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Seasonal variations of low pesticides contamination and biomarker responses in marine bivalves from French estuaries

Adélaïde Lerebours^{1§*}, Thierno Diallo^{1,2§}, Annie Lecureuil^{1,3}, Justine Receveur³, Valérie Huet¹, Julien Parinet², Thierry Guérin⁴, Stéphane Le Floch³, Hélène Thomas¹

¹UMRi CNRS LIENSs, Université de La Rochelle, Littoral Environnement et Sociétés, 2 rue Olympe de Gouges, La Rochelle, 17 000, France

²ANSES, Laboratory for Food Safety, F-94701 Maisons-Alfort, France

³Centre de documentation, de recherche et d'expérimentations sur les pollutions accidentelles des eaux (CEDRE), 715 rue Alain Colas, Brest, 29 218, France

⁴ANSES, Strategy and Programmes Department, F-94701 Maisons-Alfort, France

§: co-authors

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Corresponding Author

*Adélaïde Lerebours, phone number: +33 (0) 5 46 50 76 23, e-mail: adelaiderebours@univ-lr.fr, Address: UMR CNRS LIENSs, Université de La Rochelle, Littoral Environnement et Sociétés, La Rochelle, 17 000, France

Abstract

Biomarkers involved in detoxification process (GST), oxidative stress (SOD and MDA), immune response (Laccase) and neurotoxic disorders (AChE) were analysed in Pacific oysters and blue mussels collected from 4 locations within the Pertuis sea (France). Seasonal variations of total pesticide mean concentrations were found in seawater with metolachlor being the main pesticide measured (up to 32 ng/L). The majority of pesticide concentrations in sediment were below the LOD. Seasonal contamination differences were evidenced for chlortoluron, especially in mussels where concentrations reached 16 ng/g (wet weight) during the winter, in the Charente estuary, but no relationships with any of the biomarkers selected arisen. Actually, low concentrations of alpha-, beta-BHC and alachlor were correlated to GST activity, and low levels of hexachlorobenzene were linked to the AChE activity and MDA content in oysters. In mussels, low concentrations of methylparathion, parathion and beta-BHC were correlated to laccase.

Introduction

Coastal waters have been subjected to various contaminants leaching from urban, industrial and agricultural activities worldwide for many years. Numerous chemicals reaching the estuarine environment *via* rivers, wastewater treatment effluents and catchment leaching accumulate in seawater, sediments and/or aquatic organisms. These chemically exposed estuarine environments also represent productive shellfish farming areas. For instance, the Pertuis-Charentais littoral area, in south-west France, exposed to anthropogenic contaminants through the estuaries of the rivers Sèvre Niortaise, Charente and Seudre, produces 22% of the French shellfish production. The blue mussel, *Mytilus edulis*, and the Pacific oyster, *Crassostrea gigas*, are the most common species in the French shellfish production and have been used as sentinel species to monitor marine pollution (Beliaeff *et al.*, 1998; Beyer *et al.*, 2017; Lerebours *et al.*, 2021, 2022). Monitoring the contamination levels of the surrounding waters and the potential biological effects induced in organisms is necessary to ensure the safety and sustainability of bivalve farming. However, despite the presence of intensive farming in the catchment areas of the Pertuis estuaries, the long-term contamination levels of pesticides and their biological effects in farmed bivalves have yet to be elucidated.

Several interesting studies have assessed the contamination levels of pesticides in oysters (Luna-Acosta *et al.*, 2015a, Lerebours *et al.*, 2021), mussels (Lerebours *et al.*, 2021) and variegated scallops (*Mimachlamys varia*) (Breitwieser *et al.*, 2016, 2018) in the Pertuis seas. Species differences were observed in a recent pilot study led in the Pertuis Charentais region in February 2019 with six pesticides quantified in Pacific oysters (alpha BHC, chlorfenvinphos, chlorpyrifos, chlortoluron, metolachlor and parathion) against one (alpha BHC) in blue mussels. Those compounds and other persistent organic pollutants (POPs) were found at very low concentrations in biota, sediments and seawater. However, some pesticide concentrations were close or slightly above the environmental guidelines. For instance, the chlortoluron concentration in seawater was found above the environmental guidelines of 10 ng/L (Lerebours *et al.*, 2021). In addition, contamination levels of certain pesticides such as chlortoluron, metolachlor, chlorfenvinphos and chlorpyrifos in bivalves from the natural environment measured in this recent study were not or scarcely addressed before. Previous studies conducted in the same region focussed on older organochloride pesticides such as

DDT and/or lindane and their by-products in oysters collected in summer and winter 2008 (Luna-Acosta *et al.*, 2015a) or variegated scallops collected in winter and autumn 2014 (Breitwieser *et al.*, 2016) and found them at very low concentrations. In winter 2019, concentrations of these old pesticides were found below the limit of detection in oysters and mussels (Lerebours *et al.*, 2021). Although these previous studies were informative, only 8 or 14 pesticides were measured (Breitwieser *et al.*, 2016 and Luna-Acosta *et al.*, 2015a, respectively) or merely assessed once in a one-off pilot study (Lerebours *et al.*, 2021). Therefore, further knowledge is needed on the long-term contamination of a large number of pesticides in bivalves farmed in estuaries of the Pertuis seas.

Biochemical assays studying key molecules (or biomarkers) have been used for years to monitor the effects of exposure to pollutants on the health status of bivalves from the environment (Amiard-Triquet *et al.*, 2013). Some of those biomarkers are involved in the oxidative balance such as the superoxide dismutase (SOD) and the malonaldehyde (MDA) content (Pellerin-Massicotte, 1994; Livingstone, 2001), detoxification processes such as the glutathion-S-transferase (GST), the immune response such as the laccase (Luna-Acosta *et al.*, 2010, 2017) and neuronal disorders such as the acetylcholinesterase (AChE) (Viarengo *et al.*, 2007). In farming sites of the Pertuis seas, potential effects related to exposure to DDT, lindane and their byproducts have been assessed in oysters and correlations were established between their respective body burdens and the antioxidant, and immune responses (Luna-Acosta *et al.*, 2015b). Nevertheless, only very limited information exists in the available literature on the effects of pesticide exposure on the health of farmed bivalves in this region.

In order to fill those gaps, the objective of the study was to determine the contamination levels of thirty pesticides and five biomarker responses (SOD, MDA, GST, AChE and Laccase) in Pacific oysters and blue mussels over a year in the littoral area of the Pertuis Charentais, one of the main French shellfish production region. Concentrations of pesticides in oysters, mussels, sediments and seawater were determined in the estuaries of the three rivers: Sèvre Niortaise, Charente and Seudre, and at a farther site from the land coast in the South-West of the Ile de Ré: Le Martray. Finally, environmental parameters were recorded to assess their potential influence on pesticide contamination and/or bivalve physiology.

Methods

Experimental design

In March 2019, farmed mussels and oysters of 1.5 years were acclimated for 1 month and half to the same environmental conditions at a remote and reference site, Le Martray, in the South-West of Ile of Ré. In May 2019, 12 oysters (total length: 3.0 ± 0.5 cm) and 12 mussels (total length 3.7 ± 0.2 cm) were collected and 36 oysters and 36 mussels were left at that control site. 72 oysters and 72 mussels were relocated at the Sèvre and Charente estuaries, and 36 oysters were relocated at the Seudre estuary (Figure 1). In September 2019, January 2020 and May 2020, 12 oysters and 12 mussels were collected at Le Martray site and the three estuaries except the Seudre estuary for mussels as it was not a mussel farming area and the conditions were not suitable to grow mussels according to local farmers. 5 individuals per condition were used for pesticide analyses and 7 digestive gland samples (from 7 individuals) per condition were used for biochemical analyses. The weight and length of oysters and mussels were recorded after each sampling event and the dead were counted and discarded every month. Moreover, the condition index (CI) was calculated on 7 additional samples per condition as follows: $CI = \text{whole flesh weight} / \text{total weight} * 100$. In addition, one surface sediment sample (in the range of 0-5 cm depth and collected using a stainless-steel bowl) and one surface seawater sample (collected using a glass bottle) were collected at the reference site and the three estuaries (Figure 1) at each sampling time. Sediment sample at Ile of Ré in May 2019 could not be analysed because of a technical issue. After each sampling, mussels, oysters, sediment and water samples were frozen at -20°C until pesticide analyses. Mussels and oysters dedicated to biomarker analyses were snap frozen using liquid nitrogen in the field, transported in a dry shipper and stored at -80°C until analyses.

