Comparison of imidacloprid, propiconazole, and nanopropiconazole effects on the development, behavior, and gene expression biomarkers of the Pacific oyster (Magallana gigas)

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KEYWORDS

Embryotoxicity; gene expression; pacific oyster; pesticide; sublethal effect; swimming behavior.

HIGHLIGHTS

- Pesticide toxicity on the early-life stages of Pacific oyster was studied
- Development, behavior, and gene expression impacts were assessed
- Imidacloprid caused major changes in the gene expression
- Propiconazole had similar developmental toxicity compared to its nanoformulation
- Studied pesticides' concentrations in Arcachon Bay are safe for larval development

Abstract

Coastal areas are final recipients of various contaminants including pesticides. The effects of pesticides on non-target organisms are often unclear, especially at environmentally relevant

concentrations. This study investigated the impacts of insecticide imidacloprid (IMI) and fungicide propiconazole (PRO), some of the most detected pesticides in the Arcachon Bay in France. This work also included the research of propiconazole nanoformulation (nanoPRO). The effects were assessed studying the development of the early life stages of the Pacific oyster (*Magallana gigas*). Oyster embryos were exposed for 24, 30, and 42 h (depending on the endpoint) at 24 °C to environmentally relevant concentrations of the two pesticides as well as to nanoPRO. The research focused on sublethal endpoints such as the presence of developmental malformations, alterations of locomotion patterns, or changes in the gene expression levels. No developmental abnormalities were observed after exposure to environmental concentrations detected in the Arcachon Bay in recent years (maximal detected concentration of IMI and PRO were 174 ng/L and 29 ng/L, respectively). EC_{50} of PRO and nanoPRO were comparable, 2.93 ± 1.35 and 2.26 ± 1.36 mg/L, while EC_{50} of IMI exceeded 200 mg/L. IMI did not affect larval behavior. PRO affected larval movement trajectory and decreased average larvae swimming speed (2 µg/L), while nanoPRO increased the maximal larvae swimming speed (0.02 µg/L). PRO upregulated especially genes linked to reactive oxygen species (ROS) production and detoxification. NanoPRO effects on gene expression were less pronounced - half of the genes were altered in comparison with PRO. IMI induced a strong dose-response impact on the genes linked to the detoxification, ROS production, cell cycle, and apoptosis regulation. In conclusion, our results suggest that current pesticide concentrations detected in the Arcachon Bay are safe for the Pacific oyster early development, but they might have a small direct effect via altered gene expressions, whose longer-term impacts cannot be ruled out.

1. Introduction

Coastal waters and estuarine areas that face growing anthropogenic pressure are among the most vulnerable aquatic ecosystems. Indeed, half of the world's population resides in coastal zones located within 60 km of the ocean (UNEP, 2016). Water ecosystems in these areas are constantly affected by pollution and are a final recipient of different chemical compounds, including pesticides (Granek et al., 2016), which influence water quality and may adversely affect non-target organisms living in these ecosystems.

Arcachon Bay is an example of such an ecosystem. It is a macrotidal semi-enclosed marine lagoon on the Atlantic Coast in the South-west of France. Its emblematic organism is the Pacific oyster, *Magallana gigas* also known as *Crassostrea gigas* due to the ongoing disagreement about its name (Bayne et al., 2017), a bivalve mollusk, commercially valuable aquaculture species, and a model organism in marine/brackish ecotoxicology. Its embryo-larval stages are commonly used according to the standardized biotest (NF ISO 17244, 2015). The oyster early-life stages are a sensitive and reliable alternative toxicity model with multiple advantages such as transparency of embryo and larvae, quick development, high-throughput screening format, sensitivity to contaminants, internal feeding until the D-larvae stage (Capela et al., 2020), and it complies with the 3Rs Principle (Russell et al., 1959).

Numerous hazardous substances are regularly detected in Arcachon Bay, such as pesticides monitored by survey network REPAR (https://www.siba-bassin-arcachon.fr/actions-environnementales/les-reseaux-de-surveillance-repar-et-rempar). Pesticides are known for causing adverse effects on non-target estuarine/marine aquatic organisms (Gutiérrez et al., 2019; Parsons et al., 2020; Vignet et al., 2019), at environmentally relevant concentrations (Bechmann et al., 2020; Behrens et al., 2016; Epelboin et al., 2015; Gamain et al., 2018; Mai

et al., 2014, 2013). The REPAR monitoring network showed that the insecticide, fungicide, and herbicide with the highest detected average concentration (cf. Table 1) during years 2010-2014 in the Arcachon Bay were imidacloprid, propiconazole (as well as other fungicides like carbendazim and metabolites of dichlofluanid), and herbicide S-metolachlor (Tapie and Budzinski, 2018). The toxicity of S-metolachlor to oyster larvae has already been assessed (Gamain et al., 2017, 2016; Mai et al., 2014, 2013). Imidacloprid (IMI) is a neurotoxic insecticide of the neonicotinoid family which binds agonistically to the post-synaptic nicotinic acetylcholine receptors (nAChRs) and is highly selective for insects (Matsuda et al., 2001). Its use has been banned since 2018 by regulation of the EU Commission (European Commission, 2018) except the use in permanent greenhouses; due to the risks to honey bees and other pollinators. However, it is still widely used in other countries in the world (Butcherine et al., 2019). IMI is one of the most detected insecticides in waters usually in a range of hundreds of ng/L (Anderson et al., 2015; Morrissey et al., 2015), which is also the case of Arcachon Bay in France (Tapie and Budzinski, 2018). However, the peak concentrations of imidacloprid in waters might be high as 320 µg/L in the Netherlands (Van Dijk et al., 2013), 3.29 µg/L in California (Starner and Goh, 2012), or 0.26 µg/L in Canada (Main et al., 2014), Several studies reported toxicity to mollusks, but the effects were not evaluated at environmentally relevant conditions corresponding to the Arcachon Bay in France and only high concentrations were used (Dondero et al., 2010; Ewere et al., 2020, 2019a, 2019b; Prosser et al., 2016; Shan et al., 2020). Propiconazole (PRO), a triazole fungicide, stops the fungal growth as it inhibits the demethylation by fungal sterol 14α-demethylase and thus obstructs the biosynthesis of ergosterol, a component of fungal cell membranes (Zarn et al., 2003). Its occurrence in surface waters all around the world in a range of ng/L to µg/L is well documented (Elfikrie et al., 2020; Papadakis et al., 2018; Quintana et al., 2019; Toan et al., 2013; Van De Steene et al., 2010) with peak concentrations going up to 0.81 µg/L in China

(Peng et al., 2018). Studies on the effects of propiconazole on mollusks are even scarcer (Bringolf et al., 2007; Gottardi et al., 2018).

IMI and PRO, as well as other pesticides, are known to be toxic to non-target aquatic organisms (Souders et al., 2019; Vignet et al., 2019). Toxicity to non-target organisms as well as the use of large amounts of pesticides stimulates research of novel efficient possibilities and alternatives including e.g. nanoformulated pesticides. Pesticide nanoformulation often represents an active ingredient encapsulated in nanocarriers (Kah et al., 2018). Polymer nanocarriers are often composed of biodegradable and/or biocompatible polymers such as poly(ε-caprolactone) (PCL) (Grillo et al., 2012; Woodruff and Hutmacher, 2010). Besides lower quantities of pesticides needed and lower toxicity to non-target organisms, the nanopesticides may carry also other advantages such as higher efficiency, slow and controlled release of the active ingredient from the nanocarrier, extended lifetime, better uptake or dispersion (Kumar et al., 2019). These may ultimately lead to lower contamination of water ecosystems and lesser impact on non-target organisms. This rapid advancement of the agrochemical industry should also be accompanied by proper ecotoxicity studies. As emphasized by Kah et al. (2018), it is necessary to compare the impacts of the nanoformulation with the conventional active ingredient.

The present study investigated the sublethal toxicity of environmentally relevant concentrations of the main pesticides detected in Arcachon Bay, France - insecticide IMI and fungicide PRO on embryo-larval stages of resident Pacific oyster (*Crassostrea gigas*). Furthermore, a prospective assessment of the PRO nanoformulation was carried out. The studied parameters included apical endpoints (mortality, developmental abnormalities) of oyster larvae as well as neurobehavioral endpoints related to swimming activity (speed and trajectory type), and biochemical responses (transcription changes of selected genes). This

integrative approach allowed for a detailed examination of the sublethal toxicity of the pesticides and the nanoformulation.

