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1 **An environmentally realistic pesticide and copper mixture impacts embryonic**  
2 **development and DNA integrity of the Pacific oyster, *Crassostrea gigas***

3

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11

12 **Abstract**

13 Frequent occurrences of pesticides in the environment have raised concerns that combined  
14 exposure to these chemicals may result in enhanced toxicity through additive or synergistic  
15 interaction between compounds. Spermatozoa and embryos of the Pacific oyster, *Crassostrea*  
16 *gigas*, were exposed to different concentrations of a pesticide mixture with and without  
17 copper, mimicking the cocktail of pollutants occurring in the oyster culture area of Arcachon  
18 Bay. For the 1X exposure condition, measured concentration corresponds to a total  
19 concentration of 1.083  $\mu\text{g L}^{-1}$  for the mixture of 14 pesticides and to 6.330  $\mu\text{g L}^{-1}$  for copper  
20 (Cu). Several endpoints, including larval abnormalities, DNA damage to spermatozoa and  
21 embryo and gene expression in D-larvae were investigated. Results demonstrated that  
22 pesticide mixtures in combination with or without copper induced a dose-dependent increase  
23 in embryotoxic and genotoxic effects on D-larvae from the lowest tested dose of 0.1X.  
24 Transcription of genes involved in anti-oxidative stress (*cat*), respiratory chain (*coxI*), metal  
25 detoxification (*mt1* and *mt2*) and cell cycle arrest and apoptosis (*p53*) was found to be  
26 significantly downregulated while the xenobiotic biotransformation gene *gst* was significantly  
27 upregulated in embryos exposed to pesticide mixture with and without Cu. These findings  
28 raise the question of the possible impacts of mixtures of pesticides and metals on wild or  
29 farmed oyster populations from polluted coastal marine areas.

30 **Keywords:** pesticide mixture, copper, gene expression, genotoxicity, embryotoxicity, Pacific  
31 oyster

32

33

## 34 **1. Introduction**

35 For several decades, coastal ecosystems have been subjected to increased copper (Cu) and  
36 pesticide contamination, mainly from agricultural practices and antifouling paints used on  
37 boats (Martinez et al, 2001; Konstantinou et Albanis 2004). About 4.6 million tons of  
38 pesticides are applied yearly into the environment throughout the world (Ansari et al, 2014)  
39 and many of them inadvertently are discharged in aquatic ecosystems. Some pesticides are  
40 hydrophobic compounds, which tend to bind to suspended particulates and dissolved organic  
41 matter, accumulating in sediments and aquatic biota (Brooks et al, 2007; Thomas and Brooks  
42 2010). Others are more hydrophilic and less persistent in aquatic ecosystems (Lamoree et al,  
43 2002). Pesticides are found in water bodies such as lakes, rivers, streams and other surface  
44 waters that support aquatic life (Gilliom 2007); as a consequence, the receiving ecosystem is  
45 invariably contaminated with multiple pesticides. Since at least the 19<sup>th</sup> century, Cu has been  
46 used as a fungicide in vineyards (Komarek et al, 2010). Copper is also used in boat hull  
47 painting as a replacement for tributyltin (TBT) (Konstantinou et Albanis, 2004). As a  
48 consequence Cu can be found at concentrations up to 2000  $\mu\text{g g}^{-1}$  in sediment within severely  
49 polluted areas (Bryan and Langston 1992; Legorburu and Canton 1991). Concentrations of  
50 pesticides and copper measured in surface water and bed sediment frequently exceeded water-  
51 quality benchmarks for aquatic life (Geffard et al, 2002; Gilliom 2007). Several previous  
52 toxicity studies have demonstrated that pesticides and trace metals such as Cu can impair  
53 water quality and cause adverse effects on aquatic species, in particular at the early  
54 developmental stages of invertebrates (Akcha et al. 2012; Mai et al. 2012, Manzo et al. 2006).  
55 However, these mainly focus on the impacts of individual pesticides, but few data are  
56 available on the toxicity of mixtures of pesticides in combination with or without copper to  
57 marine molluscs. The exposure of animals in ecosystems to pesticides is never limited to a  
58 single compound, but to a complex mixture of chemicals from a variety of sources. Therefore,  
59 the frequent co-occurrence of pesticides and copper in the environment has raised concern  
60 over the combined exposure to these chemicals that would result in toxicity modulation due to  
61 synergistic, additive or antagonistic effects of chemicals (Monosson 2004).

62 Assessing the toxicity of pesticides in mixtures has been an enduring challenge in  
63 environmental health research for the past few decades (Monosson 2004). Interaction effects  
64 can be seen when small and statistically insignificant effects of separate compounds are added  
65 to induce statistically significant effects when these compounds are mixed (Gagnaire et al.

66 2006). Indeed, one chemical can affect the toxicity of others not only through molecular  
67 interactions but also by influencing their adsorption, distribution and excretion,  
68 biotransformation, and bioavailability (Altenburger et al. 2003). The persistence of a number  
69 of pesticides may be changed when used in combination with other pesticides (Vischetti et al.  
70 1996). The toxic effect of multiple chemicals has been recognized as an important factor in  
71 ecotoxicology, because mixtures can have a greater negative impact than the individual  
72 constituents of the mixture (Hernando et al. 2003, Faust et al. 2001, Poletta et al. 2011).  
73 Indeed, the effect of a mixture of pesticides on the phagocytosis competence of *C. gigas*  
74 haemocytes was truly demonstrated, while no effect was induced when compounds were  
75 tested separately (Gagnaire et al. 2007, Gagnaire et al. 2006). At present, few studies have  
76 investigated pesticide mixture or heavy metal mixture toxicity on aquatic species (Gagnaire et  
77 al. 2007; Hernando et al. 2003; Junghans et al. 2003; Manzo et al., 2008; Verslycke et al.  
78 2003), but no study has focused on the effects of pesticide mixture in combination with  
79 copper. The risk assessment process is complicated by the fact that environmental exposure  
80 frequently involves mixtures of chemicals rather than a single compound.

