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

4
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11 **KEYWORDS**

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

14 **HIGHLIGHTS**

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

20 **Abstract**

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

23 concentrations. This study investigated the impacts of insecticide imidacloprid (IMI) and
24 fungicide propiconazole (PRO), some of the most detected pesticides in the Arcachon Bay in
25 France. This work also included the research of propiconazole nanoformulation (nanoPRO).
26 The effects were assessed studying the development of the early life stages of the Pacific
27 oyster (*Magallana gigas*). Oyster embryos were exposed for 24, 30, and 42 h (depending on
28 the endpoint) at 24 °C to environmentally relevant concentrations of the two pesticides as well
29 as to nanoPRO. The research focused on sublethal endpoints such as the presence of
30 developmental malformations, alterations of locomotion patterns, or changes in the gene
31 expression levels. No developmental abnormalities were observed after exposure to
32 environmental concentrations detected in the Arcachon Bay in recent years (maximal detected
33 concentration of IMI and PRO were 174 ng/L and 29 ng/L, respectively). EC₅₀ of PRO and
34 nanoPRO were comparable, 2.93 ± 1.35 and 2.26 ± 1.36 mg/L, while EC₅₀ of IMI exceeded
35 200 mg/L. IMI did not affect larval behavior. PRO affected larval movement trajectory and
36 decreased average larvae swimming speed (2 µg/L), while nanoPRO increased the maximal
37 larvae swimming speed (0.02 µg/L). PRO upregulated especially genes linked to reactive
38 oxygen species (ROS) production and detoxification. NanoPRO effects on gene expression
39 were less pronounced - half of the genes were altered in comparison with PRO. IMI induced a
40 strong dose-response impact on the genes linked to the detoxification, ROS production, cell
41 cycle, and apoptosis regulation. In conclusion, our results suggest that current pesticide
42 concentrations detected in the Arcachon Bay are safe for the Pacific oyster early development,
43 but they might have a small direct effect via altered gene expressions, whose longer-term
44 impacts cannot be ruled out.

45 **1. Introduction**

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

52 Arcachon Bay is an example of such an ecosystem. It is a macrotidal semi-enclosed marine
53 lagoon on the Atlantic Coast in the South-west of France. Its emblematic organism is the
54 Pacific oyster, *Magallana gigas* also known as *Crassostrea gigas* due to the ongoing
55 disagreement about its name (Bayne et al., 2017), a bivalve mollusk, commercially valuable
56 aquaculture species, and a model organism in marine/brackish ecotoxicology. Its
57 embryo-larval stages are commonly used according to the standardized biotest (NF ISO
58 17244, 2015). The oyster early-life stages are a sensitive and reliable alternative toxicity
59 model with multiple advantages such as transparency of embryo and larvae, quick
60 development, high-throughput screening format, sensitivity to contaminants, internal feeding
61 until the D-larvae stage (Capela et al., 2020), and it complies with the 3Rs Principle (Russell
62 et al., 1959).

63 Numerous hazardous substances are regularly detected in Arcachon Bay, such as pesticides
64 monitored by survey network REPAR ([https://www.siba-bassin-arcachon.fr/actions-](https://www.siba-bassin-arcachon.fr/actions-environnementales/les-reseaux-de-surveillance-repar-et-rempar)
65 [environnementales/les-reseaux-de-surveillance-repar-et-rempar](https://www.siba-bassin-arcachon.fr/actions-environnementales/les-reseaux-de-surveillance-repar-et-rempar)). Pesticides are known for
66 causing adverse effects on non-target estuarine/marine aquatic organisms (Gutiérrez et al.,
67 2019; Parsons et al., 2020; Vignet et al., 2019), at environmentally relevant concentrations
68 (Bechmann et al., 2020; Behrens et al., 2016; Epelboin et al., 2015; Gamain et al., 2018; Mai

69 et al., 2014, 2013). The REPAR monitoring network showed that the insecticide, fungicide,
70 and herbicide with the highest detected average concentration (cf. Table 1) during years 2010-
71 2014 in the Arcachon Bay were imidacloprid, propiconazole (as well as other fungicides like
72 carbendazim and metabolites of dichlofluanid), and herbicide S-metolachlor (Tapie and
73 Budzinski, 2018). The toxicity of S-metolachlor to oyster larvae has already been assessed
74 (Gamain et al., 2017, 2016; Mai et al., 2014, 2013). Imidacloprid (IMI) is a neurotoxic
75 insecticide of the neonicotinoid family which binds agonistically to the post-synaptic nicotinic
76 acetylcholine receptors (nAChRs) and is highly selective for insects (Matsuda et al., 2001). Its
77 use has been banned since 2018 by regulation of the EU Commission (European Commission,
78 2018) except the use in permanent greenhouses; due to the risks to honey bees and other
79 pollinators. However, it is still widely used in other countries in the world (Butcherine et al.,
80 2019). IMI is one of the most detected insecticides in waters usually in a range of hundreds of
81 ng/L (Anderson et al., 2015; Morrissey et al., 2015), which is also the case of Arcachon Bay
82 in France (Tapie and Budzinski, 2018). However, the peak concentrations of imidacloprid in
83 waters might be high as 320 µg/L in the Netherlands (Van Dijk et al., 2013), 3.29 µg/L in
84 California (Starner and Goh, 2012), or 0.26 µg/L in Canada (Main et al., 2014). Several
85 studies reported toxicity to mollusks, but the effects were not evaluated at environmentally
86 relevant conditions corresponding to the Arcachon Bay in France and only high
87 concentrations were used (Dondero et al., 2010; Ewere et al., 2020, 2019a, 2019b; Prosser et
88 al., 2016; Shan et al., 2020). Propiconazole (PRO), a triazole fungicide, stops the fungal
89 growth as it inhibits the demethylation by fungal sterol 14 α -demethylase and thus obstructs
90 the biosynthesis of ergosterol, a component of fungal cell membranes (Zarn et al., 2003). Its
91 occurrence in surface waters all around the world in a range of ng/L to µg/L is well
92 documented (Elfikrie et al., 2020; Papadakis et al., 2018; Quintana et al., 2019; Toan et al.,
93 2013; Van De Steene et al., 2010) with peak concentrations going up to 0.81 µg/L in China

