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

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29 **Abstract**

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

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## 57 **Introduction**

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

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

85 DDT and/or lindane and their by-products in oysters collected in summer and winter 2008 (Luna-  
86 Acosta *et al.*, 2015a) or variegated scallops collected in winter and autumn 2014 (Breitwieser *et al.*,  
87 2016) and found them at very low concentrations. In winter 2019, concentrations of these old  
88 pesticides were found below the limit of detection in oysters and mussels (Lerebours *et al.*, 2021).  
89 Although these previous studies were informative, only 8 or 14 pesticides were measured (Breitwieser  
90 *et al.*, 2016 and Luna-Acosta *et al.*, 2015a, respectively) or merely assessed once in a one-off pilot  
91 study (Lerebours *et al.*, 2021). Therefore, further knowledge is needed on the long-term contamination  
92 of a large number of pesticides in bivalves farmed in estuaries of the Pertuis seas.

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

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

112

## 113 **Methods**

### 114 **Experimental design**

115 In March 2019, farmed mussels and oysters of 1.5 years were acclimated for 1 month and half to the  
116 same environmental conditions at a remote and reference site, Le Martray, in the South-West of Ile of  
117 Ré. In May 2019, 12 oysters (total length:  $3.0 \pm 0.5$  cm) and 12 mussels (total length  $3.7 \pm 0.2$  cm)  
118 were collected and 36 oysters and 36 mussels were left at that control site. 72 oysters and 72 mussels  
119 were relocated at the Sèvre and Charente estuaries, and 36 oysters were relocated at the Seudre estuary  
120 (Figure 1). In September 2019, January 2020 and May 2020, 12 oysters and 12 mussels were collected  
121 at Le Martray site and the three estuaries except the Seudre estuary for mussels as it was not a mussel  
122 farming area and the conditions were not suitable to grow mussels according to local farmers. 5  
123 individuals per condition were used for pesticide analyses and 7 digestive gland samples (from 7  
124 individuals) per condition were used for biochemical analyses. The weight and length of oysters and  
125 mussels were recorded after each sampling event and the dead were counted and discarded every  
126 month. Moreover, the condition index (CI) was calculated on 7 additional samples per condition as  
127 follows:  $CI = \text{whole flesh weight} / \text{total weight} * 100$ . In addition, one surface sediment sample (in the  
128 range of 0-5 cm depth and collected using a stainless-steel bowl) and one surface seawater sample  
129 (collected using a glass bottle) were collected at the reference site and the three estuaries (Figure 1) at  
130 each sampling time. Sediment sample at Ile of Ré in May 2019 could not be analysed because of a  
131 technical issue. After each sampling, mussels, oysters, sediment and water samples were frozen at -  
132 20°C until pesticide analyses. Mussels and oysters dedicated to biomarker analyses were snap frozen  
133 using liquid nitrogen in the field, transported in a dry shipper and stored at -80°C until analyses.

### 134 **Pesticide analyses**

135 Five bivalves from each site were thawed and homogenates of each entire bivalve were obtained by  
136 cutting the flesh into pieces. 1 g of mussel homogenate or 3 g of oyster homogenate were spiked with  
137 perdeuterated internal standards. Thirty pesticides (Table 1) were extracted from biological matrix  
138 following the QuEChERS method (Quick, Easy, Cheap, Efficient, Rugged and Safe) (Khorshid *et al.*,  
139 2015; Pfannkoch *et al.*, 2015) as briefly described: each sample was mixed with a solution of  
140 acetonitrile for 1 min at 160 rpm, then 6 g of  $MgSO_4$  and 1.5 g of sodium acetate were added and the

141 mixture incubated 10 min at 250 rpm and finally centrifuged at 4700 rpm for 5 min at 5°C. A second  
142 extraction using the Stir Bar Sorptive Extraction (SBSE) method was performed as follows: 10 mL of  
143 supernatant was collected and mixed with 40 mL of NaCO<sub>3</sub> (0.1 M). Polydimethylsiloxane-coated stir  
144 bar (film thickness 0.5 mm, length 20 mm) (Twister®, Gerstel) was inserted and the solution  
145 incubated at 750 rpm during 16h in darkness and at room temperature. After the extraction, the stir  
146 bars were carefully rinsed, dried and analysed by TD-GC-MS/MS: a gas chromatography system  
147 (Agilent 7890A) coupled to a triple quadrupole mass spectrometer (Agilent 7000) and equipped with a  
148 thermal desorption unit combined with a cooled injection system (Gerstel).