2. Materials and methods

2.1 Chemicals and reference seawater

Imidacloprid (IMI, CAS 138261-41-3, Pestanal, purity 100 %), propiconazole (PRO, CAS 60207-90-1, Pestanal, purity 100 %), and $CuSO_4$ were purchased from Sigma-Aldrich. The stock solution of PRO (5 g/L) was prepared in DMSO and was stored at 5 °C. The stock solution of IMI (200 mg/L) was prepared directly in seawater and was used for testing immediately. The solution of Cu^{++} was used as a positive control and stored at 5 °C (stock solution in milliQ water at 100 mg/L). Exposure solutions were prepared by serial dilution in seawater. Seawater was collected at beach Petit Nice (approx. 44°33'40.3"N 1°14'27.1"W), serially filtered at 0.22 µm, and passed through UV light to eliminate debris and microorganisms. Filtered seawater (FSW) was stored at 5 °C in the dark and was used typically within two days (within 7 days at the latest). It was refiltered through 0.22 µm for solution preparation and oyster spawning. The presence of pesticides and copper in FSW was verified by LC-MS/MS (cf. section 2.7 and 3.1).

All chemicals (poly-ε-caprolactone, Myritol 318, sorbital monostearate surfactant (Span 60), polysorbate 80 surfactant (Tween 80), and acetone) needed for the nanoformulation of propiconazole were purchased from Sigma-Aldrich. Internal standards for chemical analysis tebuconazole D6 and imidacloprid D4 (CAS 1015855-75-0) were purchased from LGC Standards and TRC Canada. Chemicals for the gene expression analysis including the RNA later buffer were purchased from Qiagen. Phenol and chloroform Rectapur® were purchased from Sigma-Aldrich.

2.2 Nanopropiconazole

The nanopropiconazole formulation used in this work consisted of poly-ε-caprolactone nanocapsules loaded with the fungicide. The method used for its preparation was the interfacial deposition of a preformed polymer as described by Grillo et al. (2012) with one

modification: Myritol 318, instead of Miglyoil 810, was used as the triglyceride oil. As a control, nanocapsules (nanoC) not containing the active ingredient (verified by LC-MS/MS; the used method was the same as for the nanoformulation mentioned below) were also prepared using the same method. The stock suspension of nanopropiconazole (nanoPRO) with a propiconazole concentration of 325 mg/L (determined by LC-MS/MS as described in section 2.7 after diluting the sample in acetonitrile, centrifuging it through nylon filter – 6000 rpm/6 min, and freezing at -20 °C; more details in section 2.7) was stored at ambient temperature in an amber glass vial. The average size (z-average diameter) and the zeta potential of nanoparticles was measured in the stock suspension in milliQ water (diluted 100x) by dynamic light scattering (DLS) using Malvern Instruments with software Zetasizer Ver. 6.20 with a detector at a fixed angle of 173° and the average size measurement was repeated by Cordouan Technologies SAS with software NanoQ V2.6.3.0 with a detector at a fixed angle of 135°. Cordouan Technologies SAS was also used to measure the average size of nanoparticles in seawater (only the highest exposure concentrations, 10 mg/L of loaded PRO, was measured due to the power of the laser). Both average size and zeta potential results are expressed as the means of three acquisitions.

The encapsulation efficiency characterizes the percentage of pesticide loaded into the nanocarrier in reference to the total amount of pesticide in the system. The total quantity of PRO was determined by diluting a sample suspension with acetonitrile as detailed above. The amount of fungicide associated with the nanocarriers was measured by the centrifugal ultrafiltration method: samples in triplicates were centrifuged (11,481 rpm, 30 min) using Microcon-30kDa Centrifugal Filter Unit with Ultracel-30 membrane (Millipore). A solution of propiconazole (conventional formulation) was analyzed in parallel to measure a potential loss/adsorption of propiconazole on the filter. The filtrates were then diluted in 20 %

acetonitrile and measured by LC-MS/MS to determine the free amount of propiconazole, as described in section 2.7. The encapsulation efficiency was calculated according to the formula

$$EE\ (\%) = \frac{W(associated)}{W(total)} = \frac{W(total) - W(free)}{W(total)},$$

where *EE* stands for encapsulation efficiency, and *W(total)* and *W(free)* for the total and free amount of PRO, respectively. The free amount of PRO was corrected for the loss of propiconazole in the centrifugal ultrafiltration device. In previous studies, the same procedure has been used to determine the EE of pesticides and drugs in PCL nanoparticles (Grillo et al., 2012; Moraes et al., 2011; Pereira et al., 2014).

The release of propiconazole from the nanocapsules was measured by the sample and separation method (D'Souza, 2014; Nothnagel and Wacker, 2018). In brief, a portion of nanoPRO suspension was diluted in 20 mL of seawater obtaining a propiconazole concentration of 10 mg/L. At the same time, 20 mL of 10 mg/L of pure active ingredient propiconazole solution were prepared and used as a control. Both dilutions were kept in amber glass vials at room temperature on a shaking platform for 48 h (100 rpm). Duplicate samples were taken at the beginning of the test and after 4, 8, 24, and 48 hours, and processed by the centrifugal ultrafiltration procedure, as described above. The filtrates were then diluted in 20 % acetonitrile and analyzed by LC-MS/MS as described in section 2.7. The apparent concentration of nanoPRO was corrected by the encapsulation efficiency and all results were corrected for the loss of active ingredient propiconazole reference sample in the centrifugal ultrafiltration devices.

2.3 Test organism

Pacific oyster (*Magallana gigas*, called also *Crassostrea gigas*) mature adults (5 couples for each test) were received from Guernesey Sea Farm hatchery (Guernesey, UK) and were used immediately, or kept in oxygenated FSW at 11 °C during 24 h. Oysters were placed in FSW

(12 °C, 30 min) for the acclimatization and were then subjected to alternating thermal shock in FSW at 18 °C and 28 °C for 30 min. Female spawning was facilitated by adding frozen filtered oyster sperm which contains diantlin (Dupuy et al., 1977). Detailed spawning method is described by Gamain et al. (2016). The embryos were then transferred to experimental units and kept at 24 °C in the dark until they reach the developmental stage of D-larva.

2.4 Embryo-larval test

The embryo-larval oyster test was carried out following the French guideline (NF ISO 17244, 2015) with modifications: The embryos were transferred to 24-well microplates (Greiner Bio-One, Cellstar; 225 embryos per well) and were exposed to a wide concentration range of every substance: PRO (20 ng/L, 200 ng/L, 2 µg/L, 20 µg/L, 200 µg/L, 2 mg/L, 10 mg/L), nanoPRO (20 ng/L, 200 ng/L, 2 µg/L, 20 µg/L, 200 µg/L, 2 mg/L, 10 mg/L), IMI (20 ng/L, 200 ng/L, 2 µg/L, 20 µg/L, 200 µg/L, 2 mg/L, 20 mg/L, 200 mg/L), and the nanoC control nanocarrier (suspension of empty nanocapsules diluted in the same manner as the nanoPRO to get the same amount of nanocapsules in the seven suspension dilutions). Larvae batches obtained from each of the four mature oyster couples were exposed separately for each compound. Each concentration was tested in four replicates, i.e. sixteen replicates of embryos in total (siblings from different parents were never pooled). Negative control (FSW), solvent (DMSO), and nanocarrier control (whenever relevant) were present on every microplate in four replicates as well. The concentration of DMSO (0.00002 %) and nanoC controls corresponded to their concentration in the 2 µg/L solution of PRO and nanoPRO i.e. the highest concentrations used for locomotion and gene expression analysis. Microplates were kept at 24 °C in the dark.

At 24 hpf (hours post-fertilization), the microplates were used for the locomotion analysis (cf. section 2.5). After the video capture (approximately at 30 hpf), formaldehyde (25 µL at 37 %) was added to every well (final volume 2025 µL), and the microplates were kept at 4 °C

until the analysis of developmental malformations was carried out (within 14 days). Percentage of different developmental malformations (mantle and shell malformation), arrested development, or well-developed D-shaped larvae (Figure 1) per 100 embryos per well was determined using an inverted microscope (Nikon Eclipse TS100). For all tests, validity criteria were fulfilled as follows, malformation rate lower than 20 % in the control, and EC_{50} for abnormal larvae exposed to positive control Cu^{++} between 6 and 16 µg/L (tested concentration range: 0-5-10-20-50 µg/L).

