Currently used pesticides and their mixtures : what are the risks to non-target aquatic organisms? Laboratory and in situ approaches.

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Currently used pesticides and their mixtures: what are the risks to non-target aquatic organisms?
Laboratory and *in situ* approaches.

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Ph.D. Dissertation

Masaryk University, Faculty of Science

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Par Eliška KUCHOVSKÁ

PESTICIDES LARGEMENT UTILISÉS ET LEURS MELANGES : QUELS RISQUES POUR LES ORGANISMES AQUATIQUES NON-CIBLES ?

Approche en laboratoire et *in situ*

Sous la direction de : Patrice GONZALEZ
co-directeur : Luděk BLÁHA
co-encadrement : Bénédicte Morin

Soutenue le 17 décembre 2020

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Title: Currently used pesticides and their mixtures: what are the risks to non-target aquatic organisms? Laboratory and *in situ* approaches.

Abstract:

Pesticides have enabled humankind to protect its crops from pests, intensifying thus the crop yields to sustain the growing population. However, pesticides often end up in aquatic water bodies, e.g. via field runoff, where they may harm non-target organisms. The environmental concentrations of pesticides are often considered safe for aquatic ecosystems although they might induce sublethal changes in exposed organisms. Moreover, the organisms are generally not dealing with only one pesticide issued from a nearby field but with a complex mixture of various chemical compounds, interacting amongst themselves, and creating a toxic cocktail with unknown and hardly predictable impacts. These compounds, each with different environmental fate, eventually degrade and form more or less toxic and persistent metabolites aggravating the complexity of the mixtures.

This dissertation thesis summarizes the state-of-the-art in pesticide mixture toxicity research and is composed of five research articles dealing with sublethal effects of selected pesticides on non-target aquatic species. Vulnerable embryo-larval stages of two model organisms: freshwater zebrafish (*Danio rerio*) and euryhaline bivalve Pacific oyster (*Magallana gigas*) were used to assess the sublethal toxicity of especially environmental concentrations (detected in selected European water bodies) of commonly used herbicide S-metolachlor with its two metabolites metolachlor oxanilic acid and metolachlor ethanesulfonic acid, insecticide imidacloprid, and fungicide propiconazole, alone and in a mixture. A complementary *in situ* approach was carried out to evaluate a real impact on early-life stages of the Pacific oyster in Arcachon Bay in France, a final recipient of various substances including pesticides from respective watersheds.

First, zebrafish embryo-larval stages were observed to be highly sensitive to environmentally relevant concentrations of propiconazole and to a lesser extent also to imidacloprid. In contrast, S-metolachlor and its metabolites had almost no effect on their development, neurobehavioral functions, or gene expression except for altered genes implicated in the thyroid system. A mixture of these compounds exhibited a concentration addition effect on zebrafish development. These observations imply that the development of freshwater fish may be at risk with current agricultural practice.

Second, a study with Pacific oyster embryos and larvae revealed very low toxicity of propiconazole and imidacloprid on their development and locomotion patterns. Few effects caused by these compounds were observed at the molecular level, as well as the effects caused by the mixture. The environmental concentration of the mixture induced developmental malformations in oyster larvae, however, those exposed *in situ* in Arcachon Bay did not show higher proportions of abnormal larvae suggesting that the water quality of Arcachon Bay is sufficient for oyster development. Nevertheless, oyster larvae exposed in the inner part of Arcachon Bay showed different gene expression levels than larvae from the reference site located near the ocean entrance, which may indicate consequences of a potential long-term impact.

These results documented that embryo-larval stages of zebrafish and Pacific oysters are relevant tools for the assessment of low concentrations of pesticides and pesticides in a mixture, and that laboratory studies complemented with field research are useful for (eco)toxicity assessment and of high ecological relevance.

Keywords: Ecotoxicity**,** Embryo-larval stages, Pacific oyster, Pesticide, Sublethal effect, Zebrafish

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Titre : Pesticides largement utilisés et leurs mélanges : quels risques pour les organismes aquatiques non-cibles ? Approche en laboratoire et *in situ*.

Résumé:

Les pesticides ont pour rôle de protéger les cultures des espèces nuisibles permettant ainsi d'intensifier le rendement agricole pour nourrir une population toujours en augmentation. Néanmoins, les pesticides se retrouvent souvent dans le réseau aquatique, par exemple via le ruissellement, où ils peuvent nuire aux organismes non-cibles. Les concentrations environnementales des pesticides sont souvent considérées sans risque pour les écosystèmes aquatiques, mais elles peuvent cependant induire des effets sublétaux dans les organismes exposés. De plus, les organismes ne font généralement pas face à un seul pesticide provenant d'un champ voisin, mais à un mélange complexe de différents composés chimiques qui interagissent entre eux pour former un cocktail potentiellement toxique avec des impacts inconnus et difficilement prévisibles. Ces composés, peuvent se dégrader au fil du temps et forment des métabolites plus au moins toxiques et persistants qui aggravent encore la complexité des mélanges.

Cette thèse s'intéresse à la toxicité de pesticides seuls, en mélange ou en nanoformulation sur des organismes aquatiques non-cibles. Les stades de vie précoces vulnérables de deux organismes modèles : le poisson zèbre (*Danio rerio*) d'eau douce et un bivalve euryhalin l'huître creuse (*Magallana gigas*) ont été utilisés afin d'évaluer les effets sublétauxe de concentrations environnementales (détectées dans les cours d'eau européens) de différents pesticides couramment utilisés dont l'herbicide S-métolachlore avec ses deux métabolites acides oxanilique et sulfonique du métolachlore, l'insecticide imidaclopride et le fongicide propiconazole. En complément, une approche *in situ* a été développée pour évaluer les effets toxiques sur les stades embryo-larvaires de l'huître creuse associés à la qualité de l'eau du Bassin d'Arcachon, réceptacle final de différentes substances provenant des bassins versants.

Les résultats indiquent une grande sensibilité des embryons et larves de poisson zèbre aux concentrations environnementales de propiconazole et à un degré moindre de l'imidaclopride. Au contraire, le S-métolachlore et ses métabolites ne présentent que peu d'effet sur le développement, les fonctions neurocomportementales et l'expression des gènes à l'exception des gènes impliqués dans le système thyroïdien. Ces pesticides en mélange semblent se comporter selon un modèle d'addition des concentrations si l'on considère le développement du poisson zèbre. Ces observations sont en lien avec un risque des pratiques agricoles actuelles.

Les résultats obtenus lors de ce travail montrent une faible toxicité du propiconazole et de l'imidaclopride sur le développement et le comportement des embryons et larves de l'huître creuse. Quelques effets causés par ces composés seuls ou en mélange sont observés au niveau moléculaire. La concentration environnementale du mélange a induit les malformations larvaires, néanmoins, les embryons d'huître encagés dans le Bassin d'Arcachon ne présentent pas de malformations quel que soit le site d'exposition, ce qui suggère une qualité suffisante de l'eau du Bassin pour le développement de l'huître creuse. Cependant, des différences au niveau de l'expression des gènes sont observées pour les embryons exposés dans la partie interne du bassin d'Arcachon suggérant des conséquences potentielles sur le long terme.

Ces résultats indiquent que les stades embryo-larvaires du poisson zèbre et de l'huître creuse sont des outils pertinents pour l'évaluation des faibles concentrations de pesticides seuls ou en mélange. De plus, la mise en œuvre d'expérimentations *in situ* en complément des approches de laboratoire s'avère utile dans une démarche d'évaluation des risques environnementaux.

Mots-clés : Ecotoxicité, Effet sublétal, Huître creuse, Pesticide, Poisson zèbre, Stades embryo-larvaires

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Název: Aktuálně používané pesticidy a jejich směsi: jaká představují rizika pro necílové vodní organismy? Laboratorní a *in situ* studie.

Abstrakt:

Pesticidy umožnily lidstvu chránit úrodu proti škůdcům, čímž zintenzivnily výnosy z úrody pro uživení stále rostoucí lidské populace. Pesticidy však často končí v povrchových vodách například splachem z polí, kde mohou ublížit necílovým vodním organismům. Environmentální koncentrace pesticidů jsou často považovány za bezpečné pro vodní ekosystémy, ačkoliv mohou způsobovat subletální změny v exponovaných organismech. Navíc se většinou organismy nemusí vyrovnávat jen s jedním pesticidem spláchnutým z vedlejšího pole, ale s komplexní směsí různých chemických látek, které mezi sebou interagují a tvoří chemický koktejl s neznámými a těžko předvídatelnými účinky.

Tato disertační práce shrnuje současné znalosti ve vědě o toxicitě směsí pesticidů a je složena z pěti vědeckých publikací, které se věnují problematice subletálního vlivu vybraných pesticidů na necílové vodní organismy. Zranitelná embryo-larvální stádia dvou modelových organismů: sladkovodní ryby dania pruhovaného (*Danio rerio*) a v brakických vodách žijícího mlže ústřice velké (*Magallana gigas*) byly použity pro posouzení subletální toxicity především environmentálních koncentrací, naměřených v evropských vodách, běžně používaného herbicidu S-metolachloru a jeho dvou metabolitů (metolachlor oxanilic acid, metolachlor ethanesulfonic acid), insekticidu imidaklopridu a fungicidu propikonazolu, samostatně a ve směsi. Doplňující *in situ* terénní výzkum posuzující reálný dopad na raná vývojová stádia ústřice velké byl proveden v Arcachonském zálivu ve Francii, který představuje finálního příjemce rozličných látek včetně pesticidů přinášených z odpovídajících povodí.

Zaprvé, embryo-larvální stádia danio pruhovaného byly vysoce citlivé na environmentálně relevantní koncentrace propikonazolu a v menší míře též imidaklopridu. Herbicid a jeho degradační produkty naopak neměly téměř žádný efekt na jejich vývoj, neurobehaviorální funkce a genovou expresi s výjimkou genů implikovaných v thyroidním systému. Směs zmiňovaných látek měla koncentračně adiční efekt na vývoj dania pruhovaného. Tato pozorování naznačují, že vývoj sladkovodních ryb může být ohrožen současnými zemědělskými praktikami.

Zadruhé, studie s embryi a larvami ústřice velké odhalila nízkou toxicitu propikonazolu a imidaklopridu na jejich vývoj a pohybové vzorce. Tyto látky způsobily pár efektů na molekulární úrovni, stejně jako testovaná směs. Environmentální koncentrace směsi přivodila

larvám ústřic vývojové malformace, které však nebyly pozorovány v larvách exponovaných přímo v Arcachonském zálivu, což ukazuje na dostačující kvalitu vody v zálivu pro úspěšný vývoj ústřic. Nicméně, larvy ústřic exponované ve vnitřní části zálivu měly rozdílné genové exprese od larev exponovaných na referenčním stanovišti, které bylo vybráno blíže ústí zálivu do oceánu, což může značit potenciální následky s dlouhodobým dopadem.

Výsledky obdržené při tomto výzkumu dokazují, že embryo-larvální stádia dania pruhovaného a ústřice velké jsou relevantními nástroji pro posuzování vlivu toxicity nízkých koncentrací pesticidů, jakož i pesticidů ve směsích a že laboratorní studie doplněné terénním výzkumem jsou užitečné pro (eko)toxikologické hodnocení a mají vysokou ekologickou relevanci.

Klíčová slova: Danio pruhované, Ekotoxicita, Embryolarvální stadia, *In situ*, Subletální efekt, Pesticid, Ústřice velká

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RÉSUMÉ

La pollution des écosystèmes est devenue un des enjeux prioritaires du monde actuel, impactant non seulement l'espèce humaine, mais également tous les organismes vivants et leurs habitats. Une des sources de cette pollution est l'agriculture qui utilise des pesticides afin de lutter contre les organismes nuisibles et d'augmenter le rendement agricole, mais aussi de protéger les stocks des entrepôts et limiter la propagation des maladies aux fermes. Après application des pesticides, le ruissellement, le lessivage peuvent se produire et les écosystèmes aquatiques peuvent ainsi devenir le récepteur final de ces molécules, qui peuvent nuire aux organismes non-cibles (de Souza et al., 2020) comme les poissons (Altenhofen et al., 2017; Vignet et al., 2019; Weeks Santos et al., 2019), les invertébrés (Bechmann et al., 2020; Rösch et al., 2017; Velisek et al., 2019), les plantes aquatiques (Demailly et al., 2019), etc. Les organismes dans leur stade de vie précoce sont les plus vulnérables à ces contaminations.