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

154 Prior to extraction, 10 g of each dried sediment samples (unsieved) were weighed and spiked with  
155 internal standards as previously described. 20 mL of a methylene chloride / acetone (50/50 v/v) were  
156 added to 10 g of each sediment sample and the extraction was performed using an ultrasonic bath (3  
157 times during 15 min each). After filtration with NaSO<sub>4</sub>, the extract was evaporated until 250 µL. The  
158 extract was then diluted in 10 mL of methanol and 100 mL of ultrapure water. Organic compounds  
159 were then extracted following the SBSE protocol as described above.

160 The stir bars were subsequently analysed for all samples using a gas chromatography system Agilent  
161 7890A coupled to an Agilent 7000 triple quadrupole mass spectrometer (Agilent Technologies) and  
162 equipped with a Thermal Desorption Unit (TDU) combined with a Cooled Injection System (Gerstel).  
163 Thermodesorption and GC-MS/MS analyses were performed as previously described in Lacroix *et al.*,  
164 (2014). The interface temperature was 300°C and the GC temperature gradient was 50°C (1 min) to  
165 320°C (20 min) at 3°C/min. The carrier gas was Helium at a constant flow of 1 ml/min. The capillary  
166 column used was an RXi 5-ms (Restek, Bellefonte, USA): 30 m x 0.25 mm ID x 0.25 µm film  
167 thickness. The quadrupole was used in the Electronic Impact mode (Electronic Impact: 70 eV. voltage:  
168 2000V). The extraction yield of each organic molecule was corrected using the standard reference

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

#### 174 **Biochemistry**

175 Oysters and mussels were thawed on ice, their digestive glands were harvested and homogenised in  
176 ice-cold phosphate buffer (100 mM, pH 7.2, 1100 mOsm). The homogenates were then centrifuged for  
177 15 min at 12,500 g at 4 °C and the supernatant was used for biochemical assays.

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

#### 183 **Environmental parameters**

184 Turbidity (NTU), conductivity (mS/cm), chlorophyll *a* (µg/L), salinity (PSU), temperature (°C),  
185 dissolved oxygen (mg/L) and pH were measured continuously by an immersed automated system in  
186 the Charente and Seudre estuaries using multi-parameters probes (SAMBAT sea, Nke  
187 instrumentation) from May 2019 until February 2020 (due to technical maintenance). In addition,  
188 those parameters (except for chlorophyll *a*) were recorded monthly at the four sites in surface water  
189 along the cages using portable multi-meter probe (HANNA HI 9829). Finally, daily precipitation  
190 (mm) data (source: Météo France) at La Rochelle-Ile of Ré (46°10'40"N, 1°11'34"W), Chassiron  
191 (46°02'48"N, 1°24'42"W) and Saintes (45°45'40"N, 0°39'07"W) stations were used to assess the  
192 potential relationships with the pesticide concentrations found.

#### 193 **Statistical analysis**

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



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

## 205 **Results**

### 206 **Environmental parameters**

207 *Seasonal variation.* Water temperature and salinity decreased during the winter (Table S1,  
208 Excel files S1 and S2). In addition, rainfall varied from 1 - 2 mm in spring and summer, to 2 - 8 mm in  
209 winter (Table S1). In parallel, the water flow rates reached 29, 240 and 6.6 m<sup>3</sup>/s in the winter for the  
210 Sèvre, Charente and the Seudre rivers, respectively, then, decreased down to 9.5, 60 and 2.9 m<sup>3</sup>/s in  
211 spring 2020, respectively (Table S1).