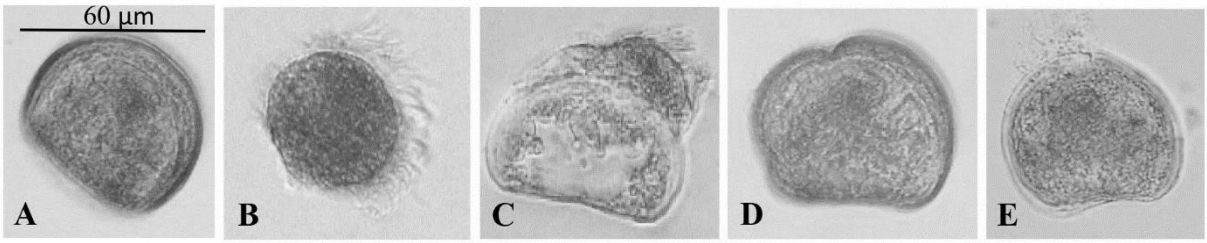


Figure 1 Different types of developmental malformations of oyster larvae (*Magallana gigas*) at 30 hpf: well-formed D-larva (A), developmental arrest (B), mantle malformation (C), (scalloped) shell malformation (D), (concave) shell malformation (E).

2.5 Locomotion analysis

Oyster embryos were exposed to three concentrations of PRO and nanoPRO (20 ng/L, 200 ng/L, 2 µg/L), and IMI (200 ng/L, 2 µg/L, 20 µg/L) The first concentration of PRO and IMI is environmentally realistic and corresponds to the concentrations detected in the Arcachon Bay in France (Table 1).

Table 1 Concentrations of pesticides of interest detected at different sampling points in Arcachon Bay during the years 2010-2014. N=669 for each pesticide. Concentrations were calculated according to the data of Tapie et al., (2018).

	PRO	**IMI**
Limit of quantification (ng/L)	1	1
Samples with detected substance (%)	20.7	34.8
Average concentration in all samples (ng/L)	0.7	2.6

Average concentration in samples with detected substance (ng/L)	3.1	7.6
Maximal concentration (ng/L)	**29.1**	**173.6**

Before adding the formaldehyde into the microplates (c.f. section 2.4) to evaluate the morphologic abnormalities, videos of larvae locomotion were captured. Videos were taken after 24 hours of incubation at the stage of the D-shaped larva. The temperature in the solutions/suspensions with larvae was maintained at 24 °C during the video capture (using room air conditioning). Two-minute video per well was captured at zoom 40x using an inverted microscope Nikon Eclipse TS100 equipped with camera Nikon DS-Fi2, and software NIS Element. The videos were then converted into 4 fps with software VirtualDub and analyzed using ImageJ to acquire the trajectory type, the average, and the maximal swimming speed of each tracked oyster larva. The ImageJ plugin and method are described in detail by Gamain et al. (2019). Three different trajectory paths were discriminated as follows: rectilinear, circular, and stationary (presented in Figure 2). The results provided by ImageJ were manually checked for artifacts (larvae exiting field of view after too short trajectory; larvae collisions influencing the speed and trajectory; larvae passing too close to each other and exchanging their tracking identities etc.).

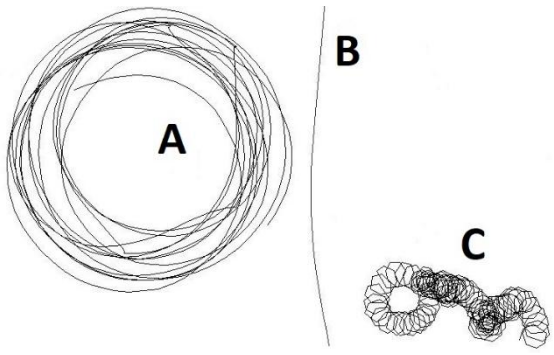


Figure 2 Different types of trajectory paths of oyster larvae observed during locomotion experiments: circular (A), rectilinear (B), stationary (C).

2.6 Oyster exposures for analysis of gene expressions

To collect enough RNA for the analysis, 500,000 embryos (originated from one oyster couple) were incubated in three-liter glass beakers (exposure to PRO and nanoPRO) or plastic bottles (exposure to IMI) at 24 °C in the dark for 42 hours. For oxygenation and for keeping embryos suspended in the water column, solutions were aerated with aquarium airstones. Dissolved oxygen was checked at the beginning and the end of the tests. The concentrations of the exposure solutions were the same as for the locomotion analysis: PRO and nanoPRO (20 ng/L, 200 ng/L, 2 µg/L), and IMI (1 µg/L, 10 µg/L, 100 µg/L). After 42 hours, the larvae were collected on a 20 µm mesh (SEFAR NITEX®) using a vacuum pump, resuspended in 5 mL of exposure solution, and kept on ice. Their concentration was calculated immediately and five replicates, each containing 30,000 larvae, were collected in 1.5 mL polypropylene microtubes tubes. These replicates were centrifuged (2 min, 1000 rpm) and the larvae pellet was resuspended in 500 µL of RNA later. Samples were then kept at -80 °C until RNA extraction.

The total RNAs were extracted using the SV Total RNA Isolation System Kit (Promega). Samples were first homogenized using vortex and 200 µL of glass beads (0.10 – 0.11 mm, acid washed, B. Braun Biotech International) in 500 µL of RNA Lysis buffer, and centrifuged (7,500 rpm, 1 min). Lysed samples were collected, 500 µL of phenol-chloroform-isoamyl alcohol (25-24-1) was added, and the tubes were vortexed. Centrifugation (13,500 rpm, 5 min) divided the samples into two phases and the upper (aqueous) was collected, mixed with 450 µl of 75 % ethanol, vortexed, and transferred onto a spin column following manufacturer's instructions with few modifications as follows: RNA samples were treated with DNase I mixture for 15 min at 37 °C, and purified RNAs were collected in 50 µL of Nuclease-Free water. The concentration and purity of collected RNA samples were checked spectrophotometrically at 260/280 nm with software Gen5 (Biotek), using a

spectrophotometer (Spectro Multivolume Epoch; BioTek). The purity of all samples was between 2.0-2.2. Reverse transcription was performed with the GoScript™ Reverse Transcription System kit (Promega) according to the manufacturer's instructions. Purified 1 µg of RNA was reversely transcribed to get the final volume of 20 µL of cDNA, which was stored at -20 °C until the quantitative PCR analysis was performed.

QPCR was carried out with the GoTaq® qPCR Master Mix kit (Promega) on a LightCycler® 480 (Roche). QPCR mix was composed of 1 µL of cDNA, 2 µL of a mix containing each reverse and forward primer (2 µM), and 10 µL of 2x GoTaq master mix, which was completed with nuclease-free water to the final volume of 20 µL. Fourteen genes in total were selected to evaluate the effects of chosen pesticides on mitochondrial metabolism (*12S*, *cox1*), regulation of the cell cycle and apoptosis (*p53*), oxidative stress defense (*cat*, *sodMn*, *sodCu/Zn*, *gpx*), detoxification (*mt1*, *mt2*), apoptosis (*bax*, *casp3*), biotransformation (*cyp1a*), growth arrest and DNA damage (*gadd45*), and DNA repair (*rad51*). Three reference genes were used in the analysis (*β-actin*, *ef1α*, and *rpl7*). The genes were chosen to evaluate non-specific toxicity and general responses of oyster larvae to pollutant stress, and to correspond with the studies of Mai et al. and Gamain et al. referenced in this publication. Sequences, references, and accession numbers are presented in Supplementary Table S1. Primers were purchased from Sigma proligo. Primer-pairs efficiencies for all genes were verified to be higher than 95 %. The PCR procedure was as follows: the pre-incubation step lasted 2 min at 95 °C, then the amplification consisted of 50 cycles with each cycle at 95 °C for 15 s and 60 °C for 1 min. The melting curve continued at 95 °C for 30 s, at 60 °C for 2 min, and 95 °C until the next cycle.