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

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

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

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

120 **2. Materials and methods**

121 **2.1 Chemicals and reference seawater**

122 Imidacloprid (IMI, CAS 138261-41-3, Pestanal, purity 100 %), propiconazole (PRO, CAS
123 60207-90-1, Pestanal, purity 100 %), and CuSO₄ were purchased from Sigma-Aldrich. The
124 stock solution of PRO (5 g/L) was prepared in DMSO and was stored at 5 °C. The stock
125 solution of IMI (200 mg/L) was prepared directly in seawater and was used for testing
126 immediately. The solution of Cu⁺⁺ was used as a positive control and stored at 5 °C (stock
127 solution in milliQ water at 100 mg/L). Exposure solutions were prepared by serial dilution in
128 seawater. Seawater was collected at beach Petit Nice (approx. 44°33'40.3"N 1°14'27.1"W),
129 serially filtered at 0.22 µm, and passed through UV light to eliminate debris and
130 microorganisms. Filtered seawater (FSW) was stored at 5 °C in the dark and was used
131 typically within two days (within 7 days at the latest). It was refiltered through 0.22 µm for
132 solution preparation and oyster spawning. The presence of pesticides and copper in FSW was
133 verified by LC-MS/MS (cf. section 2.7 and 3.1).

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

141 **2.2 Nanopropiconazole**

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

145 modification: Myritol 318, instead of Miglyoil 810, was used as the triglyceride oil. As a
146 control, nanocapsules (nanoC) not containing the active ingredient (verified by LC-MS/MS;
147 the used method was the same as for the nanoformulation mentioned below) were also
148 prepared using the same method. The stock suspension of nanopropiconazole (nanoPRO) with
149 a propiconazole concentration of 325 mg/L (determined by LC-MS/MS as described in
150 section 2.7 after diluting the sample in acetonitrile, centrifuging it through nylon filter –
151 6000 rpm/6 min, and freezing at -20 °C; more details in section 2.7) was stored at ambient
152 temperature in an amber glass vial. The average size (z-average diameter) and the zeta
153 potential of nanoparticles was measured in the stock suspension in milliQ water (diluted
154 100x) by dynamic light scattering (DLS) using Malvern Instruments with software Zetasizer
155 Ver. 6.20 with a detector at a fixed angle of 173° and the average size measurement was
156 repeated by Cordouan Technologies SAS with software NanoQ V2.6.3.0 with a detector at a
157 fixed angle of 135°. Cordouan Technologies SAS was also used to measure the average size
158 of nanoparticles in seawater (only the highest exposure concentrations, 10 mg/L of loaded
159 PRO, was measured due to the power of the laser). Both average size and zeta potential
160 results are expressed as the means of three acquisitions.

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

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

$$171 \quad EE (\%) = \frac{W(\text{associated})}{W(\text{total})} = \frac{W(\text{total}) - W(\text{free})}{W(\text{total})},$$

172 where *EE* stands for encapsulation efficiency, and *W(total)* and *W(free)* for the total and free
173 amount of PRO, respectively. The free amount of PRO was corrected for the loss of
174 propiconazole in the centrifugal ultrafiltration device. In previous studies, the same procedure
175 has been used to determine the EE of pesticides and drugs in PCL nanoparticles (Grillo et al.,
176 2012; Moraes et al., 2011; Pereira et al., 2014).

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

189 **2.3 Test organism**

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

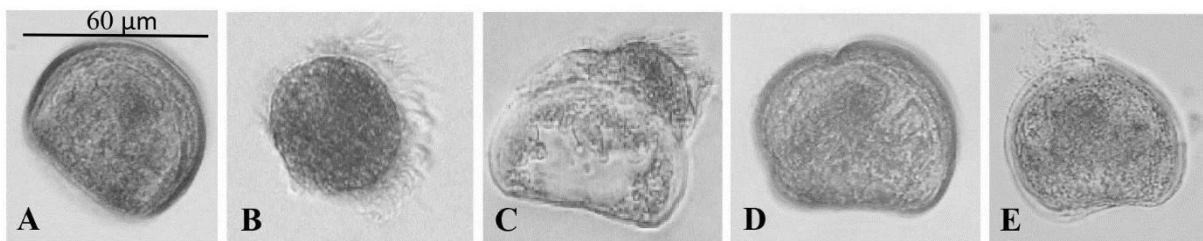
193 (12 °C, 30 min) for the acclimatization and were then subjected to alternating thermal shock
194 in FSW at 18 °C and 28 °C for 30 min. Female spawning was facilitated by adding frozen
195 filtered oyster sperm which contains diantlin (Dupuy et al., 1977). Detailed spawning method
196 is described by Gamain et al. (2016). The embryos were then transferred to experimental units
197 and kept at 24 °C in the dark until they reach the developmental stage of D-larva.

198 **2.4 Embryo-larval test**

199 The embryo-larval oyster test was carried out following the French guideline (NF ISO 17244,
200 2015) with modifications: The embryos were transferred to 24-well microplates (Greiner Bio-
201 One, Cellstar; 225 embryos per well) and were exposed to a wide concentration range of
202 every substance: PRO (20 ng/L, 200 ng/L, 2 µg/L, 20 µg/L, 200 µg/L, 2 mg/L, 10 mg/L),
203 nanoPRO (20 ng/L, 200 ng/L, 2 µg/L, 20 µg/L, 200 µg/L, 2 mg/L, 10 mg/L), IMI
204 (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
205 control nanocarrier (suspension of empty nanocapsules diluted in the same manner as the
206 nanoPRO to get the same amount of nanocapsules in the seven suspension dilutions). Larvae
207 batches obtained from each of the four mature oyster couples were exposed separately for
208 each compound. Each concentration was tested in four replicates, i.e. sixteen replicates of
209 embryos in total (siblings from different parents were never pooled). Negative control (FSW),
210 solvent (DMSO), and nanocarrier control (whenever relevant) were present on every
211 microplate in four replicates as well. The concentration of DMSO (0.00002 %) and nanoC
212 controls corresponded to their concentration in the 2 µg/L solution of PRO and nanoPRO i.e.
213 the highest concentrations used for locomotion and gene expression analysis. Microplates
214 were kept at 24 °C in the dark.