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

### 218 **Bivalve growth and mortalities**

219 Oysters and mussels grew steadily over the course of the one-year biomonitoring period. Specifically,  
220 oysters grew from  $3.2 \pm 0.1$  g and  $3.0 \pm 0.5$  cm in May 2019 to  $5.7 \pm 2.6$  g and  $7.3 \pm 1.4$  cm in May  
221 2020 (Table S2). Mussels grew from  $1.4 \pm 0.5$  g and  $3.7 \pm 0.2$  cm in May 2019 to  $5.2 \pm 2.7$  g and  $4.1 \pm$   
222  $0.5$  cm in May 2020 (Table S2). The weight, length and condition indexes remained similar between  
223 the sites (Tables S2 and S3). The condition indexes of oysters significantly decreased between  
224 September 2019 ( $14 \pm 2$ ) and January 2020 ( $11 \pm 2$ ), and, then, significantly increased in May 2020

225 (15 ± 3) ( $p < 0.05$ ) (Table S3). No significant difference between the seasons was observed in mussels  
226 (Table S3).

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

232

### 233 **Pesticide contamination in seawater and sediments**

234 The sum of pesticide concentrations in water were similar across the sites (Figure 2). Regarding the  
235 variation across the seasons, the highest values found in May and September 2019 with concentrations  
236 reaching 11 - 32 ng/L and 13 - 20 ng/L, respectively (Figure 2 and Excel file S1). Later in the year, the  
237 sum of pesticide concentrations decreased in January and May 2020 with concentrations dropping to 3  
238 - 12 ng/L and 3 - 15 ng/L respectively (Figure 2 and Excel file S1). Metolachlor concentrations were  
239 predominant for all seasons. The highest concentration found was 32 ng/L in the Charente estuary, in  
240 May 2019 (Figure 2 and Excel file S1). The majority of other pesticides concentrations (including  
241 chlortoluron) were identified below the LOD (Excel File S3) as well as in sediments, where the  
242 majority of pesticides were identified below the LOD or present at very low concentrations whatever  
243 the season and site (Figure 2 and Excel file S1).

### 244 **Seasonal pesticide contamination in marine bivalves**

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

253 the Seudre estuary were also associated to DDT and its metabolites in May 2020 as revealed by  
254 principal component analyses (PCA) (Figure S2). Finally, results from the PCA analyses showed that  
255 the Ile of Ré site was characterised by alachlor, alpha-BHC, beta-BHC and hexachlorobenzene in May  
256 2019 (Figure S2), but the corresponding contamination levels (pesticide concentration or number)  
257 were not significantly higher at that site ( $p > 0.05$ ) (Figures 4 and S3).

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

### 271 **Biomarkers' responses**

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

281 0.81;  $p < 0.05$ ), and dissolved oxygen to MDA ( $r: -0.90$ ;  $p < 0.05$ ). In oysters from the Charente site,  
282 dissolved oxygen was significantly correlated to GST ( $r: -0.96$ ;  $p < 0.05$ ). Index conditions of oysters  
283 were significantly correlated to SOD ( $r: -0.94$ ;  $p < 0.05$ ), MDA ( $r: 0.89$ ;  $p < 0.05$ ) and Laccase ( $r: -1$ ;  $p$   
284  $< 0.05$ ) at Le Martray, Charente and Seudre sites. Finally, index conditions of mussels were  
285 significantly correlated to SOD at the Sevre and Charente site ( $r: -0.83$  and  $0.94$  respectively;  $p <$   
286  $0.05$ ), 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  
287 Martray site.

### 288 **Correlations between pesticides and biomarker response**

289 In oysters, the GST activity was positively correlated to alpha BHC ( $r: 0.48$ ;  $p < 0.05$ ), beta BHC ( $r:$   
290  $0.44$ ;  $p < 0.05$ ) and alachlor concentrations ( $r: 0.49$ ;  $p < 0.05$ ) (Figure S2). Moreover, the  
291 hexachlorobenzene concentration was negatively correlated with AChE activity ( $r: -0.74$ ;  $p < 0.05$ )  
292 and positively correlated to MDA ( $r: 0.78$ ;  $p < 0.05$ ), in oysters (Figure S2). No significant correlations  
293 were found between oyster pesticide concentrations and SOD activity.

