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## **Pesticide mixture toxicity assessment through in situ and laboratory approaches using embryo-larval stages of the pacific oyster (*Magallana gigas*)**

Eliška Kuchovská, Patrice Gonzalez, Lucie Bláhová, Mathilde Barré, Corentin Gouffier, Jérôme Cachot, Alicia Roméro-Ramirez, Luděk Bláha, Bénédicte Morin

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1 **Pesticide mixture toxicity assessment through *in situ* and laboratory**  
2 **approaches using embryo-larval stages of the Pacific oyster (*Magallana***  
3 ***gigas*)**

4

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12

13

## 14 **Abstract**

15 Worsened state of oysters in French Arcachon Bay, demand an investigation of possible  
16 causes. This study evaluated the effects of an environmentally relevant mixture of five  
17 common pesticides on the early-life stages of the Pacific oyster (*Magallana gigas*).  
18 Laboratory assays with artificial mixture and *in situ* transplantation were complementarily  
19 used to investigate a series of sublethal endpoints. The laboratory exposure revealed  
20 developmental toxicity at 0.32 µg/L, which corresponds to mixture concentrations in  
21 Arcachon Bay. Downregulation of some gene transcriptions was observed at environmental  
22 level. No difference in larvae development was revealed among the three sites in Arcachon  
23 Bay. This study was the first to evaluate locomotion of oyster larvae exposed *in situ*.  
24 Suspected poor water quality in the inner part of Arcachon Bay was reflected by impairment  
25 at the molecular level. In conclusion, current concentrations of the tested pesticides in  
26 Arcachon Bay hinder larval development and affect several biological functions.

27

## 28 **KEYWORDS**

29 Arcachon Bay; Coastal waters; Developmental abnormality; Gene expression; *In situ*;  
30 Mixtures; Pacific oyster; Pesticides; Sublethal effects; Swimming behavior

31

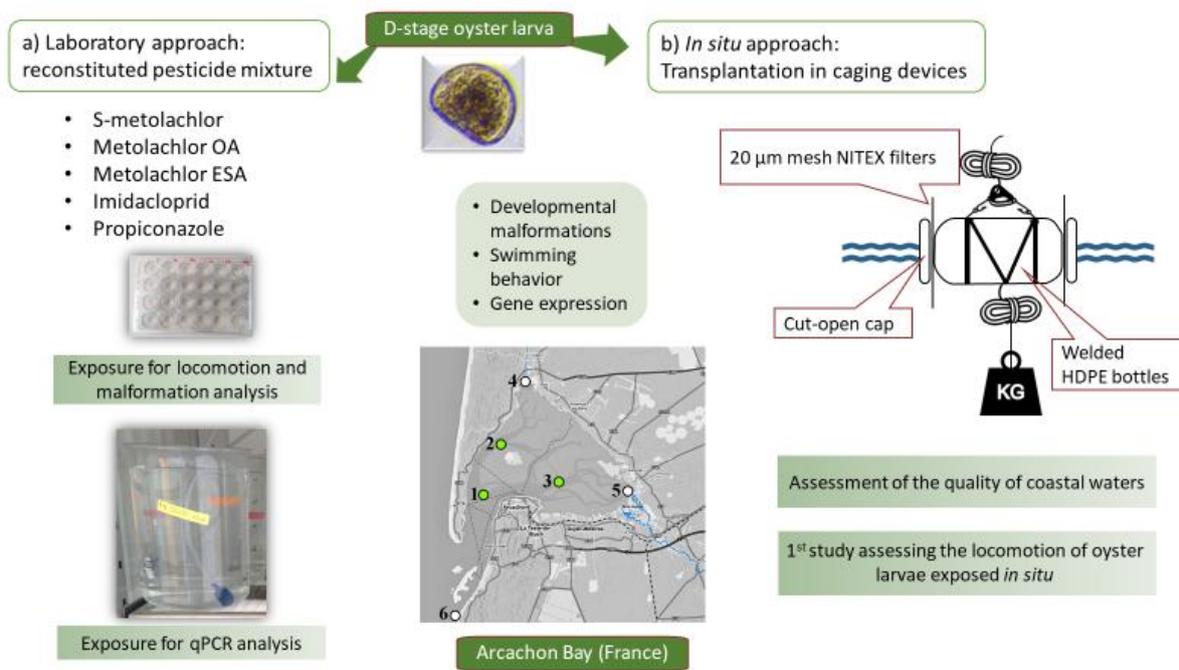
32 **HIGHLIGHTS**

- 33 • 1<sup>st</sup> study to evaluate locomotion of oyster larvae exposed *in situ*
- 34 • Pesticide mixture even at low concentration is not safe for oyster development
- 35 • Water quality in Arcachon Bay is sufficient for development of well-formed larvae
- 36 • Effects on the gene expression were observed at realistic concentrations
- 37 • Larvae caged at sites with different pollution had variable gene expressions

38

39 **Graphical abstract**

40



41

## 42 **1. Introduction**

43 Arcachon Bay is a semi-sheltered shallow lagoon (174 km<sup>2</sup>) on the Atlantic coastline in the  
44 South-west of France (Bertrand, 2014). The Pacific oyster (*Magallana gigas*, also known as  
45 *Crassostrea gigas*), an invasive species on the European coast introduced from the northwest  
46 Pacific Ocean (Troost, 2010), has become an emblematic and farmed species in Arcachon  
47 Bay. Several issues such as the decline in oyster recruitment and spat collection (Auby *et al.*,  
48 2014), decrease in the seagrass *Zostera* population (Auby *et al.*, 2011; Cognat *et al.*, 2018),  
49 and anomalies in the production of phytoplankton (Auby and Maurer, 2004) have been  
50 observed in Arcachon Bay during the last two decades. Possible causes of the oyster  
51 recruitment decline have been investigated, including the incidence of oyster herpes virus  
52 (Labbate *et al.*, 2015) and the coupled effect of climate change and pesticide pollution  
53 (Gamain, 2016). However, the described issues have not been resolved and other causes are  
54 being searched for. Numerous hazardous substances are regularly detected in the lagoon by  
55 REMPARG, the survey network for monitoring of pesticides and micropollutants  
56 ([https://www.siba-bassin-arcachon.fr/actions-environnementales/les-reseaux-de-surveillance-](https://www.siba-bassin-arcachon.fr/actions-environnementales/les-reseaux-de-surveillance-repar-et-remparg)  
57 [repar-et-remparg](https://www.siba-bassin-arcachon.fr/actions-environnementales/les-reseaux-de-surveillance-repar-et-remparg)). Pesticides are brought into the Bay by lagoon tributaries, mainly by river  
58 Leyre (Tapie and Budzinski, 2018). While the impacts of individual pesticide compounds on  
59 non-target organisms are usually studied, we still lack information about the toxicity of  
60 environmentally relevant mixtures as also emphasized in the European Green Deal (European  
61 Commission, 2020). Mixtures can result in synergistic, antagonistic, or additive effects, as  
62 documented by Gustavsson *et al.* (2017) and Cedergreen (2014). For instance, neonicotinoids  
63 are known to exert addition or synergy effects when occurring in mixtures with some  
64 fungicides (Morrissey *et al.*, 2015).

65 In Arcachon Bay, complex mixtures of tens of pesticides (out of a hundred molecules  
66 screened) are often detected (Tapie and Budzinski, 2018). According to this monitoring study,  
67 some of the most commonly found molecules in the waters of the lagoon are insecticide  
68 imidacloprid (IMI), fungicide propiconazole (PRO), and herbicide S-metolachlor (SM) with  
69 its two dominant metabolites metolachlor oxanilic acid (MOA) and metolachlor  
70 ethanesulfonic acid (MESA). IMI is a neurotoxic neonicotinoid banned in the EU, acting as  
71 an agonist on the post-synaptic nicotinic acetylcholine receptors (Matsuda *et al.*, 2001). PRO  
72 is a triazole hindering the synthesis of fungal cell membranes by inhibiting the ergosterol  
73 formation (Oliver and Hewitt, 2014), and SM is a chloroacetanilide suppressing the plant

74 growth by impairing the biosynthesis of very-long-chain fatty acids (Götz and Böger, 2004).  
75 Impact of imidacloprid on molluscs were recently thoroughly reviewed (Ewere *et al.*, 2021).  
76 Effects of the mentioned individual compounds on the embryo-larval stages of the Pacific  
77 oyster were already assessed (Gamain *et al.*, 2017, 2016; Kuchovská *et al.*, 2021; Mai *et al.*,  
78 2014, 2013), and some of these were even present in a recent complex mixture of 14  
79 pesticides toxicity evaluation (Mai *et al.*, 2020).

80 Laboratory bioassays are one possible way to assess the effects of environmental mixtures of  
81 pollutants at relevant concentrations. However, more valuable insight is gained when  
82 laboratory bioassays are coupled with *in situ* caging experiments. Results obtained by this  
83 combined approach are ecologically relevant and reflect realistic and complex site-specific  
84 conditions. These conditions include not only the chemical exposure and tidal movements,  
85 weather conditions, temperature changes, or natural presence of microorganisms, which are  
86 all factors difficult to replicate in the laboratory (Ferrari *et al.*, 2013). Overall, whereas  
87 laboratory bioassays discriminate which concentration of pesticides affect the oyster  
88 development, the *in situ* approach may reveal if the laboratory findings may be used to  
89 explain the real situation in the bay, if the effects observed by these two means are  
90 comparable, and, more importantly, the rate of successful oyster development on different sites  
91 of the bay with different pollution backgrounds. A more complex picture of the issue of oyster  
92 development in Arcachon Bay is thus obtained. There is a long tradition of using bivalves  
93 (collected in the field or deployed via transplant caging) such as mussels or oysters for *in situ*  
94 coastal monitoring programs (Besse *et al.*, 2012; Beyer *et al.*, 2017). Moreover, an ASTM  
95 (American Society for Testing and Materials) guideline for field bivalve testing exists  
96 (ASTM, 2013). However, these monitoring studies use transplanted adult (rarely juvenile)  
97 oysters (Cao and Wang, 2016; Clara Rebouças Do Amaral *et al.*, 2005; Hédouin *et al.*, 2011;  
98 Jenny *et al.*, 2016; Lee and Birch, 2016) or mussels (Benedicto *et al.*, 2011; Brooks *et al.*,  
99 2012; Cappello *et al.*, 2015; Devier *et al.*, 2005; Haynes *et al.*, 1995; Lehtonen *et al.*, 2016).  
100 One of the rare studies not employing adult organisms was carried out with a six-week-old  
101 oyster spat in *in situ* microcosms (Stachowski-Haberkorn *et al.*, 2008). To the best of our  
102 knowledge, only two biomonitoring studies (Geffard *et al.*, 2001; Quiniou *et al.*, 2007) used  
103 the embryo-larval stages of bivalves. The early life stages of bivalves have a high sensitivity  
104 to contaminants due to the body mass difference and naked body surface (Hutchinson *et al.*,  
105 1998; Beiras, 2018), quick development (D-larvae is formed after 24 h), and a convenient  
106 high-throughput screening format.

