Evaluation of the deleterious effects of heavy metals and pesticides on early life stages and gametes of the Pacific Oyster, Crassostrea gigas : application to the pollution context of the Arcachon Bay

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By: **Huong MAI**

SUBJECT OF THE THESIS

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Defense on 17 September 2013 in front of the jury constituted with:

Mr Paco Bustamente, Professor, University of La Rochelle	Reviewer
Mr Ricardo Beiras, Professor, University of Vigo, Spain	Reviewer
Ms Magalie Baudrimont, Professor, University of Bordeaux	Examiner
Ms Florence Geret, Maître de Conférences, University of Albi	Examiner
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Mr Jérôme Cachot, Professor, University of Bordeaux	Director of thesis

USTH University of Science and Technology of Hanoi, Vietnam

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THÈSE

PRÉSENTÉE A

L'UNIVERSITÉ DE BORDEAUX

ÉCOLE DOCTORALE SCIENCES ET ENVIRONNEMENTS

Par Huong MAI

POUR OBTENIR LE GRADE DE

DOCTEUR

SPÉCIALITÉ : Géochimie et Écotoxicologie

Evaluation des effets délétères des métaux et des pesticides sur les gamètes et les premiers stades de développement de l'huître creuse, *Crassostrea gigas* : Application à la problématique de la pollution du Bassin d'Arcachon

Soutenue le : 17 Septembre 2013 devant le jury formé de :

Mr Paco Bustamente, Professeur, Université de la Rochelle	Rapporteur
Mr Ricardo Beiras, Professeur, Université de Vigo, Espagne	Rapporteur
Mme Magalie Baudrimont, Professeur, Université de Bordeaux	Examinateur
Mme Florence Geret, Maître de Conférences, Université d'Albi	Examinateur
Mme Bénédicte Morin, Maître de Conférences, Université de Bordeaux	Co-Directeur de thèse
Mr Jérôme Cachot, Professeur, Université de Bordeaux	Directeur de thèse

Université des Sciences et des Technologies de Hanoi, Vietnam

1. Contexte des travaux de recherche

Les écosystèmes marins, particulièrement ceux près du littoral, sont soumis à une grande diversité de pressions anthropiques (Adams, 2005), notamment de nature chimique qui peuvent faire peser un risque réel pour la perennité des espèces aquatiques. Les contaminants sont introduits et dispersés s dans les milieux aquatiques selondifférentes voies, comprenant les rejets directs, les eaux de ruissellement, les dépôts atmosphériques, les mouvements abiotiques et biotiques et le transfert le long des réseaux trophiques (Livingstone, 1998). Le bassin d'Arcachon, lagune macrotidale située sur la façade atlantique, subit également de nombreux types de pressions anthropiques en raison des activités nautiques s et domestiques en augmentation du fait de l'accroissement du tourisme et de l'urbanisation, des activités agricoles au niveau du bassin versant et des activités industrielles. Parmi les nombreux polluants qui peuvent contribuer à la pollution aquatique, les métaux lourds (cuivre et cadmium) représentent une des formes les plus répandues et les plus graves de contamination de l'environnement (Devi et al., 1996). En plus des métaux lourds, la contamination par les pesticides s'est accrue dans les zones estuariennes au cours des dernières décennies en raison notamment de ruissellement de produits chimiques d'origine agricole (Tanguy et al., 2005). La contamination chimique du bassin d'Arcachon est bien documentée depuis quelques années en raison du grand nombre de programmes de recherche (ASCOBAR : Apports scientifiques face à la problématique conchylicole du Bassin d'Arcachon ; OSQUAR : Ostréiculture et Qualité, Approche dynamique du Bassin d'Arcachon) et le réseau de surveillance REPAR (REseau de Surveillance des Pesticides sur le Bassin d'Arcachon). Un certain nombres de molécules phytosantaires, principalement des herbicides et des molécules anti-salissures ont été detectées en concentrations non négligeables. Ces polluants peuvent avoir des conséquences écologiques et pourraient mettre en danger la survie, la croissance, la reproduction des organismes aquatiques. Le bassin d'Arcachon est le siège d'une activité ostréicole importante. Cependant depuis plusieurs années, les exploitations ostréicoles sont confrontées à une baisse de recrutement et une forte mortalité des naissains d'huître. La contamination chimique du milieu comme facteur pouvant contribuer aux effets observés sur les huîtres n'a pour l'instant pas été vérifiée. Dans ce contexte, l'objectif de cette thèse porte sur l'évaluation, à travers différentes approches, de la toxicité potentielle de métaux et pesticides sur les stades précoces de développement de l'huître creuse *Crassostrea gigas*. Les réponses embryotoxiques, génotoxiques et les niveaux de transcription de gènes d'intérêt ont été étudiés.

2. Méthodes

Plusieurs bioessais et biomarqueurs ont été utilisés pour évaluer l'impact des polluants sur les gamètes et embryons d'huître du Pacifique. Les différents pesticides (S-métolachlore, irgarol et diuron) et métaux (cuivre et cadmium) ont tout d'abord été testés séparément pour déterminer leur spectre d'effets et étudier leur mode d'action. Les solutions mères ont fait l'objet d'analyses chimiques pour confirmer la concentration des polluants avant la réalisation des bioessais.

2.1 Test embryo-larvaire

L'obtention des gamètes mâles et femelles se fait par stimulation thermique des huîtres matures sexuellement, plongées alternativement dans des bains d'eau de mer à 18 °C et 28 °C, soit par stripping de la

gonade. Le succès de fécondation est vérifié par microscopie, puis les gamètes fécondés sont transférés dans des microplaques de 24 puits et sont exposés à la substance ou à l'échantillon environmental à tester pendant 24h à 24°C. En fin de test les larves obtenues sont fixées avec du formaldéhyde à 10% et les larves D normales et anormales sont dénombrées. Les pourcentages d'anomalies sont déterminés d'après les critères de His et al. (1997).

2.2 Succès de fécondation

Les spermatozoïdes et/ou ovocytes sont exposés pendant 30 min aux contaminants. Le succès de fécondation est vérifié toutes les 15 min à partir de la mise en contact des gamètes mâles et femelles jusqu'à ce que les ovocytes subissent leur premier clivage au bout de 120 min. Les ovocytes fécondés en forme de poire sont facilement reconnaissables parmi les ovocytes non fécondés qui sont de forme ronde. Les ovocytes sont comptabilisés (≈ 300 ovocytes) avec un microscope inversé et transférés dans des microplaques de 24 puits. Les embryons sont incubés à 24 °C pendant 2 heures à l'obscurité jusqu'à ce que les embryons atteignent le stade de 2-4 cellules chez les contrôles. Les embryons sont alors fixés avec quelques gouttes de formaldéhyde à 37% pour stopper le développement et conserver les embryons. Le taux de fécondation est mesuré sous microscope inversé en dénombrant le pourcentage d'ovocytes non fécondés.

2.3 Test des comètes

Le test des comètes est une technique couramment utilisée pour mesurer les effets génotoxiques chez les organismes. Les larves sont dissociées par une action mécanique et enzymatique avec la dispase pour isoler les cellules. Le test est ensuite réalisé uniquement sur les échantillons ayant plus de 80 % de viabilité cellulaire. Un traitement alcalin permettant le déroulement de l'ADN est effectué pendant 20 minutes. Une électrophorèse est réalisée à 25 V et 300 mA pendant 20 minutes afin de faire migrer les fragments d'ADN de façon différentielle. Les molécules d'ADN intactes et donc trop "lourdes" pour être déplacées dans le champ électrique vont décrire une sphère compacte. Un ADN endommagé va, quant à lui, voir migrer ses fragments les plus courts en dehors de cette sphère, formant ainsi des figures comparables à des comètes. Le pourcentage d'ADN ayant migré dans la queue de la comète est déterminé à l'aide d'un microscope optique à fluorescence (Olympus BX 51) et un système d'analyse d'image (Komet 5.5, Kinetic Imaging Ltd).

2.4 L'expression des gènes

Les ARN totaux issus de pool d'embryons ont été extraits à l'aide de kits "Absolutely RNA ® Miniprep kit" (Stratagene, Agilent) selon les instructions du fournisseur. Le processus de rétrotranscription permet de synthétiser un brin d'ADN complémentaire (ADNc) à partir d'ARN grâce à une ADN polymérase ARN-dépendante appelée reverse transcriptase. Pour la rétrotranscription, les kits « AffinityScript™ Multiple Temperature cDNA Synthesis kit » (Stratagene, Agilent) ont été utilisés. Les amplifications ont été effectuées avec le Mx3000P (Stratagene) en utilisant les kits de réactifs « Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix » (Agilent). Les courbes de dissociation ont été obtenues en suivant la fluorescence SYBR Green. Les Ct correspondent au nombre de cycles nécessaires pour que la luminescence émise dépasse significativement le niveau basal et permette une quantification du nombre de copies initiales d'ARNm.Les facteurs d'induction (FI) seront ensuite calculés pour chaque gène d'intérêt et chaque traitement en divisant l'expression génique moyenne $(\overline{E})$ pour la condition contaminée par celle du groupe témoin :

$$FI = \frac{\overline{E}_{contaminé}}{\overline{E}_{témoin}}$$

3 . Résultats

Ce travail a montré que l'exposition de gamètes d'huîtres *C. gigas* (spermatozoïdes et ovocytes) à des concentrations environnementales de métaux et de pesticides réduit le succès de fécondation et la qualité de la descendance. Les résultats indiquent une CE_{50} du cuivre (Cu) pour les spermatozoïdes d'huître de 20 mg L^{-1} et de 830 mg L^{-1} pour le cadmium (Cd). Ces valeurs de CE_{50} sont très proches de celles retrouvées pour les spermatozoïdes d'oursins (Dinnel et al., 1989). Les résultats de cette thèse sont les premiers montrant que le S-métolachlore, le diuron et l'irgarol altèrent le succès de fécondation et la qualité de la descendance aux concentrations retrouvées dans le milieu aquatique. Les spermatozoïdes et les embryons se sont averés plus sensibles aux pesticides que les ovocytes. Ceci est cohérent avec les études antérieures dans lesquelles l'exposition aiguë d'ovocytes n'avait pas, ou peu, d'effet sur la fertilité (Bellas et al., 2001; Eyster et Morse, 1984; Fitzpatrick et al., 2008).

En terme d'embryotoxicité, les résultats de cette thèse confirment les études précédentes (Warnau et al., 1996) qui démontrent que les embryons d'huîtres du Pacifique sont très sensibles à l'exposition de polluants tels que les métaux et les pesticides. Par exemple , les valeurs de CE_{50} atteignent 12 µg L^{-1} pour le Cu et 210 µg L^{-1} pour le Cd. Ces résultats corroborent les données de CE_{50} pour ces mêmes métaux dans d'autres espèces d'invertébrés marins (Dinnel et al., 1989; King et Riddle, 2001; Nadella et al., 2009). Le cadmium, quant à lui, ne présente pas d'effet embryotoxique ou génotoxique aux concentrations habituellement retrouvées dans les milieux aquatiques. Certains pesticides dont le diuron, l'irgarol, le S-métolachlore et ses métabolites (métolachlore ESA et métolachlore OA) ont également induit des effets embryotoxiques à des concentrations très faibles. Ceci est en accord avec les études antérieures sur la toxicité de l'irgarol pour plusieurs invertébrés marins tels que les crustacés, les oursins et les moules (Bellas, 2006; Desai, 2008; Manzo et al., 2006). Récemment, Akcha et al. (2012) ont également montré que le diuron a des effets embryotoxiques sur l'huître *C. gigas* à partir de la concentration de 50 ng L^{-1}. Les métabolites du métolachlore, ESA et OA sont retrouvés dans le bassin d'Arcachon à de plus fortes concentrations que le composé parent, cependant rien n'est actuellement connu sur les effets toxiques de ces métabolites et notamment sur les larves de bivalves. À notre connaissance, notre étude est la première portant sur les effets toxiques des produits de dégradation du métolachlore sur les stades précoces de vie de l'huître. Un résultat intéressant de ces études montre que les métabolites sont moins embryotoxiques et génotoxiques sur les embryons et sur les spermatozoïdes d'huître que le métolachlore. Des variations dans l'expression des gènes impliqués dans les défenses antioxydantes sont observées pour les larves d'huître exposées au métolachlore et au métolachlore ESA. Un profil différent dans l'expression des gènes est néanmoins observé entre le métolachlore et ses métabolites. Le métolachlore, contrairement au métolachlore ESA, semble augmenter la réponse des défenses antioxydantes avec une induction de l'expression du gène de la superoxyde dismutase. Cela suggère que les mécanismes d'action conduisant à des effets embryotoxiques et génotoxiques chez les larves de *C. gigas* sont différents entre le métolachlore et ses métabolites. La toxicité d'un mélange de pesticides représentatifs de la contamination du bassin d'Arcachon en présence ou non de cuivre a ensuite été évaluée. L'exposition des embryons d'huître à

ces mélanges conduit à des défauts de développement, des dommages à l'ADN et des modifications de l'expression des gènes impliqués majoritairement dans la réponse au stress oxydant, dans le métabolisme mitochondrial et la régulation du cycle cellulaire.

Nos résultats ont montré que les substances toxiques utilisées dans cette étude en concentrations environnementales, à l'exception du cadmium, sont capables d'induire des dommages de l'ADN dans des embryons d'huîtres exposés individuellement ou en mélange. Jusqu'à présent, les informations concernant les effets génotoxiques des contaminants de l'environnement sur les premiers stades de vie et les spermatozoides de bivalves restent limitées (Bolognesi et al., 1999; . Nadella et al., 2009, Wessel et al. 2007). Bien que les mécanismes d'altération de l'ADN par les métaux aient fait l'objet de plusieurs études antérieures (Bertoncini et Meneghini, 1995; Beyersmann et Hechtenberg, 1997; Lesser, 2006; Stohs et Bagchi, 1995), les mécanismes par lesquels les pesticides provoquent des dommages primaires de l'ADN ne sont pas bien connus. Les modifications obtenues dans l'expression des gènes impliqués dans le stress oxydant chez les embryons exposés aux contaminants suggèrent une production accrue d'espèces réactives de l'oxygène pouvant conduire à des cassures de l'ADN (Akcha et al., 2012; . Meyer- Ficca et al., 2011; . Wang et al., 2004). Ces travaux ont également mis en évidence une corrélation entre la génotoxicité en terme de cassures de l'ADN et les malformations larvaires après l'exposition à deux métaux (Cu et Cd) et deux pesticides (Irgarol et S-métolachlore).

Finalement, une cartographie de la toxicité des sédiments du bassin d'Arcachon a été réalisée au cours des quatre saisons de l'année 2011 à l'aide du test embryolarvaire huître. Ce test est utilisé comme outil pour évaluer la qualité des sédiments dans les études de surveillance du milieu marin (Geffard et al., 2001). Les sédiments d'Arguin présentent une faible toxicité quelle que soit la saison considérée. En effet, Arguin, situé à l'entrée de la lagune et soumis à une forte influence océanique, est considéré comme le site le moins contaminé du bassin d'Arcachon. En revanche, les sédiments du Tès montrent une embryotoxicité plus importants au printemps et en été par rapport à la saison hivernale. Le Tès, situé dans la partie interne de la baie et à l'entrée du port d'Arcachon), est soumis à de plus fortes pressions anthropiques de par sa proximité au bassin versant et le nautisme.

4. Conclusions et perspectives

L'ensemble de ce travail a permis de mettre en évidence la toxicité sur les gamètes et les premiers stades de développement de l'huître creuse de deux métaux et de plusieurs pesticides bien représentés dans le Bassin d'Arcachon. Les seuils de toxicité obtenus sont proches voire même inférieurs aux concentrations retrouvées habituellement dans le bassin d'Arcachon. Ceci nous permet d'émettre l'hypothèse d'un risque chimique accru pour le développement des premiers stades de vie de huître creuse dans le bassin d'Arcachon. Il paraît important pour comprendre les baisses de recrutement observées dans le bassin d'Arcachon, d'étudier les conséquences à plus long terme des malformations embryonnaires notamment en ce qui concerne le succès de la métamorphose et de la fixation des larves. Il paraît également important d'étudier les effets combinés de la pollution chimique et du stress thermique en raison de l'accroissement prévisible des températures de surface.

L’ensemble de ces informations apparaît essentiel pour la sauvegarde de l’activité ostréicole dans le bassin d’Arcachon.

LIST OF PUBLICATIONS

1. Published in Marine Pollution Bulletin

Mai, H., Cachot, J., Brune, J., Geffard, O., Belles, A., Budzinski, H., Morin, B., 2012. Embryotoxic and genotoxic effects of heavy metals and pesticides on early life stages of Pacific oyster (*Crassostrea gigas*). Marine Pollution Bulletin 64, 2663-2670.

(http://www.sciencedirect.com/science/article/pii/S0025326X12004997)

2. Published in Marine Environmental Research

Huong Mai, Bénédicte Morin, Patrick Pardon, Patrice Gonzalez, Hélène Budzinski, Jérôme Cachot, 2013. Environmental concentrations of irgarol, diuron and S-metolachlor induce deleterious effects on gametes and embryos of the PaciFic oyster, *Crassostrea gigas*. Marine Environmental Research 89, 1-8.

(http://www.sciencedirect.com/science/article/pii/S0141113613000676)

3. Accepted in Journal of Xenobiotics

Huong Mai, Bénédicte Morin, Jérôme Cachot. Toxic effects of copper and cadmium on fertilization potency of gametes of Pacific oyster (*Crassostrea gigas*). Journal of Xenobiotics

4. . Accepted in Aquatic Toxicology

Huong Mai, Patrice Gonzalez, Patrice Pardon, Nathalie Tapie, Hélène Budzinski, Jérôme Cachot, Bénédicte Morin. Comparative responses of sperm cells and embryos of Pacific oyster (*Crassostrea gigas*) to exposure to metolachlor and its degradation products. Aquatic Toxicology.

ABSTRACT OF THESIS

The coastal areas are subject to multiple anthropogenic pressures including chemical pollution that can pose a real risk to the sustainability of aquatic species. The Arcachon Bay, a macrotidal lagoon located on the French Atlantic coast, is an important ecosystem for oyster farming. But for several years, the oyster farms face lower recruitment and high mortality of oyster spat. Chemical contamination of the environment is believed to contribute to the observed effects on oysters and has so far not been investigated.

The present thesis aimed at evaluating through different approaches, the potential toxicity of heavy metals and pesticides representative of the Arcachon Bay contamination on the early life stages of the Pacific oyster, *Crassostrea gigas*. Embryotoxicity, genotoxicity and gene transcription levels of eleven targeted genes were studied. Firstly, different pesticides (S-metolachlor, irgarol, and diuron) and metals (copper and cadmium) were individually tested to determine their spectrum of effects. It was shown that exposure of gametes and embryos of oyster to environmental concentrations of pesticides and copper increased developmental abnormalities and DNA damage, and reduced fertilization success and affected offpsring quality. Cadmium, meanwhile, showed no embryotoxic and genotoxic effects at the concentrations occuring in the Arcachon Bay. Metabolites of metolachlor, metolachlor ESA and metolachlor OA, are found in the Arcachon Bay at higher concentrations than their parent compound. The results showed that these metabolites were less embryotoxic and genotoxic on oyster embryos and spermatozoa than metolachor. Significant changes in transcription of genes involved in antioxidant defense were observed for oyster larvae exposed to metolachlor and metolachlor ESA. Toxicity of mixtures of pesticides representative of the Arcachon Bay contamination with and without copper was then evaluated. Exposures of oyster embryos to these mixtures lead to development defects, DNA damage and changes in the transcription of genes involved mainly in oxidative stress responses. Finally, mapping of toxicity of sediments from the Arcachon Bay was conducted at each season of 2011 with oyster embryo-larvae assay. Sediments collected from Arguin exhibited low toxicity, regardless any season. In contrast, sediments from Le Tès showed higher toxicity in spring and summer compared to winter.

From this work, it can be hypothesized that chemical contamination of the Arcachon Bay represents a threat for oyster reproduction and development.

Keywords: *pesticides, metals, Pacific oyster,embryo-larvae toxicity assay, fertilization assay, comet assay, gene expression*

RESUME DE LA THESE

Les zones côtières sont soumises à des pressions anthropiques multiples notamment de nature chimique qui peuvent faire peser un risque réel pour la pérennité des espèces aquatiques. Le bassin d'Arcachon, lagune macrotidale située sur la façade atlantique, est aussi le siège d'une activité ostréicole importante. Cependant depuis plusieurs années, les exploitations ostréicoles sont confrontées à une baisse de recrutement et une forte mortalité des naissains d'huître. La contamination chimique du milieu comme facteur pouvant contribuer aux effets observés sur les huîtres n'a pour l'instant pas été vérifiée.

L'étude présentée ici porte sur l'évaluation, à travers différentes approches, de la toxicité potentielle de métaux et pesticides sur les stades précoces de développement de l'huître creuse *Crassostrea gigas*. Les réponses embryotoxiques, génotoxiques et les niveaux de transcription de gènes d'intérêt ont été étudiés. Les différents pesticides (S-métolachlore, irgarol et diuron) et métaux (cuivre et cadmium) ont tout d'abord été testés séparément pour déterminer leur spectre d'effets et étudier leur mode d'action. Il a été montré qu'une exposition des gamètes ou des embryons d'huître aux pesticides étudiés et au cuivre conduit à une augmentation des malformations larvaires et des dommages à l'ADN, une diminution du succès de fécondation et un impact sur la qualité de la descendance à des concentrations environnementales. Le cadmium, quant à lui, ne présente pas d'effet embryotoxique ou génotoxique aux concentrations présentes dans le milieu aquatique. Les métabolites du métolachlore, ESA métolachlore et OA métolachlore sont retrouvés dans le bassin d'Arcachon à de plus fortes concentrations que le composé parent, cependant rien n'est actuellement connu sur les effets toxiques de ces métabolites. Il a été montré que ces métabolites sont moins embryotoxiques et génotoxiques sur les embryons et sur les spermatozoïdes d'huître que le métolachlore. Des variations dans l'expression des gènes impliqués dans les défenses antioxydantes sont observées pour les larves d'huître exposées au métolachlore et au métolachlore ESA. La toxicité d'un mélange de pesticides représentatifs de la contamination du bassin d'Arcachon en présence ou non de cuivre a ensuite été évaluée. L'exposition des embryons d'huître à ces mélanges conduit à des défauts de développement, des dommages à l'ADN et des modifications de l'expression des gènes impliqués majoritairement dans le stress oxydant. Finalement, une cartographie de la toxicité des sédiments du bassin d'Arcachon a été réalisée au cours des 4 saisons de l'année 2011 à l'aide du test embryolarvaire huître. Les sédiments d'Arguin présentent une faible toxicité quelle que soit la saison considérée. En revanche, les sédiments du Tès montrent un effet embryotoxique plus important au printemps et en été par rapport à la saison hivernale.

L'ensemble de ce travail permet d'émettre l'hypothèse d'un risque chimique accru pour le développement des premiers stades de vie de huître creuse dans le bassin d'Arcachon.

Mots clés : pesticides, métaux, huitre creuse, test embryo-larvaire, génotoxicité, test de fertilité, expression génique

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CONTENTS

GENERAL INTRODUCTION

Marine environments, especially those near the coastal line, are exposed to a wide variety of natural and anthropogenic stressors (Adams, 2005). Coastal systems are frequently contaminated by complex mixtures of xenobiotics issued from multiple human activities including industrial, agriculture and domestic effluents, potentially harming marine organisms (Poynton and Vulpe, 2009). There is growing needs for the development of bioassays for the identification, estimation, and comparative evaluation of environmental hazard assessment and management. Among aquatic organisms, bivalves, are frequently used to assess the toxicity of a large variety of contaminants in marine water or sediments (Rickwood and Galloway, 2004). Some studies have demonstrated that marine bivalves readily bioaccumulate both organic and inorganic pollutants (Cajaraville et al., 2000). Besides being relatively tolerant to a large series of contaminants, they are the most commonly used species as environmental quality indicators (Baumard et al., 1999).

Among the several pollutants that can contribute to aquatic pollution, heavy metals represent the most widespread and serious forms of environmental contamination (Devi et al., 1996). There are different kinds of sources from which heavy metals can be introduced in the environment. Natural sources of metals are mainly produced by erosion of natural mineral deposits present in the soil, volcanic eruptions, forest and vegetation fires (Clark, 1992). In urbanized and industrialized areas, however, the main source of metal pollution is the human activities. A large variety of anthropogenic activities can lead to metal discharge in the environment, such as mining, foundry, smelter, traffic and agriculture (Clark, 1992). There is numerous heavy metals which are considered as toxic because of their poisonous to aquatic life (EEA, 2003): including mercury, copper, cadmium, silver, nickel, lead, chromium, and zinc. In the present work special attention has been paid to copper and cadmium.

Pesticides are widely used throughout the world as agents for controlling invasive species (Banks et al., 2005; Okamura et al., 2000). Common examples of pesticides include herbicides to control weeds, insecticides to control insects, fungicides to control certain type of plant disease, and antifouling agents to inhibit the growth of fouling organisms on boat hull. Pesticides differ from many other environmental substances of concern in that they enter the environment through intentional use for specific purposes and accidents. Pesticides enter the aquatic ecosystems by a variety of routes from many different sources. Particularly, pesticide residues can be discharged in aquatic ecosystems following spray-drift, leaching or run-off from urban (parking lots and residential areas) and agriculture (farming-treated areas), contaminated soils and aquatic sediments. Surface runoff is considered as one of the most

important pathways for pesticides to enter surface waters (Larson et al., 1995; Liess et al., 1999). Pesticide contamination have been widely detected in waters and sediments of European estuarine and/or coastal areas (Sargent et al., 2000; Thomas et al., 2000; Zhou, 2008), or in Japanese coastal water (Okamura, 2002). Once pesticide residues enter the environment, they can remain in the aquatic environments from a few hours to several months or years depending on their physico-chemical properties.

Arcachon Bay, located in the South West Atlantic coast, with important oyster farming activities and tourism, is also concerned by the current environmental issues. Indeed, the exponential oyster farming, tourism boating and urbanization growing at the edge of the basin have increased anthropogenic pressure on the lagoon in the past decades, threatening more than ever the balance of this ecosystem. Since 1998, several years have been particularly unfavorable for oyster larval recruitment and high mortality of oyster spat have been recorded. One of the possible causes of these effects could be linked to the chemical contamination of the environment and had so far been studied very little. In 2007, IFREMER have published a report on insecticide and herbicide contamination in Arcachon, showing increasing pesticide concentrations year after year in the Arcachon Bay (Auby et al., 2007).

Oysters, *Crassostrea gigas,* have been postulated as ideal indicator organisms for assessing levels of environmental pollution, as they are ubiquitous sedentary filter-feeder organisms inhabiting coastal and estuarine areas. Oysters are also widely used as sentinel organisms in monitoring effects of pollutants. Their filtering habits, low metabolism and ability to bioaccumulate most pollutants make them suitable organisms to assess not only the bioavailability but also the effects of those contaminants. Therefore, there has been a considerable interest in development of *in vitro* tests from the oyster which could be used as complementary tools to assess the impact of xenobiotics on aquatic ecosystems such as estuaries and coastal areas. In this context, oyster gametes and embryos provide a unique and valuable tool for the evaluation of xenobiotic toxicity. Those early life stages have been used for more than 25 years in bioassays for chemical testing and environmental quality evaluation (Beiras and His, 1994; His et al., 1999a). Among various bioassays using oyster gametes and embryos, the oyster fertilization success test, oyster sperm toxicity test and oyster embryo test can be used as screening methods for chemicals and environmental samples because of their simplicity and sensitivity (His et al., 1999b; Orieux et al., 2011). In the meanwhite, the comet assay and real-time quantitative polymerase chain reaction (RT-qPCR) have been considered as the most sensitive techniques to detect changes at molecular level induced by

environmental contaminants in aquatic systems (Frenzilli et al., 2009; Neumann and Galvez, 2002).

Therefore, the aim of the present thesis is to evaluate fertilization, embryotoxic, genotoxic and gene expression responses in early life stages of Pacific oyster after exposure to tested chemicals. It has become increasingly clear with time that single biomarker is not able to determine the health status of a living organism. A multi-biomarker approach has been described as the best tool for identifying the effects and the mechanisms of pollutant toxicity to different biological levels of organization (Adams and Greeley, 2000; Faria et al., 2010; Viarengo et al., 2007). A combination of bioassays (embryo-larval test) and biomarkers (DNA damage and gene transcription levels) was used in this study to determine the toxic effects of pollutants on early life stages of Pacific oyster *C. gigas*. Embryotoxicity and genotoxicity by means of the comet assay can be used as screening methods because of their simplicity and large application to any eukaryotic cell (His et al., 1999b; Orieux et al., 2011).

CHAPTER 1: RESEARCH CONTEXT

1. STUDY AREA: ARCACHON BAY

1.1 Geography

Arcachon Bay is a marine lagoon of the Atlantic Ocean in the southwest coast of France in the Aquitaine region (Fig. 1). This 180-km^2 tidal shallow semi-sheltered lagoon allows important oyster farming activity but it is also a major mooring and sailing area receiving up to 15,000 pleasure boats in summer time (Arcachon is the second largest pleasure harbour on the French Atlantic West Coast) (Ruiz et al., 1996). This lagoon includes 110-km^2 of mudflats, 70 km^2 of which are colonized by a vast *Zostera noltii* seagrass bed and 10 km^2 by oyster parks (Auby and Maurer, 2004). The annual production of cultivated *Crassostrea gigas* is approximately 15,000 tons and Arcachon Bay is the main European center for the collecting of oyster spats (de Montaudoüin et al., 2001).

Arcachon Bay receives freshwater inputs mainly by the Leyre River and marine water via two channels located at the southwest end of the lagoon (Fig. 2). These fresh and oceanic water inputs as well as the slow renewal of water by tides (Plus et al., 2006) induce salinity and temperature gradients within the bay (Dang et al., 2010). Sediment composition in Arcachon Bay is mud to muddy sands.

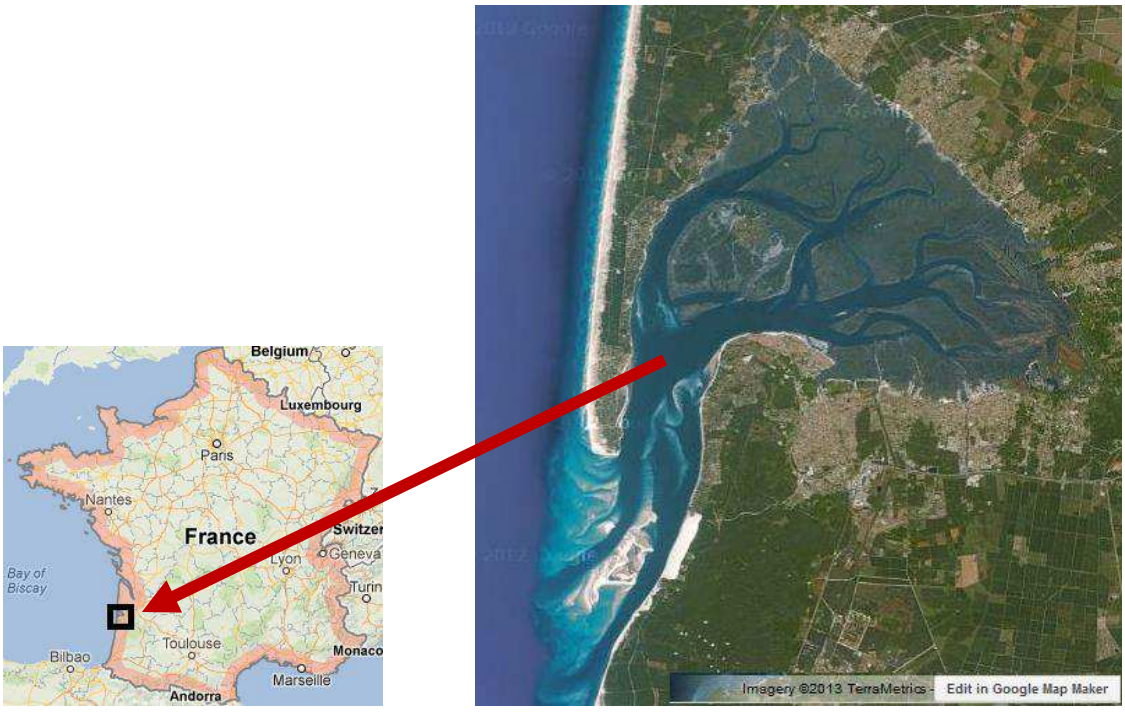


Fig. 1: Geography of Arcachon Bay, South West of France

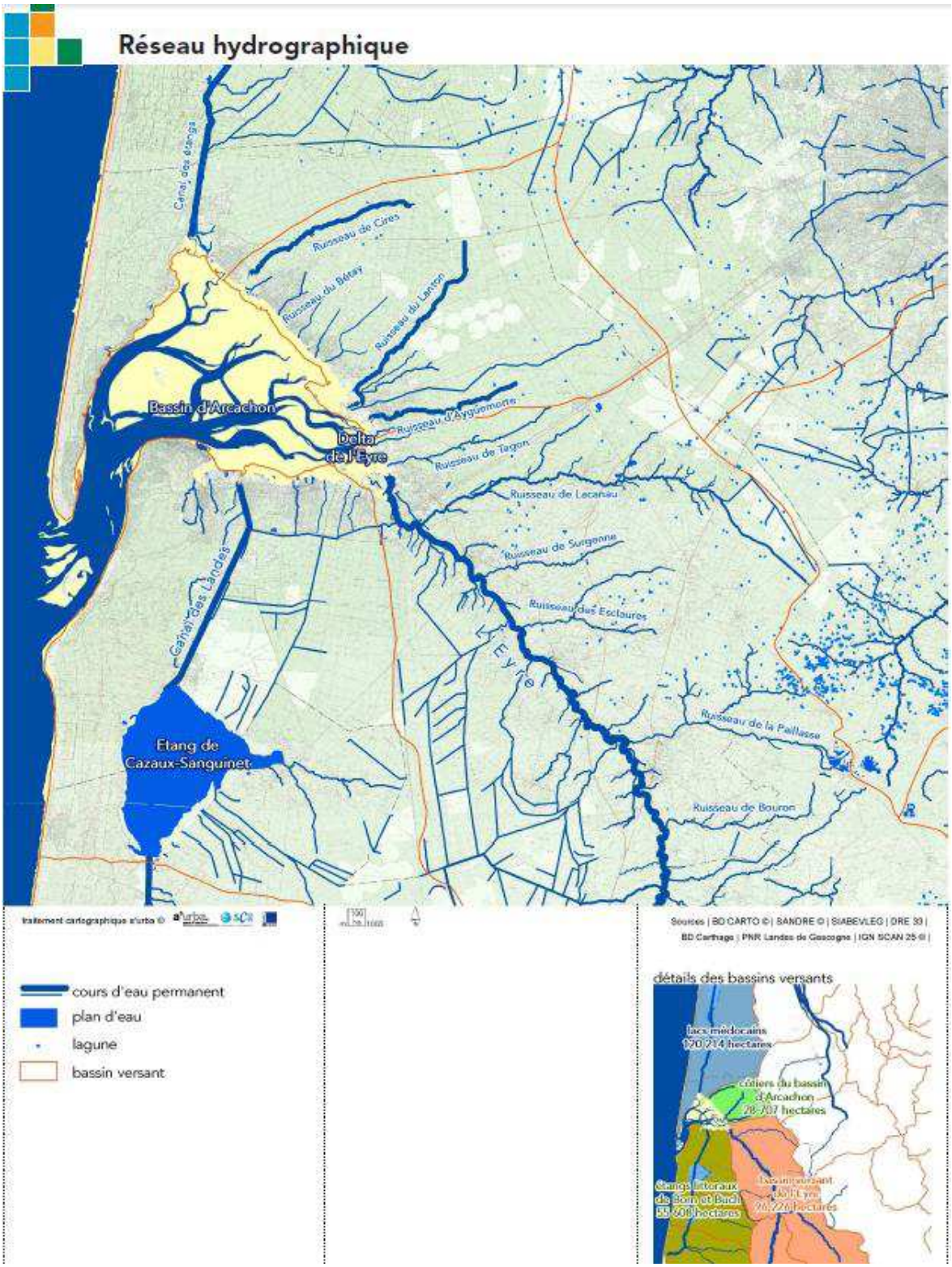


Fig. 2: Hydrographic network of the Arcachon Bay

Source : SYBARVAL, SCOT – Bassin d'Arcachon et Val de l'Eyre, état initial de l'environnement (2008)

1.2 Context of oyster farming in Arcachon Bay

Arcachon Bay is a well-known area for oyster farming with about 8,000 to 15,000 tons produced each year (de Montaudoüin et al., 2001). It provides about 60% of the French production of juvenile oysters (Auby and Maurer, 2004). The breeding season of oyster in the Arcachon Bay extends from early June to mid-September (His, 1975). For several years (2007-2011), low larval recruitment, reduced spat fall and increased oyster mortality events have been observed in the Arcachon Bay suggesting impaired reproduction, mortality or developmental defects at early life stages (Maurer et al., 2011). Exposure to pollutants could partly account for the reduction of early life stages of the Pacific oyster. Indeed, the marine environment poses particular challenges since the oyster reproduce by releasing their sperm and eggs freely into the water column so that fertilization takes place externall and also early life stages of oysters including gametes, embryos, and larvae are known to be particularly sensitive to contaminants such as metals, pesticides, PAHs and other persistent organic contaminants (Akcha et al., 2012; Geffard et al., 2003; His et al., 1999a).

The increased level of Cu in oysters of Arcachon Bay over the past 20 years is probably related to the use of this compound in antifouling paints to replace TBT (Ifremer&UB1, 2008). According to the report presented by IFREMER and the University of Bordeaux related to the potential impact of human activities on the water quality of the Arcachon Bay (2008), between 3 and 4.5 tonnes of Cu are used annually (evaluation period 1997-1999). However, during the period from 1998 to 2001, Cu amount used in antiflouing paint tended to decrease; this phenomenon can be due to a change in the method of conditioning Cu paints (Auby and Maurer, 2004). Median concentrations of Cu measured in oysters in the Arcachon Bay (less than 200 mg kg^{-1}, p.s.) were comparable the national median (Ifremer, 2008). The report of the ASCOBAR project (2010) also reported a significant bioaccumulation of Cu in oysters from different internal sites of Arcachon Bay, while the contents of Cd remain low. The oyster concentration of Cd tended to decrease since the years 1999-2000, and is now stable and well below the standard of consumption (reviewed in Barjhoux, 2011). The 2012 report of IFREMER confirmed the stability in low concentrations of Cd in oysters.

As part of the study on the reproduction of oysters in Arcachon Bay conducted by IFREMER from 1999 to 2003 (Auby and Maurer, 2004), some pesticides were monitored in several areas of the bay, during the summer months (June to September). During the period of 2005-2006, the presence of a number of contaminants, such as insecticides and herbicides, was detected in the water column as well as in oysters (Auby et al., 2007). Some substances

(metolachlor, acetochlor, diuron…) come from agricultural use or maintenance of green spaces, others have water origin (antifoulant paint protecting the boat hull). The concentrations of herbicides used in agriculture (including irgarol, diuron, alachlor and metolachlor) are generally quite low. Among pesticides found in significant concentrations, irgarol was detected at about 66 ng L^{-1} in water, 32 ng L^{-1} for diuron, and 18 ng L^{-1} for alachlor, while only 5.8 ng L^{-1} for metolachlor (Auby et al., 2007). However, in the Arcachon Bay, metolachlor and its metabolites are the main pesticides, ranging from 1 to 120 ng L^{-1} (REPAR, 2011). The authors also reported that maximal concentrations of metolachlor and diuron measured in oysters in Arcachon Bay are still lower than the mean national (e.g. 1.2 µg kg^{-1} and 1.5 µg kg^{-1}, respectively). In addition, the frequent detections of those pesticides in oysters were low, reaching only 4% for metolachlor and 17% for diuron (Auby et al., 2007).

1.3 Pollutants contamination

1.3.1 Heavy metals

Anthropogenic activities such as the release of industrial effluent and the growing use of copper as an antifoulant have resulted in Cu becoming increasingly prevalent in coastal ecosystems. Cu is extensively used in the electronic industry, roofing and building construction as wood preservation and as antifouling base in paints (EEA, 2003). Cu is mainly transported to the environment by rivers through estuaries where the amounts and chemical forms are affected by physic-chemical processes. Natural Cu concentrations in marine environments are typically < 5µg/L, but in polluted areas, Cu levels can be orders of magnitude higher than natural levels (Table 1).

Arcachon Bay, like other estuarine ecosystems, is typically subjected to a variety of stressors including complex xenobiotic pollutants. Metals such as Cu, Cd, Pb and Hg are regularly monitored in the bay. In particular, dissolved copper concentrations have been increasing in Arcachon harbour since the banning in 1982 of the use of TBT (tributylin) and all organotin-based products on boats under 25 m along the entire French coastline (Ifremer&UB1, 2008). However, copper level in Arcachon Bay was observed at lower concentration than in others European estuaries, especially estuaries of the United Kingdom (Table 1).

Table 1: Copper concentrations in water bodies as reported in the literature

Sampling area	Sampling year	Concentration (ng L^{-1})	Concentration (nM)	References
AMERICA				
Bahía Blanca Estuary, Argentina	2005	< 500-2,400	< 7.9-37.8	Fernández Severini et al. (2009)
Ing. White Port, Argentina	2005	< 610-2,000	< 9.6-31.5	Fernández Severini et al. (2009)
Massachusetts Bay, USA	1989-1991	82.6-444.5	1.3-7.0	Shine and Wallace (1995)
Eastern Adriatic coast, Croatia	2009	< 228.6-2387.6	< 3.6-37.6	Plavšić et al. (2011)
San Francisco Bay, USA	1993-2001	431.8-3295.7	6.8-51.9	Buck et al. (2007)
ASIA				
Bering Sea, Japan	1980-1981	270	4.3	Honda et al. (1987)
East Antarctica, Japan	1980-1981	240-1,170	3.8-18.4	Honda et al. (1987)
WN Pacific coast, Japan	1980-1981	300-41 0	4.7-6.5	Honda et al. (1987)
Western coast of Taiwan	1997-1998	1,300-6,300	20.5-99.2	Hung et al. (2001)
EUROPE				
Arcachon Bay, France	1999	700	11.0	Geffard et al. (2002b)
Gironde Estuary, France	1997-1998	571.5-1270	9-20	Michel et al. (2000)
Loire Estuary, France	2002	596.9-1498.6	9.4-23.6	Waeles et al. (2009)
Morlaix Estuary, France	1998-1999	63.5-1206.5	1-19	Monbet (2004)
North Biscay Bay, France	2001	114.3-508	1.8-8.0	Waeles et al. (2004)
Orwell Estuary, UK	1992-1996	20,000-39,000	315-614.2	Matthiessen et al. (1999)
Ria Vigo Estuary, Spain	2004	317.5-508	5-8	Santos-Echeandía et al. (2008)
Penzé Estuary, NW France	2001	114.3-603.3	1.8-9.5	Waeles et al. (2005)
Seine Estuary, France	1994-1995	552.5-1778	8.7-28	Chiffoleau et al. (1999)
Venice Lagoon, Italy	2003	114.3-4445	1.8-70	Delgadillo-Hinojosa et al. (2008)

Note: 1 nM Cu = 63.5 ng L^{-1}

Natural dissolved Cu concentrations in marine environments are typically low, being generally below 0.2 μg L^{-1}. In uncontaminated nearshore surface waters, levels are significantly higher, often approaching 1 μg L^{-1}. In highly polluted waters, Cu concentration may exceed 10 μg L^{-1} (Burdon-Jones and Denton, 1984) and even reaching concentration of 50 μg L^{-1} in heavily-polluted harbours (Haynes and Loong, 2002). For clean and non-geochemically enriched sediments, Cu levels are currently less than 10 μg g^{-1}, but in severely polluted areas, they can yield values of 2000 μg g^{-1} (Bryan and Langston, 1992; Legorburu and Canton, 1991).

Cadmium is an abundant and non-essential element that is continuously accumulated in the environment as a result of industrial activities. On a global scale, the ratio of anthropogenic to natural emissions of Cd is approximately 7:1 (Denton et al., 1999). Sources of Cd for freshwater and salt water ecosystems include atmospheric deposition, direct and via runoff, as well as direct discharges into water or watersheds. Thirty percent of the atmospheric

emissions fall onto water. The sources of Cd derive from by-products of anthropogenic activities including mining, metal industries and agriculture (EEA, 2003). Production of chemical stabilizers and pigments as well as of the electroplating industry also are main sources of cadmium entering the environment (EEA, 2003). Much of the Cd resulted in aquatic systems accumulates in sediments where it presents a threat to benthic biota and under certain conditions may reenter the water column. Cadmium concentrations in remote open ocean waters may be as low as 0.002 μg L^{-1} and rarely exceeds 0.5 μg L^{-1} in nearshore waters, even in heavily industrialized areas (Table 2). Non-polluted sediments normally contain 0.2 μg g^{-1} or less but levels may exceed 100 μg g^{-1} at heavily contaminated sites (Naidu and Morrison, 1994).

In 2008, ROCCH Observation Chemical Contamination coastal Network was set up to meet national and international obligations of chemical monitoring: implementation of the WFD (Water Framework Directive) and OSPAR (Oslow PARis) and Barcelona rules. This monitoring is focused on the three regulated metals (Cd, Pb and Hg) and is done on water, sediment and shellfish. Thus, the cadmium level in Arcachon Bay has been monitored for many years. In Arcachon Bay, the strengthening of regulations on the use of cadmium and the discontinuation of certain notoriously polluting activities has resulted in a general decline in levels observed presence (Ifremer, 2012); thus the level of this metal has been stable for recent years. In fact, since 2008, Cd contamination in oysters of Arcachon Bay is generally low with values generally below or closes to the national median (Ifremer, 2008).

Table 2: Cadmium concentrations in water bodies from different areas in the world

Sampling area	**Sampling year**	**Concentration**		**References**
		(ng L^{-1})	**(nM)**	
AMERICA				
Bahía Blanca Estuary, Argentina	2005	< 500-2,400	< 4.5-21.4	Fernández Severini et al. (2009)
EUROPE				
Arcachon Bay, France	1999	9	0.08	Geffard et al. (2002b)
Gironde estuary, France	1997-1998	11.2-134.9	0.1-1.2	Michel et al. (2000)
Loire estuary, France	2002	19.1-55.1	0.17-0.49	Waeles et al. (2009)
Morlaix River estuary, France	1998-1999	4.5-54	0.04-0.48	Monbet (2004)
North Biscay Bay, France	2001	12.4-29.2	0.11-0.26	Waeles et al. (2004)
Penzé estuary, NW France	2001	6.7-51.7	0.06-0.46	Waeles et al. (2005)
Ria Vigo estuary, Spain	1995	1.5-10.5	0.013-0.093	Prego et al. (2006)
Scheldt estuary, SW Netherlands	1987-1988	11.2-134.9	0.1-1.2	Zwolsman et al. (1997)
Seine estuary, France	1994-1995	19.1-28.1	0.17-0.25	Chiffoleau et al. (1999)

Note: 1 nM Cd = 112.4 ng L^{-1}

1.3.2 Pesticides

Pesticides refer to any chemicals intended to prevent, deter, destroy, or otherwise impair the ability of pest to compete with desired organisms, such as crops, animals, or humans. Pesticides can be classified in different ways, such as by their target, chemical nature, physical state, and mode of action (Yu, 2005), but classification based on the target is perhaps the most widely known (Table 3). Several pesticides have frequently been found in waters of some estuarine and coastal areas in the world (Fig. 3).

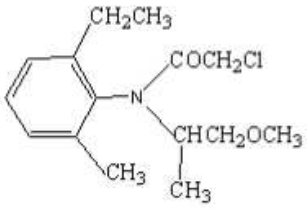
Table 3: Classification of Pesticides

Method of classification	**Examples**
By target	Insecticides, herbicides, fungicides, rodenticides, algaecides, nematocides
By chemical nature	Natural organic compounds, inorganic compounds, chlorinated hydrocarbons, organophosphates, carbamates
By physical state	Dusts, dissolved solutions, suspended solutions, volatile solids
By mode of action	Contact poisons, fumigants, stomach poisons

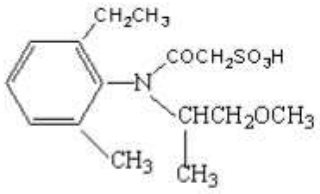
Source: Yu (2005)

Arcachon Bay, like other marine ecosystems, is typically subjected to a variety complex mixture of xenobiotics. Currently, organic pollutants, such as pesticides and their degradation products, are regularly monitored in the Bay. According to the reports on the quality of coastal marine environment, pesticide contamination of Arcachon Bay was also measured in water body and sediment as well as in oyster (Auby et al., 2007; Auby and Maurer, 2004; Belles, 2012). The potential sources of pesticides in the Arcachon Bay are mainly from agriculture (many crops including vegetable crops, corn and other cereals). The pesticides are also used in animal husbandry, home garden, anti-mosquito, and anti-termite, but rarely used on areas close to the Arcachon Bay (Auby et al., 2007; Auby and Maurer, 2004). In addition, boating is booming for several years, generating a very large attendance of the basin by boats (Ifremer&UB1, 2008). However, the knowledge of the pesticide levels in the Arcachon Bay (and more particularly in the intrabassin) remains fragmented. Specific studies as the program SURGIBA (program cofinanced by Europe, the Regional council of Aquitaine, the General Council of the Gironde and the Association of communes of the Bay of Arcachon), the insecticides and pesticides monitoring of the Arcachon Bay by the IFREMER and their environmental impact from 2005 to 2006, the ASCOBAR program (Apports Scientifiques face à la problématique Conchylicole du Bassin d'Arcachon: Etude intégrée du Bassin d'Arcachon, Projet Région 2008-2010) (ASCOBAR, 2010), the OSQUAR program

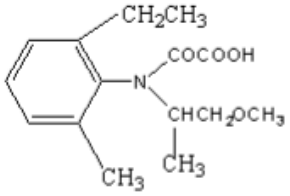
(Ostréiculture et qualité du milieu - Approche dynamique du Bassin d'Arcachon, Projet Région 2010-2012) (OSQUAR 2010-2012), highlighted the presence of a certain number of phytosanitary molecules, mainly pesticides and antifoulant molecules (REPAR, 2010; 2011). During a 5 years study (1999-2003) in the Arcachon Bay, 21 compounds of pesticides as well as their metabolite forms were detected with frequency of occurrence from 4% to 100% in water column. Herbicides including metolachlor, irgarol, atrazine and diuron occurred more frequently (> 40%) than others (Auby and Maurer, 2004; Belles, 2012). REPAR (Réseau de Surveillance des Pesticides sur le Bassin d'Arcachon) network was set up in 2010 for pesticide monitoring in Arcachon Bay. The most recent report (REPAR, 2011) showed that metolachlor and its metabolites is the major contributor to pesticide contamination of Arcachon Bay. The concentrations of pesticides measured in the water column are generally below the toxicity thresholds determined for aquatic species (see section 3.2). For instance, the pesticide contamination of oysters in Arcachon Bay was measured at below levels of the lowest maximum residue that are limit proportions allowing for consumption (Auby et al., 2007). However, pesticides are present as a complex mixture and the current guideline values are not determined for all compounds (Auby et al., 2007).