81 Embryotoxicity and genotoxicity (comet assay) assays have been widely used to estimate  
82 the deleterious effects of contaminants on bivalve mollusc larvae and embryos (His et al.  
83 1999). Indeed, oyster embryos and larvae have been proposed as model organisms for marine  
84 ecotoxicological tests (Geffard et al. 2002; His et al. 1999; Wessel et al. 2007) because of the  
85 year-round availability of fertilized eggs from adult breeding *Crassostrea gigas* oysters.  
86 However, to our knowledge, few papers have investigated molecular responses in terms of  
87 gene expression in oyster larvae and embryos exposed to pollutants (Huong et al, 2014). It is  
88 well known that combined measurement of biomarkers can offer more complete and  
89 biologically more relevant information on the potential impact of contaminants on the health  
90 of organisms (van der Oost et al. 1996). In this respect, the measurement of a large panel of  
91 biomarkers in oyster larvae may constitute a useful tool to understand the modification of  
92 oyster physiology due to the environment. In the present study, *C. gigas* embryos or sperm  
93 were exposed to environmentally realistic mixtures of pesticides both with and without added  
94 copper. These mixtures were representative of those detected in Arcachon Bay (Auby et al.  
95 2007; Budzinski et al. 2011; Diepens et al. 2017; Gamain et al. 2016, 2017a, b) and responses  
96 were investigated from the molecular level to the individual level.

97

## 98 **2. Materials and methods**

99 *2.1 Chemicals and seawater*

100 Fifteen reference toxicants (14 pesticides and CuSO<sub>4</sub>: Table 1), formalin and DMSO  
101 (dimethyl sulfoxide) with purity greater than 96% were purchased from Sigma-Aldrich  
102 Chemical (St. Quentin Fallavier, France). Dispase II, Triton X-100, low melting point (LMP)  
103 agarose, normal melting point (NMP) agarose, and MEM-alpha (Minimum Essential  
104 Medium) were purchased from Gibco (Invitrogen, Cergy Pontoise, France). Formic acid  
105 (HCOOH), ammonium acetate and organic solvents (methanol -MeOH- and acetonitrile -  
106 ACN- ULC grade) were purchased from Biosolve (Dieuze, France).

107 Seawater was collected from Grand Banc station in the Arcachon Bay (SW France) on  
108 September 2015, an area with a naturally reproducing population of oysters. Immediately  
109 after sampling, seawater was filtered at 0.2 µm to eliminate debris and microorganisms.  
110 Salinity was measured at 33 using a salinity probe (Wissenschaftlich Technische Werkstätten  
111 Multi 340i, sonde TetraCon 325). Filtered seawater (FSW) was stored at 4 °C in darkness and  
112 was used within 3 days. A few hours before experiment, FSW was filtered again at 0.2 µm.

113

114 *2.2 Animals*

115 Mature oysters (*Crassostrea gigas*, Thunberg, 1793) came from a commercial hatchery  
116 specialized in the production of mature oysters year-round (Guernsey Sea Farms, UK).  
117 Oysters were kept at around 10 °C for transportation and then acclimatized in FSW before the  
118 beginning of experiments. All oysters were used within 3 days.

119

120 *2.3 Preparation of pesticide and copper solutions*

121 Stock solutions (100, 250 or 1,000 mg L<sup>-1</sup>) were prepared in either dimethyl sulfoxide  
122 (DMSO), or acetonitrile, or milli-Q water depending on their solubility characteristics. Water  
123 was spiked with a mixture of 14 pesticides only (PM) or the same pesticide mixture in  
124 combination with copper (PM+Cu), or copper only (Cu) in a concentration range of 0 (solvent  
125 control), 0.1X, 1X, 10X and 100X with 1X representing the environmental concentration of  
126 pesticides mixture (for a total nominal concentration of 1.557 µg L<sup>-1</sup> and 2 µg L<sup>-1</sup> for copper )  
127 in Arcachon Bay (Auby et al. 2007; Budzinski et al. 2011; Diepens et al. 2017, Gamain et al.  
128 2016, 2017a, b) (Table 1). Negative control was FSW spiked with solvent DMSO and  
129 acetonitrile at final solvent total concentration less than 0.01%. Three or four replicates were  
130 performed for each tested condition.

131

## 132 *2.4 Chemical analysis*

133 Chemical analysis were performed with FSW control or spiked before the embryotoxicity  
134 test.

### 135 *2.4.1 Pesticides*

#### 136 *Sample extraction*

137 500-mL control and 100-mL spiked water samples (pH adjusted at 5 with 10% nitric acid  
138 solution) were filtered using GF/F glass microfiber filters (0.7  $\mu\text{m}$  pore size, Whatman).  
139 Before analysis, pre-concentration of the analytes was performed using Solid-Phase  
140 Extraction (SPE) with 150 mg Oasis HLB cartridges (Waters), according to the method  
141 described elsewhere (Lissalde et al. 2011; Fauvelle et al. 2015). Elutions were achieved with  
142 3 mL of methanol, followed by 3 mL of a mix of methanol: ethyl acetate (75:25, v/v). 2.5  $\mu\text{L}$   
143 of a solution of internal standards (atrazine d5, carbofuran d3, DEA d6, diuron d6,  
144 metolachlor d6, pirimicarb 6 and MCPA d3) at 1  $\text{ng } \mu\text{L}^{-1}$  was then added to the 6-mL extracts,  
145 followed by a solvent evaporation under a gentle stream of nitrogen, and then dissolved in  
146 either 250  $\mu\text{L}$  of UPW and ACN (90:10, v/v) prior to UPLC-ToF analysis or 250  $\mu\text{L}$  of UPW  
147 and ACN (5:95, v/v) prior to HILIC-MS/MS analysis.

148

#### 149 *Instrumentation and data processing*

150 UPLC-ToF analyzes were performed by liquid chromatography ACQUITY UPLC H-Class  
151 coupled to a Xevo G2-S TOF-MS (Waters) as described in Gamain et al. (2017a). Data  
152 treatment was performed with MassLynx v4.1 and a library of 45 pesticides was used for the  
153 target screening. Ion extraction was performed with 10 mD a mass window at the expected  
154 retention time ( $\pm 0.2$  min). One or two fragments were used for the confirmation (S1). HILIC-  
155 MS/MS analyses were performed with liquid chromatography Dionex Ultimate 3000 coupled  
156 to API 2000 tandem mass spectrometer (Sciex) according to the method described in Fauvelle  
157 et al. 2015.