Pesticide analyses

Five bivalves from each site were thawed and homogenates of each entire bivalve were obtained by cutting the flesh into pieces. 1 g of mussel homogenate or 3 g of oyster homogenate were spiked with perdeuterated internal standards. Thirty pesticides (Table 1) were extracted from biological matrix following the QuEChERS method (Quick, Easy, Cheap, Efficient, Rugged and Safe) (Khorshid *et al.*, 2015; Pfannkoch *et al.*, 2015) as briefly described: each sample was mixed with a solution of acetonitrile for 1 min at 160 rpm, then 6 g of MgSO_4 and 1.5 g of sodium acetate were added and the

mixture incubated 10 min at 250 rpm and finally centrifuged at 4700 rpm for 5 min at 5°C. A second extraction using the Stir Bar Sorptive Extraction (SBSE) method was performed as follows: 10 mL of supernatant was collected and mixed with 40 mL of NaCO₃ (0.1 M). Polydimethylsiloxane-coated stir bar (film thickness 0.5 mm, length 20 mm) (Twister®, Gerstel) was inserted and the solution incubated at 750 rpm during 16h in darkness and at room temperature. After the extraction, the stir bars were carefully rinsed, dried and analysed by TD-GC-MS/MS: a gas chromatography system (Agilent 7890A) coupled to a triple quadrupole mass spectrometer (Agilent 7000) and equipped with a thermal desorption unit combined with a cooled injection system (Gerstel).

One water sample for each site was used to determine the seawater exposure. 10 mL of methanol containing the internal standards (naphthalene d8, biphenyl d10, phenanthrene d10, chrysene d12, pyrene d10, benzo[a]pyrene d12 and benzo[g,h,i]perylene) was added to each sample of water (100 mL). A stir bar was then added to the solution and the extraction performed using the SBSE method (Lacroix *et al.*, 2014) as described above for the biological samples.

Prior to extraction, 10 g of each dried sediment samples (unsieved) were weighed and spiked with internal standards as previously described. 20 mL of a methylene chloride / acetone (50/50 v/v) were added to 10 g of each sediment sample and the extraction was performed using an ultrasonic bath (3 times during 15 min each). After filtration with NaSO₄, the extract was evaporated until 250 µL. The extract was then diluted in 10 mL of methanol and 100 mL of ultrapure water. Organic compounds were then extracted following the SBSE protocol as described above.

The stir bars were subsequently analysed for all samples using a gas chromatography system Agilent 7890A coupled to an Agilent 7000 triple quadrupole mass spectrometer (Agilent Technologies) and equipped with a Thermal Desorption Unit (TDU) combined with a Cooled Injection System (Gerstel). Thermodesorption and GC-MS/MS analyses were performed as previously described in Lacroix *et al.*, (2014). The interface temperature was 300°C and the GC temperature gradient was 50°C (1 min) to 320°C (20 min) at 3°C/min. The carrier gas was Helium at a constant flow of 1 ml/min. The capillary column used was an RXi 5-ms (Restek, Bellefonte, USA): 30 m x 0.25 mm ID x 0.25 µm film thickness. The quadrupole was used in the Electronic Impact mode (Electronic Impact: 70 eV. voltage: 2000V). The extraction yield of each organic molecule was corrected using the standard reference

materials (SRM 1974 c). Blank samples were considered and analytes were quantified relatively to deuterated compounds (similar in retention time and structure) using a calibration curve. Limits of quantification (LOQ) were calculated using the calibration curve method (Shrivastava et al, 2011) and limit of detection (LOD) were estimated by dividing the LOQ by 3. Concentrations measured in bivalves were expressed in ng/g wet weight (w. w.) and in sediment in ng/g dry weight (d. w.).

Biochemistry

Oysters and mussels were thawed on ice, their digestive glands were harvested and homogenised in ice-cold phosphate buffer (100 mM, pH 7.2, 1100 mOsm). The homogenates were then centrifuged for 15 min at 12,500 g at 4 °C and the supernatant was used for biochemical assays.

Oxidative stress was assessed by measuring the activity of the SOD and the levels of MDA (membrane lipid peroxidation). Potential neurotoxic effects were assessed by measuring the activity of the AChE. In addition, potential modulation of the immune response was evaluated by measuring the laccase enzyme (phenoloxidase) activity. Finally, the cell detoxification process was assessed by measuring the total GST activity. Further description is provided in Supplementary material (Text S1).

Environmental parameters

Turbidity (NTU), conductivity (mS/cm), chlorophyll *a* (µg/L), salinity (PSU), temperature (°C), dissolved oxygen (mg/L) and pH were measured continuously by an immersed automated system in the Charente and Seudre estuaries using multi-parameters probes (SAMBAT sea, Nke instrumentation) from May 2019 until February 2020 (due to technical maintenance). In addition, those parameters (except for chlorophyll *a*) were recorded monthly at the four sites in surface water along the cages using portable multi-meter probe (HANNA HI 9829). Finally, daily precipitation (mm) data (source: Meteo France) at La Rochelle-Ile of Ré (46°10'40"N, 1°11'34"W), Chassiron (46°02'48"N, 1°24'42"W) and Saintes (45°45'40"N, 0°39'07"W) stations were used to assess the potential relationships with the pesticide concentrations found.

Statistical analysis

Statistical analyses were performed using R (version 3.1.2). The potential differences between pesticides and biomarkers concentrations measured in bivalves collected at the different sites across different seasons were assessed using 1-way ANOVA. When residuals were not normally distributed,

the nonparametric Kruskal-Wallis rank test was applied. When significant, post-hoc pairwise comparisons between each site or time were assessed by the lsmean, Dunn's or Wilcoxon test using adjusted p value according to the Bonferroni method. Potential correlations between each pesticide mean concentration in oysters and mussels, environmental parameters, index conditions and biomarker responses were assessed using the Pearson or Spearman correlation test depending on the normality requirements. Principal component analyses were performed using the sum of pesticide concentrations and biomarker responses. For each pesticide measurement, 0 was assigned to values below the LOD and LOQ/2 was assigned to values <LOQ (US EPA, 2000).

Results

Environmental parameters

Seasonal variation. Water temperature and salinity decreased during the winter (Table S1, Excel files S1 and S2). In addition, rainfall varied from 1 - 2 mm in spring and summer, to 2 - 8 mm in winter (Table S1). In parallel, the water flow rates reached 29, 240 and 6.6 m³/s in the winter for the Sèvre, Charente and the Seudre rivers, respectively, then, decreased down to 9.5, 60 and 2.9 m³/s in spring 2020, respectively (Table S1).

Variation across sites. The DO, conductivity and pH data were comparable between the sites (Table S1). The turbidity was the highest at the Charente estuary site during the autumn and winter periods and reached 41.6 FNU (Table S1). The mean concentrations in chlorophyll *a* were comparable in the Charente and Seudre estuary, and were equal to 4 and 4.3 µg/L, respectively (See supplemental information in Lerebours *et al.*, 2022). Finally, the Charente River was characterised by the highest flow rate and the Seudre River, by the lowest during the year biomonitoring (Table S1).