215 At 24 hpf (hours post-fertilization), the microplates were used for the locomotion analysis (cf.
216 section 2.5). After the video capture (approximately at 30 hpf), formaldehyde (25 µL at
217 37 %) was added to every well (final volume 2025 µL), and the microplates were kept at 4 °C

218 until the analysis of developmental malformations was carried out (within 14 days).
 219 Percentage of different developmental malformations (mantle and shell malformation),
 220 arrested development, or well-developed D-shaped larvae (Figure 1) per 100 embryos per
 221 well was determined using an inverted microscope (Nikon Eclipse TS100). For all tests,
 222 validity criteria were fulfilled as follows, malformation rate lower than 20 % in the control,
 223 and EC₅₀ for abnormal larvae exposed to positive control Cu⁺⁺ between 6 and 16 µg/L (tested
 224 concentration range: 0-5-10-20-50 µg/L).



225 **Figure 1** Different types of developmental malformations of oyster larvae (*Magallana gigas*)
 226 at 30 hpf: well-formed D-larva (A), developmental arrest (B), mantle malformation (C),
 227 (scalloped) shell malformation (D), (concave) shell malformation (E).
 228

229 2.5 Locomotion analysis

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

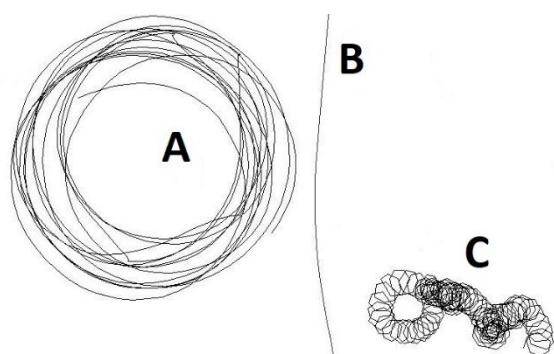
234 **Table 1** Concentrations of pesticides of interest detected at different sampling points in
 235 Arcachon Bay during the years 2010-2014. N=669 for each pesticide. Concentrations were
 236 calculated according to the data of Tapie et al., (2018).

	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

237

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



252

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

255 **2.6 Oyster exposures for analysis of gene expressions**

256 To collect enough RNA for the analysis, 500,000 embryos (originated from one oyster
257 couple) were incubated in three-liter glass beakers (exposure to PRO and nanoPRO) or plastic
258 bottles (exposure to IMI) at 24 °C in the dark for 42 hours. For oxygenation and for keeping
259 embryos suspended in the water column, solutions were aerated with aquarium airstones.
260 Dissolved oxygen was checked at the beginning and the end of the tests. The concentrations
261 of the exposure solutions were the same as for the locomotion analysis: PRO and nanoPRO
262 (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
263 were collected on a 20 µm mesh (SEFAR NITEX®) using a vacuum pump, resuspended in 5
264 mL of exposure solution, and kept on ice. Their concentration was calculated immediately and
265 five replicates, each containing 30,000 larvae, were collected in 1.5 mL polypropylene
266 microtubes tubes. These replicates were centrifuged (2 min, 1000 rpm) and the larvae pellet
267 was resuspended in 500 µL of RNA later. Samples were then kept at -80 °C until RNA
268 extraction.

269 The total RNAs were extracted using the SV Total RNA Isolation System Kit (Promega).
270 Samples were first homogenized using vortex and 200 µL of glass beads (0.10 – 0.11 mm,
271 acid washed, B. Braun Biotech International) in 500 µL of RNA Lysis buffer, and centrifuged
272 (7,500 rpm, 1 min). Lysed samples were collected, 500 µL of phenol-chloroform-isoamyl
273 alcohol (25-24-1) was added, and the tubes were vortexed. Centrifugation (13,500 rpm,
274 5 min) divided the samples into two phases and the upper (aqueous) was collected, mixed
275 with 450 µl of 75 % ethanol, vortexed, and transferred onto a spin column following
276 manufacturer's instructions with few modifications as follows: RNA samples were treated
277 with DNase I mixture for 15 min at 37 °C, and purified RNAs were collected in 50 µL of
278 Nuclease-Free water. The concentration and purity of collected RNA samples were checked
279 spectrophotometrically at 260/280 nm with software Gen5 (Biotek), using a

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

285 QPCR was carried out with the GoTaq® qPCR Master Mix kit (Promega) on a
286 LightCycler® 480 (Roche). QPCR mix was composed of 1 µL of cDNA, 2 µL of a mix
287 containing each reverse and forward primer (2 µM), and 10 µL of 2x GoTaq master mix,
288 which was completed with nuclease-free water to the final volume of 20 µL. Fourteen genes
289 in total were selected to evaluate the effects of chosen pesticides on mitochondrial metabolism
290 (*12S*, *cox1*), regulation of the cell cycle and apoptosis (*p53*), oxidative stress defense (*cat*,
291 *sodMn*, *sodCu/Zn*, *gpx*), detoxification (*mt1*, *mt2*), apoptosis (*bax*, *casp3*), biotransformation
292 (*cyp1a*), growth arrest and DNA damage (*gadd45*), and DNA repair (*rad51*). Three reference
293 genes were used in the analysis (*β-actin*, *elf1a*, and *rpl7*). The genes were chosen to evaluate
294 non-specific toxicity and general responses of oyster larvae to pollutant stress, and to
295 correspond with the studies of Mai et al. and Gamain et al. referenced in this publication.
296 Sequences, references, and accession numbers are presented in Supplementary Table S1.
297 Primers were purchased from Sigma proligo. Primer-pairs efficiencies for all genes were
298 verified to be higher than 95 %. The PCR procedure was as follows: the pre-incubation step
299 lasted 2 min at 95 °C, then the amplification consisted of 50 cycles with each cycle at 95 °C
300 for 15 s and 60 °C for 1 min. The melting curve continued at 95 °C for 30 s, at 60 °C for 2
301 min, and 95 °C until the next cycle.