Melting curves of every reaction were verified to assess reaction specificity. All data were normalized to the geometric mean of the Ct values of the three reference genes, *β-act*, *elf1a*,

304 and *rpl7*, and treated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Results are
305 shown as fold changes of the exposed group compared to the control group.

306

2.7 Chemical analysis and water quality

Salinity, pH, and dissolved oxygen were measured at the beginning and the end of the experiments, using Multi 340i probe (WTW). Oxygen saturation was always higher than 91.6 % (average 94.5 ± 1.9 %), salinity varied between 33.5 and 35.3 psu (practical salinity unit) on the first day of the test and between 34.7 and 36.0 psu on the last day of the test (higher values at the end of the test may be caused by evaporation and concentration of the seawater solutions), and pH ranged from 7.95 to 8.2. The measured parameters complied with the revised standard as reported by Leverett and Thain (2013).

Concentrations and stability of used chemical substances were verified using LC-MS/MS, as described in detail in Supplementary Material S2. Before the chemical analyses, samples were processed as follows: samples from the gene expression experiments were taken at the beginning (30 min after the addition of the chemical in the experimental unit with the aeration device) and the end of tests (at 42 h). Samples for assessing the stability of compounds in the microplates (i.e. developmental malformations and locomotion analysis tests) were taken at the beginning and the end of the test at 24 h. All samples and calibration solutions were stored at -20 °C and spiked with 10 µL of the internal standard of tebuconazole D6 and imidacloprid D4 (both dissolved in 50 % methanol). The tebuconazole D6 is used as a standard for conazole analysis in multi-parameter analyses. The samples (1.5 mL) and calibration solutions were lyophilized using a freeze dryer Alpha 2-4 LD Plus (Martin Christ Freeze Dryers). After the lyophilization, the samples were dissolved in 1 mL of 100 % acetonitrile, vortexed for 30 sec, ultrasonicated for 5 min, vortexed again (30 sec), and centrifuged (12,000 rpm, 10 °C, 10 min) in order to precipitate the salts and move the analyte to the solvent phase. The supernatant (500 µL) was transferred into a glass vial and evaporated using the nitrogen. The evaporated vial was carefully filled with 0.75 mL of 20 % acetonitrile, vortexed, and stored at -20 °C upon analysis by LC-MS/MS.

LC-MS/MS analysis was performed with a Waters Acquity LC chromatograph (Waters, Manchester, U.K.), using the Acquity BEH C18 column and gradient elution. Detection was performed on a Xevo TQ-S quadrupole mass spectrometer (Waters Manchester, U.K.) after ESI ionization in positive ion mode. The quantification of analytes was based on the external calibration of individual compounds and normalized with internal deuterium-labeled standards (imidacloprid D4 and tebuconazole D6). Calibration of standards in 20 % of acetonitrile was in the range 0.02 – 2 µg/L for propiconazole and 0.2 – 20 µg/L for imidacloprid, with limits of quantification (LOQ; S/N>10) being 0.01 µg/L for propiconazole and 0.05 µg/L for imidacloprid.

The concentration of copper in the reference seawater and the positive control was assessed using ICP-MS and ICP-OES, respectively. The samples were acidified with nitric acid before the analyses (final concentration of acid in samples was 5 %). The reported copper concentration of the reference seawater was the concentration at the moment of preparation of the exposure solution (i.e. sample collected after sampling at the beach, transport of the water in 10 L plastic canisters, filtering using the filtration system, and transporting the canisters to the laboratory). Water was stored in the dark at 5 °C.

2.8 Data analysis

Relative abundance (%) of malformed larvae (sum of all types of abnormal development i.e. developmental arrest, mantle, and shell malformations) and the results (%) of trajectory analyses were first transformed using arcsine transformation $p' = \arcsin \sqrt{\frac{p}{100}}$ (Sokal and Rohlf, 2012), and checked 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 opposite case, non-parametric Kruskal-Wallis ($P < 0.05$) with Mann-Whitney post-hoc test was carried out. All analyses were carried out using Statistica 13.3 (StatSoft,

356 USA). $EC_{50/20/30}$ were calculated from nonlinear logarithmic regression of the nominal
357 concentration-response curves, using Graph Pad Prism 5 (Graph Pad Software, USA).

358 Raw data of larval swimming speed acquired by the imaging software were converted from
359 pixel/sec to µm/sec (multiplication by 2.43; value corresponding to the microscope and zoom
360 used during the capture of videos). Data were then normalized to average control swimming
361 speed due to the high data variability of different test repetitions. Finally, normalized data
362 were compared in Statistica 13.3 using the statistical tests described above (Shapiro-Wilk test;
363 $P > 0.01$; Levene test; $P > 0.05$; ANOVA or Kruskal-Wallis; $P < 0.05$).

364 All data from the gene expression analysis were log-transformed before the analysis, treated
365 as described above (Shapiro-Wilk test; $P > 0.01$; Levene test; $P > 0.05$), and tested for
366 significant differences using ANOVA ($P < 0.05$) followed by Tukey post-hoc test. If
367 normality or homoscedasticity were not confirmed, non-parametric Kruskal-Wallis ($P < 0.05$)
368 test with Mann-Whitney post-hoc tests were performed.

3. Results

3.1. Exposure and chemical analysis

The concentration of copper in FSW used for the preparation of exposure solutions was 2.4 ± 0.8 µg/L (maximal concentration measured was 3.3 µg/L). IMI was stable in the microplate over 24 h in all tested concentrations (98.6 ± 2.3 %). Whereas PRO, a hydrophobic compound, did adsorb on the plastic microplate walls (maximal loss of 24 % of the compound at the lowest tested concentration 20 ng/L). Thus, the microplates were precoated with the appropriate concentration of propiconazole the day before the experimentation and the exposure solution was renewed an hour before the test. The recovery of propiconazole after 24 hours in precoated microplates was 101.6 ± 3.7 %. The microplate precoating was also used for the tests with nanoPRO (precoated by suspensions of nanoPRO).

The concentration of pesticides in all non-exposed variants was found to be either below the limit of detection, either as non-quantified. IMI, in treatments for the gene expression assay, was stable during the 42-hour long test (concentration at the end of the test was between 101.4 % and 107.3 % of the initial concentration) except for one of the three replicates of the concentration of 10 µg/L, where increase by 54 % was recorded. PRO, on the other hand, was not stable, and the recovery at the end of 42 h exposure varied from 0 to 100 %. Unfortunately, for practical reasons, it was not possible to precoat the beakers, as it was done for the microplates and nominal concentrations are reported but taking this caveat into account in the discussion. Complete results are shown in Supplementary Table 2.

3.2 Nanopropiconazole and nanocarrier characterization

The average size (z-average diameter) and zeta potential of nanoparticles containing propiconazole and nanocarrier in the stock suspensions dispersed in milliQ water, and diluted 100x, were measured to assess the suspension stability. After the synthesis, the particle