158

#### 159 *Method validation and quality controls*

160 Our analytical method was validated based on calibration linearity, extraction recovery,  
161 and Limits of Quantification (LOQ) according to the French standard NF T90-210.  
162 Recoveries and LOQs are shown in S1 for the detected compounds into the different samples.  
163 For quality control, SPE and POCIS blanks were routinely controlled, and the recoveries of  
164 two levels of spiked mineral water (e.g. 40 and 200 ng L<sup>-1</sup>) were evaluated for each batch.  
165 Periodic checking of two calibrating standards (e.g. 2 and 25 µg L<sup>-1</sup>, every 10 samples) and  
166 analytical blanks was also performed.

167

#### 168 *2.4.2 Copper*

169 30-mL water sample was immediately acidified with 5% final of nitric acid (Nitric acid  
170 65%, Fluka). Samples were then analysed by Inductively-Coupled Plasma Optic Emission  
171 Spectrometry (ICP-OES, Vista Pro, Agilent Technologies) and by Inductively-Coupled  
172 Plasma Mass Spectrometry (ICP-MS, Xseries2, Thermofisher Scientific) as described in  
173 Gamain et al, 2017a.

174

#### 175 *2.5 Embryotoxicity assay*

176 Embryo assays were carried out on PM and PM+Cu mixtures but not on Cu alone since  
177 data were already available (Mai et al. 2012). The oyster embryotoxicity assay has been  
178 described in detail previously (Gamain et al. 2016). Each embryotoxicity assay was  
179 performed in duplicate with two different batches of oyster embryos (N=4). A positive control  
180 was added for each embryotoxicity test with copper sulfate at 10 µg L<sup>-1</sup> corresponding to the  
181 EC<sub>-50</sub> value as mentioned in the AFNOR procedure. An important prerequisite to validate the  
182 test was the presence, in control condition (24 °C in the absence of contamination) of less  
183 than 20 % of abnormal larvae.

184

#### 185 *2.6 Comet assay*

186 Data on copper genotoxicity was published previously (Mai et al. 2012). Comet assay for  
187 all the other conditions was performed in triplicate on cells obtained from unshelled larvae.  
188 Cell dissociation was carried out as previously described in Mai et al. 2014.

189 Comet assay was also performed on oyster sperm cells. About 500,000 cells per replicate  
190 were exposed for 30 min at 24 °C in the dark to 5 mL of the tested solutions. Three replicates  
191 were performed per condition.

192 The comet assay was performed on isolated cells from larvae and sperm cells as described  
193 by Morin et al. (2011) and Mai et al. (2014).

194

## 195 *2.7 Gene expression*

196 Selected genes are involved in different cellular processes including cell cycle control and  
197 apoptosis (p53), xenobiotic biotransformation and excretion (cyp1a, gst, mxr), mitochondrial  
198 metabolism (cox, 12S), oxidative stress response (sodmt, cat) metal detoxification (mt1, mt2).  
199 Beta actin was used as a reference gene (Table 2).

200 After oyster embryos exposure to the pesticide mixture or PM+ Cu or Cu alone at 0.1X,  
201 1X and 10X for 24h, the density of larvae was determined. Three replicates for each  
202 contamination condition were performed and each replicate contained a total of 35,000 oyster  
203 larvae. Larvae solutions were then concentrated for RNA extraction, by centrifugation at  
204 4,000 g for 10 min at 4°C. The pelleted larvae were resuspended in 500 µL of “RNA later”  
205 buffer (Qiagen). Those samples were then stored at -80 °C until required.

206 Total RNA was extracted using the “Absolutely RNA<sup>®</sup> Miniprep” Kit (Stratagene,  
207 Agilent) according to manufacturer’s instructions (including Dnase I treatment). First strand  
208 cDNA was synthesized from total RNA (1 µg) using the “Affinity Script<sup>™</sup> Multiple  
209 Temperature cDNA synthesis” kit (Agilent, Stratagene).

210 After extraction and reverse transcription, real-time PCR reactions were performed using  
211 Mx3000P (Stratagene) following the manufacturer’s instructions. Detailed protocols are  
212 available in Mai et al. (2014).

213

## 214 *2.8 Statistical analysis*

215 Data is expressed as means ± standard error (S.E). Statistical software SPSS (16.0) was  
216 used for data analysis. Normality of data distribution was tested on data residues using the  
217 Shapiro-Wilk test ( $p < 0.01$ ) while homogeneity of variance was checked using Levene’s test  
218 ( $p < 0.05$ ). Once it had been confirmed that these two conditions were fulfilled, statistical  
219 analyses were performed by One-way Analysis of Variance (ANOVA). Differences among



220 conditions were then performed using the Tukey's *post hoc* test. Significance difference was  
221 accepted at  $p < 0.05$ . The  $EC_{50}$ , defined here as the toxicant doses causing 50% reduction in  
222 the embryogenesis success, and their 95% confidence intervals (CI) were calculated by  
223 PRISM 5 software (GraphPad Software, California, USA).

224

### 225 **3. Results**

#### 226 *3.1 Chemical exposure*

227 Measured concentrations of pesticides and copper in the different assays are reported in  
228 Table 1. Chlorothalonil and dichlofluanid could not be analysed by LC-ToF or LC-MS/MS,  
229 but were added to the mixture (at the nominal concentration) since they have been previously  
230 detected into the Arcachon Bay (Budzinski et al. 2011). Reference FSW from the Grand Banc  
231 station in the Arcachon Bay was shown to contain detectable but low levels of metolachlor,  
232 metolachlor ESA, acetochlor, diuron and irgarol, generally lower than the concentrations of  
233 the 0.1X condition. Background copper level measured in the reference seawater was around  
234  $3 \mu\text{g L}^{-1}$ . For the 0.1X and 1X conditions, the measured Cu concentrations reached  $4.1 \mu\text{g L}^{-1}$   
235 and  $6.3 \mu\text{g L}^{-1}$  respectively which is higher than expected since it also included the Cu  
236 initially present in the reference seawater. For all pesticides and copper concentrations, except  
237 the carbendazim, a dose-depend increase was observed from the 0.1X to the 100X exposure  
238 condition.