Compte tenu de la grande diversité des molécules de pesticides, les effets toxiques sont variés et difficilement généralisable. De plus, même si l'impact de certains pesticides pris de manière isolée est étudié et connu, les substances interagissent entre elles dans le milieu aquatique, formant ainsi des mélanges complexes dont les effets sont inconnus, difficilement prévisibles et dépendant pour leur stabilité et biodisponibilité de facteurs environnementaux variés comme la lumière, la température, le pH, …. Qui plus est, ces substances se dégradent au fil de temps et forment des métabolites, qui peuvent être même plus toxiques que le produit parent. Ces mélanges complexes peuvent affecter les organismes par des mécanismes différents des pesticides individuels avec un concept appelé « action indépendante ». En effet, le mélange peut se comporter selon plusieurs modèles : l'addition des concentrations, le synergisme ou l'antagonisme (Cedergreen, 2014). Le cocktail de pesticides final (qui peut être aussi en interaction avec d'autres polluants environnementaux) peut ainsi être plus toxique sur la communauté des organismes aquatiques. C'est pourquoi il est important d'apporter des connaissances nouvelles permettant de mesurer les risques inhérents à l'utilisation durable de pesticides tout en gardant l'assurance de pouvoir produire des ressources alimentaires en quantité suffisante pour la population humaine.

C'est dans ce contexte environnemental que s'inscrit cette thèse qui vise à contribuer aux connaissances des effets sublétaux de pesticides couramment utilisés, individuels ou en mélange, sur les stades de vie précoces de deux organismes modèles : le poisson zèbre d'eau douce *Danio rerio* et un bivalve euryhaline l'huître creuse *Magallana gigas*. Ce travail est principalement focalisé sur l'exploration des effets biologiques à des doses environnementales

de cinq substances i.e. l'herbicide S-métolachlore avec ses deux métabolites (MOA et MESA), l'insecticide imidaclopride et le fongicide propiconazole. Les concentrations des pesticides d'intérêt sont basées sur les concentrations retrouvées sur notre lieu d'étude le Bassin d'Arcachon et les rivières en République tchèque. Ce travail a été complété par une expérimentation sur le terrain : l'encagement des larves d'huître *in situ*.

Les hypothèses évoquées de ce travail sont les suivantes :

- Les pesticides d'intérêt induisent des effets mesurables même aux faibles concentrations, particulièrement au niveau biochimique/physiologique sur les stades précoces de développement de l'huître creuse et du poisson zèbre
- Les pesticides d'intérêt en mélange ont une toxicité plus importante et peuvent induire des effets qui ne sont pas prévisibles par les composés pris individuellement
- Le niveau de malformations larvaires observées au laboratoire après exposition à un mélange représentatif des pesticides présents dans le bassin d'Arcachon est similaire à celui observé après encagement des organismes *in situ*. La qualité de l'eau dans la partie interne du Bassin d'Arcachon est moins propice à un développement larvaire normal.

Deux tests embryo-larvaires normalisés ont été utilisés au cours de cette thèse : un pour les stades précoces de développement de l'huître avec la norme AFNOR (NF ISO 17244, 2015) et un autre pour le poisson zèbre suivant les recommandations de l'OCDE (OECD, 2013a).

Des poissons zèbre adultes sont maintenus dans les aquariums aux laboratoires RECETOX et sont utilisés afin d'obtenir les embryons qui seront exposés à 3 hpf (heures post fécondation) aux pesticides d'intérêt à 26 °C. La durée d'exposition dépend de l'analyse effectuée i.e. 5 dpf (jours post fécondation) pour l'analyse des malformations développementales, locomotion, l'expression des gènes ; 3 dpf pour l'analyse du battement de cœur, 22-23 hpf pour l'analyse des mouvements spontanés de la queue.

Des géniteurs maturés artificiellement d'huître creuse sont reçus de l'écloserie Guernesey Sea Farm (Grand Bretagne) ou de France Naissain (Bouin, France). Cinq couples d'huîtres matures sont stimulés par chocs thermiques afin d'obtenir des embryons qui seront exposés aux pesticides d'intérêt à 24 °C. La durée d'exposition est plus courte pour les embryons d'huître que pour les embryons de poisson. L'analyse du comportement est réalisée après 24 h d'exposition, les malformations après 30 h et l'expression des gènes à 42 h. Des larves après fécondation ont été exposées *in situ* avec des dispositifs d'encagement dans le Bassin

d'Arcachon pendant deux jours sur trois sites différents : Grand Banc en tant que site de référence et Les Jacquets et Comprian en tant que sites d'études.

Différents biomarqueurs d'effet sublétaux ont été choisis pour répondre aux différentes hypothèses et sont au cœur de ce travail : malformations larvaires, comportement et expression des gènes. Malformations des larves d'huîtres incluent les malformations du manteau, de la coquille et les arrêts de développement. Les larves de poisson zèbre sont des organismes plus complexes chez lesquelles on retrouve par exemple des malformations crâniofaciales, malformations de la squelette, œdèmes, malabsorption du sac vitellin, vessie natatoire non gonflée, etc. Des larves malformées sont en général désavantagées dans la nature, plus susceptibles de devenir une proie et leur fonctions biologiques peuvent être également affectées.

Les autres types de biomarqueurs analysés chez les larves de poisson zèbre sont le succès d'éclosion, la fréquence cardiaque et des biomarqueurs de comportement qui incluent l'activité natatoire des larves exposées successivement à des périodes de lumière et d'obscurité et une analyse des fréquences de mouvements spontanés de la queue de l'embryon. Les biomarqueurs de comportement représentent des réponses neurotoxiques au polluant et ont été mesurés également chez les larves d'huîtres en évaluant les trajectoires et les vitesses de nage.

L'expression des gènes via qPCR a également été réalisée pour évaluer les changements des transcriptions des gènes qui reflètent la quantité de protéines codées par ces gènes. Les gènes impliqués dans plusieurs fonctions ont été sélectionnés : métabolisme mitochondrial, régulation du cycle cellulaire et de l'apoptose, défense contre le stress oxydant, détoxification, biotransformation, apoptose, réparation de l'ADN, arrêt de la croissance et dommage à l'ADN, métabolisme et voie de signalisation thyroïdiens et voie de signalisation de l'acide rétinoïque.

Les résultats obtenus au cours de cette thèse ont permis d'évaluer les effets des pesticides d'intérêt sur les stades embryo-larvaires d'organismes aquatiques. Premièrement, nous nous sommes posé la question de la toxicité des concentrations environnementales des pesticides d'intérêt (c.à.d. jusqu'à 1 µg/L de S-métolachlore, ses deux métabolites et imidaclopride et jusqu'à 0,25 µg/L de propiconazole) particulièrement au niveau biochimique/physiologique. En effet, les larves de poisson zèbre se sont révélées très sensitives aux concentrations environnementales du fongicide propiconazole. Une concentration de 0,01 µg/L a notamment induit l'augmentation de la fréquence cardiaque ainsi que des mouvements spontanés de la queue. De plus, la distance parcourue par les larves à une concentration de 0,25 µg/L est significativement plus élevée que les larves non exposées et suggère un effet neurotoxique

impliqué. Des effets moins prononcés aux concentrations environnementales d'imidaclopride ont été également observés chez les larves. Au contraire, l'herbicide S-métolachlore et ses deux métabolites n'ont que peu d'effet sur le développement des larves de poisson zèbre à l'exception d'une diminution des mouvements spontanés et l'induction des gènes impliqués dans le système thyroïdien. A la différence des larves de poissons, les larves d'huîtres se sont révélées très sensitives aux concentrations environnementales du S-métolachlore et de ses deux métabolites MOA et MESA qui induisent des malformations à partir des concentrations 0,1 ; 1 et 0,1 µg/L, respectivement. Les concentrations environnementales d'imidaclopride et de propiconazole n'induisent pas de malformation, seul le propiconazole a un faible effet sur les trajectoires de nage des larves d'huîtres. Les deux pesticides ont néanmoins modifié l'expression de certains gènes, particulièrement ceux impliqués dans la défense contre le stress oxydant, le métabolisme mitochondrial et les métallothionéines.

Notre deuxième hypothèse était que les pesticides en mélange ont une toxicité plus importante et peuvent induire des effets qui ne sont pas prévisibles par les composés pris individuellement. Dans nos travaux, le mélange de pesticides a eu l'effet prévisible de l'addition des concentrations sur les larves de poisson zèbre avec des effets sur les mouvements spontanés de la queue causés déjà par la concentration environnementale. L'analyse de l'expression des gènes d'intérêts sont en cours de traitement. Sur les larves d'huîtres, les effets du mélange de pesticides ne sont pas généralisables à l'ensemble des biomarqueurs étudiés. L'augmentation des malformations semble résulter d'un effet additif du mélange, l'activité natatoire plus probablement d'un effet antagoniste alors qu'un effet synergique possible est observé dans l'expression de certains gènes.

Dans une dernière partie, une approche *in situ* a été développée pour évaluer les effets toxiques sur les stades embryo-larvaires de l'huître creuse associés à la qualité de l'eau du Bassin d'Arcachon. Il n'y a pas de différence significative sur les malformations et le comportement de nage des larves encagées entre le site de référence et les sites étudiés. Cependant, le taux de trajectoire de nage rectiligne (considéré comme normal) est plus faible pour les larves encagées sur le site de référence par rapport aux larves de référence en laboratoire. Cet effet peut être expliqué par des facteurs indépendants de la contamination du milieu (durée d'exposition plus longue *in situ*, courant, température de l'eau etc.). Au niveau moléculaire par contre, les larves encagées dans la partie interne du bassin d'Arcachon, soumise à la dynamique du bassin versant, présentent des différences dans l'expression des gènes impliqués dans la défense contre le stress oxydant, les métallothionéines et le métabolisme mitochondrial. Finalement, les effets

observés sur les trois sites sont moins sévères que ce que nous avions envisagé au départ. La qualité de l'eau de bassin d'Arcachon semble propice au bon développement larvaire de l'huître creuse.

En conclusion, ce travail a permis d'apporter des connaissances sur l'impact des concentrations environnementales de pesticides actuellement utilisés et leurs mélanges. Ces faibles concentrations sont souvent considérées sans risque et par conséquent négligées dans l'évaluation écotoxicologique. Ce travail a également illustré l'utilité et l'intérêt écologique de réaliser des études couplées d'expérimentations en laboratoire et dans le milieu naturel. Enfin cette étude a fait la preuve, de la sensitivité des stades embryo-larvaires.

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LIST OF ORIGINAL PUBLICATIONS

The overall contribution of Eliška Kuchovská is estimated to be 90 % in Publications I., II., III, and IV. (design, experimentation - laboratory and field studies, data analyses and interpretation, manuscript preparation) and 10 % in Publication V. (contribution to study design, interpretation of ecotoxicity data, manuscript editing).

Publication I.

Kuchovská, E., Morin, B., López-Cabeza, R., Barré, M., Gouffier, C., Bláhová, L., Cachot, J., Bláha, L., Gonzalez, P., 2020. Comparison of imidacloprid, propiconazole, and nanopropiconazole effects on the development, behavior, and gene expression biomarkers of the Pacific oyster (*Magallana gigas*). Sci. Total Environ. 142921. https://doi.org/10.1016/j.scitotenv.2020.142921, JIF: 6.55

Publication II.

Kuchovská, E., Gonzalez, P., Bláhová, L., Barré, M., Gouffier, C., Cachot, J., Bláha, L., Morin, B. Pesticide mixture toxicity assessment through *in situ* and laboratory approaches using embryo-larval stages of the Pacific oyster (*Magallana gigas*).

Prepared for submission in Science of the Total Environment.

Publication III.

Rozmánková, E., Pípal, M., Bláhová, L., Njattuvetty Chandran, N., Morin, B., Gonzalez, P., Bláha, L., 2020. Environmentally relevant mixture of S-metolachlor and its two metabolites affects thyroid metabolism in zebrafish embryos. Aquat. Toxicol. 221, 105444. https://doi.org/10.1016/J.AQUATOX.2020.105444, JIF: 4.34

Publication IV.

Imidacloprid, propiconazole, and pesticide mixture toxicity assessment using embryo-larval stages of zebrafish (Working title)
Kuchovská, E., Bláhová, L., Gonzalez, P., Morin, B., Bláha, L.