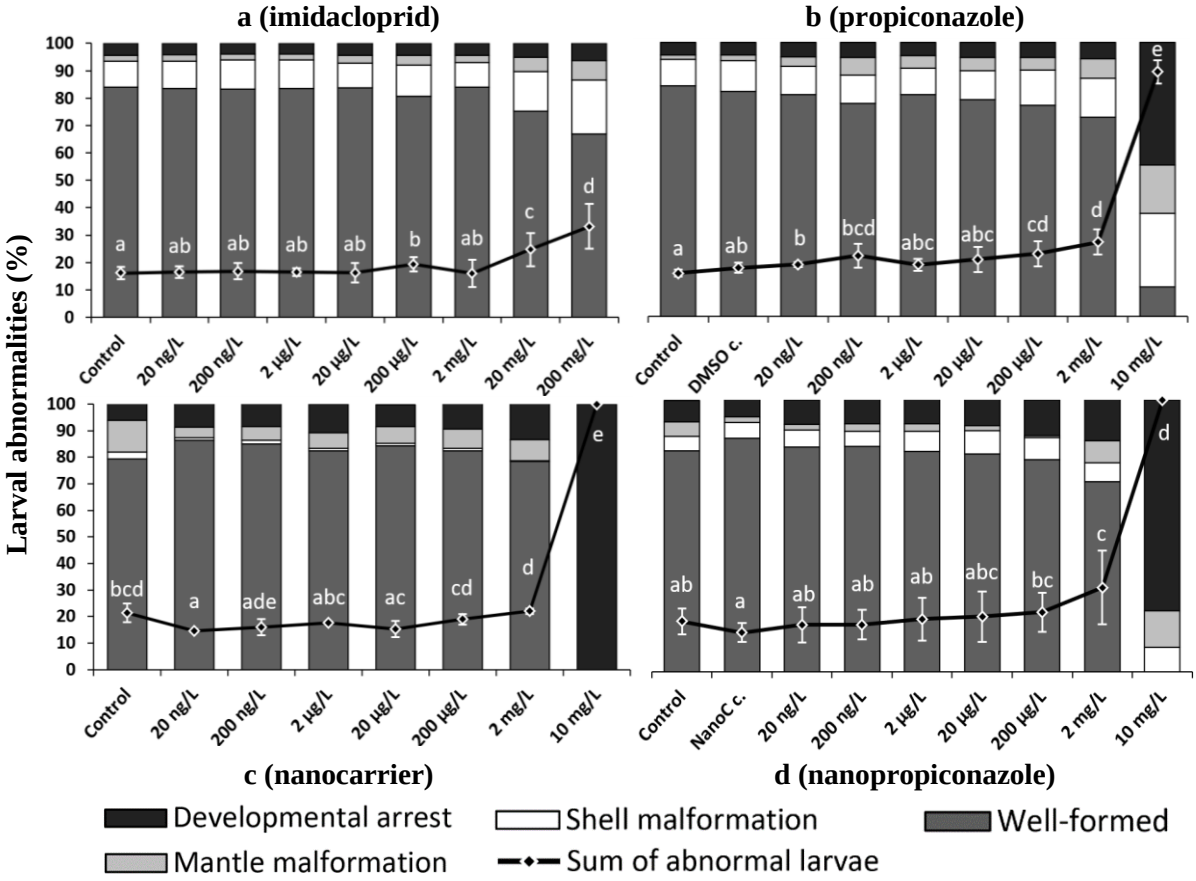
diameter in the nanoPRO and nanoC stock suspensions were 301.1 ± 1.8 nm and 263.4 ± 1.0 nm, respectively. The zeta potential measured in the nanoPRO and nanocarrier stock suspensions was -35.3 ± 0.9 mV and -35.7 ± 0.3 mV, respectively. Three months later, the properties of stock suspensions were again analyzed to check the behavior of nanoformulations in seawater, finding the z-average diameter of particles in nanoPRO and nanocarrier stock suspensions in milliQ water (diluted 100x) to be 326.4 ± 9.1 nm and 282.7 ± 3.3 nm, respectively. The slightly higher values may be caused by different instruments used (as indicated in section 2.2). The results confirm that the suspension is stable, and no aggregates of nanoparticles were formed (aggregates would have the values twice the initial size). The size of particles in the nanoformulations diluted in FSW was also measured at 0 and 24h to imitate the embryo-larval biotests in microplates. Due to the power of the laser, only the highest tested concentration (10 mg/L) was analyzed. The z-average of nanoPRO particles at 0 h and 24 h was 371.5 ± 7.8 nm and 404.0 ± 1.5 nm, respectively and the z-average of nanoC particles at 0 h and 24 h was 349.4 ± 4.2 nm and 365.1 ± 2.6 nm, respectively.

The encapsulation efficiency of the nanoPRO nanoformulation was 97.7 %. Furthermore, an analysis of the release of propiconazole from the nanocapsules in seawater found that an initial, rapid release of propiconazole occurred immediately after dilution, getting a fungicide release rate of 44 ± 1.4 %. The percentage of propiconazole released remained stable (no more propiconazole has been released after the initial burst) for the test duration of 48 h (Supplementary Figure S3).

3.3 Embryo-larval development

Validity criteria for the used bivalve embryo-larval normalized test were fulfilled with an average EC_{50} value for Cu^{++} of 9.76 ± 1.58 µg/L for all experiments.

Frequency (%) of developmental malformations and developmental arrests of oyster larvae exposed for 30 h to increasing concentrations of pesticides are shown in Figure 3. Moreover, the sum of abnormal larvae is shown for every exposure condition. These abnormal larvae proportions served for the calculations of effective concentrations (EC_x), no observable effect concentration (NOEC), and the lowest observable effect concentration (LOEC), which are shown in Table 2. In general, IMI, PRO, and nanoPRO had comparable toxicity patterns since the developmental toxicity to oyster larvae occurred only at high concentrations (at and above 200 µg/L), whereas no effect was observed after exposure to environmental concentrations of IMI and PRO. The highest tested concentration (10 mg/L) of nanoPRO malformed or arrested development of all larvae and PRO affected 89.3 ± 4.3 % of individuals. On the contrary, even the highest tested concentration of IMI (200 mg/L) affected only 33.1 ± 8.2 % larvae. Conversely, nanoC did not cause developmental malformations irrespectively of concentration and only the highest tested concentration (10 mg/L) induced developmental arrests (Figure 3), thus suggesting that whereas nanocarrier caused only developmental arrests, it was the propiconazole inside the capsules which was responsible for the developmental malformations.



435 **Figure 3** Larval abnormalities and the sum of affected individuals of oyster larvae after
436 30 hours of exposure to increasing concentrations of imidacloprid (a), propiconazole (b),
437 nanocarrier (c), and nanopropiconazole (d). Different letters indicate statistical differences
438 between variables ($P < 0.05$). Results are presented as the mean of 4 independent experiments
439 (n=3 in case of nanoC) ± SD. Solvent (DMSO) and nanocarrier (NanoC) controls are shown
440 in panels b) and d).

441 IMI was the least toxic to oyster larvae, whereas PRO, nanoPRO, and nanoC had comparable
442 EC_x values (Table 2). Interestingly, NOEC and LOEC were identical for the IMI, PRO, and
443 nanoPRO (20 and 200 µg/L, respectively). These values were within the range of µg/L, i.e.
444 higher than environmental concentrations in Arcachon Bay (Table 1). As expected, nanoC
445 was not toxic with NOEC of 2 mg/L.