302 Melting curves of every reaction were verified to assess reaction specificity. All data were
303 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

307 **2.7 Chemical analysis and water quality**

308 Salinity, pH, and dissolved oxygen were measured at the beginning and the end of the
309 experiments, using Multi 340i probe (WTW). Oxygen saturation was always higher than
310 91.6 % (average 94.5 ± 1.9 %), salinity varied between 33.5 and 35.3 psu (practical salinity
311 unit) on the first day of the test and between 34.7 and 36.0 psu on the last day of the test
312 (higher values at the end of the test may be caused by evaporation and concentration of the
313 seawater solutions), and pH ranged from 7.95 to 8.2. The measured parameters complied with
314 the revised standard as reported by Leverett and Thain (2013).

315 Concentrations and stability of used chemical substances were verified using LC-MS/MS, as
316 described in detail in Supplementary Material S2. Before the chemical analyses, samples were
317 processed as follows: samples from the gene expression experiments were taken at the
318 beginning (30 min after the addition of the chemical in the experimental unit with the aeration
319 device) and the end of tests (at 42 h). Samples for assessing the stability of compounds in the
320 microplates (i.e. developmental malformations and locomotion analysis tests) were taken at
321 the beginning and the end of the test at 24 h. All samples and calibration solutions were stored
322 at -20 °C and spiked with 10 μ L of the internal standard of tebuconazole D6 and imidacloprid
323 D4 (both dissolved in 50 % methanol). The tebuconazole D6 is used as a standard for
324 conazole analysis in multi-parameter analyses. The samples (1.5 mL) and calibration solutions
325 were lyophilized using a freeze dryer Alpha 2-4 LD Plus (Martin Christ Freeze Dryers). After
326 the lyophilization, the samples were dissolved in 1 mL of 100 % acetonitrile, vortexed for
327 30 sec, ultrasonicated for 5 min, vortexed again (30 sec), and centrifuged (12,000 rpm, 10 °C,
328 10 min) in order to precipitate the salts and move the analyte to the solvent phase. The
329 supernatant (500 μ L) was transferred into a glass vial and evaporated using the nitrogen. The
330 evaporated vial was carefully filled with 0.75 mL of 20 % acetonitrile, vortexed, and stored at
331 -20 °C upon analysis by LC-MS/MS.

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

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

348 **2.8 Data analysis**

349 Relative abundance (%) of malformed larvae (sum of all types of abnormal development i.e.
350 developmental arrest, mantle, and shell malformations) and the results (%) of trajectory
351 analyses were first transformed using arcsine transformation $p' = \arcsin \sqrt{\frac{p}{100}}$ (Sokal and
352 Rohlf, 2012), and checked for normality (Shapiro-Wilk test; $P > 0.01$) and homoscedasticity
353 (Levene test; $P > 0.05$). If confirmed, ANOVA ($P < 0.05$) followed by Tukey post-hoc test
354 was used. In the opposite case, non-parametric Kruskal-Wallis ($P < 0.05$) with Mann-Whitney
355 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 $\mu\text{m}/\text{sec}$ (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.

369 **3. Results**

370 **3.1. Exposure and chemical analysis**

371 The concentration of copper in FSW used for the preparation of exposure solutions was
372 2.4 ± 0.8 $\mu\text{g/L}$ (maximal concentration measured was 3.3 $\mu\text{g/L}$). IMI was stable in the
373 microplate over 24 h in all tested concentrations (98.6 ± 2.3 %). Whereas PRO, a hydrophobic
374 compound, did adsorb on the plastic microplate walls (maximal loss of 24 % of the compound
375 at the lowest tested concentration 20 ng/L). Thus, the microplates were precoated with the
376 appropriate concentration of propiconazole the day before the experimentation and the
377 exposure solution was renewed an hour before the test. The recovery of propiconazole after
378 24 hours in precoated microplates was 101.6 ± 3.7 %. The microplate precoating was also
379 used for the tests with nanoPRO (precoated by suspensions of nanoPRO).

380 The concentration of pesticides in all non-exposed variants was found to be either below the
381 limit of detection, either as non-quantified. IMI, in treatments for the gene expression assay,
382 was stable during the 42-hour long test (concentration at the end of the test was between
383 101.4 % and 107.3 % of the initial concentration) except for one of the three replicates of the
384 concentration of 10 $\mu\text{g/L}$, where increase by 54 % was recorded. PRO, on the other hand, was
385 not stable, and the recovery at the end of 42 h exposure varied from 0 to 100 %.
386 Unfortunately, for practical reasons, it was not possible to precoat the beakers, as it was done
387 for the microplates and nominal concentrations are reported but taking this caveat into
388 account in the discussion. Complete results are shown in Supplementary Table 2.