Manuscript draft

Publication V.

Njattuvetty Chandran, N., Fojtova, D., Blahova, L., Rozmankova, E., Blaha, L., 2018. Acute and (sub)chronic toxicity of the neonicotinoid imidacloprid on *Chironomus riparius*. Chemosphere 209, 568–577.

https://doi.org/10.1016/j.chemosphere.2018.06.102, JIF: 5.78

LIST OF CONFERENCE CONTRIBUTIONS

Platform presentations

SETAC Scicon Europe 3.5. – 7.5. 2020 – online meeting (international)
In situ evaluation of pesticide mixture effects on embryo-larval stages of the Pacific oyster (*Magallana gigas*)
Rozmankova, E., Barre, M., Blahova, L., Cachot, J., Blaha, L., Morin, B., Gonzalez, P.

ECOBIM 1.5. – 4.5. 2019 – Sousse, Tunisia (international)
Effets du S-métolachlore et de ses métabolites sur le métabolisme thyroïdien du poisson zèbre (in French)
Rozmankova, E., N. Chandran, N., Pipal, M., Blahova, L., Morin, B., Gonzalez, P., Blaha, L.

JDED (Journée des Doctorants de l'École Doctorale) 24.4. 2019 – Bordeaux, France (national)
Effets du S-métolachlore et de ses métabolites sur le métabolisme thyroïdien du poisson zèbre (in French)
Rozmankova, E., Gouffier, C., Cachot, J., Blaha, L., Gonzalez, P., Morin, B.

Poster presentations

RECETOX PhD Conference 25.5. – 29.5. 2020 – Online meeting (national)
Environmentally relevant pesticide mixture: a risk for non-target aquatic organisms? Laboratory and *in situ* approaches
Kuchovska, E.

SETAC Europe 13.5. – 17.5. 2018 – Rome, Italy (international)
Sublethal toxicity of pesticide mixtures on early life stages of non-target aquatic organisms
Rozmankova, E., N. Chandran, N., Morin, B., Cachot, J., Gonzalez, P., Blaha, L.

ECOBIM 22.5.– 25.5. 2018 – Bordeaux, France (international) **Best poster award**
Effets du propiconazole et de l'imidaclopride sur les stades précoces de développement de l'huître creuse *M. gigas* (In French)
Rozmankova, E., Gouffier, C., Cachot, J., Blaha, L., Gonzalez, P., Morin, B.

JEST Journées d'Echanges Scientifiques EPOC 6.4. 2018 – Bordeaux, France (national)
Pesticides et leurs mélanges : quels risques pour les organismes aquatiques non-cibles ? (in French)
Rozmankova, E., Gouffier, C., Blaha, L., Roumagnac, L., Cachot, J., Morin, B., Gonzalez, P.

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LIST OF ABBREVIATIONS

AFNOR	Association Française de Normalisation (French Standardization Association)
ANOVA	Analysis of variance
ASTM	American Society for Testing and Materials
ATP	Adenosine triphosphate
C	Comprian
CA	Concentration addition concept
CAT	Catalase
cDNA	Complementary DNA
CYP	Cytochrome P450 family
DMSO	Dimethyl sulfoxide
dpf	Days post fertilization
DNA	Deoxyribonucleic acid
ECHA	European Chemicals Agency
EC50	Half maximal effective concentration
EFSA	European Food Safety Agency
EMA	European Medicines Agency
EPA	Environmental Protection Agency
FSW	Filtered seawater
GB	Grand Banc
GPX	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
HDPE	High-density polyethylene
hpf	Hours post fertilization
IA	Independent action concept
ICP-MS	Inductively coupled plasma mass spectrometry
ICP-OES	Inductively coupled plasma optical emission spectrometry
IMI	Imidacloprid
ISO	International Organization for Standardization
J	Les Jacquets
LC-MS	Liquid chromatography–mass spectrometry
LC50	Half maximal lethal concentration
LOD	Limit of detection
LOEC	Lowest observed effect concentration
LOQ	Limit of quantification
MESA	Metolachlor ethanesulfonic acid
MOA	Metolachlor oxanilic acid
NGS	Next generation sequencing techniques
NOEC	No observed effect concentration
OECD	Organisation for Economic Co-operation and Development
PCL	Poly(ε-caprolactone)
POCIS	Polar organic chemical integrative sampler
PRO	Propiconazole
qPCR	Quantitative Polymerase Chain Reaction analysis
RA	Retinoic acid
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals

REMPAR	REseau de Surveillance des Micro-polluants sur le Bassin (Monitoring network of micropollutants in Arcachon Bay)
REPAR	REseau de Surveillance des Pesticides sur le Bassin (Monitoring network of pesticides in Arcachon Bay)
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SIBA	Syndicat Intercommunal de Bassin d'Arcachon (Intercommunal Association of the Arcachon Bay)
SM	S-metolachlor
SOD	Superoxide dismutase
SPE	Solid phase extraction
TBARS	Thiobarbituric acid reactive substances
TH	Thyroid hormone
T3	Triiodothyronine
T4	Thyroxine
UKZUZ	Ústřední kontrolní a zkušební ústav zemědělský (Central Institute for Supervising and Testing in Agriculture)
ZFET	Zebrafish Embryo Acute Toxicity Test
3Rs	Replacement, reduction and refinement

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INTRODUCTION

Pollution of the ecosystem has become the biggest issue in the modern world influencing not only human health but also all living beings and their habitats. One of the sources of this pollution is agriculture which uses pesticides (plant protection products) to fight against pests and increase crop yields but also to protect warehouse stock and limit the development of diseases in farms. Even though humankind was first warned about the eventual side effects of pesticides already in the sixties by the famous book Silent Spring (Carson, 1962) and keeps to be highly sensitized in the recent years, thanks to the growing knowledge of the issue, the current situation still demands a solution. Since then, the regulation of these substances has changed considerably resulting in the Directive 2009/128/EC of the European Parliament and of the Council that aims to achieve the sustainable use of pesticides. It is necessary to find an equilibrium to feed the population without worsening the state of air, soil, and water. Despite this necessity, the pesticide consumption in the European Union states did not decrease since with around 350,000 tons of pesticides used in 2018 (European Commission, 2020). After their application, spray drift, runoff, and leaching often occur and the aquatic ecosystems become the final recipient of these substances where they may harm non-target aquatic organisms (de Souza et al., 2020) such as fish (Altenhofen et al., 2017; Vignet et al., 2019; Weeks Santos et al., 2019), invertebrates (Bechmann et al., 2020; Rösch et al., 2017; Velisek et al., 2019), aquatic plants (Demailly et al., 2019), etc. Especially vulnerable are the early-life stages of organisms such as embryos and larvae.

Traditionally, the effects of pesticides on organisms were assessed using biotests with adult animals. However, mindful of the unnecessary sacrifices of organisms and their welfare, the goal is to replace these classic methods with new alternative ones such as the embryo-larval stages of organisms, *in vitro*, or *in silico* methods. The “Three Rs” principles were adopted in 1959 (Russell et al., 1959) and stand for Replace, Reduce, and Refine. This approach is promoted by the EU Directive 2010/63/EU and adopted by REACH (EU Regulation stating for Registration, Evaluation, Authorisation, and Restriction of Chemicals).

The impacts of individual molecules are well studied and understood. However, the substances interact in the aquatic ecosystem, forming complex mixtures and their stability and bioavailability are influenced by various environmental factors (light, temperature, pH, …). Moreover, they are degraded over time and form metabolites, which can be even more toxic than the parent compound. These complex mixtures may then affect organisms by different

mechanisms than individual pesticides (so-called independent action). Indeed, multiple mixture toxicity types may be exerted such as concentration addition, synergistic, or antagonistic effect (Cedergreen, 2014). The final pesticide cocktail (further combined with other environmental pollutants) may thus be more toxic to the aquatic organisms' communities. Therefore, it is important to successfully assess all the risks and to establish an equilibrium with sustainable pesticide use i.e. ensure to feed the human population without inducing any or limited adverse effect on non-target organisms.

In the light of this serious environmental issue, as emphasized by EU (European Environment Agency, 2020) this dissertation thesis aims to contribute to understanding potential sub-lethal effects of commonly occurring pesticides, individual and in a mixture, on early-life stages of two model organisms: freshwater zebrafish *Danio rerio* and marine/brackish Pacific oyster *Magallana gigas*. This work is mainly focused on the exploration of biological effects of environmentally relevant concentrations of five substances i.e. herbicide S-metolachlor with its two metabolites (metolachlor oxanilic acid and metolachlor ethanesulfonic acid), insecticide imidacloprid, and fungicide propiconazole, as detected in Arcachon Bay in France, our field of study. This work is completed by an *in situ* caging experiment.

The research included both laboratory studies and *in situ* caging experiments:

- Representatives of herbicides, fungicides, and insecticides induce detectable effects even at low concentrations, especially at biochemical/physiological levels on the embryo-larval stages of the Pacific oyster **(Publication I.** and **II.)** and the zebrafish **(Publication III.** and **IV.)**
- The effects of mixtures of the studied compounds are more pronounced and may lead to the effects that could not be predicted from individual chemicals **(Publication II.** and **IV.)**
- Effects observed in the laboratory correspond to field *in situ* observations and the pesticide contamination in the inner part of Arcachon Bay is responsible for the worsened state of oyster development **(Publication II.)**

Secondary hypotheses were:

- The toxic effects of propiconazole on the embryo-larval stages of the Pacific oyster are more severe than those of propiconazole nanoformulation **(Publication II.)**
- Low concentrations of imidacloprid impact the development of the non-target midge *Chironomus riparius* **(Publication V.)**

This manuscript is divided into several chapters. **Chapter I.** introduces state of the art knowledge of pesticide pollution in the aquatic compartment and their impact on non-target species as well as the description of used model organisms and the advantages of the use of their embryo-larval stages. Furthermore, *in situ* transplantation tests are discussed and the field of study, Arcachon Bay, is briefly described. In **Chapter II.**, used methodology and analytical strategy are succinctly introduced and are followed by **Chapters III. and IV.** where the results are presented as published articles or manuscripts in preparation. A discussion of obtained results is developed in **Chapter V**.

CHAPTER I.

RESEARCH CONTEXT

1. Plant protection products

1.1. Regulatory framework[1]

Pesticides have a significant role in ensuring enough healthy food for the growing human population. Pesticides are of various nature and may be divided into plant protection products and biocides. However, their intended use is to protect crops against pests, they may also be harmful to non-target organisms (Anderson et al., 2015; Carazo-Rojas et al., 2018). Therefore, their use is controlled by regulatory bodies throughout the world. EU pesticide regulatory frameworks are some of the strictest ones. Before a new pesticide enters the market, a rigorous scientific assessment of its active substance is carried out. The approval usually takes three years, and the substance is approved for ten years maximum. Indeed, after that period it is necessary to re-evaluate the substance to get a renewed approval of use. Directive 2009/128/EC on the sustainable use of pesticides aims to reduce the impacts of pesticides on the environment and human health. The Directive is implemented by EU member states in National Action Plans. For instance, in consequence, the Czech Republic established National Action Plan to Reduce the Use of Pesticides which aims to protect public health, groundwater and surface water, and non-target organisms (The Ministry of Agriculture of the CR, 2012). Similarly, France adopted the Ecophyto program intending to reduce the use of pesticides by 50% in 10 years by training workers and disseminating good practices of use of pesticides, by innovative farming systems, and by monitoring the impact of pesticides on non-target harmful organisms (Ministère de l'Agriculture et de la Pêche, 2008).

European Integrated Pest Management of the Directive 2009/128/EC indicates farmers useful techniques and principles to avoid unnecessary use of pesticides. Some of the principles promote the use of sustainable biological, physical, and other non-chemical methods; the pesticides should have specific toxicity; ban of aerial spraying, etc. Unfortunately, the consumption of pesticides in the EU is still increasing (European Commission, 2020) with France at the top with more than 80,000 tons of pesticides in 2018 (Figure 1). However, EU organic production has increased by 18.7% from 2012 to 2016 and the organic farming area comprises on average 7.5% of the total agricultural area in EU i.e. 13.4 million hectares of agricultural land (organic farming statistics of Eurostat). Use of biopesticides is another promising tool for sustainable pesticide use. In comparison with conventional pesticides, they

[1] This subchapter is mainly based on an online publication Agri-environmental indicators of Eurostat (European Statistical Office) accessible at https://ec.europa.eu/eurostat/statistics-explained/index.php?title=Agri-environmental_indicators and on an online factsheet on Directive 2009/128/EC of European Commission accessible at https://ec.europa.eu/food/plant/pesticides/sustainable_use_pesticides_en.

are often affecting only the target pest without substantially harming non-target species. They degrade rapidly and are effective in low quantities. They are based on natural materials such as pheromones or plant extracts or they are formed by living microorganisms like bacteria (Bozzini, 2017).

