends of Cd, Cu, and Zn in the field. *Environ. Pollut.* 138, 109–120.
- Bustamante, P., Miramand, P., 2005b. Subcellular and body distributions of 17 trace elements in the variegated scallop *Chlamys varia* from the French coast of the Bay of Biscay. *Sci. Total Environ.* 337, 59–73.
- Cerenius, L., Lee, B., Söderhäll, K., 2008. The proPO-system: pros and cons for its role in invertebrate immunity. *Trends Immunol.* 29, 263–271.
- Cotou, E., Roussis, V., Rapti, T., Vagias, C., 1998. A comparative study on the metallothionein content of six marine benthic organisms. *Rapp. Comm. Int. Mer Médit.* 35, 246–247.
- Depledge, M.H., 1993. The rational basis for the use of biomarkers as ecotoxicological tools. In: Fossi, M.C., Leonzio, C. (Eds.), *Non Destructive Biomarkers in Vertebrates*. Lewis Publishers, Boca Raton, Florida, pp. 271–295.
- Gagnaire, B., Thomas-Guyon, H., Renault, T., 2004. In vitro effects of cadmium and mercury on Pacific oyster *Crassostrea gigas* (Thunberg), haemocytes. *Fish Shellfish Immunol.* 16, 501–512.
- Galloway, T.S., Depledge, M.H., 2001. Immunotoxicity in invertebrates: measurement and ecotoxicological relevance. *Ecotoxicology* 10, 5–23.
- Gérard-Monnier, D., Erdelmeier, I., Régnard, K., Moze-Henry, N., Yadan, J., Chaudière, J., 1998. Reactions of 1-methyl-2-phenylindole with malondialdehyde and 4-hydroxyalkenals. Analytical applications to a colorimetric assay of lipid peroxidation. *Chem. Res. Toxicol.* 11, 1176–1183.
- Gofas, S., 2014. *Mimachlamys varia* (Linnaeus, 1758). Accessed through: World Register of Marine Species at <http://www.marinespecies.org/aphia.php?p=taxdetails&id=236719>
- Halliwell, B., Gutteridge, J.M.C., 1999. *Free Radicals in Biology and Medicine*. Oxford University Press, Oxford, pp. 192.
- Hannam, M.L., Bamber, S.D., Moody, A.J., Galloway, T.S., Jones, M.B., 2010. Immunotoxicity and oxidative stress in the Arctic scallop *Chlamys islandica*: effects of acute oil exposure. *Ecotoxicol. Environ. Saf.* 73, 1440–1448.
- Liu, N., Pan, L., Wang, J., Yang, H., Liu, D., 2012. Application of the biomarker responses in scallop (*Chlamys farreri*) to assess metals and PAHs pollution in Jiaozhou Bay China. *Mar. Environ. Res.* 80, 38–45.
- Lushchak, V.I., 2011. Environmentally induced oxidative stress in aquatic animals. *Aquat. Toxicol.* 101, 13–30.
- Luna-Acosta, A., Rosenfeld, E., Amari, M., Fruitier-Arnaudin, I., Bustamante, P., Thomas-Guyon, H., 2010. First evidence of laccase activity in the Pacific oyster *Crassostrea gigas*. *Fish Shellfish Immunol.* 28, 719–726.
- Luna-Acosta, A., Kanan, R., Le Floch, S., Huet, V., Pineau, P., Bustamante, P., Thomas-Guyon, H., 2011. Enhanced immunological and detoxification responses in Pacific oysters, *Crassostrea gigas*, exposed to chemically dispersed oil. *Water Res.* 45, 4103–4118.
- Luna-Acosta, A., Renault, T., Thomas-Guyon, H., Fauray, N., Saulnier, D., Budzinski, H., Le Menach, K., Pardon, P., Fruitier-Arnaudin, I., Bustamante, P., 2012. Detection of early effects of a single herbicide (diuron) and a mix of herbicides and pharmaceuticals (diuron, isoproturon, ibuprofen) on immunological parameters of Pacific oyster (*Crassostrea gigas*) spat. *Chemosphere* 87, 1335–1340.