Bivalve growth and mortalities

Oysters and mussels grew steadily over the course of the one-year biomonitoring period. Specifically, oysters grew from 3.2 ± 0.1 g and 3.0 ± 0.5 cm in May 2019 to 5.7 ± 2.6 g and 7.3 ± 1.4 cm in May 2020 (Table S2). Mussels grew from 1.4 ± 0.5 g and 3.7 ± 0.2 cm in May 2019 to 5.2 ± 2.7 g and 4.1 ± 0.5 cm in May 2020 (Table S2). The weight, length and condition indexes remained similar between the sites (Tables S2 and S3). The condition indexes of oysters significantly decreased between September 2019 (14 ± 2) and January 2020 (11 ± 2), and, then, significantly increased in May 2020

(15 ± 3) ($p < 0.05$) (Table S3). No significant difference between the seasons was observed in mussels (Table S3).

Mortalities of oysters remained very low and varied from 0 to 4% (Table 2). Contrary to oysters, the percentage of mussel mortality increased at the end of the biomonitoring with values reaching 17% at the Ile of Ré site in February-March 2020 and 42% at the Sèvre Niortaise estuary, in April-May 2020. Moreover, at the beginning of the biomonitoring in May-July 2019, mortalities at this site were higher than the mortalities observed at the other sites with 15% of dead mussels recorded (Table 2).

Pesticide contamination in seawater and sediments

The sum of pesticide concentrations in water were similar across the sites (Figure 2). Regarding the variation across the seasons, the highest values found in May and September 2019 with concentrations reaching 11 - 32 ng/L and 13 - 20 ng/L, respectively (Figure 2 and Excel file S1). Later in the year, the sum of pesticide concentrations decreased in January and May 2020 with concentrations dropping to 3 - 12 ng/L and 3 - 15 ng/L respectively (Figure 2 and Excel file S1). Metolachlor concentrations were predominant for all seasons. The highest concentration found was 32 ng/L in the Charente estuary, in May 2019 (Figure 2 and Excel file S1). The majority of other pesticides concentrations (including chlortoluron) were identified below the LOD (Excel File S3) as well as in sediments, where the majority of pesticides were identified below the LOD or present at very low concentrations whatever the season and site (Figure 2 and Excel file S1).

Seasonal pesticide contamination in marine bivalves

The pesticide concentrations in oysters whole tissue homogenates were comparable over the year biomonitoring across the seasons and sites as revealed by similar concentrations and number of pesticides measured in the oysters collected at the Ile of Ré site and in the Sèvre Niortaise, Charente and Seudre estuaries in May 2019, September 2019, January 2020 and May 2020 ($p > 0.05$) (Figures 3 and S1). An increasing trend of the sum of pesticide concentrations was observed in oysters from the Seudre estuary between January and May 2020 (Figure 3) essentially due to an increase of chlortoluron tissue concentrations that reached a mean of 17 ng/g w. w. (Figure 3; Excel file S2). Indeed, in May 2020, chlortoluron concentrations were particularly high at this site. Oysters located in

the Seudre estuary were also associated to DDT and its metabolites in May 2020 as revealed by principal component analyses (PCA) (Figure S2). Finally, results from the PCA analyses showed that the Ile of Ré site was characterised by alachlor, alpha-BHC, beta-BHC and hexachlorobenzene in May 2019 (Figure S2), but the corresponding contamination levels (pesticide concentration or number) were not significantly higher at that site ($p > 0.05$) (Figures 4 and S3).

Similarly to oysters, the concentrations and number of pesticides in mussels whole tissue homogenates were comparable across the Ile of Ré site and the Sèvre Niortaise and Charente estuaries ($p > 0.05$) (Figures 3 and S1). However, the concentrations and number of pesticides significantly increased after 8 months of exposure, in January 2020 and dropped in May 2020 ($p < 0.05$) (Figures 3 and S1). This increase of the sum of pesticide concentrations was essentially due to an increase of chlortoluron tissue concentrations; from $< \text{LOD}$ in May and September 2019 to a mean of 16 ng/g in January 2020 in mussels collected at the Charente estuary (Figure 3 and Excel file S2). This site was indeed characterised by chlortoluron and also chlorpyrifos, chlorfenvinphos, endrin, DDT and its metabolites in January 2020, as shown by principal component analyses (Figure S3). Finally, mussels from the Ile of Ré site were characterised to a lesser extent by methylparathion, parathion and beta-BHC concentrations after four months of exposure in September 2019 (Figure S3) but values were not significantly higher ($p > 0.05$) than those measured in mussels located in estuarine sites (Figures 3 and excel file S2).

Biomarkers' responses

The biomarker responses monitored in both species did not vary across the different sites ($p > 0.05$) except for laccase in oysters ($p < 0.05$). However, significant seasonal variations were found for all the biomarkers tested ($p < 0.05$) except for SOD and MDA in mussels ($p > 0.05$) (Figure 4). Finally, turbidity, dissolved oxygen, temperature (of Le Martray and Charente sites) and index conditions were correlated to certain biomarkers' responses in mussels and oysters ($p < 0.05$). Specifically, in mussels, temperature was significantly correlated to SOD at both sites ($r: -0.98$ and 0.86 ; $p < 0.05$), and to AChE ($r: -0.88$; $p < 0.05$) and GST ($r: 0.71$; $p < 0.05$) at Le Martray site. In oysters from Le Martray site, temperature was significantly correlated to all biomarkers ($r: -1$ to -0.71 ; $p < 0.05$) except for GST, turbidity was correlated to SOD ($r: -0.79$; $p < 0.05$), AChE ($r: -0.86$; $p < 0.05$) and Laccase ($r: -$

0.81; $p < 0.05$), and dissolved oxygen to MDA ($r: -0.90$; $p < 0.05$). In oysters from the Charente site, dissolved oxygen was significantly correlated to GST ($r: -0.96$; $p < 0.05$). Index conditions of oysters were significantly correlated to SOD ($r: -0.94$; $p < 0.05$), MDA ($r: 0.89$; $p < 0.05$) and Laccase ($r: -1$; $p < 0.05$) at Le Martray, Charente and Seudre sites. Finally, index conditions of mussels were significantly correlated to SOD at the Sevre and Charente site ($r: -0.83$ and 0.94 respectively; $p < 0.05$), and to SOD ($r: -0.94$; $p < 0.05$), GST ($r: 0.89$; $p < 0.05$) and AChE ($r: -0.89$; $p < 0.05$), at Le Martray site.

Correlations between pesticides and biomarker response

In oysters, the GST activity was positively correlated to alpha BHC ($r: 0.48$; $p < 0.05$), beta BHC ($r: 0.44$; $p < 0.05$) and alachlor concentrations ($r: 0.49$; $p < 0.05$) (Figure S2). Moreover, the hexachlorobenzene concentration was negatively correlated with AChE activity ($r: -0.74$; $p < 0.05$) and positively correlated to MDA ($r: 0.78$; $p < 0.05$), in oysters (Figure S2). No significant correlations were found between oyster pesticide concentrations and SOD activity.

In mussels, the laccase enzyme was negatively correlated with methylparathion ($r: -0.90$; $p < 0.05$), parathion ($r: -0.89$; $p < 0.05$) and beta BHC ($r: -0.87$; $p < 0.05$) (Figure S3). No significant correlations were found between mussel pesticide concentrations and the activities of SOD, AChE, GST and MDA levels. The percentage of mortalities were not significantly correlated to the pesticide concentrations nor the biomarkers' responses measured (Figure S3).