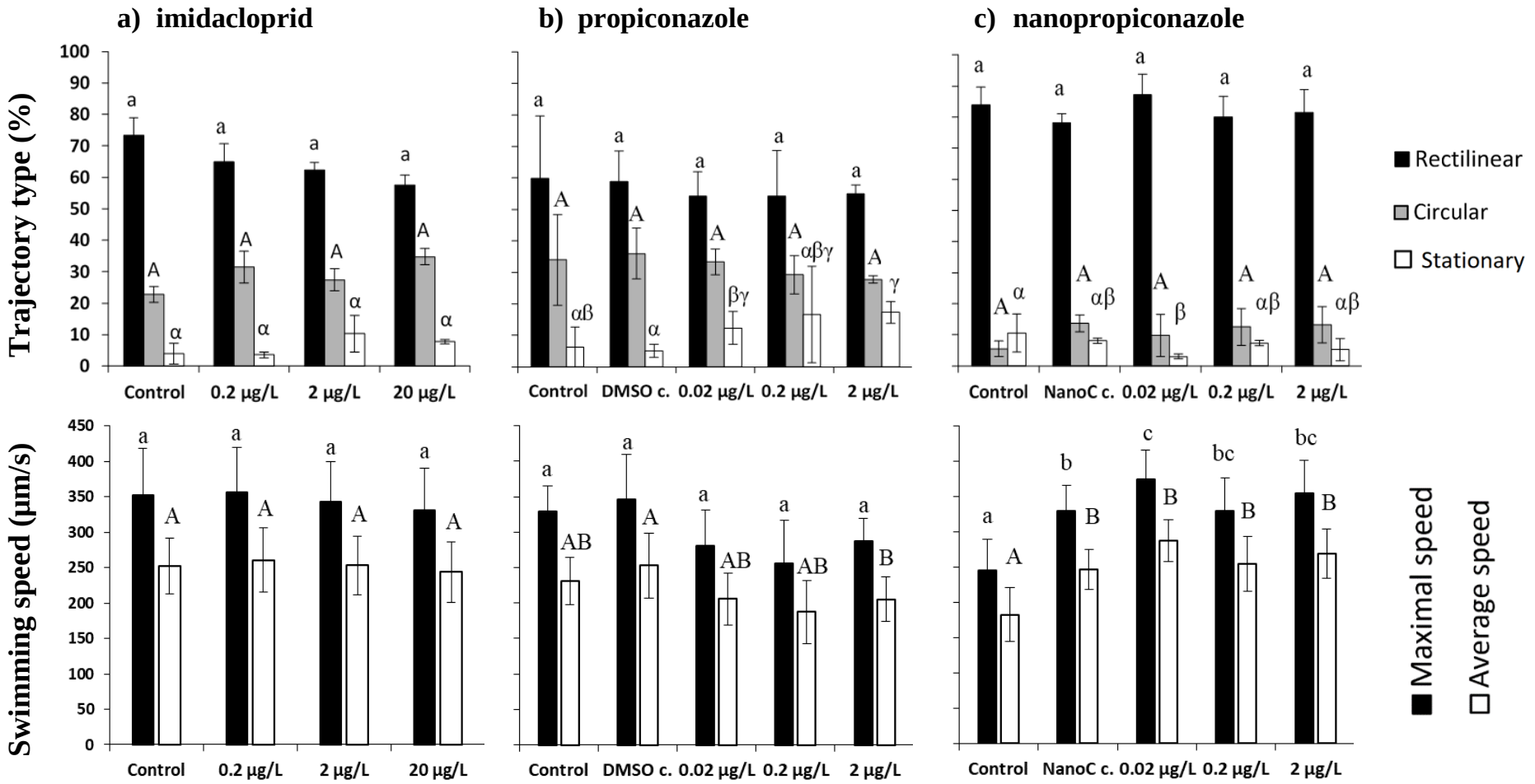
Table 2 Effective concentrations (EC_{20}, EC_{30}, EC_{50}), no observable effect concentration (NOEC), and lowest observable effect concentration (LOEC) after 30 h-long exposure of oyster larvae to imidacloprid, propiconazole, nanopropiconazole, and nanocarrier. Imidacloprid did not reach EC_{50} up to the highest tested concentration (200 mg/L).

		IMI	PRO	nanoPRO	nanoC
µg/L	**NOEC**	20	20	20	2,000
	LOEC	200	200	200	10,000
mg/L	**EC_{50} ± SE**	> 200	2.93 ± 1.35	2.26 ± 1.36	2.84 ± 1.41
	EC_{20}	6.43	0.73	0.56	0.71
	EC_{30}	70.50	1.26	0.97	1.22

3.4 Locomotion analysis

The locomotion analysis consisted of trajectory type and maximal and average speed assessment (Figure 4). The non-exposed larvae generally displayed rectilinear swimming trajectories (72.3 ± 9.9 %), less commonly the circular trajectory type (20 ± 11.7 %), and rarely they stayed stationary (6.9 ± 2.8 %). Maximum and average swimming speed employed by the non-exposed larvae were 309.3 ± 45.7 µm/s and 222.2 ± 29.1 µm/s, respectively, even though high variability was observed between the individuals. No statistically significant effects on swimming speed or trajectory were observed for IMI, but the frequency of rectilinear trajectories showed a decreasing tendency with increasing concentration (73.3 – 65.0 – 62.3 – 57.6 %) and the increase of the circular type (22.8 – 31.4 – 27.4 – 34.8 %). A statistically significant increase in stationary swimming patterns was observed in larvae exposed to 2 µg/L of PRO (17.3 ± 3.4 %) when compared to other treatments. The same effect was also observed in larvae exposed to an environmentally relevant concentration of 0.02 µg/L (12.4 ± 5.1 %) when compared to the DMSO control (5.2 ± 2.1 %). Similarly, as for PRO, nanoPRO caused no effects on the frequency of rectilinear and circular trajectories.

No effect on larvae swimming speed was observed after exposure to IMI. In contrast, PRO at 2 µg/L did cause a statistically significant decrease (205.1 ± 31.4 µm/s) in average swimming speed in comparison to the DMSO control (252.8 ± 46.0 µm/s). NanoPRO, unlike PRO, caused significant effects on both maximal as well as the average swimming speed. First, the maximal and average swimming speed of nanoC control (330.5 ± 35.4 µm/s and 246.8 ± 28.3 µm/s respectively) significantly differed from the non-exposed control (246.0 ± 43.4 µm/s and 182.9 ± 38.7 µm/s respectively). Furthermore, the low concentration of 0.02 µg/L of nanoPRO caused an even higher increase in maximal speed (374.2 ± 42.1 µm/s) statistically different from both relevant controls.



474 **Figure 4** Frequency of trajectory types and speed observed in the movement of oyster larvae after 24 h exposure to increasing concentrations of
475 imidacloprid (a), propiconazole (b), and nanopropiconazole (c). Letters indicate statistical differences ($P < 0.05$). Results are presented as the
476 mean of 3 values (n=3) i.e. independent experiments (n=4 in the case of PRO) ± SD. DMSO c. = DMSO control; NanoC c. = nanocarrier control.

3.5 Gene expression analysis

Gene expression results of fourteen pre-selected genes are shown in Table 3 as fold changes between the studied and three housekeeping genes. Expressions of *bax*, *cat*, *cox*, *cyp1a*, and *gpx* did not differ between pesticide-exposed oysters and controls. The expression of mitochondrial gene 12S RNA (*12S*) was significantly downregulated after exposure to 200 ng/L and 2 µg/L of PRO. PRO 200 ng/L caused also upregulation of *mt1*, a gene associated with detoxification, and 2 µg/L caused downregulation of *rad51*, a gene coding for a protein involved in DNA reparation. Finally, low concentrations of 20 and 200 ng/L significantly upregulated expression of copper/zinc superoxide dismutase (*sodCu/Zn*), i.e. one of the four studied genes implicated in oxidative stress defense. In contrast, nanoPRO altered the expression of fewer genes than PRO. Similarly to PRO, a low concentration of 20 ng/L nanoPRO upregulated the gene *sodCu/Zn*. However - unlike PRO - nanoPRO exposure also upregulated another oxidative stress defense gene *sodMn* (200 ng/L) and downregulated *gadd45*, linked to the growth arrest and DNA damage. IMI had the strongest disruptive effect of the tested pesticides and affected the expression of 8 genes: as with the fungicides, IMI upregulated *sodCu/Zn* (100 µg/L) but downregulated *sodMn* (1 µg/L and 100 µg/L). 100 µg/L of IMI also downregulated *casp3* and transcription factor *p53*, genes linked to apoptosis and cell cycle regulation. Genes *mt1* and *mt2* coding for two metallothioneins that are involved in protection against oxidative stress were strongly upregulated by IMI at 10 and 100 µg/L. Downregulation of *rad51* was observed at 10 µg/L and *gadd45* was upregulated at 1 µg/L and 10 µg/L of IMI.

Table 3 Expression of fourteen studied genes (relative to three housekeeping genes) involved in mitochondrial metabolism (*12S*, *cox*), regulation of the cell cycle/apoptosis (*p53*), oxidative stress defense (*cat*, *sodMn*, *sodCu/Zn*, *gpx*), detoxification (*mt1*, *mt2*), apoptosis regulation (*bax*, *casp3*), biotransformation (*cyp1a*), growth arrest and DNA damage (*gadd45*), and DNA reparation (*rad51*) in oyster larvae exposed for 72 h to different concentrations of imidacloprid, propiconazole, and nanopropiconazole. Pesticide treatments indicated by nominal concentrations; measured concentrations by LC-MS/MS shown in Supplementary Table S2. *P < 0.05; **P < 0.01; ***P < 0.001. Results are presented as the mean of 3 independent experiments ± SD. Downregulation: fold changes < 1; upregulation: fold changes > 1.