- Matthai, C., Birch, G.F., Bickford, G.P., 2002. Anthropogenic trace metals in sediment and settling particulate matter on a high-energy continental shelf (Sydney, Australia). *Mar. Environ. Res.* 54, 99–127.
- Metian, M., Bustamante, P., Hédouin, L., Oberhansli, F., Warnau, M., 2009a. Delineation of heavy metal uptake pathways (seawater and food) in the variegated scallop *Chlamys varia* using radiotracer techniques. *Mar. Ecol. Prog. Ser.* 375, 161–171.
- Metian, M., Warnau, M., Teyssié, J.-L., Oberhansli, F.R., Bustamante, P., 2009b. Delineation of Pb contamination pathways in two species of *Pectinidae*: the variegated scallop (*Chlamys varia*) and the king scallop (*Pecten maximus*). *Sci. Total Environ.* 407, 3503–3509.
- Milinkovitch, T., Sanchez, W., Ndiaye, A., Le Floch, S., Thomas-Guyon, H., 2011. Liver antioxidant responses and innate immune function in golden grey mullet (*Liza aurata*) following exposure to dispersed crude oil. *Aquat. Toxicol.* 101, 155–164.
- Munari, M., Matozzo, V., Marin, M.G., 2011. Combined effects of temperature and salinity on functional responses of haemocytes and survival in air of the clam *Ruditapes philippinarum*. *Fish Shellfish Immunol.* 30, 1024–1030.
- Pan, L., Ren, J., Liu, J., 2005. Effects of benzo(k) fluoranthene exposure on the biomarkers of scallop *Chlamys farreri*. *Comp. Biochem. Physiol. Part C* 141, 248–256.
- Pan, L.Q., Ren, J., Liu, J., 2006. Responses of antioxidant systems and LPO level to benzo(a) pyrene and benzo(k) fluoranthene in the haemolymph of the scallop *Chlamys Ferrari*. *Environ. Pollut.* 141, 443–451.
- Pan, L.Q., Ren, J., Zheng, D., 2009. Effects of benzo(a) pyrene exposure on the antioxidant enzyme activity of scallop *Chlamys farreri*. *Chin. J. Oceanol. Limnol.* 27, 43–53.
- Paoletti, F., Aldinucci, D., Mocali, A., Caparrini, A., 1986. A sensitive spectrophotometric method for the determination of superoxide dismutase activity in tissue extracts. *Anal. Biochem.* 154, 536–541.
- Rainbow, P.S., 2002. Trace metal concentrations in aquatic invertebrates: why and so what? *Environ. Pollut.* 120, 497–507.
- Rand, G.M., 1985. Behavior. In: Rand, G.M., Petrocelli, S.R. (Eds.), *Fundamentals of Aquatic Toxicology: Methods and Applications*. Hemisphere Publishing Corp., Washington DC, pp. 221–263.
- Sanudo-Wilhelmy, S.A., Flegal, A.R., 1992. Anthropogenic silver in the Southern California Bight: a new tracer of sewage in coastal waters. *Environ. Sci. Technol.* 26, 2147–2151.
- Van der Oost, R., Beyer, J., Vermeulen, N.P.E., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environ. Toxicol. Pharmacol.* 13, 57–149.
- Valavanidis, A., Vlahogiannina, T., Dassenakis, M., Scoullou, M., 2006. Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. *Ecotoxicol. Environ. Saf.* 64, 178–189.
- White, G.J., 2004. Selection of ecological indicators for monitoring terrestrial systems. In: Wiersma, G.B. (Ed.), *Environmental Monitoring*. CRC Press, Boca Raton, pp. 263–282.
- Wang, Y., Hu, M., Cheung, S.G., Shin, P.K.S., Lu, W., Li, J., 2012. Immune parameter changes of hemocytes in green-lipped mussel *Perna viridis* exposure to hypoxia and hyposalinity. *Aquaculture* 356–357, 22–29.
- Zhang, Y., Song, J., Yuan, H., Xu, Y., He, Z., Duan, L., 2010. Biomarker responses in the bivalve (*Chlamys farreri*) to exposure of the environmentally relevant concentrations of lead, mercury, copper. *Environ. Toxicol. Pharmacol.* 30, 19–25.