Discussion

The present study highlighted the predominance of chlortoluron in bivalve tissue and especially in mussels located in the Charente estuary. An increasing trend of total pesticides mean concentrations was observed in oysters in spring 2020 due to chlortoluron concentrations (that reached a mean of 17 ng/g w. w. in May 2020, in the Seudre estuary) and significant increases were observed in mussels due to higher chlortoluron concentrations that reached a mean of 16 ng/g w. w. in the Charente estuary in winter 2020 and 7 ng/g w. w. in the Sèvre Niortaise in spring 2020. Those concentrations were higher than the concentrations measured in a recent pilot study performed in the Pertuis Seas in February 2019 were authors measured 3 and 4 ng/g w. w. in oysters from the Charente and Seudre estuaries respectively, and values below the LOD of 0.5 ng/g w. w. in mussels from the

Sèvre Niortaise and Charente estuaries (Lerebours *et al.*, 2021) which is the only recent study reporting chlortoluron in marine bivalves from the environment. The present results showed that the bioaccumulation of chlortoluron did not induce a biochemical change in the selected biomarkers. Chlortoluron, is used as an herbicide to treat wheat and barley crops, and the increase observed in bivalves in the present study could be related to leaching by rainfalls after herbicide application (Pacanoski and Mehmeti, 2019). Indeed, an increase of daily mean precipitation during the winter with 8 and 4 mm was recorded in November and December 2019 respectively and the water flow from the Charente river estuary increased to reach 240 m³/s. In parallel, a decrease of salinity was recorded during that period. Therefore, those environmental conditions could explain the higher chlortoluron concentration found in mussels in January 2020 in the present study and in seawater in winter 2015 (from 48 to 84 ng/L) in the Gironde estuary (located to the south of the Pertuis seas) (Levesque *et al.*, 2018). Finally, the seawater concentrations found in the present study (below the LOD of 15 ng/L) were lower than the concentration previously reported (Levesque *et al.*, 2018; Lerebours *et al.*, 2021), yet, the results showed that this compound can accumulate in marine bivalves.

Methylparathion is an organophosphorus compound used as a broad-spectrum insecticide in crops. Concentrations of methylparathion measured in this study ranged from 0.7 to 7.4 ng/g w. w. in mussels and were below the LOD or LOQ of 0.5 and 1.7 ng/g w. w. respectively in oysters. Parathion concentrations ranged from below the LOD to 1.9 ng/g w. w. in mussels and were below the LOD or LOQ of 0.1 and 0.3 ng/g w. w. respectively in oysters. These low concentrations were similar or higher than the concentrations of methylparathion and parathion previously measured in oysters and mussels respectively in February 2019 (Lerebours *et al.*, 2021). The short half-life in biological matrices of these organophosphorus pesticides (less than a week) (Peña-Egido *et al.*, 1988; Schimmel *et al.*, 1983) could explain the low concentrations found in the present study. Studies reporting methylparathion or parathion in marine bivalves are scarce. One study carried out in Spain showed higher concentration of parathion in oysters from the bays of the Ebro delta (Spain) (Solé *et al.*, 2000). Nevertheless, the concentrations of methylparathion and parathion measured in mussels in the present study were negatively correlated with the laccase activity. Laccase enzymes are multi-copper oxidases that catalyse the oxidation of a wide range of substrates and play a role in the modulation of the

immune, antioxidant and detoxification response (Luna Acosta *et al.*, 2010; 2017). Therefore, we hypothesize that the physiology of mussels is potentially weakened, although the exact consequences of a decrease activity of the laccase enzyme are unknown. To our knowledge, there are no studies available in the literature regarding the relationship between laccase enzyme activity with methylparathion or parathion in bivalves and other marine organisms.

Metolachlor is a selective pre-emergent herbicide of the chloroacetanilide family that is widely used in field crops (wheat, corn and rape) for weed control. The present results showed that the concentration of metolachlor ranged from 0.6 ng/g (LOD) to 4.96 ng/g w. w. in oysters and were below the LOQ of 0.3 ng/g w. w. in mussels. In the Pertuis Charentais region, concentrations previously measured were lower: below the LOQ of 0.33 ng/g w. w. in oysters and between 0.5 ng/g (LOD) and 1.67 ng/g w. w. (LOQ) in mussels (Lerebours *et al.*, 2021). Therefore, the accumulation of metolachlor in bivalves was low, especially in mussels, despite being the predominant pesticide measured in seawater (up to 32 ng/L). This herbicide was banned in 2003 in France but was replaced by a similar compound: S-Metolachlor, for which the predicted no effect concentration (PNEC) for aquatic organisms is 6.7 µg/L in surface water (Anses, 2018). Finally, no association between metolachlor exposure and biomarker response was found in the present study. The environmental concentrations in the ng/L range, that are about 1000 times lower than the PNEC, might be too low to elicit effects.

The Ile of Ré site was characterised by alachlor, alpha-BHC, beta-BHC and hexachlorobenzene concentrations in oysters, in May 2019 and only beta-BHC concentration in mussels, in September 2019. However, these concentrations were not significantly higher in bivalves collected at that site and remained very low all through the year biomonitoring with values below the LOD or LOQ. This is in accordance with previous results found in the pilot study except for alpha-BHC concentrations that were quantified in oysters, but concentrations were also very low (0.6 - 0.7 ng/g w. w.) (Lerebours *et al.*, 2021). Finally, these low concentrations levels could explain the weak correlation ($r < 0.5$) with the GST activity in oysters. Considering their low concentrations in the estuarine environment, and as they are banned, those compounds are not likely to pose a threat to the health of marine bivalves in the region.

Finally, the low concentrations of hexachlorobenzene measured in oysters, a pesticide used to protect the seeds of wheat and other grains against fungus, were correlated with an inhibition of the AChE activity and an increase of the MDA levels. AChE is an enzyme specific to nervous tissue and the neuromuscular junction (Soreq and Seidman, 2001) that rapidly hydrolyses acetylcholine (neurotransmitter) into inactive choline and acetate (Serafini *et al.*, 2019). Despite this biomarker has been widely used as a pollutant-sensitive neurotoxic biomarker (Tsangaris *et al.*, 2010) there is, to our knowledge, a lack of studies dealing with the effect of this pesticide on AChE activity in bivalves. However, in coherence with our results, some authors found that exposure to hexachlorobenzene inhibits the AChE activity in the common carp (*Cyprinus carpio*) (Song *et al.*, 2006; Starek-Świechowicz *et al.*, 2017). The positive correlation found with the MDA levels indicates the peroxidation of polyunsaturated fatty acids of the membrane by free radicals (Viarengo *et al.*, 1991; Pellerin-Massicotte, 1994). Our results are corroborated by previous work that evidenced an increase of MDA levels in the estuarine crab (*Chasmagnathus granulatus*) (Chaufan *et al.*, 2006) and in the brain of common carp *C. carpio* (Song *et al.*, 2006) exposed to high dietary and waterborne concentration (50 - 200 ug/L) of hexachlorobenzene, respectively. Interestingly, the SOD activity that is involved in the regulation of the oxidative balance was not correlated to any of the pesticide concentrations measured possibly because the contamination levels were too low to modulate the activity of this biomarker. Moreover, this biomarker was found correlated to temperature in mussels and turbidity in oysters. Temperature was also correlated with MDA, AChE and Laccase activities in oysters. Turbidity was also correlated to AChE and Laccase activities in oysters. Finally, dissolved oxygen was correlated to GST and MDA in oysters. Those results revealed that temperature, dissolved oxygen and turbidity, influenced the biochemical responses (based on the selected biomarkers) of bivalve species. These findings were corroborated with previous studies that found a link between the salinity or temperature and biomarker's responses in field-collected bivalves (Viarengo *et al.*, 1991; Alves de Almeida *et al.*, 2007; Barbarin *et al.*, 2022). Therefore, the influence of temperature, turbidity and dissolved oxygen could explain the weaker correlations with the low pesticide concentrations found in the present study. The present findings revealed that environmental parameters need to be analysed as confounded factors in studies assessing the link between biomarkers' responses and

exposure to pollutants in bivalves from the natural environment. Finally, our results showed that the condition indexes of oysters varied between the autumn, winter and spring in accordance with their reproductive cycle. They were also correlated with the SOD activity in oysters. Therefore, such physiological parameters could also explain the weaker correlation found with pesticide concentrations.