	Imidacloprid			**Propiconazole**			**Nanopropiconazole**		
	1 µg/L	10 µg/L	100 µg/L	20 ng/L	200 ng/L	2 µg/L	20 ng/L	200 ng/L	2 µg/L
12S	1.0 ± 0.1	1.2 ± 0.1	1.0 ± 0.3	1.1 ± 0.4	**0.7 ± 0.1****	**0.6 ± 0.1*****	1.1 ± 0.1	1.0 ± 0.4	1.0 ± 0.3
bax	0.8 ± 0.0	1.0 ± 0.3	1.1 ± 0.1	1.0 ± 0.2	0.9 ± 0.3	0.9 ± 0.1	0.9 ± 0.1	1.0 ± 0.2	1.2 ± 0.3
casp3	0.9 ± 0.1	1.0 ± 0.1	**0.8 ± 0.2****	1.1 ± 0.3	0.9 ± 0.1	0.8 ± 0.2	1.1 ± 0.1	0.9 ± 0.2	1.1 ± 0.2
cat	0.7 ± 0.1	1.3 ± 0.3	1.1 ± 0.4	1.0 ± 0.5	1.6 ± 1.3	2.7 ± 3.1	1.0 ± 0.5	1.0 ± 0.3	1.2 ± 0.7
cox	1.0 ± 0.0	1.0 ± 0.2	1.0 ± 0.2	1.2 ± 0.4	1.0 ± 0.3	1.0 ± 0.3	1.0 ± 0.2	1.1 ± 0.2	1.0 ± 0.1
cyp1a	1.7 ± 0.8	1.4 ± 0.6	1.3 ± 0.6	1.4 ± 0.6	1.8 ± 1.3	1.5 ± 0.6	1.0 ± 0.2	1.0 ± 0.1	1.3 ± 0.3
gpx	1.0 ± 0.1	1.1 ± 0.1	1.0 ± 0.2	1.1 ± 0.2	1.0 ± 0.2	0.9 ± 0.2	1.2 ± 0.2	1.2 ± 0.1	1.3 ± 0.4
mt1	1.4 ± 0.7	**2.6 ± 0.7*****	**2.0 ± 0.5*****	1.4 ± 0.6	**1.7 ± 0.3*****	1.6 ± 0.6	1.1 ± 0.2	1.2 ± 0.2	1.1 ± 0.3
mt2	1.4 ± 0.7	**2.5 ± 0.9*****	**2.1 ± 0.3*****	1.3 ± 0.4	1.3 ± 0.4	0.9 ± 0.2	1.2 ± 0.2	1.2 ± 0.0	1.3 ± 0.4
p53	1.0 ± 0.0	1.0 ± 0.0	**0.8 ± 0.1*****	1.0 ± 0.2	1.5 ± 1.0	1.4 ± 0.8	1.0 ± 0.1	1.0 ± 0.1	1.2 ± 0.3
sodCu/Zn	1.1 ± 0.2	1.1 ± 0.0	**1.3 ± 0.1*****	**1.5 ± 0.5****	**1.3 ± 0.2*****	1.4 ± 0.5	**1.6 ± 0.7***	1.3 ± 0.7	1.4 ± 0.4
sodMn	**0.9 ± 0.1***	1.0 ± 0.2	**0.8 ± 0.1****	1.0 ± 0.3	1.5 ± 1.0	1.4 ± 0.8	1.0 ± 0.1	**1.1 ± 0.1***	1.2 ± 0.2
gadd45	**1.6 ± 0.6***	**1.4 ± 0.2*****	1.7 ± 0.8	1.0 ± 0.4	1.0 ± 0.4	1.1 ± 0.5	**0.8 ± 0.2***	0.9 ± 0.2	1.0 ± 0.4
rad51	0.9 ± 0.1	**0.8 ± 0.0****	0.9 ± 0.1	1.1 ± 0.3	0.9 ± 0.1	**0.8 ± 0.0***	1.1 ± 0.1	1.1 ± 0.2	1.2 ± 0.4

4. Discussion

In the current work, an integrative multi-endpoint approach was used to address the sub-lethal toxicity of two pesticides: IMI and PRO at environmentally relevant concentrations corresponding to the Arcachon Bay in France on embryo-larval stages of Pacific oyster and also to evaluate the effects of nano-formulated pesticide propiconazole.

IMI had mild effects on the development of oyster larvae. The environmentally relevant concentrations detected in the Arcachon Bay in France did not cause developmental abnormalities, even though some mild toxic effects on development were observed at 200 µg/L or higher concentrations, which were detected for instance in the Netherlands as reported by Van Dijk et al., (2013). To the best of our knowledge, this is the first published study assessing IMI toxicity on the early-life stages of a marine bivalve species. Prosser et al. (2016) investigated IMI toxicity on early-life stages of freshwater mollusks and showed low sensitivity of the freshwater mussel *Lampsilis fasciola* (no effect on viability at 1 mg/L) and mild effect on the freshwater pulmonate gastropod *Planorbella pilsbryi* (LC_{10} about 800 µg/L). In our study, lethal effects are represented by developmental arrests, which, however, were not affected up to the highest tested IMI concentration of 200 mg/L. Few studies evaluated the IMI toxicity on adult bivalves. Shan et al. (2020) reported that chronic (30 d) exposure to 2 mg/L of IMI caused sublethal histological changes in adult freshwater clam (*Corbicula fluminea*), including degeneration of digestive tubules and contractions and adhesions in the hemolymphatic vessels. The concentration of 2 mg/L of IMI did not cause any mortality of adult Sydney rock oysters (*Saccostrea glomerate*) in a recent study of Ewere et al. (2019a).

In the present study, IMI did not cause any effect on the behavior (swimming speed and trajectory type) of oyster larvae at concentrations up to 20 µg/L. On the contrary, in another

study 20 µg/L of IMI caused behavioral alterations in adult freshwater clams (*Corbicula fluminea*) with a decreased filtration rate and burrowing activity (Shan et al., 2020). Similarly, filtration activity of adult Sydney rock oysters (*S. glomerata*) was also decreased in the 4-day exposures to 0.5 and 1 mg/L of IMI (Ewere et al. (2019a). However, these concentrations were much higher than those used in the present study.

Gene expression was affected by IMI exposure, progressively with increasing concentration. The strongest effects (upregulation) were observed for metallothionein (*mt1, mt2*) expression, influencing thus the larvae's capacity to regulate metal content. Metallothionein proteins are originally known for binding and interacting with toxic metals (Coyle et al., 2002) but some studies reported their induction even after organic pesticide exposure (Erdoğan et al., 2011; Lim et al., 2015; Migliaccio et al., 2020). The direct link between IMI exposure and metallothionein induction was not studied in this work but it is clear that metallothioneins do not detoxify IMI since it is biotransformed by the CYP enzyme family (Wang et al., 2018). These ubiquitous proteins have diverse functions which may also include protective stress responses ((Ruttkay-Nedecky et al., 2013). Özdemir et al. (2018) linked the metallothionein *mt1* gene induction after exposure of common carp (*Cyprinus carpio*) to IMI to the presence of reactive oxygen species (ROS). On the contrary, no measurable oxidative stress (no response at the molecular level using microarray and no accumulation of lipid peroxidation by-products) was observed in the adult marine mussel *Mytilus galloprovincialis* exposed for four days to IMI (1.8 mg/L), although the gene expressions of two metallothioneins (*mt10, mt20*) was also induced (Dondero et al., 2010). In the present study, ROS production was indirectly detected by the changed expression of genes encoding proteins involved in oxidative stress defense, with IMI inducing overexpression of copper/zinc superoxide dismutase as well as repression of manganese superoxide dismutase. These enzymes use different metals to transform the superoxide anion radical. *SodCu/Zn* was upregulated

simultaneously with both metallothionein proteins *mt1* and *mt2* (100 µg/L of IMI), which seems interesting considering that metallothionein proteins also regulate copper and zinc metabolism (Krężel and Maret, 2017). While copper/zinc superoxide dismutase is cytosolic and extracellular, the manganese superoxide dismutase is found in mitochondria (Miller, 2012). Thus, suspected ROS production caused by imidacloprid exposure could have been limited to the cytoplasm, which might correspond to the known role of ROS in neonicotinoid toxicity as described in the recent review on neonicotinoid impact on oxidative stress (Wang et al., 2018). Corresponding to these findings, Ewere et al., (2020) detected signs of oxidative stress in adult Sydney rock oysters such as upregulation of proteins implicated in oxidative stress after exposure to 10 µg/L of IMI or elevated presence of glutathione-S-transferase (GST) in hemolymph after exposure to 100 µg/L of IMI (GST is an enzyme protecting against xenobiotics such as ROS).