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

### 299 **Discussion**

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

309 Sèvre Niortaise and Charente estuaries (Lerebours *et al.*, 2021) which is the only recent study  
310 reporting chlortoluron in marine bivalves from the environment. The present results showed that the  
311 bioaccumulation of chlortoluron did not induce a biochemical change in the selected biomarkers.  
312 Chlortoluron, is used as an herbicide to treat wheat and barley crops, and the increase observed in  
313 bivalves in the present study could be related to leaching by rainfalls after herbicide application  
314 (Pacanoski and Mehmeti, 2019). Indeed, an increase of daily mean precipitation during the winter with  
315 8 and 4 mm was recorded in November and December 2019 respectively and the water flow from the  
316 Charente river estuary increased to reach 240 m<sup>3</sup>/s. In parallel, a decrease of salinity was recorded  
317 during that period. Therefore, those environmental conditions could explain the higher chlortoluron  
318 concentration found in mussels in January 2020 in the present study and in seawater in winter 2015  
319 (from 48 to 84 ng/L) in the Gironde estuary (located to the south of the Pertuis seas) (Levesque *et al.*,  
320 2018). Finally, the seawater concentrations found in the present study (below the LOD of 15 ng/L)  
321 were lower than the concentration previously reported (Levesque *et al.*, 2018; Lerebours *et al.*, 2021),  
322 yet, the results showed that this compound can accumulate in marine bivalves.

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

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

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

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

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

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

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

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418 professionals for their technical support.

419

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515 **Figure and Table Legend.**

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

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

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

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

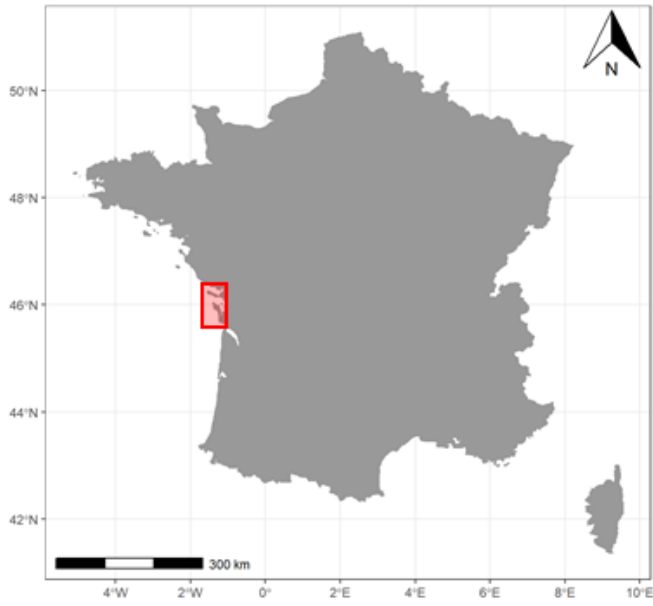
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523 **Table 1.** The 30 pesticides analysed.

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

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526 **Figure 1.**



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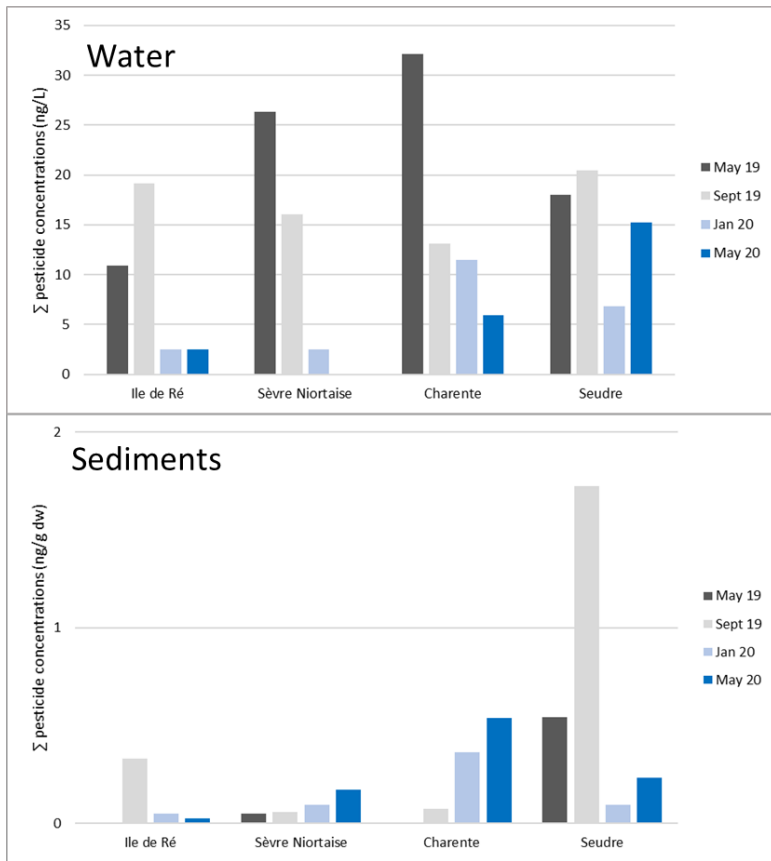
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532 **Figure 2.**