To conclude, the present study determined the concentration levels of thirty pesticides in two bivalve species, seawater and sediments from the estuarine environment of the Pertuis seas during four seasons. Metolachlor was predominant in seawater for all seasons. Seasonal variations of total pesticide concentrations were especially observed in mussels with chlortoluron reaching the highest concentration during the winter likely due to higher precipitations. Low pesticide concentrations were correlated with GST, AChE and MDA in oysters and with laccase in mussels. Our results showed that mussels' mortalities were not correlated to any of the pesticides nor biomarkers studied. Other organic pollutants than the 30 pesticides analysed, and metals, could potentially affect the physiology and biochemistry of bivalves as previously shown in previous studies (Luna-Acosta *et al.*, 2015b; Breitwieser *et al.*, 2017). Thus, the present study encourages the development of new analytical approaches by liquid/gas chromatographic coupled to high resolution mass spectrometry in order to screen a larger number of pesticides, pharmaceuticals and other xenobiotic contaminants.

Acknowledgements

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Figure and Table Legend.

Figure 1. Location of all the samples collected in the Pertuis-Charentais region, France.

Figure 2. Sum of the pesticide concentrations in water (ng/L) and sediment samples (ng/g, d. w.) over a year biomonitoring at each sampling site.

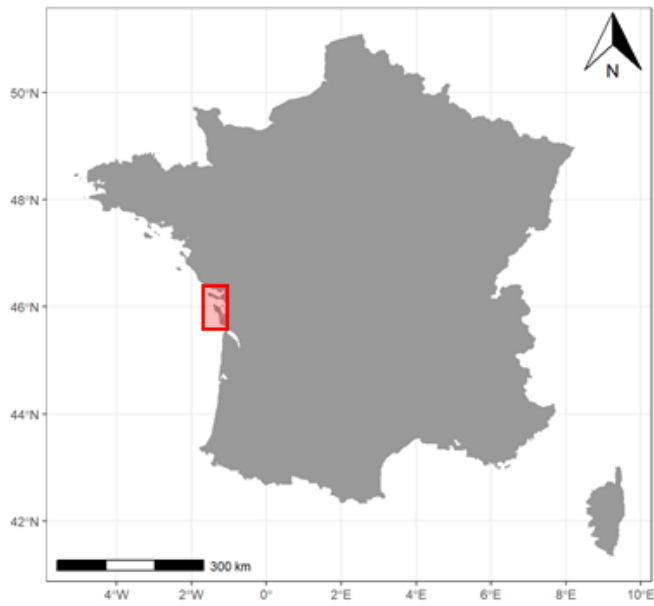
Figure 3. Sum of the pesticide concentrations in oysters and mussels (ng/g, w. w.) over a year biomonitoring at each sampling site.

Figure 4. Biomarker responses in oysters and mussels on different sampling seasons ad sites.

Table 1. The 30 pesticides analysed.

Table 2. Percentage of mortalities (%) in oysters and mussels on different sampling occasions.

Figure 1.



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532 **Figure 2.**

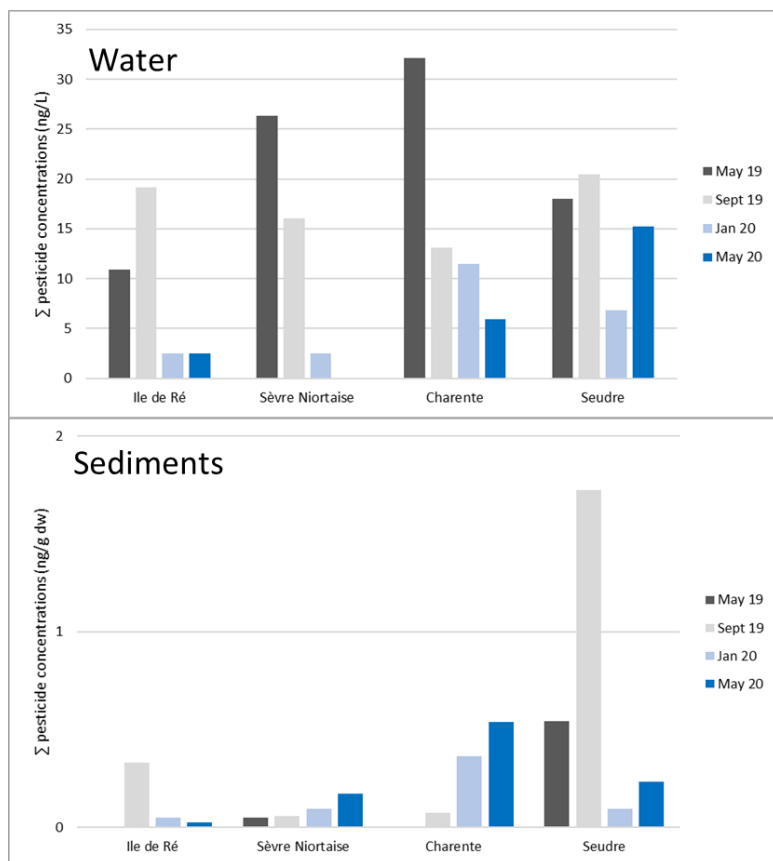


Figure 3.

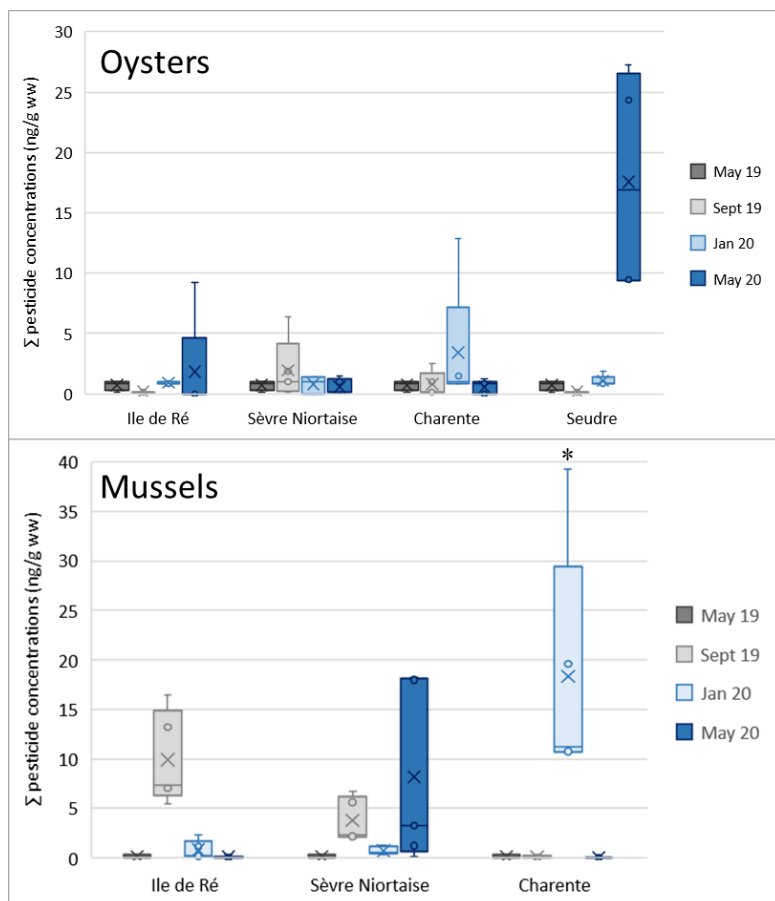


Figure 4.