389 **3.2 Nanopropiconazole and nanocarrier characterization**

390 The average size (z-average diameter) and zeta potential of nanoparticles containing
391 propiconazole and nanocarrier in the stock suspensions dispersed in milliQ water, and diluted
392 100x, were measured to assess the suspension stability. After the synthesis, the particle

393 diameter in the nanoPRO and nanoC stock suspensions were 301.1 ± 1.8 nm and
394 263.4 ± 1.0 nm, respectively. The zeta potential measured in the nanoPRO and nanocarrier
395 stock suspensions was -35.3 ± 0.9 mV and -35.7 ± 0.3 mV, respectively. Three months later,
396 the properties of stock suspensions were again analyzed to check the behavior of
397 nanoformulations in seawater, finding the z-average diameter of particles in nanoPRO and
398 nanocarrier stock suspensions in milliQ water (diluted 100x) to be 326.4 ± 9.1 nm and
399 282.7 ± 3.3 nm, respectively. The slightly higher values may be caused by different
400 instruments used (as indicated in section 2.2). The results confirm that the suspension is
401 stable, and no aggregates of nanoparticles were formed (aggregates would have the values
402 twice the initial size). The size of particles in the nanoformulations diluted in FSW was also
403 measured at 0 and 24h to imitate the embryo-larval biotests in microplates. Due to the power
404 of the laser, only the highest tested concentration (10 mg/L) was analyzed. The z-average of
405 nanoPRO particles at 0 h and 24 h was 371.5 ± 7.8 nm and 404.0 ± 1.5 nm, respectively and
406 the z-average of nanoC particles at 0 h and 24 h was 349.4 ± 4.2 nm and 365.1 ± 2.6 nm,
407 respectively.

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

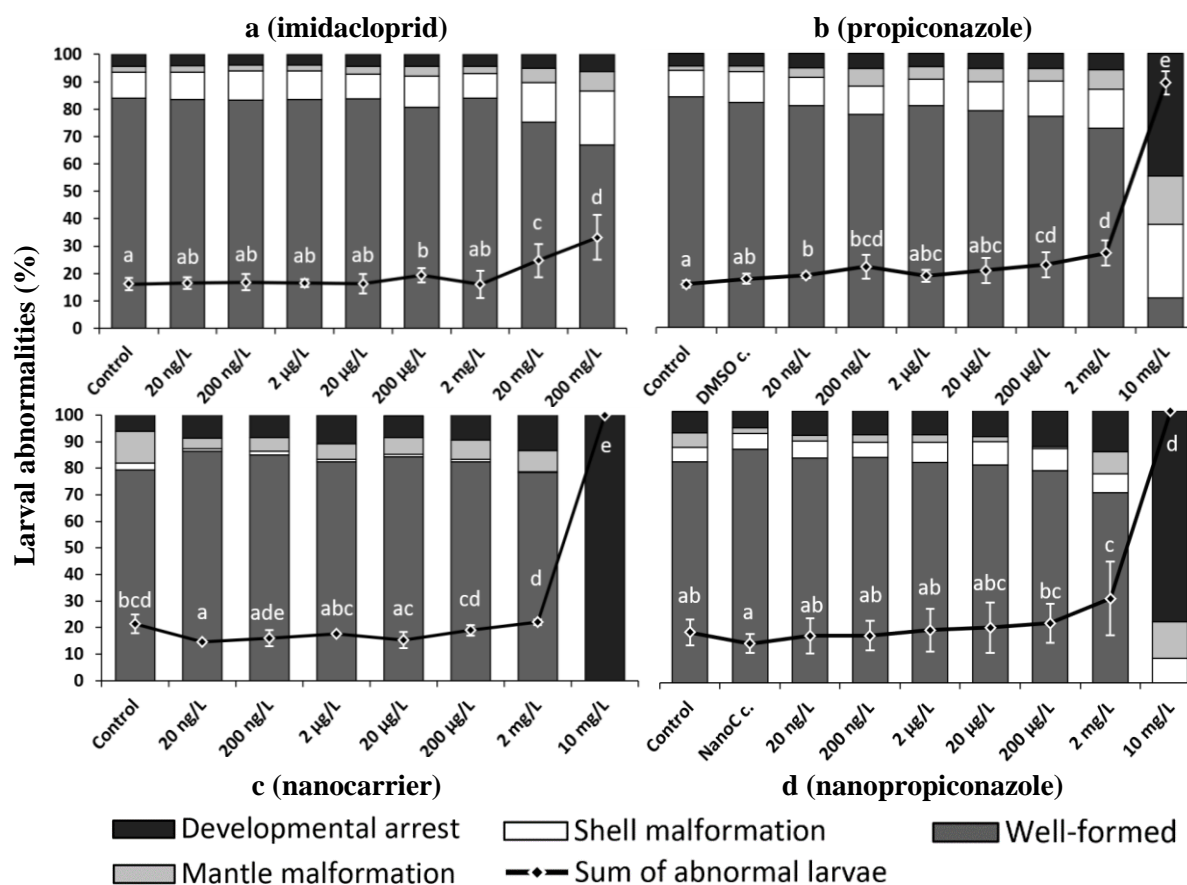
414

415 **3.3 Embryo-larval development**

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

418 Frequency (%) of developmental malformations and developmental arrests of oyster larvae
419 exposed for 30 h to increasing concentrations of pesticides are shown in Figure 3. Moreover,
420 the sum of abnormal larvae is shown for every exposure condition. These abnormal larvae
421 proportions served for the calculations of effective concentrations (EC_x), no observable effect
422 concentration (NOEC), and the lowest observable effect concentration (LOEC), which are
423 shown in Table 2. In general, IMI, PRO, and nanoPRO had comparable toxicity patterns since
424 the developmental toxicity to oyster larvae occurred only at high concentrations (at and above
425 200 $\mu\text{g/L}$), whereas no effect was observed after exposure to environmental concentrations of
426 IMI and PRO. The highest tested concentration (10 mg/L) of nanoPRO malformed or arrested
427 development of all larvae and PRO affected $89.3 \pm 4.3\%$ of individuals. On the contrary,
428 even the highest tested concentration of IMI (200 mg/L) affected only $33.1 \pm 8.2\%$ larvae.
429 Conversely, nanoC did not cause developmental malformations irrespectively of
430 concentration and only the highest tested concentration (10 mg/L) induced developmental
431 arrests (Figure 3), thus suggesting that whereas nanocarrier caused only developmental
432 arrests, it was the propiconazole inside the capsules which was responsible for the
433 developmental malformations.