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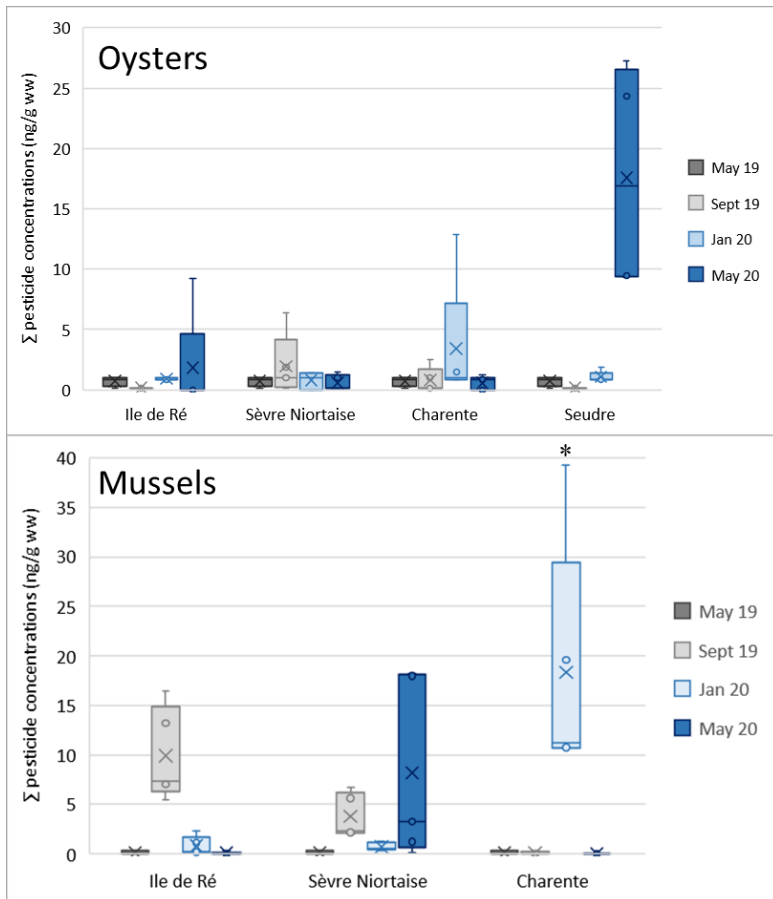
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547 **Figure 3.**



