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Huong Mai, Jérôme Cachot, Christelle Clérandeau, Martin Caroline, Nicolas Mazzella, et al.. An environmentally realistic pesticide and copper mixture impacts embryonic development and DNA integrity of the Pacific oyster, *Crassostrea gigas*. *Environmental Science and Pollution Research*, 2020, 27 (4), pp.3600-3611. 10.1007/s11356-018-3586-6 . hal-03066007

HAL Id: hal-03066007

<https://hal.inrae.fr/hal-03066007>

Submitted on 5 Jan 2021

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

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Abstract

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

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

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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 19th 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.

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

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

2. Materials and methods

2.1 Chemicals and seawater

Fifteen reference toxicants (14 pesticides and CuSO₄: Table 1), formalin and DMSO (dimethyl sulfoxide) with purity greater than 96% were purchased from Sigma-Aldrich Chemical (St. Quentin Fallavier, France). Dispase II, Triton X-100, low melting point (LMP) agarose, normal melting point (NMP) agarose, and MEM-alpha (Minimum Essential Medium) were purchased from Gibco (Invitrogen, Cergy Pontoise, France). Formic acid (HCOOH), ammonium acetate and organic solvents (methanol -MeOH- and acetonitrile -ACN- ULC grade) were purchased from Biosolve (Dieuze, France).

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

2.2 Animals

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

2.3 Preparation of pesticide and copper solutions

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

2.4 Chemical analysis

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

2.4.1 Pesticides

Sample extraction

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

Instrumentation and data processing

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

Method validation and quality controls

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

2.4.2 Copper

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

2.5 Embryotoxicity assay

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

2.6 Comet assay

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

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

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

2.7 Gene expression

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

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

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

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

2.8 Statistical analysis

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

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

3. Results

3.1 Chemical exposure

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

3.2 Embryotoxic effects

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

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

value of 0.7X for PM+Cu, whereas the EC₅₀ for the PM alone was 5.2X. The EC₅₀ value was 12.5 µg L⁻¹ for Cu alone (Mai et al. 2012).

3.3 Effects on DNA integrity (comet assay)

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

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

3.4 Gene transcription levels

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

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

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

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

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

4. Discussion

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

4.1 Embryotoxicity

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

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

4.2 DNA damage in oyster sperm and embryos

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

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

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

4.3 Gene expression

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

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

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

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

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

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

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

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

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

5. Conclusion and perspectives

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

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

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

Acknowledgements

The authors would like to acknowledge the French and Vietnamese Governments. This study was supported by the French National Research Agency, under reference “ANR- 09-CESA-005”, Aquitaine Region (OSQUAR project) and CPER A2E. This project is co-financed by the European Union with the European fund of regional development. Finally, authors acknowledge James Emery for his help in English proofreading.

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689

690 Table 1: Measured and nominal concentrations of pesticides and copper (ng L⁻¹) 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 ^a	1	1 ^a	10 ^a	100 ^a
Dichlofluanid	na	0.1 ^a	1	1 ^a	10 ^a	100 ^a
DMST	<2	<2	10	5	69	1508
Cu	3160	4180	2000	6330	23500	199000

692 * Environmental concentrations in the Arcachon Bay. na: not analysed. ^a nominal concentration

693

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
Mitochondrial metabolism			
12S	AF034688	CTCAGTCTTGCGGGAGG	GGTTATGCGGAACCGCC
cox1	AB033687	GTGCCAACTGGTATTAAGGTGT	ACACCGCACCCATTGAT
Oxidative stress response			
cat	EF687775.1	GTCGTGCCCCCTTACAACC	CGCCCGTCCGAAGTTT
sodmt	EU420128	ACAAAGTCAATCAGTGCCCT	CCATTGCCTCTGCCAGT
gpx	EF692639	ATCGAACGCTGCACCA	AGCTCCGTCGCATTGT
Detoxification			
mt1	AJ242657	TGTCTGCTCTGATTCGTGTCC	GGTCCTTTGTTACACGCACTCATT
mt2	AJ297818	TCCGGATGTGGCTGCAAAGTCAAG	GGTCCTTTGTTACACGCACTCATT
mxr	AJ422120	AGGAAGGGCAGTTGAGTG	CGTTGGCCTCCTTAGCG
Metabolization			
cyp1A1	EF645271	AGGCATAGGGCTCCAC	CTGGTTTCGCGGGTTTCAT
gst	AJ557140	AGGCTACCGAAATGGCTG	CTCTGACTTGTAATAGGCCGC
Cell regulation			
P53	AM236465	CCCTCAAAGCAGTCCCCA	TGTAGCGATCCACCTGATT
Reference			
<i>β-actin</i>	AB071191	AGTACCCCATTTGAACACGG	TGGCGGGAGCGTTGAA

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

Table 3: EC₅₀ values ($\pm$ CI 95%) obtained with the oyster embryo-larval assay for Cu alone, pesticide mixture and pesticide mixture plus Cu.

Compounds	EC ₅₀ value
Cu (ng L ⁻¹)	12,500 (11,000-14,200) ^a
Pesticide mixture	5.2X (2.8X-9.9X)
Pesticide mixture + Cu	0.7X (0.2X-2.0X)

Note: 1X was set at 1,085 ng L⁻¹ for the sum of all tested pesticides and 6,330 ng L⁻¹ for copper.

^a Mai et al. (2012)

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.

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

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

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

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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).

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