239

#### 240 *3.2 Embryotoxic effects*

241 The embryo-larval assay revealed that pesticide mixtures (PM) and PM+Cu induced  
242 embryotoxic effects on oyster embryos, with some differences in the level of responses (Fig.  
243 1). Embryotoxicity was detected at the lowest tested dose of 0.1X for PM and PM+Cu ( $p <$   
244  $0.0001$ ). In addition, a dose-dependent increase of abnormal D-larvae was observed. When  
245 comparing both pesticide mixtures with or without spiked Cu, the percentage of abnormal  
246 larvae was not statistically different even for the two lowest concentrations (0.1X and 1X).  
247 Larval abnormalities reached 100% at the highest tested dose (100X) for the PM+Cu  
248 exposure, while it reached approximately 78.1% for the same dose of PM exposure.

249 Values causing a 50% reduction in embryogenesis success ( $EC_{50}$ ) and their 95%  
250 confidence intervals (CI) were reported in Table 3. The mixture of pesticides gave an  $EC_{50}$

251 value of 0.7X for PM+Cu, whereas the EC<sub>50</sub> for the PM alone was 5.2X. The EC<sub>50</sub> value was  
252 12.5 µg L<sup>-1</sup> for Cu alone (Mai et al. 2012).

253

### 254 3.3 Effects on DNA integrity (comet assay)

255 A statistically significant increase in DNA damage in exposed-embryos was observed from  
256 the lowest dose of PM and PM+Cu (0.1X) compared to the control groups ( $p < 0.001$ ) (Fig.  
257 2A). The percentage of tail DNA also increased in a dose-dependent manner for both  
258 mixtures. DNA damage appeared significantly higher at the highest dose (100X) for PM+Cu  
259 in comparison to PM treatment. It was not the case at lower doses. Indeed, at the highest  
260 tested dose (100X), PM exposure induced 27% of tail DNA, against 37% for the same dose  
261 group of PM+Cu.

262 In the exposed-sperm experiment, a significant dose dependent increase of DNA damage  
263 was observed for both PM and PM+Cu exposures ( $p < 0.001$ ) (Fig. 2B). A significant increase  
264 of tail DNA levels was measured from the lowest dose of both PM and PM+Cu exposures.  
265 Statistical analysis revealed no significant differences in DNA damage between PM and  
266 PM+Cu treatments.

267

### 268 3.4 Gene transcription levels

269 Transcription levels of 11 genes involved in several cellular mechanisms were analysed by  
270 quantitative RT-PCR in embryos after exposure to PM and PM+Cu mixtures. The *β-actin*  
271 gene was used as a reference gene. The results of this analysis are reported in Table 4.

272 For oyster embryos exposed to Cu only, no transcription modulation of selected genes  
273 involved in mitochondrial metabolism (*coxI*, *l2s*), cell cycle arrest (*p53*) and detoxification  
274 (*mt1*, *mt2*, *mxr*) was observed at any tested concentrations. However, Cu induced  
275 overexpression of *cyp1A* gene (IF = 1.8) at the highest tested concentration (10X).

276 For oyster embryos exposed to the pesticide mixture, a significant repression of *coxI*, *mt1*,  
277 *mt2* and *p53* genes at all tested doses was noted. Surprisingly, a significant repression of *cat*  
278 gene was observed only at 0.1X and 1X concentrations of PM.

279 Exposure of oyster embryos to PM+Cu resulted in a significant induction of *gst* gene ( $p <$   
280 0.05), with IF values from 3.1 to 5.5. In contrast, the *cat* gene was significantly repressed (IF  
281 = 0.1-0.4) at all tested doses ( $p < 0.05$ ). The two metallothionein genes (*mt1* and *mt2*), *coxI*

282 gene and *cat* gene were overexpressed at 1X and 10X doses. Finally, the *p53* gene was  
283 significantly repressed at 0.1X and 10X doses ( $p < 0.05$ ).

284

## 285 **4. Discussion**

286 Pesticides are designed to selectively eliminate various pests including fungi, plants or  
287 animals. They usually target specific biological functions of the pest through disruption of  
288 vital cellular pathways. Their mechanisms of action are tightly linked to their chemical  
289 structures which are highly diverse. Although animals are exposed to complex mixtures of  
290 pollutants in the environment, most laboratory experiments are based on the use of a unique  
291 or binary mixture of molecules. Studies on pesticide mixtures (Faust et al. 2001, Poletta et al.  
292 2011) have shown that mixtures generally exhibit higher toxicity than single compounds.  
293 Indeed, the effect of a mixture of pesticides on the phagocytosis competence of *C. gigas*  
294 haemocytes was truly demonstrated, while no effect was induced when compounds were  
295 tested separately (Gagnaire et al. 2007, Gagnaire et al. 2006). At present, few studies have  
296 investigated pesticide mixture or heavy metal mixture toxicity on aquatic species (Gagnaire et  
297 al. 2007; Hernando et al. 2003; Junghans et al. 2003; Manzo et al., 2008; Verslycke et al.  
298 2003), but no study has focused on the effects of pesticide mixture in combination with  
299 copper.

300

### 301 **4.1 Embryotoxicity**

302 The toxicity of copper alone, metolachlor and its metabolites, irgarol and diuron for oyster  
303 embryos has already been investigated in previous studies (Mai et al. 2012, 2013). EC50  
304 values of 12.5, 2332, 196 and 672  $\mu\text{g L}^{-1}$  were obtained for copper (Mai et al., 2012), diuron,  
305 irgarol and metolachlor (Mai et al. 2013), respectively. In the present study a hundred percent  
306 of abnormal larvae was obtained at the highest dose group (100X) of PM+Cu and 78.1% at  
307 the same dose of PM without spiked Cu. At concentrations occurring in the Arcachon Bay  
308 (1X), 54% of abnormalities were obtained for the PM+Cu treatment and 32.4% for the PM  
309 without Cu. These results clearly indicate: (i) the higher toxicity of mixture of pollutants in  
310 comparison to pollutants alone and (ii) the likelihood of deleterious effects of environmental  
311 concentrations of mixture of pollutants. Faust et al. (2001) and Poletta et al. (2011) have  
312 reported higher toxicity for mixture of pesticides than for the single compounds. In the case of  
313 cumulative toxic effects, each chemical in the mixture can contribute to the overall toxicity in