107 The present study aimed to investigate and compare the sublethal effects (developmental  
108 malformations, neurobehavioral locomotion patterns, and gene expression levels) on Pacific  
109 oyster larvae (*Magallana gigas*) in two complementary exposure conditions, *i.e.* i) laboratory  
110 exposure to an environmentally relevant reconstituted mixture of pesticides and ii) *in situ*  
111 transplantation in caging devices for two days on three different sites in Arcachon Bay. This  
112 study is the first to combine *in situ* and laboratory approaches with embryo-larval stages of  
113 Pacific oyster.

## 114 **2. Materials and methods**

### 115 **2.1 Chemicals and reference seawater handling**

116 Imidacloprid (IMI, CAS 138261-41-3, Pestanal, purity 100 %), propiconazole (PRO, CAS  
117 60207-90-1, Pestanal, purity 100 %), S-metolachlor (SM, CAS 87392-12-9, Pestanal,  
118 purity  $\geq$  98.0 %), metolachlor oxanilic acid (MOA, CAS 152019-73-3, Pestanal,  
119 purity  $\geq$  98.0 %), metolachlor ethanesulfonic acid (MESA, CAS 947601-85-6, Pestanal,  
120 purity  $\geq$  95.0 %), and CuSO<sub>4</sub> were purchased from Sigma-Aldrich. Concerning gene  
121 expression analysis, primers were purchased from Sigma proligo, RNA later buffer was  
122 purchased from Qiagen, and phenol and chloroform Rectapur ® were purchased from Sigma.  
123 Copper solution (stock solution of 100 mg/L in milliQ water) was used as a positive control in  
124 laboratory experiments and was stored at 5 °C, as well as PRO solution, which was prepared  
125 in DMSO (5 g/L) and other pesticide solutions (SM, IMI, MOA, MESA) which were prepared  
126 in milliQ water (50 mg/L). Dilutions in final exposure solutions were carried out using  
127 reference seawater collected at beach Petit Nice (approx. 44°33'40.3"N 1°14'27.1"W).  
128 Seawater was transported in 10 L plastic containers, filtered at 0.22 µm, and passed through  
129 UV light to eliminate microorganisms. Filtered seawater (FSW) was stored at 5 °C in the dark  
130 and was used usually within a few days. Few hours before the experimentations, FSW was  
131 filtered again at 0.22 µm. The background concentration of pesticides in FSW was measured  
132 by LC-MS/MS (cf. section 2.6).

133

### 134 **2.2 Embryo-larval test: a laboratory approach**

135 Five couples of mature pacific oysters (*Magallana gigas*, also called *Crassostrea gigas*  
136 (Bayne *et al.*, 2017)) were obtained from Guernesey Sea Farm hatchery (Guernesey, UK).  
137 Oysters were immediately used on the day of the arrival or they were kept in oxygenated  
138 FSW at 11 °C for 24 h. A detailed version of the used method for oyster spawning (alternating

139 thermal shocks) is described in Kuchovská *et al.* (2021) and Gamain *et al.* (2016) and follows  
 140 the French guideline (NF ISO 17244, 2015). Because not all the oysters did spawn, multiple  
 141 shipments of oysters were needed during the years 2018 and 2019. Obtained embryos were  
 142 then incubated in experimental units (vessel type is depending on the measured endpoint as  
 143 described below) at 24 °C in the dark until they reached the developmental stage of D-larva.  
 144 After the exposures, developmental malformations, locomotion (section 2.4), and gene  
 145 expression analyses (section 2.5) were carried out. To assess the developmental  
 146 malformations, an embryo-larval test was carried out according to the French guideline (NF  
 147 ISO 17244, 2015) in 24-well microplates (Greiner Bio-One, Cellstar).The microplates were  
 148 precoated 24 h before the test with the corresponding concentration of mixture solution due to  
 149 propiconazole hydrophobicity, as confirmed by chemical analyses during our previous study  
 150 (Kuchovská *et al.*, 2021). Embryos from one oyster couple were considered as one  
 151 independent experiment; each oyster couple formed 4 analytical replicates (wells) per  
 152 concentration in the microplates with approximately 225 ± 10 % embryos per well. Seven  
 153 oyster couples per condition were used for the assessment of developmental malformations.  
 154 Negative control (FSW) and solvent control (0.016 % DMSO) were present on every  
 155 microplate. The embryos were exposed to increasing concentrations of the pesticide mixture  
 156 (MIX) composed of IMI, PRO, SM, MOA, and MESA (total concentration of five pesticides  
 157 corresponding to 0.32 µg/L, 1.6 µg/L, 8 µg/L, 40 µg/L, 200 µg/L, 1 mg/L, 5 mg/L, 25 mg/L;  
 158 individual concentrations are listed in Table 1).

159 **Table 1** Concentrations of different pesticides in the reconstituted mixture used to expose  
 160 embryos of oyster *Magallana gigas*.

| Code | PRO         | IMI        | SM          | MOA        | MESA       | Total concentration |
|------|-------------|------------|-------------|------------|------------|---------------------|
| C1   | 10 ng/L     | 100 ng/L   | 10 ng/L     | 100 ng/L   | 100 ng/L   | 0.32 µg/L           |
| C2   | 50 ng/L     | 500 ng/L   | 50 ng/L     | 500 ng/L   | 500 ng/L   | 1.6 µg/L            |
| C3   | 0.25 µg/L   | 2.5 µg/L   | 0.25 µg/L   | 2.5 µg/L   | 2.5 µg/L   | 8 µg/L              |
| C4   | 1.25 µg/L   | 12.5 µg/L  | 1.25 µg/L   | 12.5 µg/L  | 12.5 µg/L  | 40 µg/L             |
| C5   | 6.25 µg/L   | 62.5 µg/L  | 6.25 µg/L   | 62.5 µg/L  | 62.5 µg/L  | 200 µg/L            |
| C6   | 31.25 µg/L  | 312.5 µg/L | 31.25 µg/L  | 312.5 µg/L | 312.5 µg/L | 1 mg/L              |
| C7   | 156.25 µg/L | 1.563 mg/L | 156.25 µg/L | 1.563 mg/L | 1.563 mg/L | 5 mg/L              |
| C8   | 781.25 µg/L | 7.825 mg/L | 781.25 µg/L | 7.825 mg/L | 7.825 mg/L | 25 mg/L             |

161

162 The lowest used concentration (C1) is reflecting the usual concentrations often measured in  
 163 Arcachon Bay in France (cf. Supplementary Table S1). Concentration C2 approximately  
 164 corresponds to the highest concentrations measured in Arcachon Bay. The treatments C3 and  
 165 C4 may be considered as maximal environmental concentrations measured in the surface  
 166 waters in the world (Table 2). Higher concentrations (C5-C8) were used to allow for  
 167 estimation of the EC<sub>50</sub> or as a prediction of acute peak contamination periods.

168

169 **Table 2** Detected concentrations of pesticides of interest in surface waters in various locations  
 170 in the world.

| Substance     | Detected concentration   |   |
|---------------|--|---|
| Propiconazole | 0.7 µg/L Vietnam<br>(Toan <i>et al.</i> , 2013)                    | 0.81 µg/L China<br>(Peng <i>et al.</i> , 2018)          |
| Imidacloprid  | 3.29 µg/L California (USA)<br>(Starner and Goh, 2012)              | 320 µg/L Netherlands<br>(Van Dijk <i>et al.</i> , 2013) |
| Metolachlor   | 10.5 µg/L South Georgia (USA)<br>(Glinski <i>et al.</i> , 2018)    | 16.5 µg/L Italy<br>(Meffe and de Bustamante, 2014)      |
| MOA           | 1.21 µg/L Mississippi River Basin<br>(Rebich <i>et al.</i> , 2004) | 5.3 µg/L Iowa (USA)<br>(Kalkhoff <i>et al.</i> , 2012)  |
| MESA          | 2.51 µg/L Mississippi River Basin<br>(Rebich <i>et al.</i> , 2004) | 10.3 µg/L Iowa (USA)<br>(Kalkhoff <i>et al.</i> , 2012) |

171

172 After the incubation of embryos (24 h at 24 °C) exposed to the mentioned concentrations, the  
 173 microplates were used for the non-invasive video capture (cf. 2.4 Locomotion analysis). After  
 174 the video capture (at approximately 30 h), formaldehyde (25 µL at 37 %) was added to every  
 175 well (final volume 2025 µL) to stabilize the larvae state for the developmental malformation  
 176 analysis. The microplates were kept at 4 °C in the dark. An inverted microscope (Nikon  
 177 Eclipse TS100) was used to assess different forms of developmental malformations in  
 178 100 embryos per well, *i.e.* mantle malformation, scalloped shell malformation, concave shell  
 179 malformation, developmental arrest. Photos of different malformations, as well as well-  
 180 formed larvae, are published in Kuchovská *et al.* (2021) in Figure 1. According to the ISO

