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Eliška Kuchovská, Patrice Gonzalez, Lucie Bláhová, Mathilde Barré, Corentin Gouffier, et al.. Pesticide mixture toxicity assessment through in situ and laboratory approaches using embryo-larval stages of the pacific oyster (*Magallana gigas*). *Marine Environmental Research*, 2021, 169, pp.105390. 10.1016/j.marenvres.2021.105390 . hal-03434109

HAL Id: hal-03434109

<https://hal.science/hal-03434109>

Submitted on 25 Nov 2021

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

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Abstract

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

KEYWORDS

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

HIGHLIGHTS

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

Graphical abstract

1. Introduction

Arcachon Bay is a semi-sheltered shallow lagoon (174 km²) on the Atlantic coastline in the South-west of France (Bertrand, 2014). The Pacific oyster (*Magallana gigas*, also known as *Crassostrea gigas*), an invasive species on the European coast introduced from the northwest Pacific Ocean (Troost, 2010), has become an emblematic and farmed species in Arcachon Bay. Several issues such as the decline in oyster recruitment and spat collection (Auby *et al.*, 2014), decrease in the seagrass *Zostera* population (Auby *et al.*, 2011; Cognat *et al.*, 2018), and anomalies in the production of phytoplankton (Auby and Maurer, 2004) have been observed in Arcachon Bay during the last two decades. Possible causes of the oyster recruitment decline have been investigated, including the incidence of oyster herpes virus (Labbate *et al.*, 2015) and the coupled effect of climate change and pesticide pollution (Gamain, 2016). However, the described issues have not been resolved and other causes are being searched for. Numerous hazardous substances are regularly detected in the lagoon by REMPARG, the survey network for monitoring of pesticides and micropollutants (<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 Leyre (Tapie and Budzinski, 2018). While the impacts of individual pesticide compounds on non-target organisms are usually studied, we still lack information about the toxicity of environmentally relevant mixtures as also emphasized in the European Green Deal (European Commission, 2020). Mixtures can result in synergistic, antagonistic, or additive effects, as documented by Gustavsson *et al.* (2017) and Cedergreen (2014). For instance, neonicotinoids are known to exert addition or synergy effects when occurring in mixtures with some fungicides (Morrissey *et al.*, 2015).

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

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

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

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

2. Materials and methods

2.1 Chemicals and reference seawater handling

Imidacloprid (IMI, CAS 138261-41-3, Pestanal, purity 100 %), propiconazole (PRO, CAS 60207-90-1, Pestanal, purity 100 %), S-metolachlor (SM, CAS 87392-12-9, Pestanal, purity ≥ 98.0 %), metolachlor oxanilic acid (MOA, CAS 152019-73-3, Pestanal, purity ≥ 98.0 %), metolachlor ethanesulfonic acid (MESA, CAS 947601-85-6, Pestanal, purity ≥ 95.0 %), and CuSO_4 were purchased from Sigma-Aldrich. Concerning gene expression analysis, primers were purchased from Sigma proligo, RNA later buffer was purchased from Qiagen, and phenol and chloroform Rectapur® were purchased from Sigma. Copper solution (stock solution of 100 mg/L in milliQ water) was used as a positive control in laboratory experiments and was stored at 5 °C, as well as PRO solution, which was prepared in DMSO (5 g/L) and other pesticide solutions (SM, IMI, MOA, MESA) which were prepared in milliQ water (50 mg/L). Dilutions in final exposure solutions were carried out using reference seawater collected at beach Petit Nice (approx. 44°33'40.3"N 1°14'27.1"W). Seawater was transported in 10 L plastic containers, filtered at 0.22 μm , and passed through UV light to eliminate microorganisms. Filtered seawater (FSW) was stored at 5 °C in the dark and was used usually within a few days. Few hours before the experimentations, FSW was filtered again at 0.22 μm . The background concentration of pesticides in FSW was measured by LC-MS/MS (cf. section 2.6).

2.2 Embryo-larval test: a laboratory approach

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

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

Table 1 Concentrations of different pesticides in the reconstituted mixture used to expose 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

The lowest used concentration (C1) is reflecting the usual concentrations often measured in Arcachon Bay in France (cf. Supplementary Table S1). Concentration C2 approximately corresponds to the highest concentrations measured in Arcachon Bay. The treatments C3 and C4 may be considered as maximal environmental concentrations measured in the surface waters in the world (Table 2). Higher concentrations (C5-C8) were used to allow for estimation of the EC₅₀ or as a prediction of acute peak contamination periods.