Induction of the *gadd45* regulator gene might indicate both toxic effect of IMI (possibly caused by induced ROS) as well as a potential adaptive response – i.e. growth arrest that might minimize eventual cell damage (Crawford and Davies, 1994). Lastly, the highest tested concentration of IMI (100 µg/L) caused downregulation of the genes *casp3* and *p53* which suggests a modification of the regulation of the cell cycle and an anti-apoptotic effect. Downregulation of caspase-3 implies lesser apoptotic activity, which, however, may hinder the development of the nervous system (D'Amelio et al., 2010).

In both treatments with nanoformulations (nanoPRO and nanoC), the average particle size did not change significantly over the three months that elapsed from their preparations to their use in different tests. The slight differences observed could also be attributed to the use of different instrumentations at the place of preparation (Masaryk University, Brno, Czech Republic) and use of the nanoformulation (University of Bordeaux, France). The z-average diameter of particles in the nanoformulations was slightly greater when the nanoformulation

was diluted in FSW. However, this could be related to the presence of ions in the seawater that could also affect DLS analysis. Overall, both nanoformulations remained stable without the formation of aggregates over time. The zeta potential values were greater than ± 30 mV, which means that the surface charge is high enough to produce a strong repulsive interaction between the particles and to avoid the forming of aggregates (Tamjidi et al., 2013). In addition, our nanoformulations contain a surfactant (PVA) that can be adsorbed on the surface of nanoparticles, preventing the aggregation of particles by steric effect (de Oliveira et al., 2015). In general, the contribution of the steric effect in the colloidal stabilization is more important than the electrostatic effect, therefore the stability of the nanoformulations of this work cannot be attributed to the surface electrostatic repulsion but to steric hindrance (Bhattacharjee, 2016; de Oliveira et al., 2015).

PRO and its nanoformulation induced more significant effects on the development of oyster larvae compared to IMI. Although LOECs were identical, PRO and nanoPRO caused greater effects at higher concentrations. Fractions of abnormal larvae increased only at the highest, yet not environmentally relevant concentrations measured in the Arcachon Bay in France and elsewhere in the world as referenced in the introduction. As non-target organisms, Pacific oyster larvae seem to be more sensitive to propiconazole toxicity than freshwater mussels. Bringolf et al. (2007) reported the EC_{50} of propiconazole around 20 mg/L for acute (24 and 48 h) toxicity test with glochidia of mussel *Lampsilis siliquoidea*, and EC_{50} of 10 mg/L for 96 h toxicity test with juveniles of the same species. The propiconazole toxicity observed in the present study is comparable to that of other early-life stages of non-target aquatic species e.g. LC_{50} of 20.4 mg/L for zebrafish (*Danio rerio*) in a 5-day-long test (Coors et al., 2012) and LC_{50} of 5 mg/L for water fleas *Daphnia magna* in a 48 h-long test (Kast-Hutcheson et al., 2001).

603 The swimming behavior of oyster larvae was slightly affected by PRO and nanoPRO. At
604 lower concentrations, the PRO-only treatment did not seem to affect this endpoint. Whereas
605 the nanoPRO enhanced the larvae swimming speed which might be explained as the
606 combined effect of the active ingredient and the nanocapsules. To the best of our knowledge,
607 no work studying the behavioral effects of PRO on mollusks has been reported so far. In a
608 study of Souders et al. (2019), early-life stages of zebrafish (*Danio rerio*) exerted hypoactive
609 swimming behavior after exposure to 10 µM (3.4 mg/L) of PRO but no effect was seen at
610 0.1 µM (34 µg/L), a concentration still 17-times higher than the highest tested in the present
611 study (2 µg/L). Adult freshwater mussel (*Unio tumidus*) exposed to a high concentration of
612 10 mg/L of tebuconazole, a fungicide with a similar mode of action as PRO, manifested
613 decreased shell opening rate and daily activity time (Chmist et al., 2019). Nevertheless, oyster
614 larvae swimming behavior seems to be a sensitive endpoint revealing the effects at low ng/L
615 environmental concentrations (Gamain et al., 2020). Therefore, it seems that the nervous
616 system controlling the behavior function is probably not an important toxicity target of PRO
617 at low concentrations (0.02 – 2 µg/L) as based on the assessment of larvae swimming
618 behavior. However, it cannot be fully excluded before other behavioral biomarkers are
619 assessed (larvae feeding, capture success, settlement behavior).

620 PRO showed a lesser effect on gene expression in comparison with IMI, and only a few genes
621 were affected (*12S*, *mt1*, *sodCu/Zn*, *rad51*). Moreover, nanoPRO altered expression of only 3
622 genes (*sodCu/Zn*, *sodMn*, *gadd45*). This suggests that nanoformulations such as nanoPRO
623 should be explored as an alternative with potentially lower toxic impact on oyster larvae. The
624 most important impact of PRO was the induction of copper/zinc superoxide dismutase and
625 repression of the gene coding for the mitochondrial small ribosomal unit (*12S*). As in the case
626 of IMI, the induction of superoxide dismutase might be related to the production of ROS
627 supported by the observed induction of *mt1*, a scavenger of ROS. PRO is indeed known to

trigger ROS production as shown previously (Li et al., 2011; Nesnow et al., 2011). On the other hand, repression of *12S* suggests an impact on the mitochondrial metabolism and mitochondria count. However, according to the literature, environmental pollution by metals or pesticides is often accompanied by upregulated *12S* keeping the mitochondria quantity and maintaining the level of ATP necessary when facing chemical stress (Kim Tiam et al., 2012; Moisset et al., 2015).

The present study aimed to investigate the sublethal effects of exposure to IMI and PRO at low environmental concentrations (complemented with higher concentrations to establish EC_{50} for the developmental malformations). However, only rough estimations are possible for nanoPRO because it has not (yet) been used in the field. Prediction and impacts of any nanopesticide in aquatic environments depend on various factors such as the type of the polymer used for the encapsulation and environmental conditions, which affect its bioavailability, degradability, persistence, and bioaccumulation. Shakiba et al. (2020) in their review summarize various – often contradictory - studies addressing the role of encapsulation in nano-formulated pesticides transport from soils into water. Nevertheless, the present study showed that nanoPRO had a comparable impact on the development of the oyster larvae compared to the active ingredient alone, but it increased the swimming speed of the oyster larvae movement. NanoPRO was less toxic on the molecular level than PRO but kept some comparable toxicity patterns such as the induction of one of the ROS associated genes. As demonstrated by the release experiment, the suspension of nanoPRO is in reality a combination of encapsulated and free PRO (44 % of released PRO in the 10 mg/L dilution in seawater). This might explain the lower direct toxicity of nanoPRO. On the other hand, the nanocapsules themselves may also have an effect – as seen by the increased swimming speed of oyster larvae. Whilst lower toxicity on the molecular level of nanoPRO might indicate lower risk to non-target organisms, it must be noted that the polymeric nanocapsules may

degrade over time releasing the fungicide in a delayed fashion. This might prolong the exposure of non-target organisms, and further research is needed to elucidate potentially associated chronic toxicity to non-target organisms.

5. Conclusion

Our work brings important findings of the effects of environmentally relevant concentrations of the insecticide imidacloprid and fungicide propiconazole (and its nanoformulation) on the early life stages of the Pacific oyster. Imidacloprid caused no effect on the development or behavior of oyster larvae, but had complex and dose-dependent impacts at the molecular level altering scavenger capacity, ROS regulation as well as cell cycle and apoptosis regulation. The embryotoxicity of propiconazole was comparable between the active ingredient alone and its nanoformulation, and abnormal swimming behavior was observed after exposures to environmentally relevant propiconazole concentrations. Gene expression analysis indicated a sub-cellular impact of propiconazole on genes involved in ROS detoxification and decreased mitochondrial metabolism, and these effects were much less pronounced for the nanoformulation of the same active ingredient. In conclusion, the actual environmental concentrations of IMI and PRO in Arcachon Bay in France (in a low range of tens of ng/L) might be considered safe for the development of early life stages of oyster, but the alterations at the molecular level suggest possible sublethal changes of some important biological functions, which should be elucidated by further research which might also consider environmentally relevant mixtures.

CRediT authorship contribution statement

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

Compliance with ethical standards

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

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