434



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) \pm 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 μ g/L, respectively). These values were within the range of μ g/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.

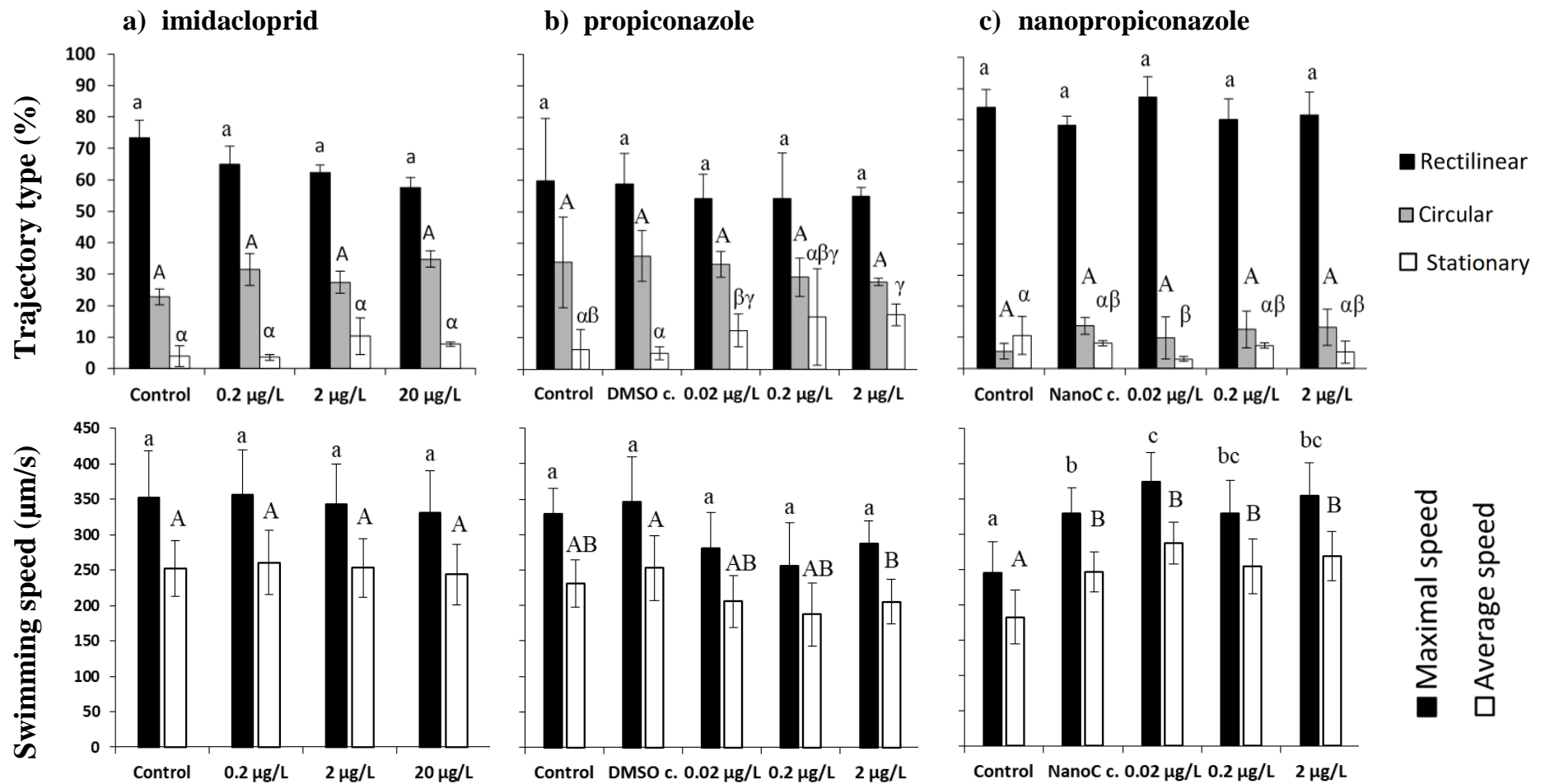
446 **Table 2** Effective concentrations (EC₂₀, EC₃₀, EC₅₀), no observable effect concentration
 447 (NOEC), and lowest observable effect concentration (LOEC) after 30 h-long exposure of
 448 oyster larvae to imidacloprid, propiconazole, nanopropiconazole, and nanocarrier.
 449 Imidacloprid did not reach EC₅₀ 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
	EC₅₀ ± SE	> 200	2.93 ± 1.35	2.26 ± 1.36	2.84 ± 1.41
mg/L	EC₂₀	6.43	0.73	0.56	0.71
	EC₃₀	70.50	1.26	0.97	1.22

450 **3.4 Locomotion analysis**

451 The locomotion analysis consisted of trajectory type and maximal and average speed
 452 assessment (Figure 4). The non-exposed larvae generally displayed rectilinear swimming
 453 trajectories (72.3 ± 9.9 %), less commonly the circular trajectory type (20 ± 11.7 %), and
 454 rarely they stayed stationary (6.9 ± 2.8 %). Maximum and average swimming speed employed
 455 by the non-exposed larvae were 309.3 ± 45.7 µm/s and 222.2 ± 29.1 µm/s, respectively, even
 456 though high variability was observed between the individuals. No statistically significant
 457 effects on swimming speed or trajectory were observed for IMI, but the frequency of
 458 rectilinear trajectories showed a decreasing tendency with increasing concentration (73.3 –
 459 65.0 – 62.3 – 57.6 %) and the increase of the circular type (22.8 – 31.4 – 27.4 – 34.8 %). A
 460 statistically significant increase in stationary swimming patterns was observed in larvae
 461 exposed to 2 µg/L of PRO (17.3 ± 3.4 %) when compared to other treatments. The same
 462 effect was also observed in larvae exposed to an environmentally relevant concentration of
 463 0.02 µg/L (12.4 ± 5.1 %) when compared to the DMSO control (5.2 ± 2.1 %). Similarly, as
 464 for PRO, nanoPRO caused no effects on the frequency of rectilinear and circular trajectories.