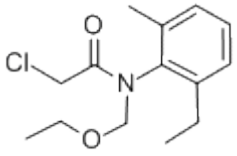
Metolachlor



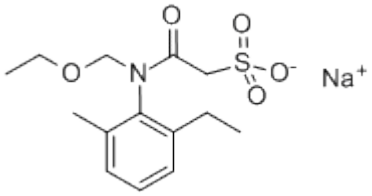
Metolachlor ESA



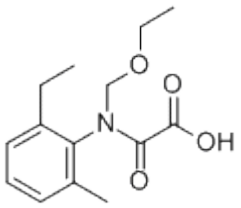
Metolachlor OA



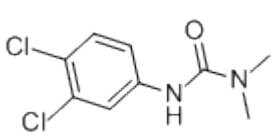
Acetochlor



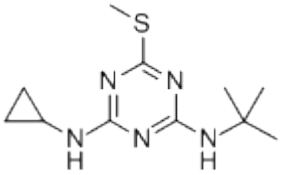
Acetochlor ESA



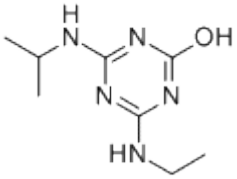
Acetochlor OA



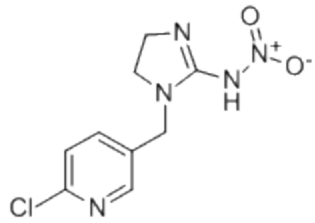
Diuron



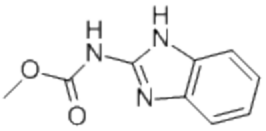
Irgarol



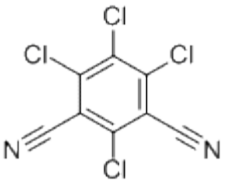
Atrazine-2-hydroxy



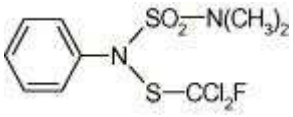
Imidacloprid



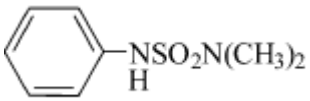
Carbendazim



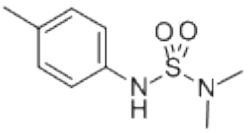
Chlorothalonil



Dichlofluarid



DMSA



DMST

Fig. 3: Structure of pesticides frequently found in coastal areas

- *Herbicide contamination*

Irgarol (2-methylthio-4-*tert*-butylamino-6-cyclopropylamino-s-triazine) is the most widely used antifouling biocide in paint formulations that are applied to the hulls of ships. Due to the presence of both *tert*-butyl and the cyclopropyl group, irgarol is the most hydrophobic compound of the family of the triazines. Zhou (2008) reported that the release rate of irgarol from paint residues is very low. It is classified as a persistent organic pollutant due to its long half-life (with a half-life of approximately one year), and its long residence time (over 10 years) in the marine system (Ranke, 2002). Irgarol can undergo biological and chemical degradation, and photodegradation. The compound 2-methylthio-4-*tert*-butylamino-6-amino-s-triazine (called GS26575) is major degradate of irgarol by fungi (Liu et al., 1997; Liu et al., 1999a; Okamura, 2002; Okamura et al., 1999). In addition, three photodegradation products ("RT5.8"; "RT7.8"; and "RT8.7") were also identified, of which two degradation products RT5.8 and RT8.7 were much more stable than RT7.8, even after 6 months irradiation (Okamura et al., 1999). According to those authors, its rate of photodegradation in natural sea water and river water were much higher than those in pure water and buffered solutions (pH 5, 7, and 9).

Currently, irgarol has been found in both water column and sediment of marinas, ports, coastal water, estuaries, and fishery harbors (Albanis et al., 2002; Basheer et al., 2002; Dahl and Blanck, 1996; Gardinali et al., 2002; Lamoree et al., 2002; Liu et al., 1997; Martinez et al., 2001; Readman et al., 1993; Scarlett et al., 1999; Toth et al., 1996). The occurrence of irgarol has been reported in European coastal areas as well as in USA, Japan and Singapore

(Table 4). Compared to other European coastal areas, irgarol concentrations were lower and tended to decrease during the time of from 1999 to 2003 in Arcachon Bay (Table 4).

Table 4: Irgarol in water bodies of estuarine or coastal areas in different areas in the world

Sampling area	Sampling year	Conc. (ng L^{-1})	References
AMERICA			
Biscayne Bay, USA	1999-2000	< 1-15.2	Gardinali et al. (2002)
Biscayne Bay, USA	2001	12.2-144.2	Owen et al. (2002)
ASIA			
Coastal areas, Singapore	2000	3,020-4,200	Basheer et al. (2002)
Seto Inland Sea, Japan (Marinas and fishery harbours)	1997	12-264	Liu et al. (1999b)
Seto Inland Sea, Japan (Marinas and fishery harbours)	1998	55-296	Okamura et al. (2000)
Seto Inland Sea, Japan (Ports, Marinas and fishery harbours)	1999	< 10-262	Okamura et al. (2003)
EUROPE			
Arcachon Bay, France	1999	< 2.5-95	Auby and Maurer (2004)
Arcachon Bay, France	2000	< 2.5-40	Auby and Maurer (2004)
Arcachon Bay, France	2001	< 2.5-47	Auby and Maurer (2004)
Arcachon Bay, France	2002	< 2.5-50	Auby and Maurer (2004)
Arcachon Bay, France	2003	< 2.5-22	Auby and Maurer (2004)
Arcachon Bay, France	2011	< 2.5	REPAR (2011)
Blackwater estuary, UK	1999	<150-350	Voulvoulis et al. (2000)
Brighton marina, UK	2003-2004	< 3.1-102	Gatidou et al. (2007)
Brighton marina, UK	2004-2005	< 3.1-22	Zhou (2008)
Conwy marina, UK	1999	7-543	Sargent et al. (2000)
Dutch coast	2000	20-90	Lamoree et al. (2002)
Gosport marina, UK	2004-2005	< 3.1-15	Zhou (2008)
Hamble estuary, UK	1998	< 1-141	Thomas et al. (2001)
Orwell estuary, UK	1998-1999	5.6-201.4	Boxall et al. (2000)
Shoreham port, UK	2003-2004	< 3.1-136	Gatidou et al. (2007)
Shoreham harbor, UK	2004-2005	< 3.1-45	Zhou (2008)
Southern coast, UK	1999	< 1-1,421	Thomas et al. (2001)
Southampton water estuary, UK	2000	< 1-305	Thomas et al. (2002)
Spanish coast	1996-2000	330	Martinez et al. (2001)

Diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea) is a registered herbicide. Diuron is a substituted urea herbicide and has been associated with general weed control on land. Diuron has also been used as an antifouling agent in many European countries (Boxall et al., 2000; Martinez et al., 2001). This compound is commonly detected in areas of high boating or yachting activity and is considered to be relatively persistent in seawater (Konstantinou and Albanis, 2004), with a slow breakdown (from one month to another) (Okamura, 2002) and half-lives of about 60 days (Harino et al., 2005) into metabolite DCPMU which are the main

diuron metabolite (REPAR, 2011). In Dutch and British coastal areas, diuron is present at higher concentrations than irgarol and other biocides (Lamoree et al., 2002; Thomas et al., 2002). Depending on the time or concentration of exposure and the considered species, diuron has shown toxic effects on various organisms such as algae, crustaceans and fish (Fernández-Alba et al., 2001; Fernández-Alba et al., 2002; Okamura, 2002). The concentrations of diuron in some estuarine or coastal areas are described in Table 5. Among the studied estuarine and coastal areas, Arcachon Bay showed lower levels of contamination in comparison to the coasts in the United Kingdom, Spain, Dutch and Sweden (Table 5).

Table 5: Diuron in water bodies of estuarine or coastal areas in the world

Sampling area	**Sampling year**	**Conc. (ng L^{-1})**	**References**
ASIA			
Seto Inland Sea, Japan (Marinas and fishery harbor)	1999	< 3054	Okamura et al. (2003)
EUROPE			
Arcachon Bay, France	1999	< 2.5-95	Auby and Maurer (2004)
Arcachon Bay, France	2000	< 10-186	Auby and Maurer (2004)
Arcachon Bay, France	2001	< 10-290	Auby and Maurer (2004)
Arcachon Bay, France	2002	< 10-153	Auby and Maurer (2004)
Arcachon Bay, France	2003	< 2.5-33	Auby and Maurer (2004)
Arcachon Bay, France	2011	< 2.5	REPAR (2011)
Catalan coast, Spain	1999	2190	Martinez et al. (2001)
Crouch estuary, UK	1998	5-305	Thomas et al. (2001)
Dutch coast	2000	90-1130	Lamoree et al. (2002)
Hamble estuary, UK	1998	1-613	Thomas et al. (2001)
Orwell Estuary, UK	1998-1999	21.9-768	Boxall et al. (2000)
Southampton estuary, UK	1998	<1-6742	Thomas et al. (2001)
Southampton estuary, UK	2000	26-1249	Thomas et al. (2002)
Spanish Mediterranean coast	1996-2000	2190	Martinez et al. (2001)
Sutton Harbour, UK	1998	1-334	Thomas et al. (2001)
West coast, Sweden	1993-1994	10-100	Dahl and Blanck (1996)

Metolachlor (2-Chloro-N-(2-ethyl-6-methylphenyl)- N-(2-methoxy-1-methylethyl)-acetamide) is one of the most intensively used chloroacetamide herbicide in agriculture to control annual grasses and broadleaf weeds in the field corn, soybeans, and peanuts. In the US, approximately 8.5 million kilogram per year of metolachlor are used in the agriculture sector (Kiely et al., 2004). Transport by water runoff and leaching may cause the metolachlor contamination of surface and ground water. Metolachlor is highly persistent in water over a wide range of water acidity (Stamper and Tuovinen, 1998). Its half-life at 20°C is more than 200 days in highly acidic waters, and is 97 days in highly basic waters (U.S. Environmental Protection Agency, 1987). In the condition of natural light and pH of 7, metolachlor can

persist for 335 days (Hayes and Mlaws, 1991). Amongst the acetanilide herbicides, metolachlor appears to be the most persistent and has the potential to leach to ground water because of its relatively high water solubility (530 mg L^{-1} at 20ºC) and low K_{oc} (200 mL g^{-1}) (Rivard, 2003). Metolachlor contamination in worldwise estuaries is presented in Table 6. Similar to the irgarol and diuron contamination levels reported in Arcachon Bay, metolachlor concentration was low during the monitoring survey from 1999 to 2003 compare to other areas but the lagoon imprints are widely dominated by the metolachlor and its metabolites (Auby and Maurer, 2004; REPAR, 2010, 2011).

Table 6: Metolachlor in water bodies of estuarine or coastal areas in the world

Sampling area	Sampling year	Conc. (ng L^{-1})	References
AMERICA			
Chesapeake Bay, Maryland	1997	< 0.5-58	Lehotay et al. (1998)
Canal 111 Bay, USA	1999-2000	4.2-62	Carriger and Rand (2008)
Mississippi Bay, Gulf of Mexico	1991-1997	50	Clark et al. (1999)
South Biscayne Bay, USA	1999-2000	5.7-7.2	Carriger and Rand (2008)
EUROPE			
Arcachon Bay, France	1999	< 2.5-13.9	Auby and Maurer (2004)
Arcachon Bay, France	2000	< 2.5-7	Auby and Maurer (2004)
Arcachon Bay, France	2001	< 2.5-5	Auby and Maurer (2004)
Arcachon Bay, France	2002	< 2.5-4	Auby and Maurer (2004)
Arcachon Bay, France	2003	< 2.5-5	Auby and Maurer (2004)
Arcachon Bay, France	2011	< 2.5	REPAR (2011)

Once in the soil, pesticides undergo degradation processes that give rise to a complex pattern of degradates, which can be transported to ground water and streams. Degradation products can be more persistent and mobile than their parent compounds, which can lead to frequent detection and increased concentration in ground and surface water. Metolachlor ethane sulfonic acid (ESA) and metolachlor oxanilic acid (OA) are the two most common degradates of metolachlor. Unlike the parent compound, the concentration of those metabolites was only studied in freshwater ecosystems and no information for marine ecosystems in the literature is available. Kalkhoff et al. (1998) showed that metabolites of ESA and OA in streams in eastern Iowa were generally present from 3 to 45 times more frequently than the parent compound. Another study in Mississippi Basin has shown that concentrations of metolachlor's metabolites are typically higher from 3 to 5-fold than corresponding parent compound (Kolpin et al., 2009). A survey of 355 water samples from 12 stream sites in Eastern Iowa also found metolachlor ESA in 99.7% of samples, metolachlor OA in 94.3% of samples and the parent compound in 54.1% of samples (Kalkhoff and

Thurman, 1999). A study of degradates in tile drain discharge from agricultural fields in central New Yord indicated that metolachlor ESA and OA can persist in agricultural soils for 3 or more years after application, and that metolachlor ESA concentrations in tile-drain discharge exceeded metolachlor OA from 2 to 5 times (Eckhardt et al., 1999). It can be concluded that degradates of metolachlor are more persistent in aquatic ecosystems than parent compound and are found in higher concentrations and more frequently in surface and ground water than metolachlor itself. In Arcachon Bay, the concentrations of metabolites were reported at about less than 2.5 to 70 ng L^{-1} for metolachlor OA and from 2.5 to 120 ng L^{-1} for metolachlor ESA depending on the inner parts of the Arcachon Bay (REPAR, 2011).

Acetochlor (2-chloro-N-(ethoxymethyl)-N-(2-ethyl-6-methylphenyl)-acetamine) is also a common chloroacetanilide herbicide in agricultural environments. Previous studies have demonstrated the presence of acetochlor in surface water (Hladik et al., 2008), soil (Chao et al., 2007), and sediments (Xue et al., 2005). Measurable concentrations of acetochlor ranged from 0.05 to 2.5 µg/L in surface water (Boyd, 2000; Hladik et al., 2008; Kolpin et al., 1996; Maloschik et al., 2007). The environmental concentrations of acetochlor and its metabolites are summerized in Table 7. Compared to estuaries in the North America, Arcachon Bay shows much less contaminated by acetochlor. Indeed, acetochlor was detected at only 2.5-3 ng/L in the Arcachon Bay (Auby and Maurer, 2004). As most pesticides, the occurrence of acetochlor's metabolites (16.3%) was generally greater than their parent compound (about 1.2%) (Kolpin et al., 2009; Yokley et al., 2002). The most recent survey in Arcachon Bay (REPAR, 2011) showed that metabolites of acetochlor were present in higher concentration than acetochlor with concentration ranging from a few ng L^{-1} up to 20 ng L^{-1}. Their presence varied with the sampling site and time.

Table 7: Acetochlor and its metabolites in water bodies of estuarine or coastal worldwide

Sampling area	Sampling year	Conc. (ng L^{-1})	References
AMERICA			
Mississippi Bay, Gulf of Mexico	1991-1997	50[a]	Clark et al. (1999)
Mississippi Bay, Gulf of Mexico	1999-2001	< 50 – 1,660[a]	Rebich et al. (2004)
		< 200 – 1,540[b]	
		< 200 – 2,040[c]	
EUROPE			
Arcachon Bay, France	2003	< 2.5-3[a]	Auby and Maurer (2004)
Arcachon Bay, France	2011	< 2.5[a]	REPAR (2011)
Arcachon Bay, France	2011	< 2.5-20[b]	REPAR (2011)
Arcachon Bay, France	2011	< 2.5-15[c]	REPAR (2011)

[a]Acetochlor; [b]Acetochlor ESA; [c]Acetochlor OA

- *Other pesticides*

Hydroxy-2-atrazine (HA) is the main metabolite products of atrazine (2-chloro-4-(ethylamino)-6-(isopropylamino)-s-triazine), which is commonly used to control broad leaf weeds in a variety of crops. Although atrazine used has been forbidden for many years, the presence of atrazine is still detected in the aquatic ecosystems because of its slow degradation over a period of weeks to months through hydrolytic reactions and microbiological transformation (reviewed by Sabik et al., 1995). The hydroxylated degradation products are generally more persistent in the aquatic environment because they are absorbed by the organic matter. The hydroxy-2-atrazine was found in waters ranging from 50 to 150 ng L^{-1} (Sabik et al., 1995). Recently, this metabolite was found in greater concentrations in several estuaries. For example, for the period of 1999-2001, hydroxy-2-atrazine concentration ranged from 200 to 2180 ng L^{-1} in the Mississippi Bay of Mexico Gulf (Rebich et al., 2004) and still lower concentrations were detected in the northern Adriatic Sea (39.4-65.5 ng L^{-1}) (Carafa et al., 2007). However, up to now, there is little information about hydroxy-2-atrazine contamination reported for the Arcachon Bay. Recently REPAR (2011) reported the concentration of hydroxy-2-atrazine in Arcachon Bay at about 1 to 10 ng L^{-1}.

Fungicides and insecticide have been used worldwide and may be available in ecosystems, (Cuppen et al., 2000). However, the environmental occurence of those pesticides were more difficult to determine due to the limited contamination data available (Table 8). Although a project was implemented to study the contamination of those pesticides including carbendazim, chlorothalonil, dichlofluarid, DMSA, DMST, and imidacloprid, but there is still scarce information about their occurrence in the Arcachon Bay (Auby et al., 2007). Recently, those pesticides have been monitored in Arcachon Bay (REPAR, 2010; 2011).

Among fungicides, carbendazim (methyl 1H-benzimidazol-2-ylcarbamate) proved to be very persistent in the water column and has a solubility of 8 mg/L at pH 7 and 20°C (Cuppen et al., 2000; Satapornvanit et al., 2009). In the environment, carbendazim has been frequently detected in surface waters, with degradation half-lives of 2 months under aerobic conditions, and 25 months in the absence of oxygen (Tomlin, 2009). A few ng L^{-1} carbendazim was detected in the Arcachon Bay (REPAR, 2011).

Chlorothalonil (2,4,5,6-tetrachloro-isophthalonitrile) is a protective fungicide widely used in agriculture. Its presence in seawater is partly due to soil leaching by drainage waters from inland areas (Cima et al., 2008). However, high concentration of chlorothalonil in both water

and sediment observed in June seemed to be related to agricultural run-off (Voulvoulis et al., 2000) and can be degraded after four weeks in natural seawater (Albanis et al., 2002; Callow and Willingham, 1996). Indeed, chlorothalonil was found in all sediments at the Blackwater Estuary (Voulvoulis et al., 2000) and at Greek marine sediments (Albanis et al., 2002). According to Cima et al. (2008), higher concentrations of chlorothalonil can be found in marinas and harbours than in wild coastal areas.

Dichlofluanid (N-Dichlorofluoromethylthio-N'N'-dimethyl-N-phenylsulfamide) is also a fungicide and highly unstable in the water phase (van Wezel and van Vlaardingen, 2004). Due to dichlofluanid known high hydrolytic degradation rate, the level of dichlofluanid was below the detection limit in any analyzed seawater samples (Hamwijk et al., 2005; van Wezel and van Vlaardingen, 2004). However, high concentration of dichlofluanid was detected in sediments after boating season (Voulvoulis et al., 2000). In Arcachon Bay, dichlofluanid could not be detected in water or sediment as it is immediately degraded to metabolite demethylamiosulfanilide (DMSA) (Schouten et al., 2005).

Metabolite DMSA is stable to further hydrolysis (Schouten et al., 2005). DMSA was detected in seawater at very low concentration, varying from less than 3 to 36 ng L^{-1} (Hamwijk et al., 2005). Similarly, DMSA concentration was less than 2.5 ng L^{-1} in the Arcachon Bay (REPAR, 2011).

DMST (N,N-Dimethyl-N′-tolylsulfonyldiamide) is a main metabolite of tolylfluanid. Tolylfluanid hydrolyses rapidly into DMST at alkaline pH with a half-life of < 10 min at pH 9 and 22°C. Surface water may be contaminated through overspray, spray drift and/or run-off. DMST may accumulate in water up to about 4.5 mg L^{-1} with a half-life between 42.1 and 76.4 days (APVMA, 2000). In Arcachon Bay, DMST concentration was about 10-12 ng L^{-1} (REPAR, 2011).

Finally, insecticide imidacloprid (N-[1-[(6-Chloro-3-pyridyl)methyl]-4,5-dihydroimidazol-2-yl]nitramide) is mainly used to control sucking insects on crops (Tomizawa and Casida, 2005). Although imidacloprid is not intended for use in water, it may transfer into ecosystems by spray drift, accidental spills, or by run-off after application, leading to local point-source contaminations. According to Phillips and Bode (2002), the concentration of imidacloprid ranged from 70 to 200 ng L^{-1} in surface waters in New York. However, Fossen (2006) reviewed that the concentration of imidacloprid was greater (1,000 ng L^{-1}) in surface water in Florida. In the future, higher environmental levels of imidacloprid should be expected due to its increasing use and physio-chemical properties. Study in the Arcachon Bay over the period

2005-2006 stated the contaminations of several insecticides (Auby et al., 2007), but there was no specific information about imidacloprid contamination in this Bay. However REPAR (2011) reported the concentration of imidacloprid at about 1-10 ng L^{-1}.

Table 8: Ranges of pesticide fungicides and DMSA in waters worldwide

Sampling area	Sampling year	Conc. (ng L^{-1})	References
Chlorothalonil			
Chesapeake Bay	1997	< 0.97-235	Lehotay et al. (1998)
Blackwater Estuary, UK	1999	< 200-810	Voulvoulis et al. (2000)
Blackwater Estuary, UK	1998	< 200-1,380	Voulvoulis et al. (2000)
Spanish Mediterranean coast	1996-2000	< 1	Martinez et al. (2001)
Dichlofluanid			
Blackwater Estuary, UK	1999	< 240	Voulvoulis et al. (2000)
Blackwater Estuary, UK	1998	< 240	Voulvoulis et al. (2000)
Greek marinas	2003	< 3- <10	Hamwijk et al. (2005)
DMSA			
Greek marinas	2003	< 3-36	Hamwijk et al. (2005)
Arcachon Bay, France	2011	< 2.5	REPAR (2011)

2. STUDY SPECIES: PACIFIC OYSTER *CRASSOSTREA GIGAS*

2.1 Biology

The Pacific oyster or Japanese Oyster, or Miyagi Oyster (*Crassostrea gigas*, Thunberg, 1793), is an oyster native to the Pacific coast of Asia and is a bivalve oyster from Ostreidea family. It has become an introduced species in North America, Australia, Europe, and New Zealand. The Pacific oyster, *Crassostrea gigas*, is one of the world's most economically important bivalves with 4.2 million metrics tons annual production and worth $ 3.5 billion (Hedgecock et al., 2005).

C. gigas is an estuarine species but can also be found in intertidal and subtidal zones. They prefer to attach to hard or rocky surfaces in shallow or sheltered waters up to 40 m but have been known to attach to muddy or sandy areas when the preferred habitat is scarce. The Pacific oyster can also be found on the shells of other animals. Larvae often settle on the shell of adults, and great masses of oysters can grow together to form oyster reefs. The Pacific oyster can be tolerant to salinities as high as 35‰. The Pacific oyster is also a very temperature tolerant species as it can withstand a range from -1.8 to 35°C.

Pacific oyster broodstock in hatcheries are kept in optimum conditions so that the production of large amounts of high quality eggs and sperm can be achieved (FAO, 2005).

Gametogenesis begins at around 10°C and for salinities between 15 and 32‰ and is rarely completed at higher salinities (FAO, 2005). Spawning generally occurs at above 20°C and rarely at 15-18°C. Pacific oyster females are very fecund and individuals of 70-100 grams live weight can produce up to 50-200 million eggs (with a rate at 5-10 times a minute) in a single spawning (FAO, 2005). These individuals can be induced into spawning by thermal shock treatment (FAO, 2005). Yet, it is more common for the eggs from a small sample of females to be stripped from the gonads using Pasteur pipettes and fertilized by sperm from a similar number of males.

Once released from the gonads, the eggs move through the suprabranchial chambers (gill), are then pushed through the gill ostia into the mantle chamber and finally they are released in the water forming a small cloud. In males, the sperm is released at the opposite end of the oyster along with the normal exhalent stream of water (Quayle, 1969). A rise in water temperature is thought to be the main factor in the initiation of spawning, as the onset of higher water temperatures in the summer results in earlier spawning in the Pacific oyster (Grangeré et al., 2009).

The Pacific oyster is the most frequently cultivated bivalve species in France and is typically reared in coastal areas that have become increasingly threatened by exposure to pollutants. Due to bio-ecological particularities of (e.g. filter-feeding activity, ubiquity, susceptible bioaccumulation of pollutants), this species has become an ideal species for pollution monitoring in marine environment. Literature has demonstrated that oyster is one of the most sensitive species to micro-pollutants (Geffard et al., 2002a; His et al., 1999a; McPherson and Chapman, 2000; Quiniou et al., 2007). Thus, this species has been selected as biological model for many ecotoxicological studies.

The use of oyster as bioindicator to assess pollution impact is of increasing importance and can allow early detection of environmental problems. As other marine bivalves, oysters live in close contact with the sediment in the intertidal zone of estuarine or coastal areas. These characteristics make oysters to be a useful species for pollution studies. The choice of the suitable life stages (Fig. 4) for bioassay must take into account many factors, such as sensitivity and reliability, distribution and environmental relevance, availability over the course of the year. Furthermore, a toxicity assay also requires a reproducible endpoint that can be accurately, predictably and reliably measured (Chapman, 2002). The early life stages of marine bivalves, especially Pacific oysters, are usually more susceptible to environmental

toxicants than the adult forms (His et al., 1999a); consequently, they are widely used in ecotoxicological bioassays (His et al., 1999a; Nipper, 2000).

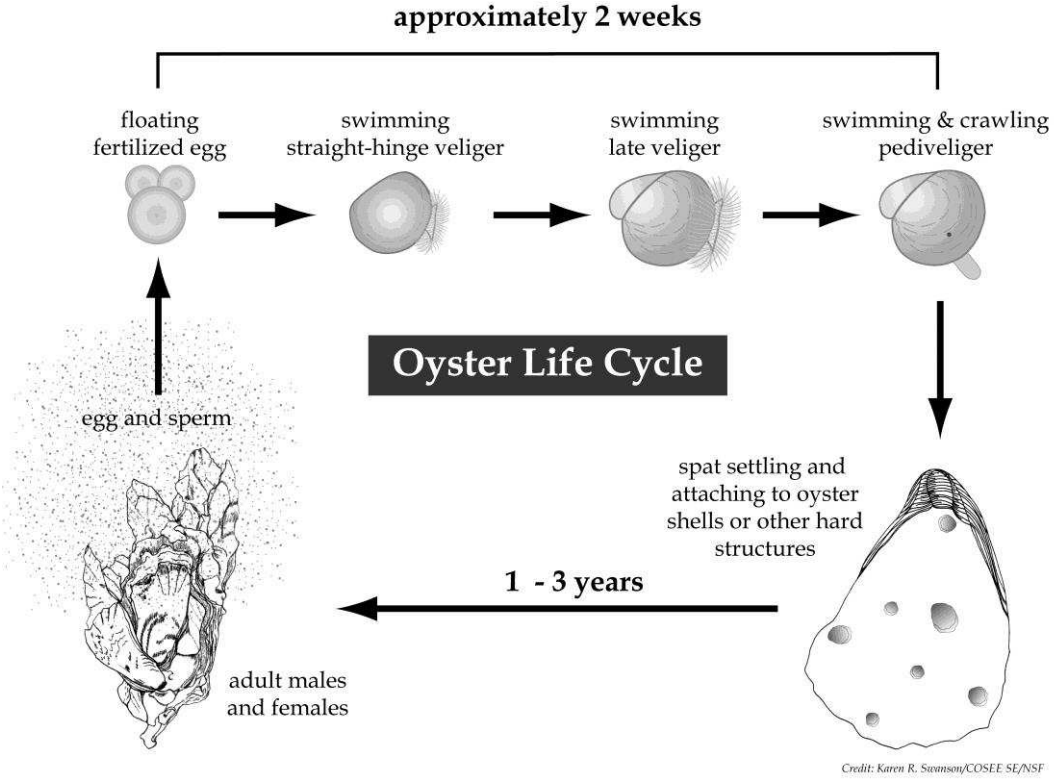


Fig. 4: Life cycle of the Pacific oyster (http://www.cggc.duke.edu/pdfs/CGGC_Oyster-Reef-Restoration.pdf)

2.2 Pollutant metabolism in oyster

Bivalve mollusks, such as Pacific oyster have been proposed as ideal bioindicator organisms for monitoring programs to assess the spatial and temporal trends in chemical contamination in estuarine and coastal areas. *C. gigas* is used as a bioindicator organism due to the following characteristics: (1) they are sedentary filter-feeder organisms and thus, they are better integrators than mobile species of chemical contamination in a given area; (2) they are filter-feeders able to accumulate pollutants in their tissues; (3) in comparison to fish and crustacean, they have a very low xenobiotic extraction ability, and thus contaminant concentration in the tissues of oysters more accurately reflect the magnitude of environmental contamination. Thereafter, Pacific oyster was demonstrated to be a suitable organism not only to assess the bioavailablity of contaminants, but also to measure the effects of those contaminants. In this context, *C. gigas* has been widely used as sentinel organisms in monitoring pollution program (Cajaraville et al., 2000; His et al., 1999b).

Processes associated with pollutant uptake, transfer and deputation are integrally linked to both environmental conditions and the intrinsic biological functions of organisms (Deb and Fukushima, 1999). Studies, to date, have identified the importance of the chemical speciation in controlling pollutant uptake and a number of the biological mechanisms involved in pollutant regulation and metabolism. Aquatic organisms utilize a variety of mechanisms to accumulate or eliminate pollutants from their bodies (Katagi, 2010). Final contaminant body burden in bivalves results of chemical uptake and depuration mechanisms. It is generally assumed that a high concentration in a bivalve mollusc corresponds to an elevated concentration in its environment (Sericano and Wade, 2011). The overall process is species-specific, organ and tissue-specific, and pollutant and ligand- specific. At the individual level, the kinetic of pollutant release is expected to be very complex and depend on the diverse compartments from which pollutants must be mobilized.

In marine bivalves, metals can be complexed by mucous secretion in secretory granules, or bound to cytosolic components such as transport protein or metallothionein, or incorporated into mineralized granules, or immobilized in tertiary lysosomes (reviewed by Gómez-Mendikute, 2006). Metallothioneins (MTs) are a class of cysteine-rich proteins which are involved in binding and detoxifying of heavy metals. MTs have been identified in a wide range of aquatic species and are inducible by certain metals such as Cd, Cu, Hg, Ag and Zn (Sheehan and Power, 1999). MTs have previously been shown to be concentrated in oyster embryos (Ringwood and Brouwer, 1995) and can be induced by either direct metal exposure or antioxidant production that inhibits an increase of oxyradicals (Haq et al., 2003). For instance, following AgNP exposure, oyster embryos exhibited high MTs upregulation compared to adults and seemed to be able to limit metal toxicity over a wide range of low concentrations (Ringwood et al. 2010). Above threshold concentration, development was nearly completely blocked (Ringwood et al., 2010). Upon metal exposure, if oxygen radicals react with lipids from membrane, impairment of structure and function of cell membrane could be observed (Cajaraville et al., 2007). The importance of MTs in reducing metal toxicity has been well documented in embryo-larval stage of oyster *Crassostrea virginica* (Roesijadi et al., 1996). The increased levels of MTs and DNA damage indicated an increased metabolism of heavy metal at adult clam *Mya arenaria* collected from contaminated sites in the Sagueney Fjord (Gagné et al., 2002). The expression of MTs in organisms exposed to toxicants can be measured by quantitative RT-PCR analysis (Asselman et al., 2012).

Xenobiotic metabolism is one of the most important factors that govern the bioconcentration, bioaccumulation of pollutants. It commonly divided into three distinct phases: Phase I – functionalization reactions, Phase II – conjugative reactions, and Phase III – excretion (reviewed in Gómez-Mendikute, 2006). Phase 1 of detoxification is catalyzed by a complex multigene family of enzymes comprising the cytochrome P-450 system (CYP) (Schlenk, 1998). The function of Phase I is to prepare the xenobiotic for conjugation to a variety of water-soluble compounds (Livingstone, 1998; Lüdeking and Köhler, 2002). Several studies have demonstrated that exposure to a variety of contaminants induces the activitiy of enzymes of the cytochrome P-450 system (Lüdeking and Köhler, 2002). In mussels, the level of cytochrome P-450 activity is generally lower than in mammalian and fish species (Livingstone and Pipe, 1992; Sheehan and Power, 1999). In addition, it is less inducible in response to xenobiotic exposure (Cajaraville et al., 2000; Cajaraville et al., 2007). The main purpose of Phase II is the addition of a hydrophilic moiety onto the xenobiotic which may be recognized by Phase III transmembrane pumps (Sheehan and Power, 1999). Those authors have reported that Phase II involves a range of enzyme activities which conjugate xenobiotic to endogenous substrates (Lüdeking and Köhler, 2002). In mussels, glutathionein S-transferases (GST), which conjugate glutathione to xenobiotics, play a major role in Phase II reaction (Lüdeking and Köhler, 2002). The glutathionein S-transferases are a family of enzymes that are mainly cytosolic and catalyse a range of conjugation reaction between glutathione (GSH) and electrophilic substrates. Phase III of detoxification results in unidirectional excretion of the xenobiotic metabolite from the cell driven by hydrolysis of ATP. This phenomenon was named multixenobiotic resistance (MXR) mechanism (Cornwall et al., 1995; Kurelec, 1992). The MXR mechanism represents a defense system directed against numerous xenobiotic, preventing the intracellular accumulation and potentially toxic effect of those xenobiotics (Cornwall et al., 1995; Eufemia and Epel, 2000; Kurelec, 1992; Smital et al., 2003). MXR has already been described in marine bivalves such as the oysters *Crassostrea gigas* and *Crassostrea virginica* (Keppler and Ringwood, 2001; Minier et al., 2002). This protein acts as a pumps involved in the export of xenobiotics out of the cell.

3. TOXICITY OF METALS AND PESTICIDES TO BIVALVES AND OTHER AQUATIC ORGANISMS

Coastal and estuarine areas, such as Arcachon Bay, serve as reproductive and nursery grounds for many invertebrate and fish species and should be preserved. However, these areas are under high pressure and are constantly subjected to the introduction of natural and

anthropogenic pollutants, which are mostly adsorbed onto suspended particles and subsequently accumulated in the sediments. The inputs of diverse chemicals in ocean system may threaten the ecosystem's health and marine biodiversity. Persistent contaminants such as heavy metals and pesticides of anthropogenic origin ultimately enter into coastal areas and estuaries and have short term or long term negative biological effects (Wang and Rainbow, 2008; Yu, 2005). Pollutants can exert different kinds of toxicity from molecular to physiological levels, triggering potential consequences at individual or community scale. In fact, the bioavailability of contaminants depends on many factors such as the properties of the contaminants itself, the characteristics of the environment, and that of the organism used in the bioassay (Borgmann, 2000).

Benthic and epibenthic species such as bivalves are the most heavily exposed to contaminants originating from sediments or to those adsorbed onto particles, as well as to those that are dissolved in interstitial water. Pelagic organisms can also be threaten by contaminants from sediments, when the contaminants from sediments are not only remobilized into the sea water and dissolved in the water or adsorbed in the particles, but also through the contamination of the food web (Chapman and Long, 1983; Fichet et al., 1998; Miller et al., 2000). During the last decades, many programs and studies have been implemented to study adverse response of aquatic organisms and environmental health resulting from toxic substance exposures. In the present study, chemicals, including metals (e.g. Cd and Cu), herbicides (irgarol, diuron, metolachlor and its metabolites, acetochlor and its metabolites, atrazine-2-hydroxy), insecticide (imidacloprid), fungicides (carbendazim, chlorothalonil, dichlofluanid, DMST) and pesticide DMSA, have been used to stand for the contaminants currently detected in Arcachon Bay and in mainly coastal areas.

3.1 Heavy metals

Metal pollution has been an environmental issue in many developed and developing countries for decades, and there is a substantial need to understand toxicity of metals to aquatic organisms. Many studies dealing with toxicity of metals were performed in laboratories or in fields and most of them found negative effects of metals to aquatic organisms (Cavet et al., 2003; Rensing et al., 2000; Rensing and Grass, 2003). Such studies have evaluated changes in growth and development, mortality and aspects of physiology and biochemistry in aquatic organisms. They can, thus, provide critical information for assessing the metal toxicity in aquatic environments. For instance, over the past decades, several studies have demonstrated toxic effects produced in different life stages of invertebrate species by

copper and cadmium exposures (Table 9). The deleterious effects frequently involved in fertilization success, offspring quality, mortality and changes in physiological functions.

Table 9: Toxic effects of Cu and Cd on bivalves and other invertebrate species

Species	Metals	Life stage	Effects	References
BIVALVES				
Crassostrea gigas	Cd, Cu	Adult	Hemocyte aggregation	Auffret and Oubella (1997)
Crassostrea gigas	Cu	Embryo	Larval development	Mamindy-Pajany et al. (2010)
Crassostrea iradalei	Cu, Cd	Larvae	Survival and larval development	Ramachandran et al. (1997)
Crassostrea virginica	Cu, Cd	Larvae	Metallothionein induction	Roesijadi et al. (1997)
Crassostrea virginica	Cu, Cd	Embryo	Survival of embryos	Calabrese et al. (1973)
Isognomon californicum	Cu, Cd	Gametes, embryo	Fertilization and embryo development, larval growth	Ringwood (1992a)
Mytilus edulis	Cd	Larvae	Embryonic development	Martin et al. (1981)
Mytilus edulis	Cu	Adult	Mortalities	Nelson et al. (1988)
Mytilus edulis	Cd	Adult	Nuclear integrity and DNA repair efficiency	Pruski and Dixon (2002)
Mytilus galloprovincialis	Cd	Larvae	Metallothionein induction	Pavicic et al. (1994)
Mytilus trossulus	Cu	Gametes, embryo	Sperm motility, fertilization success and larval development	Fitzpatrick et al. (2008)
Mytilus trossolus	Cu, Cd	Embryo	Larval development	Nadella et al. (2009)
Perna perna	Cu	Spat	Physiological and cellular	Vosloo et al. (2012)
CRUSTACEAN				
Cancer anthonyi	Cu, Cd	Embryo	Larval survivorship	Macdonald et al. (1988)
Cancer magister	Cu	Larvae	Embryonic development	Martin et al. (1981)
Scylla seratta	Cu, Cd	Larvae	Survival and larval development	Ramachandran et al. (1997)
POLYCHAETE				
Hydroides elegans	Cd	Gametes, embryo	Fertilization success, larval development, larval release and larval settlement	Gopalakrishnan et al. (2008)
Galeolaria caespitosa	Cu	Sperm	Fertilization and exacerbates Allee effects	Hollows et al. (2007)
SEA URCHIN				
Diadema setosum	Cu, Cd	Larvae, sperm	Survival and larval development	Ramachandran et al. (1997)
Echinometra mathaei	Cu, Cd	Gametes, embryo	Fertilization and embryo development, larval growth	Ringwood (1992a)
Paracentrotus lividus	Cu, Cd	Gametes, embryo	Fertilization and developmental defects	Warnau et al. (1996)
Paracentrotus lividus	Cu	Embryo	Larval development	His et al. (1999b)
Paracentrotus lividus	Cu	Sperm, embryo	Fertilization success and Offspring quality	Manzo et al. (2008)
Sterechinus neumayeri	Cu, Cd	Embryo	Larval development	King and Riddle (2001)

Among heavy metals, Cu is an ubiquitous element present in all compartments of the marine environment, and required by most organisms for normal metabolic function. It may exist in many different chemical species from free copper ion, inorganic salts and organically bound Cu. Copper, depending on its concentration, can be either an essential nutrient or be toxic to marine organisms; it can catalyse the synthesis of reactive oxygen species, which, through protein oxidation can lead to severe damage to cytoplasm and membrane, cleavage of DNA and RNA, and lipid peroxidation (Garcia et al., 2002; Halliwell and Gutteridge, 1984). Copper is also an important co-factor for many enzymes involved indifferent cellular processes such as radical detoxication, oxidative phosphorylation and iron metabolism (Tsivkovskii et al., 2003). Cadmium is abundant and is a non-essential element, but it is well known that Cd is highly toxic to most animal species (Moore, 1991). Therefore, concerns are growing that Cu and Cd could induce adverse effects because of their elevated levels in ecosystems (White and Rainbow, 1985).

3.2 Pesticides

Generally, pesticide is a term for a wide variety of products designed to control and manage pests. Despite significant improvements during the last decade, the intensive use of pesticides remains a concern for the health of aquatic systems. Pesticides toxicity may occur in a broad range of non-target aquatic organisms, both in plants from microalgae to macrophytes (Ma et al., 2006) and in animals from microinvertebrates to fish (Jemec et al., 2007). It has been showed that pesticides have a more serious impact on marine environments and organisms than previously recognized (Zhou et al., 2006). Thus, numerous pesticides have been studied in many previous ecotoxicology studies.

In the modern agriculture, new pesticides such as metolachlor and acetochlor, which show a more specific mode of action and have a higher polarity and lower persistence than old ones, have been developed. Those pesticides can be transformed in the environment, and living organisms into a large number of metabolite products. Currently, there is an increasing concern regarding the formation of transformation/metabolite products since there are evidences indicating that these products can be more or less toxic (Belfroid et al., 1998; Roubeix et al., 2012) and persistent than their parent compounds (Aga and Thurman, 2001). Depending on the time/dose of exposure and the species which were exposed, pesticides can induce a large of spectrum of toxic effects to various aquatic organisms such as fish, algae, sea urchin, crustacean, and molluscs (Fernández-Alba et al., 2001; Fernández-Alba et al., 2002; Manzo et al., 2006; Okamura, 2002).

- *Herbicide*

Table 10: Toxicity of irgarol on bivalve, crustacean, echinoidea and fish

Species	Effect	Criterion	Results ($\mu g\ L^{-1}$)	References
BIVALVE				
Mytilus edulis		48h-EC_{50}	1540	Bellas (2006)
CRUSTACEANS				
Daphnia magna	Immobility	48h-EC_{50}	8,100	Toth et al. (1996)
	Lethality	24h-LC_{50}	16,000	Okamura et al. (2000a)
		48h-LC_{50}	8,300	
	Reproduction	24h-EC_{50}	1,300	Fernández-Alba et al. (2002)
		48h-EC_{50}	1,050	
	Mortality	48h-EC_{50}	7,300	Hernando et al.(2003)
Daphnia pulex	Lethality	24h-LC_{50}	5,700	Okamura et al. (2000)
Thamnocepharus	Lethality	24h-LC_{50}	12,000	
Artemia salina	Lethality	24h-LC_{50}	> 40,000	
Artemia salina	Mortality	24h-LC_{50}	1,620	Panagoula et al. (2002)
Palaemonetes pugio	Larval lethality	96h-LC_{50}	1,520	Key et al. (2007b)
	Adult lethality	96h-LC_{50}	2,460	
Balanus amphitirite	Mortality	48h-LC_{50}	1,352	Desai (2008)
ECHINOIDEA				
Anthocidaris crassispina	Fertilization	60min-NOEC	100	Kobayashi and Okamura (2002)
Paracentrotus lividus	Fertilization	20min-EC_{50}	9,040	Manzo et al. (2006)
		20min-NOEC	100	
	Offspring quality	48h-EC_{50}	990	
	Embryotoxicity	48h-EC_{50}	4021	Bellas (2006)
	Embryotoxicity	48h-EC_{50}	990	Manzo et al. (2008)
		48h-NOEC	10	
Paracentrotus lividus	Fertilization	48h-EC_{50}	9,040	Manzo et al. (2008)
		48h-NOEC	100	
FISH				
Oncorhynchus tshawytscha	Cell viability	24h-EC_{50}	< 100,000	Okamura et al. (2002)
Oncorhynchus mykiss	Lethality	96h-LC_{50}	860	Toth et al. (1996)
	Mortality	7d-LC_{50}	25,000	Okamura et al. (2002)
		14d-LC_{50}	7,400	
		21d-LC_{50}	2,500	
		28d-LC_{50}	880	
	Growth	98d-LOEC	< 29	

The herbicide irgarol is the first booster biocide to gain prominence as an environmental contaminant. Irgarol has been reported toxic to algae and higher plants species because of its mode of action by inhibiting photosysnthesis (Dahl and Blanck, 1996; Macinnis-Ng and Ralph, 2003; Nyström et al., 2002; Okamura et al., 2000; Owen et al., 2002). However, the toxicity and persistence of the antifouling booster biocide irgarol is now of major concerns

due to its negative effects on the health of aquatic organisms and human life (Fernández-Alba et al., 2001; Horvat et al., 2005). Because of adverse environmental concerns (toxicity on non-target species at low concentrations and persistence), irgarol has been banned in some EU countries (UK, Sweden, Denmark) (Konstantinou and Albanis, 2004). According to previous toxicity studies of irgarol to aquatic animals, irgarol was showed as moderately toxic to crustacean, echinoidea, fish and bivalve species (Table 10).

Table 11: Toxicity of diuron on bivalve on various aquatic species

Species	**Effect**	**Criterion**	**Result ($\mu g\ L^{-1}$)**	**References**
BIVALVE				
Crassostrea gigas	Embryotoxicity	24h-NOEC	< 0.05	Akcha et al. (2012)
	Genotoxicity	24h-NOEC	< 0.05	
	Sperm ultrastructure	24h-NOEC	> 1.5	
CRUSTACEAN				
Daphnia magna	Mortality	48h-EC_{50}	8,600	Hernando et al. (2003)
	Reproduction	48h-EC_{50}	8,600	Fernández-Alba et al. (2002)
Daphnia pulex	Adult survival	96h-EC_{50}	17,900	Nebeker and Schuytema (1998)
		7d-LC_{50}	7,100	
	Reproduction	7d-NOEC	4,000	
Hyalella azteca	Adult survival	96h-LC_{50}	19,400	Nebeker and Schuytema (1998)
		10d-LC_{50}	18,400	
	Growth	10d-NOEC	7,900	
Paleamon serratus	Larval mortality	24h-LC_{50}	0.35	Bellas et al. (2005)
		48h-LC_{50}	0.22	
ECHINOIDEA				
Paracentrotus lividus	Fertilization	20min-EC_{50}	5,090	Manzo et al. (2006)
		20min-NOEC	500	
	Offspring quality	48h-EC_{50}	2,390	
		48h-NOEC	250	
	Embryotoxicity	48h-EC_{50}	2,390	Manzo et al. (2008)
	Fertilization success	48h-EC_{50}	5,090	
	Embryotoxcity	48h-EC_{50}	5,500	Bellas et al. (2005)
Anthocidaris crassispina	Embryotoxicity	32h-LOEC	1,000	Kobayashi and Okamura (2002)
FISH				
Oncorhynchus tshawytscha	Cell viability	24h-EC_{50}	52,000	Okamura et al. (2002)
Oncorhynchus mykiss	Mortality	7d-LC_{50}	74,000	Okamura et al. (2002)
		14d-LC_{50}	15,000	
		21d-LC_{50}	5,900	
		28d-LC_{50}	230	
Pimephales promelas	Embryotoxicity	7d-LC_{50}	11,700	Nebeker and Schuytema (1998)
	Juvenile growth	10d-LC_{50}	27,100	

Diuron is reported to be highly toxic for some non-target organisms (Nebeker and Schuytema, 1998; Simon et al., 1998; Teisseire et al., 1999). Depending on the time and concentration of exposure and the considered species, diuron has shown moderate toxic effects on various organisms such as crustacean, echinoidea, fish, and bivalve species (Table 11). However, diuron may have a higher toxic effects on oyster recruitment, because it is able to significantly affect embryo-larval development from the lowest tested concentration of 0.05 µg/L, i.e. an environmentally realistic concentration (Akcha et al., 2012).