314 proportion to its toxic unit and ratio even if it is present at concentrations below the threshold  
315 of statistically detectable effects (Jacobsen et al. 2012; Silva et al. 2002). For compounds with  
316 dissimilar modes of action, the effects of each individual chemical may be directly opposed  
317 and the effects of the mixtures are more difficult to predict (Jacobsen et al. 2012). For  
318 compounds with similar mode of action, mixture could lead to much higher toxicity than  
319 predicted for additive effects. For example, in binary combination metolachlor elevated the  
320 toxicity of chlorpyrifos by 1.5-fold in the aquatic midge (Jin-Clark et al. 2008). However,  
321 Manzo et al. (2008) reported that irgarol, diuron and copper in ternary mixtures were more  
322 toxic than diuron and less toxic than irgarol and copper when tested alone for sea urchin  
323 embryos. These authors also noted that the mixture was less toxic than the single  
324 contaminants when considering the NOEC obtained on embryos. The knowledge of a  
325 chemical mode of action is essential for understanding how mixtures may act jointly. Mode of  
326 action of certain pesticides are well known, in particular herbicides in photosynthetic  
327 organisms such as algae species (Backhaus et al. 2004; Ranke and Jastorff 2000), but there is  
328 much little information about the mechanisms of action of pesticides on aquatic animal  
329 species.

330

#### 331 ***4.2 DNA damage in oyster sperm and embryos***

332 Gametes are released directly into seawater and are thus exposed to all environmental  
333 pollutants present during spawning. It is of particular interest to study because this creates the  
334 potential for contaminants to disrupt fertilization processes. A significant increase of DNA  
335 damage in both exposed-sperm and exposed-embryos was observed following the exposure to  
336 PM and PM+Cu from the lowest tested dose (0.1X). The PM+Cu treatment induced a slightly  
337 higher DNA damage level than PM at the higher tested dose (100X) for both oyster  
338 spermatozoa and embryos. However, combination of copper and mixture of pesticides did not  
339 significantly increase DNA damage in comparison to copper exposure alone. Zhou et al.  
340 (2006) observed, that synergistic effects of binary mixture of antifouling chemicals and  
341 copper in *Vibrio fischeri* were due to the presence of Cu<sup>2+</sup>. These authors suggested that Cu  
342 could induce the formation of more lipophilic organic copper complexes, which diffused  
343 across the plasma membrane more easily. The mix of PM+Cu could give rise to chemical  
344 complexes that could influence the bioavailability and the activity of mixture components  
345 (Dinku et al. 2003; Metcalfe et al. 2006; Singh et al. 2002). In addition, Rouimi et al. (2012)

346 reported that mixtures of pesticides (atrazine, chlorpyrifos and endosulfan) could inhibit the  
347 cleavage of the PARP protein involved in DNA repair and programmed cell death processes.

348 A great proportion of sperm DNA damage (19.3-22.6% tail DNA) was observed from the  
349 lowest tested dose (0.1X) for PM and PM+Cu exposures, compared to the control group  
350 (7.2% tail DNA). Deleterious effects of trace metals (Cu, Zn, Cd) and organic xenobiotics  
351 (phenol, PCB, butyltin) on sperm cells are well documented for several invertebrate species  
352 such as sea urchin, mussel and oyster (Au et al. 2000, 2003; Yurchenko et al. 2009), but not in  
353 mixtures. Au et al. (2000) reported that low concentrations of individual trace metals or  
354 organic substances can induce ultra-structure abnormalities in spermatozoa of mussel and sea  
355 urchin. In addition, exposure to neonicotinoid pesticides has also been shown to alter  
356 chromatin structure of sperm cells (Gu et al. 2013; Ramazan et al. 2012). Gharred et al.  
357 (2015) also reported that sea urchin sperm cells (*Paracentrotus lividus*) exposed to a mixture  
358 of copper and deltamethrin show a high increase in mitotic division and asymmetric and/or  
359 asynchronous cell divisions. Therefore, PM or PM+Cu mixtures could lead to higher toxicity,  
360 even if each individual toxicant in mixtures are present at very low concentrations (less than  
361  $10 \text{ ng L}^{-1}$  for individual pesticides). However, Manzo et al. (2008) also observed antagonistic  
362 effects of a ternary mixture of two antifouling chemicals and copper in sperm cells of the sea  
363 urchin *Paracentrotus lividus*. Sperm DNA damage induction has been also associated with  
364 high levels of reactive oxygen species (Tamburrino et al. 2012; Zini and Libman 2006).  
365 However, the functional relationship between reactive oxygen species production and DNA  
366 damage induction has never been demonstrated in oyster sperm. Sperm cells are generally  
367 considered to have little capacity for DNA repair (Lacaze et al. 2011; Lewis and Galloway  
368 2009). Spermatozoa are therefore considered a sensitive target for genotoxic compounds, and  
369 sperm DNA integrity is pointed out as one of the major risk factors for abnormal development  
370 of progeny and for the reduction in the number of offspring.

### 371 **4.3 Gene expression**

372 The rapid evolution of molecular techniques has led to a new approach in environmental  
373 science and risk assessment to link molecular and ecotoxicological responses. A well-founded  
374 identification and understanding of underlying molecular mechanisms will lead to a more  
375 effective risk assessment (Amiard et al. 2006; Watanabe et al. 2007). Here, we applied  
376 molecular tools to investigate stress responses in oyster embryos exposed to a pesticide  
377 mixture with and without copper. Eleven genes known to be involved in antioxidant defences,  
378 mitochondrial metabolism, detoxification, biotransformation process, and cell cycle arrest and

379 apoptosis were investigated by quantitative real-time PCR. Several of these genes showed  
380 significant transcription modulation after exposure to either PM or PM+Cu exposures. It is  
381 worth nothing that a lot of genes were downregulated following PM or PM+Cu exposures  
382 such as *p53*, *coxI*, *mt* and *cat*. Those genes could be the first targets towards major disruptions  
383 to cell homeostasis (Asselman et al. 2012; Pereira et al. 2010; Dondero et al. 2006; Banerjee  
384 et al. 2001; Liu and Kulesz-Martin 2001).