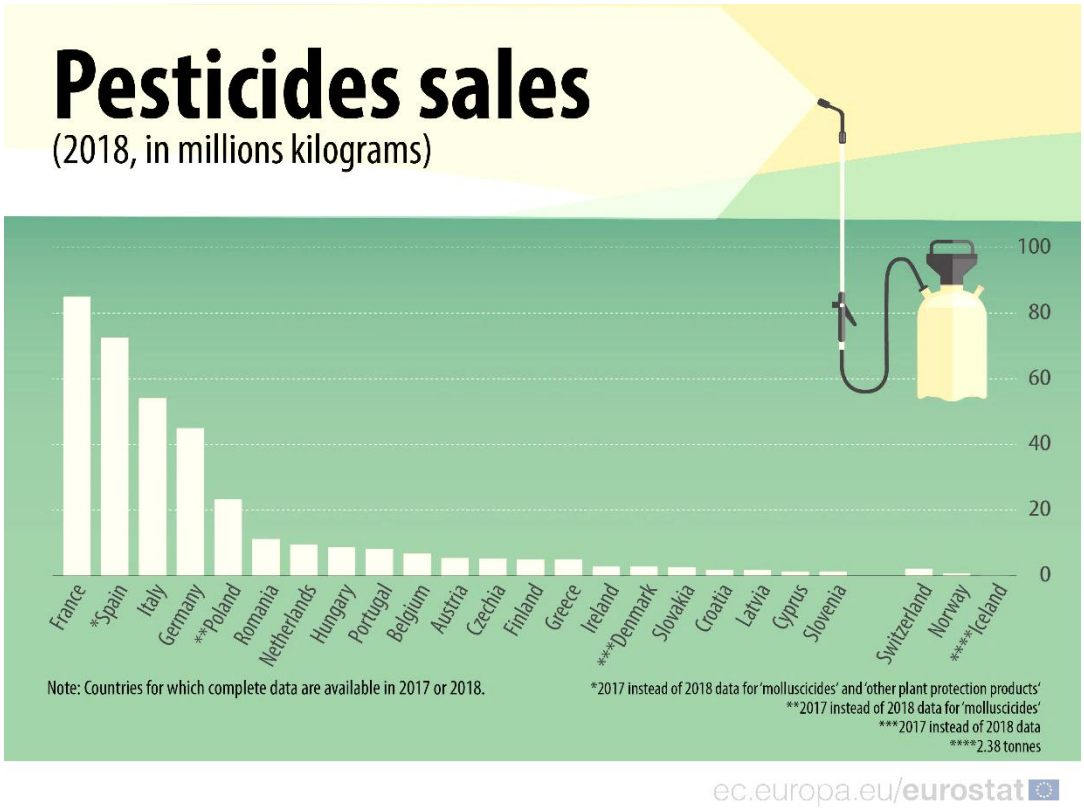

Figure 1 Consumption of pesticides in the member states of EU (source: https://ec.europa.eu/eurostat/statistics-explained/index.php?title=Agri-environmental_indicators)

1.2. Pesticide pollution and its risk to non-target aquatic organisms

Pesticides are directly applied to agricultural land. However, they may end up in aquatic ecosystems via various pathways, including drifts during (aerial) application and accidental spilling, they may be washed off during rain events, leached into the groundwater, or be transported to surface water through irrigation water drains (Figure 2). Less commonly, pesticides may also be applied directly into aquatic ecosystems to control pests of aquatic organisms e.g. in fish farms (Muñoz et al., 2010). That was for example the case of an environmental issue in the Washington state, where burrowing shrimps, pests of the Pacific oyster, which is extensively farmed in Willapa Bay and Grays Harbor, were controlled for 40

years using carbaryl (carbamate pesticide) which was applied directly on the sediments at low tide, thus polluting the estuaries. It was supposed to be replaced by neonicotinoid imidacloprid but in 2018 the Washington state did not authorize such imidacloprid use to protect environmental health and safety[2] (Dumbauld et al., 2001; Iliff et al., 2019).

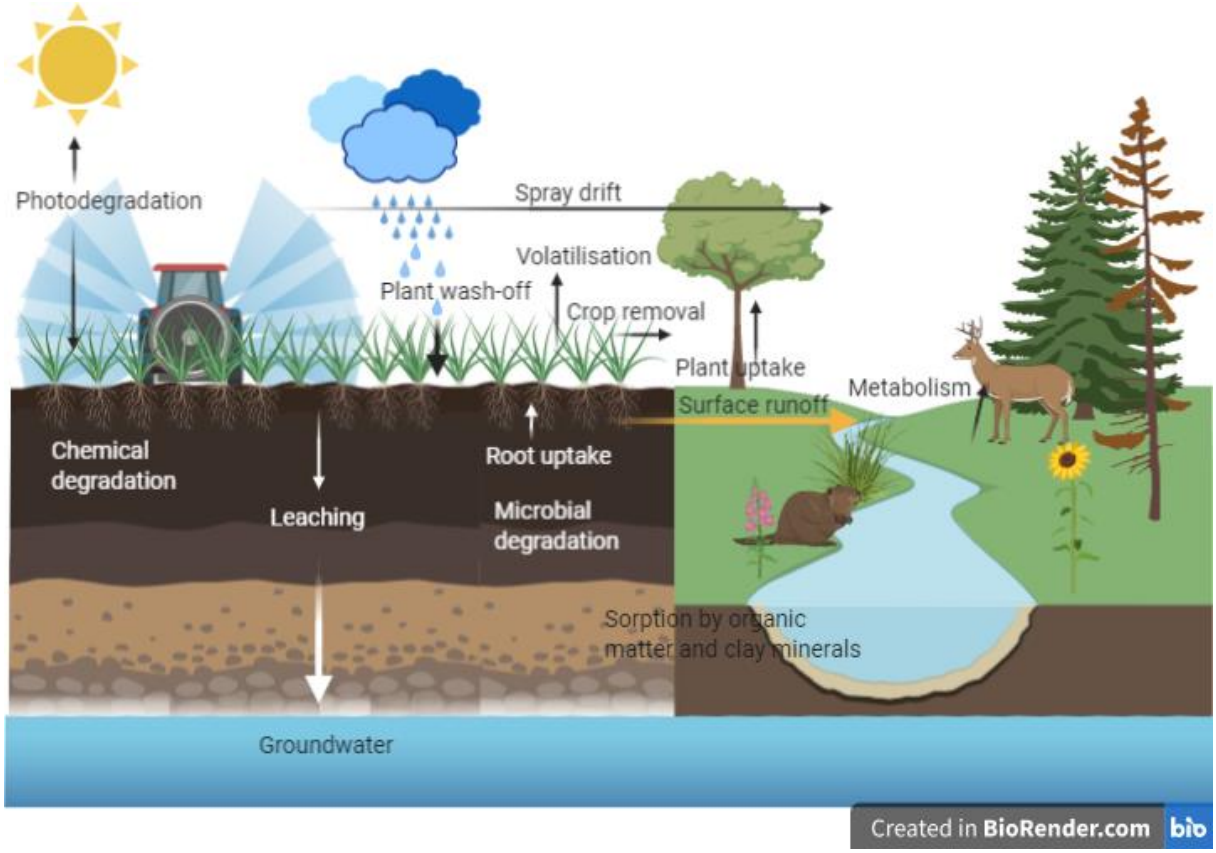

Figure 2 Fate of pesticides and their transfer in waters; Created with Biorender.com

The main route of pesticide transfer to surface water is the surface runoff, which is influenced by multiple factors, mainly climatic (intensity of rainfall) and geological (soil type) but also by the properties of the pesticide itself (solubility, hydrophobicity), crop on site, etc. (Stanley and Preetha, 2016). Furthermore, pesticides in aquatic ecosystems may degrade and form metabolites via multiple ways e.g. photodegradation, hydrolysis, or microbial degradation. These metabolites may also exert biological effects as the parent compound. Sometimes they can be even more toxic as shown for example for the metabolites of fipronil (Weston and Lydy, 2014). However, more commonly, lower toxicity of metabolites is observed as shown for example for metabolites of atrazine (Ralston-Hooper et al., 2009). When assessing the toxicity of a pesticide, it is necessary to also evaluate its metabolites.

[2] Decision of the Department of Ecology, State of Washington, accessible at: https://ecology.wa.gov/Regulations-Permits/Permits-certifications/Aquatic-pesticide-permits/Burrowing-shrimp-control-Imidacloprid

Once in the aquatic ecosystem, the pesticides may harm non-target aquatic species. Non-target species are those, which the pesticide was not intended to control. Pesticide exposure in surface waters may be extremely variable since the pesticide concentrations usually peak (peak concentrations higher by factor 10-100 times) after a rain event and are subjected to seasonal variations (Cedergreen and Rasmussen, 2017). Usually, herbicides and fungicides have a slow mode of action unlike the insecticides, which have in addition probably the highest impact on aquatic ecosystems (Yu, 2015).

Depending on the pesticides' physico-chemical properties, they tend either to stay in the water column, be adsorbed on inorganic particles (sediments), or organic substrates (algae, etc.). Routes of exposure for aquatic organisms are several i.e. dermal exposures through the skin, exposure via breathing (fish gills), or oral exposure by ingesting water or contaminated food. The toxicity is then dependent on the dose, duration of exposure and its type, concerned species, the substance itself, and on various environmental factors such as pH, temperature, salinity, etc. (Stanley and Preetha, 2016). Low pesticide concentrations in the range of ng/L, or peak concentrations of several µg/L, which are usually detected in the surface waters (Cedergreen and Rasmussen, 2017), usually do not impact directly the survival of aquatic non-target organisms. However, numerous sublethal effects have been documented, such as the impact on behavior (Crosby et al., 2015a; Denoël et al., 2013; Gamain et al., 2020; Velki et al., 2017), development (Liang et al., 2015; Monteiro et al., 2019; Velisek et al., 2019), immunity (Oluah et al., 2020; Raibeemol and Chitra, 2020), growth (Monteiro et al., 2019; Velisek et al., 2019), hormonal system (Liang et al., 2015; Raibeemol and Chitra, 2020; Suvetha et al., 2015), etc.

1.3. Mixture toxicity

The aquatic environment is commonly a final recipient of contamination and thus contains multiple substances simultaneously. Usually, co-exposure to ten to twenty different pesticides is observed (Cedergreen and Rasmussen, 2017). Due to the possible interactions between molecules, the toxicity of mixtures can be higher than that of the individual compounds (Cedergreen, 2014) and can be defined by several concepts. The most common mixture toxicity type is the concentration addition concept (CA), where the molecules in the mixture act by the same mechanism of action. More than 90% of all mixtures seem to exert the CA effect (Cedergreen and Rasmussen, 2017). Secondly, the independent action concept (IA) describes mixtures with different molecules still without interaction but with different mechanisms of

action. On the contrary, synergism and antagonism can occur in mixtures with interactions between the substances. In the case of a synergistic mixture, the total toxicity is higher than that predicted by CA. On the contrary, the effects of the antagonistic mixture are lower than the summed toxicity of individual compounds (European Chemicals Agency, 2014). A synergistic mixture is defined as a mixture of substances with the EC_{50} less than two-fold smaller than the EC_{50} which would be predicted by CA. Rarely, the EC_{50} was observed to be less than a 10-fold decrease (Cedergreen and Rasmussen, 2017). In the past, the research tended to assess the toxicity of single compounds, hence the effects of mixtures are often not known. Therefore, there are strong needs to assess the impact of mixtures on the environment, which is even emphasized in the European Green Deal as stated in the document "Chemicals Strategy for Sustainability Towards a Toxic-Free Environment" (EC, 2020). Indeed, in the domain of risk assessment, it is important to know with which type of mixture toxicity is relevant, otherwise, the overall toxicity can be underestimated (synergy) or overestimated (antagonism). Another problem of interpretation may also occur whilst distinguishing CA and IA concepts. For instance, we can encounter "something from nothing" effect in the case of low environmental concentrations of substances characterized by CA (and not by IA), when the mixture is composed of compounds below their NOECs (no observed effect concentration) but together, the effect is pronounced (Silva et al., 2002). The knowledge of a chemical mode of action is essential for understanding how mixtures may act jointly and is typically known for herbicides acting on photosynthetic organisms like algae (Backhaus et al., 2004). However, little knowledge exists on the mechanisms of action of pesticides on aquatic animal species (Mai et al., 2014).