Table 12: Toxicity of metolachlor on various aquatic species

Species	**Effect**	**Criterion**	**Results (µg L^{-1})**	**References**
AQUATIC INSECT				
Chironomus tentans	Midges affected	72h-NOEC	100	Jin-Clark et al. (2008)
		72h-LOEC	1,000	
	AchE residual activity	72h-NOEC	100	
		72h-LOEC	1,000	
CRUSTACEAN				
Daphnia magna	Acute and chronic toxicities	24h-LC_{50}	51,200	Liu et al. (2006)
		24h-NOEC	100	
		24h-LOEC	500	
		48h-LC_{50}	80,000	Wan et al. (2006)
	Acute toxicity	48h-EC_{50}	13,000	
Orconectes rusticus	Agonistic behaviour	96h-NOEC	70	Cook and Moore (2008)
		96h-LOEC	80	
FISH				
Oncorhynchus kisutch	Acute toxicity	24h-LC_{50}	> 20,000	Wan et al. (2006)
		48h-LC_{50}	15,000	
		72h-LC_{50}	9,000	
		96h-LC_{50}	9,000	
Oncorhynchus mykiss	Acute toxicity	24h-LC_{50}	19,000	Wan et al. (2006)
		48h-LC_{50}	15,000	
		72h-LC_{50}	14,000	
		96h-LC_{50}	13,000	
Oncorhynchus tshawytscha	Acute toxicity	24h-LC_{50}	44,000	Wan et al. (2006)
		48h-LC_{50}	15,000	
		72h-LC_{50}	13,000	
		96h-LC_{50}	13,000	

Metolachlor is one of the most intensively used chloroacetamide herbicides in agriculture and is moderately toxic to aquatic animals (Cook and Moore, 2008; Jin-Clark et al., 2008; Lizotte et al., 2009; Wan et al., 2006), such as coldwater and warmwater fish, including

rainbow trout, carp, and bluegill sunfish (Ahrens et al., 1994; Hall et al., 1999; Rivard, 2003). Benthic invertebrates and fish (3,103 and 4,334 µg/L, respectively) were less sensitive to metolachlor than plants (106 µg/L) when calculation of geometric means by tropic group (Hall et al., 1999). Recently, the toxic effects for species-specific and risk analysis of metolachlor was reported in several studies and review papers (Table 12).

Acetochlor has been found to be a very hazardous xenobiotic, being 500 to 10,000 times more toxic for fish than for mammals (Kovriznych and Urbancikova, 1998). Using molecular approaches, Li et al. (2009) have demonstrated that larval development and adult brain of rare mirrow, *Gobiocypris rarus*, could be affected by acetochlor at environmentally relevant concentrations. However, no literature has been reported about toxicity of acetochlor to marine invertebrate species, in particular bivalve species (Table 13).

Table 13: Toxicity of acetochlor on various aquatic species

Species	**Effect**	**Criterion**	**Results ($\mu g\ L^{-1}$)**	**References**
ALGAE				
Raphidocelis subcapitata	Inhibition	96h-EC_{50}	1,588.7	Ma et al. (2006)
AMPHIBIANS				
Rana ridibunda	Sciatic nerve	24h-EC_{50}	59.3	Zafeiridou et al. (2006)
		24h-NOEC	27	
Bufo raddei	Survival	14d-NOEC	17	Liu et al. (2006)
		14d-LOEC	34	
	DNA damage	14d-NOEC	< 17	
FISH				
Gobiocypris rarus	Thyroid hormones of larvae	*d1*, *d2*, *me*, and *nis* mRNA level	Down-regulated	Li et al. (2009)
Danio rerio	Aldult lethality	96h-LC_{50}	370	Kovriznych and Urbancikova (1998)
	Embryo lethality	96h-LC_{50}	610	
Poecilia reticulate	Adult lethality	96h-LC_{50}	1,700	
	Juvenile lethality	96h-LC_{50}	1,300	

Ecotoxicological data for parent compounds of herbicides such as, metolachlor, acetochlor and atrazine is relatively available in the literature, but the toxic effects of their degradation products, metolachlor ESA, metolachlor OA, acetochlor ESA, acetochlor OA, and hydroxy-2-atrazine on non-target aquatic organisms, remain to be elucidated. However, it is well known that degradation products are of similar or less toxicity than their parent compounds (Battaglin et al., 2003; Boxall and Sinclair, 2002). For instance, in the phototrophic microorganism

toxicity test, hydroxy-2-atrazine was found to be nontoxic towards most of the cultured tested and did not inhibit the photosynthesis and growth of microorganisms (Pugh, 1994).

- *Other pesticides*

Despite the wide application of fungicides and insecticides, published information on their toxic effects on marine ecosystems is very scarce. In the present thesis, the selected fungicides and insecticides include carbendazim, chlorothalonil, dichlofluanid, DMST, imidacloprid and DMSA; all being used in the Aquitain Region and Arcachon Bay (REPAR, 2010, 2011).

For fungicide carbendazim, up to date, a few studies have dealt with the effects of carbendazim on freshwater ecosystems (Daam et al., 2009; Ferreira et al., 2008; Satapornvanit et al., 2009; van Wijngaarden et al., 1998). In fact, carbendazim can affect nucleus division and inhibits the activity of the enzyme acetylcholinesterase (Cuppen et al., 2000). This fungicide was also reported to act very slowly and only induce adverse effects at high concentrations (Table 14). Recently, a study focused on the brain tissue and blood plasma of fish also indicate a marked neurotoxic effect of carbendazim (Govindassamy et al., 2011).

Table 14: Toxicity studies for carbendazim on aquatic animals

Species	**Effect**	**Criterion**	**Results ($\mu g\ L^{-1}$)**	**References**
CLITELLATA				
Stylaria lacustris	Response to tactile stimulation	48h-EC_{50}	1,060	van Wijngaarden et al. (1998)
		96h-EC_{50}	219	
		48h-LC_{50}	2,035	
		96h-LC_{50}	821	
CRUSTACEAN				
Daphnia magna	Immobility	48h-EC_{50}	428	van Wijngaarden et al. (1998)
	Feeding inhibition	24h-EC_{50}	97.54	Ferreira et al. (2008)
		48h-LC_{50}	156.7	
Gammarus pulex	Mortality	48h-LC_{50}	77	van Wijngaarden et al. (1998)
Macrobrachium rosenbergii	Feeding inhibition	24h-EC_{50}	> 100,000	Satapornvanit et al. (2009)
		24h-LC_{50}	> 100,000	
Simocephalus vetulus	Immobility	48h-EC_{50}	4,948	van Wijngaarden et al. (1998)

Another fungicide, chlorothalonil and its metabolites was shown to be highly toxic to fish (Caux et al., 1996; Davies and White, 1985; Ernst et al., 1991; Gallagher et al., 1992) and aquatic invertebrates (Davies et al., 1994). Chlorothalonil is known to form complexes with thiol compounds and to be an inhibitor of mitochondrial electron transport (Fernández-Alba et

al., 2002; Lebailly et al., 1997). However, most *in vivo* studies in different species showed no genotoxic effects of this compound (FAO, 2006). The toxic effects of chlorothalonil on marine species are represented in Table 15.

Table 15: Toxicity of chlorothalonil in marine species. (*) (Cima et al., 2008)

Species	**Effect**	**Criterion**	**Results ($\mu g\ L^{-1}$)**	**References**
ASCIDIAN				
Botrullus schlosseri	Immunotoxicity	60min-NOEC	< 26.6	Cima et al. (2008)
Ciona intestinalis	Embryotoxicity	24h-EC_{50}	33	Bellas (2006)
	Inhibition of larval settlement	48h-EC_{50}	42	
BIVALVE				
Crassostrea virginica	Growth	96h-EC_{50}	26	Mayer (1987)*
	Growth	96h-EC_{50}	5	Montforts (1999)*
	Embryotoxicity	96h-EC_{50}	3.6	U.S. EPA Office of Pesticide Programs (2000)*
Mytilus edulis	Embryotoxicity	48h-EC_{50}	8.8	Bellas (2006)
	Mortality	96h-LC_{50}	5,940	Ernst et al. (1991)
CRUSTACEAN				
Cancer magister	Behaviour	48h-EC_{50}	170	Armstrong et al. (1976)*
		96h-EC_{50}	100	
	Larval mortality	48h-LC_{50}	560	
		96h-LC_{50}	140	
Daphnia magna	Reproduction	24h-EC_{50}	70	Fernández-Alba et al. (2002)
		48h-EC_{50}	28	
ECHINOIDEA				
Paracentrotus lividus	Embryotoxicity	48h-EC_{50}	6.7	Bellas (2006)
FISH				
Anguilla japonica	Mortality	24h-LC_{50}	110	Yokoyama et al. (1988)
Galaxias auratus	Mortality	96h-LC_{50}	29	Davies and White (1985)
Galaxias maculates	Mortality	24h-LC_{50}	23.7	
Galaxias truttaceus	Mortality	96h-LC_{50}	16	
Pseudaphritis urvillii	Juvenile mortality	10d- LC_{50}	8.2	Davies et al. (1994)

An overview of the aquatic toxicity tests of dichlofluanid is given in Table 16. Many freshwater species including crustacean and fish were tested for acute and chronic toxicity of dichlofluanid (EC, 2006; Fernández-Alba et al., 2002; Hernando et al., 2003). However, only one study stated the dichlofluanid toxicity to embryos of *Mytilus edulis* (Bellas, 2006). Based on this review, dichlofluanid is very toxic to aquatic organisms and much more toxic to fish than crustacean. It was known that the dichlofluanid's mode of action is related to carcinogenic/mutagenic (Fernández-Alba et al., 2002).

Table 16: Toxicity studies for dichlofluanid on aquatic animals

Species	Effect	Criterion	Results ($\mu g\ L^{-1}$)	References
BIVALVE				
Mytilus edulis	Embryotoxicity	48h-EC_{50}	81.3	Bellas (2006)
CRUSTACEAN				
Daphnia magna	Immobility	48h-EC_{50}	420	EC (2006)
	Reproduction, Length & weight	21d-NOEC	2.7	
	Reproduction	24h-EC_{50}	1,300	Fernández-Alba et al. (2002)
		48h-EC_{50}	1,050	
	Mortality	48h-EC_{50}	1,050	Hernando et al.(2003)
ECHINOIDEA				
Paracentrotus lividus	Embryotoxicity	48h-EC_{50}	626.8	Bellas (2006)
FISH				
Pimephales promelas	Length & weight	33d-NOEC	4.1	EC (2006)
Salmo gairdneri	Mortality	96h-LC_{50}	10	EC (2006)
	Mortality	21d-NOEC	4.6	

However, the dichlofluanid metabolite DMSA is considered as non-toxic or slightly toxic compound, very safe and well tolerated, with few side-effects (Hamwijk et al., 2005). In ecotoxicological tests (Table 17), DMSA was shown to be no or very slightly toxic to crustacean and fish (EC, 2006).

Table 17: Toxicity studies for DMSA on aquatic animals

Species	Effect	Criterion	Results (µg/L)	References
CRUSTACEAN				
Daphnia magna	-	48h-EC_{50}	> 95,600	Hamwijk et al. (2005)
	Immobility	48h-EC_{50}	95,600	EC (2006)
Chironomus riparius	Development	28d-EC_{50}	9,700	EC (2006)
FISH				
Oncorhynchus mykiss	Mortality	96h-LC_{50}	> 100,000	EC (2006)
	Weight & length	21d-NOEC	10,000	

The biochemical mode of action has not been fully elucidated for DMST, although it is assumed that its parent compound tolylfluanid, interferes with several enzymes by reaction with -SH bonds. However, APVMA (2000) reported that in chronic exposures, DMST was only from very slightly to slightly toxic to aquatic species, such as fish and daphnia species

(Table 18), despite high toxicity and very high toxicity reported for its parent compound to the same aquatic species (APVMA, 2000).

Table 18: Toxicity of DMST in aquatic species

Species	**Effect**	**Criterion**	**Results ($\mu g\ L^{-1}$)**	**References**
CRUSTACEAN				
Daphnia sp.	Lethality	48h-LC_{50}	30,800	APVMA* (2000)
		21d-NOEC	10,000	
		21d-LOEC	5,600	
FISH				
Leuciscus idus		96h-EC_{50}	> 10,000	APVMA* (2000)
Oncorhynchus mykiss	Chronic toxic to juvenile	21d-NOEC	58,000	APVMA* (2000)
		21d-LOEC	9,200	

(*) Australian Pesticides and Veterinary Medicines Authority (http://www.apvma.gov.au)

The imidacloprid is a systemic insecticide belonging to a class of chloronicotinyl insecticides, acting on the nicotinic acetylcholine receptors (nAChRs) which are common to many invertebrate taxa, and insects in particular (Tomizawa and Casida, 2003). Fleeger et al. (2003) have showed significant contamination of coastal waters, and is highly toxic on an acute basis to aquatic invertebrates (freshwater and estuarine/marine) (Federoff et al., 2008). To date, only few toxicity studies have been performed on the effects of imidacloprid on aquatic organisms, despite its increasing use (Jemec et al., 2007). In 2009, Tisler and colleagues found out that its toxicity to crustacean *Daphnia magna* or fish *Danio rerio* was relatively low (Tišler et al., 2009). In fact, imidacloprid is not expected to be chronically hazardous, unless it is accidentally spilled in a small pond (Jemec et al., 2007). However, due to the increasing use of imidacloprid, further ecotoxicological studies with different aquatic organisms are needed for this insecticide. Table 19 sums up ecotoxicological studies for imidacloprid carried out on several aquatic animals.

Table 19: Toxicity of imidacloprid in aquatic species

Species	Effect	Criterion	Results ($\mu g\ L^{-1}$)	References
AQUATIC INSECTS				
Sericostoma vittatum	Feeding behavior	96h-LOEC	3.9	Pestana et al. (2009)
Chironomus riparius	Respiration	96h-LOEC	3.9	
	Burrowing behaviour	96h-LOEC	7.8	
CRUSTACEAN				
Palaemonetes pugio	Larval mortality	96h-LC_{50}	309	Key et al. (2007a)
		96h-LOEC	200	
		96h-NOEC	100	
	Aldult mortality	96h-LC_{50}	563.5	
Daphnia magna	Immobility	24h-EC_{50}	97,900	Tišler et al. (2009)
		48h-EC_{50}	56,600	
	Mortality	48h-LC_{50}	17,360	Song et al. (1997)
	Mortality	21d-LOEC	40,000	Jemec at al. (2007)
	Immobility	48h-EC_{50}	8,500	Ashauer et al. (2011)
Gammarus pulex	Mortality	24h-EC_{50}	103.3	Ashauer et al. (2011)
		48h-EC_{50}	109.9	
		72h-EC_{50}	103.5	
		96h-EC_{50}	131.4	
Aedes aegypti	Mortality	48h-LC_{50}	44	Song et al. (1997)
Aedes taeniorhynchus	Mortality	48h-LC_{50}	13	
Artemia sp.	Mortality	48h-LC_{50}	361,230	
FISH				
Danio rerio	Mortality	24h-LC_{50}	241,000	Tišler et al. (2009)

4. BIOASSAYS USING BIVALVE EARLY LIFE STAGES FOR ENVIRONMENT QUALITY ASSESSMENT

Advances in analytical chemistry provide increasingly accurate measurements of the amount of anthropogenic pollutant present in continental and oceanic water and sediments. However, they are inadequate for detecting all possible pollutants and their degradation products, therefore only living systems are able to integrate the various complex effects of contaminants and to assess the level of pollution that have detrimental effects on coastal areas (Chapman and Long, 1983). Numerous bioassays have been developed in invertebrates over the several past decades (Geffard et al., 2002b; His et al., 1999a). Bioassays using common aquatic organisms are needed for toxicity evaluation and for monitoring deleterious effects of pollution in living organisms. Bioassays can evaluate the toxicity of contaminants that are really bioavailable and, therefore, are a valuable tool to study the quality of ecosystems, especially estuaries and coastal areas. However, it has become clear that no single bioassay is

able to describe the global environmental quality. Each bioassay use different endpoints to detect the effects of contaminant. The combined use of several assays increases the power of the data and thus may increase confidence in results. Furthermore, using a multi-bioassay approach is a best tool for identifying the effects and the mechanisms of pollutant toxicity to different biological levels of organization (Adams and Greeley, 2000; Faria et al., 2010; Viarengo et al., 2007).

4.1 Fertilization assay

Typically, in gamete toxicity tests, pollutant toxicity is assessed by exposing gametes to the toxic agents for a short time period (in minutes), and then fertilizing them together and allowing embryos to develop. One advantage of the fertilization assay is the short time-frame, since the assay can be completed in a couple of minutes or hours.

Table 20: Fertilization assay using invertebrate species

Species	Chemicals	Life stage	References
ANNELIDA			
Hydroides elegans	Cd, Hg, Pb, Ni, Zn	Gametes	Gopalakrishnan et al. (2008)
CNIDARIA			
Acropora sp.	Cu, Cd	Gametes	Reichelt-Brushett and Harrison (2005)
Galeolaria caespitosa	Cu	Spermatozoa	Hollows et al. (2007)
Goniastrea aspera	Cu, Cd	Gametes	Reichelt-Brushett and Harrison (1999)
Goniastrea sp.	Cu, Cd	Gametes	Reichelt-Brushett and Harrison (2005)
Lobophytum compactum	Cu	Gametes	Reichelt-Brushett and Harrison (2005)
ECHINOIDEA			
Paracentrotus lividus	Cd	Gametes	Pagano et al. (1982)
	As, Cd, Cr, Ni, Pb, Cu, Zn, Hg	Spermatozoa	Novelli et al. (2003)
	Tributyltin	Spermatozoa	Moschino and Marin (2002)
	Irgarol, Diuron	Spermatozoa	Manzo et al. (2006)
Strongylocentrotus sp.	Cu, Ag, Cd, Zn, Endrin, Endosulfan, Dieldrin, DDT	Spermatozoa	Dinnel et al. (1989)
Dendraster excentricus	Cu, Ag, Cd, Zn, Endrin, Endosulfan, Dieldrin, DDT	Spermatozoa	Dinnel et al. (1989)
Anthocidaris crassispina	Cd	Spermatozoa	Vaschenko et al. (1999)
		Gametes	Au et al. (2001)
MOLLUSCA			
Mylitus trossulus	Cu	Gametes	Fitzpatrick et al. (2008)
Spisula solidissima	Ag	Gametes	Eyster and Morse (1984)

The use of gametes in toxicity assay have previously used sea urchin and coral (Manzo et al., 2006; Moschino and Marin, 2002; Novelli et al., 2003; Reichelt-Brushett and Harrison,

1999; Vaschenko et al., 1999). However, very few studies have examined how toxicants decrease fertilization success of marine bivalves. The early study was developed to examine the effect of metals on gametes of the surf clam *Spisula solidissima* (Eyster and Morse, 1984) and bivalve *Isognomon californicum* (Ringwood, 1992a). Recently, gametes of *Mytilus trossulus* were subjected for 1h to various concentrations of copper in order to assess the toxic effects of copper on fertilization success (Fitzpatrick et al., 2008). Because it is sensitivi, easy to use, time saving and ecologically relevant (Fitzpatrick et al., 2008; His et al., 1999a; Ringwood, 1992a), fertilization assay is now increasingly used for toxicity testing of chemicals (Table 20).

4.2 Embryotoxicity assay for water and sediment quality assessments

Knowledge of the life cycles and the control breeding of bivalve species allowed development of embryotoxicity tests in various invertebrate species (Chapman and Morgan, 1983; Connor, 1972; His et al., 1999a; His et al., 1997). Embryotoxicity assay is simple, inexpensive and rapid as it lasts only 24h for *Crassostrea* sp. and less than 48h for *Mytilus* sp. (His et al., 1999a) (Fig. 5). Those authors also reported that embryotoxicity assay is environmentally relevant because fertilization is performed in the presence of the toxicant that mimic natural environmental conditions. The abnormal development of embryos, expressed as D-larvae with mantle and/or shell abnormalities (Fig. 6), is a toxic effect criterion (His et al., 1999a). Developmental stages of oyster from fertilized eggs to straight-hinge D-larvae represent critical life stages for toxicological studies (His et al., 1999a; Martin et al., 1981). In fact, embryotoxicity assay is now currently used to assess micropollutant toxicity such as heavy metals (Beiras and His, 1994; Calabrese et al., 1977; Martin et al., 1981; Ringwood, 1992b), and organic pollutants such as pesticides (Akcha et al., 2012; Manzo et al., 2006). Particularly, embryos of Pacific oyster, *Crassostrea gigas,* are among the most sensitive test organisms and have frequently been used as sentinel organisms in environmental toxicological studies (Table 21).

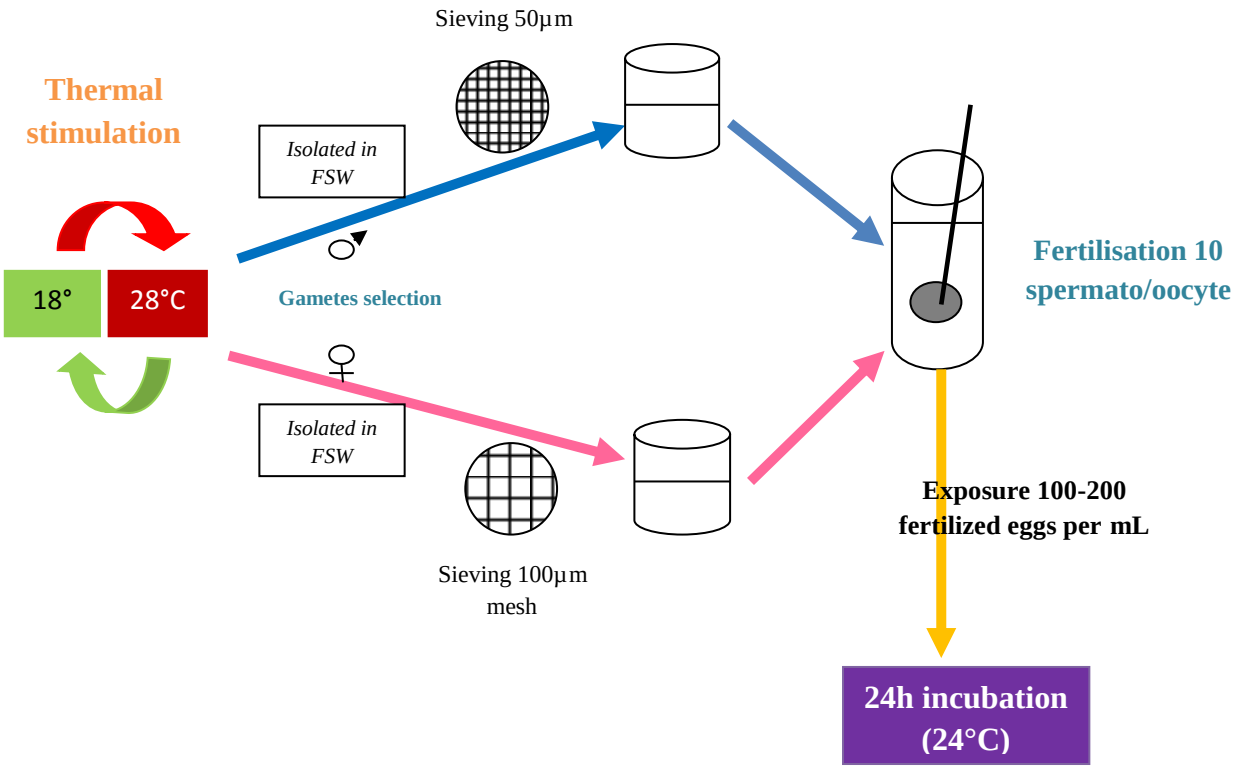


Fig. 5: Protocol for embryotoxicity of bivalve species (application for both water quality assessment and sediment assessment)

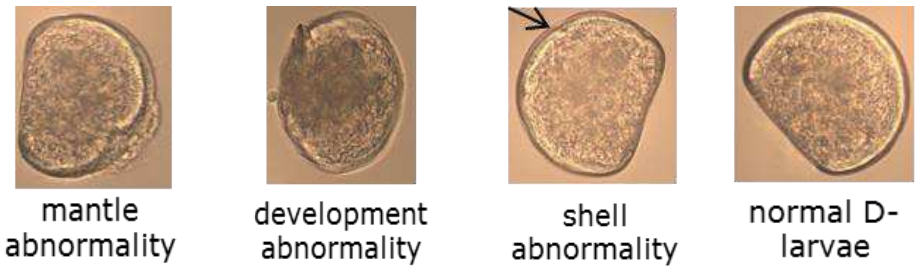


Fig. 6: The different abnormalities observed in D-larvae of bivalve species

Table 21: Embryotixicity assays with *Crassostrea gigas* for metals and pesticides testing

Agents	Conc. (µg/L)	Response	References
METALS			
Zn	125 – 500	+	Brereton et al. (1973)
Cu	10 – 100	+	Okazaki (1976)
Hg, Se	3 – 10,000	+	Glickstein (1978)
Cu	5 – 100	+	Knerovich et al. (1981)
Cu, Zn, Ni, Pb, Cr, Se	<5.3 - >10 000	+	Martin et al. (1981)
Cu, Ag	2 – 18	+	Coglianese (1982)
Cd	10-50	+	Robert and His (1985)
Hg	2 – 128	+	Beiras and His (1994)
BCl	50 – 250	+	His et al. (1996)
Hg, Cu, Pb	10 – 1,200	+	His et al. (1999b)
PESTICIDES			
Dinoterbe	50-250	+	His et al. (1999b)
Endosulfan	0.5 – 500*	+	Wessel et al. (2007)
Diuron, Glyphosate	0.05 – 5	+	Akcha et al. (2012)
Mercaptodimethur, Glyphosate	50 – 200	-	His et al. (1999b)
Roundup	0.5 – 5	-	Akcha et al. (2012)
Glyphosate, Roundup	0.1 – 100,000	+	Mottier et al. (2013)

(+) Increase abnormality; (-) non-effect developmental embryos; (*) Unit = nM

Sediment represent an important sink for contaminants in the aquatic systems and may pose a threat to pelagic and benthic species (Chapman and Long, 1983). Thus, assessing the biological quality of the coastal sediments is now a major necessity for many coastal areas in the world because they serve as breeding and nursery ground for a large number of economically important fish and shellfish species. Embryotoxicity assays using various marine invertebrates, such as sea urchin (Geffard et al., 2001; Novelli et al., 2003), and mussels (Beiras and Bellas, 2008; Jha et al., 2000; Phillips et al., 2003), have been proposed to assess the toxicity of sediments in coastal areas. Among the bioassays, the embryotoxicity test with the oyster *Crassostrea gigas* has been recognized as one of the most sensitive and has been used during the last few decades to study the effects of the sedimentary contamination on benthic organisms (Beiras and His, 1995; Chapman and Morgan, 1983; Geffard et al., 2001; Geffard et al., 2002b; Mamindy-Pajany et al., 2011; Mamindy-Pajany et al., 2010; Poirier et al., 2007). Embryotoxicity tests with the oyster *C. gigas* have been performed on different sediments collected from several coastal areas (Table 22).

Table 22: Sediment testing using embryotixicity assays with oyster *Crassostrea gigas*

Location	Agents	Response	References
Puget Sound	-	+	Chapman and Morgan (1983)
Ria de Ferrol	Hydrocarbon-polluted (in fresh sediment)	-	Beiras and His (1995)
Ria de Ferrol	Hydrocarbon-polluted (in freeze-dried sediment)	+	Beiras and His (1995)
Arès (Arcachon Bay), Bidassoa Estuary	Metals, PAHs, and PCBs	-	Geffard et al. (2001)
Arès, Bidassoa, Dunkerque	Metals and PAHs	+	Geffard et al. (2002b)
Fier d'Ars	Alamethicin and Peptaibols	+	Poirier et al. (2007)
Venice Lagoon	Metals, PAHs, pesticides, PCBs	+	Libralato et al. (2008)
French Mediterranean coast	Pesticides, PAHs, metals	+	Mamindy-Pajany et al. (2010)

Note: PAH = Polycyclic Aromatic Hydrocarbon; PCB = polychlorinated biphenyl; (+) Increase abnormality; (-) non-effect

4.3 Genotoxicity tests by comet assay

The single cell gel electrophoresis assay, more commonly known as the comet assay, has extensively been used in the evaluation of genotoxicity of pollutants (Cotelle and Férard, 1999; Lee and Steinert, 2003; Mitchelmore and Chipman, 1998). The comet assay has an extensive history in detection of single and double strand DNA breaks, alkali-labile sites, and incomplete DNA repair of organisms resulting from exposure to a wide variety of genotoxic chemicals (Fairbairn et al., 1995). Comet assay offers considerable advantages over other cytogenetic methods for DNA damage detection, like chromosome aberrations, sister chromatid exchanges and micronucleus test, because the cells studied need not to be mitotically active. Briefly, the assay depends on the relaxation of supercoiled DNA in agarose-embedded nucleoids (the residual bodies remaining after lysis of cells with detergent and high salt), which allows the DNA to be drawn out towards the anode under electrophoresis, forming comet-like images as seen under fluorescence microscopy. The relative amount of DNA in the comet tail indicates DNA break frequency (Fig. 7).

The comet assay is a very sensitive DNA damage assay. However, the simplicity of the comet assay is deceptive. Care must be taken over practical details, and there are also some theoretical issues to consider. Variations in the basic comet assay protocol can influence the results of an experiment quite profoundly. Two recent papers (Azqueta et al., 2011; Ersson and Möller, 2011) independently identified the most critical factors. The most common form

of the assay is the alkaline version, although there is not yet a definitive alkaline assay protocol which allows the detection of single and double strand DNA breaks and alkali-labile sites (Mateuca et al., 2006). This method has been used with a wide variety of cell types from many different species and provides a sensitive indication of response to genotoxic exposure (Albertini et al., 2000; Frenzilli et al., 2001; Nacci et al., 1996). Thus it has gained in popularity as a standard technique for evaluation of DNA damage/repair, biomonitoring of genotoxicity tests.

Several studies have used comet assay as a tool to investigate the toxicity of chemicals or sediment quality of coastal areas (Table 23). The comet assay is a useful method for measuring genotoxic effects to organisms like fish, bivalves and sea urchin living in polluted environments (Dixon et al., 2002). Exposure of individuals to genotoxic pollutants has been associated to the occurrence of DNA damage in aquatic animals (Ohe et al., 2004). As a consequence, reduced growth, abnormal development and decreased survival of embryo and adults were reported (Lee and Steinert, 2003). As far as this assay was adapted to numerous aquatic species and cells (Morin et al., 2011; Pérez-Cadahía et al., 2004). Therefore, the comet assay represents a useful test to evaluate the biological consequences of environmental pollution on marine organisms (Gabbianelli et al., 2003; Taban et al., 2004).

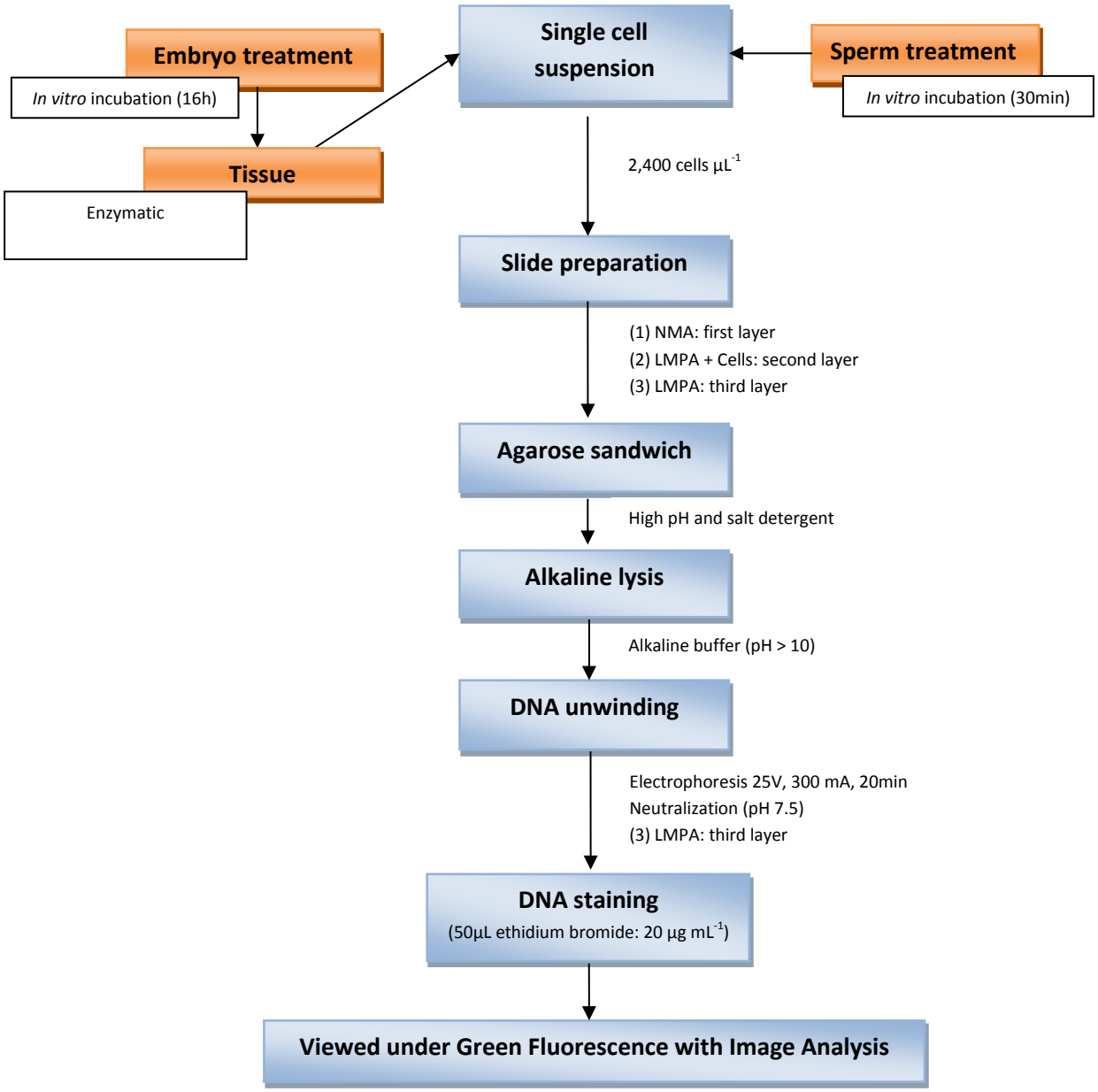


Fig. 7: Outline of the comet assay protocol (modified protocols described by Fairbairn et al. 1995 and Mitchelmore et al. 1998). LMPA = low melting point agarose; NMA = normal melting agarose

Table 23: Studies using the comet assay to detect DNA damage in bivalve species after exposuring to toxicants

Species	Cell type	Agent	Effects	References
Cerastoderma edule	Haemocytes	H_2O_2	+	Cheung et al. (2002)
Crassostrea gigas	Spermatozoa	Diuron,	+	Akcha et al. (2012)
Crassostrea virginica	Hemocytes	H_2O_2	+	Gielazyn et al. (2003)
Dreissena polymorpha	Haemocytes	PCP, wastewater outlet	+	Pavlica et al. (2001)
Dreissena polymorpha	Haemocytes	River Sava	+	Klobucar et al. (2003)
Limnoperna fortunei	Haemolymph	Copper, PCP	D-R	Villela et al. (2006)
Limnoperna fortunei	Haemocytes	Guaiba Basin	+	Villela et al. (2007)
Mercenaria mercenaria	Hemocytes	H_2O_2	+	Gielazyn et al. (2003)
Mya arenaria	Haemocytes, digestive gland	Hydrocarbon fractions	+	Hamoutene et al. (2002)
Mytilus edulis	Gill	Vitamine E	+	Wilson et al. (1998)
Mytilus edulis	Haemocytes, digestive gland	Hydrocarbon fractions	+	Hamoutene et al. (2002)
Mytilus edulis	Gill	Cadmium	-	Pruski and Dixon (2002)
Mytilus edulis	Gill, hemocytes	Methyl methansulfonate	D-R	Rank and Jensen (2003)
Mytilus edulis	Haemocytes	Crude oil	+	Taban et al. (2004)
Mytilus edulis	Gill, haemocyte	Reykjavik harbour	+	Halldórsson et al. (2004)
Mytilus edulis	Gill, haemolymph	Koge bay, Denmark	+	Rank et al. (2005)
Mytilus edulis	Haemocytes			Cheung et al. (2006)
Mytilus galloprovincialis	Gills	Prestige oil spill	+	Laffon et al. (2006)
Mytilus galloprovincialis	Haemocytes	Kasteta Bay	+	Klobucar et al. (2008)
Perna viridis	Haemocytes	B[a]P	-	Siu et al. (2004)
Scapharca inaequivalvis	Erythrocytes	Organotin compounds	+	Gabbianelli et al. (2006)
Scapharca inaequivalvis	Erythrocytes	Copper	+	Gabbianelli et al. (2003)
Scrobicularia plana	Blood	(xeno-)oestrogens	+	Petridis et al. (2009)

(+) Increase DNA damage; (-) decrease/non-effect DNA damage; (D-R) dose-response

5. BIOMOLECULAR RESPONSES USING qRT-PCR ANALYSIS

The quantitative real-time polymerase chain reaction (RT-PCR) is specifically designed to amplify short nucleotide sequences from small amounts of tissue and can generate the thousands to millions of copies of a particular DNA sequence (Fig. 8). The quantitative RT-PCR technique has been proved to be a fast and sensitive tool for investigating differential expression of interested genes (Bustin et al., 2005; Kubista et al., 2006). This technique can be applied to all kinds of organs and their explants, as well as to cultured cells (Lüdeking and Köhler, 2002). Those authors also reported that the quantitative RT-PCR is a promising tool for identifying characteristic differential gene expression patterns in aquatic invertebrates at the gene regulatory level. Thus, gene expression analyses are increasingly used in contaminated environments biomonitoring (Viarengo et al., 2007).

The selectivity of PCR results from the use of primers that are designed to specifically amplify selected genes. The use of primers to conversed protein domains is a powerful approach for the identification of new expressed gene sequences from different gene families. The most attractive feature of molecular biomarker is that it can forecast changes in higher levels of biological organization (Cajaraville et al., 2000). Molecular biomarker responses reflect the actual physiological status of organism at molecular level (Dondero et al., 2006). Several studies have used PCR techinique to identify and quantify the changes of molecular biomarkers in oyster larvae and embryos. Tanguy et al. (2005) investigated the expression of *Cg*GSII gene encoding cytosolic glutamine synthetase in different early developmental stages of *C. gigas*, such as trochophore, D-stage, eyed, and metamorphosed. The expression of immune-related genes during ontogenesis in the oyster *C. gigas* was also analyzed using RT-PCR (Tirapé et al., 2007). RT-PCR assay was also used to demonstrate pesticide effects on adult oysters at molecular level (Gagnaire et al., 2007). Recently, RT-PCR technique was used to quantify the genes involved in metabolism, immunity and cellular stress during a massive mortality in larvae of the American oyster *C. virginica* (Genard et al., 2012). However, compared to studies in adults, few studies have focused on gene transcription measurement using RT-PCR in oyster embryos or larvae.

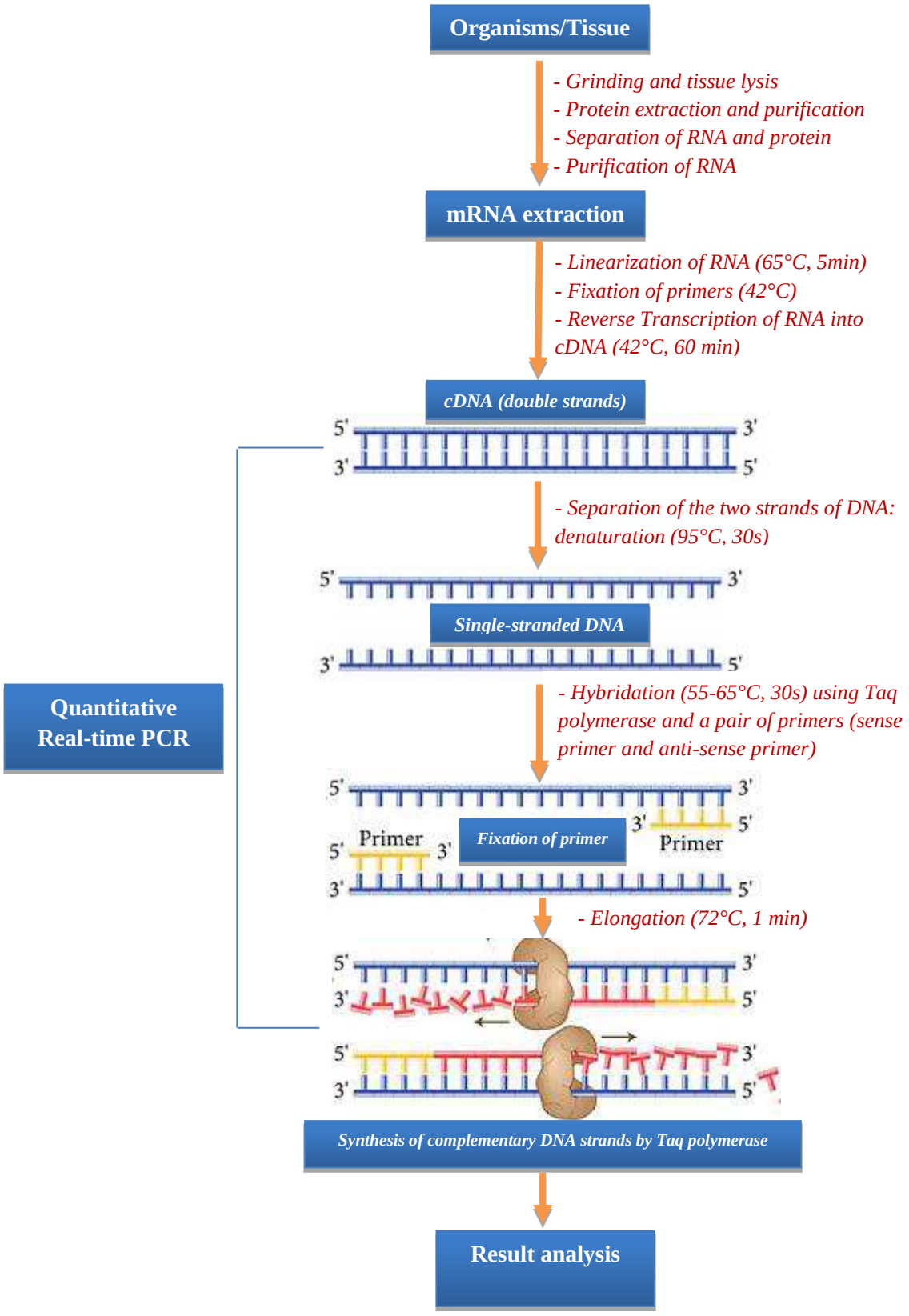


Fig. 8: General schematic of RT-PCR analysis

HYPOTHESIS AND OBJECTIVE OF THE WORK

The hypothesis we intend to demonstrate in the present work is that the deleterious effects of heavy metals and pesticides can be assessed using a sensitive battery of *in vitro* assays using early life stages of oyster *Crassostrea gigas*. These tests can be providing a complementary tool in environmental monitoring and risk assessment of coastal and estuarine ecosystems.

In order to prove this hypothesis, the present report attempts to address the following general objective:

- Using *in vitro* tests based on early life stages of oysters, including gametes and embryos, to assess the toxicity and the environmental risk of heavy metals and pesticides on oyster reproduction and development.

This general objective has been subdivided into a series of partial objectives that are shown below and are addressed in the different parts of the Results and Discussion section:

- First, the toxicity effects of individual pesticides (metolachlor, diuron and irgarol) and heavy metals (copper and cadmium) on fertilization success and embryonic development of oyster are investigated using the fertilization success and offspring quality as endpoints.
- In the second step, the relationship between embryotoxicity and genotoxicity of oyster embryos after exposing to selected metals and pesticides is also investigated.
- Following, to compare the toxic levels of the herbicide metolachlor and its metabolites, bioassays including comet assay and embryotoxicity assay are performed on sperm cells and embryos of *C. gigas*. The study of the transcription levels of targeted genes involved in different physiological mechanisms using RT-qPCR technique is also investigated to determine the molecular mechanism responsible for toxic effects of those.
- Next step, to evaluate the synergic or antagonistic effects of pesticide mixture in combination with or without copper on early life stages of *C. gigas*, the endpoints of D-larvae abnormalities, DNA damage and transcriptional responses of targeted genes are assessed.
- Finally, embryotoxicity assay is applied to analyse the biological quality of sediments collected at different locations and different seasons in the Arcachon Bay.

CHAPTER 2: RESULTS AND DISCUSSION

1. EVALUATION OF DELETERIOUS EFFECTS OF REFERENCE TOXICANTS ON FERTILIZATION SUCCESS AND DEVELOPMENTAL EMBRYOS OF THE PACIFIC OYSTER (*CRASSOSTREA GIGAS*)

Water polluted with pesticides and metals constitutes an important environmental problem as increasing volumes of aqueous solutions of these hazardous chemicals are being generated. In this context, gametes and embryo-larval bioassays with Pacific oyster (*Crassostrea gigas*) were developed to assess the toxic effects of individual pollutants. Two complementary studies were carried out to determine the thresholds of those toxicants:

Firstly, gametes of *C. gigas*, released directly into seawater, are unprotected and exposed to any environmental contaminants present during spawing, so the potential for contaminants to disrupt fertilization process is high. Therefore, two different bioassays, including spermiotoxicity and oocyte toxicity assays, have been developed using the fertilization success and offspring quality as endpoints (percentages of successful fertilized eggs and fertilized eggs developing into abnormal D-larvae, respectively) to evaluate pollutant sensitivity of *C. gigas* gametes to tested toxicants: metals (copper and cadmium - see section 1.1, Chapter 2) and pesticides (metolachlor, irgarol and diuron – see section 1.2, Chapter 2).

Secondly, the experiments (see section 1.3, Chapter 2) have been conducted to assess the genotoxic effect of two metals (copper and cadmium) and two pesticides (metolachlor and irgarol) to *C.gigas* embryos at environmentally relevant concentration. We also investigated the effects of genotoxicant exposures in embryos to find a possible correlation between genotoxicity (by means of the comet assay) and developmental impairment (using embryo larval assay).

1.1. Toxic effects of copper and cadmium on fertilization potency of gametes of Pacific oyster (Crassostrea gigas)

This section has been accepted in:

Huong Mai, Bénédicte Morin, Jérôme Cachot, *in press*. Toxic effects of copper and cadmium on fertilization potency of gametes of Pacific oyster (*Crassostrea gigas).* Journal of Xenobiotics.

Parts of this section have been presented at:

- ECOBIM 2012 - EA4689 Interactions Animal-Environnement on 5-8 June 2012, Reims-France. Morin Bénédicte, Mai Huong, Hélène Budzinski, Cachot Jérôme. *Toxicity evaluation of relevant pollutants in Arcachon Bay on early life stage of Pacific oyster (Crassostrea gigas)*

Abstract

In the present study, the effects of copper (Cu) and cadmium (Cd) to the Pacific oyster *Crassostrea gigas* gametes were investigated. Exposure to metals concentrations of 1, 5, 10, 20, 40 µg.L^{-1} for Cu–and 100, 200, 400, 800, 1600 µg.L^{-1} for Cd for 30 min in seawater caused significant adverse effects on the fertilization success of sperm (EC50 = 20 µg.L^{-1} for Cu, EC50 = 830 µg.L^{-1} for Cd) and oocytes (EC50 = 57 µg.L^{-1} and 1350 µg.L^{-1}, respectively). Lowest observed effect concentrations (LOEC) for spermiotoxicity assay were 1 µg.L^{-1} for Cu and 100 µg.L^{-1} for Cd. However, LOEC values for fertilization success of Cu-exposed and Cd-exposed oocytes were obtained at higher concentrations: 10 µg.L^{-1} and 400 µg.L^{-1} for Cu and Cd respectively. It can therefore be concluded that Cu was more toxic than Cd to oyster *C.gigas* gametes where spermatids were seemingly more sensitive than oocytes. The study showed that these metals can adversely affect reproduction at environmentally realistic concentrations.

Key words: metals, spermatids, oocytes, fertilization success, Pacific oyster

1. Introduction

Heavy metals have long been recognized as hazardous pollutants for aquatic organisms in particular at an early developmental stage. Copper (Cu) and cadmium (Cd) are among the most dangerous metals for bivalve[1,2], shrimp[3] and sea urchin larvae[4]. Although Cu is an essential micronutrient for aquatic animals, it can be toxic to marine organisms at elevated concentrations. Background Cu levels in surface water are usually low (0.03–0.23 $\mu g.L^{-1}$), but can exceed 100 $\mu g.L^{-1}$ in severely polluted coastal areas[5]. Solomon[6] reported that when parental sea scallops were exposed to environmentally realistic concentrations of Cu at 10-20 $\mu g.L^{-1}$, sperm and oocyte production was decreased. In contrast to Cu, Cd is a non-essential element which can accumulate in polluted environments reaching $\mu g/L$[7]. Cd has been shown to be highly toxic to bivalves embryos from *Ruditapes decussatus* and *M. galloprovincialis*[8].