385 This study has shown that oyster embryos exposed to PM and PM+Cu exhibited significant  
386 repression of the *p53* gene. For instance, the expression of *p53* gene was significantly reduced  
387 after a 24h exposure to either PM or PM+Cu at almost all tested concentrations. p53 protein is  
388 involved in key cellular processes such as cell cycle control, DNA repair and apoptosis  
389 (Elmore 2007; Liu and Kulesz-Martin 2001). p53 protein expression and function are tightly  
390 controlled by a feedback loop involving at least MDM2 protein (Wu et al. 1993). Some  
391 authors reported the inactivation of p53 protein after exposure of human HepG2 cells to  
392 copper or other agents that induce oxidative damage (Tassabehji et al. 2005). According to  
393 Hainaut and Milner (1993), redox conditions in cells influence the conformational folding of  
394 p53 protein, through oxidation/reduction of specific thiol groups (-SH) in its DNA binding  
395 domain. Sandrini et al. (2009) reported that the repression of *p53* gene could be explained by  
396 the inhibition of p53 protein due to a direct interaction with copper or indirect synergistic  
397 effect through ROS oxidation of the thiol (-SH) residues.

398 Expression of *coxI* gene was strongly repressed at higher doses of PM+Cu. Given that the  
399 COXI protein is involved in the electron transport chain, our results suggest that  
400 mitochondrial metabolism can be affected by pesticides exposure. The number of copies of  
401 *12s* ARNm is indicative of the amount of mitochondria per cell. The ratio *coxI/12s* can be  
402 used as an indicator of relative changes in the number of mitochondria. This ratio did show  
403 significant lower values in exposed embryos than in control embryos ( $p < 0.05$ ) (Table 5).  
404 Pereira et al. (2010) reported that pesticides could affect the transcription of mitochondrial  
405 proteins and ATP synthesis-related proteins.

406 Ringwood and Brouwer (1995) and Roesijadi et al. (1996) have shown that embryos of  
407 *Crassostrea* sp. were able to induce metallothionein synthesis after exposure to trace metals  
408 such as copper and cadmium. A comprehensive review of the multifaceted role of  
409 metallothioneins emphasized that the metals, Zn, Cu, Cd, Hg, Au and Bi all induce MT  
410 synthesis (Coyle et al. 2002). Unexpectedly, in this study *mt1* and *mt2* gene transcriptions  
411 were strongly repressed in embryos exposed to both PM and PM+Cu, except for the lowest

412 tested dose of 0.1X PM+Cu (100 ng L<sup>-1</sup> Cu and from 0.1 ng L<sup>-1</sup> to 45 ng L<sup>-1</sup> of each pesticide).  
413 In addition, when oyster embryos were exposed to copper only, no modulation of gene  
414 expression was observed for genes *mt1* and *mt2*. Recently, Asselman et al. (2012) reported  
415 that the *mt1* expression was influenced by herbicide exposure regardless the concentrations.  
416 We hypothesize that the repression of genes *mt1* and *mt2* may be due to toxic effects of  
417 pesticides on oyster embryos. The expression ratio shows that *mt1* and *mt2* genes were  
418 repressed at the same level. This result could suggest that *mt1* and *mt2* genes are both targeted  
419 by pesticides and could play the same physiological roles in oyster embryos.

420 In the present study, *gst* gene transcription level tends to be overexpressed in oyster  
421 embryos exposed to PM and PM+Cu. GST protein is involved in xenobiotic metabolism  
422 by the formation of glutathione acetanilide conjugates (Lüdeking and Köhler 2002, Tanguy et  
423 al. 2005). Overexpression of this gene could be an adaptive response by oyster embryos to  
424 allow biotransformation and elimination of lipophilic xenobiotics such as certain pesticides.

425 Our experiments showed that *cat* gene transcription was significantly repressed by PM  
426 and/or PM+Cu exposures, which could lead to a decrease of protection against oxidative  
427 stress. On the contrary, the *cat* gene was shown to be significantly up-regulated in mussels  
428 exposed to a copper contamination gradient (Dondero et al. 2006). Damiens et al. (2004)  
429 reported that CAT activity was inhibited in oyster larvae after exposure to pesticides. There is  
430 therefore evidence to suggest that pesticide mixtures with or without copper induce activation  
431 of the antioxidant systems resulting from an adaptive response to a likely oxidative stress in  
432 exposed oysters at an early life stage.

433 Finally, expression of genes encoding for proteins involved in anti-oxidative stress (*gpx*),  
434 multixenobiotic resistance (*mxr*) and biotransformation process (*cyp1A*) was unchanged  
435 regardless the doses of PM and PM+Cu exposures compared with control groups. The basal  
436 expression levels of these genes may suggest either oyster embryos were not under stress or  
437 they were under stress but this set of genes were not specifically involved in response to  
438 pesticides or copper exposures in *C. gigas* oyster early life stages.

439

## 440 **5. Conclusion and perspectives**

441 Our experimental approach was consistent with environmental exposure conditions of  
442 oyster embryos living in polluted coastal areas chronically impacted by leaching from  
443 agricultural run-off. In this study, exposures of oyster embryos to pesticide mixtures with or

444 without copper lead to developmental defects and DNA damage even at very low  
445 environmental concentrations. Changes in gene expression were mainly observed following  
446 exposure to pesticide mixture with or without Cu but not for Cu alone. Several genes involved  
447 in key cellular functions such as cell cycle control, mitochondria metabolism, anti-oxidative  
448 defence and metal detoxification were down regulated indicating likely major impact of  
449 pesticides on cell homeostasis.

450 To our knowledge, this study is one of the first to investigate the combined effects of  
451 mixtures of pesticides at environmentally relevant concentrations on embryos and sperm cells  
452 of Pacific oyster. In their natural habitat, organisms are exposed not only to mixtures of  
453 chemicals, but also to multiple stressors such as fluctuating physico-chemical parameters  
454 (T°C, pH, oxygen, turbidity...) or infectious agents (bacteria, virus and parasites...). Therefore,  
455 for future studies, the combined effects/risk resulting from the interaction between chemical,  
456 physical and biological stressors should be taken into account.