Previously recognized mixtures exerting pesticide synergy are those that contain azole fungicides (where the biotransformation processes are altered by inhibiting the cytochrome P450), organophosphate and carbamate insecticides (cholinesterase inhibitors), and pyrethroid insecticides mixed with azoles (Cedergreen, 2014; Gottardi et al., 2018; Rösch et al., 2017).

1.4. Nanopesticides

Toxicity to non-target organisms and the need to use lower quantities of pesticides stimulates the scientific search for new substances and new alternatives of conventional pesticides. One of the novel pesticide alternatives may be nanoformulated pesticides. They are emerging technological development, which could revolutionize agriculture, but they may also have an

unpredictable environmental impact. The definition of nanopesticides differs in various reviews and even in periodically updated legislation. We consider nanopesticides, as defined by Kah et al., (2013), a pesticide formulation which contains a substance in the nanometer size range (up to 1000 nm), is labeled with "nano" prefix and/or have novel properties due to the small size of its entities. Multiple types of nanoformulations are designed, the most common are polymer-based nanocarriers with the active ingredient encapsulated inside, nanometals, and nanoemulsions. The polymer-based nanocapsules may be biodegradable, such as those composed of poly(ε-caprolactone) (PCL) (Woodruff and Hutmacher, 2010). These polymers have low cytotoxicity and rather small and short-term environmental hazard (Shakiba et al., 2020). The desired nanopesticide advantages may include slower release, targeted delivery, higher water solubility, protection against premature degradation such as reduced hydrolysis or volatilization, enhanced uptake, faster decomposition in soil, etc. (Kah et al., 2013; Kah and Hofmann, 2014; Kookana et al., 2014). Most importantly, nanopesticides should be more efficient against pests with a lower quantity of pesticide used and/or should be less harmful to non-target organisms. In any case, their authorization on the market must be accompanied by rigorous ecotoxicity studies, and a comparison of their effects, risk, and impacts on the environment with the conventional active ingredient (Kah et al., 2018).

Despite their promising qualities and rapidly evolving research in this area, the nanopesticide market is not expanding yet, mostly because of the unknown environmental impacts and possible slow acceptance by the public being skeptic about "nano" products. The first nanoformulation biocides on the market were nano-silver particles with antimicrobial ability, which are added for example to clothing (Seltenrich, 2013). However, several other pesticide products are already on the market such as AZteroid FC and Bifender FC, an azoxystrobin fungicide, and bifenthrin insecticide, both encapsulated in nanopolymer, produced by Vive Crop Protection[3].

1.5. Pesticides of interest

For research in this thesis, pesticides were selected according to the environmental relevancy (cf. Chapter I. section 2 Areas of study).

Metolachlor, 2-chloro-N-(2-ethyl-6-methylphenyl)-N-(1-methoxypropan-2-yl)acetamide, is a selective pre-emergent herbicide of the chloroacetanilides family, first registered in 1976. It is

[3] www.vivecrop.com/products/

an example of a re-evaluation of the use of pesticides because, since 1997, only its active enantiomer **S-metolachlor,** (Figure 3) is utilized. S-metolachlor has become one of the most widely used pesticides in the world (Atwood and Paisley-Jones, 2017). Its adverse effects on non-target animals are reduced whilst the biological activity of the pesticides is maintained. This process is called a "chiral switch" (Poiger et al., 2002) and permitted to reduce the amount of sprayed pesticide by 35% (Shaner et al., 2006) to 40% (Blaser and Spindler, 1997).

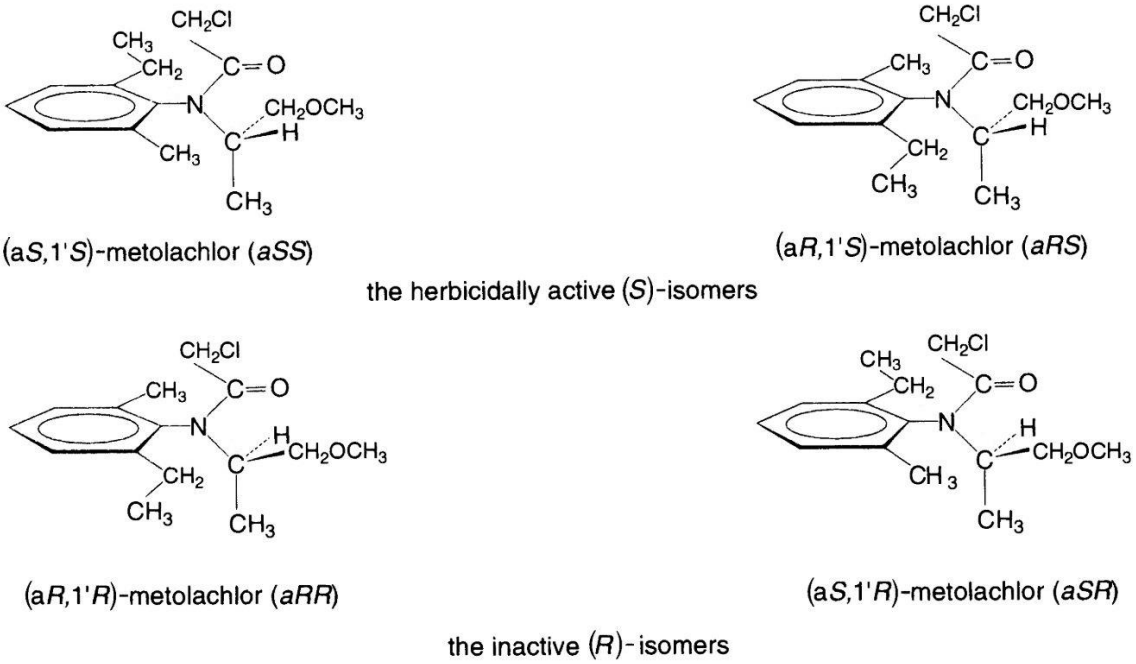

Figure 3 Chemical structure of the four stereoisomers of metolachlor. Modified from Poiger et al. (2002).

S-metolachlor is mostly spread in cornfields but also cereals or beet fields to control grassy weeds and undesired broadleaf plants. S-metolachlor affects the growth of plants by inhibiting enzymes involved in the biosynthesis of gibberellins and very-long-chain fatty acids (Götz and Böger, 2004; Rose et al., 2016). The main route of metolachlor transport from fields is the agricultural runoff (Krutz et al., 2005), leading to often observed contamination of water bodies by S-metolachlor (Glinski et al., 2018; Kapsi et al., 2019; Meffe and de Bustamante, 2014; Ryberg et al., 2014; Tapie and Budzinski, 2018). The main degradation pathway of metolachlor (Figure 4) is mediated by microbial activity in the soil when the two main metolachlor metabolites are formed by the glutathione conjugation: metolachlor oxanilic acid (MOA) and metolachlor ethanesulfonic acid (MESA) (Zemolin et al., 2014). The chlorine atom from the parent molecule is removed and the water solubility of metabolites is highly enhanced (Postle

et al., 2004). The minor degradation pathway is photolysis, however, its efficacy is low in comparison with the microbial one (Zemolin et al., 2014).

Although the acute LC_{50} (concentration that causes a mortality of 50% of studied organisms) of S-metolachlor for fish is relatively high: 3.9 – 10.0 mg/L depending on specie (Munn et al., 2006), the environmentally relevant concentrations as low as 10 ng/L have led to measurable sublethal effects such as abnormal larval development and DNA damage of oyster embryos (Mai et al., 2014) and delayed development, slower growth and altered behavior of early-life stage crayfish (Velisek et al., 2019).

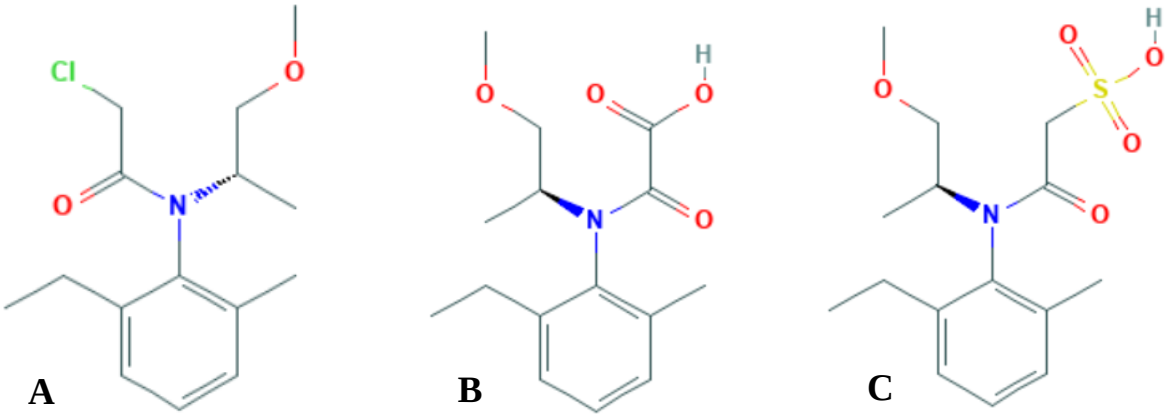

Figure 4 A Metolachlor ($C_{15}H_{22}ClNO_2$) with its two main degradation products: **B** MOA ($C_{15}H_{21}NO_4$) and **C** MESA ($C_{15}H_{23}NO_5S$). Source: https://pubchem.ncbi.nlm.nih.gov.

Propiconazole (Figure 5A), a mixture of four stereoisomers, fungicide of conazole class is a triazole pesticide that is widely used to protect cereals and oil plants from fungi contamination. Propiconazole stops the fungal growth by inhibiting the synthesis of ergosterol forming the fungal (and yeast) cell membranes (fungistatic action). The azoles target the steroidogenesis by blocking two enzymes: sterol 14α-demethylase and aromatase, both members of the cytochrome P450 family, encoded by *cyp51* and *cyp19*, respectively. However, enzyme sterol 14α-demethylase is also active in other organisms and participate in the metabolic pathway of cholesterol in animals (Zarn et al., 2003). By affecting the steroidogenesis (inhibition of estrogen and androgen biosynthesis), the azoles exert endocrine-disrupting effects. However, propiconazole seemed less potent *in vitro* than other tested triazoles such as epoxiconazole and tebuconazole or imidazoles (Kjærstad et al., 2010).

Propiconazole is mainly degraded by biotransformation. It may contaminate the aquatic environment by field runoff and spray drift. It is stable in water but prefers to move from water and adsorb to sediments or soil (Gad and Pham, 2014). Propiconazole was observed to have sublethal effects in fish (Hemalatha et al., 2016; Li et al., 2010; Skolness et al., 2013; Souders et al., 2019a; Teng et al., 2020, 2019) as well as in aquatic invertebrates (Bringolf et al., 2007; Kast-Hutcheson et al., 2001; Rösch et al., 2017).

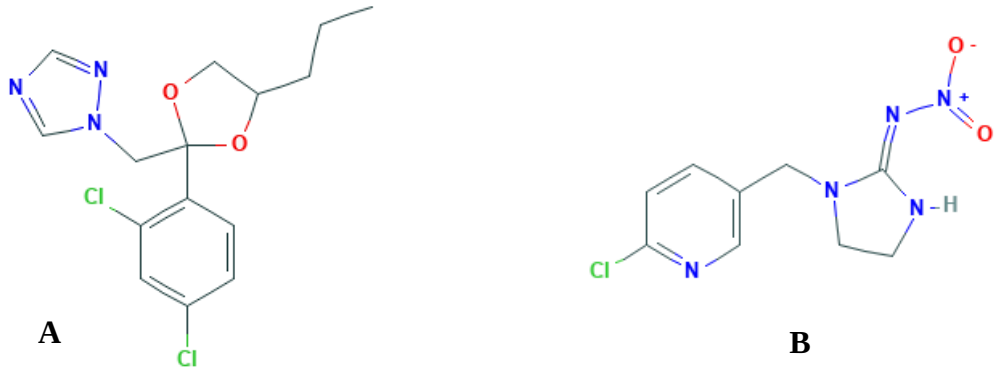

Figure 5 Structure of **A** propiconazole ($C_{15}H_{17}Cl_2N_3O_2$) **B** imidacloprid ($C_9H_{10}ClN_5O_2$). Source: https://pubchem.ncbi.nlm.nih.gov.