Bioassays play a crucial role in assessing the hazard of pollutants in marine organisms. Pacific oysters, particularly in their highly sensitive early life stages, are commonly used to assess the toxicity of a large variety of pollutants[1,9,10]. However, previous studies on heavy metal embryotoxicity in the Pacific oyster have been mainly focused on the effect of metals on embryogenesis (from fertilization to D-shape larvae) with only limited information available on Cu or Cd toxicity to sperm and oocytes fertilizing capabilities. In the present paper, we studied the fertilizing potency of spermotozoa and oocytes of *C. gigas* exposed to Cu and Cd. Our aims were to establish a dose-response relationship for each tested metal and to assess the pollutant sensitivity of both gamete types in comparison to embryos.

2. Materials and Methods

Analytical grade of copper sulfate, cadmium chloride and formalin solution were purchased from Sigma-Aldrich Chemical (St. Quentin Fallavier, France). Seawater was collected from Arcachon Bay (SW of France), an area where oysters reproduce naturally. Immediately after sampling, seawater was filtered using 0.2 µm-pore membrane filter. Filtered seawater (FSW) was stocked at 4°C in the dark and was used within 3 days. Mature oysters (*Crassostrea gigas*) came from a commercial hatchery specialized in the year-round production of mature oysters (Guernsey Sea Farms, UK). All oysters were used within 3 days.

Stock solutions of metals (500 $mg.L^{-1}$ for Cd and 250 $mg.L^{-1}$ for Cu) were prepared in Milli-Q water. Test concentrations of either metal were prepared by diluting the stock solution in FSW. The test concentrations were 1, 5, 10, 20, 40 $\mu g.L^{-1}$ for Cu and 100, 200, 400, 800, 1600 $\mu g.L^{-1}$ for Cd. In each experiment, FSW was used as negative control. All glasswares were acid-washed before the experiments. Experimental solutions were acidified with 1%

final v/v 65% nitric acid and were then analyzed for Cd or Cu content by ICP-AES (Varian Vista ProAxial, Agilent Technologies, USA) using standard conditions (Table 1). Detection limits were 1 µg.L^{-1} for water samples.

Table 1: Copper and cadmium concentrations (µg.L^{-1}) determined for exposure solutions.

	Copper						Cadmium					
Nominal	0[a]	1	5	10	20	40	0[a]	100	200	400	800	1600
Measured	<1	2.0	6.1	12.6	21.1	51.7	<1	98	200	410	910	1610

[a] control seawater

The spermiotoxicity and oocyte toxicity tests have been described in details previously[10]. Briefly, sperms cells and oocytes were exposed to metals for 30-min before they were used for fertilization. Two fertilization assays were then conducted. For assay (1), 1.0 mL of exposed sperm solution was added to 10 mL of FSW containing unexposed oocytes. For assay (2) 1.0 mL of unexposed sperm solution was added to 10 mL of FSW containing exposed oocytes. Embryos were incubated at 24°C for 2 hours until the 2-4 cell-stage was attained in the control treatment. To calculate the fertilization rate (FR), unfertilized oocytes were scored under an inverted microscope (Olympus, magnification x 200) among 100 oocytes.

Data are expressed as means ± SEM (standard error mean). Differences in fertilization success were assessed for significance by one-way analysis of variance and Tukey post hoc test. The EC50 defined here as the toxicant concentrations causing 50% unsuccessful fertilization or abnormal larval development were calculated by PRISM 5 software (GraphPad Software, California, USA).

3. Results and discussion

Nominal and measured concentrations of Cu and Cd for the different applied treatments were determined (Table 1). Measured concentrations were within 10-23% of the nominal concentrations. Therefore, nominal concentrations were used for presentation and calculation of toxicity parameters. The background level of unfertilized eggs in the various controls was low as well as the response variability between replicates (ca 7 %). A significant decrease of the fertilization rate was observed after sperm exposure (Assay 1) to the lowest Cu and Cd concentrations tested (1 µg.L^{-1} and 100 µg.L^{-1}, respectively) as compared to the control ($p < 0.05$) (Fig.1). The fertilization success was reduced to 20% at the highest concentration tested for both Cu and Cd. Calculated EC_{50} were 20 µg.L^{-1} for Cu and 827 µg.L^{-1} for Cd (Table 2). The NOEC for Cu and Cd in this assay was below 1 and 100 µg.L^{-1} respectively. For oocyte exposure (Assay 2), Cu inhibited fertilization from 10 µg.L^{-1} concentration and Cd impaired

fertilization at concentration of 400 µg.L^{-1} (Fig. 1). The EC_{50} and NOEC data for fertilization rate are summarized in Table 2.

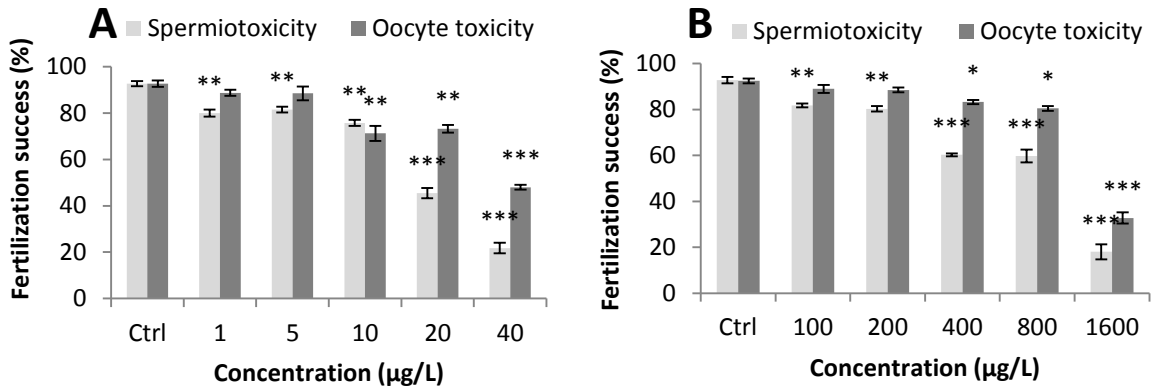


Fig. 1: Percentages (Mean ± SEM) of fertilization success after gamete exposure to **(A)** Cu or **(B)** Cd for 30 min. Asterisks indicate statistical differences between control and exposed treatments at *p < 0.05, **p < 0.01, ***p < 0.001.

Table 2: Mean effective concentrations (EC_{50}) and their 95% coefficients of variation (CV), and NOEC values for different toxicity assays with *C. gigas* for Cd and Cu. Less than (<) values are given for cases where NOEC could not be calculated.

Bioassays	**Cd** (µg.L^{-1})		**Cu** (µg.L^{-1})	
	EC_{50}	**NOEC**	**EC_{50}**	**NOEC**
Spermiotoxicity	827 (681-1005)	< 100	20.3 (17.8-23.1)	< 1
Oocyte toxicity	1346 (1258-1440)	200	57.1 (37.1-88.0)	5
Embryotoxicity[a]	212 (162-277)	< 100	12.5 (11.0 -14.2)	< 1

[a] Embryotoxicity data were published previously (Mai et al., 2012)

To date, most bioassays using bivalves have been performed using either embryos and larvae or adults, while the sensitivity of spermatozoa and oocytes to toxicants are less understood. Most of the existing data on the effects of metals on sperm and eggs have been obtained with the sea-urchin[11,12] and to a lesser extent with blue mussel[13].

Herein it is shown that Cu and Cd can induce deleterious effects in *C. gigas* gametes. Cu pre-exposure did significantly decrease fertilization capacity of oyster spermatozoa at very low concentrations (1 µg.L^{-1}) consistent with those measured in harbor environment. Sperm cells of Pacific oyster seem to be more sensitive to Cu than spermatozoa from other marine invertebrates such as blue mussel[13] and sea squirt[14] with LOEC at 100 µg.L^{-1} and 1,024 µg.L^{-1}, respectively. In the present study, the deduced EC_{50} of Cu for oyster sperm is 20 µg.L^{-1}. This EC_{50} value is very close to that reported for spermatozoa of various sea urchins[15]. Indeed, they reported an EC_{50} values of 25 µg.L^{-1} Cu for purple sea urchin and 59 µg.L^{-1} Cu for green sea urchin. Cd was less toxic to Pacific oyster sperm cells than Cu with significant

adverse effects observed after 30-min Cd exposure at 100 µg.L^{-1} Cd. The Cd concentration causing 50% unsuccessful fertilization on spermatozoa was 830 µg.L^{-1} in this study (Table 2), whereas EC_{50} values for several sea urchin species[15] ranged from 8,000 to 26,000 µg.L^{-1}. Unlike spermiotoxicity, eggs fertilization was only altered after exposure to high exposure concentrations: 10 µg.L^{-1} for Cu and 400 µg.L^{-1} for Cd. This is consistent with previous studies in which acute exposure of oocytes had no, or limited, effect on fertility[10,13,14]. A likely explanation is that the complex envelop of oocyte may act as a protective barrier against metals or other contaminants accumulation into eggs[14]. As previously reported[9], Pacific oyster embryos are also very sensitive to Cu and Cd inducing embryotoxicity at 0.1 µg.L^{-1} for Cu and 10 µg.L^{-1} for Cd. The EC_{50} values for embryotoxicity reached 12 µg.L^{-1} for Cu and 210 µg.L^{-1} for Cd (Table 2). The results are in good agreement with existing data on EC_{50} values of embryotoxicity assays of those metals in other marine invertebrate species[2,15]. The results confirm previous studies[11] demonstrating that sea urchin were more sensitive than sperm or oocytes to pollutant exposure. Gametes and embryos were relatively resistant to Cd toxicity compared to Cu. The impact of Cd has apparently no ecological relevance as the effects on oyster early life stages take place only at environmentally unrealistic concentrations. We can, however, expect that Cu could represent a threat for the reproduction of wild or cultivated Pacific oysters in particular in the Arcachon Bay where concentrations exceeding 0.7 µg L^{-1} are currently measured[16].

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1.2. Environmental concentrations of irgarol, diuron and S-metolachlor induce deleterious effects on gametes and embryos of the Pacific oyster, Crassostrea gigas

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Environmental concentrations of irgarol, diuron and S-metolachlor induce deleterious effects on gametes and embryos of the Pacific oyster, *Crassostrea gigas*

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- The 2nd International Conference on Environmental Pollution, Restoration and Management: Enhancing Environmental Research and Education in Developing Countries on 4-8 March 2013, Hanoi, Vietnam. Huong Mai, Bénédicte Morin, Jérôme Cachot. *Environmental concentrations of irgarol, diuron and S-metolachlor induce deleterious effects on gametes and embryos of the Pacific oyster,* Crassostrea gigas

Abstract

Irgarol and diuron are the most representative "organic booster biocides" that replace organotin compounds in antifouling paints, and metolachlor is one of the most extensively used chloroacetamide herbicides in agriculture. The toxicity of S-metolachlor, irgarol and diuron was evaluated in Pacific oyster (*Crassostrea gigas*) gametes or embryos exposed to concentrations of pesticides ranging from 0.1X to 1000X, with 1X corresponding to environmental concentrations of the three studied pesticides in Arcachon Bay (France). Exposures were performed on (1) spermatozoa alone (2) oocytes alone and (3) both spermatozoa and oocytes, and adverse effects on fertilization success and offspring development were recorded. The results showed that the fertilizing capacity of spermatozoa was significantly affected after gamete exposure to pesticide concentrations as low as 1X of irgarol and diuron and 10X of metolachlor. The offspring obtained from pesticide-exposed spermatozoa displayed a dose-dependent increase in developmental abnormalities. In contrast, treating oocytes with pesticide concentrations up to 10X did not alter fertilization rate and offspring quality. However, a significant decline in fertilization success and increase in abnormal D-larvae prevalence were observed at higher concentrations 10X (0.1 µg L^{-1}) for S-metolachlor and 100X for irgarol (1.0 µg L^{-1}) and diuron (4.0 µg L^{-1}). Irgarol, diuron and S-metalolachlor also induced a dose-dependent increase in abnormal D-larvae prevalence when freshly fertilized embryos were treated with pesticide concentrations as low as concentration of 1X (0.01 µg L^{-1} for irgarol or S-metolachlor, and 0.04 µg L^{-1} for diuron). The two bioassays on *C. gigas* spermatozoa and embryos displayed similar sensitivities to the studied pesticides while oocytes were less sensitive. Diuron, irgarol and S-metolachlor induced spermiotoxicity and embryotoxicity at environmentally relevant concentrations and therefore might be a threat to oyster recruitment in coastal areas facing chronic inputs of pesticides.

Keywords: ***Pacific oyster, gametes, embryos, fertilization success, developmental abnormalities, pesticides***

1. Introduction

Pesticides are commonly used throughout the world as agents for controlling adverse species in agriculture or as antifouling biocides in paint formulations (Banks et al., 2005; Okamura et al., 2000a). They may enter the environment by a variety of routes including through urban (parking lots and residential areas) and agricultural (treated farming areas) run-off, leaching or spray drift, contaminated soils and aquatic sediments. Pesticide contamination has now been widely recorded in waters and sediments from various European estuaries, coastal areas, and lakes (Sargent et al., 2000; Thomas et al., 2000). Once pesticide residues have been discharged in aquatic ecosystems, they can persist from a few months to several years (Hayes and Mlaws, 1991; Ranke, 2002).

Aquatic organisms accumulate pollutants from contaminated water or by ingesting contaminated food; therefore the pollutants may lead to the contamination not only of the aquatic organisms themselves but also of the entire ecosystem, including mankind, through the food chain. There are also numerous compounds such as pesticides that do not bioaccumulate at all and thus do not leave clear fingerprint, but do injure living organisms. For this reason bioindicators, such as invertebrate species, are very useful to study the effects of pollutants in the aquatic environment (His et al., 1999). Bioassays are also very useful to measure the toxicity of chemicals or effluents and to estimate the safe concentration of pollutants which is acceptable in the environment. The early developmental stages of bivalves are particularly suitable for these kinds of test because they are easily produced and maintained in controlled laboratory conditions and because they develop quickly in a few hours or days, depending on the species (Beiras and His, 1994; Chapman and Morgan, 1983). They are also sensitive to a large range of pollutants (His et al., 1999). For all these reasons they are widely used to provide accurate biological criteria on the toxicity of pollutants, in particular pesticides (Armstrong and Millemann, 1974).

According to Apte and Day (1998), pesticides used in grass and broadleaf weed control in agriculture or in antifouling boat paint are the most ecotoxicologically relevant to marine species. S-metolachlor is one of the most intensively used chloroacetamide herbicide in agriculture and is moderately toxic to freshwater and estuarine animals (Cook and Moore, 2008; Jin-Clark et al., 2008; Lizotte et al., 2009; Wan et al., 2006). For instance, the low acute toxicity of metolachlor exposure was found for *Oncorhynchus* sp. and *Daphnia magna*, with 24h-LC_{50} values of 19,000-44,000 µg L^{-1} and 80,000 µg L^{-1}, respectively. Metolachlor is regularly detected in European surface waters during the crop-growing season (Buser et al.,

2000). Diuron is a substituted urea herbicide and has been associated with general weed control on land. The use of this compound as an herbicide has been restricted in France since 2002 (JO n°80 April 5, 2002). Diuron has also been used as an antifouling agent in many European countries (Boxall et al., 2000; Martinez et al., 2001). This compound is commonly detected in areas of high boating or yachting activity and is considered to be relatively persistent in seawater (Konstantinou and Albanis, 2004), with a slow breakdown (from one month to one year) (Okamura, 2002) and half-lives of about 60 days (Harino et al., 2005). In Dutch and British coastal areas, diuron is present at higher concentrations than irgarol and other biocides (Lamoree et al., 2002; Thomas et al., 2002). Depending on the time or concentration of exposure and the considered species, diuron has shown toxic effects on various organisms such as algae, crustaceans and fish (Fernández-Alba et al., 2001; Fernández-Alba et al., 2002; Okamura, 2002). Irgarol is a herbicidal additive used in copper-based antifouling paints and it is estimated that its overall residence time in the marine systems is over 10 years (Ranke, 2002). If so, irgarol can be considered as a persistent organic pollutant. High levels of irgarol were reported in April-May when the high boating season starts at the west coast of Sweden (Dahl and Blanck, 1996). Early studies reported that although irgarol was found to be highly toxic to algae and higher plants, as it is a potent inhibitor of photosynthesis (Dahl and Blanck, 1996; Macinnis-Ng and Ralph, 2003; Nyström et al., 2002; Owen et al., 2002), it is comparatively less toxic to marine bacteria and crustaceans as well as in fish cell culture (Okamura et al., 2000b; Okamura et al., 2002). However, to our knowledge, the literature relating to the toxic effects of metolachlor, irgarol and diuron on marine invertebrate species, particularly the Pacific oyster, is still limited.

The Arcachon Bay is a marine lagoon of the Atlantic Ocean on the Southwest coast of France in the Aquitaine region. The bay covers an area of about 150 km² at high tide and 40 km² at low tide and receives mainly fresh water inputs from the Leyre River (about 20 $m^3 s^{-1}$). This area is a well-known place for oyster farming with about 8,000 to 10,000 tons produced each year and provides 60% of the French production of juvenile oysters (Auby and Maurer, 2004). For several years (2007-2011), low larval recruitment, reduced spat fall and increased oyster mortality events were observed in the Arcachon Bay suggesting impaired reproduction, mortality or developmental defects at early life stages (Maurer et al., 2011). Exposure to pollutants could partly account for the reduction of early life stages of the Pacific oyster. Indeed, early life stages of oysters including gametes, embryos, and larvae are known to be particularly sensitive to contaminants such as metals, pesticides, PAHs and persistent organic

contaminants (Akcha et al., 2012, His et al., 1999). The most recent data available for the Arcachon Bay (Table 1) indicate concentrations up to 40 ng L^{-1} for diuron, 22 ng L^{-1} for irgarol and 5 ng L^{-1} for metolachlor (Auby and Maurer, 2004)

Pacific oyster embryos have been used for more than one decade to assess both chemical toxicity and water quality (His et al., 1999; Beiras and His, 1994; Geffard et al., 2001). It is well-known that Pacific oyster *(Crassostrea gigas)* gametes are freely spawned and that fertilization occurs externally in the seawater. Therefore, exposure to pollutants can occur as soon as gametes are released in the water column. In this study, chemical exposure was performed either on gametes or on freshly fertilized embryos from *C. gigas*. The purpose of this study was both (i) to evaluate the toxicity of commonly used herbicides on *C. gigas* and (ii) to compare the sensitivity of both gamete types and embryos to pesticide exposure.

2. Materials and Methods

2.1 Chemicals and reference seawater

Analytical grade S-metolachlor, irgarol and diuron were purchased from Sigma-Aldrich (St Quentin Fallavier, France). Seawater was collected from Eyrac station in Arcachon Bay (SW of France), an area where oysters reproduce naturally. Immediately after sampling, seawater was sieved (0.2 µm mesh) to eliminate debris and microorganisms. The filtered seawater (FSW) was stored at 4°C and was used within 3 days. A few hours before the 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 year-round production of mature oysters (Guernsey Sea Farms, UK). Oysters were kept at around 10°C for transportation and then acclimatized in FSW for 2 hours at 18°C before the beginning of the experiments. All oysters were used within 3 days.

2.3 Pesticide solutions

Since irgarol and diuron have a relatively low water solubility (7 mg L^{-1} and 42 mg L^{-1} respectively), their stock solutions (100 mg L^{-1}) were prepared in DMSO, whereas the stock solution of metolachlor (100 mg L^{-1}) was prepared in Milli-Q water. 100 µg L^{-1} (10000X) working solutions were obtained from the dilution of the stock solutions in FSW. Five test solutions at 0.1X, 1X, 10X, 100X and 1000X were then obtained by diluting the working solutions in FSW. 1X represents the environmental concentration of pesticides in Arcachon

Bay according to (Auby and Maurer, 2004) (see Table 1). The negative control consisted of FSW with 0.01% DMSO (the highest DMSO concentration used in the test solutions) except for metolachlor exposure where control was carried out at a final Milli-Q water concentration of 0.01%. All treatments were performed using four replicates in 24 well-plates.

Table 1: Pesticides concentrations (ng L^{-1}) determined for reference seawater from the Arcachon Bay and working solutions used in this study

	Measured		
	Diuron	Irgarol	Metolachlor
Arcachon Bay[a]	5-40	2.5-22	2.5-5
Reference seawater [b]	1	2	1
Working solution 10000 X	406,000	75,000	90,000

[a] Auby and Maurer (2004)
[b] Seawater collected in Eyrac (Arcachon Bay) in April 2011

2.4 Pesticides analysis

The working pesticide solutions (10000X) were chemically analysed before the toxicity test for confirmation of pollutant concentrations. Diuron, irgarol and S-metolachlor were extracted via solid-phase extraction (SPE), using Oasis HBL cartridges (3cc, 60 mg) and analysed by LC/MS/MS. The analytical procedure was adapted from Alder et al. (2006). The cartridges were conditioned successively with 3 mL of MeOH and 3 mL of acidified water (pH 2). Water samples were acidified (HCl, pH 2), and spiked with internal standards (diuron d6, irgarol d9 and carbofuran d3) before percolation under vacuum. The cartridges were dried for 30 min by application of a gentle vacuum. Finally, the analytes were eluted with 3 mL of MeOH and concentrated to 100 µL using nitrogen stream evaporator. In addition the individual contamination solutions (10000X) were characterized to define experimentally the exact introduced pesticide amount in embryotoxicity tests. 200 µL of each individual solution were mixed with 200 µL of mixture of internal standards in methanol (nominal concentrations 1 µg g^{-1}) and were directly analysed by LC/MS/MS.

Analyses were performed using a HPLC/MS/MS system from Agilent Technologies company (HPLC 1290 system coupled to 6440 mass spectrometer) (mobile phase: water (5mM ammonium acetate + 0.1% acetic acid) / methanol (100% / 0% to 0% / 100% within 17 min at 0.5 mL/min); column : Kinetex C18 100 mm x 2.1mm x 1.7 mm; ionization mode: ESI +). Procedural blanks were performed to ensure the absence of laboratory-contamination. Recovery and reproducibility were determined using spiked water samples (at nominal concentration of 100 ng L^{-1}) processed at the same time as the samples to be characterized and were the followings 105±10% (n=10). Detection limits were 0.1 ng L^{-1} for water samples.

2.5 Embryotoxicity test

Embryotoxicity test was only performed in this study with diuron. Data on irgarol and S-metolachlor embryotoxicity was published previously (Mai et al., 2012). Male and female oysters were induced to spawn by thermal stimulation (alternating immersion in seawater of 18°C and 28°C for 30 minutes) or gonad stripping. Spawning males and females were individually isolated in beakers with 0.2 µm FSW. They were left undisturbed for 15 minutes and were then removed from the beakers. Oocytes and spermatozoa from two individuals were selected to give a single pairing. Spermatozoa and oocytes were sieved separately through 50 µm and 100 µm meshes, respectively. Sperm mobility was checked and the number of oocytes was counted under a microscope (Leica DME) using a Malassez's counting cell.

The embryotoxicity bioassay was detailed by His et al. (1999), Quiniou et al. (2005) and Mai et al. (2012). Oocytes were fertilized with spermatozoa in ratio of 1:10 (oocyte:spermatozoa). Fertilization success was verified under a microscope (Leica DME, magnification x400) and embryos were counted and transferred to 24-well microplates for embryotoxicity assays. Fertilized oocytes (approximately 300 oocytes/well and four replicates/condition) were exposed in wells containing 1.8 mL of toxicant solution. Microplates were incubated at 24°C for 24 hours in the dark. Following exposure, 50 µL of 37% buffered formalin were added in each well and the percentage of abnormal oyster larvae was then recorded under an inverted microscope (Olympus, magnification x200). The percentage of abnormal D-shell larvae was scored out of 100 individuals per well. The abnormalities (D-larvae presenting mantle and/or shell abnormalities) were determined according to the criteria described in His et al. (1999) and Quiniou et al. (2005). Normally developed larvae exhibited a fully developed and symmetrical shell and complete soft structure including the velum. Convex hinge, indented shell margin, incomplete shell and protruding mantle and developmental arrest were considered and scored as developmental abnormalities. In accordance to His et al. (1999), results from the embryotoxicity assay were rejected when normal larval development was less than 70% in the negative control.

2.6 Spermiotoxicity and oocyte toxicity tests

A freshly collected sperm solution (3.0 mL at 1,750,000 sperm cells per mL) was added in the vials containing 10 mL of each pesticide solution at the appropriate concentration in FSW. An oocyte aliquot (1,500 oocytes) was added in the vials containing 10 mL of each toxicant concentration. Sperm cells and oocytes were exposed to pesticide solutions for 30 min in the

same manner as previously reported for sea urchins and oysters (Manzo et al., 2006; Geffard et al., 2001) before they were used for fertilization at 24°C in the dark. Three fertilization assays were then conducted (Table 2). Assay (A) 1.0 mL of exposed sperm solution was added to 10 mL of FSW containing unexposed oocytes (1,500 oocytes). Assay (B), 1.0 mL of unexposed sperm cell suspension was added to 10 mL of test solution containing exposed oocytes (1,500 oocytes). Assay (C), 1.0 mL of exposed sperm solution was added to 10 mL of test solution containing oocytes (1,500 oocytes) exposed to the same concentration of pesticide.

Table 2: Experimental design for gamete toxicity and offspring quality tests on Pacific oyster

	Exposed sperm cells	Unexposed sperm cells
Unexposed oocytes	Assay A	Control
Exposed oocytes	Assay C	Assay B

In all sets of assays a positive control was added by mixing unexposed sperm with unexposed oocytes from the same couple of oysters to control the variability of fertilization efficiency between the experiments.

Fertilization success was checked every 15 min after sperm cells and oocytes were mixed, until the oocytes underwent their first cleavage within 120 min (His et al., 1999). Pear-shaped fertilized oocytes are easily recognizable among unfertilized oocytes which are round-shaped. Fertilized oocytes were counted (≈ 300 oocytes) under an inverted microscope (Olympus, magnification x200) and transferred to 24-well microplates. Embryos were incubated at 24°C for 2 hours in the dark. When the 2-4 cell stage was attained in the control treatment, a few drops of 37% buffered formalin were added to all treatments to stop fertilization and preserve the developing embryos. To calculate the fertilization rate (FR), unfertilized oocytes were scored under an inverted microscope (Olympus, magnification x200) among 100 oocytes.

2.7 Offspring quality assessment

In parallel, continuous exposures from fertilization to zygote (24 hours) were conducted at 24°C in the dark, as described in part 2.5, to assess the offspring quality, expressed as abnormal D-larvae prevalence. Following exposure, 50 µL of 37% buffered formalin were added in each well and the percentage of abnormal oyster larvae was then recorded under an inverted microscope (Olympus, magnification x200). The percentage of abnormal D-shell larvae was scored out of 100 individuals per well. Abnormal D-larvae was determined as described above (see section 2.5).

2.8 Statistical analysis

All data were expressed as means ± standard error (S.E). Statistical analyses were conducted using the SPSS version 16.0 statistical software. Homogeneity of variance was checked with Levene's test and differences between treatments were tested for significance by means of one-way analysis of variance (ANOVA). When differences among the groups were significant the Tukey's test was used for paired comparisons between the control group and each treated groups. Significance was accepted when $p < 0.05$. Lowest Observed Effective Concentration (LOEC) and the No Observed Effective Concentration (NOEC) were then deduced from the statistical analysis. The EC_{50} defined here as the toxicant concentrations causing 50% reduction in the embryogenesis success was calculated by PRISM 5 software (GraphPad Software, California, USA).

3. Results

3.1 Chemical analysis of tested pollutant and reference seawater

Table 1 shows diuron, irgarol and metolachlor concentrations determined in the contamination solution. The reference seawater from the Arcachon bay used in the different bioassays and also to prepare the test solutions presents very low pesticide contamination (1-2 ng L^{-1}).

3.2 Embryotoxicity

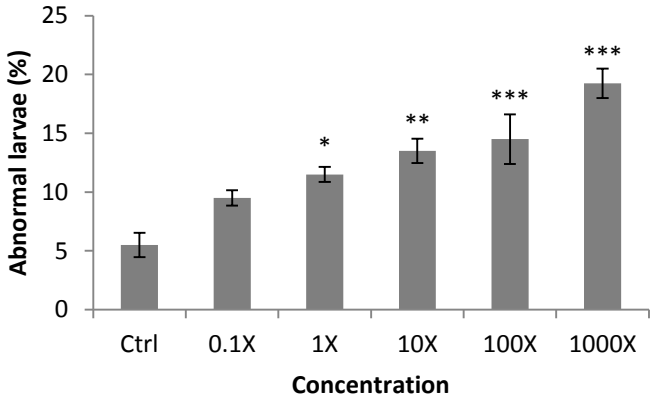

Fig. 1: Percentages of abnormal D-larvae (Mean ± S.E.) after 24h exposure to different concentrations of Diuron. Asterisks indicate the statistical differences observed between control and exposed groups. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$, one-way ANOVA, and *post hoc* Tukey's test, N=4.

After a 24h exposure to diuron, a significant increase of the percentage of abnormal D-larvae was observed from 1X (0.04 µg L^{-1}) ($p < 0.05$), in comparison with the control (Fig. 1). Larval abnormalities reached a 3-4-fold increase at the highest tested concentrations of 1000X (10-40 µg L^{-1}) ($p<0.01$). The EC_{50} values were 2332 µg L^{-1} for diuron, 196 µg L^{-1} for irgarol, and 672 µg L^{-1} for S-metolachlor (Table 3).

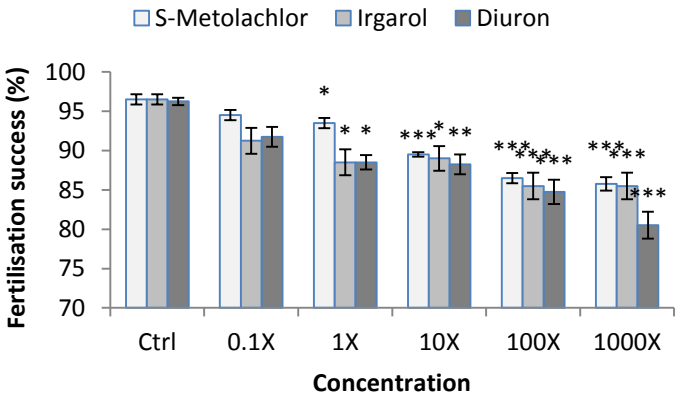

Fig. 2: Percentages of fertilization success (Mean ± S.E.). Only spermatozoa of *C. gigas* were exposed to different concentrations of three pesticides for 30 minutes prior to fertilization assay. Asterisks indicate the statistical differences between control and exposed groups. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$, one-way ANOVA, and *post hoc* Tukey's test, N=4.

3.3 Spermiotoxicity and oocyte toxicity tests

The prevalence of unfertilized eggs in the various controls varied between 3 and 4% (Fig. 2 and Fig. 3). A significant decrease of the fertilization rate was observed after sperm exposure (Assay A) to pesticide concentrations as low as 1X, as compared to the control ($p < 0.05$ for irgarol and S-metolachlor, $p < 0.01$ for diuron) (Fig. 2). The deduced NOEC and LOEC for the three studied pesticides were very similar, i.e. 0.1X (0.001-0.004 µg L^{-1}) and 1X (0.01-0.04 µg L^{-1}) respectively (Table 3). After a 30-minute exposure of sperm cells to the highest pesticide concentrations (1000X), the percentage of fertilization success decreased to 85.8% with S-metolachlor, 85.5% with irgarol and 80.5% with diuron.

For oocyte exposure (Assay B) the fertilization success significantly differed according to the considered concentrations and pesticides (Fig. 3). Metolachlor inhibited fertilization at concentrations of 10X, but this was only a 9% decrease in comparison with the control. Irgarol and diuron impaired fertilization at concentrations between 100X and 1000X (Fig.3). Fertilization rate decreased approximately to 85%, 86% or 80% when oocytes were exposed

to 1000X for S-metolachlor, irgarol or diuron, respectively. NOEC and LOEC values for fertilization rate are summarized in Table 3.

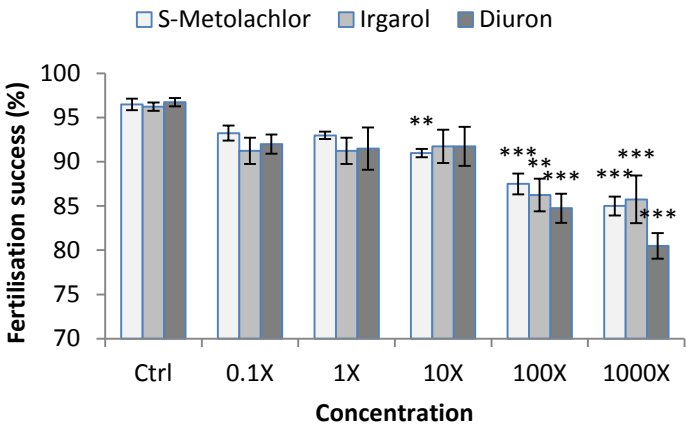

Fig. 3: Percentages of fertilization success (Mean ± S.E.). Only oocytes of *C. gigas* were exposed to different concentrations of three pesticides for 30 minutes prior to fertilization assay. Asterisks indicate the statistical differences between control and exposed groups. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$, one-way ANOVA, and *post hoc* Tukey's test, N=4.

Very similar results to assay A were obtained after simultaneous exposure of spermatozoa and oocytes to pesticides (Assay C) (Fig.4, Table 2).

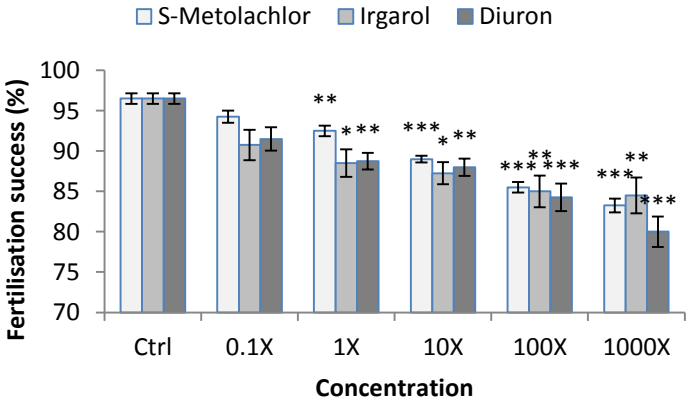

Fig. 4: Percentages of fertilization success (Mean ± S.E.). Both spermatozoa and oocytes of *C. gigas* were exposed to different concentrations of three pesticides for 30 minutes prior to fertilization assay. Asterisks indicate the statistical differences between control and exposed groups. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$, one-way ANOVA, and post hoc Tukey's test, N=4.

3.4 Offspring quality

Larvae abnormalities 24 hours after sperm and/or oocytes exposure to the three studied pesticides are shown in Fig. 5 to 7. Prevalence of abnormal larvae in the control group ranged from 4 to 5%. The offspring of sperm treated (Assay A) with S-metolachlor, irgarol and diuron showed a significant increase in abnormal D-larvae prevalence from concentrations as low as 1X ($p < 0.05$), as compared to the control group (Fig. 5). The dose-response curve showed a significant increase of the percentage of abnormal larvae with increasing concentrations of pesticides ranging from 1X to 1000X ($p < 0.001$). At the highest concentration tested (1000X), the percentage of abnormal larvae reached 25% for metolachlor, 29% for irgarol and 37% for diuron. Therefore, exposure to the three tested pesticides induces first impairment of oocyte fertilization and then developmental defects in embryos.

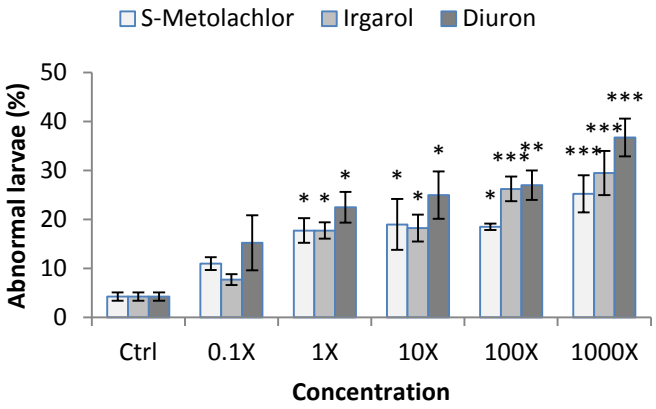

Fig. 5: Percentages of abnormal D-larvae (Mean ± S.E.) 24h after sperm exposure to different concentrations of three pesticides for 30 minutes prior to fertilization. Asterisks indicate the statistical differences observed between control and exposed groups. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$, one-way ANOVA, and *post hoc* Tukey's test, N=4.

Treating oocytes (Assay B) with low concentrations (< 10X) of the three studied pesticides did not alter the offspring quality (Fig. 6). Indeed, the occurrence of abnormal larvae significantly increased ($p < 0.01$) from 10X of S-metolachlor or diuron and from 100X of irgarol (Fig. 6). A significant dose-dependent increase in abnormal larvae was also observed (Fig. 6). It is interesting to note that the offspring quality is affected after oocyte exposure to diuron at 10X even if the fertilization rate is not altered.

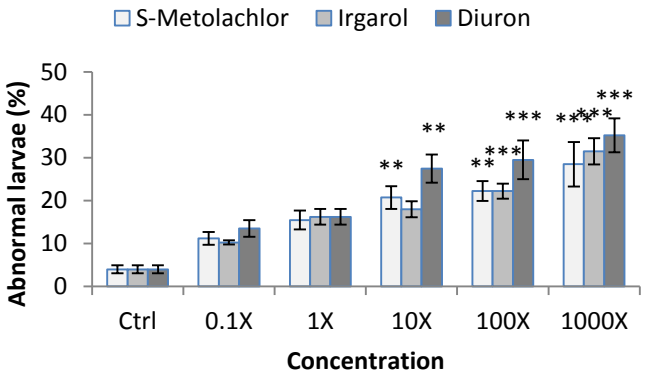

Fig. 6: Percentages of abnormal D-larvae (Mean ± S.E.) 24h after oocytes exposure to different concentrations of three pesticides for 30 minutes prior to fertilization assay. Asterisks indicate the statistical differences observed between control and exposed groups. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$, one-way ANOVA, and *post hoc* Tukey's test, N=4.

Following simultaneous exposure of spermatozoa and oocytes to S-metolachlor, irgarol and diuron (Assay C), a significant increase in abnormal larvae prevalence was observed at concentrations as low as 1X ($p < 0.05$), in comparison with the negative controls (Fig. 7). A clear dose-dependent increase in developmental defects was observed from 4% in the controls to 32.5, 33.5 and 40.8% for the highest tested concentrations of S-metholachlor, irgarol and diuron, respectively (Fig. 7). Once again, the dose-effect curve was very similar for assays A and C (Fig. 5 and 7). The estimated NOECs and LOECs of the three tested pesticides as regards developmental defects were similar when treated sperm cells were crossed with either untreated oocytes (Assay A) or treated oocytes (Assay C), at 0.1X and 1X, respectively. However, when untreated sperm cells were crossed with treated oocytes (Assay B), NOEC and LOEC values for S-metolachlor and diuron increased up to 1X and 10X, respectively while for irgarol NOEC and LOEC reached 10X and 100X respectively (Table 3).

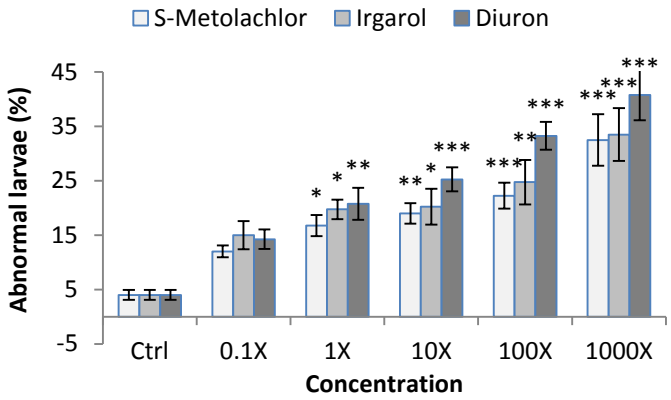

Fig. 7: Percentages of abnormal D-larvae (Mean ± S.E.) 24h after sperm and oocytes exposure to different concentrations of three pesticides for 30 minutes prior to fertilization assay. Asterisks indicate the statistical differences observed between control and exposed groups. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$, one-way ANOVA, and *post hoc* Tukey's test, N=4.

Table 3: NOEC, LOEC and EC_{50} values (µg L^{-1}) obtained from Pacific oyster gametes and embryos exposed to the three studied pesticides

Toxicant	**Indicators**	**Assays**	**NOEC**	**LOEC**	**EC_{50}**
Diuron	Abnormal D-larvae	Embryotoxicity	0.004	0.04	2332
	Fertilization success	Assay A	0.004	0.04	
		Assay B	0. 4	4	
		Assay C	0.004	0.04	
	Offspring quality	Assay A	0.004	0.04	
		Assay B	0.04	0. 4	
		Assay C	0.004	0.04	
Irgarol	Abnormal D-larvae	Embryotoxicity	0.001*	0.01*	196
	Fertilization success	Assay A	0.001	0.01	
		Assay B	0.1	1	
		Assay C	0.001	0.01	
	Offspring quality	Assay A	0.001	0.01	
		Assay B	0.1	1	
		Assay C	0.001	0.01	
Metolachlor	Abnormal D-larvae	Embryotoxicity	0.001*	0.01*	672
	Fertilization success	Assay A	0.001	0.01	
		Assay B	0.01	0.1	
		Assay C	0.001	0.01	
	Offspring quality	Assay A	0.001	0.01	
		Assay B	0.01	0.1	
		Assay C	0.001	0.01	

* Mai et al. (2012)

4. Discussion

To date, the majority of bioassays using bivalves were performed using either embryos and larvae or adults, while the sensitivity of spermatozoa and oocytes to toxicants remained

largely unknown. In addition, the toxic effects of pesticides on oyster reproduction potency including fertilization success and embryonic development are poorly documented. Indeed most existing data concern the effects of metals on sperm, eggs and embryos of the blue mussel (Fitzpatrick et al., 2008) and sea urchin (Moschino and Marin, 2002; Warnau et al., 1996) and only a few focused on toxic effects of organic pollutants on oyster gametes (Geffard et al., 2001; Favret and Lynn, 2010; Akcha et al., 2012). Sensitivity to pollutants widely varies according to the species and the stages of development tested (Chapman and Long, 1983). Although spermiotoxicity assays using sea-urchin species have already been used to assess the toxicity of heavy metals (Manzo et al., 2006), very few studies report the use of oyster sperm cells with pesticides (Akcha et al., 2012).

4.1 Effects of pesticides on fertilization success of Pacific oyster

For oysters and most aquatic invertebrates, fertilization is an external process and gametes are freely emitted in the aquatic environment. Because of the quick dilution of gametes and rapid loss of fertilization potency of the sperm cells, oocytes must be fertilized only a few minutes after spawning (reviewed in His et al., 1999). Any process altering the quality and/or quantity of emitted gametes, gamete survival, sperm mobility, DNA integrity may dramatically reduce the success of fertilization and consequently impair the reproduction success. In the present study, it was demonstrated that sperm cells exposed to concentration as low as 1X of irgarol, S-metolachlor or diuron (Assay A) significantly reduced sperm cell fertilization ability. It was reported that irgarol and diuron at concentrations around 10 µg L^{-1} and 1 mg L^{-1} respectively were able to inhibit the fertilization rate of sea urchin *Paracentrotus lividus* (Manzo et al., 2006). Another study suggested that pesticides such as tributyltin could impair fertilization by reducing successful sperm-oocyte interactions of the ascidian *Phallusia mammillata* (Franchet et al., 1999). Furthermore, triphenyltin, used as antifouling compound, has been shown to alter sperm cell membrane by inhibiting the plasma membrane Ca^{2+}-ATPase which regulates calcium homeostasis in cells (Moschino and Marin, 2002; Rurangwa et al., 2002). This antifouling compound can also cause a drop in intracellular ATP concentration which is essential to sperm motility, leading to a decrease of sperm-oocyte contacts (Rurangwa et al., 2002). We suppose that the tested pesticides may proceed in a similar manner on oyster spermatozoa by reducing sperm-oocyte interactions. However, in a recent study no decrease of ATP content in oyster sperm cells after diuron exposure to concentration up to 0.5 µg L^{-1} was observed. Meanwhile, sperm DNA damage was reported at

concentration as low as 0.05 µg L^{-1}, which can affect sperm fertilization capacity (Akcha et al., 2012).

In the present study, exposing oocytes to pesticide concentrations below 100X (except for S-metolachlor) did not influence fertilization success. The oocyte fertilization failure can probably result from the impairment by pesticides of the sperm signal transduction into the oocytes (Franchet et al., 1999). However, the percentage of fertilized oocytes only decreased by 15-20% at the highest tested pesticide concentrations of 1000X. At this concentration, the percentage of abnormal D-larvae obtained after fertilization of exposed oocytes reached the same order of magnitude than those obtained after fertilization of exposed sperm (28.5% for S-metolachlor, 31.5% for irgarol and 35.3% for diuron).

Exposure of both sperm cells and oocytes (Assay C) to pesticides does not decrease the fertilization rate in comparison to sperm exposure alone (Fig.4). We can thus assume that the negative effects of pesticides on fertilization success mainly result from the effects on sperm cells.

When comparing data from oocytes and sperm cells, it was noteworthy, regarding the fertilization success, that oocytes were about 10 times less sensitive than sperm cells to S-metolachlor and about 100 times less sensitive to irgarol and diuron. These results may be at least partially explained by membrane permeability differences between the gamete types. Another putative explanation could be the existence in oocytes but not in sperm cells of proficient defense systems allowing either elimination of toxicants or repair of the induced damage. It was recently shown that sperm cells are much sensitive to DNA damage because of defects in their repair systems (Akcha et al., 2012).

4.2 Effects of pesticides on the embryonic development of Pacific oyster

It was previously reported (Mai et al., 2012), that oyster embryos are very sensitive to metolachlor and irgarol. In the present study, a similar sensitivity to diuron was shown with significant embryotoxic effects from 0.01 µg L^{-1} (Mai et al., 2012). This result is in accordance with the recent data obtained by Akcha and collaborators (2012).

The effects of gamete exposure on offspring quality were also investigated in this study. The results showed that the offspring quality of pesticide-exposed oocytes was affected at higher concentrations than for pesticide-exposed sperm cells. Both S-metolachlor and diuron significantly affected the embryonic development at concentrations as low as 0.1 µg L^{-1} in the oocyte toxicity experiment. However, irgarol did not affect offspring quality until 1.0 µg L^{-1}.

Although less sensitive to toxicants than exposed spermatozoa, it is difficult to assert that exposed oocytes are not impacted at all. According to Sedano et al. (1995), the stressed oocytes may face biochemical changes which could result in abnormal development of embryos.

The present results suggest that irgarol is probably less toxic to oyster oocytes than S-metolachlor or diuron. However, a similar toxic potency has been reported in eggs and embryos of sea urchins *Anthocidaris crassispina* (Kobayashi and Okamura, 2002) and daphnia (Fernández-Alba et al., 2002) exposed to diuron and irgarol. Similar developmental defects have been observed in oyster larvae obtained after fertilization of treated sperm cells with either untreated oocytes or treated oocytes. Pesticides might interfere with sperm recognition and attachment on oocytes because pesticides can affect the sulfhydryl groups (-SH) that control sperm activation and motility (Dinnel et al., 1987). The number of abnormal D-larvae increased significantly after sperm exposure to concentrations as low as 1X (0.01 µg L^{-1} for irgarol or metolachlor, and 0.04 µg L^{-1} for diuron) of the three tested pesticides. These results are more or less similar to those previously reported by Akcha et al. (2012) and Mai et al. (2012) as regards embryo exposure. Indeed, significant embryotoxicity and genotoxicity were reported following exposure of oyster embryos to 0.05 µg L^{-1} of diuron (Akcha et al., 2012) and 0.01 µg L^{-1} of metolachlor and irgarol (Mai et al., 2012). It can be hypothesized that the tested pesticides affected sperm cell integrity and exerted transmissible damage (damage and/or epigenetic modifications on DNA) to oocytes that could affect the offspring quality through embryo-larval developmental defects.

4.3 Comparative sensitivity of gametes and embryos to pesticides

Strikingly, the NOECs (0.1X – 1X) and LOECs (1X – 10X) obtained in the present study for both fertility and developmental parameters are much lower than those reported in the literature for other marine species exposed to the same pesticides. Manzo et al. (2006) studied the effects of diuron on sea urchin *P. lividus* sperm cells and embryos. In their study, the NOEC for sperm and LOEC for embryos exposed to diuron were 0.5 mg L^{-1}, while irgarol had no toxic effect up to 0.01 mg L^{-1} on sea urchin sperm. Irgarol had a NOEC value for crustacean spermatozoa at 0.1 mg L^{-1} (Konstantinou and Albanis, 2004). Recently, Akcha et al. (2012) showed that diuron had embryotoxic effects on oyster embryos at concentrations of 0.05 µg L^{-1} which is very consistent with our study.

In the present study, metolachlor, irgarol and diuron impaired fertilization success and offspring quality following sperm exposure at the same concentrations of 1X. Similar

sensitivity was observed for oyster embryos exposed to diuron (present study), S-metolachlor and irgarol (Mai et al., 2012). Comparatively to sperm cells and embryos, oocyte fertilization potency was less impacted by pesticide treatment since LOEC values reach 0.1 µg L^{-1} (10X) for S-metolachlor, and 1 µg L^{-1} (100X) for irgarol and 4 µg L^{-1} (100X) for diuron. Therefore, we can assume that sperm cells and embryos of oyster are more sensitive to pesticides than oocytes. This is consistent with the reports of His et al. (1999) and Manzo et al. (2006). The present results highlight that the use of a battery of bioassay is more useful and reliable in describing the effects of toxic compounds.