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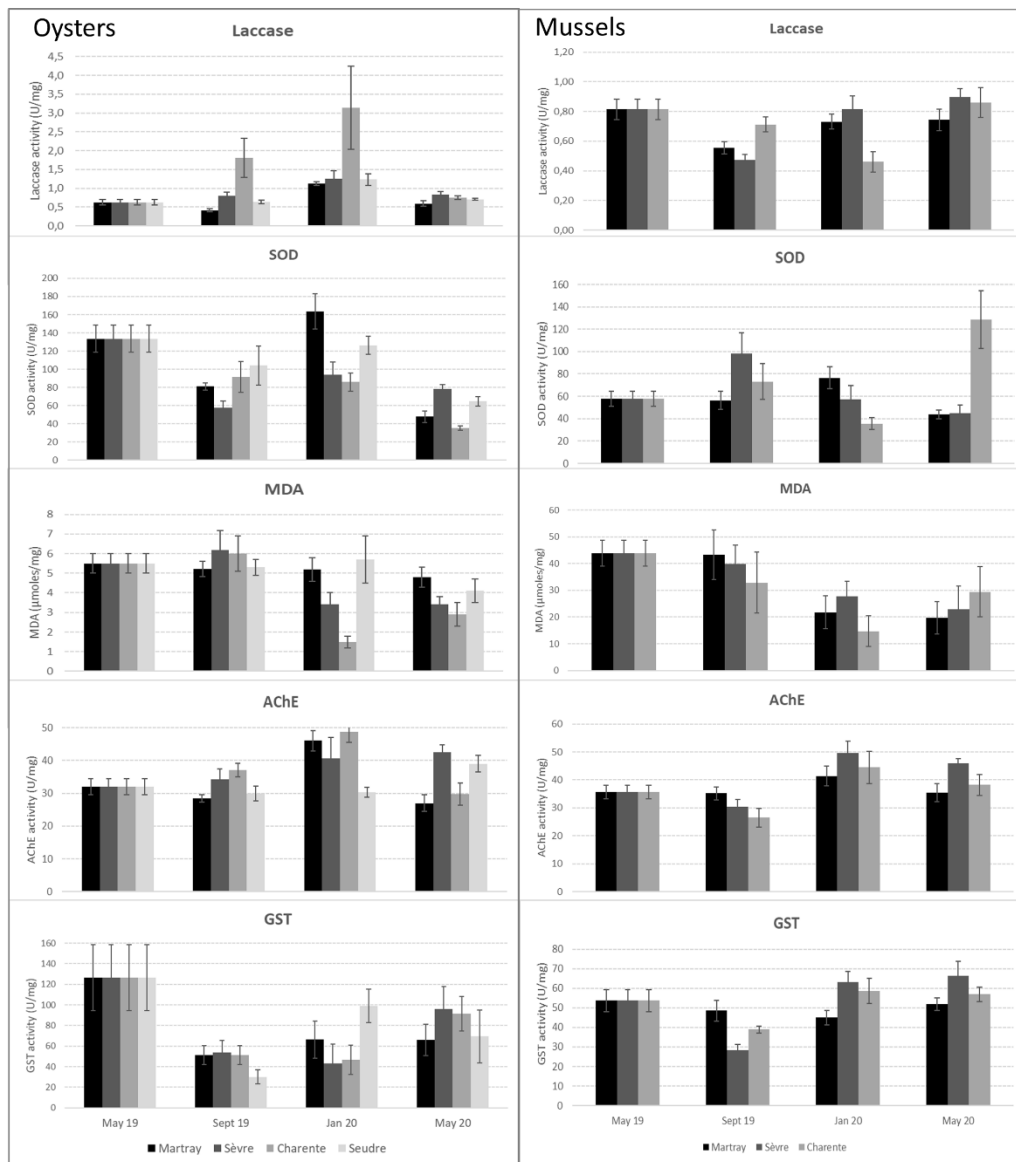
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561 **Figure 4.**



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571 **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
<b>Oysters</b>						
<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
<b>Mussels</b>						
<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**

584 **Text S1.** Biochemical analyses.

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

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

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

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

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

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

597 **Table S2.** Fresh weight (g) and total length (cm) of oysters and mussels collected over the year  
598 biomonitoring (mean ± SD).

599 **Table S3.** Index conditions of oysters and mussels.

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610 **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  $\text{Cu}^{2+}$  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,  $\text{MnCl}_2$  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.