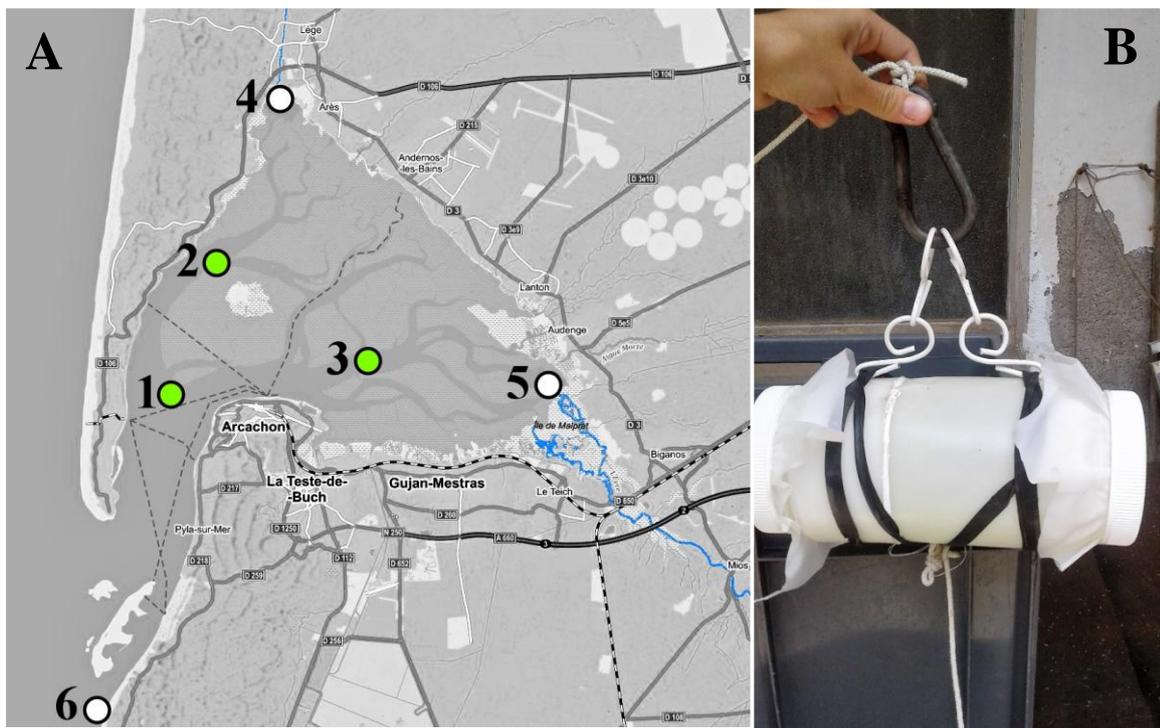
181 norm (NF ISO 17244, 2015), the malformation rate in the negative control cannot exceed  
182 20 % and the EC<sub>50</sub> for abnormal larvae exposed to Cu<sup>++</sup> should be between 6 and 16 µg/L.

### 183 **2.3 *In situ* approach**

184 Three sites were chosen for the *in situ* study. The reference site “Grand Banc” (GB) is located  
185 in Teychan channel (44° 39, 914 N; 001° 13, 076 W) near the mouth of Arcachon Bay. The  
186 Passes is a narrow (2-3 km) but long (12 km) channel connecting Arcachon Bay with the Bay  
187 of Biscay. Therefore, water quality (concerning pesticides) at GB is in a relatively good state  
188 as monitored in years 2010-2016 (Tapie and Budzinski, 2018). The two other sites are  
189 situated in the inner part of Arcachon Bay, thus undergoing higher anthropogenic pressure:  
190 “Les Jacquets” (J) and “Comprian” (C) are in channel Chenal de l'Île (44° 42, 831 N; 001° 11,  
191 235 W) and Teychan channel (44° 40, 833 N; 001° 07, 096 W), respectively. The location of  
192 the sites is shown in Figure 1A.

193 Adult oysters producing embryos for the *in situ* caging approach were purchased from France  
194 Naissain (Bouin, France). The spawning of the mature oysters was carried out in the same  
195 manner as for the laboratory approach (cf. section 2.2). Obtained embryos were transported to  
196 the three selected sites (Figure 1A) in FSW and the caging devices were deployed at the site  
197 approximately at 2 hpf (hours post fertilization) using a quick small boat. The device was  
198 inspired by the caging device of Geffard *et al.* (2001) and was made of two welded 4 L HDPE  
199 bottles with open caps at each side (Figure 1B). Both caps were holding a 20 µm mesh filter  
200 (SEFAR NITEX®) to let the water freely circulate. Devices were held under the water surface  
201 using a weight and were attached to a buoy with a rope (approximately 2 meters long to keep  
202 the device underwater surface even at low tide). Four devices containing approximately  
203 666,000 embryos from four mature oyster couples were deployed at each of the three sites.  
204 Before adding the embryos to the device, water from the site was slowly poured into the  
205 device through the 20 µm mesh filter to keep the water in the device clean from larger debris  
206 that would have corrupted the ulterior locomotion analysis. The transplantation was carried  
207 out on the 24<sup>th</sup> July 2019 between 11h and 13h15 local time (summertime is the period of  
208 oyster reproduction in Arcachon Bay). Underwater view of used devices is attached to this  
209 research paper. The water temperature in the waters of Arcachon Bay oscillated during the  
210 experimentation approximately between 19 and 24 °C as measured 1.5 m under the water  
211 surface at Comprian site and buoy 13 (temperature data obtained from SOMLIT database at  
212 somlit.fr). Although the exposure during the laboratory approach lasted 1 day, the exposure in  
213 the field was prolonged to 2 days to ensure the successful development of D-larva. Indeed, the

214 constant temperature of 24 °C kept in the laboratory is ideal for the development of D-larva.  
215 However, in the field, the embryos are subjected to varying temperatures, often below the  
216 24 °C which slow the development. After being deployed two days (approx. 46 hours) in the  
217 field, the devices were transported in beakers into the laboratory and the larvae were gently  
218 collected on a 20 µm mesh and transferred into 24-well microplates in the approximate  
219 concentration of 225 embryos per well (4 replicates per caging device) to perform the video  
220 capture (section 2.4) followed by formaldehyde stabilization (as in the laboratory approach),  
221 and ulterior developmental malformation analysis. The rest of the larvae was collected in a  
222 polypropylene microtube for the gene expression analysis (section 2.5).



223  
224 **Figure 1** A Map of Arcachon Bay (France) with selected sites for the field transplantation  
225 experiment and other important points: 1 Site Grand Banc (GB); 2 Site Les Jacquets (J); 3  
226 Site Comprian (C); 4 Mouth of the river Canal des Étangs; 5 Mouth of the river Leyre; 6  
227 Beach Petit Nice – sampling point of the reference seawater. **B** Photo of the caging device.  
228 (Map modified from mapy.cz)

## 229 2.4 Locomotion analysis

230 In the laboratory approach, oyster embryos were exposed to three environmentally relevant  
231 concentrations of the pesticide mixture (total nominal concentrations of five pesticides  
232 0.32 µg/L, 1.6 µg/L, and 8 µg/L; for individual concentrations cf. Table 1) for 24 h in the dark  
233 in 24-well microplates (Greiner Bio-One, Cellstar; 225 embryos per well). In the *in situ*

234 approach, oyster larvae were gently collected at the end of the experiment (48 h) on a 20  $\mu$ m  
235 mesh and transferred into 24-well microplates in the approximately same concentration of  
236 embryos per well (4 replicates per caging device). 2 min video of each well was taken at  
237 zoom 40x using an inverted microscope Nikon Eclipse TS100 equipped with camera Nikon  
238 DS-Fi2, and software NIS Element. The temperature of solutions in the microplates was kept  
239 at 24 °C all the time for both experimental approaches. The detailed method (including  
240 conversion of videos by VirtualDub and their analysis by ImageJ) is described in Gamain *et*  
241 *al.* (2019). At the end of the ImageJ analysis, the maximal and average swimming speed, and  
242 trajectory type (rectilinear, circular, and stationary) of each larva are determined. Graphical  
243 representation of different types of trajectory paths is published in Kuchovská *et al.* (2021) in  
244 Figure 2.

## 245 **2.5 Gene expression analysis**

246 Oyster embryo laboratory exposure for gene expression analysis is described in detail in  
247 Kuchovská *et al.* (2021). In brief, half of the million embryos issued from one oyster mature  
248 couple were incubated in three-liter glass beakers at 24 °C in the dark for 42 h (it was not  
249 possible to expose the organisms for 48 hours because the handling of larvae would have to  
250 be done during the night) with an oxygenation system (plastic tube with an aquarium air  
251 stone). The total pesticide exposure concentrations were 0.32  $\mu$ g/L, 1.6  $\mu$ g/L, and 8  $\mu$ g/L (for  
252 concentrations of individual compounds cf. Table 1). Larvae samples from the laboratory  
253 exposure in glass beakers and the *in situ* caging devices were collected on a 20  $\mu$ m mesh  
254 (SEFAR NITEX®) and distributed in five replicates containing 30,000 larvae each. The  
255 samples were stored in RNA later at -80 °C until RNA extraction.

256 The RNA extraction, transcription, and qPCR analyses were carried out as described  
257 previously (Kuchovská *et al.*, 2021). Briefly, SV Total RNA Isolation System Kit, Reverse  
258 Transcription System kit, and GoTaq® qPCR Master Mix kit (Promega) were used. The  
259 purity of all isolated RNA samples at 260/280 nm was between 2.14 and 2.18. Primer-pairs  
260 efficiency was checked beforehand (> 95 %). qPCR was carried out using the LightCycler480  
261 (Roche). Results are expressed as fold changes of the exposed group compared to the control  
262 group (laboratory tests) or sampling site compared to the site Grand Banc (*in situ* tests).

263 Three reference genes ( *$\beta$ -actin*, *ef1a*, and *rpl7*) and fourteen genes of interest were used in the  
264 analysis. Genes of interest were implicated in mitochondrial metabolism (12S, *cox1*),  
265 regulation of the cell cycle/apoptosis (*p53*), oxidative stress defense (*cat*, *sodMn*, *sodCu/Zn*,

266 *gpx*), metal regulation (*mt1*, *mt2*), apoptosis (*bax*, *casp3*), biotransformation (*cyp1a*), growth  
267 arrest and DNA damage repair (*gadd45*, *rad51*). Sequences, references, and accession  
268 numbers of all genes can be found in Kuchovská *et al.* (2021).