4.4 The use of the oyster sperm assay to assess pollutants toxicity

Table 4: Review of studies using the sperm assay in various invertebrate species for toxicity testing of chemicals

Phylum	**Species**	**Toxicants**	**Endpoints**	**Toxicity (µg L^{-1})**	**Reference**
Annelida	*H. elegans*	Cd, Hg, Pb, Ni, Zn	Fertilization	EC_{50}: 32.1-2458.9	Gopalakrishnan et al. (2008)
Cnidaria	*Goniastrea* sp.	Cu, Pb, Zn, Ni, Cd	Fertilization	NOEC: 10-5455	Reichelt-Brushett and Harrison (2005)
	Acropora sp.	Cu, Pb, Zn, Ni, Cd	Fertilization	NOEC:15.3-2000	Reichelt-Brushett and Harrison (2005)
Mollusca	*M. trossulus*	Cu	Sperm motility, fertilization	NOEC: 32	Fitzpatrick et al. (2008)
	S. solidissima	Ag	Fertilization	NOEC: 0.6	Eyster and Morse (1984)
	C. gigas	Diuron, Glyphosate	Sperm viability, enzyme activity, sperm capacity, sperm DNA damage	LOEC: 0.05	Akcha et al. (2012)
Echinoidae	*P. lividus*	Cd	Fertilization	NOEC: 11240	Pagano et al. (1982)
		As, Cd, Cr, Ni, Pb, Cu, Zn, Hg	Fertilization	EC_{50}: 17-16210	Novelli et al. (2003)
		Tributyltin	Fertilization	LOEC: 0.1	Moschino and Marin (2002)
		Irgarol, Diuron	Fertilization	NOEC: 100-500	Manzo et al. (2006)
	Strongylocentrotus sp.	Cu, Ag, Cd, Zn, Endrin, Endosulfan, Dieldrin, DDT	Fertilization	EC_{50}: 1.9-502	Dinnel et al. (1989)
	D. excentricus	Cu, Ag, Cd, Zn, Endrin, Endosulfan, Dieldrin, DDT	Fertilization	EC_{50}: 8.0-441	Dinnel et al. (1989)
	A. crassispina	Cd	Fertilization	EC_{50}: 1700 LOEC : >100	Vaschenko et al. (1999) Au et al. (2001)

The sperm assay was developed by Eyster and Morse (1984) to study silver toxicity. Because it is sensitive, easy to use, time saving and ecologically relevant, sperm bioassay is now increasingly used for toxicity testing of chemicals (Table 4). The sperm assay has been developed in various invertebrate species and through the measurement of different endpoints such as fertilization success, sperm viability, motility and DNA integrity (Table 4). Fertilization success is the most widely used endpoint (Table 4).

Oyster sperm is used to assess the toxicity of chemicals but it can also be used to monitor the quality of coastal marine waters and to study the adverse effects of pollution on oyster recruitment. Indeed, oysters are freely spawned and fertilization occurs externally in the seawater. Thus, the fertilization success and offspring quality depend on the sperm-oocyte interaction within the water column, and any changes in water quality such as accidental or chronic pollutant inputs can be detrimental to the oyster population recruitment.

5. Conclusion

It was shown herein that Pacific oyster gametes and embryos are sensitive to low concentrations of S-metolachlor, irgarol and diuron, which significantly reduce fertilization success and increase developmental defects. Regarding range of concentrations currently measured in coastal areas such as the Arcachon Bay, we can assume that these pesticides could represent a threat for the reproduction of wild or cultivated Pacific oysters. The results presented herein may be of high relevance in the management of coastal areas where different types of economic activities cohabitate.

Acknowledgement

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1.3. Embryotoxicity and genotoxicity effects of heavy metals and pesticides on early life stages of Pacific oyster, (Crassostrea gigas)

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Embryotoxic and genotoxic effects of heavy metals and pesticides on early life stages of Pacific oyster (*Crassostrea gigas*)

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- ECOBIM 2012 - EA4689 Interactions Animal-Environnement on 5-8 June 2012, Reims- France. Morin Bénédicte, Mai Huong, Hélène Budzinski, Cachot Jérôme. *Toxicity evaluation of relevant pollutants in Arcachon Bay on early life stage of Pacific oyster (Crassostrea gigas).*

Abstract

This study evaluated embryotoxicity and genotoxicity of two dissolved metals copper and cadmium (Cu and Cd) and two pesticides (metolachlor and irgarol) occurring in Arcachon Bay (SW France) in Pacific oyster (*Crassostrea gigas*) larvae and investigated the relationship between those two endpoints. Embryotoxicity was measured by calculating the percentage of abnormal D-shaped larvae and genotoxicity was evaluated with DNA strand breaks using the comet assay. After 24 h exposure, significant increases of the percentage of abnormal D-larvae and the DNA strand breaks were observed from 0.1 µg L^{-1} for Cu, 10 µg L^{-1} for Cd and 0.01 µg L^{-1} for both irgarol and metolachlor in comparison with the controls. A strong positive relationship between embryotoxicity and genotoxicity was recorded for Cu, Cd and metolachlor. The current study suggests that copper, irgarol and metolachlor can induce larval abnormalities and DNA damage in a population of exposed oysters at environmentally relevant concentrations.

Key words: G*enotoxicity, Embryotoxicity, Pacific oyster, Heavy metals, Irgarol, Metolachlor*

1. Introduction

An unfortunate consequence of industrialization and urbanization is the generation of undesirable toxic wastes and effluents. The discharge of these wastes into surface waters constitutes a major source of water pollution (Chen and White, 2004). Pollution has deleterious effects on aquatic organisms through interference with a range of biological and biochemical pathways (Widdows et al., 2002). Aquatic organisms can accumulate metals or pesticides directly from contaminated water or indirectly by the ingestion of contaminated feed sources (Hung et al., 2001); leading not only to the contamination of the aquatic organisms themselves but also of the entire ecosystem and humans through the food chain.

Among the several pollutants that can contribute to aquatic pollution, heavy metals (e.g. copper and cadmium) represent one of the most widespread and serious form of environmental contamination (Devi et al., 1996). The copper and cadmium mean concentrations in seawater of the French Atlantic coast were 0.7 and 0.009 µg L^{-1} respectively (Geffard et al., 2002) (Table 1). Copper is an essential micronutrient required by all living organisms for a variety of physiological and biochemical processes. However, the increasing copper concentration in marine ecosystems is of high concern because at elevated levels copper is one of the most toxic heavy metals (White and Rainbow, 1985). Thus, copper can be potentially toxic to aquatic organisms when available in excess in the water. In contrast to copper, cadmium is an abundant, non-essential element that is continuously accumulated in the environment as a result of industrial activities. Cadmium has been classified as a human and animal carcinogen by the International Agency for Research on Cancer (IARC, 1997).

Pesticides, such as metolachlor and irgarol, are organic compounds that are widely used as herbicide or antifouling in boat paint (Apte and Day, 1998). Metolachlor is one of the most intensively used chloroacetamide herbicides in agriculture and is slightly and moderately toxic to freshwater and estuarine animals (Cook and Moore, 2008). Metolachlor concentrations in Ontario Lake (Canada) ranged from < 0.1 to 1.83 µg L^{-1} (Byer et al., 2011) and can reach 80 µg L^{-1} after rainfalls during the spring and summer in Ohio River in the United States (Cook and Moore, 2008). Although, the available data on metolachlor concentrations in coastal and estuarine areas are limited, metolachlor has been reported at low level around < 2.5 - 5 ng L^{-1} in Arcachon Bay, France (Table 1) (Ifremer, 2004). Irgarol is a herbicidal additive for use in copper-based antifouling. The overall residence time for irgarol in the marine systems is over 10 years (Ranke, 2002) and consequently irgarol can be considered as a persistent organic pollutant. Since the first report of coastal contamination by

irgarol (Readman et al., 1993), this herbicide has been found in numerous aquatic environments around the globe. Although, irgarol was not detected in the Canadian aquatic environment (either ports, marinas and fishery harbors) during a two-year study (1996–1997) (Liu et al., 1999), irgarol has been frequently reported in estuarine, coastal and lake waters in Europe (reviewed by Konstantinou and Albanis, 2004). Indeed, irgarol has been detected in British coastal areas, with concentrations ranging from 0.201 to 1.421 µg L^{-1} (Boxall et al., 2000; Thomas et al., 2001). Monitoring of surface waters from the ports and marinas in French Mediterranean (Côte d'Azur), concentration of irgarol was reported at 1.7 µg L^{-1} (Readman et al., 1993). The most recent data (Table 1) in the Arcachon Bay (SW France) show concentrations of up to 22 ng l^{-1} of irgarol (Ifremer, 2004). Earlier studies reported that although irgarol has been found to be highly toxic for algae and higher plants due to its inhibition potency of photosynthesis activity (Macinnis-Ng and Ralph, 2003; Owen et al., 2002), it has comparatively low toxicity towards marine bacteria and crustaceans and fish cell culture (Okamura et al., 2000a; Okamura et al., 2002).

Table 1: Environmental concentrations of tested toxicants in Arcachon Bay (ng L^{-1})

	Copper	Cadmium	Metolachlor	Irgarol
Previous works	700[a]	9[a]	2.5 – 5[b]	< 2.5 – 22[b]
This study[c]	810	nd	1	2

[a] Geffard et al. (2002)
[b] Ifremer (2004); nd: not determined
[c] Seawater was collected in April 2011
nd: not determined

Aquatic bioassays are useful tools in assessing hazardous and potential impacts of pollutants on the natural environment (in order to determine the admissible concentration of pollutants in the environment). Bivalve species have been shown as suitable bioindicators to study the effects of these pollutants in the aquatic environment (O'Connor, 2002). Pacific oyster (*Crassostrea gigas*), especially in early development stages, is commonly used as sentinel organisms to assess the toxicity of a large variety of pollutants in marine bioassays because of their high sensitivity to a large range of pollutants (Geffard et al., 2002; His et al., 1999; Wessel et al., 2007) and year-around availability of fertilized eggs from adult breeding oyster. However, there is no data on the literature about genotoxic effects of copper, cadmium, metolachlor and irgarol on embryos of Pacific oyster. There is also little information about the correlation between genotoxicity and embryotoxicity for those pollutants in bivalves. The comet assay is considered as a good indicator of genotoxicity and is now widely used for environmental biomonitoring (Mitchelmore and Chipman, 1998). This assay has proved to be useful for measuring DNA damage in marine and freshwater species in the presence of genotoxic compounds (Kim and Hyun, 2006; Nacci et al., 1996).

This study was conducted to quantify toxicity of environmental concentrations of two model metals (copper and cadmium) and two pesticides (metolachlor and irgarol) to *C.gigas* embryos as well as to analyze the relationship between embryotoxicity (D-larvae abnormalities) and genotoxicity (comet assay) in oyster embryos exposed to metals and pesticides.

2. Materials and Methods

2.1 Chemicals and seawater

Reference toxicants ($CuSO_4$, $CdCl_2$, S-metolachlor, irgarol), formalin and DMSO (dimethyl sulfoxide) 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).

Seawater was collected from Arcachon Bay (SW France), an area which has naturally reproducing population of oysters. Immediately after sampling, seawater was filtered using membrane filter of 0.2 µm to eliminate debris and microorganisms. Filtered seawater (FSW) was stored at 4°C 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 metal and pesticide solutions

Depending on the assays, three or four replicates were performed for each condition tested. The range of experimental concentrations was chosen on the basis of preliminary studies.

The metal solutions were made up from analytical grade copper sulfate ($CuSO_4$) and cadmium chloride ($CdCl_2$). Stock solutions (100 mg L^{-1}) of the two metals were prepared in pure Milli-Q water (Millipore). Test solutions were then obtained diluting the stock solutions in FSW. Three concentrations of exposure were selected for metals: 0.1, 1 and 10 µg L^{-1} for copper and 10, 100 and 1000 µg L^{-1} for cadmium. A preliminary study was conducted to

define the lowest concentration of cadmium (exposure to 0.01, 0.1, 1 and 10 µg L^{-1}) which can adversely affect the development of oyster embryos.

Since irgarol has a relatively low water solubility (7 mg L^{-1}), the stock solution (100 mg L^{-1}) was prepared in dimethyl sulfoxide (DMSO), while stock solution (100 mg L^{-1}) of metolachlor was made in Milli-Q water. From stock solution, 100 µg L^{-1} working solutions were then obtained diluting the stock solution in FSW and were chemically analyzed. Test solutions were then obtained diluting the stock solutions in FSW. The following concentrations were used for test solutions, 0.01, 0.1, 1 and 10 µg L^{-1}. Negative control was FSW with 0.01% Milli-Q water (the highest one in the test solutions) except for irgarol exposure where control was carried out at a final DMSO concentration of 0.01% (the highest one in the test solutions).

2.4 Heavy metals and pesticides analysis

The stock solutions were chemically analyzed before the toxicity test for confirmation of pollutant concentrations. For chemical analysis of metals, about 3 mL of each solution (stock solutions and FSW) was acidified with 1%final v/v 65% nitric acid. Samples were then analyzed for Cd or Cu content by ICP-AES (Varian Vista ProAxial, Agilent Technologies, Santa Clara, USA) using standard conditions. Method blanks were added to each set of sample. The quality of the analytical method was checked by analyzing the certified international reference water – (BCR403).

S-metolachlor and irgarol were extracted via solid-phase extraction (SPE), using Oasis HBL cartridges (3cc, 60 mg) and analyzed by LC/MS/MS. The analytical procedure was adapted from Alder et al. (2006). The cartridges were conditioned successively with 3 mL of MeOH and 3 mL of acidified water (pH 2). Water samples were acidified (HCl, pH 2), and spiked with internal standards (carbofuran d3 and irgarol d9) before percolation under vacuum. The cartridges were dried for 30 min by application of a gentle vacuum. Finally, the analyses were eluted with 3 mL of MeOH and concentrated to 100 µL using nitrogen stream evaporator. In addition the individual contamination solutions (nominal concentrations. 100 µg.L^{-1} in water) were characterized to define experimentally the exact introduced pesticide amount in embryotoxicity tests. 200 µL of each individual solution were mixed with 200 µL of mixture of internal standards in methanol (nominal concentrations 1 µg.g^{-1}) and were directly analysed by LC/MS/MS. Analyses were performed using a HPLC/MS/MS system from Agilent Technologies company (HPLC 1290 system coupled to 6440 mass spectrometer) (mobile phase: water (5mM ammonium acetate + 0.1% acetic acid) / methanol

(100% / 0% to 0% / 100% within 17 minutes at 0.5 mL/min); column : Kinetex C18 100 mm x 2.1 mm x 1.7 mm; ionization mode: ESI +). Procedural blanks were performed to ensure the absence of laboratory-contamination. Recovery and reproducibility were determined using spiked water samples (at nominal concentration of 100 $ng.L^{-1}$) processed at the same time as the samples to be characterized and were the followings 105±10% (n=10). Detection limits were 0.1 $ng.L^{-1}$ for water samples.

2.5 Embryotoxicity assay

Male and female oysters were induced to spawn by thermal stimulation (alternating immersion in seawater of 18°C and 28°C for 30 minutes) or by stripping the gonad. Spawning males and females were individually isolated in beakers with 0.2 µm FSW. They were left undisturbed for 15 minutes and were then removed from beakers. Eggs and sperm from two individuals were selected to give a single pairing. Sperms and eggs were sieved separately through a 50 µm and 100 µm meshes, respectively. Sperm mobility was checked and the number of eggs was counted under the microscope (Leica DME). Eggs were fertilized with sperm in ratio of 1:10 (egg:sperm). Fertilization success was verified under microscope, and embryos were then counted and transferred to 24-well microplate for embryotoxicity assays and/or to 250 mL beaker for comet assays.

The embryotoxicity bioassay has been described in details by His et al. (1999) and Quiniou et al. (2005). Fertilized eggs (500 eggs) were exposed in wells containing 1.8 ml of toxicant solution. These microplates were incubated at 24°C for 24 hours in the dark. After incubation, 50 µl of 1% buffered formalin were added and the percentage of abnormal oyster larvae was recorded. Hundred individuals per well were directly observed under inverted microscope (Olympus, magnification x200) to determine the number of abnormal D-shell larvae. The abnormalities (D-shell larvae presenting mantle and/or shell abnormalities) were determined according to the criteria described in His et al. (1999) and Quiniou et al. (2005).

2.6 Comet assay

For genotoxicity assays, embryos were incubated in 250 mL beakers for 16h at 24°C in the dark. This exposure period allows to recover unshelled larvae which can be enzymatically dissociated to obtain viable cells for the comet assay. Three replicates were performed per condition and each replicate contains a total of 1,000,000 oyster larvae.

Cell isolation was performed prior to comet analysis. Following 16 hours of exposure, the embryos were recovered by sieving at 40 µm. One mL of embryo suspension (about 12000

embryos) was incubated with 1 mL of Dispase II diluted in MEM at final concentration of 1 g L^{-1} for 20 min at 37°C with gentle shaking (150 rmp). The reaction was stopped by centrifugation for 10 min at 1000 rmp and 4°C. The cell pellet was then suspended in 1 mL of MEM at a final cell density of about 2000 x 10^3 cells mL^{-1}. Cell viability was determined for each sample by trypan-blue exclusion test. Comet assay experiments were only carried out with cell suspension with more than 80% cell viability.

The comet assay was performed on isolated cells as described by Morin et al. (2011) with slight modifications. 50 µL of cell suspension (about 120 x 10^3 cells) were added to 100 µL of 1% LMP agarose and two gels of 50 µL were laid down on a pre-coated slide. Alkaline treatment was performed for 20 minutes to allow DNA unwinding. Electrophoresis was carried out at 25 V, 300 mA for 20 minutes.

Slides were analyzed at x400 magnification using an optical fluorescence microscope (Olympus BX 51) and an image analysis system (Komet 5.5, Kinetic Imaging Ltd.). DNA damage was expressed as percentage of "Tail DNA", which is the percentage of total DNA that has migrated from the head. A hundred randomly selected nucleoids were analyzed on two replicate gels.

2.7 Statistical analysis

All data are expressed as means ± standard error (S.E). Statistical software, SPSS (12.0) and Microsoft Excel (2010) were used for data analysis. Homogeneity of variance (Levene'test) was checked and statistical analysis was performed by One-way Analysis of Variance (ANOVA). Differences among tested concentration means were then performed using Tukey *post hoc* test. The Lowest Observed Effective Concentration (LOEC) and the No Observed Effective Concentration (NOEC) were then determined. Significance was accepted when $p < 0.05$. The EC50 defined here as the toxicant concentrations causing 50% reduction in the embryogenesis success, and their 95% confidence intervals (CI) were calculated by PRISM 5 software (GraphPad Software, California, USA). Regression linear analysis was used to assess relationships between DNA damage and the percentage of abnormal oyster embryos after pollutant exposure.

3. Results

3.1 Chemical analysis of tested pollutant solution

Table 2 shows Cd, Cu, irgarol and metolachlor concentrations determined in the contamination solution. Measured concentrations were within 10-25% of the nominal

concentrations. Therefore, nominal concentrations were used for presentation and calculation of toxicity parameters.

Table 2: Metal and pesticide concentrations in working solutions

Compounds	Nominal (µg L^{-1})	Measured (µg L^{-1})	% Recovery
Copper	100	77	77
Cadmium	1000	1097	110
Metolachlor	100	90	90
Irgarol	100	75	75

3.2 Background levels

In the experiments carried out, the seawater controls showed 79.03 ± 2.6 % of normally developed D-larvae of *C. gigas*. Overall, the background level of DNA damage in non-exposed embryos was low (9.56 ± 0.59%).

3.3 Metal exposures

3.3.1 Copper

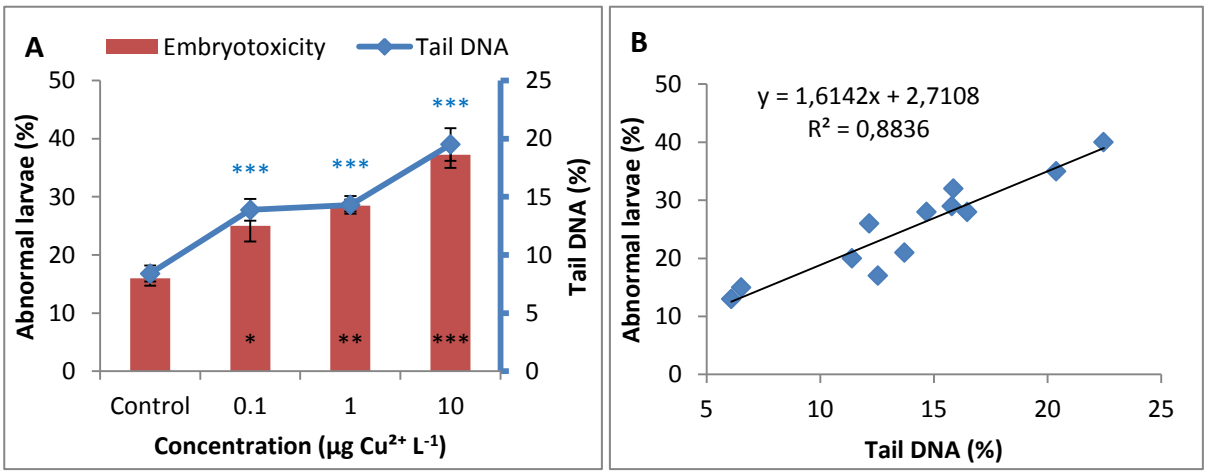

Fig. 1: (**A).** Percentages (Mean ±S.E.) of abnormal D-larvae and tail DNA following oyster embryos exposed to different concentrations of Cu. Asterisks indicate sinificant differences between exposed and control treatment (* $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$). **(B).** Relationship between Tail DNA and D-larvae abnormalities in oyster ($p = 0.000006$).

Copper had a significant effect on oyster embryo development, inducing a significant increase of the percentage of abnormal D-shell larvae ($p < 0.05$). Embryotoxicity was observed from the lowest tested concentration at 0.1 µg L^{-1} copper ($p < 0.05$) and was concentration-dependent (Fig. 1A). At 10 µg L^{-1} of copper, the percentage of abnormal D-larvae reached 37.2%. Copper inhibited the embryonic development of *C. gigas* with EC_{50} value of 12.5 µg L^{-1} (Table 3). The level of DNA damage significantly increased with copper

concentration, from 13.9% at the lowest studied concentration (0.1 µg L^{-1} of copper) to 19.5% at the highest studied concentration (10 µg L^{-1}) in comparison to negative control (Fig. 1A). A strong positive correlation after copper exposure (R^2 = 0.88, p = 0.000006) between DNA damage and percentage of abnormal D-larvae was observed (Fig 1B).

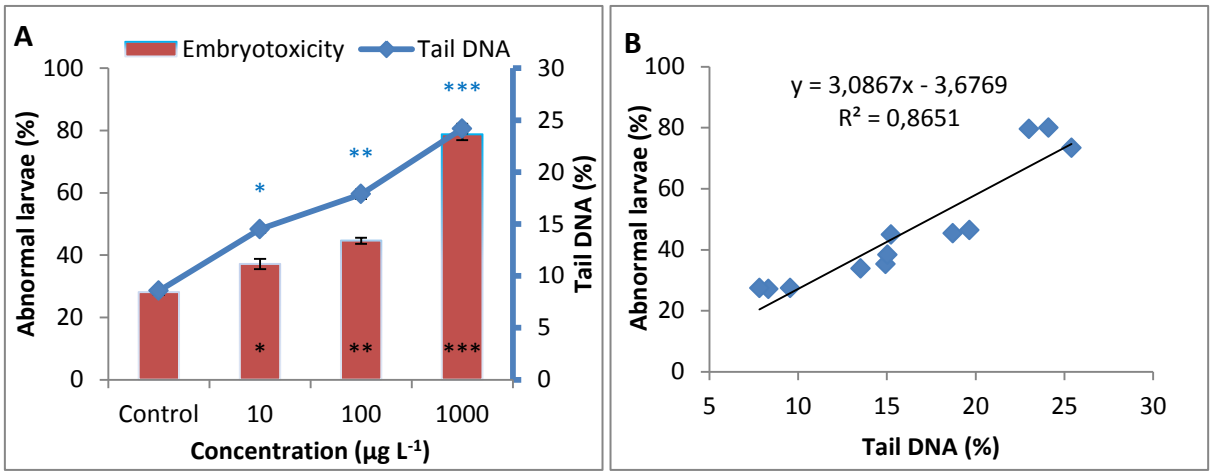

Fig. 2: (**A).** Percentages (Mean ±S.E.) of abnormal and tail DNA following oyster embryos exposed to different concentrations of Cd. Asterisks indicate significant differences between exposed and control treatment (* p < 0.05, ** p < 0.01, ***p < 0.001). (**B).** Relationship between Tail DNA and D-larvae abnormalities in oyster (p = 0.000011).

3.3.2 Cadmium

At the lowest tested concentration (10 µg L^{-1}) the percentage of abnormal D-shell larvae and the level of DNA damage were significantly higher than that of control groups (p < 0.05) (Fig. 2A). At 100 µg L^{-1} Cd, the level of DNA damage was significantly increased 2-fold in comparison to the control treatment (p < 0.01) and 3-fold at 1000 µg L^{-1} Cd (p < 0.001). Similar trend was observed for the frequency of abnormal D- shell larvae. At highest tested concentration of 1000 µg L^{-1}, the percentage of embryotoxicity increased 3-fold in comparison to the control group. The EC50 for Cd was 212.3 µg L^{-1} (Table 3). However, at low concentrations of cadmium (less than10 µg L^{-1}), no threat at environmental dose to oyster embryos in terms of both embryotoxicity and genotoxicity was found (data not shown). Interestingly, a strong positive relationship (Fig. 2B) was observed between the percentage of DNA damage and the percentage of abnormal D-shell larvae (R^2 = 0.87, p = 0.000011).

3.4 Pesticide exposures

3.4.1 Metolachlor

The response of oyster embryos to different metolachlor doses is shown in Fig. 3A. The DNA strand breaks significantly increased following exposure to metolachlor ($p < 0.001$). A dose-dependent increase of DNA damage was observed from 0.01 µg L^{-1} of metolachlor. Tail DNA significantly increased from 12.1% at 0.01 µg L^{-1} to 18.3% tail DNA at 10 µg L^{-1} of metolachlor. A significant increase of abnormal D-larvae over background was observed at the lowest tested concentration of 0.01 µg L^{-1} ($p < 0.05$) (Fig 3B). Larval abnormalities reached 47.4% at the highest tested concentration of 10 µg L^{-1} ($p < 0.001$). A strong relationship was observed between the percentage of DNA damage and the percentage of abnormal D-shell larvae ($R^2 = 0.71$, $p = 0.00008$) (Fig. 3B).

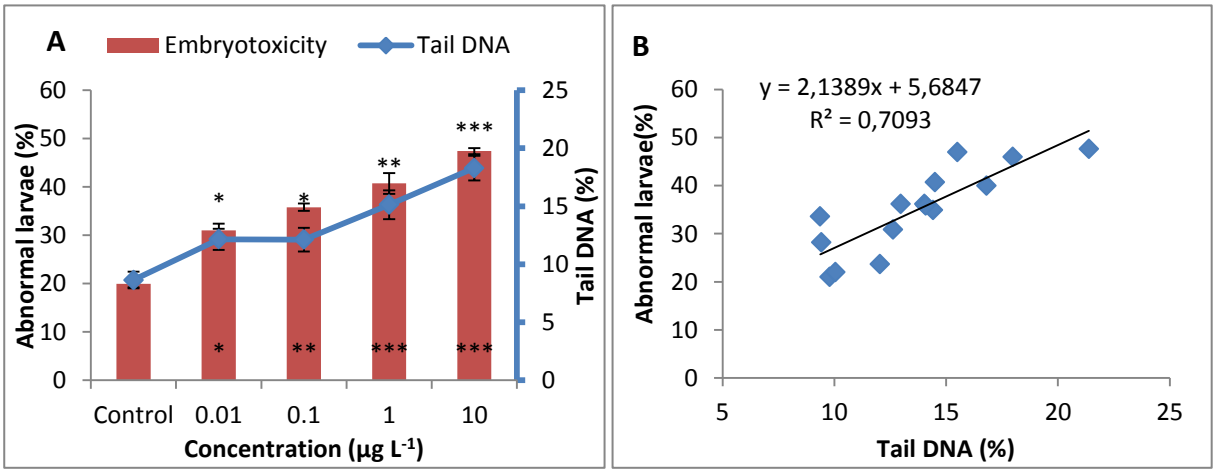

Fig. 3: **(A).** Percentages (Mean ±S.E.) of abnormal and tail DNA following oyster embryos exposed to different concentrations of metolachlor. Asterisks indicate significant differences between exposed and control treatment (* $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$). **(B).** Relationship between Tail DNA and D-larvae abnormalities in oyster ($p = 0.00008$).

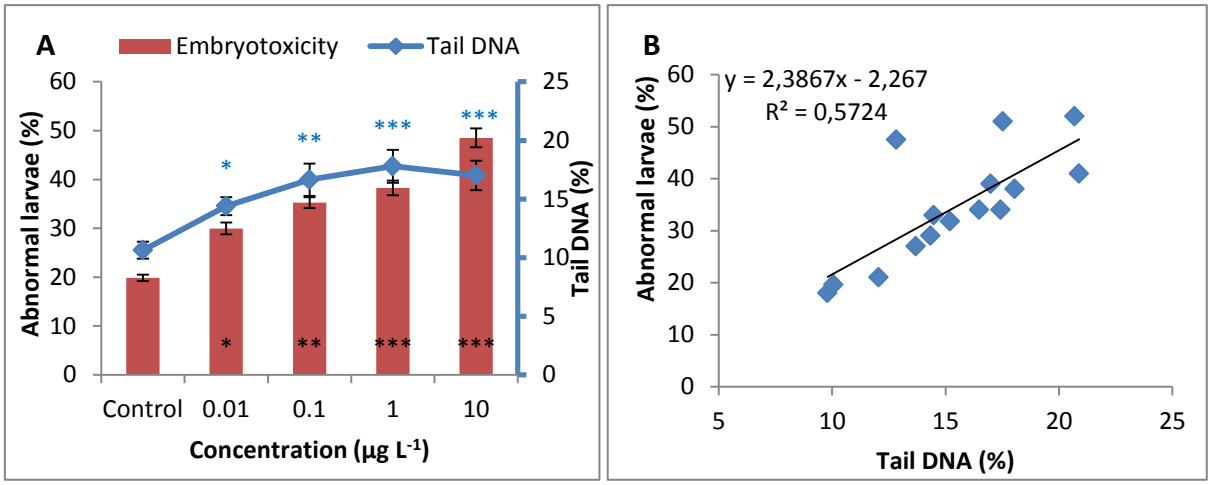

Fig. 4: (**A).** Percentages (Mean ±S.E.) of abnormal and tail DNA following oyster embryos exposed to different concentrations of irgarol. Asterisks indicate significant differences between exposed and control treatment (* $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$). (**B).** Relationship between Tail DNA and larvae abnormalities in oyster ($p = 0.0019$).

Table 3: EC_{50} (µg L^{-1}), LOEC and NOEC (ng L^{-1}) values obtained from oyster embryos toxicity test

Compound	**EC_{50} (µg L^{-1})**	**LOEC (ng L^{-1})**	**NOEC (ng L^{-1})**
Cu	12.5 (11.0-14.2)	100	nd
Cd	212.3 (162.5-277.3)	10000	1000
Irgarol	> 10	10	1
Metolachlor	> 10	10	1

The 95% confidence intervals (95CI) are given in the brackets. Nd: not determined

3.4.2 Irgarol

The genotoxicity and embryotoxicity of irgarol were evaluated in oyster larvae (exposed for 16 hours and 24 hours, respectively). Fig. 4A presents the percentages of abnormal D-shell larvae and DNA damage. Significant increase in DNA damage was observed after irgarol exposure ($p < 0.05$). This genotoxic effect was noticed from the lowest tested concentration of 0.01 µg L^{-1} ($p < 0.05$) and was dose-dependent, with an increase in percentage tail DNA to 14.4% at 0.01 µg L^{-1} and to 17.01% at 10 µg L^{-1} of irgarol (Fig. 3A) compared to control. The percentage of abnormal D-shell larvae was also significantly increased from the lowest tested dose of 0.01 µg L^{-1} irgarol ($p < 0.05$) compared to the control. After exposure to 10 µg L^{-1} irgarol, the percentage of abnormal larvae was 2-fold over control values ($p < 0.001$). There was also a positive relationship between genotoxic and embryotoxic effects in oyster embryos exposed to irgarol, but it was not as strong as observed for copper, cadmium and metolachlor, with $p = 0.0019$ and $R^2 = 0.57$ (Fig. 4B).

4. Discussion

The contamination of aquatic ecosystems by heavy metals and pesticides has gained increasing attention in recent decades (Boxall et al., 2000; Byer et al., 2011; His et al., 1999). Chemical analysis of pollutants in water is needed for pollution monitoring but is not able to evaluate potential risks for living organisms. Biological monitoring using 'target species' could allow a sensitive approach to predict the potential risk of persistent pollutants like heavy metals or pesticides. Although the effects of heavy metals (Cu and Cd) and pesticides (metolachlor and irgarol) on marine species have been well documented in terms of their toxicity and bioaccumulation, data on the genotoxicity and embryotoxicity of these compounds in the Pacific oysters are still scarce in the literature. The present study aims to demonstrate the sensitivity biomarker of genotoxicity and embryotoxicity in Pacific oyster larvae to detect heavy metal and pesticide effects.

The copper contamination of the French Atlantic coast has been studied by Geffard et al. (2002). Copper concentrations ranged from 70 to 158 µg g^{-1} in sediments, and 0.7 µg L^{-1} in seawater (5-10.6 µg L^{-1} in unfiltered elutriates and 0.4-1.1 µg L^{-1} in filtered elutriates). Embryotoxic effects of copper have been documented in the blue mussel *Mytilus trossolus* (Fitzpatrick et al., 2008; Nadella et al., 2009). Fitzpatrick et al. (2008) reported that copper concentration between 3.2 and 10 µg L^{-1} dramatically increased the abnormal development of embryos. Embryotoxicity data obtained (on Pacific oyster, *C. gigas*) in the present study are in agreement with these previous results. Indeed, in our study, the percentage of abnormal D-shell oyster larvae increased by 21% at the highest tested copper concentration of 10 µg L^{-1}. Similar results were also obtained for embryos of American oyster (*C. virginica*) with an increase of 29% in the level of abnormal larvae after exposure to 20 µg L^{-1} of copper (MacInnes and Calabrese, 1979). The highest sensitivity to copper was obtained for blue mussel embryos (*M. trossolus*), with 40% increase of abnormal larvae following exposure to 10 µg L^{-1} of copper (Nadella et al., 2009). However, the blue mussel embryos were exposed for 48h compared to only 24h for oyster embryos in the present study. In contrast, another invertebrate species, sea urchin (*Paracentrotus lividus*), did not exhibit any embryotoxic effects at copper concentration of 5 µg L^{-1}, a significant effect was recorded only from 15 µg L^{-1} (Manzo et al., 2008). The present findings also indicate that embryos of Pacific oyster are extremely sensitive to copper with embryotoxicity threshold observed at concentration as low as 0.1 µg L^{-1}. 24 h-EC_{50} value of copper was 12.5 µg L^{-1}. This result was compared with the previously reported EC_{50} data on the Cu from various bivalve marine organisms. The values

for sea urchin *P. lividus* (Manzo et al., 2008) and oyster *C. gigas* (Quiniou et al., 2005) were 46.0 and 32.9 µg L^{-1}, respectively. Nadella et al. (2009) reported that 48h-EC_{20} value for abnormal development in embryos of blue mussel was 0.3 µg L^{-1} Cu. Copper's toxicity on sea urchin and oyster was at almost the same level as the values in our experiments.

Although copper is a bio-essential metal for the normal functioning of cells, it can also catalyze the formation of hydroxyl free radical in the Fenton and metal-catalyzed Haber-Weiss reactions, resulting in oxidative DNA damage and DNA strand excision (Lesser, 2006). There is evidence demonstrating that excess copper can induce oxidative stress which can result in free radical attacks, possibly arising from DNA-bound metals participating in Fenton-reactions (Bertoncini and Meneghini, 1995). Up to date, information regarding genotoxic effects of this metal to marine species using comet assay are still scarce in the literature, especially for early life stage. Only few genotoxic studies have been done with adult mussel (Bolognesi et al., 1999). According to this study, DNA strand breakages were observed in gill cells of mussels treated with 40 µg L^{-1} of Cu. In the present study, DNA damage has been significantly induced in oyster embryos after 16h exposure to Cu at concentration as low as 0.1 µg L^{-1}.

The genotoxicity and embryotoxicity of cadmium has been demonstrated in numerous studies and in a variety of marine invertebrate species such as mussel and oyster (Bolognesi et al., 1999; Emmanouil et al., 2007; Nadella et al., 2009; Pruski and Dixon, 2002). It was observed here that Cd-exposure impaired oyster embryonic development (Fig. 2A). Furthermore, oyster embryos were shown to be slightly more sensitive to Cd-exposure than other bivalve embryos, with EC_{50} values of 212 µg L^{-1} for the oyster (the present work), of 342.5 µg L^{-1} for the sea urchin *Strongylocentrotus purpuratus* (Phillips et al., 2003), of 502 µg L^{-1} for the mussel *M. trossolus* (Nadella et al., 2009) and of 1200 µg L^{-1} for *M. edulis* (Martin et al., 1981). The present EC_{50} is in accordance with the EC_{50} value of 211-316 µg L^{-1} reported for *Crassostrea rhizophorae* embryos (da Cruz et al., 2007). In addition, DNA damage in Pacific oyster embryos were induced after 16 hours of exposure to 10 µg L^{-1} of Cd. Lower concentrations (< 10 µg L^{-1} Cd) did not significantly increase DNA damage as well as did not cause abnormal development of oyster larvae in comparison to the control group (data not shown). Cd-induced DNA damage may be due to the increase of lipid peroxidation in various tissues (Stohs and Bagchi, 1995). Although cadmium is regarded as a weak genotoxic metal for adult organisms, particularly in mammals (Filipic et al., 2006; Pruski and Dixon, 2002), Cd may interfere with DNA repair processes and thus may enhance the genotoxicity of

directly acting mutagens (Beyersmann and Hechtenberg, 1997). Previous studies have also reported that cadmium may directly induce damage on DNA through the induction of single strand breaks (Hassoun and Stohs, 1996), or indirectly by the inhibition of DNA repair (Beyersmann and Hechtenberg, 1997), or also by a reduction in the level of glutathione (Shimizu et al., 1997). Bolognesi et al. (1999) reported significant increase of DNA damage in the haemocytes of *Mytilus galloprovinciallis* after 5 days of exposure to 112 µg L^{-1} of Cd. It was also shown that the level of DNA damage increased 1.5 times in *M. edulis* gill cells exposed to 200 µg L^{-1} of cadmium in comparison to control group (Pruski and Dixon, 2002).

In addition to metals, two commonly used pesticides, metolachlor and irgarol, were used to assess the susceptibility of the Pacific oyster embryos. A dose-depended behaviour was observed in the comet assay and embryotoxicity tests. After 16 hours of exposure to 0.01, 0.1, 1 and 10 µg L^{-1} of metolachlor or irgarol, a significant and dose-dependent increase of DNA damage and developmental abnormalities were observed in oyster larvae.

Metolachlor is an acetanilide pesticide and may cause toxicity to aquatic organisms through non-point source pollution (Lin et al., 1999). Up to date, few studies of metolachlor toxicity have been conducted on aquatic species and little is known about the impact of pulse contamination of metolachlor on the marine invertebrate species. Data from this study showed that metolachlor at concentration as low as 0.01µg L^{-1} is able to elicit embryotoxicity and genotoxicity in oyster embryos. This toxicity threshold was compared to the literature on aquatic species. Hall et al. (1999a) documented effects of metolachlor in the different aquatic species, with low-observed-effect concentration (LOEC) of: 106 µg L^{-1} in plant, 3,103 µg L^{-1} in benthos and 4,334 µg L^{-1} in fish. For genotoxicity, Clements et al. (1997) reported that DNA damage was significantly induced in erythrocyte of the American bullfrog (*Rana catesbeiana*) following 24h-exposure to 270 µg $L^{-1}$of metolachlor. The high sensitivity of oyster embryos was asserted by many authors (His et al., 1999; Quiniou et al., 2005). Oyster embryos seem to be much more sensitive than other aquatic species to metolachlor with toxicity threshold (LOEC) at 0.01 µg L^{-1}. Metolachlor toxicity may arise from inhibition of protein synthesis by chloroacetamide functional group, resulting in inhibition of normal development of organisms (Weisshaar and Böger, 1987; Weisshaar et al., 1988). Hence, the chloroacetamide in metolachlor might affect protein synthesis of oyster embryos and cause developmental defects and DNA damage.

Irgarol is a widely used “organic boosted biocide” which replaced organotin compounds in antifouling paints. As a result of their continued use as antifouling compounds, relatively high

concentrations have been found in marine environment. Recently, irgarol has been subjected to restrictions in some European countries because of its environmental stability and high toxicity (Thomas et al., 2001). Numerous studies on the toxic effects of this compound on marine organisms such as crustaceans, sea urchin and mussel have been done (Bellas, 2006; Desai, 2008; Manzo et al., 2006). It was shown here that oyster embryos exposed to 0.01 µg L^{-1} of irgarol exhibited significant increase of abnormal D-shell larvae and DNA damage. At the highest concentration (10 µg L^{-1}) 48.5% of abnormal D-shell larvae and 17.0% of DNA damage were induced. Earlier probabilistic investigations on the ecological risk of irgarol by Hall et al. (1999b) reported the concentration of irgarol toxicity to aquatic ecosystems to be around 1.693 µg L^{-1} in coastal waters of Netherland, but 4.2 µg L^{-1} of irgarol has been reported in Singapore coastal waters (Basheer et al., 2002). Desai (2008) reported that a concentration of 1 µg L^{-1} irgarol caused significant reduction in the number of diatom cells and mortality of nauplii *Balanus amphitrite*. The data reported here indicate that oyster embryos are more sensitive after exposure to irgarol even at very low concentrations, although irgarol has been shown much more toxic to algae and higher plant species (Okamura et al., 2000b).

To our knowledge, only one study has demonstrated the potential relationship between embryotoxicity and genotoxicity in invertebrate species at early life of development (Wessel et al., 2007). Relatively high correlation ($R^2 = 0.652$, $p < 0.001$) between embryotoxicity and genotoxicity was reported after oyster embryos exposure to benzo[a]pyrene, 17[α]-ethinylestradiol and endosulfan. In the present study, it was also shown a strong positive correlation between embryotoxicity and genotoxicity ($R^2 = 0.71$-0.88, $p < 0.001$) for two metals (Cu and Cd) and one pesticide (metolachlor), whereas moderate correlation was found for irgarol ($R^2 = 0.5724$, $p = 0.0019$). Léonard et al. (1983) showed that DNA strand breaks occurring during cell division can lead to abnormal development of organisms such as fish. Wells et al. (1997) also suggested that DNA oxidation may be a critical event in teratogenesis. The mechanisms responsible for DNA integrity alteration of metolachlor and irgarol remain unknown. Further studies should be implemented to fulfill a gap in knowledge about the link between embryotoxicity and genotoxicity in oyster embryos.

5. Conclusion

The present study confirms the high sensitivity of Pacific oyster embryos to heavy metals and pesticides. DNA damage was detected at concentration as low as 0.1, 0.01 and 0.01 µg L^{-1} for copper, metolachlor and irgarol, respectively. For cadmium, concentration threshold that

caused DNA damage was higher than for other tested pollutants at concentration of 10 µg L^{-1}. Similar dose-response and toxicity threshold were obtained for genotoxicity and embryotoxicity. This indicates that the toxicity effects caused by those pollutants are equally detected by both endpoints. This study also shows that the pollutant concentrations that caused toxic effects on oyster embryos were in the range concentrations that are present in Arcachon Bay except for cadmium. A strong relationship between embryotoxicity and genotoxicity has been found with most of the pollutants tested in this study, suggesting that developmental defects observed were likely the results of DNA damage. In view of the high toxicity of metolachlor and irgarol on oyster embryos, one could hypothesize possible impact of these kinds of compounds on native and cultivated oysters.

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2. EFFECTS OF PESTICIDE METABOLITES ON OYSTER SPERM CELLS AND EMBRYOS

Most herbicides applied in agriculture are transformed by physical, chemical and biological processes into one or more metabolite products. Metolachlor ethane sulfonic acid (MESA) and metolachlor oxanilic acid (MOA) are the main degradation products of metolachlor. In Arcachon Bay, the MESA and MOA are present in higher concentration than other pesticides, with concentration ranging from few to hundred ng L^{-1} depending on the sampling time and sites. However, the toxic effects of those metabolites on non-target aquatic organisms such as *C. gigas*, remain to be elucidated. Thus, to compare the toxic effects of parent pesticide (S-metolachlor) and its metabolites (MESA and MOA), genotoxicity (comet assay) and embryotoxicity have been performed on sperm cell and embryos of *C. gigas*. In addition, for a better understanding of the molecular mechanism responsible for toxic effects of those pesticides, the study of the transcription levels of targeted genes involved in different physiological mechanisms has been conducted using RT-qPCR technique. The experimental data obtained has been presented in detail in this section.

2.1. Comparative responses of sperm cells and embryos of Pacific oyster (Crassostrea gigas) *to exposure to metolachlor and its degradation products*

This section has been accepted for publication in Aquatic Toxicology:

Huong Mai, Patrice Gonzalez, Patrice Pardon, Nathalie Tapie, Hélène Budzinski, Jérôme Cachot, Bénédicte Morin. *Comparative responses of sperm cells and embryos of Pacific oyster (*Crassostrea gigas*) to exposure to metolachlor and its degradation products.*

Parts of this section have been presented at:

- Poster PRIMO 17: Pollutant Responses in Marine Organisms to be held in the University of Algarve (Faro, Portugal) from 5th to 8th May 2013. Huong Mai, Bénédicte Morin, Patrice Gonzalez, Jérôme Cachot. *Deleterious effects of metolachlor and its metabolites on spermatozoa and embryos of Pacific oyster* (Crassostrea gigas).

- ECOBIM 2013 - EA4689 Interactions Animal-Environnement on 27-30 June 2013, Montréal-Canada. Huong Mai, Bénédicte Morin, Patrice Gonzalez, Jérôme Cachot. *Comparative toxicities of metolachlor and its metabolites on spermatozoa and embryos of Pacific oyster* (Crassostrea gigas).

Abstract

Metolachlor is one of the most intensively used chloroacetanilide herbicides in agriculture. Consequently, it has been frequently detected in coastal waters as well as its major degradation products, metolachlor ethane sulfonic acid (MESA) and metolachlor oxanilic acid (MOA) which are encountered at higher concentrations than metolachlor. Although a few studies of metolachlor toxicity have been conducted on marine organisms, little is known about the environmental toxicity of metolachlor degradation products. In this study, the deleterious effects of metolachlor and its degradation products on spermatozoa and embryos of *Crassostrea gigas* have been compared using biomarkers of developmental defects, DNA damage and gene transcription levels. After 24h exposure, significant increases in the percentage of abnormal D-larvae and DNA damage were observed from 0.01 µg L^{-1} for S-metolachlor and 0.1 µg L^{-1} for MESA and MOA. Results showed that S-metolachlor was more embryotoxic and genotoxic than its degradation products. Oyster sperm was also very sensitive to metolachlor exposure and followed the pattern: metolachlor (0.01 µg L^{-1}) > MOA (0.1 µg L^{-1}) > MESA (1 µg L^{-1}). Metolachlor and MESA mainly triggered variations in the transcription level of genes encoding proteins involved in oxidative stress responses (mitochondrial superoxide dismutase and catalase). Overall, no significant variation in transcription levels could be detected in *C. gigas* embryos exposed to MOA. This study demonstrates that metolachlor and its main degradation products have the potential to impact several steps of oyster development and therefore recruitment in coastal areas exposed to chronic inputs of pesticides.

Keywords: metolachlor, degradation products, gene transcription, genotoxicity, embryotoxicity, Crassostrea gigas

1. Introduction

Most herbicides applied in agriculture are transformed by physical, chemical and biological processes into one or more metabolite products. The application of herbicides by distribution over the land crop introduces them into the environment including ground water, surface water and sediment. Among chloroacetanilide herbicides, metolachlor is one of the most important pesticides applied to corn and other crops for controlling broadleaf and grass weeds. Metolachlor is ranked as intermediate in terms of environmental mobility and persistency with a half life degradation in soil of 6 to 10 weeks (Hostetler and Thurman, 2000).

Metolachlor ethane sulfonic acid (MESA) and metolachlor oxanilic acid (MOA) are the major degradation products of metolachlor. A study of degradation products in tile drain discharge from agricultural fields in central New York indicated that MESA and MOA can persist in agricultural soils for 3 or more years after application (Phillips et al., 1999). Previous studies showed that metolachlor degradation products were generally present 3 to 45 times more frequently than the parent compound (Boyd, 2000; Kalkhoff et al., 1998; Phillips et al., 1999; Rebich et al., 2004). For example, study of 355 water samples from 12 stream sites in Eastern Iowa found MESA in 99.7% of samples, MOA in 94.3% of samples and the parent compound in 54.1% of samples (Kalkhoff et al., 1998). Recently, Rebich et al. (2004) also reported that MESA was detected in all sampling sites of the Mississippi River Basin and more frequently than its parent compound, whereas MOA was found in 89% of the samples. Concentrations of metolachlor from ppt (ng L^{-1}) to sub-ppm (mg L^{-1}) were frequently found in surface and groundwater surveys throughout North America (Kolpin et al., 2002). For instance, in surface water in the Mid-Western US in 2003, average concentrations of MESA and MOA were 1.55 and 0.73 µg L^{-1} respectively (Battaglin et al., 2003).