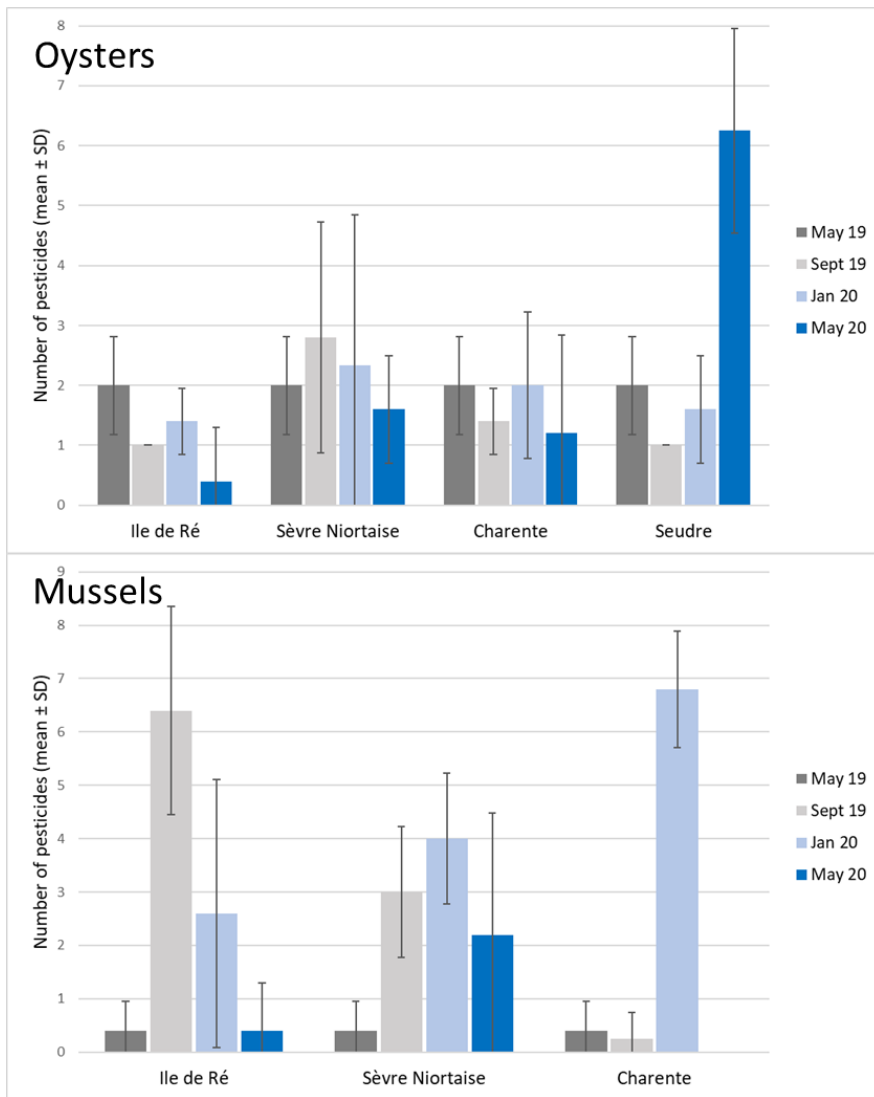
634 Environmental stress with Laccase assay