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

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

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

norm (NF ISO 17244, 2015), the malformation rate in the negative control cannot exceed 20 % and the EC₅₀ for abnormal larvae exposed to Cu⁺⁺ should be between 6 and 16 µg/L.

2.3 *In situ* approach

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

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

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

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

2.4 Locomotion analysis

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

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

2.5 Gene expression analysis

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

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

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

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

2.6 Chemical analysis and water quality

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

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

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

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

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

2.7 Data analysis

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

All pre-treated data were then compared using Statistica 13.3 (StatSoft, USA). Data were controlled for normality (Shapiro-Wilk test; $P > 0.01$) and homoscedasticity (Levene test; $P > 0.05$). If confirmed, ANOVA ($P < 0.05$) followed by Tukey post-hoc test was used. In the other case, a non-parametric Kruskal-Wallis ($P < 0.05$) with Mann-Whitney post-hoc test was carried out. EC₅₀ was calculated using nonlinear logarithmic regression of the nominal concentration-response curves, using Graph Pad Prism 5 (Graph Pad Software, USA).

3. Results

3.1 Chemical analysis

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

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

Site	Sampling 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

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

3.2 Embryo-larval development and observed malformations

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

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

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

3.3 Behavioral analysis

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

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

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

3.4 Gene expression analysis

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

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

	a) MIX – laboratory approach			b) <i>In situ</i> approach	
	0.32 μ g/L	1.6 μ g/L	8 μ g/L	J:GB	C:GB
12S	0.7 $\pm$ 0.2**	0.9 $\pm$ 0.1*	0.9 $\pm$ 0.2	1.2 $\pm$ 0.5	1.3 $\pm$ 0.2**
<i>bax</i>	1.0 $\pm$ 0.1	1.0 $\pm$ 0.3	0.8 $\pm$ 0.0**	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	3.4 $\pm$ 1.1***	2.7 $\pm$ 1.2***
<i>cox1</i>	1.5 $\pm$ 0.5	1.0 $\pm$ 0.3	1.1 $\pm$ 0.2	1.3 $\pm$ 0.3**	1.2 $\pm$ 0.4
<i>cyp1a</i>	0.7 $\pm$ 0.2***	0.8 $\pm$ 0.2***	0.9 $\pm$ 0.1*	0.9 $\pm$ 0.5	0.8 $\pm$ 0.2
<i>gadd45</i>	0.8 $\pm$ 0.4*	0.7 $\pm$ 0.1***	0.7 $\pm$ 0.2**	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	0.7 $\pm$ 0.1***	0.6 $\pm$ 0.1***
<i>mt1</i>	1.6 $\pm$ 1.0	1.5 $\pm$ 0.7	1.5 $\pm$ 1.0	1.1 $\pm$ 0.7	0.6 $\pm$ 0.5***
<i>mt2</i>	0.8 $\pm$ 0.4	0.9 $\pm$ 0.4	0.7 $\pm$ 0.3*	0.8 $\pm$ 0.5*	0.5 $\pm$ 0.3***
<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	0.8 $\pm$ 0.2*	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	1.2 $\pm$ 0.2*

4. Discussion

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

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

concentration of pesticide mixture (0.32 µg/L) in the laboratory approach led to a higher number of malformed larvae or larvae with arrested development. The pesticide concentration measured *in situ* was lower than the lowest concentration used in the laboratory. Moreover, other unknown beneficial factors in the field may have played a role (oxygenation, ...). Concerning the developmental malformations, the LOEC for the mixture laboratory experiment was the lowest used C1 concentration. The LOECs for individual compounds were already determined *i.e.* PRO and IMI 200 µg/L in our previous study (Kuchovská *et al.*, 2021), SM 10 ng/L (Gamain *et al.*, 2016), and MOA and MESA 100 ng/L (Mai *et al.*, 2014). Data on SM, MOA, and MESA embryotoxicity has thus been previously published but additional experiment with these three substances was repeated in this study to compare toxicity with IMI, PRO, and MIX within the same experiment: LOEC of SM, MOA, and MESA was calculated to be 100 ng/L for SM and MESA and 1000 ng/L for MOA based on additional experimentation (cf. Supplementary Figure S1). MOA, MESA, and SM are thus probably the main drivers behind the toxicity of the pesticide mixture. Because the EC₅₀ was not achieved for some compounds, the comparison of EC₃₀ indicates that the pesticides in the studied mixture acted rather through additive toxicity without synergistic or antagonistic interactions. Indeed, the EC₃₀ of the mixture was calculated to be 1.44 mg/L of imidacloprid and the metabolites, and 335 µg/L of S-metolachlor and propiconazole (when divided proportionally between the compounds), while S-metolachlor, MOA, and MESA had EC₃₀ around 10 ng/L, 100 ng/L, and between 10 and 100 ng/L, respectively, as visible from figures of Mai *et al.* (2014). EC₃₀ of imidacloprid and propiconazole were calculated to be 70.50 and 1.26 mg/L, respectively (Kuchovská *et al.*, 2021). The effect of the mixture thus seemed to be slightly attenuated but insufficiently to be classified as antagonism, which is defined by a minimum two-fold difference (Cedergreen, 2014)