The widespread occurrence of metolachlor and its degradation products in aquatic systems could represent a threat for aquatic species. Indeed, small number of studies showed acute toxicity in the crustacean *Daphnia magna* and fish *Oncorhynchus sp.* (Wan et al., 2006) or reduced activities of detoxification enzymes (cytochrome P450 O-deethylation and glutathione S-transferases) in metolachlor treated midge larvae, *Chironomus tentans* (Jin-Clark et al., 2008). We previously demonstrated adverse effects on fertilization success, offspring development, and DNA integrity in the Pacific oyster (Mai et al., 2012; 2013). However, the toxic effects of the major degradation products of metolachlor, on non-target aquatic organisms, remain to be characterized. Therefore, the aim of the present study is to

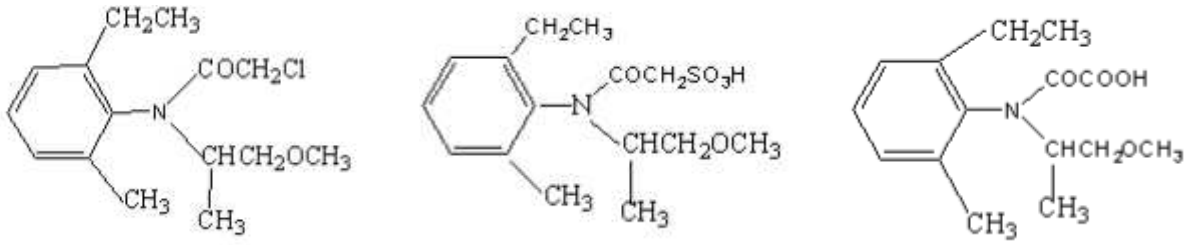
compare the adverse effects of metolachlor and its main degradation products MESA and MOA following a 24h-exposure of Pacific oysters at their embryonic stage. The early life stages of Pacific oyster *Crassostrea gigas* have been selected as a biological model because of their high sensitivity to a large range of pollutants (Geffard et al., 2002; His et al., 1999a; Wessel et al., 2007) and year-around availability of fertilized eggs from adult breeding oysters.

Over time, it has become increasingly clear that a single biomarker is not able to determine the health status of a living organism. A multi-biomarker approach has been reported as the best tool for identifying the effects and the mechanisms of pollutant toxicity to different biological levels of organization (Adams and Greeley, 2000; Faria et al., 2010; Viarengo et al., 2007). A combination of bioassays (embryo-larval test) and biomarkers (DNA damage and gene transcription analyses) were used in this study to investigate the toxic effects of metolachlor and its degradation products on the early life stages of Pacific oyster *C. gigas*. Embryotoxicity and genotoxicity by means of the comet assay can be used as screening methods due to their simplicity and wide application to any eukaryotic organisms (His et al., 1999b; Orieux et al., 2011). In recent years, real-time quantitative polymerase chain reaction (RT-qPCR) has been considered one of the most sensitive techniques in detecting changes in gene transcription induced by environmental contaminants in aquatic systems (Neumann and Galvez, 2002). Therefore, RT-qPCR was used to analyse transcription levels of a panel of 11 genes involved in xenobiotic metabolism, antioxidant defense, mitochondrial metabolism, cell cycle regulation and apoptosis. The response to oxidative stress was studied through mitochondrial superoxide dismutase (*sodmt*), catalase (*cat*), glutathion peroxidase (*gpx*) and metallothionein isoforms *mt1* and *mt2* gene transcription. The mitochondrial metabolism was investigated by analysis the cytochrome C oxidase subunit 1 (*cox1*) transcript levels. The quantity of mitochondria in the cells was estimated using mitochondrial 12S ribosomal transcript levels. Activation of metolachlor and its degradation products phase I and II metabolism was studied using cytochrome P450 1A (*cyp1A*) transcripts and glutathion S-transferase (*gst*) transcripts. Metolachlor-induced apoptosis was studied through *p53* gene transcription levels. Finally, multixenobiotic resistance gene (*mxr*) involved in cell detoxication was also investigated.

2. Materials and Methods

2.1 Chemicals and seawater

Reference toxicants, including S-metolachlor, MESA and MOA (Fig.1), and 37% formalin 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).



Metolachlor **Metolachlor ESA (MESA)** **Metolachlor OA (MOA)**

Fig.1: Structures of metolachlor and its metabolites

Seawater was collected from Arguin in Arcachon Bay (SW France), an area which has a naturally reproducing population of oysters and has been frequently used in the laboratory for ecotoxicological tests. Immediately following sampling, seawater was filtered using a 0.2 µm-pore membrane filter to eliminate debris and microorganisms. Filtered seawater (FSW) was stocked at 4°C in the dark and was used within 3 days. A few hours before the 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 year-round production of mature oysters (Guernsey Sea Farms, UK). Oysters were kept at around 10 °C for transportation and kept in FSW for 1h before the start of the experiment. All oysters were used within 3 days.

2.3 Pesticide solutions

Depending on the assays, three to four replicates were performed for each condition. Experimental concentrations were chosen on the basis of previous results (Mai et al., 2012).

Due to their high solubility in water (e.g. 530 mg L^{-1} for metolachlor), stock solutions (100 mg L^{-1}) of metolachlor and its degradation products (metolachlor ESA and metolachlor OA)

were prepared in Milli-Q water. Working solutions of each pesticide were prepared by diluting the stock solution in FSW. The negative control for all experiments was FSW.

2.4 Pesticide analysis

All contamination solutions were controlled in concentration as well as reference seawater used to prepare the test solutions. Metolachlor and its degradation products were extracted via solid-phase extraction (SPE), using Oasis HLB cartridges (3cc, 60 mg) and analyzed by LC/MS/MS. The analytical procedure was adapted from Alder et al. (2006). The cartridges were conditioned successively with 3 mL of MeOH and 3 mL of acidified water (pH 2). Water samples were acidified (HCl, pH 2), and spiked with internal standards (carbofuran d3) before percolation under vacuum. The cartridges were dried for 30 min by application of a gentle vacuum. Finally, the analytes were eluted with 3 mL of MeOH and concentrated using a nitrogen stream evaporator. 200 µL of each individual solution was mixed with 200 µL of mixture of internal standards in methanol (nominal concentrations 1 µg g^{-1}) and were directly analysed by LC/MS/MS to determine response factors.

Analyses were performed using a HPLC/MS/MS system from Agilent Technologies (HPLC 1290 system coupled to 6460 mass spectrometer) (mobile phase: water (5mM ammonium acetate + 0.1% acetic acid) / methanol (100% / 0% to 0% / 100% within 17 minutes at 0.5 mL/min); column : Kinetex C18 100 mm x 2.1mm x 1.7 mm; ionization mode: ESI +). Procedural blanks were performed to ensure the absence of laboratory-contamination. Recoveries and reproducibility were determined using spiked water samples (at a nominal concentration of 100 ng L^{-1}) processed at the same time as the samples to be characterized and were the following for respectively metolachlor, MESA and MOA : 105±10% (n=10), 90±15% (n=10), 102 ±12% (n=10). Detection limits were 0.1 ng L^{-1} for metolachlor and 1 ng L^{-1} for MOA and MESA in water sample.

2.5 Embryotoxicity assay

Embryotoxicity tests were performed in this study with metolachlor degradation products. Data on metalochlor embryotoxicity has been previously published (Mai et al., 2012) but a new experiment with metolachlor was repeated in this study to compare toxicity with metolachlor degradation products within the same experiment. The embryotoxicity bioassay has been described in detail by His et al. (1997), Quiniou et al. (2005) and recently by Mai et al. (2012, 2013). Briefly, after fertilization, oocytes (500 oocytes) were exposed in 24-well microplate (Greiner Bio-One, polystyrene with physical treatment surface) containing 1.8 mL

of the toxicant solution. These microplates were incubated at 24 °C for 24 hours in the dark. After incubation, 50 µL of 1% buffered formalin were added and the percentage of abnormal oyster larvae was recorded. A hundred individuals per well were directly observed under an inverted microscope (Olympus, magnification x200) to determine the number of abnormal D shaped larvae. The abnormalities (D larvae presenting mantle and/or shell abnormalities) were determined according to the criteria described in His et al. (1999b) and Quiniou et al. (2005).

2.6 Comet assay

Comet assay was performed in this study with metolachlor degradation products. Data on the DNA damage of oyster embryos exposed to metalochlor was previously published (Mai et al., 2012), but a comet assay was repeated here with metolachlor to ensure the same exposure conditions for all contaminants. Embryos were incubated in 250 mL beakers (Pyrex^{TM} glass) for 16h at 24 °C in the dark. This exposure period allows the recovery of unshelled larvae which were enzymatically dissociated for the comet assay. Three replicates were performed per condition and each replicate contained a total of 1,000,000 oyster larvae. Cell isolation and the comet assay on oyster embryos were performed as previously described (Mai et al., 2012).

Sperm cells (150 µL ≈ 1.5 10^6 sperm cells per mL) were collected from male oysters induced to spawn by thermal stimulation (alternating immersion in seawater at 18°C and 28°C for 30 minutes) or by stripping the gonad (Mai et al, 2012) and were immediately exposed to 5 mL contaminant solutions in 15 mL tubes for 30 min at 24 °C in the dark. Three replicates were performed per condition and each replicate contains a total of 525,000 sperm cells.

The comet assay was performed on sperm cells as described by Morin et al. (2011) with slight modifications. 50 µL of cell suspension (about 120 x 10^3 cells) was added to 100 µL of 1% LMP agarose and two agarose gels of 50 µL were laid on a pre-coated slide. Alkaline treatment was performed for 20 minutes to allow DNA unwinding. Electrophoresis was carried out at 25V, 300mA for 20 minutes.

The slides were stained with 20µL of ethidium bromide (20µg/mL) and were analyzed at x400 magnification using an optical fluorescence microscope (Olympus BX 51) and an image analysis system (Komet 5.5, Kinetic Imaging Ltd.). DNA damage was expressed as percentage of “Tail DNA”, i.e. the percentage of total DNA that has migrated from the head. A hundred randomly selected nucleoids were analyzed on two replicate gels.

2.7 Gene expression analysis

After oyster embryos exposure to metolachlor and its degradation products for 24h at 24 °C, the density of larvae was determined using a Malassez's counting cell. 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 (Quiagen). Those samples were then stored at -80 °C until required.

Extraction of RNA: Total RNAs were extracted using the "Absolutely RNA® Miniprep" Kit (Strategene, Agilent) according to manufacturer's instructions. The quality of all RNA extraction was evaluated by electrophoresis on a 1% agarose-formaldehyde gel, and their concentration determined by spectrophotometry.

cDNA synthesis: First-strand cDNA was synthesized from total RNAs (3 µg) using the "AffinityScript™ Multiple Temperature DNAc synthesis" kit (Agilent, Stratagene). In each of the above three tests, the techniques were carried out according to the manufacturer's instructions. The cDNA mixture was stored at -20 °C, until required.

Real-time PCR: After extraction and reverse transcription, real-time PCR reactions were performed with an Mx3000P (Stratagene) following the manufacturer's instructions. Primer sequences for all of the eleven studied genes and the housekeeping gene are reported in Table 2. Real-time PCR was performed in a total volume of 20 µL with 1 µL cDNA, 1 µL of reverse and forward primers (200 µM, each), 7 µL of distilled water and 10µL of GoTaq® qPCR Master Mix (Promega). The amplification program consisted of one cycle at 95°C for 10 min followed by 40 amplification cycles at 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds. PCR specificity was determined for each reaction from the dissociation curve of each PCR product. The cycle threshold (CT) value corresponded to the number of cycles at which the fluorescence emission monitored in real-time exceeded the threshold limit. Transcription levels of the selected genes were normalized according to the expression of the housekeeping gene *β-actin* which was observed to be expressed at the same level in our experimental conditions. Relative expression of a gene was calculated using the $2^{-\Delta CT}$ method as described by Livak and Schmittgen (2001) where ΔCT represents the difference between the cycle threshold of a specific gene and the cycle threshold of the *β-actin* gene. Therefore, the Induction factor (IF) of each gene compared with control corresponds to the following equation: $IF = 2^{-\Delta CT}(\text{Treatment})/ 2^{-\Delta CT}(\text{Control})$.

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

Gene	Sequence 5'-3'	Function	Accession number (EMBL or GenBank)
β-actin	AGTACCCCATTGAACACGG[a] TGGCGGGAGCGTTGAA[b]	Cytoskeletal gene (housekeeping gene)	AF026063
p53	CCCTCAAAGCAGTCCCCA[a] TGTAGCGATCCACCTGATT[b]	Cell cycle arrest/apoptosis	AM236465.2
12S	CTCAGTCTTGCGGGAGG[a] GGTTATGCGGAACCGCC[b]	Mitochondrial metabolism	EF484875
coxI	GTGCCAACTGGTATTAAGGTGT[a] ACACCGCACCCATTGAT[b]	Mitochondrial metabolism	AB033687
sodmt	ACAAAGTCAATCAGTGCCCT[a] CCATTGCCTCTGCCAGT[b]	Mitochondrial oxidative stress	EU420128
cat	GTCGTGCCCCTTTACAACC[a] CGCCCGTCCGAAGTTT[b]	Oxidative stress	EF687775.1
gpx	ATCGAACGCTGCACCA[a] AGCTCCGTCGCATTGT[b]	Oxidative stress	EF692639
mt1	TGTCTGCTCTGATTCGTGTCCAGC[a] GGTCCTTTGTTACACGCACTCATTT[b]	Detoxification	AJ242657
mt2	TCCGGATGTGGCTGCAAAGTCAAG[a] GGTCCTTTGTTACACGCACTCATTT[b]	Detoxification	AJ297818
mxr	AGGAAGGGCAGTTGAGTG[a] CGTTGGCCTCCTTAGCG[b]	Detoxification	AJ422120
gst	AGGCTACCGAAATGGCTG[a] CTCTGACTTGTAATAGGCCGC[b]	Biotransformation	AJ557140
cyp1A	AGGCATAGGGCT...ACA[a] CTGGTTTCGCGGGTTTCAT[b]	Biotransformation	EF645271

[a] Forward primer, [b] Reverse primer

2.8 Statistical analysis

All data are expressed as means ± standard error (S.E). Statistical software SPSS (16.0) was used for data analysis. Normality of the data distribution was tested on data residues using the Shapiro-Wilk test ($p < 0.01$). Homogeneity of variance was checked by the Levene test. As both conditions were always verified for embryotoxicity and genotoxicity, statistical data comparisons were performed by One-way Analysis of Variance (ANOVA). Differences among tested concentration means were performed using Tukey *post hoc* test. Significant differences in gene expression between exposed and control treatments were also statistically analyzed using Tukey's test. Significance was accepted at $p < 0.05$. The statistical tests were conducted separately for each studied compounds. The Lowest Observed Effective

Concentration (LOEC) and the No Observed Effective Concentration (NOEC) were then deduced from the statistical analysis.

3. Results

3.1 Pesticides analysis

Nominal and measured concentrations of S-metolachlor and its degradation products (MESA and MOA) for the different applied treatments were determined (Table 1). Background contaminant levels measured in reference filtrated seawater were less than 10 ng L^{-1} for S-metolachlor and less than 100 ng L^{-1} for its degradation products (Table 1), confirming that the tests were done with water sampled in a clean area. Pesticide concentrations in seawater were in accordance with those expected for the three highest concentrations tested (0.1, 1, 10 µg L^{-1}) and were within 1-25% of the nominal concentrations. However, at the two lowest nominal concentrations (1 ng L^{-1} and 10 ng L^{-1}), the measured concentrations were higher than the nominal concentrations because they also included the pesticide concentration already present in the reference FSW.

Table 1: Measured concentrations of metolachlor and its metabolites in working solutions (ng L^{-1})

Nominal	**RSW***	**1**	**10**	**100**	**1,000**	**10,000**
Metolachlor	8	11	24	134	1,110	8,056
Metolachlor ESA	26	22	30	139	998	7,479
Metolachlor OA	60	56	86	190	1,290	12,653

Note: * Reference seawater (RSW) was collected from Arguin site of Arcachon Bay in May 2012

3.2 Embryotoxicity

Embryotoxicity for *C.gigas* exposed to metolachlor has been previously investigated (Mai et al., 2012). Similar results were obtained in the present study, with a significant increase in abnormal D-larvae over background levels at the lowest tested concentration of 0.01 µg L^{-1} metolachlor ($p < 0.05$). Fig. 2 shows the levels of abnormal D-larvae after exposure of oyster embryos to metolachlor degradation products (MESA and MOA). Compared with the control group, the percentage of abnormal D-larvae significantly increased from 0.1 µg L^{-1} of MESA or MOA. Larval abnormalities reached a 2 to 3-fold increase at the highest tested concentrations of 1 and 10 µg L^{-1}. The estimated LOEC values for oyster embryos are reported in Table 3. LOEC was 10 times less for metolachlor than for its two degradation products.

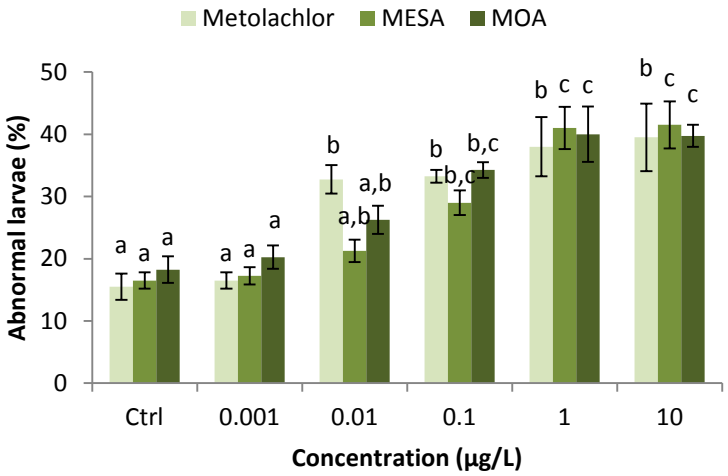


Fig. 2: Percentage of abnormal larvae of *C. gigas* following a 24-hour exposure to metolachlor, MESA, and MOA. Values are mean ± S.E of four replicates. Different letters indicate significant differences between exposed and control treatments for each studied compounds ($p < 0.05$; Tukey's test, N=4).

3.3 Comet assay for oyster spermatozoa and embryos

The genotoxic effects of metolachlor and it degradation products on oyster spermatozoa are depicted in Fig. 3. The background level of DNA damage in non-exposed sperm cells was low (10 – 12%). Following 30 min exposure to metolachlor, a significant and dose dependant increase in DNA damage was observed by the comet assay from the lowest tested concentration 0.01 µg L^{-1}.

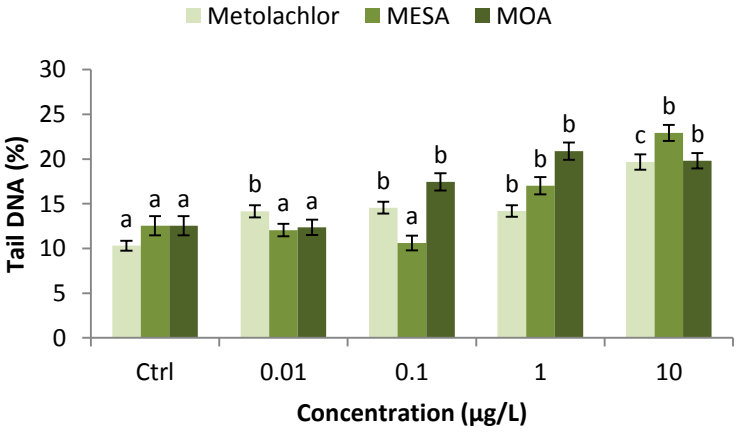


Fig. 3: Levels of DNA damage in oyster sperm cells following a 30 min exposure to metolachlor, MESA, and MOA. Values are mean ± S.E of three replicates. Different letters

indicate significant differences between exposed and control treatments for each studied compounds ($p < 0.05$; Tukey's test, N=4*)*.

For the degradation products MESA and MOA, a significant and dose dependant increase of DNA strand breaks could also be observed but at higher concentrations than for metolachlor e.g. 1 µg L^{-1} for MESA and 0.1 µg L^{-1} for MOA (Fig. 3). At the highest tested concentration of 10 µg L^{-1}, DNA damage reached 19.7% for metolachlor, 22.9% for MESA, and 19.8% for MOA.

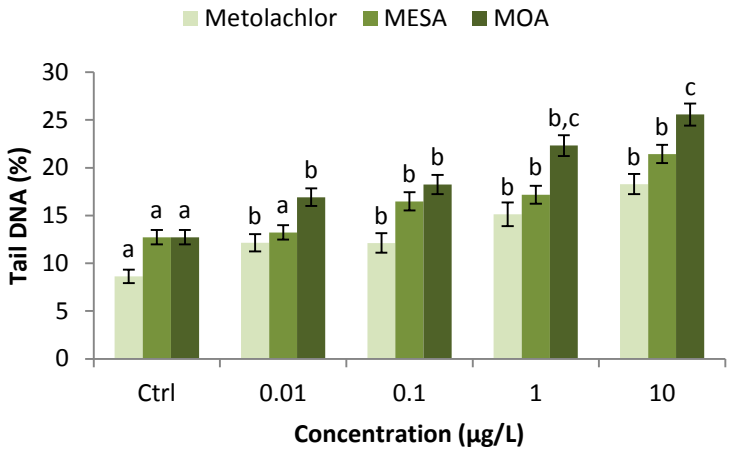


Fig. 4: Levels of DNA damage in oyster embryos following a 16h exposure to metolachlor, MESA, and MOA. Values are mean ± S.E of three replicates. Different letters indicate significant differences between exposed and control treatments for each studied compounds ($p < 0.05$; Tukey's test, N=4*)*.

LOEC values for DNA integrity of *C. gigas* spermatozoa and embryos are summarized in Table 3. LOEC values for metolachlor are equal or much lower (10 to 100 times) than for its two degradation products. MESA had the highest LOEC values regardless of the considered endpoints. Genotoxicity thresholds are overall higher in sperm than in embryos.

Table 3: LOEC values for embryotoxic and genotoxic assays (µg L^{-1})

Pollutants	**Embryotoxicity**	**Genotoxicity-embryos**	**Genotoxicity-sperm**
Metolachlor	0.01	0.01	0.01
MESA	0.1	0.1	1.0
MOA	0.1	0.01	0.1

LOEC = Lowest Observed Effective Concentration.

3.4 Gene expression

During the subsequent qPCR amplifications, the output cycle corresponding to the *β-actin* was examined. This output was always obtained around the same value; i.e. 20.8 ± 0.43 (mean ± SE, n =3) for control, 20.6 ± 0.51 (mean ± SE, n = 12) for metolachlor-exposed embryos and 20.7 ± 0.29 (mean ± SE, n = 18) for metolachlor metabolites (MOA and MESA)-exposed embryos, demonstrating the relevance of the *β-actin* as reference gene in our conditions. Therefore, the transcriptions levels of the target genes were normalized using the *β-actin* gene. Induction factors of each gene in oyster embryos exposed to metolachlor, MESA and MOA in comparison to the control are represented in Table 4.

Table 4: Induction Factors (IF) of the 11 studied genes in oyster embryos exposed to metolachlor, MESA and MOA (N=3 in each treatment condition).

Functions	**Genes**	**Metolachlor (µg L^{-1})**				**MESA (µg L^{-1})**			**MOA (µg L^{-1})**		
		0.001	0.01	0.1	1	0.01	0.1	1	0.01	0.1	1
Cells cycle arrest/apoptosis	*p53*	/	/	3.2*	/	/	/	/	/	/	/
Mitochondrial metabolism	*12s*	/	/	0.1*	/	/	/	/	/	/	/
	coxI	/	/	/	/	0.3*	/	/	0.3*	/	0.2*
Oxidative stress	*sodmt*	10.7*	14.7*	0.9*	0.8*	/	/	/	/	/	/
	cat	/	2.2	2.2	2.9	0.1*	0.01*	0.1*	/	/	/
	gpx	/	/	/	/	/	/	/	/	/	/
Detoxification	*mt1*	/	/	/	/	/	/	/	/	/	/
	mt2	/	/	/	/	/	/	0.2*	/	/	/
	mxr	/	/	/	/	/	/	/	/	/	/
Biotransformation	*gst*	/	/	/	/	/	/	4.1*	/	/	/
	cyp1A	/	/	/	/	/	/	/	/	/	/

The results are given in the form of induction (> 2) or repression (< 0.5) compared to control. Asterisk indicates significant difference in expression difference between exposed and control treatments by Tukey's test ($p < 0.05$). The sign / means equal to control.

Following the metolachlor exposure, a significant difference in gene transcription levels was observed for targeted genes, *sodmt* (mitochondrial superoxide dismutase), 12S and *p53* (tumor suppressor gene P53). Strong induction of the *sodmt* gene involved in oxidative stress defense was observed for low metolachlor concentrations of 0.001 and 0.01 µg L^{-1}. At higher metolachlor concentrations of 0.1 and 1 µg L^{-1}, slight but significant transcript repression was observed. Exposure to 0.1 µg L^{-1} metolachlor significantly induced *p53* transcript expression. Finally, expression of 12S gene was down regulated by exposure to 0.1 µg L^{-1} metolachlor.

Cat (catalase) transcript expression was significantly down regulated by metolachlor ESA at all tested concentrations. Down regulation was also observed for mitochondrial transcript

coxI (cytochrome C oxidase subunit I) at 0.01 µg L^{-1} and for *mt2* (metallothionein) gene at 1 µg L^{-1}. In contrast, a strong induction of *gst* transcription (IF = 4.1) was noticed at 1 µg L^{-1} .

Following exposure of oyster embryos to MOA, only the *coxI* gene was repressed at 0.1 and 1 µg L^{-1}.

4. Discussion

Metolachlor, MOA and MESA contamination in the spiked seawater

In the present study, metolachlor was examined as it is one of the most intensively used herbicides in agriculture. Its degradation products are more persistent and found in higher concentrations and more frequently in coastal water than metolachlor itself. For instance, pesticide contamination in the Arcachon bay (South West France), although present at low levels among coastal areas, is mainly dominated by metolachlor and its degradation products (Auby et al. 2007, REPAR, 2011). These molecules were respectively detected at 0.01 and 0.1 µg L^{-1} in this lagoon depending on sampling sites and seasons as reported in a 2011 study (REPAR, 2011). Among those sites, Arguin, located at the entrance of the lagoon, is subjected to oceanic influence and is characterized by a naturally reproducing population of *C. gigas*. It is the least polluted site in the Arcachon Bay exhibiting a low pesticide and metal concentrations compared to other bay locations (REPAR, 2010; REPAR 2011). Several authors used artificial sea water to perform embryolarvae tests in order to ensure good water quality and no variability over time (His et al, 1997; Anselmo et al, 2011). However, the use of artificial seawater prepared with commercial sea salt (Instant Ocean® and Red Sea Salt®) was inadequate for conducting our embryo larval assay, producing a very high level of abnormal larvae in the controls (above 50%; unpublished data). Moreover, using synthetic seawater is a disadvantage because conditioning and monitoring of the water is required when using dry salt (Anselmo et al, 2011). Furthermore, synergistic effects of dry salts with toxicants are not well understood and the effects may not be reflective of the receiving environment (Jonczyk et al., 2011). In this study, natural sea water taken in Arguin was used as a control and contained a low amount of metolachlor and its degradation products (Table 1). Given the low larval abnormality frequencies in the controls (16.8 ± 3.8 %) (Fig. 2) and the significant advserse effects of tested pesticides, the embryolarvae test could be validated (His et al, 1997; Quiniou et al, 2005). Moreover, the chemical values obtained were consistent with those expected from this sampling area. As shown in Table 1, chemical analyses confirmed the concentrations of S-metolachlor and its degradation products in the exposure solutions for the three highest concentrations tested (0.1, 1, 10 µg L^{-1}). Regarding the two

lowest concentrations used, which were in the same order of magnitude as the reference seawater, the measured concentrations were higher than the nominal ones due to the additive effect of the reference seawater. Preliminary experiments were performed to compare the concentration values recorded at the beginning of the experiment with those measured after 24h of exposure in 24-multiwell plates (polystyrene) in the absence of larvae (data not shown). An average loss of 33% for metolachlor, 18% for MOA and 13% for ESA was found. Such results could be explained by herbicide adsorption by the container walls.

Embryotoxic effects of metolachlor, MOA and MESA

In terms of embryotoxicity, abnormal D-shell larvae have been described as one of the most useful toxicity endpoints in Pacific oysters (His et al., 1999a). Different types of abnormalities could be distinguished: shell abnormality (convex hinge, indented shell margin, incomplete shell), mantle abnormality (protruding mantle) and retarded/arrested development (His et al., 1999a). In this study, no distinction between the different types of abnormality was made. However, retarded/arrested development and mantle deformities appeared to be more severe compared to shell abnormalities, leading to larvae mortality. It would be interesting to determine whether shell deformities could lead to increasing dead larvae or could reduce larvae seetlement at a later development stage.

Recently, the embryotoxic effects of several pesticides, including diuron, irgarol and glyphosate have been investigated in Pacific oysters (Akcha et al., 2012; Mai et al., 2012; Mottier et al., 2013). These authors reported toxic effects in *C. gigas* embryos at environmental concentrations of diuron (40 ng L^{-1}) and irgarol (7 ng L^{-1}). The results from our previous work (Mai et al., 2012) combined with this study have demonstrated the embryotoxicity of metolachlor to oyster embryos at environmentally realistic concentrations. The same toxicity threshold (LOEC) at 0.01 µg L^{-1} was obtained from this study and our previous work (Mai et al., 2012) showing the reliability of the experiment. It should be emphasized that the effects of metolachlor on oyster embryos were detected at concentrations notably lower than on other invertebrate species such as crayfish and aquatic midge in the studies of Cook and Moore (2008) and Jin-Clark et al. (2008). Being an herbicide, metolachlor is much more toxic to plants, it is acting as a growth rate inhibitor. Studies of chloroacetanilide herbicides on higher plants and microalgae show that metolachlor primarily inhibits the synthesis of very long chain fatty acids by inactivating one of the enzymes involved in this pathway. The resulting imbalance of the very long chain fatty acids interferes with the normal cell division (Bach et al, 2011; Thakkar et al, 2013). Other recent studies with

HepG2 cells, an immortalized human cell line, exposed to 50 ppb metolachlor showed a decrease in cell number. Proteins that are involved in cell cycle progression are altered in the presence of metolachlor leading to cell cycle arrest/slowdown (Lowry et al, 2013; Hartnett et al., 2013). *p53* transcripts were significantly overexpressed in oyster embryos exposed to 0.1 µg L^{-1} of metolachlor. *p53* induction could result in cell cycle arrest before S phase allowing repair of DNA damage prior to DNA replication or the induction of apoptosis when the DNA damage are too severe to be properly repaired. We speculate that metolachlor affect protein synthesis of oyster embryos and decrease cell proliferation, growth and hence cause retarded or arrested development.

Relatively few investigations have addressed the impact of pesticide degradation products on bivalve larvae. The toxicity of degradation products of irgarol and diuron have been studied in several aquatic species such as crustaceans, algae and higher plants (Mestankova et al., 2011; Okamura et al., 2000). According to Okamura et al. (2000), irgarol's degradation products appear to have lower toxicity to crustacean species, with 24h-LC_{50} values from 17 to 40 mg L^{-1}, compared to irgarol. To our knowledge, the present study is the first relating to the embryotoxic effects of metolachlor's degradation products on oyster early life stages. An interesting finding of the present experiments is the fact metolachlor breakdown products appeared less toxic than the parent compound. Ecotoxicological tests carried out on river periphytic diatoms exposed to metolachlor and its degradation products, demonstrated the low toxic impact of the degradation products on diatom growth in spite of the high doses applied (100 µg L^{-1} to 10 mg L^{-1}) (Roubeix et al., 2012). Metabolite production involves the dechlorination of the chlororoacetanilides. This indicates that the chlorine element probably plays an important role in the toxicity of the parent herbicide. Indeed, glutathione has been suggested to play an important detoxification role through its reaction with electrophilic xenobiotics compounds to form typically less toxic, water-soluble, and excretable products. The initial steps in metolachlor metabolism proceed via removal of chlorine through a glutation-S-transferase reaction with glutathione. Gene transcription analysis performed on oyster embryos exposed to MESA at the highest tested concentration revealed a significant induction of *gst* gene transcription. In accordance with this, it was shown that degradation products of the acetanilide herbicides (metolachlor, acetochlor and propachlor) could be detoxified by the formation of glutathione-acetanilide conjugates mediated by glutathione-S-transferase (Stamper and Tuovinen, 1998; Field and Thurman, 1996). No significant variation

of transcription of other gene involved in xenobiotics detoxification (*mt1*, *mt2*, *mxr*) or biotransformation (*cyp1a*) was observed.

Impact of metolachlor, MOA and MESA exposure on transcription of oxidative stress defense and mitochondrial metabolism genes

For metolachlor and MESA exposures, transcription deregulation was mainly observed for genes involved in anti-oxidant responses. But a striking difference can be observed between these two compounds. Metolachlor at low or moderate concentrations induced significant increase of *sodmt* transcript levels which can be interpreted as a defense response to reactive oxygen species (ROS) production. In contrast, MESA exposure even at low concentrations led to strong repression of *cat* gene transcription probably indicating toxic effects. Catalase and mitochondrial superoxide dismutase proteins are known to play a key role as ROS scavengers and enhanced activity of those proteins represent an important defense mechanism to pollutant-induced oxidative stress (Richardson et al., 2008). Significant decrease of *cox1* transcript levels in embryos exposed to MESA et MOA has been observed in our study, suggesting an impact on mitochondrial metabolism. COXI is the subunit one of complex IV of the mitochondrial respiratory chain which plays a critical function during respiration by transferring electrons from Cytochrome c to oxygen and contributing to ATP generation. Indeed, it has been demonstrated that complex IV inhibitors cause a rapid and severe depletion of cellular ATP content resulting in acute cell death in rat hepatocytes (Zhang et al., 2001). Moreover, it has been shown that inhibition of Cytochrome c oxydase enhanced ROS production (Wang et al., 2004), triggering overexpression of genes involved in anti-oxidative stress response (Al Kaddissi et al., 2011).

Table 5: Expression ratio *coxI*/12S and *mt2/mt1* in oyster embryo after exposure to S-metolachlor, MESA and MOA

Expression ratio		**Metolachlor (µg L^{-1})**				**MESA (µg L^{-1})**			**MOA (µg L^{-1})**		
	Ctrl	0.001	0.01	0.1	1	0.01	0.1	1	0.01	0.1	1
coxI/12S	0.59	0.11	0.11	0.29	0.18	0.08	0.07	0.43	0.3	0.45	0.04*
mt2/mt1	0.94	1.14	1.07	0.98	0.86	0.74	0.73	0.13*	0.81	1.63	1.35

Asterisk indicates significant difference between exposed and control treatments (Tukey's test, $p < 0.05$).

The decrease of *cox1* gene transcripts could reflect either a decrease of mitochondria cell number or a repression of the *coxI* gene. The gene encoding for RNA 12s (12S) is expressed equally in all mitochondria of the same tissue and provided a baseline for the number of mitochondria in cells. In the present work, *cox1*/12S ratio was constant for all treatments and

did not show any significant difference compared with the control group, except for metolachlor MOA at 1 µg L^{-1} (Table 5), suggesting that the decreased of *coxI* transcripts is probably due to a decrease of the mitochondrial network in cell.

Overall results seemed to indicate that MOA and MESA induce impairment of the cellular energetic metabolism in exposed embryos which could lead to developmental defects and cell death. Metolachlor exposure could lead to ROS overproduction, as supported by the transcription induction of antioxydant gene following metolachlor exposure.

DNA damage induction in embryos by metolachlor, MOA and MESA

Single cells are needed for the comet assay, thus an isolation technique that does not injure DNA is required. DNA damage in control cell did not exceeded 10-12 % DNA in tail, indicating that the enzymatic and mechanical procedure for cell isolation used herein has a minimal effect on cell survival and DNA integrity. Similar background level for oyster embryos was previously observed (Mail et al, 2012; Akcha et al, 2012). DNA damage was seen after metolachlor exposure in several *in vitro* studies. Erythrocytes of *Rana catesbeiana* tadpoles (270 ppb; Clements et al., 1997) and mouse embryos incubated in metolachlor (100 ppb; Greenlae et al., 2004) exhibited a significant increase in the percentage of apoptosis. Metolachlor was shown to be genotoxic at concentration of 0.01 µg L^{-1} and upwards in oyster embryos (Mai et al., 2012, this study), but no data was available for its degradation products. DNA damage could be the result of ROS overproduction as suggested by *cox1* and *sodmt* transcripts modulation. As discussed previously in this study, metolachlor exposure led to ROS overproduction, as supported by the transcription induction of *sodmt* gene following metolachlor exposure, which would be responsible for primary DNA damage induction such as strand breaks. Moreover, a significant increase of *p53* transcriptional level was shown in embryos exposed to 0.1 µg.L^{-1} metolachlor. This also suggests the presence of DNA damage requiring cell cycle arrest for the activation of DNA repair mechanisms. MOA and MESA induce impairment of the electron transport chain in exposed embryos, as suggested by *cox1* transcripts down regulation, possibly enhancing ROS production (Wang et al., 2004) and altering DNA integrity (Barjhoux, 2011).

DNA damage induction in oyster sperm by metolachlor, MOA and MESA

Gametes are released directly into seawater and thus are exposed to all environmental pollutants present during spawning, so the potential for contaminants to disrupt fertilization processes exists. The genotoxic effects of metolachlor and its degradation products on the sperm cells of bivalves, particularly of Pacific oyster, are presently unknown. However,

several recent laboratory experiments have demonstrated the potential for environmental stressors to disrupt sperm function in marine invertebrates. All of these experiments identified mechanisms that impaired sperm swimming or induced DNA damage in sperm (Au et al., 2000; Au et al., 2003; Yurchenko et al., 2009). In the present study, DNA damage in oyster spermatozoa was observed at low concentrations of metolachlor (0.01 µg L^{-1}), MESA (1.0 µg L^{-1}) and MOA (0.1 µg L^{-1}). Akcha et al. (2012) reported that diuron was able to alter oyster sperm DNA integrity at concentrations of 0.05 µg L^{-1}. The mechanisms by which metolachlor and its degradation products induced DNA damage are not known. Sperm is generally considered to have limited capacity for DNA repair and antioxidant defenses, thus this cell type is potentially more susceptible to genotoxicant effects than oocytes and somatic cells (Lacaze et al., 2011; Lewis and Galloway, 2009). Damage to sperm DNA is a very important issue as it can affect reproductive success and offspring quality such as defective embryonic development and high morbidity (Aitken and De Iuliis, 2007). However it is difficult to accurately assess whether the damaged DNA can be transmitted to oocytes. As previously described, the fertilizing capacity of spermatozoa was significantly affected after 30 minutes gamete exposure to metolachlor concentrations as low as 0.1 µg L^{-1} and offspring displayed a dose-dependent increase in developmental abnormalities (Mai et al., 2013). It can be hypothesized that the tested pesticides affected sperm motility, hence decreasing fertilization success. They can also impair sperm cell and DNA integrity which can be transmitted to oocytes. Both mechanisms of sperm toxicity could result in a reduction in the number of offspring or their quality.

5. Conclusion

This study highlighted that metolachlor and its degradation products can induce developmental defects, DNA damage and alter expression of several genes in the early life stages of *C. gigas* at environmentally relevant concentrations. A different pattern was observed in the transcriptional response of studied genes between metolachlor and its degradation products. Metolachlor appeared to enhance antioxidant responses by inducing *sodmt* expression, while MOA and MESA did not. It suggests that these degradation products act through different mechanisms to produce embryotoxicity or genotoxicity in *C. gigas* larvae. However, due to the limited availability of *C. gigas* genome resources at this time, we have studied only a reduced number of genes, which are not representative of all potential molecular targets of metolachlor and its degradation products. It was shown that gene

deregulation was a more sensitive endpoint than embryotoxicity and genotoxicity to detect the effects of metolachlor and its degradation products. Therefore, the study of gene expression by RT-PCR could be a promising tool for evaluating exposure or effects of pollutants in oyster early life stages. The study also showed that embryos of the Pacific oyster are highly sensitive to metolachlor and its degradation products confirming that it is a good bioindicator species for pollution monitoring and ecological risk assessments. Our results showed that metolachlor and its degradation products can induce DNA strand breaks in sperm which could lead to poor fertilization rates and induction of developmental abnormalities. Future work is planned to investigate the reproductive consequences of parent exposure to genotoxicants on both fertilization capacities and development success, notably the role of paternal DNA damage in the disruption of early developmental processes.

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3. EFFECTS OF MIXTURE PESTICIDES AND METAL ON OYSTER SPERM CELLS AND EMBRYOS

In Chapter 2 (Section 1 and 2), we have demonstrated the embryotoxic, genotoxic and spermiotoxic effects of individual contaminants (heavy metals and pesticides) and some of their metabolites present in the Arcachon Bay at environnmentally relevant concentrations to oyster *C. gigas*. Over the past decades, much attention has been paid to the toxicity of single chemicals rather than to complex mixtures. Most of the knowledge and comprehensive of chemical contamination effects in aquatic organisms is based upon the effects of individual pesticides, but the ecosystem is invariably contaminated with multiple biocides. The toxic effect of multiple chemicals has been recognized as an important factor in ecotoxicology because chemical mixture can provoke some dramatic consequences due to additive, synergistic or antagonistic effects. In this context, in the next section, we have studied the combined effects of 15 pesticides with or without copper which are currently detected in the Arcachon Bay upon oyster *C. gigas* spermatozoa and embryos. The toxic effects of those complex mixtures have been assessed throughout the endpoints of D-larvae abnormalities, DNA damage and transcriptional responses of targeted genes.

3.1. Combined toxicity effects of pesticides and copper on early life stages of Pacific oyster, (Crassostrea gigas)

This section is prepared for submission

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Abstract

The frequent occurrence of pesticides and copper in the environment has raised the concerns that combined exposure to these chemicals would result in enhanced toxicity by synergistic interaction between compounds. Spermatozoa and embryos of a sentinel species, *Crassostrea gigas,* were exposed to different concentrations of the environmentally relevant pesticide mixtures in combination with or without copper. For the 1X exposure condition, concentration of the 15 tested pesticides was set at 1,518 ng L^{-1} and 1,000 ng L^{-1} for copper. Several biomarkers including larval abnorrmalities, DNA damage on spermatozoa and embryos and gene expression in D-larvae. Results demonstrated that pesticide mixture in combination with or without copper induced a dose-depent increase of embryotoxic and genotoxic effects from the lowest tested dose of 0.1X. Similar pattern in genotoxicity was observed for exposed spermatozoa. Gene expression analysis indicated that genes involved in anti-oxidative stress *cat,* respiratory chain *coxI,* metal detoxification *mt1, mt2* and cell cycle arrest *p53* were found to be significantly repressed in pesticides and pesticides + Cu exposed oyster embryos. In contrast, biotransformation gene *gst* was significantly overexpressed in oyster embryos exposed to pesticide mixture in combination with copper. In conclusion, this is the first study reporting toxic effects of pesticide mixture in combination with or without copper on early life stages of Pacific oysters. Deleterious effects were detected at below environmental concentrations for individual chemical. Therefore, these findings raise the questions of level possile empacts of mixtures of pesticides and metals on wild or farm oyster population.

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

1. Introduction

For several decades, coastal ecosystems have been subjected to increased copper and pesticide contamination, mainly from agricultural practices and antifouling paints in boating. Many of the more than 1000 pesticides currently used in most of countries throughout the world inadvertently reach aquatic ecosystems. Some pesticides are hydrophobic compounds and tend to bind to suspended particulates and dissolved organic matter and to accumulate in sediments and aquatic biota; while others are more hydrophobic and less persistent in aquatic ecosystems. Pesticides are found in water bodies such as lakes, rivers, streams and other surface waters that support aquatic life (Gilliom, 2007); as a consequence, the receiving ecosystem is invariably contaminated with multiple pesticides. For copper, since the prohibition on the use of TBT (tributyltin) as an antifouling agent and Cu was used as replaced substance, the copper contamination for ecosystems is paid more attention (Ifremer&UB1, 2008), because it can accumulate up to 2000 μg g^{-1} in sediment of severely polluted areas (Bryan and Langston, 1992; Legorburu and Canton, 1991). Concentrations of pesticide compounds and copper measured in surface water and bed sediment frequently exceeded water-quality benchmarks for aquatic life (Geffard et al., 2002; Gilliom, 2007). Up to date, there are several toxicity studies that demonstrated that pesticides and heavy metals such as copper can impair water quality and cause adverse effects on aquatic species, in particular at the early developmental stages (Akcha et al., 2012; Mai et al., 2012; Manzo et al., 2006). However, these mainly focus on the impacts of individual pesticides, but few data are available on the toxicity of mixtures of pesticides in combination with or without copper to marine molluscs. Moreover, the exposure of animals in ecosystems to pesticides is never limited to a single pollutant, but to a complex mixture of chemicals from a variety of sources. Therefore, the frequent co-occurrence of pesticides and copper in the environment has raised the concern that combined exposure to these chemicals would result in toxicity modulation due to synergistic or antagonistic effects of chemicals (Monosson, 2004).

Assessing the cumulative toxicity of pesticides in mixtures has been an enduring challenge in environmental health research for the past few decades (Monosson, 2004). Cumulative effects can be seen when small and statistically insignificant effects of separate compounds are added 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 by molecular interactions but also its influence on adsorption, distribution and excretion, biotransformation, and bioavailability (Altenburger et al., 2003). Moreover, the persistence of

a number of pesticides may be changed when used in combination with other pesticides (Vischetti et al., 1996). The toxic effects of multiple chemicals has been recognized as an important factor in ecotoxicology because mixture can have a greater negative impact than the individual constituents of the mixture (Hernando et al., 2003). The risk assessment process is complicated by the fact that environmental exposures frequently involve 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, embryos and larvae of oysters of the genus *Crassostrea* have been proposed as sentinel organisms for marine ecotoxicological tests (Geffard et al., 2002; His et al., 1999; Wessel et al., 2007) and year-around availability of fertilized eggs from adult breeding oyster. However, to our knowledge, very few papers have investigated molecular responses in terms of gene expression in oyster larvae and embryos exposed to pollutants. 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 assess the modifications of the environment due to contaminants. In the present study, *Crassostrea gigas* embryos or sperm were exposed to environmentally realistic mixture of pesticides with or without copper addition (Table 1) and deleterious or adaptative responses were investegated from the molecular level to individual level.

2. Materials and methods

2.1 Chemicals and seawater

Sixteen reference toxicants (15 pesticides and copper: Table 1), formalin and DMSO (dimethyl sulfoxide) were purchased above 96% purity 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).

Seawater was collected from Eyrac station, Arcachon Bay (SW France) on May 2012, an area which has naturally reproducing population of oysters. Immediately after sampling, seawater was filtered using membrane filter of 0.2 μm to eliminate debris and

microorganisms. Filtered seawater (FSW) was stored at 4°C 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

Depending on the assays, three or four replicates were performed for each condition tested. The range of pesticide concentrations was chosen on the basis of preliminary studies (Auby et al., 2007; Belles, 2012).

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 (Table 1). Tested solutions were a mixture of 15 pesticides only (PM), or pesticide mixture in combination with copper (PM+Cu), or copper only and were prepared by diluting the stock solutions in FSW. The following concentrations were tested: 0.1X, 1X, 10X and 100X, 1X being the environmental concentration in the Arcachon Bay (Table 1). Negative control was FSW spiked with solvent DMSO and acetonitrile at final concentration of 0.01% (the highest concentration in the test solutions).

Table 1: Nominal concentrations of pesticide mixture in combination with or without copper (ng L^{-1})

Chemicals	**100X**	**10X**	**1X***	**0.1X**	**Ctrl.**
Acetochlor	500	50	5	0.5	0
Acetochlor ESA	45,000	4,500	450	45	0
Acetochlor OA	1,000	100	10	1	0
Metolachlor	3,000	300	30	3	0
Metolachlor ESA	45,000	4,500	450	45	0
Metolachlor OA	45,000	4,500	450	45	0
Diuron	400	40	4	0.4	0
Irgarol	500	50	5	0.5	0
Atrazine 2 hydroxy	1,500	150	15	1.5	0
Imidachloprid	8,000	800	80	8	0
Carbendazim	400	40	4	0.4	0
Chlorotalonil	100	10	1	0.1	0
Dichlofluanid	100	10	1	0.1	0
DMSA	300	30	3	0.3	0
DMST	1,000	100	10	1	0
Cu	100,000	10,000	1,000	100	0

* Environmental concentrations in Arcachon Bay.

2.4 Embryotoxicity assay

Male and female oysters were induced to spawn by thermal stimulation (alternating immersion in seawater of 18°C and 28°C for 30 minutes) or by stripping the gonad. Spawning males and females were individually isolated in beakers with 0.2 µm FSW. They were left undisturbed for 15 minutes and were then removed from beakers. Eggs and sperm from two individuals were selected to give a single pairing. Sperms and eggs were sieved separately through a 50 µm and 100 µm meshes, respectively. Sperm mobility was checked and the number of eggs was counted under the microscope (Leica DME, magnification x10). Eggs were fertilized with sperm in ratio of 1:10 (egg:sperm). Fertilization success was verified under microscope, and embryos were then counted and transferred to 24-well microplate for embryotoxicity assays.