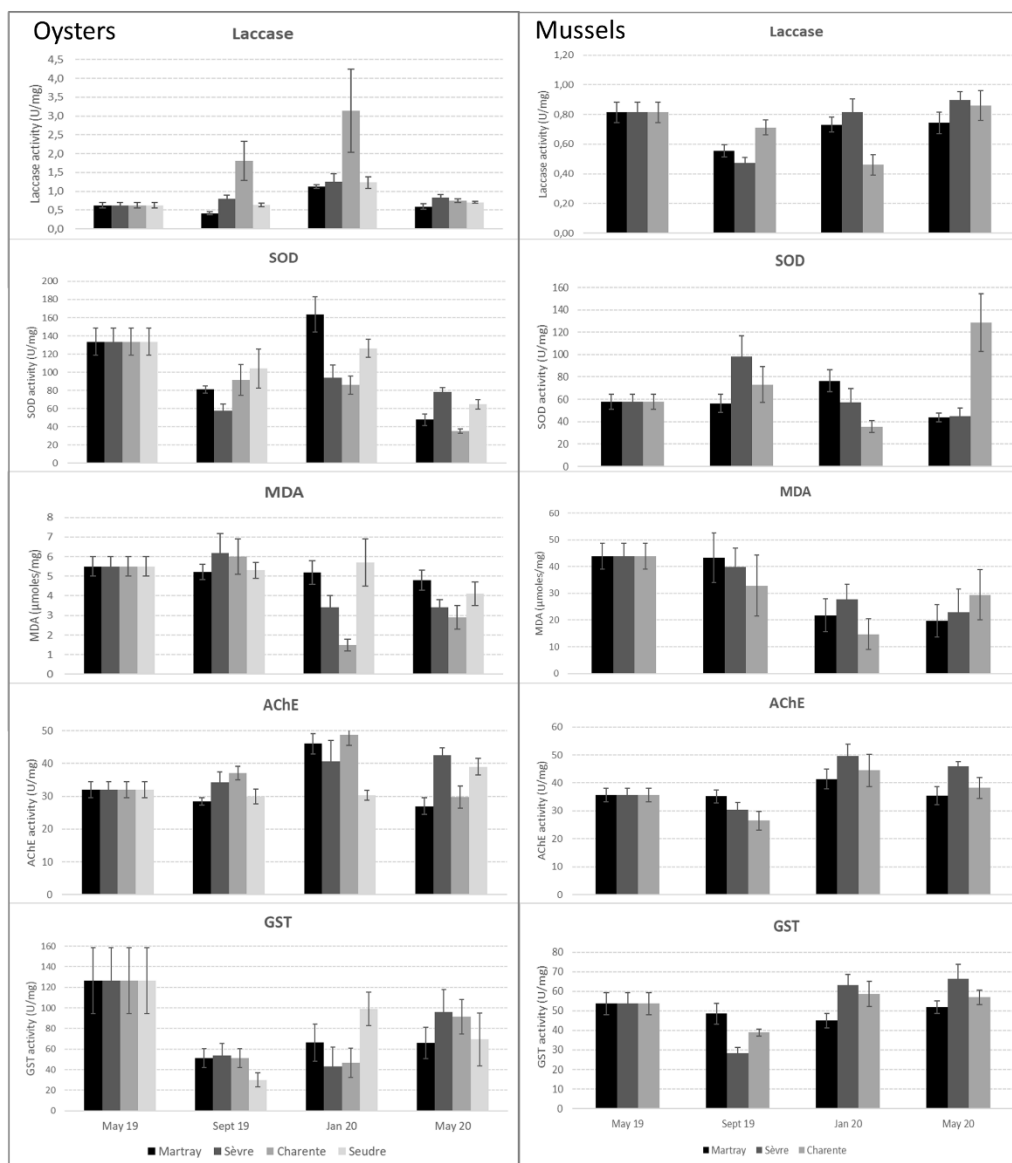


Table 1.

Pesticides analysed		
acetochlor	diazinon	endosulfan beta
alachlor	dieldrine	endosulfan sulfate
aldrine	diuron	endrine
alpha-BHC	2,4'-dde	hexachlorobenzene
beta-BHC	4,4'-dde	isodrine
gamma-BHC	2,4'-ddd	methylparathion
delta-BHC	4,4'-ddd	metolachlor
chlorfenvinphos	2,4'-ddt	metazachlor
chlorpyrifos	4,4'-ddt	oxadiazon
chlortoluron	endosulfan alpha	parathion

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576 **Table 2.**

	May - Jul 19	Aug - Sept 19	Oct - Nov 19	Dec - Jan 20	Feb - Mar 20	Apr - May 20
Oysters						
<i>Ile de Ré</i>	0	2	1	0	0	1
<i>Sèvre N.</i>	1	2	2	0	1	0
<i>Charente</i>	1	5	1	0	1	1
<i>Seudre</i>	4	0	2	2	1	0
Mussels						
<i>Ile de Ré</i>	7	6	2	4	17	20
<i>Sèvre N.</i>	15	5	3	2	2	42
<i>Charente</i>	4	6	2	1	2	5

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583 **Supplementary material**

Text S1. Biochemical analyses.

Figure S1. Number of pesticides in oysters and mussels over a year biomonitoring at each site.

Figure S2. Principal component analyses and correlations of pesticide concentrations and biomarker responses in oysters.

Figure S3. Principal component analyses and correlations of pesticide concentrations and biomarker responses in mussels. Percentages of mussel mortalities were also considered.

Excel File S1. Concentrations of the thirty pesticides measured in seawater and sediments' samples.

Excel File S2. Concentrations of the thirty pesticides measured in oysters and mussels' samples.

Table S1. Environmental data recorded from May 2019 to February 2020 : monthly mean of daily precipitations (mm) (source: Meteo France) at La Rochelle-Ile of Ré (46°10'40"N, 1°11'34"W), Chassiron (46°02'48"N, 1°24'42"W) and Saintes (45°45'40"N, 0°39'07"W) stations, and temperature (°C), Dissolved Oxygen (DO) (%), conductivity (mS/cm), pH, turbidity (FNU) and salinity (PSU) measured using the multi-meter probe (HANNA HI 9829) at the four studied sites.

Table S2. Fresh weight (g) and total length (cm) of oysters and mussels collected over the year biomonitoring (mean \pm SD).

Table S3. Index conditions of oysters and mussels.

Text S1.

611 Determination of total protein concentrations

612 Total protein concentrations were determined adapting the BCA Kit methodology (Bicinchononique
613 Acid Kit, Sigma Aldrich ®). The BCA kit contained bovine serum albumin (BSA) as a standard and
614 involved the reduction of alkaline Cu^{2+} by proteins (Smith et al., 1985) at absorbance 562 nm using a
615 spectrofluorometer (SAFAS Flx- Xenius ®).

616 Detoxification, antioxidant and defence enzyme activities

617 The SOD activity was assessed in the FF using the method developed by Paoletti et al. (1986). The
618 assay, involving EDTA, MnCl_2 and mercaptoethanol, measured the decrease in nicotinamide adenine
619 dinucleotide (NADH) oxidation. The inhibition of NADH oxidation, function of the SOD activity, was
620 monitored at 340 nm (using the SAFAS Flx-Xenius spectrofluorimeter ®). One SOD unit
621 corresponded to 50% inhibition of NADH oxidation. Results are presented in U of SOD/mg of protein.