Insecticides are not generally used in such quantities as herbicides or fungicides but may have deleterious effects on non-target organisms as well. For instance, neonicotinoids were linked to globally recorded bee declines (Sureda Anfres, 2016). One of the neonicotinoids, **imidacloprid** (Figure 5 B) was recently banned because of its harmful effects on bees and other non-target insects in the European Union by regulation of the EU Commission (European Commission, 2018) except the use in permanent greenhouses. However, it can still be largely used in other parts of the world, where it may harm non-target species (Butcherine et al., 2019; Morrissey et al., 2015). Imidacloprid is a systemic neurotoxic insecticide that kills pests by disrupting neural transmission (agonistic binding to the post-synaptic acetylcholine receptors) in the central nervous system of invertebrates (Simon-Delso et al., 2015). It has higher affinity for the insect target site than the vertebrate one (Matsuda et al., 2001). Nevertheless, adverse impact on non-target aquatic organisms was observed. Imidacloprid water contamination affected non-target aquatic insects (Cavallaro et al., 2017), mollusks (Dondero et al., 2010; Ewere et al., 2020; Prosser et al., 2016; Shan et al., 2020), and fishes (Crosby et al., 2015a; Özdemir et al., 2018; Vignet et al., 2019). Neonicotinoids are also known to be additively or synergistically toxic when mixed together or when mixed with fungicides inhibiting the detoxification by cytochrome P450 (Anderson et al., 2015; Morrissey et al., 2015).

2. Areas of study

2.1. Pesticide pollution in Arcachon Bay

In addition to laboratory experimental studies, research presented in this dissertation thesis included field studies. Arcachon Bay is situated in south-west France near the city of Bordeaux (Figure 6). Arcachon Bay is a 174 km^2 mesotidal shallow lagoon connected to the Atlantic Ocean by a narrow channel "Passes du Bassin" (Bertrand, 2014). The lagoon is under considerable anthropogenic pressure e.g. extensive boat traffic, aquatic recreational activities, runoff from surrounding agriculture areas with the most pesticide pollution brought by the river Leyre (Fauvelle et al., 2018). Since the middle of the 19th century, it has become popular for its extensive oyster farming with cultivated specie Pacific oyster *Magallana gigas* (also called *Crassostrea gigas*). Its importance for Arcachon Bay is not only economic but also ecologic. Indeed, oysters are considered as sentinel species and anomalies in the development of their larvae that are very sensitive to pollution may be used for early warning in the case of ecological dysfunction of Arcachon Bay (Auby and Maurer, 2004).

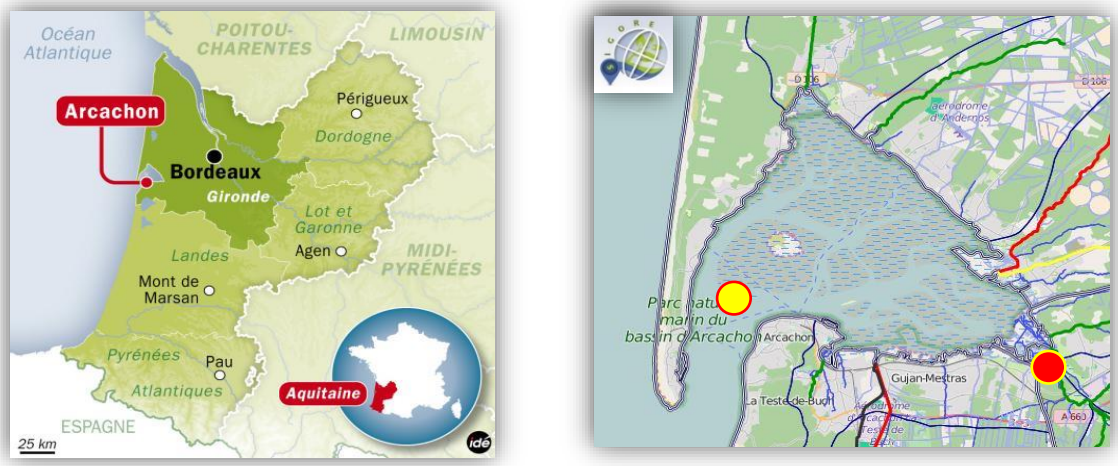

Figure 6 Location of Arcachon Bay in France (©Carte Idé) and its water system (rivers emphasized with colors; red circle: mouth of the river Leyre, yellow circle: Grand Banc) (©SIGORE Gironde)

In the recent years, several reports regarding Arcachon Bay fauna and flora indicated a decreased of collected oyster spat and recruited oysters (Auby et al., 2014), a decline in the seagrass *Zostera* population (Auby et al., 2011; Cognat et al., 2018), and phytoplankton richness and amount anomalies, which serve as the main feed for oyster larvae (Auby and Maurer, 2004).

The presence of pesticides in Arcachon Bay is regularly assessed by REPAR[4] (REseau de Surveillance des Pesticides sur le Bassin – Monitoring network of pesticides in the Bay) of SIBA[5] (Syndicat Intercommunal de Bassin d'Arcachon – Intercommunal Association of Arcachon Bay). As seen in Figure 7, the most common plant protection products detected in waters of Arcachon Bay are the metabolites of herbicide metolachlor and acetochlor, as presented in the report of REPAR (Tapie et al., 2012).

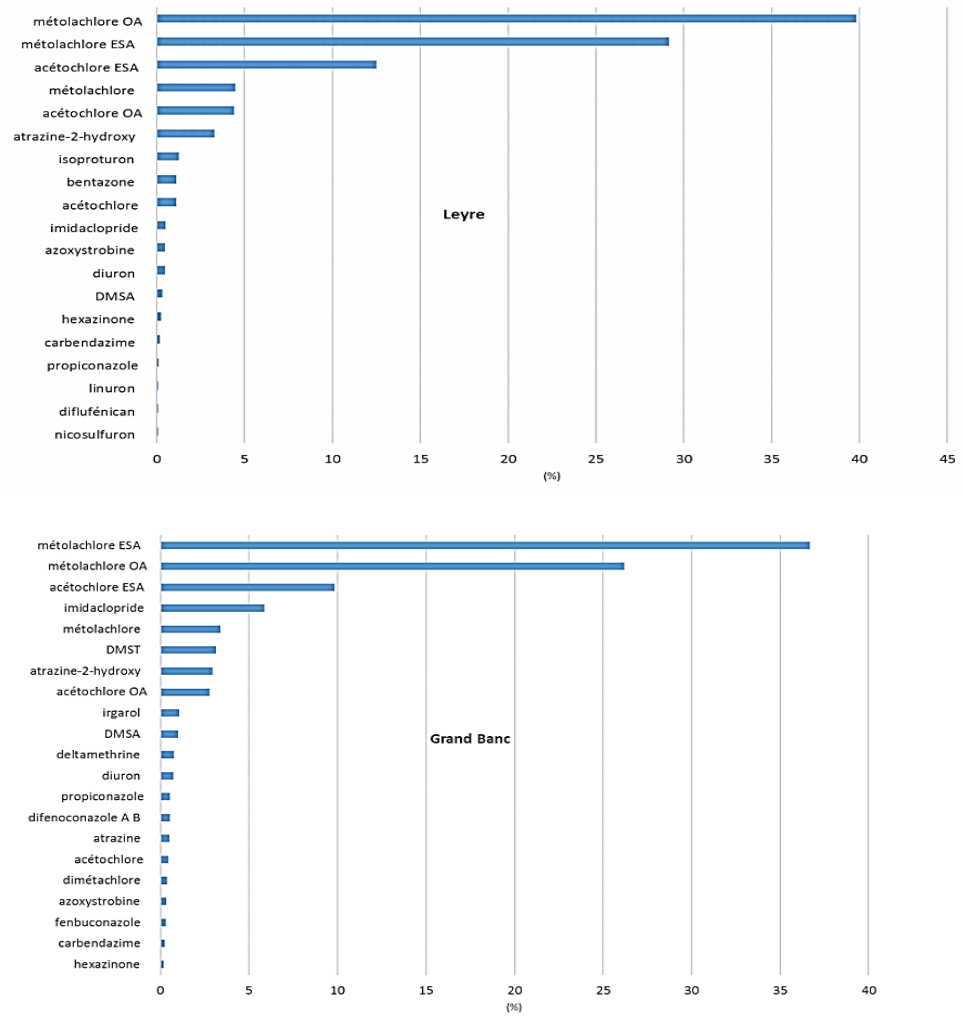

Figure 7 The most commonly detected pesticides (expressed as % of the total pesticide concentration) in the river Leyre, the biggest tributary of Arcachon Bay, and at Grand Banc near the mouth of the lagoon (for locations see Figure 6). Reprinted from Tapie et al. (2012).

To our knowledge, several studies investigated the toxicity of pesticides at environmentally relevant concentrations on indigenous non-target organisms of Arcachon Bay such as seagrass *Zostera nolteii* (Gamain et al., 2018) or oyster *Magallana gigas*. The recent studies addressed

[4] https://www.siba-bassin-arcachon.fr/actions-environnementales/les-reseaux-de-surveillance-repar-et-rempar
[5] https://www.siba-bassin-arcachon.fr/

for example the toxicity of herbicide S-metolachlor (Gamain et al., 2016), metabolites of S-metolachlor (Mai et al., 2014), and herbicides irgarol and diuron (Mai et al., 2013) on Pacific oyster.

Complex mixtures containing pesticides and other pollutants are regularly detected in Arcachon Bay (Tapie and Budzinski, 2018). In the recent years, the most detected herbicide, insecticide, and fungicide in Arcachon Bay were metolachlor with its metabolites MOA and MESA, imidacloprid, and propiconazole, respectively. Fungicide carbendazim and metabolites of dichlofluanid were also often detected. Table 1 summarizes maximum and average concentration measured on different sampling locations in Arcachon Bay in the tributaries during 2010-2014 by REPAR (Tapie and Budzinski, 2018). These reported concentrations were considered the selection of the studied concentrations used for the experimentations in this dissertation work.

Table 1 Concentrations (maximum, average, and average on the specific location with a maximum concentration of the pesticide) of S-metolachlor, MOA, MESA, imidacloprid, and propiconazole detected by chemical analyses in Arcachon Bay during years 2010-2014. (Calculated based on data of Tapie et al., (2018)).

Compounds	Maximum concentration (ng/l)	Average concentration (ng/l)	Average concentration on the sampling point with max conc. (ng/l)
S-metolachlor	1695.9	31.9	274.3
MOA	1609.9	163.5	727.1
MESA	1059.2	117.6	424.4
Imidacloprid	173.6	2.6	7.8
Propiconazole	29.1	0.7	2.7

2.2. Pesticide pollution in the Czech Republic

To complement the marine/brackish environment with a freshwater one, our second area of concern was the hydrological system in the Czech Republic. Although the Czech Republic is a smaller consumer of pesticides compared to France (Figure 1), in 2015 the Czech Republic used more than 4843 tons of active substances of plant protection products (UKZUZ, 2015).

Metolachlor, similarly in Arcachon Bay, is widely detected in the streams of the Czech Republic. Indeed, in 2014, metolachlor was present in almost 18% of all the surface water samples in the Czech Republic (data obtained from the Czech Hydrometeorological Institute

database[6]). Due to its degradation properties (cf. Chapter I. section 1.5), its metabolites are detected even more frequently: metolachlor OA is found in one-third of all samples and metolachlor ESA in almost 65% of all samples and exceeds the arbitrary threshold concentration of 0.1 μg/l in almost 19% of sampling locations (data obtained from Czech Hydrometeorological Institute database).

Propiconazole is not among the most widely used fungicides in the Czech Republic (unlike e.g. tebuconazole) but, it is also utilized with reported 56 tons in 2015 (UKZUZ, 2015). Its concentrations in surface waters exceeded the 0.1 μg/l threshold at 3 stations (out of 215 locations tested) in 2014 according to Czech Hydrometeorological Institute databases.