457

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689

690 Table 1: Measured and nominal concentrations of pesticides and copper (ng L<sup>-1</sup>) in the  
 691 different pesticide mixtures tested

	Control (FSW)	0.1X	1X* (nominal concentration)	1X*	10X	100X
Acetochlor	1	3	5	8	48	513
Acetochlor ESA	<5	57	450	404	3466	34061
Acetochlor OA	<5	<5	50	17	84	862
Metolachlor	13	23	30	21	74	952
Metolachlor ESA	7	57	450	231	3778	25201
Metolachlor OA	<2	53	450	273	4219	28795
Diuron	1	3	5	5	40	415
Irgarol	2	2	5	5	37	369
Hydroxy-atrazine	<1	1	15	28	185	1728
Imidacloprid	<5	14	80	83	684	7516
Carbendazim	<0.5	<0.5	5	3	5	3
Chlorothalonil	na	0.1 <sup>a</sup>	1	1 <sup>a</sup>	10 <sup>a</sup>	100 <sup>a</sup>
Dichlofluanid	na	0.1 <sup>a</sup>	1	1 <sup>a</sup>	10 <sup>a</sup>	100 <sup>a</sup>
DMST	<2	<2	10	5	69	1508
Cu	3160	4180	2000	6330	23500	199000

692 \* Environmental concentrations in the Arcachon Bay. na: not analysed. <sup>a</sup> nominal concentration

693

695 Table 2: Nucleotide sequences of primers used in real-time PCR analysis of *C. gigas* larvae

Gene function and name	Accession number	Forward primer	Reverse primer
<b>Mitochondrial metabolism</b>			
<b>12S</b>	AF034688	CTCAGTCTTGC GGGAGG	GGTTATGCGGAACCGCC
<b>cox1</b>	AB033687	GTGCCAACTGGTATTAAGGTGT	ACACCGCACCCATTGAT
<b>Oxidative stress response</b>			
<b>cat</b>	EF687775.1	GTCGTGCCCTTTACAACC	CGCCCGTCCGAAGTTT
<b>sodmt</b>	EU420128	ACAAAGTCAATCAGTGCCCT	CCATTGCCTCTGCCAGT
<b>gpx</b>	EF692639	ATCGAACGCTGCACCA	AGCTCCGTCGCATTGT
<b>Detoxification</b>			
<b>mt1</b>	AJ242657	TGCTGCTCTGATTCTGTGCC	GGTCCTTTGTTACACGCACTCATT
<b>mt2</b>	AJ297818	TCCGATGTGGCTGCAAAGTCAAG	GGTCCTTTGTTACACGCACTCATT
<b>mxr</b>	AJ422120	AGGAAGGGCAGTTGAGTG	CGTTGGCCTCCTTAGCG
<b>Metabolization</b>			
<b>cyp1A1</b>	EF645271	AGGCATAGGGCTCCAC	CTGGTTTCGCGGGTTTCAT
<b>gst</b>	AJ557140	AGGCTACCGAAATGGCTG	CTCTGACTTGTAATAGGCCGC
<b>Cell regulation</b>			
<b>P53</b>	AM236465	CCCTCAAAGCAGTCCCCA	TGTAGCGATCCACCTGATT
<b>Reference</b>			
<b><i>β-actin</i></b>	AB071191	AGTACCCATTGAACACGG	TGGCGGGAGCGTTGAA

696 Abbreviations: *mt*: metallothionein; *coxI*: cytochrome C oxidase subunit I; *gpx*: glutathione peroxidase; *sod*:  
697 superoxide dismutase; *cat*: catalase; *sodmt*: mitochondrial superoxide dismutase; *mxr*: multixenobiotic  
698 resistance, *gst*: glutathione S transferase, *cyp*: cytochrome P450.  
699

700

701 Table 3: EC<sub>50</sub> values ( $\pm$  CI 95%) obtained with the oyster embryo-larval assay for Cu alone,

702 pesticide mixture and pesticide mixture plus Cu.

703

<b>Compounds</b>	<b>EC<sub>50</sub> value</b>
Cu (ng L <sup>-1</sup> )	12,500 (11,000-14,200) <sup>a</sup>
Pesticide mixture	5.2X (2.8X-9.9X)
Pesticide mixture + Cu	0.7X (0.2X-2.0X)

704 Note: 1X was set at 1,085 ng L<sup>-1</sup> for the sum of all tested pesticides and 6,330 ng L<sup>-1</sup> for copper.

705 <sup>a</sup> Mai et al. (2012)

706

707

708 Table 4: Induction factors (IF) of gene transcription for the eleven studied genes in oyster D-  
 709 larvae following a 24h-exposure to copper alone or to pesticide mixture with or without  
 710 copper (N =3 for each treatment condition).

Functions	Genes	Copper			Pesticide mixture			Pesticide mixture + Cu		
		0.1X	1X	10X	0.1X	1X	10X	0.1X	1X	10X
Cells cycle arrest/apoptosis	<i>p53</i>	/	/	/	0.3*	0.3*	0.4*	0.3*	/	0.4*
Mitochondrial metabolism	<i>l2s</i>	/	/	/	/	/	/	/	/	/
	<i>coxI</i>	/	/	/	0.3*	0.3*	0.4*	/	0.3*	0.3*
Oxidative stress response	<i>sodmt</i>	/	/	/	/	/	/	/	/	/
	<i>cat</i>	/	/	/	0.02*	0.1*	/	0.2*	0.4*	0.1*
	<i>gpx</i>	/	/	/	/	/	/	/	/	/
Metal detoxification	<i>mt1</i>	/	/	/	0.03*	0.04*	0.1*	/	0.1*	0.04*
	<i>mt2</i>	/	/	/	0.04*	0.1*	0.1*	/	0.1*	0.04*
	<i>mxr</i>	/	/	/	/	/	/	/	/	/
Xenobiotic biotransformation	<i>gst</i>	/	/	/	/	/	/	5.5*	3.1*	3.1*
	<i>cyp1A</i>	/	/	1.8*	/	/	/	/	/	/

711 The results are given in the form of induction (>2.0) or repression (< 0.5) compared to control group.  
 712 Asterisks indicate significant difference in gene transcription level between exposed and control  
 713 treatments by Tukey's test (p < 0.05). The sign / means no significant change in gene expression  
 714 compared to control.