465 No effect on larvae swimming speed was observed after exposure to IMI. In contrast, PRO at
466 2 $\mu\text{g/L}$ did cause a statistically significant decrease ($205.1 \pm 31.4 \mu\text{m/s}$) in average swimming
467 speed in comparison to the DMSO control ($252.8 \pm 46.0 \mu\text{m/s}$). NanoPRO, unlike PRO,
468 caused significant effects on both maximal as well as the average swimming speed. First, the
469 maximal and average swimming speed of nanoC control ($330.5 \pm 35.4 \mu\text{m/s}$ and 246.8 ± 28.3
470 $\mu\text{m/s}$ respectively) significantly differed from the non-exposed control ($246.0 \pm 43.4 \mu\text{m/s}$
471 and $182.9 \pm 38.7 \mu\text{m/s}$ respectively). Furthermore, the low concentration of $0.02 \mu\text{g/L}$ of
472 nanoPRO caused an even higher increase in maximal speed ($374.2 \pm 42.1 \mu\text{m/s}$) statistically
473 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) \pm SD. DMSO c. = DMSO control; NanoC c. = nanocarrier control.

477 **3.5 Gene expression analysis**

478 Gene expression results of fourteen pre-selected genes are shown in Table 3 as fold changes
479 between the studied and three housekeeping genes. Expressions of *bax*, *cat*, *cox*, *cyp1a*, and
480 *gpx* did not differ between pesticide-exposed oysters and controls. The expression of
481 mitochondrial gene 12S RNA (*12S*) was significantly downregulated after exposure to
482 200 ng/L and 2 µg/L of PRO. PRO 200 ng/L caused also upregulation of *mt1*, a gene
483 associated with detoxification, and 2 µg/L caused downregulation of *rad51*, a gene coding for
484 a protein involved in DNA reparation. Finally, low concentrations of 20 and 200 ng/L
485 significantly upregulated expression of copper/zinc superoxide dismutase (*sodCu/Zn*), i.e. one
486 of the four studied genes implicated in oxidative stress defense. In contrast, nanoPRO altered
487 the expression of fewer genes than PRO. Similarly to PRO, a low concentration of 20 ng/L
488 nanoPRO upregulated the gene *sodCu/Zn*. However - unlike PRO - nanoPRO exposure also
489 upregulated another oxidative stress defense gene *sodMn* (200 ng/L) and downregulated
490 *gadd45*, linked to the growth arrest and DNA damage. IMI had the strongest disruptive effect
491 of the tested pesticides and affected the expression of 8 genes: as with the fungicides, IMI
492 upregulated *sodCu/Zn* (100 µg/L) but downregulated *sodMn* (1 µg/L and 100 µg/L). 100 µg/L
493 of IMI also downregulated *casp3* and transcription factor *p53*, genes linked to apoptosis and
494 cell cycle regulation. Genes *mt1* and *mt2* coding for two metallothioneins that are involved in
495 protection against oxidative stress were strongly upregulated by IMI at 10 and 100 µg/L.
496 Downregulation of *rad51* was observed at 10 µg/L and *gadd45* was upregulated at 1 µg/L and
497 10 µg/L of IMI.

498

499 **Table 3** Expression of fourteen studied genes (relative to three housekeeping genes) involved in mitochondrial metabolism (*12S*, *cox*), regulation
500 of the cell cycle/apoptosis (*p53*), oxidative stress defense (*cat*, *sodMn*, *sodCu/Zn*, *gpx*), detoxification (*mt1*, *mt2*), apoptosis regulation (*bax*,
501 *casp3*), biotransformation (*cyp1a*), growth arrest and DNA damage (*gadd45*), and DNA repair (*rad51*) in oyster larvae exposed for 72 h to
502 different concentrations of imidacloprid, propiconazole, and nanopropiconazole. Pesticide treatments indicated by nominal concentrations;
503 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
504 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
<i>12S</i>	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
<i>bax</i>	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
<i>casp3</i>	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
<i>cat</i>	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
<i>cox</i>	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
<i>cyp1a</i>	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
<i>gpx</i>	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
<i>mt1</i>	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
<i>mt2</i>	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
<i>p53</i>	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
<i>sodCu/Zn</i>	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
<i>sodMn</i>	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
<i>gadd45</i>	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
<i>rad51</i>	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

505 **4. Discussion**

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

510 IMI had mild effects on the development of oyster larvae. The environmentally relevant
511 concentrations detected in the Arcachon Bay in France did not cause developmental
512 abnormalities, even though some mild toxic effects on development were observed at
513 200 µg/L or higher concentrations, which were detected for instance in the Netherlands as
514 reported by Van Dijk et al., (2013). To the best of our knowledge, this is the first published
515 study assessing IMI toxicity on the early-life stages of a marine bivalve species. Prosser et al.
516 (2016) investigated IMI toxicity on early-life stages of freshwater mollusks and showed low
517 sensitivity of the freshwater mussel *Lampsilis fasciola* (no effect on viability at 1 mg/L) and
518 mild effect on the freshwater pulmonate gastropod *Planorbella pilsbryi* (LC₁₀ about
519 800 µg/L). In our study, lethal effects are represented by developmental arrests, which,
520 however, were not affected up to the highest tested IMI concentration of 200 mg/L. Few
521 studies evaluated the IMI toxicity on adult bivalves. Shan et al. (2020) reported that chronic
522 (30 d) exposure to 2 mg/L of IMI caused sublethal histological changes in adult freshwater
523 clam (*Corbicula fluminea*), including degeneration of digestive tubules and contractions and
524 adhesions in the hemolymphatic vessels. The concentration of 2 mg/L of IMI did not cause
525 any mortality of adult Sydney rock oysters (*Saccostrea glomerate*) in a recent study of Ewere
526 et al. (2019a).