Finally, imidacloprid was used in the Czech Republic mostly to protect hops. Information about its presence in the streams of the Czech Republic are scarce with only 18 locations screened in the year 2014 (year was chosen to compare the observed values with those found in France). However, in 2017, 5 locations out of 233 exceeded 0.1 μg/l (data obtained from Czech Hydrometeorological Institute database).

Table 2 presents maximum detected concentration and percentage of values exceeding the threshold of 0.1 μg/l of pesticides of interest in the Czech Republic in 2014.

Table 2 Maximum concentrations and percentage of values exceeding the threshold of 0.1 μg/l of metolachlor, MOA, MESA, imidacloprid, and propiconazole detected in water bodies in the Czech Republic in 2014. (Based on online database of the Czech Hydrometeorological Institute[7]).

Compound	Maximum concentration (ng/l)	% of values > 100 ng/L
Metolachlor	5800	2.5
MOA	4200	5.1
MESA	4200	22.6
Imidacloprid[8]	420	0.3
Propiconazole	1610	0.1

[6] A total of more than 3000 samples; available at http://hydro.chmi.cz/pasporty/
[7] http://hydro.chmi.cz/pasporty/
[8] Values for 2017

3. Model organisms

Fish have important functions in aquatic communities and are privileged tools for evaluating the impacts of eventual contamination of aquatic ecosystems. Their importance is without a doubt based on their unique position at the top of the ecological food web of aquatic ecosystems and certain similarities with human physiology and development. However, in light of animal welfare and their protection from unnecessary sacrifices or pain, the "Three Rs" principles was adopted in 1959 (Russell et al., 1959). Three Rs stand for Replace, Reduce, and Refine. This ethical principle aims to reduce the number of animals needed and refine the methods of breeding, keeping, and testing animals to minimize their pain or stress during their life span. Most importantly, the goal is to replace the standard toxicity tests on vertebrates with **alternative tests** such as *in silico* (computer predictions) or *in vitro* (cell culture) methods, or by substituting vertebrate models with invertebrates which supposedly feel less pain or using early life stages of vertebrate models such as embryos or larvae of fish (Fenwick et al., 2009). All these alternative methods grew substantially in importance during the last few decades. In 2013, EU Directive 2010/63/EU, which is based on the Three Rs tenet, was adopted in the European Union. It protects, as stated in the Directive, (a) live non-human vertebrate animals, including (i) independently feeding larval forms; and (ii) fetal forms of mammals as from the last third of their normal development; (b) live cephalopods. The same approach to the welfare of animals has been adopted also by other regulations including REACH, European regulation of industrial chemicals and their impact on human health and the environment, which promotes alternative tests for hazard assessment (ECHA, 2017). It is also reflected in principles applied by European Food Safety Agency (EFSA, 2019) and European Medicines Agency (EMA, 2016).

3.1. Pacific oyster (*Magallana gigas*)

Pacific oyster, a bivalve, is known as *Magallana gigas* or *Crassostrea gigas* due to the on-going scientific discussion (Bayne et al., 2017; Salvi et al., 2014; Salvi and Mariottini, 2017). The Pacific oyster is a primordial species in Arcachon Bay, both from the economic and ecological point of view. It is a euryhaline organism, relatively tolerant to changes in salinity and temperature (His et al., 1989). Its early life stages, embryos, and larvae are sensitive to aquatic pollution. Therefore, they provide a suitable model for assessing the toxicity of chemical compounds. Furthermore, biotest with oyster early life stages is standardized by French norm AFNOR (NF ISO 17244) and has been successfully used to assess the toxicity of numerous

chemicals, including pesticides (Akcha et al., 2011; Gamain et al., 2017; Geffard et al., 2001; Mai et al., 2013; Rondon et al., 2016).

Oyster embryos present numerous advantages: can be obtained in great numbers (one pair of adult oysters can produce millions of embryos at once), develop rapidly (so-called D larva is formed after 24 hpf, hours post-fertilization), feed endotrophically during the first two days, can be easily assessed for developmental malformations due to its typical D shape, sensitive behavioral tests showing neurotoxic potential can be carried out, etc. (Capela et al., 2020; Gamain, 2016; His et al., 1997). The life cycle of the Pacific oyster is shown in Figure 8. D larva at 24 hpf measures around 60 μm.

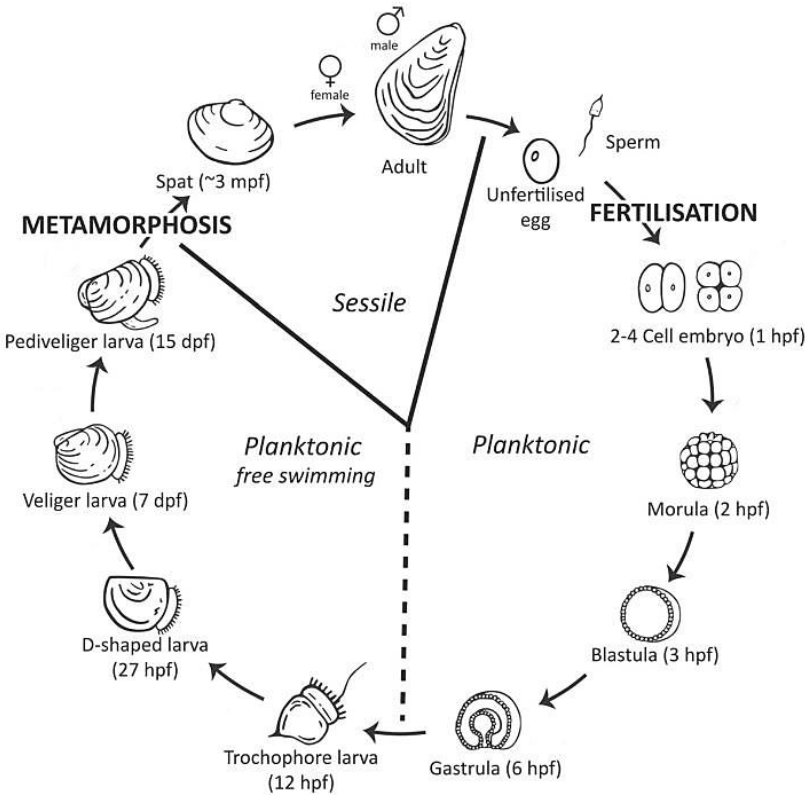

Figure 8 Life cycle of Pacific oyster (*Magallana gigas*) reprinted from (Vogeler et al., 2016).

3.2. Zebrafish (*Danio rerio*)

Zebrafish is a freshwater fish of the *Cyprinidae* family. It is broadly accepted vertebrate model in (eco)toxicology research as well as in other disciplines such as biomedicine, genetics, neurophysiology, etc. Its early life stages are used in alternative toxicity studies and comply with the Three Rs principle. Moreover, biotest with early life stages of zebrafish is standardized by OECD guideline no. 210 (2013). This alternative test is commonly used to assess the toxicity

of pesticides (Crosby et al., 2015b; Mu et al., 2016; Quintaneiro et al., 2017; Stevanovic et al., 2017; Tu et al., 2013).

Zebrafish ecology is described in detail in the review of Spence et al. (2008). This tropical fish originates in South Asia (Ganges and Brahmaputra river basins) but is often kept as aquarium fish throughout the world. It is around 40 mm long and has typical longitudinal dark blue stripes. Zebrafish is tolerant to range of environmental conditions e.g. capable of living in temperatures ranging from 6 to 38 °C. It prefers slow-moving or lentic water bodies. Zebrafish are omnivorous, feeding mostly on zooplankton which is abundantly present in rice fields where we can often find dwelling the zebrafish populations. Hence its name "danio" from the Bengali language "of the rice field" (Talwar and Jhingran, 1991).

Zebrafish is easy and quick to breed and its genome has been fully sequenced. Another advantage of using zebrafish is the possibility of high-throughput screening (Love et al., 2004) and possibility to choose a specific strain or zebrafish mutant according to assessed biomarker (Rafferty, 2018). Furthermore, vertebrate biology, i.e. similarities between zebrafish and human development and physiology, allow for extrapolation of results e.g. regarding thyroid metabolism disruption (Spaan et al., 2019) or use of zebrafish in human disease research (Van Dam and De Deyn, 2011; Willemsen et al., 2011).

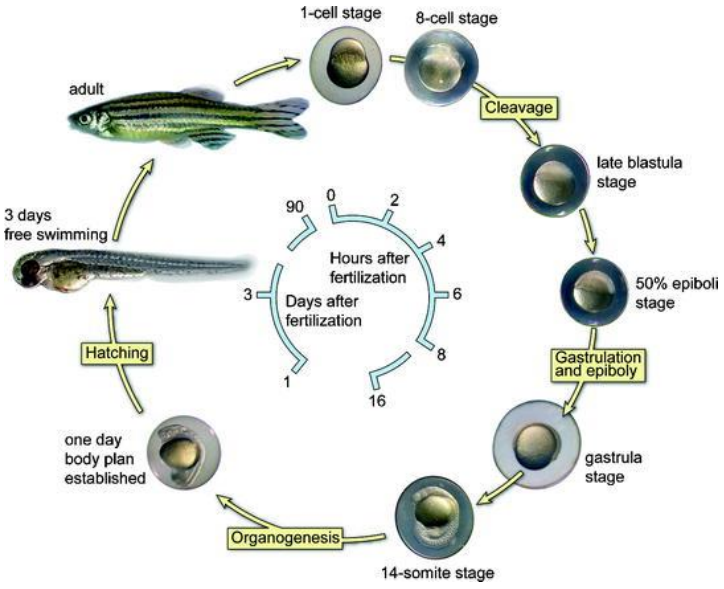

Figure 9 Life cycle of zebrafish (*Danio rerio*) reprinted from (Willemsen et al., 2011).

Female zebrafish can spawn eggs (0.7 mm) a few times a week and may lay several hundred eggs per one spawning. The fertilization is external. Embryos are transparent thus facilitating

their manipulation and evaluation of their fast development. First, embryos undergo cleavage and are nourished via yolk sac until 120 hpf (hours post fertilization). At 48 hpf primary organ systems are formed, and embryos start to hatch. Larvae start actively to swim approximately at 72 hpf, when the swim bladder inflates (Kimmel et al., 1995). Acute toxicity tests are done usually until 4 or 5 dpf (days post-fertilization) since the larvae are exempt from the Directive 2010/63/EU. The life cycle of zebrafish is shown in Figure 9.

4. *In situ* bioassays

Conventional laboratory bioassays have advantages of standardization, good control of known conditions (temperature, pH, oxygen), and reproducibility. However, they also have several disadvantages such as less ecological relevancy, impossibility to replicate the real environmental conditions observed in the field (meteorological events, natural water content, natural stressors, ultraviolet light, etc.). Standardized laboratory models also cannot fully reflect complex pollutant mixtures detected in the field (Amiard-Triquet, 2015; Ferrari et al., 2013). Therefore, in some situations, bioassays carried out in the field may be uniquely advantageous, fill the knowledge gaps, and complement the conventional laboratory bioassays. There are several approaches to carry out field experiments. First, environmental samples (water, sediments, etc.) are collected and organisms are exposed to them or their dilutions in the laboratory. Second, organisms (ideally indigenous species) are exposed in the field and can be collected after exposure and brought in the laboratory, where the assessment of chosen biomarkers is carried out. Alternatively, exposed organisms are screened in the field by an online biomonitoring system (Amiard-Triquet, 2015). On the other hand, also field studies have limitations of their own. It is difficult to directly link observed effects with the cause and there is sometimes limited control and replicability of the results (Buchwalter et al., 2017). The comparison of the above-mentioned approaches is summarized in Figure 10.