622 The Glutathione S-Transferase activity in the digestive glands was determined by adapting a Sigma
623 assay kit. The GST assay kit resorted to 1-chloro-2,4-dinitrobenzene (CDNB), suitable for the broadest
624 range of GST isozymes. Upon conjugation of the thiol group of glutathione to the CDNB substrate, an
625 increase in absorbance at 340 nm (SAFAS Flx-Xenius ®) was observed. Results are presented in U of
626 GST/mg of protein (Mannervik et al., 1988; Habig et al., 1974).

627 Lipid peroxidation

628 Lipid peroxidation was measured by assaying the Malondialdehyde (MDA) concentration in the FF
629 (Gerard-Monnier et al., 1998). A commercially available MDA assay kit (Oxis International) was
630 used. The method is based on the MDA reaction with a chromogenic reagent (n-methyl-2-
631 phenylindole). The blue product was quantified by measuring absorbance at 586 nm (Gerard-Monnier
632 et al., 1998) with a SAFAS Flx-Xenius spectrofluorimeter ®. Results are presented in $\mu\text{M}/\text{mg}$ of
633 protein.

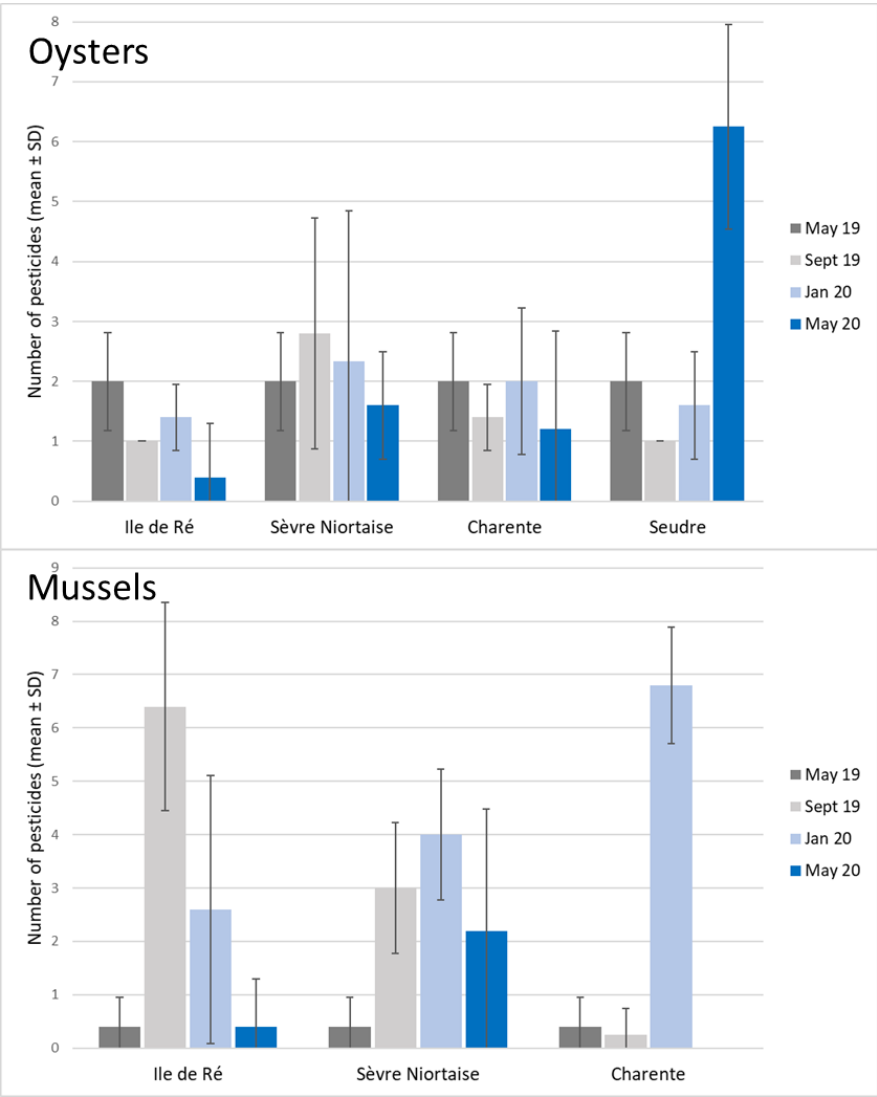
634 Environmental stress with Laccase assay

635 The phenoloxidase (Laccase) activity highlighted alterations to the immune system. Indeed, the
636 enzymatic activity is sensitive to contaminants (Gagnaire et al., 2004; Luna-Acosta et al., 2010a and
637 Breitwieser et al., 2016) and plays an important role in the oyster immune defence mechanism. The
638 Laccase activity was measured in the FF, using a method developed by Luna-Acosta et al. (2015). The
639 latter relied on the oxidation of PPD (p-phenylenediamine) being catalysed by laccase. The reaction
640 was measured at 420 nm using a SAFAS Flx-Xenius spectrofluorometer ®. Laccase activity was
641 expressed in enzymatic units per mg of protein (U/mg of protein).

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643 **Figure S1.**

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654 **Figure S2.**

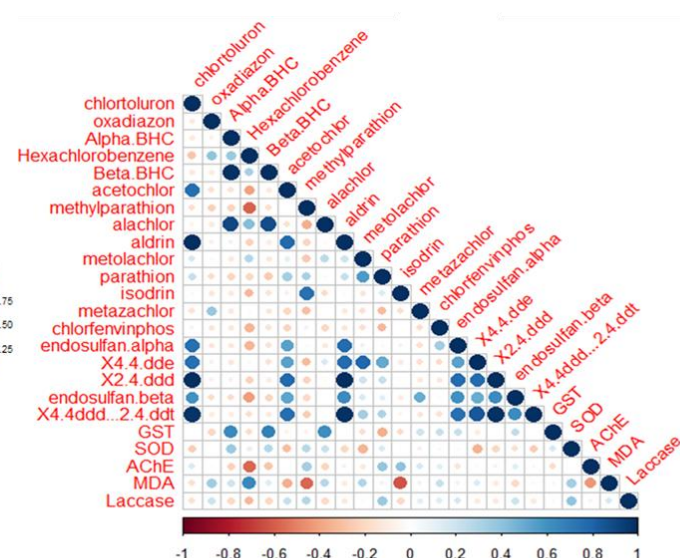
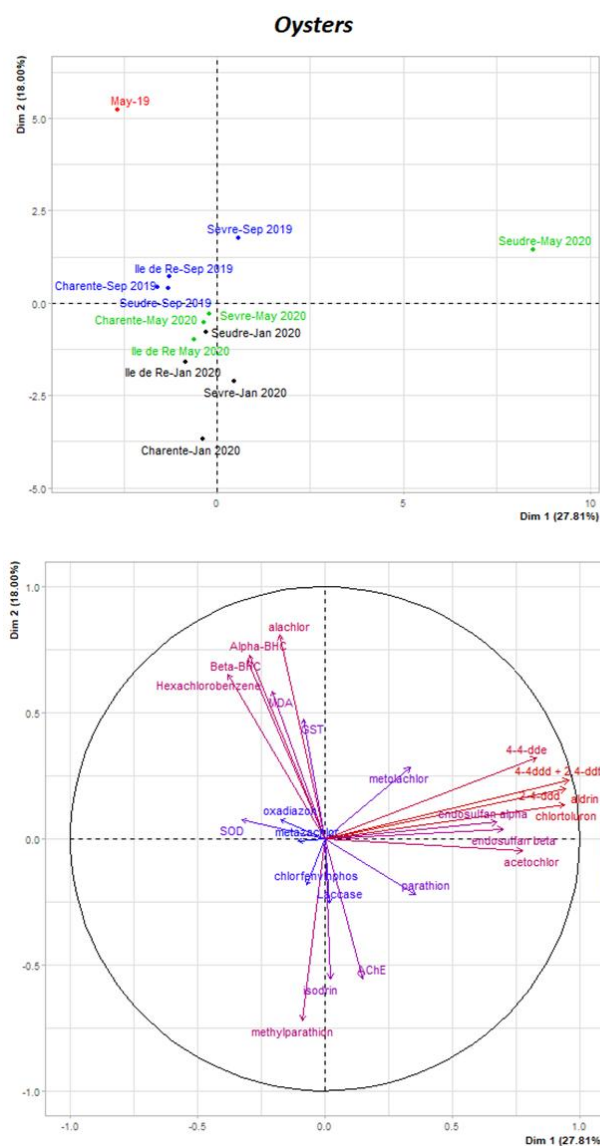


Figure S3.