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

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

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

554 simultaneously with both metallothionein proteins *mt1* and *mt2* (100 µg/L of IMI), which
555 seems interesting considering that metallothionein proteins also regulate copper and zinc
556 metabolism (Krężel and Maret, 2017). While copper/zinc superoxide dismutase is cytosolic
557 and extracellular, the manganese superoxide dismutase is found in mitochondria (Miller,
558 2012). Thus, suspected ROS production caused by imidacloprid exposure could have been
559 limited to the cytoplasm, which might correspond to the known role of ROS in neonicotinoid
560 toxicity as described in the recent review on neonicotinoid impact on oxidative stress (Wang
561 et al., 2018). Corresponding to these findings, Ewere et al., (2020) detected signs of oxidative
562 stress in adult Sydney rock oysters such as upregulation of proteins implicated in oxidative
563 stress after exposure to 10 µg/L of IMI or elevated presence of glutathione-S-transferase
564 (GST) in hemolymph after exposure to 100 µg/L of IMI (GST is an enzyme protecting against
565 xenobiotics such as ROS).

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

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

579 was diluted in FSW. However, this could be related to the presence of ions in the seawater
580 that could also affect DLS analysis. Overall, both nanoformulations remained stable without
581 the formation of aggregates over time. The zeta potential values were greater than ± 30 mV,
582 which means that the surface charge is high enough to produce a strong repulsive interaction
583 between the particles and to avoid the forming of aggregates (Tamjidi et al., 2013). In
584 addition, our nanoformulations contain a surfactant (PVA) that can be adsorbed on the surface
585 of nanoparticles, preventing the aggregation of particles by steric effect (de Oliveira et al.,
586 2015). In general, the contribution of the steric effect in the colloidal stabilization is more
587 important than the electrostatic effect, therefore the stability of the nanoformulations of this
588 work cannot be attributed to the surface electrostatic repulsion but to steric hindrance
589 (Bhattacharjee, 2016; de Oliveira et al., 2015).

590 PRO and its nanoformulation induced more significant effects on the development of oyster
591 larvae compared to IMI. Although LOECs were identical, PRO and nanoPRO caused greater
592 effects at higher concentrations. Fractions of abnormal larvae increased only at the highest,
593 yet not environmentally relevant concentrations measured in the Arcachon Bay in France and
594 elsewhere in the world as referenced in the introduction. As non-target organisms, Pacific
595 oyster larvae seem to be more sensitive to propiconazole toxicity than freshwater mussels.
596 Bringolf et al. (2007) reported the EC_{50} of propiconazole around 20 mg/L for acute (24 and
597 48 h) toxicity test with glochidia of mussel *Lampsilis siliquoidea*, and EC_{50} of 10 mg/L for
598 96 h toxicity test with juveniles of the same species. The propiconazole toxicity observed in
599 the present study is comparable to that of other early-life stages of non-target aquatic species
600 e.g. LC_{50} of 20.4 mg/L for zebrafish (*Danio rerio*) in a 5-day-long test (Coors et al., 2012)
601 and LC_{50} of 5 mg/L for water fleas *Daphnia magna* in a 48 h-long test (Kast-Hutcheson et al.,
602 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 μ M (3.4 mg/L) of PRO but no effect was seen at
610 0.1 μ M (34 μ g/L), a concentration still 17-times higher than the highest tested in the present
611 study (2 μ g/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 μ g/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

628 trigger ROS production as shown previously (Li et al., 2011; Nesnow et al., 2011). On the
629 other hand, repression of *I2S* suggests an impact on the mitochondrial metabolism and
630 mitochondria count. However, according to the literature, environmental pollution by metals
631 or pesticides is often accompanied by upregulated *I2S* keeping the mitochondria quantity and
632 maintaining the level of ATP necessary when facing chemical stress (Kim Tiam et al., 2012;
633 Moisset et al., 2015).

634 The present study aimed to investigate the sublethal effects of exposure to IMI and PRO at
635 low environmental concentrations (complemented with higher concentrations to establish
636 EC₅₀ for the developmental malformations). However, only rough estimations are possible for
637 nanoPRO because it has not (yet) been used in the field. Prediction and impacts of any
638 nanopesticide in aquatic environments depend on various factors such as the type of the
639 polymer used for the encapsulation and environmental conditions, which affect its
640 bioavailability, degradability, persistence, and bioaccumulation. Shakiba et al. (2020) in their
641 review summarize various – often contradictory - studies addressing the role of encapsulation
642 in nano-formulated pesticides transport from soils into water. Nevertheless, the present study
643 showed that nanoPRO had a comparable impact on the development of the oyster larvae
644 compared to the active ingredient alone, but it increased the swimming speed of the oyster
645 larvae movement. NanoPRO was less toxic on the molecular level than PRO but kept some
646 comparable toxicity patterns such as the induction of one of the ROS associated genes. As
647 demonstrated by the release experiment, the suspension of nanoPRO is in reality a
648 combination of encapsulated and free PRO (44 % of released PRO in the 10 mg/L dilution in
649 seawater). This might explain the lower direct toxicity of nanoPRO. On the other hand, the
650 nanocapsules themselves may also have an effect – as seen by the increased swimming speed
651 of oyster larvae. Whilst lower toxicity on the molecular level of nanoPRO might indicate
652 lower risk to non-target organisms, it must be noted that the polymeric nanocapsules may

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

656 **5. Conclusion**

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

673

674 **CRedit authorship contribution statement**

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

683 **Compliance with ethical standards**

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

685

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