## 269 **2.6 Chemical analysis and water quality**

270 Dissolved oxygen, pH, and salinity were measured in the experimental units for gene  
271 expression (laboratory approach) at the beginning and the end of the laboratory experiments,  
272 using a probe Multi 340i (WTW). All checked parameters complied with the revised norm by  
273 Leverett and Thain (2013). In brief, oxygen saturation varied between 91.6 % and 98.2 % (on  
274 average 95.0 % and 93.8 % respectively at the beginning and the end of the test); pH values  
275 oscillated between 7.96 and 8.2; salinity ranged from 34.2 to 35.7 psu (on average 35.0 psu).

276 Salinity in the *in situ* samples varied greatly, depending on the location of the sampling site  
277 and tide level. Samples from GB had salinity 28.8 and 34.6 psu at the beginning and the end  
278 of the experiments, respectively, samples from J site 22.4 and 23.5 psu, and samples from C  
279 site 17.1 and 18.7 psu. The tidal coefficient at the city of Arcachon was low: 50 on the first  
280 day and 41 on the last of the experiment.

281 LC-MS/MS was used to measure the concentrations of pesticides of interest. Samples of the  
282 *in situ* experiment were taken under the water surface (approx. 40 cm) at each sampling point  
283 at the beginning and the end of the *in situ* experiment and transferred in glass bottles using a  
284 sampling stick. Calibration solutions and all samples were spiked with 10 µL of the internal  
285 standard of tebuconazole D6 and imidacloprid D4 (both dissolved in 50 % methanol) and  
286 stored at -20°C. The samples (50 mL each) were lyophilized using a freeze dryer Alpha 2-4  
287 LD Plus (Martin Christ Freeze Dryers). The samples were then dissolved in 3 mL of 100 %  
288 acetonitrile and processed as described in Kuchovská *et al.* (2021). The quantification of  
289 analytes was based on the external calibration (0.01 – 50 µg/L in 20 % of acetonitrile) and  
290 normalized with internal deuterium-labeled standards (imidacloprid D4 and tebuconazole  
291 D6). The limits of quantification (LOQ; S/N>10) in the samples for LC-MS/MS for IMI,  
292 PRO, SM, MOA, and MESA were 0.05, 0.01, 0.01, 0.1, and 0.1 µg/L, respectively. However,  
293 the samples were concentrated 33 times, thus the concentrations of *in situ* samples ultimately  
294 quantifiable (LOQ) were 1.5, 0.3, 0.3, 3.0, and 3.0 ng/L, for IMI, PRO, SM, MOA, and  
295 MESA respectively. The applied method is described in detail in Supplementary material S1.

296 Copper concentration was measured in positive control spiked samples (ICP-OES), and in the  
297 *in situ* samples (ICP-MS). The *in situ* seawater samples (100 mL) were taken with a sampling

298 stick with a plastic vial at the end of the exposure and carefully filtered through a filter into  
299 plastic falcons (all the material was beforehand cleaned with acid and was rinsed three times  
300 with seawater at the sampling point). The samples were then acidified with 5% final  
301 concentration of nitric acid in each sample and stored in the dark at 5 °C.

## 302 **2.7 Data analysis**

303 Total malformed larvae (p) *i.e.* the sum of mantle malformation, shell malformation, and  
304 developmental arrest; and type of larval trajectory (p) were transformed using the arcsine  
305 transformation  $p' = \arcsin \sqrt{\frac{p}{100}}$  (Sokal and Rohlf, 2012) before the statistical analysis (p is  
306 expressed in %). Because of the high variability of different test repetitions of larvae  
307 swimming speed data, the values ( $\mu\text{m}/\text{sec}$ ) were normalized to the control (each replicate was  
308 divided by the average of four control replicates of the respective independent test repetition)  
309 before the statistical analysis. Gene expression results ( $2^{-\Delta\Delta\text{CT}}$ ) were first log normalized. All  
310 data are shown with standard deviations ( $\pm$  SD) and corresponding numbers of independent  
311 values (N; indicated at each results presentation).

312 All pre-treated data were then compared using Statistica 13.3 (StatSoft, USA). Data were  
313 controlled for normality (Shapiro-Wilk test;  $P > 0.01$ ) and homoscedasticity (Levene test;  
314  $P > 0.05$ ). If confirmed, ANOVA ( $P < 0.05$ ) followed by Tukey post-hoc test was used. In the  
315 other case, a non-parametric Kruskal-Wallis ( $P < 0.05$ ) with Mann-Whitney post-hoc test was  
316 carried out.  $\text{EC}_{50}$  was calculated using nonlinear logarithmic regression of the nominal  
317 concentration-response curves, using Graph Pad Prism 5 (Graph Pad Software, USA).

## 318 **3. Results**

### 319 **3.1 Chemical analysis**

320 Pesticide and copper concentrations measured in the *in situ* samples are shown in Table 3. All  
321 pesticide concentrations were found below a hundred ng/L except for SM at the Comprian  
322 site. Copper concentration at all sites was low and unlikely to cause any substantial  
323 developmental malformations.

324

325 **Table 3** Measured concentrations of selected pesticides (and metabolites) and copper at three  
 326 different sites of Arcachon Bay (GB: Grand Banc, J: Les Jacquets, C: Comprian) at the time  
 327 of transplanting of the oyster embryos in the field and at the end of the exposure; LOD  
 328 (MOA, MESA) = 1.5 ng/L.

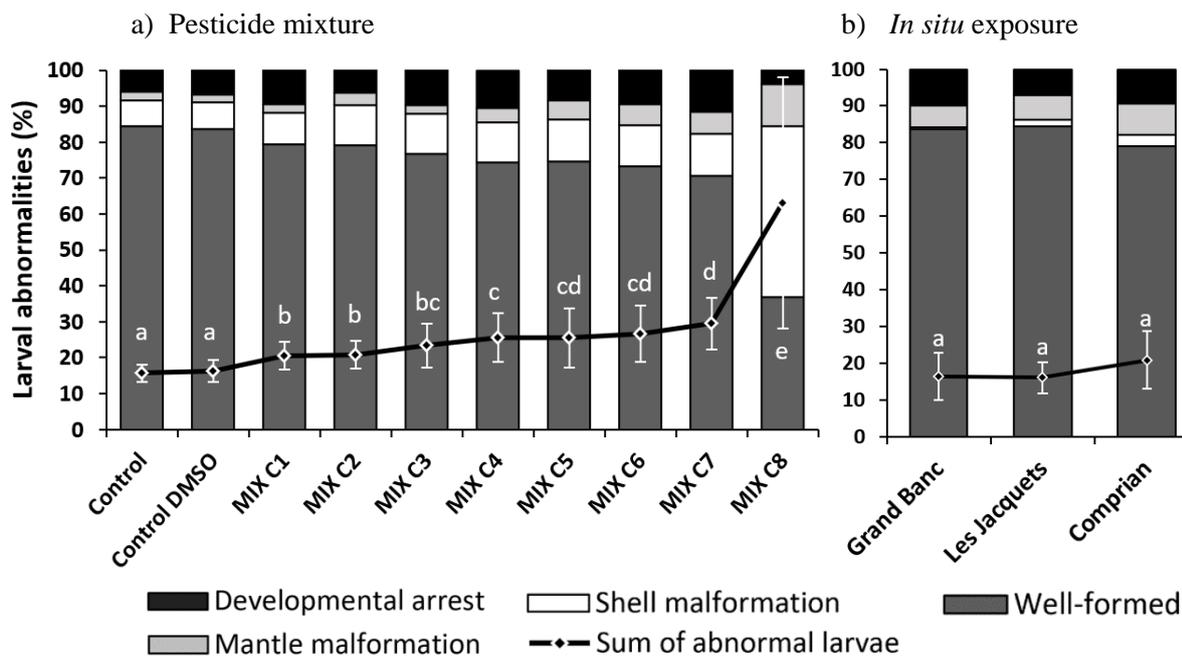
| Site | Sampling<br>time (h) | ng/L  |       |        |      |       | μg/L |
|------|----------------------|-------|-------|--------|------|-------|------|
|      |                      | PRO   | IMI   | SM     | MOA  | MESA  | Cu   |
| GB   | 0                    | 6.30  | 26.96 | 72.59  | <LOD | <LOD  | 0.59 |
|      | 48                   | 5.67  | 9.14  | 72.54  | <LOD | 37.17 | 0.54 |
| J    | 0                    | 12.69 | 14.63 | 97.20  | <LOD | <LOD  | 0.42 |
|      | 48                   | 8.73  | 10.08 | 92.75  | <LOD | 54.54 | 0.79 |
| C    | 0                    | 9.27  | 10.89 | 119.79 | <LOD | <LOD  | 0.65 |
|      | 48                   | 16.74 | 16.83 | 138.29 | <LOD | 26.01 | 0.49 |

329 The stability of used compounds in seawater was verified in previous studies. Imidacloprid  
 330 and propiconazole (with precoating) were perfectly stable in microplates used for the embryo-  
 331 larval exposures. Imidacloprid was also stable in gene expression 3-liter beakers, however,  
 332 propiconazole concentrations slightly varied due to the impossibility to precoat the beakers  
 333 (Kuchovská *et al.*, 2021). Metolachlor was also stable in seawater as shown by Gamain *et al.*  
 334 (2016).

### 335 **3.2 Embryo-larval development and observed malformations**

336 Positive control (Cu<sup>2+</sup>) in the embryo-larval test revealed oyster sensitivity with EC<sub>50</sub> of  
 337 9.76 ± 1.58 μg/L which corresponds to the value range (6 - 16 μg/L) preconized by the norm  
 338 (NF ISO 17244, 2015). Proportions of different larvae abnormalities caused by pesticide  
 339 mixture in the laboratory approach and by water in Arcachon Bay in the *in situ* experiment are  
 340 presented in Figure 2.