The embryotoxicity bioassay has been described in details by His et al. (1999) and Quiniou et al. (2005). For one replicate of each exposure condition, fertilized eggs (500 oocytes) were exposed in wells containing 1.8 ml of toxicant solution. Microplates were then incubated at 24°C for 24 hours in the dark. After incubation, 50 µl of 1% buffered formalin were added and the percentage of abnormal oyster larvae was recorded. 100 individuals per well were directly observed under inverted microscope (Olympus, magnification x200) to determine the number of abnormal D-larvae. The abnormalities (D-shell larvae presenting mantle and/or shell abnormalities) were determined according to the criteria described in His et al. (1999) and Quiniou et al. (2005).

2.5 Comet assay

Embryos were incubated in 250 mL beakers for 16h at 24°C in the dark. Unshelled larvae were recovered during the period of incubation and were digested by enzyme for the comet assay. Three replicates were performed per condition and each replicate contains a total of 1,000,000 oyster larvae.

Cell isolation was performed prior to comet analysis. Following 16 hours of exposure, the embryos were recovered by sieving at 40 µm. One mL of embryo suspension (about 12,000 embryos) was incubated with 1 mL of Dispase II diluted in MEM at final concentration of 1 g L^{-1} for 20 min at 37°C with gentle shaking (150 rpm). The reaction was stopped by centrifugation for 10 min at 1,000 rpm and 4°C. The cell pellet was then suspended in 1 mL

of MEM at a final cell density of about 2,000 x 10^3 cells mL^{-1}. Cell viability was determined for each sample by trypan-blue exclusion test. Comet assay experiments were only carried out with cell suspension with more than 80% cell viability.

Sperm cells were exposed for 30 min at 24°C in the dark to tested solutions in 15 mL falcon containing 5 mL of solution. Three replicates were also performed per condition and each replicate contains a total of 525,000 sperm cells.

The comet assay was performed on isolated cells from embryos and sperm cells as described by Morin et al. (2011) with slight modifications. 50 µL of cell suspension (about 120 x 10^3 cells) was added to 100 µL of 1% LMP agarose and two gels of 50 µL were laid down on a pre-coated slide. Alkaline treatment was performed for 20 minutes to allow DNA unwinding. Electrophoresis was carried out at 25V, 300mA for 20 minutes at 4°C in the dark.

Slides were analyzed at x400 magnification using an optical fluorescence microscope (Olympus BX 51) and an image analysis system (Komet 5.5, Kinetic Imaging Ltd.). DNA damage was expressed as percentage of "Tail DNA", which is the percentage of total DNA that has migrated from the head. A hundred randomly selected nucleoids were analyzed on two replicate gels for each sample.

2.6 Gene expression

Total RNA was extracted from 35,000 embryos exposed to tested concentrations of 0.1X, 1X and 10X (n = 3 for each exposure condition) using the "Absolutely RNA® Miniprep" Kit (Stratagene, Agilent) according to manufacturer's instructions. The quality of all RNAs extracted was evaluated by electrophoresis on a 1% agarose-formaldehyde gel, and their concentration determined by spectrophotometer.

First-strand cDNA was synthesized from total RNA using the "AffinityScript™ Multiple Temperature cDNA synthesis" kit (Agilent, Stratagene). In each of the above three tests, the techniques were carried out according to the manufacturer's instructions. The cDNA mixture was stored at -20°C, until required.

After extraction and reverse transcription, real-time PCR reactions were performed using Mx3000P (Stratagene) following the manufacturer's instructions. Primer sequences for all studied and housekeeping genes are reported in Table 2. Real-time PCR was performed in a total volume of 20 µL with 1 µL cDNA, 2 µL of each pair primers, 7 µL distilled water and

10µL of GoTaq® qPCR Master Mix. The amplification program consisted of one cycle at 95°C for 10 min followed by 40 amplification cycles at 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds. PCR amplification specificity was determined for each reaction from dissociation curve of the PCR product. The dissociation curve was obtained by following the SYBR Green fluorescence. Cycle threshold (CT) value corresponding to the number of cycles at which the fluorescence emission monitored in real-time exceeded the threshold limit were calculated. Relative quantification of each gene expression level was normalized according to the expression of the housekeeping gene *β-actin*. Relative expression of a specific gene was calculated using the $2^{-\Delta CT}$ method as described by Livak and Schmittgen (2001) where ΔCT represents the difference between the CT of a specific gene and the CT of the *β-actin* gene. Therefore, the Induction's Factor (IF) of each gene compared with control corresponds to the following equation: IF = $2^{-\Delta CT}$(Treatment)/ $2^{-\Delta CT}$(Control).

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

Primer	**Sequence 5'-3'**	**Function**
β-act	AGTACCCCATTGAACACGG[a]	Cytoskeletal gene (housekeeping gene)
	TGGCGGGAGCGTTGAA[b]	
p53	CCCTCAAAGCAGTCCCCA[a]	Cell cycle arrest/apoptosis
	TGTAGCGATCCACCTGATT[b]	
12S	CTCAGTCTTGCGGGAGG[a]	Mitochondrial metabolism
	GGTTATGCGGAACCGCC[b]	
coxI	GTGCCAACTGGTATTAAGGTGT[a]	
	ACACCGCACCCATTGAT[b]	
Sodmt	ACAAAGTCAATCAGTGCCCT[a]	Oxidative stress defense
	CCATTGCCTCTGCCAGT[b]	
Cat	GTCGTGCCCCTTTACAACC[a]	
	CGCCCGTCCGAAGTTT[b]	
Gpx	ATCGAACGCTGCACCA[a]	
	AGCTCCGTCGCATTGT[b]	
mt1	TGTCTGCTCTGATTCGTGTCCAGC[a]	Detoxification
	GGTCCTTTGTTACACGCACTCATTT[b]	
mt2	TCCGGATGTGGCTGCAAAGTCAAG[a]	
	GGTCCTTTGTTACACGCACTCATTT[b]	
Mxr	AGGAAGGGCAGTTGAGTG[a]	
	CGTTGGCCTCCTTAGCG[b]	
Gst	AGGCTACCGAAATGGCTG[a]	Biotransformation
	CTCTGACTTGTAATAGGCCGC[b]	
cyp1A	AGGCATAGGGCT...ACA[a]	
	CTGGTTTCGCGGGTTTCAT[b]	

[a] Forward primer; [b] Reverse primer

2.7 Statistical analysis

All data are expressed as means ± standard error (S.E). Statistical software SPSS (16.0) was used for data analysis. Normality of the data distribution was tested on data residues by the Shapiro-Wilk test ($p < 0.01$). Homogeneity of variance (Levene's test) was checked and statistical analysis was performed by One-way of Analysis of Variance (ANOVA). Differences among conditions were then performed using Tukey *post hoc* test. Significance 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 Effects on embryotoxicity

3.1 Embryotoxic effects

The two independent embryo-larval bioassays (Assay 1 and Assay 2) revealed that PM and PM+Cu induced embryotoxic effects on oyster embryos, with some differences in level of responses (Fig. 1A & B). In both deplicated experiments, embryotoxicity was detected at the lowest tested dose of 0.1X for PM and PM+Cu ($p < 0.0001$). In addition, in both experiments and for both mixtures, a dose-dependent increase of abnormal D-larvae was observed. When comparing both mixtures (Fig. 1A&B), the percentage of abnormal larvae was not statistically different even for the two lowest concentrations (0.1 and 1X). Larvae 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.

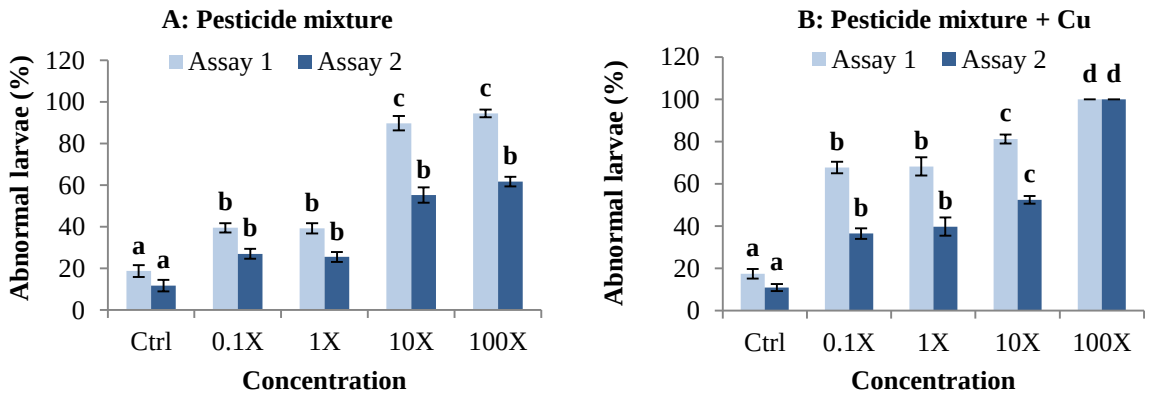

Fig. 1: Developmental abnormalities in oyster D-larvae following exposure to **(A)** pesticide mixture and **(B)** pesticide mixture in combination with copper. Different letters indicate significant differences between treatments ($p < 0.0001$, Tukey's test).

The values that causing 50% reduction in the embryogenesis success (EC_{50}) and their 95% confidence intervals (CI) were reported in Table 3. In Assay 1, a mixture of 15 pesticides only induced embryotoxicity with EC_{50} value of 1.9X, and 1.5X for PM+Cu exposure. Similar trend was observed in Assay 2, but higher EC_{50} value of 12.9X for PM exposure and 3.6X for PM+Cu exposure. When taking into account the data from the two embryotoxic assays, PM exposure impaired embryonic development of *C. gigas* with EC_{50} value of 5.2X, whereas the EC_{50} for exposure to PM+Cu was 0.7X.

Table 3: EC_{50} values (CI 95%) obtained from oyster embryos toxicity tests

	Assay 1	**Assay 2**	**Average**
Pesticide mixture	1.9X (1.1X-3.5X)	12.9X (9.1X-34.9X)	5.2X (2.8X-9.9X)
Pesticide mixture + Cu	1.5X (0.6X-4.2X)	3.6X (1.4X-9.2X)	0.7X (0.2X-2.0X)

Note: 1X was set at 1,518 ng L^{-1} for all tested pesticides and 1,000 ng L^{-1} for copper.

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. 2). The percentage of tail DNA also increased in dose-dependent manner for both mixtures. DNA damage appeared significantly higher in the higher dose (100X) for PM+Cu in comparison to PM treatments. It was not the case for lower doses. Indeed, at the highest tested dose (100X), PM exposure induced 27% of tail DNA, against inducing 37% for the same dose group of PM+Cu.

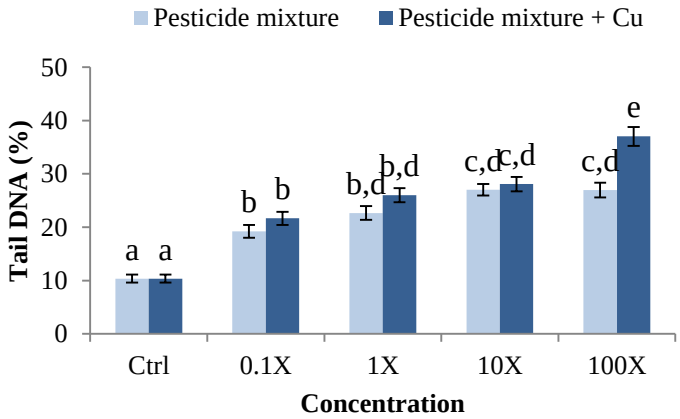

Fig. 2: Percentages (Mean ±S.E.) of tail DNA following oyster embryos exposed to pesticide mixture and pesticide mixture in combination with copper. Different letters indicate significant differences between treatments ($p < 0.001$, Tukey's test).

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. 3). 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 exposures.

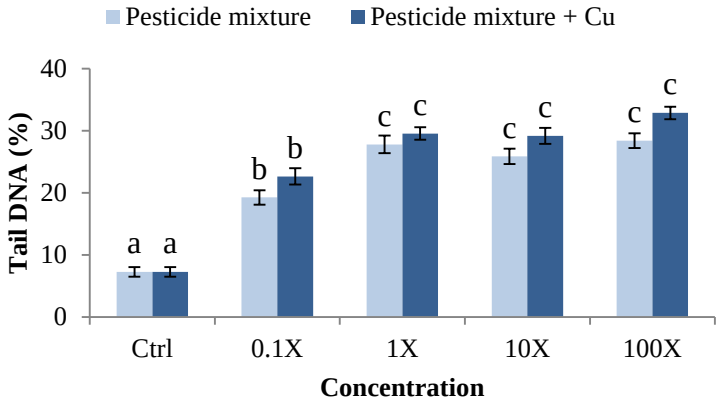

Fig. 3: Percentages (Mean ±S.E.) of tail DNA following oyster sperm exposure to pesticide mixture and pesticide mixture in combination with copper. Different letters indicate significant differences between treatments ($p < 0.001$, Tukey's test).

3.4 Gene transcription levels

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

For oyster embryos exposed to Cu only, no transcription modulate of selected genes involved in mitochondrial metabolism (*coxI, 12s*), cell cycle arrest (*p53*) and detoxification (*mt1, mt2, mxr*) was observed at any tested concentrations. However, Cu induced overexpression of *gst* gene (IF = 4.4), cat gene (IF = 3.8) and cyp1A gene (IF = 1.8) at the highest concentration (10X).

For oyster embryos exposed to pesticide mixtures, 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 low concentrations of 0.1X and 1X. No significant difference in gene expression was observed for xenobiotics metabolism gene *gst* compared to control ($p > 0.05$), despite a clear induction pattern at any tested concentrations (IF = 2.1-6.1). The

expression level for oxidative stress response gene *sodmt* was not significantly increased at any dose ($p > 0.05$).

Table 4: Induction factors (IF) of gene expression for the eleven studied genes in oyster embryos following exposures to copper and pesticide mixture in combination with and without 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	*p53*	/	/	/	0.3*	0.3*	0.4*	0.3*	/	0.4*
Mitochondrial metabolism	*12s*	/	/	/	/	/	/	/	/	/
	coxI	/	/	/	0.3*	0.3*	0.4*	/	0.3*	0.3*
Oxidative stress response	*sodmt*	/	/	/	/	/	2.8	2.4	2.3	/
	cat	/	/	3.8	0.02*	0.1*	/	0.2*	0.4*	0.1*
	gpx	/	/	/	/	/	/	/	/	/
Detoxification	*mt1*	/	/	/	0.03*	0.04*	0.1*	/	0.1*	0.04*
	mt2	/	/	/	0.04*	0.1*	0.1*	/	0.1*	0.04*
	mxr	/	/	/	/	/	/	/	/	/
Biotransformation	*gst*	/	/	4.4	3.0	2.1	6.1	5.5*	3.1*	3.1*
	cyp1A	/	/	1.8*	/	/	/	/	/	/

The results are given in the form of induction (> 2.0) or repression (< 0.5) compared to control group. Asterisk indicates significant difference in gene expression between exposed and control treatments by Tukey's test ($p < 0.05$). The sign / means no significant change in gene expression compared to control.

Exposure of oyster embryo to PM+Cu resulted in significant induction of *gst* gene ($p < 0.05$), with IF values from 3.1 to 5.5. The *sodmt* gene was not significantly amplified at low tested concentrations of 0.1X and 1X ($p > 0.05$). In contrast, *cat* gene was significantly suppressed (IF = 0.1-0.4) at all tested doses ($p < 0.05$). The two metallothionein genes (*mt1* and *mt2*), *cox* gene and *cat* gene were suppressed at 1X and 10X doses. Finally, the gene *p53* was significantly repressed at concentrations of 0.1X and 10X ($p < 0.05$).

4. Discussion

Pesticides are designed to selectively eliminate various pests among 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 widely diversified. Although animals are exposed to complex mixtures of pollutants in the environment, most of laboratory experiments are based on the use of a unique molecule. Studies on pesticides mixtures (Faust et al., 2001; Poletta et al., 2011) have shown that mixture of pollutants was higher toxicity than the chemicals tested individually. Indeed, the effect of a mixture of pesticides on the phagocytosis competence of *C. gigas*

hemocytes was truly demonstrated, while no effect was induced when tested separately (Gagnaire et al., 2007; Gagnaire et al., 2006). Up to date, several 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 have already been investigated in previous studies (Mai et al., 2012; Mai et al., 2013). Our present study showed a hundred percent of abnormal larvae at the highest dose group of PM+Cu exposure, whereas only 78.1% of abnormal larvae were observed at the same dose of PM exposure. This indicates that Cu can be predominantly involved in developmental defects induced by PM+Cu mixture. Our previous studies on embryo exposure to copper have shown that embryo development was entirely affected at the concentration of 40 µg L^{-1} Cu^{2+} (Mai et al., 2012). Poletta et al. (2011) reported higher toxicity for mixture of pesticides than for the single molecules. Because toxic effect are cumulative mixture, can contribute to the overall in proportion to its toxic unit, even if it presents at below 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 chemical may be in opposite direction (increase *vesus* decrease) and the effects of the mixtures are more difficult to predict (Jacobsen et al., 2012). For example, in binary combination of each herbicide with insecticide, metolachlor elevated the toxicity of chlorpyrifos by 1.5-fold at the EC_{50} levels, indicating the synergistic effect of metolachlor on the toxicity of chlorpyrifos insecticide (Jin-Clark et al., 2008). However, Manzo et al. (2008) reported that the irgarol, diuron and copper in ternary mixtures were more toxic than diuron and less toxic than irgarol and copper. These authors also stated that the mixture was less toxic than the single contaminants when considering the embryo NOEC. The knowledge of a chemical mode of action is essential for understanding how mixtures may act jointly. Some pesticides modes of action are well known, in particular in photosynthetic organisms such as algae species (Backhaus et al., 2004; Ranke and Jastorff, 2000), but there is scarce information about the mechanisms of those pesticides for complex cellular systems of aquatic animal species.

4.2 DNA damage for oyster spermatozoa and embryo exposures

A great proportion of sperm DNA damage (19.3-22.6% tail DNA) was observed from the lowest tested dose (0.1X) for pesticides and pesticides + Cu exposures, compared to the control group (7.2% tail DNA). Deleterious effects of heavy metals (Cu, Zn, Cd) and organic xenobiotics (phenol, PCB, butyltin) on sperm cells is well documented for several invertebrate species such as sea urchin, mussel and oyster (Au et al., 2000; Au et al., 2003; Yurchenko et al., 2009), but not in mixtures. Au et al. (2000) reported that low concentrations of individual heavy metals or organic substance can induce ultra-structure abnormalities in spermatozoa of mussel and sea urchin. In addition, exposure to pesticides has also been shown to alter chromatin structure of sperm cells (Sánchez-Peña et al., 2004). Therefore, tPM or PM+Cu mixtures could enhance the toxicity or a synergistic interaction (Monosson, 2004), even if each individual toxicants in mixtures are present from very low concentration (less than 1 ng L^{-1} for pesticides) to 100 ng L^{-1} for copper in the present study. However, Manzo et al. (2008) observed, for sperm cells of sea urchin *Paracentrotus lividus*, that antagonistic effects occurred in ternary mixture of two antifouling chemicals and copper. 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 reaction oxygen species production and DNA damage induction has never been shown for oyster sperm.

A significant increase of DNA damage for both exposed-sperm and exposed-embryos was observed of both mixtures of PM and PM+Cu. However, the PM+Cu showed a slightly greater DNA damage increase for both oyster spermatozoa and embryos for the high dose groups (100X), indicating a possible synergistic interaction among the contaminants. Zhou et al. (2006) observed, for *Vibrio fischeri*, that synergistic effects occurred in binary mixture of antifouling chemicals and copper were due to the presence of Cu^{2+}. These authors suggested that it was due to formation of more lipophilic organic copper complexes, which diffused across the plasma membrane more easily. Our mixture of PM+Cu could probably give rise to chemical complexes that could influence bioavailability changing 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 mixture of pesticides (atrazine, chlorpyrifos and endosulfan) can inhibit the cleavage of the PARP protein involved in DNA repair and programmed cell death residual contaminants. Interestingly, combination of copper and mixture of pesticides did not significantly increase DNA damage in comparison to copper exposure alone.

4.3 Gene expression

The rapid evolution of molecular techniques has initiated a new approach in environmental science and risk assessment to link molecular and exotoxicological 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 apply molecular tool to investigate stress responses in oyster embryos exposed to pesticide mixture with and without copper. Eleven genes known to be involved in antioxidant defenses, mitochondrial metabolism, detoxification, biotransformation process, and cell cycle arrest were investigated by quantitative real-time PCR and normalized according to *actin* gene expression. Several of these genes showed significant transcription modulation after exposure to either PM or PM+Cu exposures.

It was shown that expression of genes encoding for proteins involved in 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 that either oyster embryos were not under stress or they were under stress but this set of genes were not specifically induced in response to pesticides or copper exposures.

Lü et al. (2004) reported that herbicide could induce an increase in both enzyme CAT and SOD activities. In the present study, exposure for 24h to 10X PM induced overexpression of *sodmt* gene. However, overexpression of this gene was only observed at low doses (0.1X and 1X) of PM+Cu. The overexpression of *sodmt* gene could protect cells by scavenging free radicals, generated by pesticide exposure (Banerjee et al., 2001). 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. The *cat* gene was also shown to be significantly up-regulated in mussel along a copper contamination gradient (Dondero et al., 2006). Damiens et al. (2004) reported that CAT activity decreased in oyster larvae after exposute to pesticide, demonstrating the toxicity of pesticide which appear to inhibit CAT. Thus, there is lines of evidence that pesticide mixtures with or without copper induce oxidative stress in exposed oyster early life stage.

In the present study, gene expression *gst* involved in xenobiotic metabolism tends to be overexpressed in oyster embryos exposed to PM and PM+Cu by the formation of glutathione acetanilide conjugates in exposed invertebrates (Lüdeking and Köhler, 2002; Tanguy et al., 2005). Overexpression of this gene could be an adaptive response of oyster embryos to allow

biotranformation and elimination of lipophilic xenobiotics. Study in *Daphnia magna* (Pereira et al., 2010) exposed to several herbicides and insecticides induced the transcription of genes involved in specific processes such as defense mechanism. Thus, overexpression of *gst* gene is probably related to the strong contamination by chemicals in the mixtures.

Ringwood and Brouwer (1995) and Roesijadi et al. (1996) have shown that embryos of *Crassostrea* genus were able to induce metallothioneins synthesis after exposure to heavy metals such as copper and cadmium. A comprehensive review of the multifaceted role of metatllothioneins emphasized that the metals, Zn, Cu, Cd, Hg, Au and Bi all induce MT (Coyle et al., 2002). Unexpectedly, in this study *mt1* and *mt2* genes expressions 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} for each presticide). 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 regardless the concentrations. We also may 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 levels. This result could suggest that *mt1* and *mt2* genes are both target by pesticides and could play the same physiological roles in oyster embryos.

Expression of *coxI* gene was strongly repressed at the higher doses of PM+Cu. The coxI protein being involved in the electron transport chain, our results suggest that mitochondrial metabolism can be affected by pesticides and pesticides + Cu exposures. The *12s* gene expression is indicative of the number 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 affected the transcriptional changes involved in mitochondrial proteins and ATP synthesis-related proteins.

Table 5: Expression ratios of *coxI/12s* and *mt2/mt1* in oyster embryos after 24h of 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
coxI/12s	1.9	1.6	1.3	0.2*	0.1*	0.2*	0.2*	0.1*	0.2*
mt2/mt1	0.8	1.0	0.9	1.1	1.0	1.0	1.4	1.0	1.0

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

Expression profile of oyster embryo exposed to PM and PM+Cu showed significant repression for *p53* gene. For instance, expression of *p53* gene was significantly reduced after 24h exposure to either PM or PM+Cu at almost all tested concentrations, which could impair key cellular processes such as cell cycle control, DNA repair and appotosis. Some authors described the inactivation of *p53* gene after metal exposure 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* gene, through oxidation/reduction of specific thiol groups (-SH) in the DNA binding domain. Moreover, Sandrini et al. (2009) reported that the repression in *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 from individual pesticide in mixtures.

5. Conclusion and perspectives

Our experimental approach was consistent with exposure conditions of oyster embryos may encounter in their native habitat polluted, for instance by leaching from agricultural runoff. It is well known that higher toxicity for the mixture of pollutants than for the single pollutant, even if each pollutant presents at below concentrations of the threshold of statistically detectable effects. In this study, exposures of oyster embryos to these mixtures lead to development defects, DNA damage. Changes in gene expression of oyster embryos exposed to mixture pesticides exposures were mainly observed for genes involved in oxidative stress defense. In addition, the present study also demonstrated that the presence or absence of metal copper in mixtures did not generally alter the gene expression profile for oyster embryos exposed. Thus, the expression of genes involved in oxidative stress suggests that pesticide contaminations represented as a single or mixtures might stimulate a response to oxidative stress in the oyster embryos exposed.

To our knowledge, this study is one of the first to investigate the combination effects of mixture of chemicals at environmentally relevant concentrations on embryos and sperm cells of Pacific oyster. However, in the environment, organisms may exposed not only to mixtures of chemicals, but also to multiple stressors such as physical properties (t°C, pH, turbility...) or infectious agents (bacteria, virus, and parasites...). Therefore, the combined effects/risk resulting from the interaction between chemical, physical and biological stressors should be assessed in further studies.

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4. EFFECT OF ENVIRONMENTAL SAMPLES

In Chapter 2 (section 3), we showed the toxic effects of a pesticide mixture, combined with or without copper, representative of the Arcachon Bay contamination to spermatozoa and embryos of oyster *C. gigas*. Sediments are both a sink and a source of organic contaminants and trace metals. Toxic chemicals accumulated in sediments can be released back into the adjacent ecosystem and consequently degrade the quality of ecological resources. *Crassostrea gigas* are one of the most ubiquitous and abundant marine benthic invertebrates. In the next section 4 in Chapter 2, natural sediments from three locations (Le Tès, Bélisaire and Arguin) in the bay of Arcachon, during different seasons of year 2011, were analyzed with respect to their physical characteristics, concentrations of ammonia and of dissolved organic carbon, and their biological quality were assessed by bioassays with embryos of oyster, *Crassostrea gigas*.

4.1. Embryogenesis of the Pacific oyster, Crassostrea gigas*: implication for assessing the sediment quality of Arcachon Bay*

This section is prepared for submission

Huong Mai, Benedicte Morin, Christelle Clerandeau,Jerome Cachot*. *Embryogenesis of the Pacific oyster* Crassostrea gigas*: implication for assessing the sediment quality of Arcachon Bay*.

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Parts of this section have been presented at:

- ECOBIM 2012 - EA4689 Interactions Animal-Environnement on 5-8 June 2012, Reims- France. Morin Bénédicte, Mai Huong, Hélène Budzinski, Cachot Jérôme. *Toxicity evaluation of relevant pollutants in Arcachon Bay on early life stage of Pacific oyster (Crassostrea gigas)*

Abstract

Sediments have traditionally been viewed as a sink for contaminants in marine ecosystems. Embryotoxicity test on the Pacific oyster, *Crassostrea gigas*, were used to investigate the sediment quality of Arcachon Bay at three different stations and four different seasons. The oyster embryos exposed by sediment contacts were quantified by considering the rates of abnormalities in D-shape larvae after 24h exposure. The results showed that the higher development defect was observed in the period from April to July 2011 and low sediment concentrations of 0.5 and 1.0 g L^{-1}. The levels of ammonium and particulate organic carbon were low in Arcachon Bay and did not seem to affect to the development of oyster embryos, regardless the sampling times.

Key words: sediment contact, embryotoxicity, Pacific oyster, Arcachon Bay

1. Introduction

Coastal ecosystems are currently subject to the impact of numerous human activities that lead to the input of a variety of pollutants of agricultural, urban or industrial origin. After entering estuarine or coastal areas, most contaminants of anthropogenic origin may have short term (e.g. heavy metals and chlorophenol) or long term negative biological effects (e.g. polychlorinated biphenyls - PCBs, polycyclic aromatic hydrocarbons - PAHs, and pesticides) (Burton, 1992; Mottier et al., 2013). These substances tend to be absorbed by particulate matters and finally accumulate in the sediment, which can thus be considered both the main sink and source of contaminants in marine coastal areas (Chapman and Long, 1983; Libralato et al., 2008). Assessing the 'biological quality' of the sedimentary compartment is now a major necessity for many countries in the world and can be assessed using a variety of approaches. The chemical and the biological approaches are both essential and complementary methods for the assessment of environmental contamination and its biological effects (Chapman et al., 1987).

In France, coastal sediments are far less monitored in comparison to freshwater ecosystems and little data exist describing contamination of marine or coastal sediments by contaminants. The Arcachon Bay (South West France) is a well known farming area for the Pacific oysters *Crassostrea gigas* and is thus assumed to have a good environmental quality. A research by Baumard et al. (1998) showed a moderate contamination of sediments in the Bay of Arcachon in 1995, with total PAH concentrations within the lagoon between 0.9 ng g^{-1} and 4.1 µg g^{-1}. Sediments from this Bay contained relatively low metal concentrations, with 0.2 µg Cd g^{-1}, 33 µg Cu g^{-1} and 180 µg Zn g^{-1} (Damiens et al., 2006). However, during the period of 2009-2011, low larval recruitment, reduced spat fall and increased oyster mortality events were observed in the Arcachon Bay suggesting impaired reproduction, mortality or developmental defects at early life stages (Maurer et al., 2011). It is well known that sediments from coastal areas, estuaries and bays are contaminated with toxic chemicals that can adversely affect aquatic organisms (Matthiessen and Law, 2002). Indeed, early life stages of oysters including gametes, embryos, and larvae are known to be particularly sensitive to contaminants including those accumulated in sediments (Beiras and His, 1995; Chapman and Morgan, 1983; Geffard et al., 2001; Geffard et al., 2002; Mamindy-Pajany et al., 2011; Mamindy-Pajany et al., 2010; Poirier et al., 2007).

In recent years, ecotoxicological tests have been used more and more frequently and are now standardized in inter-laboratory comparisons (ASTM, 2008; US-EPA, 2000). Amongst

marine bivalves, oyster are one of the most ubiquitous and abundant marine benthic invertebrate species; as they live in direct contact to sediments and feed on sediment particles, they would be directly exposed to toxicants. Thus, embryo-larval stages of Pacific oyster have been used ecotoxicological study for more than 25 years (His et al., 1999a; His et al., 1997; His et al., 1999b). Since the 90s oyster embryos have been used for chemical toxicity testing (Beiras and His, 1994; His et al., 1999b), water (His et al., 1996), and sediment (Butler et al., 1992; Geffard et al., 2001; van den Hurk et al., 1997) quality assessment.

Bioassay based on *C. gigas* at embryo-larval stage was applied to assess sediment contamination in Arcachon Basin. Sediment contact is one of the best indicators of sediment toxicity; organisms may be exposed simultaneously to the sediment, water and interstitial water from the sediment-water interface during the test (Geffard et al., 2001). The hypothesis of the toxic effect of sediment collected from the Arcachon Bay was thus tested by observing the embryo-larval development of the Pacific oyster throughout the endpoint of abnormality rates.

2. Material and Methods

2.1 Sampling sites and sediment collection

Sediments was collected in 3 stations (Le Tes, Belisaire, and Arguin) in Arcachon Bay (Fig.1) in April, July and October 2011, and January 2012. Only the oxidized part (the first two centimeters) of the sediments was collected. Immediately after sampling, sediments were homogenised and then stored in the dark at -20^{0}C in glass bottles.

Fig. 1: Location of the sampling sites within the Arcachon Bay

Seawater was collected from Eyrac station of Arcachon Bay in France. Immediately after sampling, seawater was sieved using 0.2 µm mesh to eliminate debris and microorganisms. Filtered seawater (FSW) was stored in bottles at 4°C and was used within 3 days. A few hours of before performing experiments, FSW was filtered again at 0.2 µm.

2.2 Preparation of sediment contact solution

The sediment was sieved to remove debris and native wildlife and placed in a glass liter beaker. Sediments were freeze-dried using centrifugation of at 4,000 rpm for 10 min. Suspending freeze-dried sediments were then prepared in filtered seawater (0.2 µm) to establish the concentrations. Sediment suspensions of 0 (control) and 0.5, 1.0, 2.5 and 5 g/L were prepared in 500 mL flasks and the volumes of suspension required for embryotoxicity bioassays (30 mL) were transferred into the test vessels. To avoid any mechanical effects, the sediments were allowed to settle for 1 hour before before adding oyster embryos; this period was shown to eliminate 99% of clay particles from suspension, reducing the turbility of the water to negligible level (Geffard et al., 2001). FSW (0) was used as a negative control treatment.

2.3 Embryotoxicity assay

2.3.1 Parental oysters

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.

2.3.2 Gamete collection

Male and female oysters were induced to spawn by thermal stimulation (alternating immersion in seawater at 18°C and 28°C for 30 minutes) or by stripping the gonad. Spawning males and females were individually isolated in beakers that contained 0.2 µm FSW. They were left undisturbed for 15 minutes and were then removed from beakers. Oocytes and spermatozoa from two individuals were selected to give a single pairing. Spermatozoa and oocytes were sieved separately through a 50 and 100 µm meshes, respectively.

2.3.3 Exposure protocol

Fifteen minutes after fertilization, about 1,200 fertilized eggs were transferred into 50-ml glass tubes, which contained the different concentrations of sediment (three replicates for each

treatment). Embryos were incubated in the sediment suspension for 24 hrs at 24°C ± 1 in the dark. Following exposure, 50 µl of 37% buffered formalin were added in each well and the percentage of abnormal oyster larvae was then recorded under inverted microscope (Olympus, magnification x200). The percentage of abnormal D-larvae was scored on 100 individuals per well. D-larvae presenting mantle and/or shell abnormalities were recorded according to the criteria described in His et al. (1999a) and Quiniou et al. (2005). Normal larvae exhibited a fully developed and symmetrical shell and complete soft structure including the velum. Convex hinge, indented shell margin, incomplete shell and protruding mantle were considered and scored as developmental abnormalities. In the case where the result is not very readable, a few µl Rose Bengal that allow the unique coloring of organic cells was added in the wells.

2.4 Physical and Chemical analysis of sediments

2.4.1 Ammonium analysis

The pore water was removed from the sediments by centrifugation at 4,000 rpm for 10 minutes. Sediment and FSW were mixed together at the maximum tested concentration of 5 g L^{-1} as performing for embryo test. Sediment solutions were shaken for 24h at 250 rpm and then decanting by centrifugation at 4,000 rpm for 5 min. Supernatant was then removed carefully in order to have aqueous for ammonium analysis.

Indophenol blue method has been used to analyze ammonium concentrations in all samples (Grasshoff and Johannsen, 1972; Koroleff, 1969). According to those authors, this measurement technique has been applied for the determination of low concentrations and the percentage of error with this method is less than 5%. Blank and standard solutions were prepared using the ammonium form monochloramine to have standard curve and to compute concentration ammonium of samples by comparing sample absorbance with the standard. For each analysis, 700 µl sample solution was added with thorough mixing after each addition as follow: 20 µL of phenol nitroprusside solution, and 20 µL of dichloroisocyanurate solution. Phenol nitroprusside solution was prepared by dissolving 3.5 g cold phenol and 40 mg of sodium nitroprusside, and then made volumn up to 100 mL by MilliQ water. Dichloroisocyanurate solution was dissolved using 28 g of cold trisodium citrate, 500 mg dichloroisocyanurate and 2.2 g sodium (for seawater) and then made volumn up to 100 mL by MilliQ water. Finally, let all samples develop blue color at room temperature (22-27°C) for overnight and then they are measured using spectroporesis at λ = 630 nm.

2.4.2 Particulate organic carbon analysis

Sediments were dried at 42°C for 24h and then ground to homogenize them. About 100 mg of sediment for each sample was balanced and stored in crucible. All crucibles were taken place in the heater plate at 60°C under the fume hood. Few drops (0.5 mL/drop) of HCl 2N was added into sediment of each crucible to decarbonate the samples because carbonates would effervesce with acids. If there is still effervescence in sediment, it was added a few drops of HCl 2N and then drying the sediment at the heater plate (60°C) under the fume hood.

Five blanks and five standard samples were included in each series of analysis to calibrate measurement system before performing the analysis of organic carbon. For blank samples, each sample contained 100 mg accelerator of Iron Ship and 100 mg Lecosel I were added. For standard samples, beside the accelerator of Iron Ship and Lecosel I, one piece of carbon (0.85% carbon) and sulfur (0.0079% sulfur) in steel was added.

After acidifying sediment samples with HCl 2N, 100 mg accelerator of Iron Ship and 100 mg Lecosel I were added in each sample. Organic carbon in blank and standard samples as well as sediment samples was analyzed using LECO CS 125 equipment and an image analysis system (CS 200).

2.4 Statistical analysis

All data were expressed as means ± standard error (S.E). Statistical analyses were conducted using the SPSS version 16.0 statistical software. Normality of the data distribution was tested on data residues by the Shapiro-Wilk test ($p < 0.01$). Homogeneity of variance (Levene'test) was checked and differences between treatments were tested for significance by means of one-way analysis of variance (ANOVA). When differences among groups were significant the post-Hoc Tukey's test was used for paired comparisons between control group and each treated groups. Null hypothesis was rejected when $p < 0.05$.

In order to calculate the Net Percentage of Abnormality (NPA), data were normalized to the control mean percentage of larval abnormality using Abbott's formula:

$$NPA = (P_{exp.} - P_{ctrl})/(100 - P_{ctrl})*100$$

where: P_{ctrl} and $P_{exp.}$ are control and experimental percentages of response, respectively.

3. Results and discussion

3.1 Physical parameters in sediment suspensions

Generally, no seasonal variations of all parameters were observed for sediment contacts of three stations in Arcachon Bay at initiation of embryotoxicity tests (Table 1). Temperature

and pH values were around 19-20°C and 7.2-7.7, respectively for all sediment exposures. Similarly, salinity values were from 34.1 to 35.4‰. However, the oxygen saturation values (73.4-82.6%) of Arguin sediment were slightly lower than that of La Tes and Belisaire, 83.1-94.9% and 77.7-91.6%, respectively. Thus, it can be stated that all physical parameters are within the optimum range for oyster embryo development (FAO, 2005; His et al., 1999a).

Table 1: Water quality parameters measured during the assay for the different sediments collected in the Arcachon Bay

Locations	Sampling time	Temp (°C)	Oxygen saturation (%)	pH	Salinity (‰)
Le Tes	Apr. 2011	19.4	94.9	7.6	
	Jul. 2011	19.0	90.7	7.3	
	Oct. 2011	19.3	83.1	7.3	34.1
	Jan. 2012	20.2	83.4	7.4	
Belisaire	Apr. 2011	20.2	91.6	7.7	
	Jul. 2011	20.0	77.7	7.6	
	Oct. 2011	19.4	85.0	7.5	35.1
	Jan. 2012	20.4	85.1	7.5	
Arguin	Apr. 2011	20.3	82.6	7.7	
	Jul. 2011	20.6	73.4	7.5	
	Oct. 2011	20.1	78.1	7.3	35.4
	Jan. 2012	20.2	74.8	7.2	

3.2 Ammonium and particulate organic carbon in sediments

Ammonium content in sediments collected in Le Tes, Belisaire and Arguin of the Arcachon Bay were measured (Table 2). While there was little seasonal variation of the ammonium concentration in Le Tes sediments (29.3-39.2 µM), the ammonium concentration was lowest in July 2011 for Belisaire sediment (12.9 µM), and in April 2011 for Arguin sediment (7.1 µM). Mollusc species were shown to be the most sensitive tested invertebrate group to ammonia, but less sensitive than fishes (Arthur et al., 1987). Keppler (2007) reported that the chronic ammonium exposure could result eventually in an increased susceptibility to contaminants stress in both adults and embryos of oyster *C. virginica*. However, according to Huber et al. (1997) ammonium concentrations up to 2.3 mg/L did not affect the growth of the bivalve *Mulinia lateralis* and concentration up to 8.2 mg/L had no effect on survival. In the present study, the ammonium concentrations were relatively low (7.1-39.2 µM ≈ 0.114-0.627 mg L^{-1}) throughout the course of the exposures, regardless of seasonal samplings for all sampling sites. Thus, it can be assumed that the ammonium concentrations measured in sediments collected from the Arcachon Bay might not affect the development of embryos of the Pacific oyster. The low ammonium concentrations in sediments can be explained by the twice-daily tidal flushing avoiding water stagnation (Plus et al., 2009) and/or by ammonium fluxes from the sediment to the water column (Bouchet et al., 2007). Further studies are

needed to determine potential toxic effects that may occur in oyster early life stages exposed to low concentrations of ammonium over a longer period exposure.

In surface sediments, particulate organic carbon (POC) is well recognized for its binding ability of metals, and increased concentrations of POC have been demonstrated to reduce the toxicity of many metals (Chapman et al., 1998; Strom et al., 2011). In the present study, low concentrations of particulate organic carbon (less than 1%) were measured in all sediments analyzed (Table 2). Particularly, Arguin sediments had lower content of particulate organic carbon (0.04 – 0.16%), while Le Tes and Belisaire had higher POC content, with 0.17-0.63% and 0.08-0.55%, respectively. Furthermore, the metal and/or organic pollutant contaminations in Arcachon Bay are generally low, with values below or close to the nation median (Auby et al., 2007; Auby and Maurer, 2004; Ifremer, 2008). Thus, the low concentrations of particulate organic carbon in the Arcachon sediments are unlikely to significantly influence the development of oyster embryos exposed.

Table 2: Seasonal variations of ammonium (µM) and organic carbon (%) concentrations in sediments collected at three locations and four seasons in the Arcachon Bay

	Ammonium (µM)			**Organic carbon (%)**		
Time	**Le Tes**	**Belisaire**	**Arguin**	**Le Tes**	**Belisaire**	**Arguin**
Arp. 2011	29.3	20.0	**7.1**	0.29	**0.55**	**0.04**
Jul. 2011	34.7	**12.9**	38.3	**0.63**	0.21	0.08
Oct. 2011	39.2	22.5	28.9	**0.17**	**0.08**	0.11
Jan. 2012	38.7	24.4	38.2	0.26	0.23	0.16

3.3 Embryotoxicity assay

Embryotoxicity generally provided a more sensitive assessment of ecological risk than other early life stages such as spermitoxicity bioassay (His et al., 1999a; Ringwood, 1992). The embryotoxicity test, which is quite inexpensive and easy to apply, provide interesting and ecologically relevant results, and this test can be recommended for ecotoxicological evaluation of contamination. In particular, His et al. (1997) and Geffard et al. (2001) found in their investigations with sediments that oyster embryos are much more sensitive than sperm alone.

Embryotoxicity assays presented in this study could be validated since they full-filled the validation condition required by the standardized procedure (Quiniou et al., 2005). Indeed, the rates of normal larvae must reach at least 80%. In our experiments, the normal D-larvae were more than 82% (data not shown).

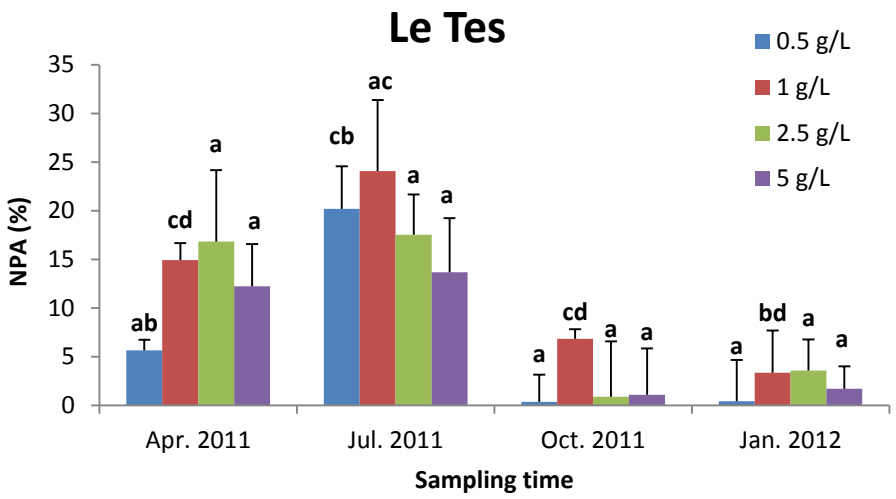

Fig. 2: Seasonal variations of percentages of abnormal D-larvae (mean ± SE) following 24h exposure to Le Tes sediment. Different letters indicate significant differences between different sampling times and different sediment concentration ($p < 0.05$). NPA = Net Percentage of Abnormality

Embryotoxicity of sediments collected from Le Tes showed a clear temporal pattern with higher larval abnormality levels in April and July (spring and summer times) than in October and January (Fig. 2). In contrast, no significant seasonal variations were observed for the toxicity of Arguin and Belisaire sediment exposures (Fig. 3 & 4). The highest net percentage of abnormal development (24.1%) was recorded in July 2011 for Le Tes sediment (Fig. 2), but only 13.9% of NPA was recorded at Belisaire (Fig. 3) and 12% for Arguin (Fig. 4). Higher toxicity of sediment from Le Tès in April and July compared to October and January could be related to pollutant discharge by boating activities during spring and summer periods. In addition, Le Tes is located in the inner part of the Arcachon Bay and receives direct inputs of freshwater from many little streams, while Arguin and Belisaire are at the mouth of the Bay to direct connections to the sea. Thus, we can assume that Le Tes sediment might be much more contaminated than Arguin or Belisaire sediments. Indeed, according to the report of REPAR (2011), Arguin had lower pesticide contamination than other inner parts of the Arcachon Bay.

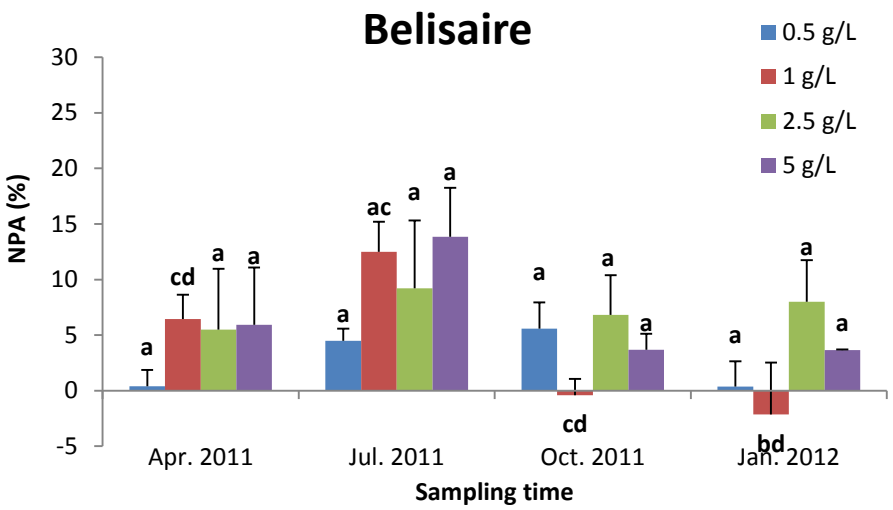

Fig. 3: Seasonal variations of percentages of abnormal D-larvae (mean ± SE) following 24h exposure to Belisaire sediment. Different letters indicate significant differences between different sampling times of each sediment concentration ($p < 0.05$). NPA = Net Percentage of Abnormality

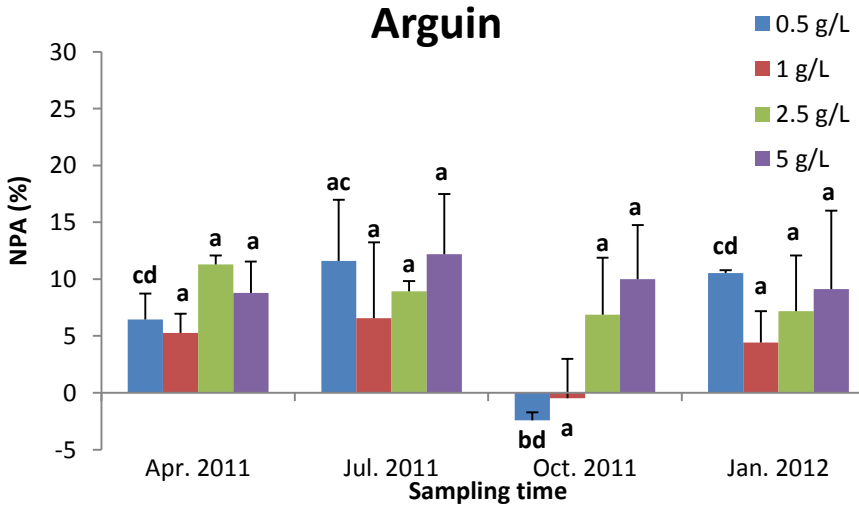

Fig. 4: Seasonal variations of percentages of abnormal D-larvae (mean ± SE) following 24h exposure to Arguin sediment. Different letters indicate significant differences between different sampling times of each sediment concentration ($p < 0.05$). NPA = Net Percentage of Abnormality

When the different sediment concentrations were compared, higher sediment toxicity was observed at low and medium sediment concentrations (0.5 and 1.0 g/L). Nadella et al. (2009) demonstrated that high concentrations of dissolved organic carbon could reduce the metal toxicity to developing embryo of blue mussel. The analysis of particulate organic carbon in

sediments of the Arcachon Bay demonstrated that this bay has relatively poor particles which have important role in uptaking contaminants, particularly metals (Turner et al., 2004). Thus, it can assume that low tested sediment concentrations (0.5 and 1.0 g/L) can result in low particle levels that causing the higher abnormal D-larvae development of oyster was observed. Although the Arcachon Bay display low contamination by metals or organic contaminants (Auby et al., 2007; Auby and Maurer, 2004; Ifremer, 2008), further study are needed to determine the physical and/or chemical characteristics of sediments or hydrodynamic which can govern the contaminant contents in the sediments.