715

716

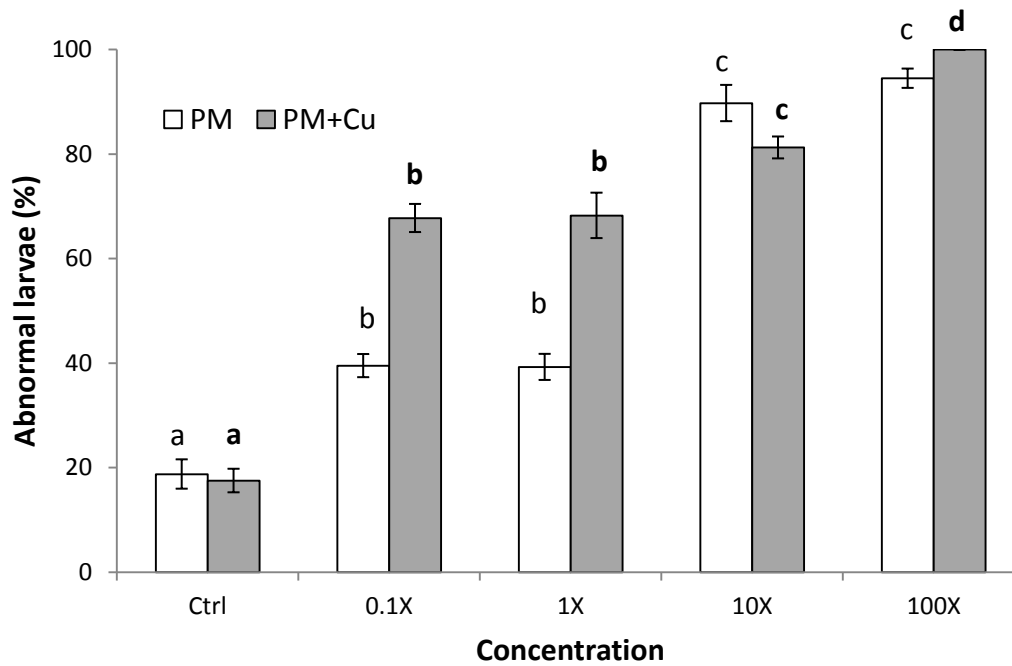
717 Table 5: Expression ratios of *coxI/12s* and *mt2/mt1* in oyster embryos after a 24h-exposure to  
718 pesticide mixture, or pesticide mixture + Cu, or Cu alone

719

Expression ratio	Cu			Pesticide mixture			Pesticide mixture + Cu		
	0.1X	1X	10X	0.1X	1X	10X	0.1X	1X	10X
<i>coxI/12s</i>	1.9	1.6	1.3	0.2*	0.1*	0.2*	0.2*	0.1*	0.2*
<i>mt2/mt1</i>	0.8	1.0	0.9	1.1	1.0	1.0	1.4	1.0	1.0

720 Asterisks indicate significant difference between exposed and control treatments (Tukey's test, p <  
721 0.05)

722



724

725 Fig.1: Developmental abnormalities in oyster D-larvae following a 24h-exposure to pesticide  
726 mixture (PM) and pesticide mixture in combination with copper (PM+Cu). Different letters  
727 indicate significant differences between treatments (N=4,  $p < 0.0001$ , Tukey's test).

728

729

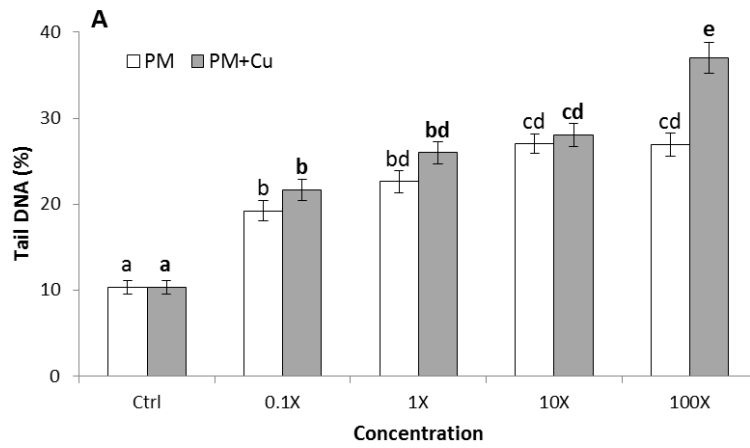
730

731

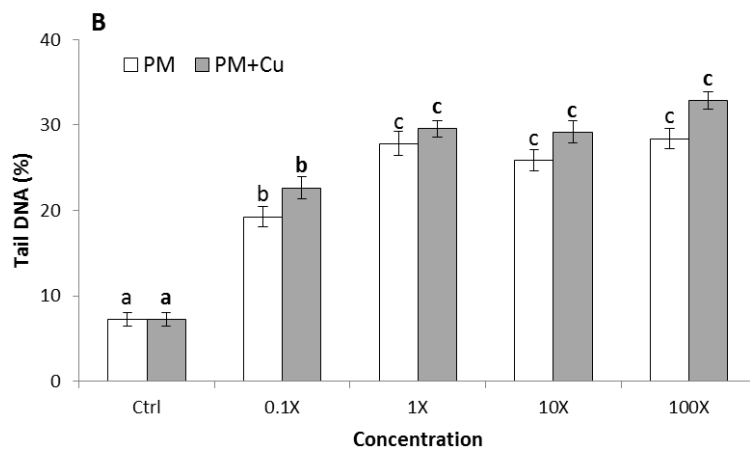
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736

737 Fig.2: Percentages (Mean  $\pm$  S.E.) of tail DNA in A) oyster larvae following a 16h-exposure or  
 738 B) oyster sperm after 30 min-exposure to pesticide mixture (PM) and pesticide mixture in  
 739 combination with copper (PM+Cu). Different letters indicate significant differences between  
 740 treatments (N=3,  $p < 0.001$ , Tukey's test).

741