341

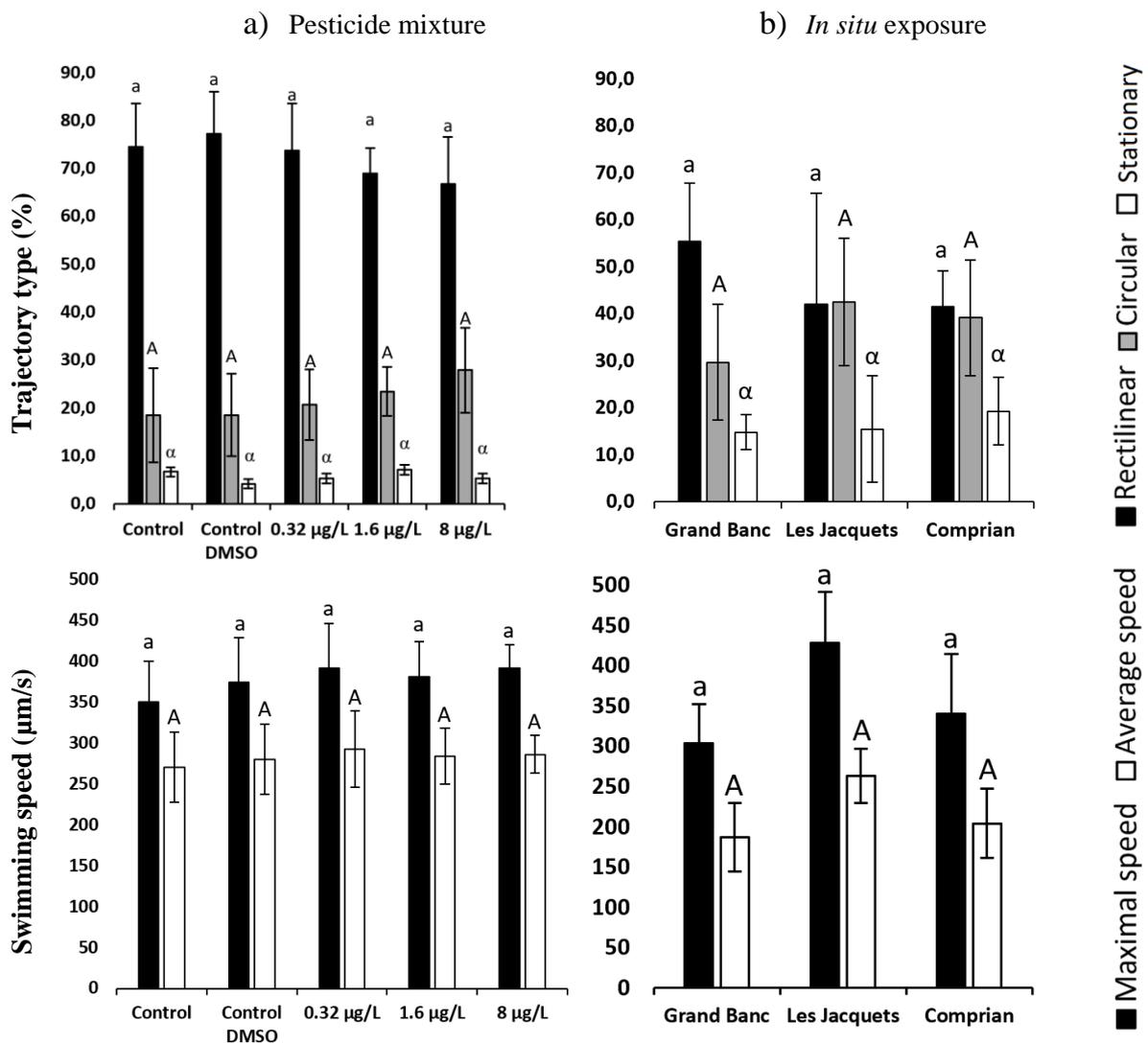


342 **Figure 2** Larval abnormalities and the sum of affected oyster larvae a) after 30 hours of  
 343 exposure to increasing concentrations of the MIX (individual concentrations of individual  
 344 chemicals are shown in Table 1) and b) deployed in cages for two days *in situ* on three  
 345 different sites of Arcachon Bay. Different letters indicate statistical differences between  
 346 variables ( $P < 0.05$ ). Results are presented as the mean ( $\pm$  SD) of 7 independent experiments  
 347 in the case of pesticide mixture and 4 in the case of *in situ* caging.

348 High variability in the sum of abnormal larvae caused by the highest concentration of the  
 349 MIX in the laboratory mixture experiment was possibly due to the fact that 4 experiments  
 350 were carried out in 2018 and 3 in 2019. The sensitivity to the positive control ( $EC_{50}$ ) was,  
 351 however, in the correct value range as preconized by the ISO norm (NF ISO 17244, 2015) for  
 352 both years ( $11.81 \pm 1.18 \mu\text{g/L}$  and  $8.80 \pm 1.25 \mu\text{g/L}$  in 2018 and 2019, respectively). The sum  
 353 of abnormal larvae served for the calculation of NOEC ( $< 0.32 \mu\text{g/L}$ ), LOEC ( $0.32 \mu\text{g/L}$ ), and  
 354  $EC_{50}$  ( $10.70 \pm 1.32 \text{ mg/L}$ ) of the pesticide mixture (concentrations are expressed as the sum of  
 355 nominal concentrations of the 5 pesticides). Even the lowest tested concentration of the  
 356 pesticide mixture induced a significant increase of abnormal larvae ( $20.61 \pm 3.84 \%$ ) from  
 357 controls ( $15.66 \pm 2.28 \%$  and  $16.38 \pm 3.08 \%$  of abnormal larvae in control FSW and DMSO  
 358 control, respectively). On the other hand, no difference was observed between the three sites  
 359 in Arcachon Bay.

### 360 3.3 Behavioral analysis

361 The behavioral assessment comprised different trajectory paths used by the oyster larvae and  
 362 their maximal and average swimming speeds. The results are shown in Figure 3. Absolute  
 363 values of the larvae exposed *in situ* swimming speed are shown in Supplementary Table S2.  
 364 No differences were observed after the laboratory exposure to the mixture of pesticides,  
 365 neither between the different sites in Arcachon Bay. However, a general decrease of  
 366 rectilinear trajectories may be observed at all *in situ* sites compared to the results obtained in  
 367 the laboratory.  
 368



369

370 **Figure 3** Trajectory types (%; upper graphs) and swimming speed (µm/s; lower graphs)  
 371 observed in oyster larvae a) after 24 h exposure to three concentrations of the MIX  
 372 (concentrations of individual chemicals are shown in Table 1) and b) after 2 days of

373 transplantation at three different sites in Arcachon Bay. Different letters indicate statistical  
374 differences ( $P < 0.05$ ). Results are presented as the mean ( $\pm$  SD) of 5 (pesticide mixture) and 4  
375 (*in situ*) independent experiments.

376

### 377 **3.4 Gene expression analysis**

378 Transcriptions of 14 selected genes known to be involved in mitochondrial metabolism,  
379 detoxification, antioxidant defenses, biotransformation process, cycle cellular arrest and  
380 apoptosis, and DNA damage repair were investigated. The results of the gene expression level  
381 analysis are shown in Table 4. In the laboratory exposure study, only 5 genes were found to  
382 be downregulated. Genes 12S, *cyp1a*, and *gadd45* showed comparable levels of  
383 downregulation in oyster larvae after exposure to the two lowest (environmental)  
384 concentrations of the MIX. In addition, one of the metallothionein genes (*mt2*) and *bax* gene  
385 were downregulated in oyster larvae after exposure to 8  $\mu\text{g/L}$  of the MIX. *In situ* approach  
386 revealed more affected genes than the laboratory approach and enabled a comparison between  
387 the three sites. No differences between the sites were observed at the level of *bax*, *casp3*,  
388 *cyp1a*, *gadd45*, *p53*, and *sodCu*. Importantly, both C and J sites had highly induced *cat* and  
389 downregulated *gpx* and *mt2* compared to the site GB.

390

391 **Table 4** Gene expressions in the oyster larvae a) exposed in the laboratory for 42 h to three  
 392 low, environmental concentrations of pesticide mixture (for individual concentrations cf.  
 393 Table 1) and b) caged for two days at the three sampling sites. Results are shown as fold  
 394 changes between target and housekeeping genes for a) MIX -laboratory approach or as the  
 395 ratio between target and GB site for b) *In situ* approach. Results are presented as the mean  
 396 ( $\pm$  SD) of 3 (pesticide mixture) and 4 (*in situ*) independent experiments. Statistically different  
 397 results are highlighted in bold. \*P < 0.05; \*\*P < 0.01; \*\*\*P < 0.001. Downregulation: fold  
 398 changes < 1; upregulation: fold changes > 1. Statistical differences obtained by nonparametric  
 399 Kruskal-Wallis except for *cat* and *sodMn*, where normality and homoscedasticity conditions  
 400 were met and thus ANOVA was used.