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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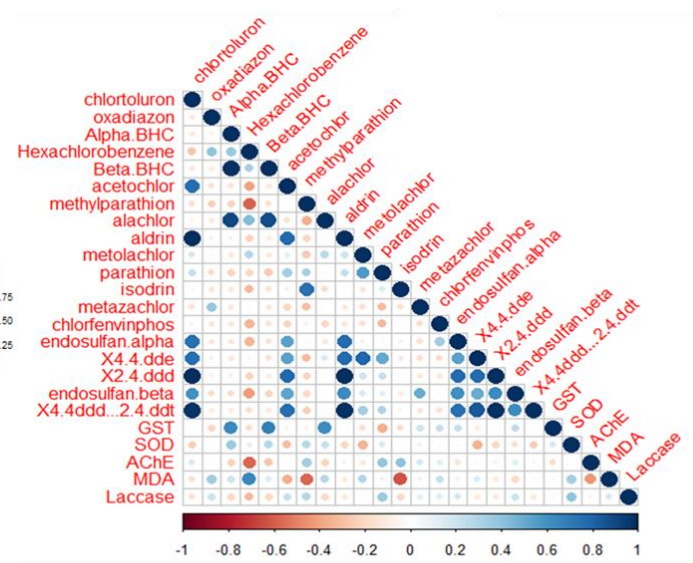
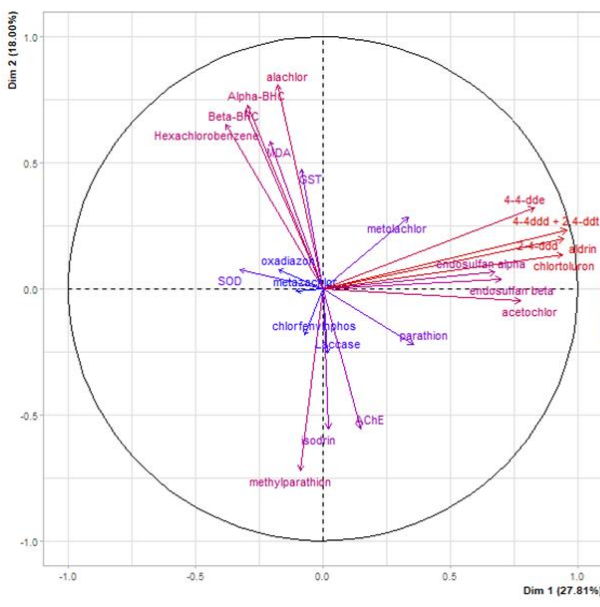
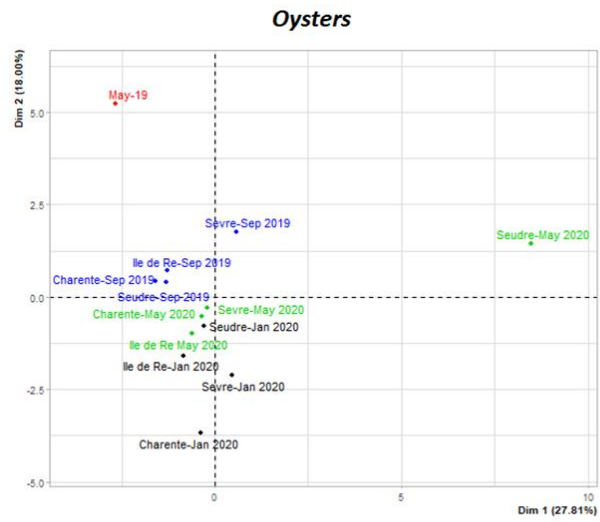
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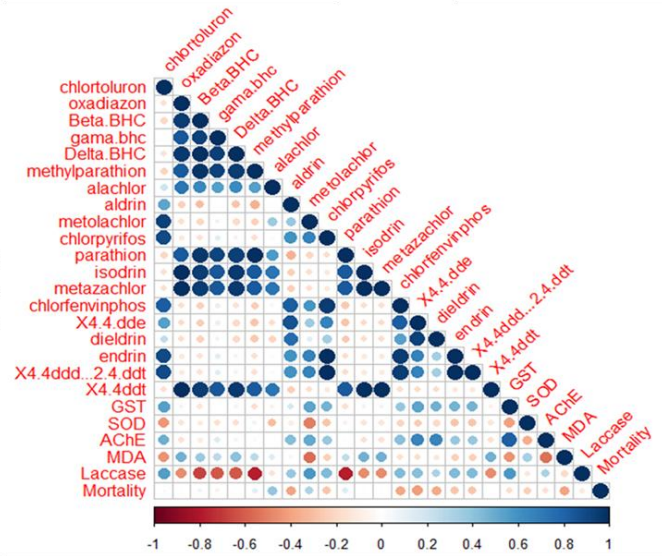
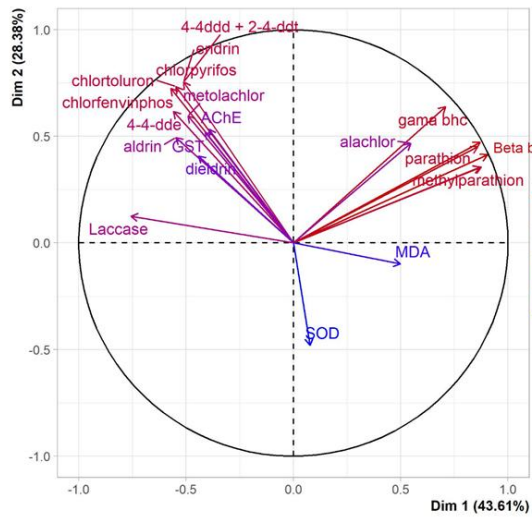
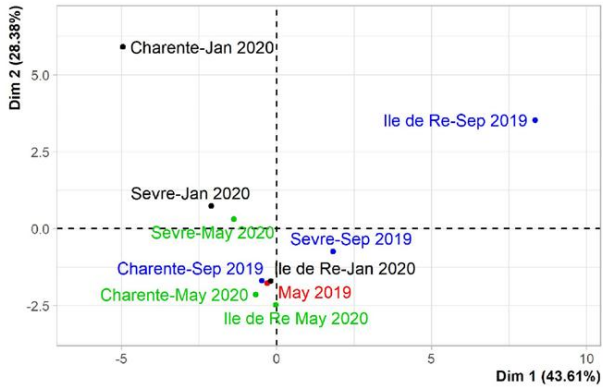
654 **Figure S2.**



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**Figure S3.**

### Mussels



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678 **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
<b>Oysters</b>					
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
<b>Mussels</b>					
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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