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CHAPTER 3: GENERAL DISCUSSION

Pollutants entering into aquatic ecosystems can pose a threat to not only benthic organisms living in direct contact to sediments, but also to pelagic species due to the potential release of contaminants into the water column. The aim of this thesis was to apply different bioassays on early life stages of the Pacific oyster, *Crassostrea gigas,* for assessing the toxicity of pollutants in particular metals and pesticides. First, the toxicity of individual pollutants including metals and pesticides was investigated using *C. gigas* early life stage bioassays at phenotypic, genotypic as well as molecular levels. In a second step, these bioassays were applied to assess the potential toxic effects of environmental mixture of pollutants. Finally, embryotoxicity assay on *C. gigas* was applied to evaluate the toxicity of natural sediments collected from three sites of the Arcachon Bay.

In the present thesis, the acute and sub-acute toxicity of pollutants were addressed through several bioassays (fertilization assay, embryotoxicity assay, comet assay) and biomarker (gene transcription levels). Those bioassays and biomarker should be considered to yield a complete picture of the oyster embryo developmental status in environmental monitoring. The fertilization assays are sensitive to a large set of pollutants and could be used as the first screening method in a monitoring program (Fitzpatrick et al., 2008; Gopalakrishnan et al., 2008; Manzo et al., 2006). The gene transcription level and DNA damage by comet assay could represent the good biomarker tools to evidence environmental pollution effects by complex pesticides and copper in sentinel organisms such as oyster embryos. In the meanwhile, the responses of embryotoxicity could allow detection of the abnormal development at early life stage of oyster.

The present thesis has shown that exposure of Pacific oyster gametes (spermatozoa and oocytes) to environmental concentration of metals and pesticides reduced fertilization success and offspring quality. The results showed that the deduced EC_{50} of Cu for oyster sperm is 20 µg L^{-1} and 830 µg L^{-1} for Cd. Those EC_{50} values are very close to that reported for spermatozoa of various sea urchins (Dinnel et al., 1989). Interestingly, the results of this thesis are the first showing that S-metolachlor, irgarol and diuron impaired fertilization success and offspring quality following sperm exposure at the environmental concentrations. In contrast, oocytes fertilization was only altered after exposure to high concentrations of toxicants (see section 1.1 and 1.2 in Chapter 2). This is consistent with previous studies in which acute exposure of oocytes had no, or limited, effect on fertility (Bellas et al., 2001; Eyster and Morse, 1984; Fitzpatrick et al., 2008).

In terms of embryotoxicity, the results of the present thesis confirm previous studies (Warnau et al., 1996) demonstrating that Pacific oyster embryos were very sensitive to pollutant exposures such as metals (Cu and Cd) and pesticides (diuron, irgarol and S-metolachlor). For instance, the EC_{50} values reached 12 µg L^{-1} for Cu and 210 µg L^{-1} for Cd (see section 1.1 and 1.3 in Chapter 2). The results are in good agreement with existing data on EC_{50} values of embryotoxicity assays of those metals in other marine invertebrate species (Dinnel et al., 1989; King and Riddle, 2001; Nadella et al., 2009).

Selected pesticides including diuron, irgarol, S-metolachlor and its metabolites (metolachlor ESA and metolachlor OA) also induced embryotoxic effects at very low concentrations (see in section 1.2 & 2 in Chapter 2). This is in accordance with the previous studies on irgarol toxicity for several marine invertebrates such as crustaceans, sea urchin, mussel (Bellas, 2006; Desai, 2008; Manzo et al., 2006). Recently, Akcha et al. (2012) showed that pesticide diuron had embryotoxic effects on oyster embryos at concentrations of 0.05 µg L^{-1} which is very consistent with our study. However, relatively few investigations have addressed the impact of pesticides degradation products on bivalve larvae. To our knowledge, the present study is the first relating to the embryotoxic effects of metolachlor's degradation products on oyster early life stages. An interesting finding of the present experiments is the fact metolachlor breakdown products appeared less toxic than the parent compound.

This thesis also demonstrated that multiple toxicants induced more embryotoxicity than single toxicant (see section 3 in Chapter 2). This finding is in agreement with the study of Poletta et al. (2011). It is well known that the cumulative toxicity of pesticides in mixtures can provoke some dramatic consequences of embryotoxicity due to additive, synergistic or antagonistic effects (Altenburger et al., 2003; Hernando et al., 2003; Vischetti et al., 1996). However, the contribution of any compound in mixtures to its toxic unit for oyster embryo has not been detected. In addition, it is difficult to predict the effects of the mixture because it also depends on the compound mode of action: similar or dissimilar modes of action (Jacobsen et al., 2012). Therefore, the present thesis is the first effort to assess the toxic effects of pesticide mixtures in early development of oyster.

The sensitive response of embryos to genotoxicants by implementing the comet assay has been shown in mussel (Bolognesi et al., 1999; Nadella et al., 2009). Up to date, information regarding genotoxic effects of tested toxicants to oyster using comet assay is still scarce in the literature, especially for early life stages and spermatozoa. Our results showed that toxicants

used in this study with environmental concentrations, are able to induce DNA damage in exposed oyster embryos either individually or in mixture, except for exposure to Cd.

Although the mechanisms of DNA damage induction caused by metals were demonstrated in several previous studies (Bertoncini and Meneghini, 1995; Beyersmann and Hechtenberg, 1997; Lesser, 2006; Stohs and Bagchi, 1995), the mechanisms by which pesticides induced DNA damage are not well known. However, in the present study, DNA damage to embryos is likely to link with the changes in the transcription levels of targeted genes involved in oxidative stress and mitochondrial metabolism. The results showed that transcription deregulation for oyster embryos exposed to either single pesticide (e.g. metolachlor and metolachlor ESA) or mixture of pesticides was mainly observed for genes involved in oxidative stress defense. This could lead to ROS overproduction which would be responsible for primary DNA damage induction such as strand breaks (Akcha et al., 2012; Meyer-Ficca et al., 2011; Wang et al., 2004).

DNA damage in oyster sperm cells after exposure to individual pesticides or in mixture was also identified using comet assay (see in section 2 & 3 in Chapter 2). The results of this thesis are in good agreement with previous studies which stated that the environmental stressors or pollutants can disrupt sperm functioning or induce sperm DNA damage in marine invertebrates (Akcha et al., 2012; Au et al., 2000; Au et al., 2003; Yurchenko et al., 2009). Damage to sperm DNA is a very important issue as it can affect reproductive success and offspring quality such as defective embryonic development and high morbidity.

The present thesis highlighted the correlation between genotoxicity (by means of the comet assay) and developmental impairment (using embryo-larval assay) after exposing to two metals (Cu and Cd) and to two pesticides (irgarol and S-metolachlor) (see in section 1.3 in Chapter 2). Up to date, there is still little information that possibly explains the relationship between genotoxicity and embryotoxicity at early life of development in aquatic species (Léonard et al., 1983; Wells et al., 1997). However, the developmental defects observed in the present thesis were likely the results of DNA damage, which is consistent with the recent finding of Wessels et al. (2007) for oyster embryos exposed to PAHs and a pesticide endosulfan. Further studies should be implemented to fulfill a gap in knowledge about the link between embryotoxicity and genotoxicity in oyster embryos.

Thanks to the many benefits of the oyster embryo-larvae assay, it has been used to assess the quality of the marine sediments in Arcachon Bay (see section 4 in Chapter 2). Geffard et al. (2001) used this assay as a tool in routine monitoring studies on the biological quality of

sediments in different sites of Arcachon Bay. In this study, mapping of toxicity of sediments from the Arcachon Bay (Le Tès, Belisaire and Arguin) was conducted at each season of 2011. Sediments collected from Arguin exhibited low toxicity regardless any season. In contrast sediments from Le Tès showed higher toxicity in spring and summer compared to winter. Among those sites, Arguin, located at the entrance of the lagoon, is subjected to oceanic influence and is the least pollluted site in the Arcachon Bay exhibiting a low pesticides and metal concentrations compared to other bay locations (REPAR, 2012, 2011). On the contrary, Le Tès is located at the inner part of the bay in front of the harbor entrance which generate higher anthropogenic activities than Belisaire and Arguin. The ammonium and particulate organic carbon concentrations in the selected sediments of Arcachon Bay was determined and showed low values for both those chemical characteristics. Accordingly to the study of Geffard et al. (2002b), this level of ammonium concentrations is unlikely to significantly influence the development of oyster embryos. The low variability in particulate organic carbon concentrations of the studied sediments is similar to that found by Geffard et al. (2002b) for other inner parts of the Arcachon Bay such as Ile aux Oiseaux, Arcachon harbor and Gujan. Those authors also reported that those particulate organic carbon concentrations have no adverse effects on the development of oyster embryo.

Overall, the usefulness of oyster early life stage assays in marine monitoring program was confirmed in the present thesis. Our experimental approaches in term of pollutant concentrations were consistent with exposure conditions encountered by oysters in their native environment. From this work, it can be hypothesized that chemical contamination of the Arcachon Bay represents a threat for reproduction and development of wild or cultivated Pacific oysters.

CHAPTER 4: CONCLUSION AND PERSPECTIVES

The gamete toxicity assays are sensitive to a large set of pollutants and have good ecological significance. Moreover, this assay is time-saving and cost-effective. The present thesis has shown that exposure of Pacific oyster sperm to environmental concentrations of metals and pesticides reduced fertilization success and offspring quality. In contrast, oocytes were not affected at environmental concentrations by pollutant exposure. Reduced fertilization rate subsequent to exposure to pollutants, together with decreased offspring quality, may magnify any deleterious impacts that pollutants exert on recruitment of any aquatic species. However, despite the recognized high sensitivity of oyster spermatozoa, spermiotoxicity may not allow forecasting of potential effects of contaminants because of the short-term exposure to pollutants. In addition, it is not clear whether reduced fertilization rates and offspring quality observed in those tests were caused by impaired sperm function or due to early exposure of the developing embryo to pollutants.

Among the different studied developmental stages of *C. gigas*, embryos were the most sensitive and best suited to study the toxicity of pollutants. It can be used in embryotoxicity assay for a wide range of pollutants such as heavy metals or organic pollutants. Although the embryotoxicity assay is sensitive to a large set of pollutants, it can only give a rough measure of the toxicity of the environment because they do not reflect true environmental conditions. Results showed that larval abnormalities of *C. gigas* are correlated to the concentration of contaminants. The experiments carried out in this thesis are intended to close the gap between the effective pollutant concentrations determined in the laboratory conditions and the ecologically relevant concentrations occuring in the marine environment. The limitation of this work is the lack of information about bioavailablity of contaminants to *C. gigas* embryos since no chemical measurement was performed in embryos.

Our results demonstrated that the comet assay is highly sensitive to genotoxicants such as metals and pesticides, whatever these compounds were tested in single or in mixture at environmental concentrations. Moreover, the comet assay has been successfully applied on two different cells of the Pacific oyster: somatic cells (obtained from embryos) and spermatozoa. Of particular interest is the significant induction of DNA damage in spermatozoa exposed to environmental concentrations of pesticides. However, DNA damage can arise through various mechanisms and many factors can affect the basal level of DNA damage. It is possible that certain heavily damaged cells are generated during the protocol cell dissociation and/or during the implementation of the comet assay. In addition, multiple

biological factors (e.g. age, life stage, reproduction stage and interindividual variability) can also influence the outcome of DNA damage.

qRT-PCR analysis was carried out to measure transcription levels of genes involved in a wide range of physiological processes. Furthermore, to our knowledge, this thesis is the first report of the use qRT-PCR for gene transcription analysis in oyster embryos exposed to pollutants. This study highlighted some molecular targets particularly impacted in the presence of complex mixture of pollutants, such as the changing of the transcription levels of genes involved in oxidative stress responses. However, the application of qRT-PCR in oyster embryos seems less relevant for assessing the toxicity of individual pesticides at environmentally realistic concentrations. Efforts should continue to standardize the techniques in terms of extraction of mRNA and reverse transcription of cDNA. In addition, the size of the biological sample should be optimized because it may affect the outcome of PCR analysis.

In the present thesis, the *C. gigas* early life stage bioassays were applied to analyze the toxicity of two metals such as Cu and Cd. Both metals induced acute toxicity in gametes and embryos of the Pacific oyster. However, while Cu increased developmental defects and DNA damage, and reduced fertilization success and offspring quality at environmental relevant concentrations, Cd showed no embryotoxic and genotoxic effects at the same concentrations. Lowest observed effect concentrations (LOEC) for Cu-exposed sperm cells and oocytes were at 1 µg L^{-1} and 10 µg L^{-1}, respectively. However, for most studied genes, gene expression was not affected by environmental concentrations of Cu. This lack of response can be explained by absence of toxic effects or adaptative responses or by different target genes or pathways that those investigated in this study.

The application of *C. gigas* early life stage bioassays to assess the toxicity of individual pesticides, including metolachlor, irgarol and diuron, has highlighted significant toxic effects on oyster spermatozoa and embryos. Our results showed a reduction in fertilization success and offspring quality, when exposing oyster gametes to pesticides at environmentally relevant concentrations. LOEC values of the tested pesticides as regards fertilizing capacity and developmental defects were estimated to concentrations as low as 0.01 µg L^{-1} of irgarol and metolachlor, and 0.04 µg L^{-1} of diuron. The offspring obtained from pesticide-exposed spermatozoa displayed a dose-dependent increase in developmental abnormalities.

The toxicity of pesticide mixtures in combination with or without copper was also evaluated in this work. Exposures of oyster embryos to these mixtures lead to development defects, DNA damage induction and changes in the expression of genes involved mainly in

oxidative stress. Changes in gene expression of oyster embryos exposed to either single pesticide exposures (e.g. metolachlor or metolachlor ESA) or mixture of pesticides were mainly observed for genes involved in oxidative stress defense. This result confirmed that oxidative stress is one of the main reaction pathways following pesticide exposure. Moreover, the present study demonstrated that the presence or absence of metal copper in mixtures did not generally alter the gene expression profile for exposed oyster embryos. Thus, the changes in transcription levels of genes involved in oxidative stress suggests that pesticide contaminations represented as a single or mixtures might stimulate a response to oxidative stress in the exposed oyster embryos. However, for metolachlor and its metabolites MESA exposures, they had low effects on targeted gene expression profile at any tested concentrations. In contrast, mixtures of pesticides in combination with or without copper showed higher effects on gene expression profile from very low concentrations, probably due to synergistic effects. This hypothesis should be confirmed by further analysis to measure more precisely toxicity of mixtures of pesticides.

In present work, embryotoxicity assay with *C. gigas* was carried out with sediments from the Arcachon Bay to measure their toxic potency. In our study, larval abnormalities are correlated to the sampling season and sampling site as well as to the sediment quality. Oyster embryos seemed to be more sensitive to sediments collected during spring and early summer and for low concentrations of sediment. In the present study, parental oysters for the toxicity tests were obtained from an oyster farm (Guernsey Sea Farms, UK). Thus it is possible that gametes or embryos from those parental oysters can be more sensitive to chemicals or to natural sediments than those reared in the Arcachon Bay. For further studies, local oysters should be used for embryotoxicity tests; this will provide more relevant information for monitoring the sediment quality of the Arcachon Bay. This thesis also showed that environmental concentrations of ammonium and particulate organic carbon did not affect the development of oyster embryos. However, other non-chemical factors (e.g. sediment composition and turbidity) and chemical factors (e.g. H_2S) can also impact normal development of oyster embryos. Thus, analysis of such factors needs to be addressed to better understand sediment toxicity.

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ANNEXES

ANNEX 1 : PROPERTIES OF TESTED TOXICANTS

Common name	Chemical name	Purity (%)	Molecular (g/mol)	Solubility in water (mg/L)

Acetochlor	2-Chloro-N-(ethoxymethyl)-N-(2-ethyl-6-methylphenyl)acetamide	96.8	269.7	223
Acetochlor ESA	2-[(Ethoxymethyl)(2-ethyl-6-methylphenyl) amino]-2-oxo-ethanesulfonic acid sodium salt	98.3	337.3	10 times of Acetochlor
Acetochlor OA	[(Ethoxymethyl)(2-ethyl-6-methylphenyl)amino]oxo-acetic acid	97.7	265.3	10 times of Acetochlor
Atrazine	2-chloro-4-(ethylamino)-6-(isopropylamino)-s-triazine	97.4	215.6	70
Atrazine-2-hydroxy	2-Hydroxyatrazine	98.8	197.2	5.9
Carbendazim	Methyl 1H-benzimidazol-2-ylcarbamate	99.2	191.1	8
Chlorothalonil	2,4,5,6-tetrachloroisophthalonitrile	99.3	265.9	0.9
Dichlofluanid	N-Dichlorofluoromethylthio-N' N'-dimethyl-N-phenylsulfamide	99.6	333.2	1.3
DMSA	Dimethylaminosulfanilide	≈ 98	200.2	1300
Diuron	(1,1-dimethyl, 3-(3',4'-dichlorophenyl)	99.5	233.0	42 (25°C)
DMST (100 ng/µL)	N,N-Dimethyl-N′-tolylsulfonyldiamide	97	214.2	97 ng/µl
Metolachlor	2-Chloro-*N*-(2-ethyl-6-methyl-phenyl)-*N*-(1-methoxypropan-2-yl)acetamide	97.6	283.7	530
Metolachlor ESA	2-[(2-Ethyl-6-methylphenyl)(2-methoxy-1-methylethyl)-amino]-2-oxo-ethanesulfonic acid sodium salt	95.8	351.3	Water soluble
Metolachlor OA	2-[(2-ethyl-6-methylphenyl)(2-methoxy-1-methylethyl)amino]-2-oxoacetic acid	98.3	279.3	8500 g/L
Imidacloprid	N-[1-[(6-Chloro-3-pyridyl)methyl]-4,5-dihydroimidazol-2-yl]nitramide	99.9	255.6	610
Irgarol	2-(tert-Butylamino)-4-(cyclopropylamino)-6-(methylthio)-s-triazine	98.6	253.3	6
Copper	Copper (II) sulfate pentahydrate	> 98	249.7	208 g/L
Cadmium	Cadmium chloride dihydrate	96	219.3	

ANNEX 2 : PROTOCOLS OF BIOASSAYS

1. EMBRYOTOXICITY ASSAY

MATERIALS

- 4 supports a filter Nalgene container with superior
- Millipore filter 1.2 µm RAWP
- Millipore filter 0.2 µm RAWP
- 4 glass bottles 1L
- Basin (x4)
- Jars
- Specimens (1L)
- Filter SEFAR NITEX 100 µm
- Filter SEFAR NITEX 50 µm
- Microscope
- Thermostated cabinet
- Microplates (24 wells)
- Filtered seawater (EMF)
- Oyster *Crassostrea gigas* to its optimum stage of maturity (milky)
- Sperm and ovum of frozen oysters

SELECTION OF BROODSTOCK

This choice is crucial for the success and quality of the bioassay. The broodstock should be selected at their peak of maturity to ensure the sexual maturity of gametes harvested. For this, there is a function of the season and means of locomotion different possibilities to recover the mature oyster.

Sources from

- Of the oyster Basin (Partnership with Ms. Angelica HERMANN, La Teste)
- The hatchery in Guernsey Sea Farms (UK)
- Best fish markets (Vents et Marees, Barriere de Begles, Bordeaux)

The hatchery of oyster farm can provide mature oysters throughout the year but with some deductions for the period October to December. It turned out that oysters delivered to that time cannot recover gamete of very good quality. We therefore call on them from January to September

Note: Transport conditions influence the quality of spawning. The transport water should preferably be attached directly to the storage place for oysters from the hatchery: place them in a bath of EMF for an hour before starting the thermal stimulation

PROTOCOL

Recovery of gametes

There are 2 methods to get the male and female gametes

- Induction heat
- By stripping

Induction heat

This is to stimulate the oysters by variations in temperature of the baths where they are:

- Leave approximately (depending on size and shellfish) 6 to 12 oysters per bowl
- Place them in the bath EMF "cold" 18^0C for 30 min
- Then place the broodstock in the bath EMF 'hot' 28^0C for 30 min
- Repeat the operation every 30 min (alternating cold bath / hot bath), stirring the bowl regularly.
- Isolate the progenitors of the beginning of spawning. The isolation is realized in a jar containing the EMF to the mother that the bath temperature that triggered the spawning

Note: The spawning of the oyster male is characterized by a steady stream white (milky) while the female gametes ejected by clapping his violent (white cloud)

- The onset of spawning may take up to 5 or 6 thermal shock. To expedite the process, it can stimulate spawning gametes with previously harvested and frozen at -20^0C.
- Once spawning ended sexing check under the microscope. The oocytes were elongated pear-shaped
- Filter the contents of each jar on the filters 100 or 50 µm depending on sexing
    - Filtration of 100 µm for oocytes
    - Filtration of 50 µm for sperm

Pouring the filtrate in large specimens of 1 L for females

By stripping

In the case of thermal stimulation did not work, it's possible to recover the by stripping gametes. This is to open the progenitor and scarify the gonads. Only drawback: the sacrifice of broodstock.

- Protect your hands before opening the oysters
- Take oysters and start by cutting the adductor muscle. It is located on the left when looking at an oyster in front
- Once the oyster open, put a knife to the heart
- Verify that the oyster has not already laid or start laying. To do this, check that the gonad is large, white and streaked
- Cut the lung tissue to clear the gonad
- Take a glass stirrer with round toe
- Make slight movements of pressure from top to bottom all the way to the gonad maintaining the oyster on the surface of a jar of EMF
- The mature gametes normally flow naturally in the jar
- Otherwise, take a scalpel and cut the gonad. Make the same movements of pressure so as to leave the gametes
- Realize sexing microscope
- Filter the contents of jars with the same filter size as the gametes RECOVERED by thermal induction.

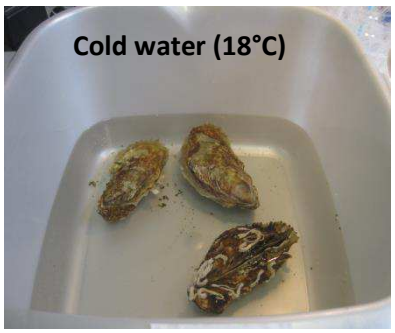
FERTILIZATION

- Once filtration is complete, check the good quality of gametes
- Then ensure the microscope ratio Oocytes / Sperm 1:10
- Otherwise, add a further few mL of sperm

INCUBATION DEVELOPMENT

- Once the ratio obtained, controlling the microscope during the first 20 minutes of departure polar body to ensure proper fertilization
- When a large portion (platform-all) of the polar cells is gone, it's possible to inoculate the eggs fertilized in the test media. The test media are either in multi well plates or in glass tubes according to the contaminant
- Whatever the medium, the media tested are placed in a thermostatic chamber at 24^0C for 24h

- At the end of the period of exposure, the larvae are normally reached the stage of larva D. We can then fix the larvae by adding 1% formaldehyde by volume



Thermal stimulation

Stripping

Spawning

Microplate, 24-well

Inverted microscope

2. PROTOCOL OF FERTILIZATION ASSAY

MATERIALS

- 4 supports a filter Nalgene container with superior
- Millipore filter 1.2 µm RAWP
- Millipore filter 0.2 µm RAWP
- 4 glass bottles 1L
- Basin (x4)
- Jars
- Specimens (1L)
- Filter SEFAR NITEX 100µm
- Filter SEFAR NITEX 50µm
- Microscope
- Thermostated cabinet
- Microplates (24 wells)
- Filtered seawater (EMF)
- Oyster *Crassostrea gigas* to its optimum stage of maturity (milky)
- Sperm and ovum of frozen oysters

GAMETE RECOVER

The oocytes were fertilized by sperm –dense suspension to raise a ratio of 10 spermatozoa per 1 oocyte. Fifteen minutes after fertilization, embryo were counted and dispatched in each assay beaker at a final concentration of 50,000 fertilized oocytes per litter.

EXPOSURE PROTOCOL

- Add 3.0 mL of sperm in the vials containing 10 mL of each toxicant at the appropriate concentration in FSW.
- Add 1500 oocytes in the vials containing 10 mL of each toxicant concentration.
- Sperm cells and oocytes were exposed to toxicants for 30 min in the same manner at 24°C in the dark.

Three fertilization assays were then conducted.

- (1) 1.0 mL of exposed sperm solution was added to 10 mL of FSW containing unexposed oocytes (1500 oocytes).
- (2) 1.0 mL of unexposed sperm cell suspensionwas added to 10 mL of test solution containing exposed oocytes (1500 oocytes).

- (3) 1.0 mL of exposed sperm solution was added to 10 mL of test solution containing oocytes (1500 oocytes) exposed to the same concentration of a toxicant.

Note: In all sets of assays a positive control was added by mixing unexposed sperm with unexposed oocytes from the same couple of oysters to control the variability of fertilization efficiency between the experiments.

- Fertilization success was checked every 15 min after sperm cells and oocytes were mixed
- Counting about 300 fertilized oocytes under an inverted microscope (Olympus, magnification x200) and transferring to 24-well microplates.
- Incubating at 24°C for 2 h in the dark.
- When the 2-4 cell stage was attained in the control treatment, a few drops of 37% buffered formalin to stop fertilization and preserve the developing embryos.
- Unfertilized oocytes were scored under an inverted microscope (Olympus, magnification x200) among 100 oocytes.

Note: When oocytes underwent their first cleavage within 120 min => stop exposure. Pear-shaped fertilized oocytes are easily recognizable among unfertilized oocytes which are round-shaped.

3. PROTOCOL FOR COMET ASSAY

Materials: ***Supplier (Catalogue Number):***

Normal Melting Agarose (NMA)	- HiMedia (RM273)
Low Melting Point Agarose (LMPA)	- Sigma (A9414)
Methanol	- Qualigens
Coverslips (No. 1, 24 x 60 mm)	- Blue Star
Microcentrifuge Tubes	- Tarsons (500010)
Micropipettor and Tips	- Tarsons
Microscope Slides, Conventional /	
Micro gel electrophoresis (MGE) slides	- Blue Label or Es Em Inc (NS 0001)
Coplin jars (opaque)	- Tarsons (480000)
Horizontal Gel Electrophoresis Apparatus	- GIBCO BRL, Life Technologies
Electrophoresis Power Supply	- Techno Source
Microscope Slide Tray (aluminum)	

Gamate recorvery and fertilization

The oocytes were fertilized by sperm –dense suspension to raise a ratio of 10 spermatozoa per 1 oocyte. Fifteen minutes after fertilization, embryo were counted and dispatched in each assay beaker at a final concentration of 50,000 fertilized oocytes per litter.

Dissociation of embryo cells

- Following 16h of exposure, the larvae were recovered by sieving at 40 mm and incubated in 1.0 mL of enzyme digestion (Dispase II) solution of 1%.
- The reaction was stopped by centrifugation for 10 min at 1000 rmp and 4°C.
- Cell viability was determined for each sample by a trypan-blue exclusion test at least equal to 75% for allowing the conduction of the comet assay.

REAGENTS

Solution stocks (conservation at 4°C)

- Tris-HCl 1M: 78.8 g Tris-HCl in 500 mL mQ water (purer than distilled water)
- EDTA 0.2M: 74.44 g EDTA in 1000 mL mQ water
- NaOH 10M: 400 g NaOH in 1000 mL mQ water

Ethanol absolute at -20°C

Solution of staining

- Ethidium Bromide (EtBr; 10X Stock - 20 µg/mL): add 10 mg in 50 mL H2O, store at room temperature. For 1X stock - mix 1 mL with 9 mL dH2O.
- Mother solution: 2 mg/ml in mQ water
- Work solution at 20 µg/ml: 10µl of the mother solution in 1 ml of mQ water

Solution of lysis

Tris-HCl 10mM, NaCl 2.5M, EDTA 100mM, triton X-100 1%, pH 10

- 14.61 g NaCl in 50 mL of the solution EDTA 0.2M
- Add 1mL Tris-HCl 1M
- Adjust pH 10 by NaOH 10M (about 2 ml)
- Add mQ water qsp 100 ml
- Extemporaneous (immediately) add 1 ml of Triton X-100

Note: 100ml of the solution of lysis per jar: 8 lames per jar

Solution of electrophoresis

NaOH 0.3M, EDTA 1mM pH >13

- 30ml of solution NaOH 10M
- Add 5ml EDTA 0.2M
- Check that the pH is 13
- Add mQ water to 1L

Note: 1L solution for small tank (12-15 lames). 2L solution for large tank (24 lames)

Solution of neutrolisation

Tris-HCl 0.4M pH 7.5

- 40 ml of Tris-HCl 1M
- Adjust pH at 7.5 by NaOH 10M (about 650 µL)
- Add mQ water to 100 mL

Note: 100ml solution of lysis per jar 8 lames/jar – 3 neutrolisation

SLIDE PREPARATIONS: FIRST LAYER OF AGAROSE

Preparation of Agarose solution 1%

- Dry 250 mL erlenmeyer
- Weigh 0.8 agarose in 800 mL of PBS (phosphate buffered saline)
- Raise the total mass of all
- Heat the mixture to microwaves in order to obtain a solution of NMP agarose

Note : Heat in small duration (10 seconds) to avoid any splash that are quickly becoming the gel on the edges of the beaker

- Once the clear solution obtained, weighed again and complete mQ water until the initial mass obtaining

Deposit first layer

- Complete 50-mL beaker in a preheated bath at 37^0C the solution NMP agarose
- Soak the slides in the beaker STARFROST
- Wipe the face that does not hang (back of the blade)
- Deposit slides into plateaus, covered with paper towel and covered with foil

Preparation of Agarose solution (LMP: Low melting point)

- Preparation is identical to that of the NMP agarose
- Make aliquots of 2 mL was stored at 4^0C

Preparation of cell suspension

- The isolated cell are extracted from oyster embryos
- After observing and counting under the microscope and it then performs a cell dilution to obtain a solution to 2.10^4 cells per 50 µL of gel
- It then takes 50 µL of the suspension are placed in an Eppendorf kept in ice

Preparation and filing of the second layer

- 6 Eppendorf containing 100 µL of the LMPA are prepared and placed in a water bath at 37^0C to keep the agarose solution
- Is added into each Eppendorf 50 µL of cell suspension after centrifugation
- 2 solutions are mixed by Up and Down realized with the dropper

Note: Always make sure to leave a third of the solution in the Eppendorf at 37^0C to keep a solution and not a gel. The LMPA is used pout just stay in liquid state at 37^0C and therefore does not damage cells

- Take 50 µL of cell suspension in the LMPA at 37^0C
- Make 2 deposits of 50 µL by blades
- Immediately deposit coatings in each of a lamella 18 * 18
- The slides are then placed at -4^0C for 5-7 min to solidify the gel

(The slides have been referenced in prior noting on the upper blade with a pencil the location, number of the oyster, the mission time and date)

Depositing a third layer

- Carefully remove the slides by dragging them with your fingertips
- 50 µL of LMPA deposit only at the second gel
- Put a coverslip
- Solidification of the gel -4^0C for 5 to 7 minutes

Cell lysis

(Note: All phases must be performed under conditions that limit exposure to light. Indeed, exposure to sunlight or from the neon lights can cause damage to DNA and Dausse our study)

- It removes the last strip and put on the gels were placed back to back Blades (2 by 2) in a jar containing lysis solution
- Duration of lysis: 1 hour in the fridge -4^0C

Alkaline treatment

- Fill the tank of the electrophoresis solution electrophoresis. Adjust the generator under the conditions of electrophoresis (25V, 15 min, i < 400 mA). Launch blank to verify that the current is about 300 mA. Constraints in the case fill the tank a little more.
- Place slides in the following sense on the bottom of the tank
- Duration of alkaline treatment: 20 min without plugging the generator

Processing time and alkaline electrophoresis is determined by multiple tests

- Indeed, the longer alkali treatment, the longer the migration is long and the greater the comets
- But if time becomes too long, so we end up with too large comets in the witnesses

- And in the case, or the time is too short, there are not enough donations migration and loss of some sensitivity of the study

Electrophoresis

- Allow the strips to the bottom of the electrophoresis tank
- Verify that the tank is level
- Duration of electrophoresis: 15 min

Neutralisation

- Turn off the generator
- Remove slides from the tank. Attention, they can be glued to the bottom of the tank so the drag
- Wipe the back of the blades
- Store blades 2 by 2 back to back in a jar filled with the solution neutralisation
- Allow 5 minutes in the fridge to-4^0C
- Empty the tank and refill the jar (x2)

Drying

- Empty the jars containing the solution of neutralisation
- Wipe the back of the blades
- Fill the jars of absolute ethanol stored at -20^0C
- Allow 20 minutes in the refrigerator at -4^0C
- Remove slide from the jar and place on paper kitchen roll overnight

4. PROTOCOL OF RNA EXTRACTION

(Absolutely RNA Miniprep kit, Stratagene, 50 preparations)

all the materials used must be RNase-Free and Autocalves before use

careful not to touch the inside of the microtubes and their cap even with gloves

Before the start of handling large and wash put the bench in 70% EtOH and the RNase AWAY

Preparation of the extraction kit

1. **Solution RNase-FREE DNase I (new kit)**
    - Lyophilized DNase + 2x145 µL DNase Reconsitution Buffer (Short spin) + Up / Down against walls + bottle return
    - Identify the tube, store at -20°C
2. **Solution High-Salt Wash Buffer (new Kit)**
    - High-salt wash buffer + 16 mL absoluted ethanol
    - Check the label, store at room T°C
3. **Solution Low-Salt Wash Buffer (new Kit)**
    - Low-salt wash buffer + 68 mL absoluted ethanol
    - Check the label, store at room T°C
4. **75% ethanol solution of RNase-FREE (under manipulation.)**: *In 50 mL sterile Falcon tube:*
    - 30 mL absoluted ethanol + 10 mL DEPC water
    - Store at – 20°C
5. **Mix DNase digestion buffer - RNase-FREE DNase I (under manipulation.)**: *In Microtube 1.5 mL*

QSP 12 samples: 60 µL RNase-FREE DNase I + 600 µL DNase Digestion Buffer (short spin if necessary) + Up/Down

QSP 20 samples: 100 µL RNase-FREE DNase I + 1000 µL DNase Digestion Buffer (short spin if necessary) + Up/Down

PROTOCOL FOR EXTRACTION OF TOTAL RNA

Prepare: A series of Microtubes 1.5 mL (not supplied) and a series of Microtubes with blue column (kit)

1. **Grinding and tissue lysis (ice)**

In a series of Microtubes 1.5 mL

- 4.2 µL of β-mercaptoethanol (Toxic) + 600 µL of Lysis buffer + biological material (20 -40 mg of tissue) Note: working at area of fume cupboard
- 2-3 reversal of the tube (tissue impregnation)
- Grind the tissue with a micro-potte (1 potte/ 1 sample) _ ground material homogeneous / viscous, free pieces (Note potte rinsed by ethanol and autoclave before using)
- Transfer 600 µL of ground material _ filtration blue columns (blue column put inside the Microtube 1.5 mL)
- Centrifugation of Microtubes + blue columns at Vmax/13500 rmp for 5 min (If clogging additional 2-3 min)

Prepare: 2 series of Microtubes 1.5 mL (no supplied) and activating 2 baths at 37°C and 60°C

- Discard blue columns (throw away blue column)
- Transfer filtrates of sample solution into a new series of 1.5 mL microtubes

2. **Protein extraction and purification first**

Sorbonne in hoods with cone filter

- Add 600 µL of Phenol-Chloroforme alcool isoamylique (25:24:1) Note working under fume cupboard and using orange glove.
- Mix well for 3-10 sec
- Centrifugation by Microtubes Vmax for 5 min, at 13500 rmp

Prepare: Ethanol solution of 75% RNase-FREE and a series of Microtubes with white column (get it from kit)

- Take supernatant (600 µL of sample by P200) to 1.5 mL Microtubes (1 new series of Microtubes and make sure to take any oil)

3. **Separation of RNA and protein**

- Phases taken from + 600 µL Ethanol 75%
- Vortex 5 sec (quik centrifugation by Vortex)
- Transferring 600 µL mix solution into affinity columns white (RNA and DNA capture, facilitated by EtOH)

- Centrifugation of miroctubes + white columns at Vmax/13500 rmp for 1 min
- Throw elutriate in Microtube (keep white column) + replace columns on tube + transferring rest of mix sample solution into white columns
- Centrifugation of miroctubes + white columns at Vmax/13500 rmp for 1 min
- Throw elutriate + replace columns on tube + 600 µL Low-Salt Wash Buffer
- Centrifugation of miroctubes + white columns at Vmax/13500 rmp for 1 min
- Throw elutriate in Microtube (keep white column) + replace columns into new Microtubes
- Centrifugation of miroctubes + white columns at Vmax/13500 rmp for 2 min (drying columns)
- Throw elutriate in Microtube (keep white column) + replace columns on tube (Nevertheless, DNA and RNA trapped on columns)

  Prepare: Mix DNase Digestion Buffer – Rnase-FREE DNase I: ***50 µL DNase Digestion Buffer + 5 µL RNase-Freee DNase I which to be prepared before and to be stored at -20°C.***

4. Purification of RNA

- White columns + 54 µL Mix DNase Digestion Buffer – RNase-FREE DNase I (drop at the center columns)
- Incubate in Bain-Marie at 37°C for at least 15 min (switch on Bain-Marie 37°C before 30 min)
- *Prepare: Put Elution Buffer in Bain-Marie at 60°C and possibly, prepare RT* (switch on Bain-Marie 60°C after 37°C switch on about 5-10 min)
- Add 600 µL High-Salt Wash Buffer into the thite columns (causes DNA fragments and DNase)
- Centrifugation of miroctubes + white columns at Vmax/13500 rmp for 1 min
- Throw elutriate in Microtubes (keep white colums) + replace columns on new Microtube tubes
- Add 300 µL Low Salt Wash Buffer into white column (recover normal salinity)
- Centrifugation of miroctubes + white columns at Vmax/13500 rmp for 2 min (drying column + return normal salinity)

  Prepare: 1 new series of collected Microtubes 1.5 mL (kit) with identity samples)

- Put white column into a new series of collected Microtubes + 30 µL hot Elution Buffer (at the centre of column) Note: Elution Buffer put in Bain-Marie 60°C before
- Incubate at room T°C for 2 min
- Centrifugation of miroctubes + white columns at Vmax/13500 rmp for 1 min
- Discard columns (keep microtube with sample solution) + sample solution to be stored -20 ° C to REVERSE TRANSCRIPTION or -80 ° C (long-term storage)

REVERSE TRANSCRIPTION OF RNA INTO DNAc

(AffinityScript Multiple Temperature DNAc synthesis kit (Stratagene, 50 preparations)

all the materials used must be Rnase-Free and Autocalves before use

careful not to touch the inside of the microtubes and their cap even with gloves

Before the start of handling large and wash put the bench in 70% EtOH and the RNase AWAY

Kit is stored at -20°C

PROTOCOL

Prepare: 1 series of Microtubes 0.3 mL (not supplied) with identity samples (label)

In each 0.3 mL Microtube: 1 µL Oligo(dT) primers + 1 µL Random primers + 0.8 µL dNTPs + 2 µL Affinity Script RT Buffer + 14 µL RNA (sampe solution which to be extracted total RNA before) + Up/Down to mix all solutions very well.

1. **Linearisation of RNA (65°C) and fixation of primers (45°C)**

- Place of sample (incubation) in themorcycle at 65°C for 5 min by EPPENDORF: *Patrice/RT/rt65/start/Tubes/Start.* Finish the cycle the temperature decreasing at 45°C (hybridization of the primers)
- Add into each microtube:
    - 0.5 µL RNase block
    - 1 µL RT (Reserve Transcription)

Note: add inside the sample solution and mix solution by Up/Down several times by new cone

2. **Reverse Transcription of RNA into DNAc (42°C)**

- Incubate microtubes in thermocycle at 42°C for 60 min by EPPENDORF: *Patrice/RT/rt42/OK/Tubes/Start*
- Store DNAc solution at -20°C or -80°C for long storage

AMPLIFICATION BY STRATAGENE

all the materials used must be Rnase-Free and Autocalves before use

careful not to touch the inside of the microtubes and their cap even with gloves

Before the start of handling large and wash put the bench in 70% EtOH and the RNase AWAY

KIT: Brillant III Ultra Fast SYBR Green QPCR Master Mix (Agilent, Strategene)

1. **Materials**
   - Strategene
   - Personnel cones for DNA
   - Kit (Tampon [Tp] 2x Brillant III)+ plates + caps
   - Primers for interest genes
   - Ultra pure H2O
2. **Attention**
   - Clean the bench with ethanol
   - Thaw primers
   - Tp 2x Brillant III kit to be stored at -20°C right way after opening
   - Centrifugation of primer tubes before utilising
   - Prepare 96-well microplate
   - Attention with pipets bacause working with microvolumes
   - Between each run: keep the mixes primers, reagents, and DNAc sample solution at 4°C
   - The lamp of the unit must be heated for 20 min before the beginning of the first run
3. **Protocol**
   - In order to prepare one mix primer for whole day, it depends on the number of samples (or number of runs). Each run will take about 2 hours.
   - Prepare **MIX PRIMER** (stock solution primer-S and primer-AS = 100 µM): prepare mix primers every day.
     - For 48 samples, should prepare final volumn of 50 µL: 1µL primer-S + 1µL primer-AS + 48 µL ultra pure H2O. And then UP/DOWN and to be stored at 4°C for using whole day

    - For 98 samples, should prepare final volumn of 100 µL: 2µL primer-S + 2µL primer-AS + 96 µL ultra pure H2O. And then UP/DOWN and to be stored at 4°C for using whole day
- Prepare **MIX REACTION** solution (prepare everyday)
    - For 01 microplate (prepare in microtube 1.5ml): 1000 µL Tp 2x + 700 mL ultra pure H2O. And then UP/DOWN
- Deposit into each well of 96-well microplate:
    - 17 µL of mix reaction
    - 2 µL of mix primer
    - 1 µL DNAc sample solution (Note, change cone for each well to avoid mix DNA for wells)

Note: Check all wells with the same volumn

- Cover microplate by cover lips
- Centrifugation for microplate at 3000 rmp for 30 sec
- Place the microplate in STRATEGENE unit (about 2 hours)
- Opening sofware for PCR
    - Openning Mx pro and Selection of SYBR Green (with dissociation curve)
    - Tab: *Plate Setup/Import/2011/"Name"/Place of reference*
    - Tab: *Thermal Profil/Import/2011/"Name"/ Reference Protocol*
    - Note: select option of "Turn off lamp at the end"
    - Start run/ Enter the name of run
        - 10 min at 95°C for ACTIVATION of Taq
        - 30 sec at 95°C for DENATURATION
        - 30 sec between 55°C and 60°C for HYBRIDISATION of PRIMERS
        - 30 sec at 72°C for ELONGATION
        - Curve of fusion from 60°C to 95°C

LIST OF REFERENCE PRIMERS COULD PLACE IN ONE 96-WELL MICROPLATE

	1	2	3	4	5	6	7	8	9	10	11	12
A	Act	Gpx	Gst	Mxr	Cat	Cyp	Cox	Mt1	Mt2	SodMt	p53	12s
B	Act	Gpx	Gst	Mxr	Cat	Cyp	Cox	Mt1	Mt2	SodMt	p53	12s
C	Act	Gpx	Gst	Mxr	Cat	Cyp	Cox	Mt1	Mt2	SodMt	p53	12s
D	Act	Gpx	Gst	Mxr	Cat	Cyp	Cox	Mt1	Mt2	SodMt	p53	12s
E	Act	Gpx	Gst	Mxr	Cat	Cyp	Cox	Mt1	Mt2	SodMt	p53	12s
F	Act	Gpx	Gst	Mxr	Cat	Cyp	Cox	Mt1	Mt2	SodMt	p53	12s
G	Act	Gpx	Gst	Mxr	Cat	Cyp	Cox	Mt1	Mt2	SodMt	p53	12s
H	Act	Gpx	Gst	Mxr	Cat	Cyp	Cox	Mt1	Mt2	SodMt	p53	Blanc

Row: [1....12] different referent genes of one sample

Column: [A....H] the same gene of different samples

EXAMPLES OF INDUCTION FACTORS OF GENE EXPRESSIONS

Mitochondrial metabolism	coxl 12s
Metabolisation/Detoxication	mt1 mt2
Oxydative stress defense	sodmt cat gpx
Cell cycle arrest/apotosis	p53
Biotransformation	gst cyp1a
Multixenobiotic resistence	mxr

5. PREPARATION SEDIMENT CONTACTS

MATERIALS

- Glass bottles of 500 mL
- Variomag
- Electric pipette
- 50ml glass tube
- Falcon 50ml
- Centrifugation
- Filtered seawater
- Sediments fees levied

PROTOCOL

It is asked to prepare five concentrations of sediment contacts

5g/L	**2.5g/L**	**1 g/L**	**0.5g/L**	**0 g/L**

- Remove the pore water of a falcon (50 mL) filled with sediment by centrifugation at 4000 rpm for 10 minutes
- Raising the overlying water
- Weigh the masses needed to establish the concentrations for half a liter of filtered seawater
- Add and stir the masses to Variomag at 700 rpm
- Then take 30 mL of each concentration using a pipette electric and pour into glass tubes of 50 mL

Caution is always important to take the mother height relative to the surface. It is therefore necessary to set a mark in front of the graduation of the graduated pipette and place the scale on the surface always. For reading the results of sediment contacts, remove a volume of the bottle after you have correctly allocated stirred. The volume of the order of a few ml (2 mL) is up wells of microplates. In the case where the result is not very readable, add a few µl Rose Bengal. This will allow the unique colouring of organic cells.

6. PROTOCOL OF AMMONIUM ANALYSIS

(Koroleff, 1969; Grasshoff and Johannsen, 1972)

In slightly basic medium, the ammonium form monochloramine with one dichloroisocyanuric acid. The latter reacts with the phenol to form a compound of blue Indo-phenol whose intensity is proportional to the concentration of ammonium. The reaction is accelerated by nitroprusside. This measurement has been applied for the determination of low concentrations. The percentage of error with this method is less than 5%.

Reagents

R1: Solution of phenol nitroprusside: Dissolve 3.5 g cold phenol and 40 mg of sodium nitroprusside, and then made volumn up to 100 mL by MilliQ water.

R2: dichloroisocyanurate solution: Dissolve 28 g of cold trisodium citrate, 500 mg dichloroisocyanurate and 2.2 g sodium (for seawater) and then made volumn up to 100 mL by MilliQ water. This reagent keeps cool for 1 to 2 months.

Note: The proportions shown here are those given for the analysis of sea water. To fresh water, the amount of sodium should be reduced to 1.4 g per 100 mL of reagent.

Dosage

A standard range must be performed beforehand. Add to cuvette of 2 mL as below:

- 60 µL of R1
- 2 mL of water sample
- 60 µL of R2

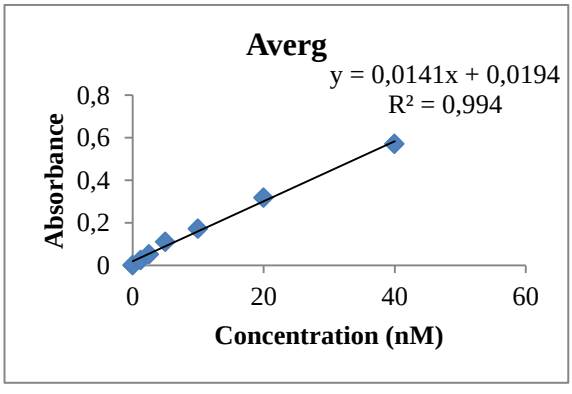
Stir and put in the dark (covered with aluminum foil) under a fumehood overnight.

Absorbance is read with λ = 630 by spectrophotomater.

The concentration is determined using a calibration curve prepared from standard solutions which were measured absorbance values. Low nutrient seawater (nutrients less than 1 micromole per litre – http:/www.osil.co.uk) is used to dilute the standard solutions.

Standard solution

Conc. (nM)	Abs1
0	0
1.25	0.0245
2.5	0.0515
5	0.1105
10	0.1715
20	0.317
40	0.5705



7. PROTOCOL OF PARTICULATE ORGANIC CARBON ANALYSIS

- Sediments were dried in oven at 42°C for 24h
- Then grill to homogenize sediment.
- Weight about 100 mg of sediment for each sample and store in crucible.
- Crucibles were taken place in the heater plate at 60°C under the fume hood.
- Few drops of HCl 2N was added into sediment of each crucible to decarbonate the samples because carbonates would effervesce with acids.

Note: If there is still effervescence in sediment, it was added more few drop of HCl 2N and then drying the sediment at the heater plate under the fume hood.

- Preparation of 5 blank and 5 standard samples is to calibrate measurement system before performing the analysis of particulate organic carbon.
- For bank samples, each sample contained 100 mg accelerator of Iron Ship and then add 100 mg Lecosel I.
- For standard samples, beside the accelerator of Iron Ship and Lecosel I (as blank sample), each standard one was added 1 piece of carbon and sulfur in steel.
- Each sample was added 100 mg accelerator of Iron Ship and 100 mg Lecosel I.
- Organic carbon in blank and standard samples as well as sediment samples was analysed using LECO CS 125 equipment and an image analysis system (CS 200).

LECO CS 125 equipment