|               | a) MIX – laboratory approach       |                                    |                                   | b) <i>In situ</i> approach         |                                    |
|---------------|------------------------------------|------------------------------------|-----------------------------------|------------------------------------|------------------------------------|
|               | 0.32 $\mu$ g/L                     | 1.6 $\mu$ g/L                      | 8 $\mu$ g/L                       | J:GB                               | C:GB                               |
| 12S           | <b>0.7 <math>\pm</math> 0.2**</b>  | <b>0.9 <math>\pm</math> 0.1*</b>   | 0.9 $\pm$ 0.2                     | 1.2 $\pm$ 0.5                      | <b>1.3 <math>\pm</math> 0.2**</b>  |
| <i>bax</i>    | 1.0 $\pm$ 0.1                      | 1.0 $\pm$ 0.3                      | <b>0.8 <math>\pm</math> 0.0**</b> | 1.0 $\pm$ 0.1                      | 1.0 $\pm$ 0.1                      |
| <i>casp3</i>  | 0.9 $\pm$ 0.1                      | 1.0 $\pm$ 0.1                      | 0.9 $\pm$ 0.1                     | 0.9 $\pm$ 0.3                      | 1.1 $\pm$ 0.1                      |
| <i>cat</i>    | 1.0 $\pm$ 0.5                      | 0.7 $\pm$ 0.2                      | 1.3 $\pm$ 0.5                     | <b>3.4 <math>\pm</math> 1.1***</b> | <b>2.7 <math>\pm</math> 1.2***</b> |
| <i>cox1</i>   | 1.5 $\pm$ 0.5                      | 1.0 $\pm$ 0.3                      | 1.1 $\pm$ 0.2                     | <b>1.3 <math>\pm</math> 0.3**</b>  | 1.2 $\pm$ 0.4                      |
| <i>cyp1a</i>  | <b>0.7 <math>\pm</math> 0.2***</b> | <b>0.8 <math>\pm</math> 0.2***</b> | <b>0.9 <math>\pm</math> 0.1*</b>  | 0.9 $\pm$ 0.5                      | 0.8 $\pm$ 0.2                      |
| <i>gadd45</i> | <b>0.8 <math>\pm</math> 0.4*</b>   | <b>0.7 <math>\pm</math> 0.1***</b> | <b>0.7 <math>\pm</math> 0.2**</b> | 1.4 $\pm$ 0.8                      | 1.1 $\pm$ 0.5                      |
| <i>gpx</i>    | 1.1 $\pm$ 0.1                      | 0.8 $\pm$ 0.1                      | 1.0 $\pm$ 0.2                     | <b>0.7 <math>\pm</math> 0.1***</b> | <b>0.6 <math>\pm</math> 0.1***</b> |
| <i>mt1</i>    | 1.6 $\pm$ 1.0                      | 1.5 $\pm$ 0.7                      | 1.5 $\pm$ 1.0                     | 1.1 $\pm$ 0.7                      | <b>0.6 <math>\pm</math> 0.5***</b> |
| <i>mt2</i>    | 0.8 $\pm$ 0.4                      | 0.9 $\pm$ 0.4                      | <b>0.7 <math>\pm</math> 0.3*</b>  | <b>0.8 <math>\pm</math> 0.5*</b>   | <b>0.5 <math>\pm</math> 0.3***</b> |
| <i>p53</i>    | 1.5 $\pm$ 1.1                      | 1.8 $\pm$ 1.0                      | 1.5 $\pm$ 0.9                     | 1.0 $\pm$ 0.3                      | 1.1 $\pm$ 0.2                      |
| <i>rad51</i>  | 0.9 $\pm$ 0.2                      | 1.0 $\pm$ 0.2                      | 0.8 $\pm$ 0.2                     | <b>0.8 <math>\pm</math> 0.2*</b>   | 1.0 $\pm$ 0.1                      |
| <i>sodCu</i>  | 1.5 $\pm$ 1.0                      | 1.4 $\pm$ 0.9                      | 1.6 $\pm$ 0.5                     | 1.0 $\pm$ 0.4                      | 1.1 $\pm$ 0.3                      |
| <i>sodMn</i>  | 1.4 $\pm$ 1.0                      | 1.4 $\pm$ 0.8                      | 1.3 $\pm$ 0.5                     | 1.2 $\pm$ 0.2                      | <b>1.2 <math>\pm</math> 0.2*</b>   |

401

## 402 **4. Discussion**

403

404 GB site, as expected, presents the lowest pesticide concentrations in Arcachon Bay (except for  
405 IMI during the installation of the device). However, the differences in concentrations between  
406 the three sites were rather low (no more than 3 times higher at J or C sites in comparison with  
407 GB; cf. Table 3). Interestingly, unlike in previous years, concentrations of MOA and MESA  
408 were lower than concentrations of the parent SM or below the limit of detection. During  
409 2010-2016, metolachlor with its metabolites was responsible for 79 % of total pesticide  
410 concentration on GB site, moreover, the parent compound represented only 5 % of the total  
411 concentration of these three molecules (Tapie and Budzinski, 2018). Variations throughout  
412 the year were observed with average concentrations between 500 and 1700 ng/L (sum of the  
413 three substances) with the lowest values recorded in autumn. Nevertheless, the average  
414 detected concentration in July during 2010-2016 was approx. 800 ng/L (sum of the three  
415 molecules). However, we are not aware of any change in agricultural practices concerning S-  
416 metolachlor consumption in the last ten years. S-metolachlor was still one of the most used  
417 herbicides in France during this work and its purchase in France stayed comparable (around  
418 1.5 tons) in 2012 and 2019 (Veyrac, 2021). The difference in obtained values may be  
419 explained by different sample processing (HDPE bottles used in 2010-2016 monitoring  
420 instead of glass vials used in this study because of the hydrophobicity of propiconazole and  
421 the concentration of samples using solid-phase extraction method instead of lyophilization  
422 method used in this study) and compounds were quantified with grab sampling only giving a  
423 snap-shot of contaminants present at the exact sampling time. We suggest that it would be  
424 best to use a passive sampling method (Tapie *et al.*, 2011) to provide an accurate time  
425 integrative estimation taking into account episodic environmental changes (such as accidental  
426 spills and meteorological conditions). The coupling of bivalve caging and passive samplers  
427 was already successfully used (Turja *et al.*, 2015). Unfortunately, passive samplers like  
428 POCIS usually need at least several days of sampling (Brooks *et al.*, 2012), so their suitability  
429 for short embryo-larval oyster caging experiments may not be adequate. Although new types  
430 of passive samplers with shorter integration periods are currently being developed such as  
431 PTFE- EC POCIS for neonicotinoids like imidacloprid sampling (Noro *et al.*, 2020), they  
432 were not operational at the time of our study.

433 The effects of the pesticide mixture on the development of oyster embryo-larval stages were  
434 evaluated. Whereas no difference was observed between the three sites, even the lowest tested

435 concentration of pesticide mixture (0.32  $\mu\text{g/L}$ ) in the laboratory approach led to a higher  
436 number of malformed larvae or larvae with arrested development. The pesticide concentration  
437 measured *in situ* was lower than the lowest concentration used in the laboratory. Moreover,  
438 other unknown beneficial factors in the field may have play a role (oxygenation, ...).  
439 Concerning the developmental malformations, the LOEC for the mixture laboratory  
440 experiment was the lowest used C1 concentration. The LOECs for individual compounds  
441 were already determined *i.e.* PRO and IMI200  $\mu\text{g/L}$  in our previous study (Kuchovská *et al.*,  
442 2021), SM 10  $\text{ng/L}$  (Gamain *et al.*, 2016), and MOA and MESA 100  $\text{ng/L}$  (Mai *et al.*, 2014).  
443 Data on SM, MOA, and MESA embryotoxicity has thus been previously published but  
444 additional experiment with these three substances was repeated in this study to compare  
445 toxicity with IMI, PRO, and MIX within the same experiment: LOEC of SM, MOA, and  
446 MESA was calculated to be 100  $\text{ng/L}$  for SM and MESA and 1000  $\text{ng/L}$  for MOA based on  
447 additional experimentation (cf. Supplementary Figure S1). MOA, MESA, and SM are thus  
448 probably the main drivers behind the toxicity of the pesticide mixture. Because the  $\text{EC}_{50}$  was  
449 not achieved for some compounds, the comparison of  $\text{EC}_{30}$  indicates that the pesticides in the  
450 studied mixture acted rather through additive toxicity without synergistic or antagonistic  
451 interactions. Indeed, the  $\text{EC}_{30}$  of the mixture was calculated to be 1.44  $\text{mg/L}$  of imidacloprid  
452 and the metabolites, and 335  $\mu\text{g/L}$  of S-metolachlor and propiconazole (when divided  
453 proportionally between the compounds), while S-metolachlor, MOA, and MESA had  $\text{EC}_{30}$   
454 around 10  $\text{ng/L}$ , 100  $\text{ng/L}$ , and between 10 and 100  $\text{ng/L}$ , respectively, as visible from figures  
455 of Mai *et al.* (2014).  $\text{EC}_{30}$  of imidacloprid and propiconazole were calculated to be 70.50 and  
456 1.26  $\text{mg/L}$ , respectively (Kuchovská *et al.*, 2021). The effect of the mixture thus seemed to be  
457 slightly attenuated but insufficiently to be classified as antagonism, which is defined by a  
458 minimum two-fold difference (Cedergreen, 2014)

459 No effects were found on the swimming speed or the trajectory paths in this study even  
460 though SM (10 and 1000  $\text{ng/L}$ ) decreased the rectilinear trajectories in the study of Gamain *et al.*  
461 *al.* (2020). The rectilinear trajectory is considered as the major one at this developmental  
462 stage of the oyster larva, unlike later stages, when helical swimming is the most useful for  
463 hunting prey, finding a settlement, or avoiding predators (Maciejewski *et al.*, 2019). Results  
464 from this study are in agreement with the previous study reporting no effects on the same  
465 endpoints after exposure to IMI (up to 20  $\mu\text{g/L}$ ) and PRO (up to 2  $\mu\text{g/L}$ ) (Kuchovská *et al.*  
466 2021). To the best of our knowledge, no other behavioral studies with embryo-larval stages of  
467 bivalves exposed to pesticides have been conducted. Concerning other embryo-larval stages,

468 Rozmánková *et al.* (2020) evaluated the effects of SM and its metabolites on total distance  
469 swam in light/dark locomotor test of zebrafish (*Danio rerio*) larvae. This study reported no  
470 impact by individual substances (up to 300 µg/L) and also by the mixture of SM, MOA, and  
471 MESA (up to 30 µg/L of each substance in the mixture). However, a decrease in spontaneous  
472 tail coiling in zebrafish embryo was observed after exposure to SM (1 µg/L) and the mixture  
473 (1 µg/L of each substance). The *in situ* study showed an increase in both maximal and average  
474 swimming speeds especially at J site ( $428 \pm 63$  and  $263 \pm 34$  µm/s, respectively) in  
475 comparison with GB ( $304 \pm 49$  and  $187 \pm 43$  µm/s, respectively), but these differences were  
476 not significantly different (summarizing table can be found in Supplemental Table S3). More  
477 field campaigns should be carried out to get a complete and more representative picture. *In*  
478 *situ* results of trajectory paths showed a non-significant decrease in rectilinear paths and an  
479 increase in circular and stationary paths of larvae at J and C sites in comparison with larvae of  
480 the site GB. Moreover, a clear difference is observed between the path results from the site  
481 GB (n=4) and in laboratory non-treated controls (n=16) from all the experimentations done  
482 during this project (described in this publication and in Kuchovská *et al.* 2021),. The  
483 swimming paths proportions follows: GB: rectilinear – circular - motionless:  $55.5 \pm 12.3$  % -  
484  $29.7 \pm 12.4$  % -  $14.8 \pm 3.77$  %; laboratory controls:  $74.5 \pm 9.29$  % -  $18.5 \pm 10.9$  % -  
485  $6.91 \pm 2.39$  %, respectively. This is an interesting finding which should be 1. explored further  
486 in the future to assess if it is a usual proportion of swimming paths of larvae exposed *in situ*  
487 and 2. compared with larvae of the same age exposed in the laboratory to find out if it is a  
488 baseline locomotive activity at that age, or 3. if it is a direct effect of the water quality.  
489 Gamain *et al.* (2020) established a link between erratic larval behavior and the presence of  
490 developmental malformations. In this study, negative control containing about 17 % of  
491 abnormal larvae exhibited predominantly (around 80 %) rectilinear, normal, trajectories,  
492 whereas 100 ng/L of S-metolachlor induced around 44 % of abnormal larvae with mostly  
493 circular trajectories. In the present study, the malformation rate for larvae exposed *in situ* was  
494 comparably less than 20 %. Therefore, we can assume that the locomotion patterns of larvae  
495 exposed *in situ* may be a result of a direct effect on the metabolism and energetic reserves of  
496 the larvae or of the water quality of Arcachon Bay on the nervous system of oyster larvae.  
497 The nervous system is already developing in D-shaped (veliger) larva and is comprised of a  
498 compact apical organ with apical cells, dendrites, neurites (Yurchenko *et al.*, 2018).