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

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

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

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

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

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

It should be highlighted, that the MIX exposure in the laboratory was only a partial representation of the complex situation in Arcachon Bay. Although selected pesticides are the most representative ones of their respective classes (herbicides, insecticides, fungicides), the actual environmental pollution of Arcachon Bay is likely more complex including not only other pesticides but also other pollutants as pharmaceuticals, UV filters, trace metals, organotins, etc. (Besse *et al.*, 2019). Consequently, we will not discuss the situation *in situ* compared to the laboratory but only the differences between the three sites. The strongest effect was seen on the *cat* gene, coding for catalase, an enzyme located in cytoplasm and peroxisomes and catalyzing the decomposition of H₂O₂ into water and oxygen. Both sites (J and C) highly induced its expression in comparison with the site GB, thus demonstrating a possible induction of defense mechanism against ROS (Gebicka and Krych-Madej, 2019). This finding agrees with the induction of *sodMn* (coding for manganese superoxide dismutase), another gene linked to the stress defense, at C site. On the other hand, downregulation of another ROS linked gene, *gpx* (coding for glutathione peroxidase), was observed at both sites. This suggests that whilst some antioxidant activities may protect cells against increased ROS production, the mitochondria may be more susceptible to the adverse effects along with the *gpx* suppression and absence of catalase in mitochondria (Ighodaro and Akinloye, 2018). Metallothionein gene expression (*mt1*, *mt2*) was strongly downregulated at both sites (J and C) in comparison with GB site. The downregulation of *mt1*, *mt2* (along with *gpx*) generally show lower scavenging and protective capacity of the cells in oysters exposed in sites J and C (Ruttikay-Nedecky *et al.*, 2013; Takahashi, 2015). Genes *cox1* (upregulated at J site) and 12S (upregulated at C site) are both linked to mitochondrial metabolism. *Cox1*, coding for the enzyme cytochrome c oxidase subunit 1, upregulation suggests increased creation of ATP probably to effectively defend the organism against pollutants (Kadenbach, 2018), whilst upregulation of 12S indicates increased ribosomal activity in mitochondria or increased number of mitochondria which ultimately also leads to increased energy production (De Silva *et al.*, 2015). Gene *rad51*, coding for a protein involved in DNA repair, was

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

5. Conclusion

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

CRedit authorship contribution statement

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

Compliance with ethical standards

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

Acknowledgments

The authors would like to thank especially the sailor Stéphane Bujan for his assistance during the fieldwork in Arcachon Bay. We also thank Christelle Clérandeau for technical assistance with seawater handling, Guillemine Daffe for her advice in the PCR laboratory, and Alexandra Coynel and Pierre-Yves Gourves for copper chemical analyses. This research was supported by funding of Campus France (doctoral scholarship), the research infrastructure project from the Czech Ministry of Education (grant number LM2018121), and the Inter-municipal Union of Arcachon Bay (SIBA).

Funding

This research was supported by funding of Campus France (doctoral scholarship), the research infrastructure project from the Czech Ministry of Education (grant number LM2018121), and the Inter-municipal Union of Arcachon Bay (SIBA).

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Supplementary Materials

Supplementary Table S1:

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

	PRO	IMI	Metolachlor	MOA	MESA
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 (ng/L)	0.7	2.6	31.9	163.5	117.6
Average concentration in samples with detected substance (ng/L)	3.1	7.6	35.4	186.9	133.8
Maximal concentration (ng/L)	29.1	173.6	1695.9	1609.9	1059.2

Supplementary material S1:

LC-MS/MS analysis of pesticides

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

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

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Supplementary Figure S1:

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

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 ($\mu\text{m/s}$)</i>	
	<i>maximal</i>	<i>average</i>
<i>Grand Banc</i>	304 ± 49	187 ± 43
<i>Les Jacquets</i>	428 ± 63	263 ± 34
<i>Comprian</i>	341 ± 74	204 ± 43

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