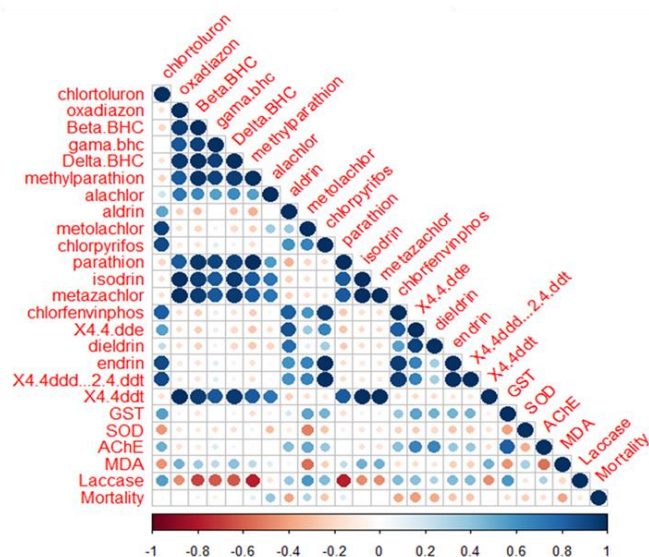
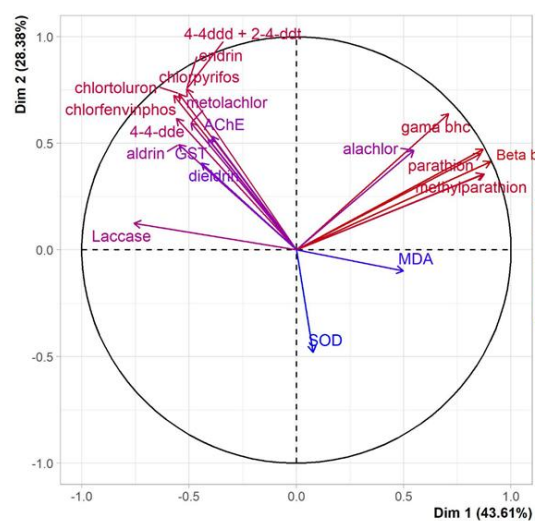
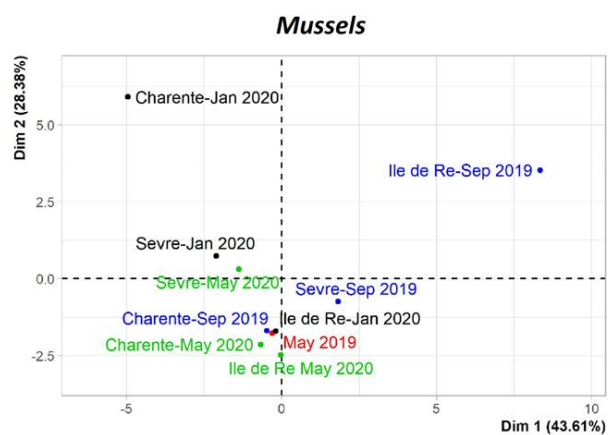


Table S1.

	2019							2020					
	May	June	July	Aug	Sept	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May
Precipitation (mm)	1	2	1	1	2	4	8	4	2	2	3	1	3
<i>Ile of Ré</i>													
T (°C)	15	23		24	17	16	14	10	12	14	16	15	20
DO (%)	46	32			90	79	103	78	76	104	104	64	97
Cond (mS/cm)	57	57		59	49	47	45	43	44	43	44	46	46
pH	8.4	8.4		8.2	8.0	8.0	7.9	7.3	8.2	8.2	8.4	8.2	8.5
Turb (FNU)	56	52			9	20	61	75	11	30	83	180	63
TDS (ppm)		29		29	24	23	22	19	22	22	22	23	23
Sal (psu)		38		39	32	31	29	27	28	29		30	30
<i>Charente</i>													
Flow rate (m/s)			28 - 10				11 - 240				132 - 60		
T (°C)	15			22	21		12	9.8	10.9			15	21
DO (%)	37				90	77	91	71	70	68	73.2	57	45
Cond (mS/cm)	46			59	49	32	22	22	35	15		43	43
pH	8.2			8.0	7.8	8.0	8.0	8.0	7.9	8.0	7.8	8.0	7.8
Turb (FNU)					500	1000	300	1000		1000		236	215
TDS (ppm)	28			29	25	15	11	11	17	12		20	22
Sal (psu)	38			39	32							25	28
<i>Sèvre Niortaise</i>													
Flow rate (m/s)			5 - 1.8				1.9 - 29				99 - 9.5		
T (°C)			28		22	19	11		12	14	15	21	23
DO (%)			19		104	81	96		74	67	90	89	60
Cond (mS/cm)			62		51	39	36		33	18	43	42	43
pH			8.7		8.3	8.2	7.9		7.3	8.0	8.3	8.3	8.5
Turb (FNU)			50		123	76	238		140	170	47	51	339
TDS (ppm)			31		26	23	18		16	13	22	21	22
Sal (psu)			41		34	33					28	27	28
<i>Seudre</i>													
Flow rate (m/s)			0.6 - 0.05				0.1 - 6.6				4.9 - 2.9		
T (°C)					19	17	11			11	13	16	18
DO (%)					93	70	90		74	83	83	48	43
Cond (mS/cm)					47	48	35		38	40	36	42	36
pH					7.8	7.9	7.9		8.0	9.0	8.0	7.9	7.8
Turb (FNU)					191	45	226		200	166	280	52	724
TDS (ppm)					23	24	17		19	20	18	21	18
Sal (psu)					30	31				26		27	

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681 **Table S2.**

		May 19	Sept 19	Jan 20	May 20
Oysters	Weight (g)	3.2 ± 1.0	7.7 ± 3.0	2.6 ± 0.9	5.7 ± 2.6
	Length (cm)	3.0 ± 0.5	4.0 ± 0.4	7.4 ± 0.9	7.3 ± 1.4
Mussels	Weight (g)	1.4 ± 0.5	1.6 ± 0.4	1.1 ± 0.4	5.2 ± 2.7
	Length (cm)	3.7 ± 0.2	3.9 ± 0.4	4.2 ± 0.4	4.1 ± 0.5

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683 **Table S3.**

	May19	Sept19	Jan20	May20
Oysters				
Le Martray		13 ± 2	12 ± 2	17 ± 1
Sèvre Niortaise	14 ± 3	13 ± 2	11 ± 1	16 ± 4
Charente		16 ± 3	10 ± 1	12 ± 4
Seudre		14 ± 2	12 ± 2	14 ± 1
Mussels				
Le Martray		24 ± 5	21 ± 2	27 ± 3
Sèvre Niortaise	24 ± 6	22 ± 4	23 ± 3	25 ± 6
Charente		26 ± 3	21 ± 9	27 ± 15

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