499 Concerning the impacts on the molecular level, the MIX of pesticides changed expressions of  
500 several genes of oyster larvae exposed in the laboratory. The gene 12S was downregulated,

501 affecting the synthesis of proteins needed for the mitochondrial membrane and, therefore,  
502 resulting in a decreased ribosomal activity for creating ATP, which is necessary for cell  
503 viability and defense against pollutants (De Silva *et al.*, 2015). The downregulation of the 12S  
504 gene was also observed in oyster larvae after exposure to 100 ng/L of metolachlor (Mai *et al.*,  
505 2014) and 200 ng/L and 2 µg/L of propiconazole (Kuchovská *et al.*, 2021). Both observations  
506 were made in higher concentrations of individual pesticides (10 and 20 times, respectively)  
507 than those used in the MIX (cf. Table 1) suggesting thus possible synergistic effect of the  
508 MIX on the expression of the 12S gene. Cytochrome P450 *cyp1a* was strongly downregulated  
509 as well. On the contrary, exposure to individual pesticides from the MIX (up to 1 µg/L of  
510 metolachlor, MOA, and MESA, up to 2 µg/L of PRO, and up to 100 µg/L of IMI) did not  
511 induce any alterations in its expression (Kuchovská *et al.*, 2021; Mai *et al.*, 2014). Therefore,  
512 possible interaction effects may have occurred among the components of the mixture. Azoles  
513 compounds like propiconazole are known to inhibit cytochrome P450 enzymes  
514 (monooxygenases). P450 enzymes participate in the detoxification of xenobiotics (Gottardi *et*  
515 *al.*, 2018), such as neonicotinoids like IMI or acetanilides as SM, by displacing the chlorine  
516 atom by glutathione (Roberts *et al.*, 1998). This might be an important mechanism of PRO  
517 toxicity towards the Pacific oyster. Moreover, the main mechanism of action of azoles  
518 towards their target organisms is to inhibit steroidogenesis and thus obstruct the creation of  
519 fungal cell membranes. However, it is possible that this mechanism do not affect the Pacific  
520 oyster, since its sterols are likely mostly derived from its prey such as microalgae (Knauer *et*  
521 *al.*, 1998).

522 Gene *gadd45*, coding for a stress response protein that regulates growth arrest and DNA  
523 damage repair, was downregulated in oyster larvae in all laboratory conditions starting with  
524 exposure to 0.32 µg/L of MIX. Interestingly, this gene was reported to be upregulated after  
525 exposure to 1 µg/L of IMI contrary to the finding of this study, whilst exposure to PRO did  
526 not affect this gene (Kuchovská *et al.*, 2021). To the best of our knowledge, the effect of  
527 metolachlor and its two metabolites on the expression of *gadd45* is not known, as well as any  
528 other chloroacetanilide herbicide. The observed downregulation of *gadd45* hinders correct  
529 regulation of the cell cycle and suggests possible effects on the DNA integrity (E. Tamura *et*  
530 *al.*, 2012). Moreover, the highest tested concentration of MIX (8 µg/L) downregulated the  
531 expressions of *bax* and *mt2*. *Bax* downregulation corroborates the suspected anti-apoptotic  
532 effects of the MIX as suggested also by the downregulation of *gadd45*. The downregulation of  
533 *mt2*, a gene coding for a multifunctional protein regulating metal homeostasis but also ROS

534 (reactive oxygen species) scavenging (Migliaccio *et al.*, 2020), after exposure to the MIX is in  
535 concordance with the downregulation of the same gene after exposure to 1 µg/L of MESA  
536 (Mai *et al.*, 2014). However, the exposure to high concentrations (10 and 100 µg/L) of IMI  
537 caused the opposite effect (Kuchovská *et al.*, 2021). Interestingly, no alteration of expression  
538 of genes linked to ROS content regulation was observed, whilst the effects of individual  
539 substances of the MIX caused contradictory results (Kuchovská *et al.*, 2021; Mai *et al.*, 2014).

540 It should be highlighted, that the MIX exposure in the laboratory was only a partial  
541 representation of the complex situation in Arcachon Bay. Although selected pesticides are the  
542 most representative ones of their respective classes (herbicides, insecticides, fungicides), the  
543 actual environmental pollution of Arcachon Bay is likely more complex including not only  
544 other pesticides but also other pollutants as pharmaceuticals, UV filters, trace metals,  
545 organotins, etc. (Besse *et al.*, 2019). Consequently, we will not discuss the situation *in situ*  
546 compared to the laboratory but only the differences between the three sites. The strongest  
547 effect was seen on the *cat* gene, coding for catalase, an enzyme located in cytoplasm and  
548 peroxisomes and catalyzing the decomposition of H<sub>2</sub>O<sub>2</sub> into water and oxygen. Both sites (J  
549 and C) highly induced its expression in comparison with the site GB, thus demonstrating a  
550 possible induction of defense mechanism against ROS (Gebicka and Krych-Madej, 2019).  
551 This finding agrees with the induction of *sodMn* (coding for manganese superoxide  
552 dismutase), another gene linked to the stress defense, at C site. On the other hand,  
553 downregulation of another ROS linked gene, *gpx* (coding for glutathione peroxidase), was  
554 observed at both sites. This suggests that whilst some antioxidant activities may protect cells  
555 against increased ROS production, the mitochondria may be more susceptible to the adverse  
556 effects along with the *gpx* suppression and absence of catalase in mitochondria (Ighodaro and  
557 Akinloye, 2018). Metallothionein gene expression (*mt1*, *mt2*) was strongly downregulated at  
558 both sites (J and C) in comparison with GB site. The downregulation of *mt1*, *mt2* (along with  
559 *gpx*) generally show lower scavenging and protective capacity of the cells in oysters exposed  
560 in sites J and C (Ruttikay-Nedecky *et al.*, 2013; Takahashi, 2015). Genes *cox1* (upregulated at  
561 J site) and 12S (upregulated at C site) are both linked to mitochondrial metabolism. *Cox1*,  
562 coding for the enzyme cytochrome c oxidase subunit 1, upregulation suggests increased  
563 creation of ATP probably to effectively defend the organism against pollutants (Kadenbach,  
564 2018), whilst upregulation of 12S indicates increased ribosomal activity in mitochondria or  
565 increased number of mitochondria which ultimately also leads to increased energy production  
566 (De Silva *et al.*, 2015). Gene *rad51*, coding for a protein involved in DNA repair, was

567 downregulated at J site, suggesting impairment of the DNA repair process (Laurini *et al.*,  
568 2020). Altogether, several lines of evidence from gene expression analyses indicate that both  
569 C and J sites showed a worse state of oyster larvae. This can be linked to their location in the  
570 inner part of the bay and the proximity of the C site with river Leyre, which is the main  
571 entrance of the herbicides into Arcachon Bay.

572

## 573 **5. Conclusion**

574 The present work successfully demonstrated the added value of joining two complementary  
575 approaches, *i.e.* laboratory and *in situ* to evaluate the sublethal impact of water pollution on  
576 Pacific oyster larvae (*Magallana gigas*). Early life stages were exposed either in the  
577 laboratory to environmentally relevant reconstituted pesticide mixture or transplanted *in situ*  
578 in caging devices and exposed in Arcachon Bay. Whereas the laboratory mixture, composed  
579 of 5 pesticides, caused developmental toxicity already at the environmental concentration of  
580 0.32 µg/L (corresponding to current concentration in Arcachon Bay in France), it did not  
581 impair larval swimming. Several effects caused by environmental concentrations of MIX  
582 were found at the molecular level such as downregulation of genes linked to mitochondrial  
583 metabolism, biotransformation, growth arrest, and DNA damage repair. Moreover, higher  
584 concentration also downregulated genes linked to apoptosis and metal regulation. *In situ*  
585 approach allowed to a comparison of three different sites in Arcachon bay: Grand Banc,  
586 Comprian, and Les Jacquets, each of them under different contamination pressure. No  
587 differences in larvae swimming or development were observed between sites, however, gene  
588 expression analysis revealed a worsened state at both sites Comprian and Les Jacquets in  
589 comparison with Grand Banc. At these sites, we have observed possible elevation of oxidative  
590 stress, downregulated metallothionein function, and enhanced mitochondrial metabolism.  
591 However, the obtained *in situ* results do not explain the issues with oyster development  
592 observed in Arcachon Bay in recent years and further investigation should be carried out. This  
593 is the first study that successfully complemented laboratory tests with *in situ* approach, and  
594 for the first time, it investigated the swimming of oyster larvae after *in situ* caging exposures.

595

596 **CRedit authorship contribution statement**

597 **Eliška Kuchovská:** Conceptualization, Investigation, Validation, Data curation, Formal  
598 analysis, Writing - original draft, Funding acquisition. **Patrice Gonzalez:** Supervision,  
599 Funding acquisition, Conceptualization, Writing - review & editing. **Lucie Bláhová:**  
600 Investigation, Methodology, Validation, Writing - review & editing. **Mathilde Barré:**  
601 Investigation. **Corentin Gouffier:** Investigation. **Jérôme Cachot:** Conceptualization, Writing  
602 - review & editing. **Alicia Romero-Ramirez:** Software, Writing - review & editing. **Luděk**  
603 **Bláha:** Writing - review & editing, Funding acquisition. **Bénédicte Morin:** Supervision,  
604 Funding acquisition, Conceptualization, Writing - review & editing.

605

606 **Compliance with ethical standards**

607 This work was done in compliance with the Publishing Ethics policy of Elsevier.

608

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621

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846 [web/download/publication/publie/Chd2104/cd2021-4\\_VentePPP.pdf](https://agreste.agriculture.gouv.fr/agreste-web/download/publication/publie/Chd2104/cd2021-4_VentePPP.pdf)
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- 850

851

## Supplementary Materials

852

853 **Supplementary Table S1:**

854 Concentrations of pesticides of interest detected on multiple sites in Arcachon Bay in the  
 855 years 2010-2014. N=669 for each pesticide. Concentrations were calculated according to the  
 856 data of Tapie *et al.*, (2018).

857

|  | <b>PRO</b>  | <b>IMI</b>   | <b>Metolachlor</b> | <b>MOA</b>    | <b>MESA</b>   |
|--|-------------|--------------|--------------------|---------------|---------------|
| Limit of quantification (ng/L)                                     | 1           | 1            | 0.5                | 3             | 2             |
| Samples with detected substance (%)                                | 20.7        | 34.8         | 90.3               | 87.6          | 88.0          |
| Average concentration in all samples<br>(ng/L)                     | 0.7         | 2.6          | 31.9               | 163.5         | 117.6         |
| Average concentration in samples with<br>detected substance (ng/L) | 3.1         | 7.6          | 35.4               | 186.9         | 133.8         |
| Maximal concentration (ng/L)                                       | <b>29.1</b> | <b>173.6</b> | <b>1695.9</b>      | <b>1609.9</b> | <b>1059.2</b> |

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860

861 **Supplementary material S1:**

862 **LC-MS/MS analysis of pesticides**

863 The analysis was performed with a Waters LC chromatograph (Waters, Manchester, U.K.)  
864 and the chromatographic separation was achieved using a column Acquity BEH C18 of 100 x  
865 2.1 mm ID and 1.7  $\mu\text{m}$  particle size. A gradient elution method was set with phase A (0.1 %  
866 formic acid in water) and phase B (0.1 % formic acid in acetonitrile). The gradient elution  
867 started with 20 % B, increased to 90 % B over 9 min, followed by 3 min of washing (100 %  
868 B, then decreased to 20 % B and held for 4 min to equilibrate the system before the next  
869 injection. The flow rate was set at 0.3 mL/min and the injection volume of samples in 20 % of  
870 acetonitrile at 8  $\mu\text{L}$ . The column and sample temperatures were set at 35°C and 10°C,  
871 respectively.

872 Detection was performed on a Xevo TQ-S quadrupole mass spectrometer (Waters  
873 Manchester, U.K.) and analytes after ESI ionisation were detected in positive ion mode using  
874 tandem mass spectrometry. The following  $m/z$  transitions were monitored, for imidacloprid:  
875  $m/z$  256.1 > 175.0, (quant.: cone voltage 25V; collision energy 20V) and  $m/z$  256.1 > 209.0  
876 (qual.: cone voltage 25V; collision energy 12 V); for propiconazole:  $m/z$  342.1 > 159.0  
877 (quant.: cone voltage 42 V; collision energy 30 V) and  $m/z$  342.1 > 69.0 (qual.: cone voltage  
878 42 V; collision energy 15 V); for imidacloprid D4:  $m/z$  260.1 > 213.0 (quant.: cone voltage  
879 25 V; collision energy 12 V); for tebuconazole D6:  $m/z$  314.3 > 72.1 (quant.: cone voltage 35  
880 V; collision energy 20 V); for S-metolachlor:  $m/z$  284.1 > 176.1, (quant.: cone voltage 30V;  
881 collision energy 25V) and  $m/z$  284.1 > 252.1 (qual.: cone voltage 30V; collision energy 15 V);  
882 for metolachlor OA:  $m/z$  280.3 > 146.2, (quant.: cone voltage 20V; collision energy 25V);  $m/z$   
883 280.3 > 119.0 (qual.: cone voltage 20V; collision energy 30 V) and  $m/z$  280.3 > 131.0 (qual.:  
884 cone voltage 20V; collision energy 30 V); for metolachlor ESA:  $m/z$  330.4 > 160.2, (quant.:

885 cone voltage 25V; collision energy 30V);  $m/z$  330.4 > 132.1 (qual.: cone voltage 25V;  
886 collision energy 50 V );  $m/z$  330.4 > 145.5 (qual.: cone voltage 25V; collision energy 50 V)  
887 and  $m/z$  330.4 > 174.1 (qual.: cone voltage 25V; collision energy 35 V). The capillary voltage  
888 was set at 3 kV and the cone, desolvation and collision gas flows were set at 150 (L/h), 700  
889 (L/h) and 0.15 (mL/min), respectively. The source and desolvation temperature were set at  
890 150°C and 400°C, respectively. Data were processed by MassLynx™ software (Manchester,  
891 U.K.). The limit of detection (LOD, signal to noise ratio S/N>3) for imidacloprid,  
892 propiconazole, S-metolachlor, metolachlor OA, and metolachlor ESA were 0.02, 0.005,  
893 0.005, 0.05, and 0.05 µg/L, respectively. The limit of quantification (LOQ, signal to noise  
894 ratio S/N>10) for imidacloprid, propiconazole, S-metolachlor, metolachlor OA, and  
895 metolachlor ESA were 0.05, 0.01, 0.01, 0.1 and 0.1 µg/L, respectively. The analytes were  
896 quantified using external calibration (0.01 – 50 µg/L in 20 % of acetonitrile) and normalized  
897 with internal deuterium labelled standards (imidacloprid D4 for imidacloprid, MOA and  
898 MESA and tebuconazole D6 for SM and propiconazole).

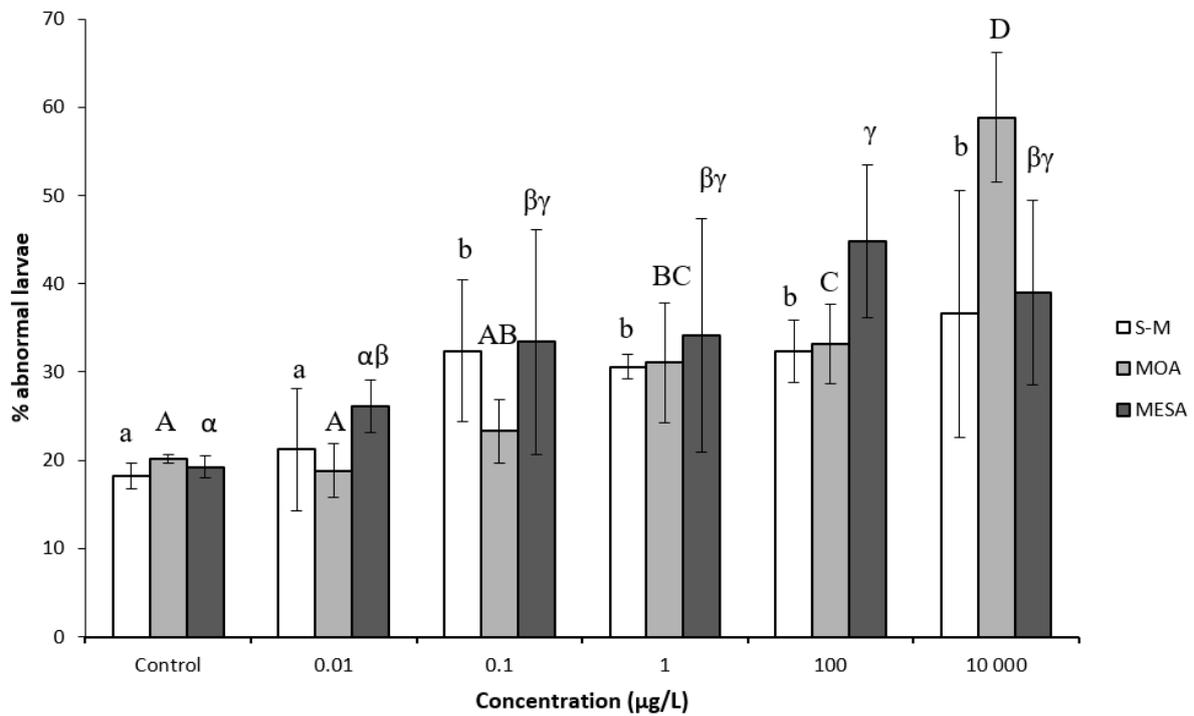
899

900 **Supplementary Figure S1:**

901 Sum of abnormal oyster larvae (arrested development and different malformation types) after  
902 30 hours of exposure to increasing concentrations of SM, MOA, and MESA. Different letters  
903 indicate statistical differences between variables ( $P < 0.05$ ). Results are presented as the mean  
904 of 3 independent experiments  $\pm$  SD.

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908

909 **Supplementary Table S2:**

910 Swimming speed ( $\mu\text{m/s}$ ) observed in oyster larvae after 2 days of transplantation at three  
911 different sites in Arcachon Bay. Results are presented as the mean of 4 independent  
912 experiments  $\pm$  SD.

913

| <i>Sampling site</i> | <i>Speed (<math>\mu\text{m/s}</math>)</i> |                                |
|----------------------|---|--------------------------------|
|                      | <i>maximal</i>                            | <i>average</i>                 |
| <i>Grand Banc</i>    | <b><math>304 \pm 49</math></b>            | <b><math>187 \pm 43</math></b> |
| <i>Les Jacquets</i>  | <b><math>428 \pm 63</math></b>            | <b><math>263 \pm 34</math></b> |
| <i>Comprian</i>      | <b><math>341 \pm 74</math></b>            | <b><math>204 \pm 43</math></b> |

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