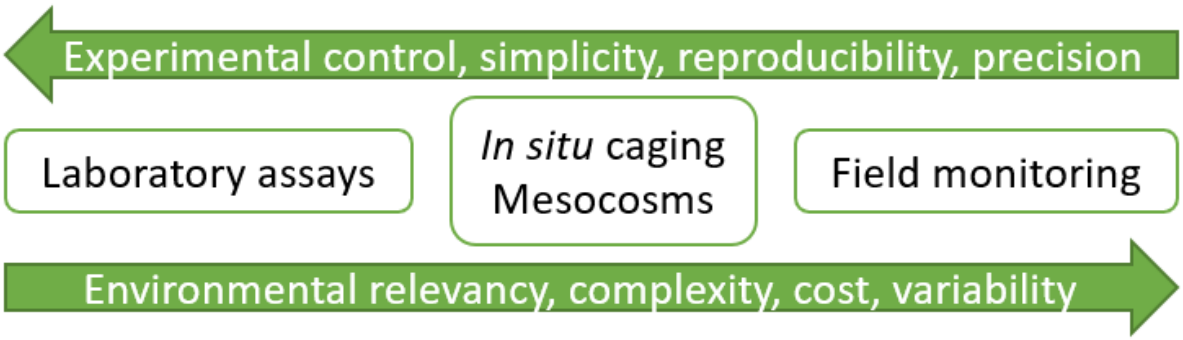

Figure 10 Relationship and properties between the main ecotoxicological approaches in toxicity assessment.

In this work, the *in situ* caging bioassay is designed as an experiment carried out in the field during which organisms are held in appropriate caging devices and are being affected by real environmental conditions. This allows to measure the effects inflicted by the quality of the (polluted) environment on selected organisms (Ferrari et al., 2013).

However, few obstacles need to be addressed in *in situ* bioassays. First, the reference site should be carefully chosen because of the risk of contamination or presence/absence of other (unknown) factors such as nutrition, etc. Second, the caging device must be well designed, appropriate for the type and number of organisms used, and should be protected against vandalism (Burton et al., 2005; Crane et al., 2007).

Bivalve organisms such as mussels and oysters are routinely used in *in situ* bioassays (Beyer et al., 2017; Brooks et al., 2012; Haynes et al., 1995; Hédouin et al., 2011; Jenny et al., 2016; Lee and Birch, 2016; Lehtonen et al., 2016; Morroni et al., 2018; Stachowski-Haberkorn et al., 2008; Turja et al., 2015) and have even a standardized procedure for their caging experiments (ASTM, 2013). Often, adult bivalves are used in caging studies to assess bioaccumulation and other biomarkers, since they are filter-feeders and are easy to collect and handle (Salazar and Salazar, 1996).

In conclusion, small-scale laboratory experiments have lower relevance for environmental issues but when combined with field testing they provide a complex profound understanding and insight (Buchwalter et al., 2017).

CHAPTER II.

ANALYTICAL STRATEGY

General design of the studies

Study I.

A spectrum of biological effects of separate exposures of pesticides imidacloprid and propiconazole on the early-life stages of Pacific oyster was evaluated. Furthermore, the impact of a novel synthesized propiconazole nanoformulation was also assessed.

Study II.

Mixture toxicity of five pesticides routinely detected in Arcachon Bay in France on early-life stages of Pacific oyster was assessed. The laboratory assessment was coupled with *in situ* oyster caging study on three different sites of Arcachon Bay undergoing different pesticide pressure.

Study III.

Sublethal effects of herbicide S-metolachlor, its two metabolites, and their mixture on zebrafish early-life stages were analyzed. Particular focus was placed on alterations of the thyroid metabolism and signaling.

Study IV.

Mixture toxicity of five pesticides, as well as individual toxicity of imidacloprid and propiconazole on early-life stages of zebrafish, was assessed.

Study V.

Sublethal acute and (sub)chronic toxicity of environmental (and higher) concentrations of imidacloprid on larvae of non-target aquatic species *Chironomus riparius* was investigated.

Husbandry of organisms and embryo collection

Two main model organisms were used in the mentioned studies: zebrafish and oyster. Selected life stages for the bioassays were the embryo-larval early-life stages. Work with both zebrafish and oysters is described in this chapter. Moreover, larvae of midge *Chironomus riparius* were used to complement our studies, thus they will be briefly described as well.

Zebrafish (*Danio rerio*)

Zebrafish used for our studies was held in glass aquariums with tap water in RECETOX laboratories, Masaryk University, Czech Republic following appropriate guidelines (ISO, 2008; OECD, 2013b). Water temperature was set to 26 ± 1 °C and the photoperiod to 14 h light and 10 h dark. Fish were fed with live brine shrimp (Artemia salina) once a day and twice a day with a mixture of dry aquarium fish feed composed of spirulina, micro flakes, Gammarus shrimps, and tubifex worms.

For study III., a wild type unspecified zebrafish strain was used. Wild type zebrafish strain AB, which was received as a gift from J. Legradi, Vrije Universiteit Amsterdam, was used for study IV.

Embryos were collected in collection boxes which are placed in the aquariums the evening before the experimentation. In the morning, embryos were meticulously sorted and exposed at 3 hpf. The bioassays last 120 hpf, at 26 °C, and the solutions were not renewed during the test. Zebrafish larva reaches around 3.9 mm at 120 hpf. The bioassay was carried out following the OECD guideline (OECD, 2013a).

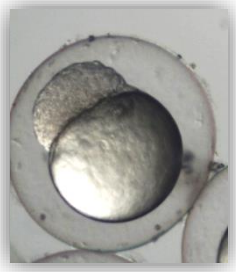

Figure 11 Zebrafish embryo at 3 hpf (original image by the author of the dissertation).

Pacific oyster (*Magallana gigas*)

Mature adult oysters were received from Guernesey Sea Farm hatchery (Guernesey, UK) and were used for the laboratory approach in studies I. and II. For the *in situ* approach in study II., mature adult oysters were purchased from France Naissain (Bouin, France).

The bioassays were carried out in EPOC Laboratory, University of Bordeaux (France) with the early-life stages of Pacific oyster from embryos until D larva stage (which is around 60 µm long). Experiments followed the French guideline (NF ISO 17244, 2015) and the revised version of the oyster embryo-larval bioassay (Leverett and Thain, 2013). Mature oysters were thus subjected to thermal shocks (Figure 12) of 18 and 28 °C to induce spawning, which is also enhanced by adding diantlin, a hormone present in sperm (Dupuy et al., 1977). Embryos are then collected and kept at 24 °C during the duration of the bioassay.

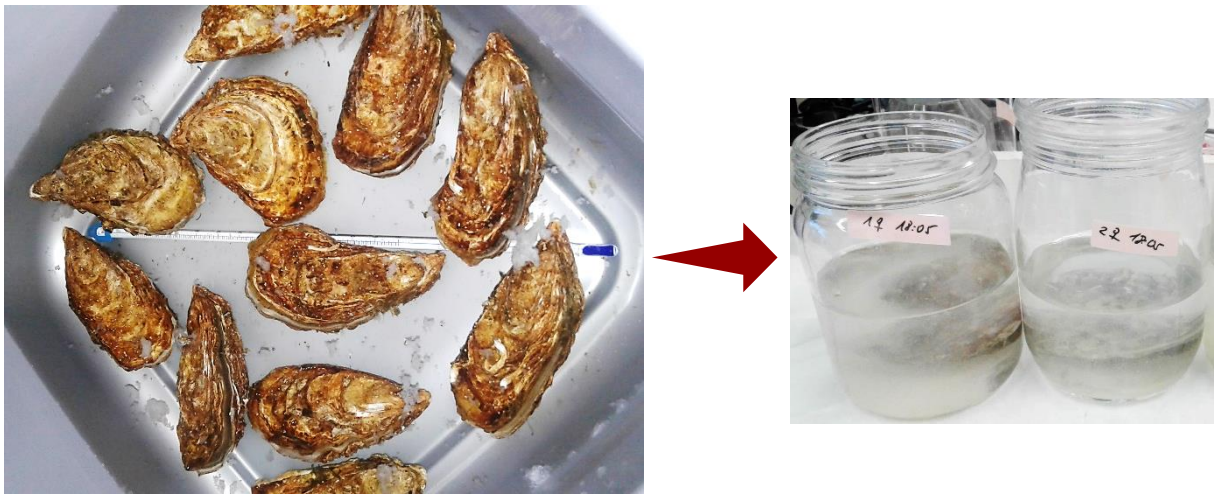

Figure 12 Oyster spawning induced by alternating thermal shocks (original images by the author of the dissertation).

The overview of exposure of the two major model organisms: Pacific oyster and zebrafish used in this dissertation thesis is summarized in Table 3.

Midge *Chironomus riparius*

Larvae of *Chironomus riparius* are routinely used in ecotoxicity testing and possess many advantages such as short lifecycle, simple maintenance, and sensitivity to pollutants. Moreover, the larvae have an important position in the food web, with fish as their typical predators (Ha and Choi, 2008). Larvae of this midge were used in study V.

The nonbiting midge culture was maintained in aquaria at 20 ± 0.5 °C and the photoperiod of 16 h light and 8 h dark at RECETOX laboratories, Czech Republic.

Table 3 Summary of exposure conditions of organisms

Endpoint	Organism	Duration	Exposure chamber	Volume and type of medium per rep.	N° of embryos per rep.
Malformations	Oyster	30 hpf	Plastic 24 well plate*	2 mL, seawater	225
	Zebrafish	5 dpf	Glass crystallization dishes	20 mL, ISO medium	20
Swimming patterns	Oyster	24 hpf	Plastic 24 well plate*	2 mL, seawater	225
Locomotion light-dark test	Zebrafish	5 dpf	Glass crystallization dishes > transferred to plastic 96 well plate in the evening of 4 dpf	200 µL after the transfer, ISO medium	1 per well, 32 per condition in the microplate
Spontaneous movement	Zebrafish	21 hpf	Glass crystallization dishes	20 mL, ISO medium	20
Heartbeat	Zebrafish	3 dpf	Glass crystallization dishes	20 mL, ISO medium	20
Gene expression	Oyster	42 hpf	Glass and plastic beakers (depending on the compound)	3 L, seawater	500,000
	Zebrafish	5 dpf	Glass crystallization dishes	20 mL, ISO medium	20
In situ	Oyster	2 dpf	HDPE caging device > transferred into plastic 24 well plate after the exposure	4 L, seawater	666,000

*Precoated when testing with a hydrophobic compound

Effect biomarkers of interest

Effect biomarkers can be defined as "indicators or signaling events in biological systems or samples of measurable changes at the molecular, biochemical, cellular, physiological, pathological, or behavioral levels in response to xenobiotics" (Gupta, 2014).

The sublethal biomarkers, which are generally early and sensitive indices of chemical stress (Amiard-Triquet and Berthet, 2015), were the focus of this dissertation thesis. However, any eventual mortality was also checked every day of the bioassays (in the case of zebrafish).

Developmental abnormalities

The developmental abnormalities are a biomarker of good development of the early-life stages, which are critical and often very sensitive to harmful substances. While zebrafish embryos have advantage (in comparison with some other species) by the presence of a protective layer chorion during the first days of their life, Pacific oyster embryos, on the other hand, start developing a shell after 10 hpf (trochophore stage; shown in Figure 12 B) and the shell is fully formed between 24 and 48 hpf at the stage of D shell larvae (Waldbusser et al., 2013). Indeed, malformations of the shell (concave shell and scalloped shell) or malformations of the mantle are amongst those often evaluated. The developmental arrest of the oyster embryo is considered a lethal effect. Different types of oyster developmental abnormalities as well as a well-formed D-larva are shown in Figure 13.

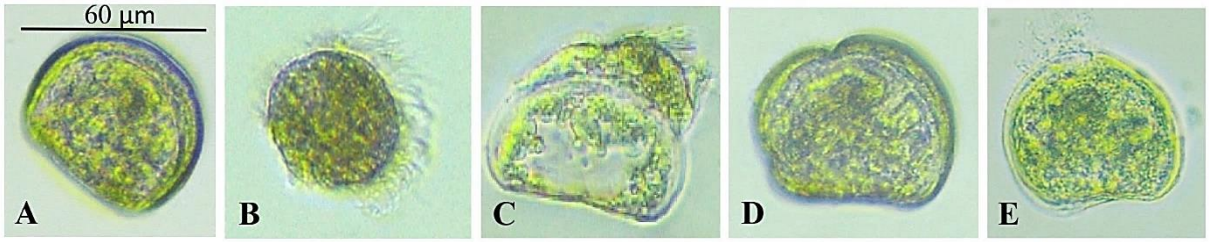

Figure 13 Different types of developmental malformations of oyster larvae (*Magallana gigas*) at 30 hpf: well-formed D-larva (A), developmental arrest (B), mantle malformation (C), (scalloped) shell malformation (D), (concave) shell malformation (E). (Original images by the author of the dissertation).

Malformations of zebrafish were evaluated at 120 hpf when the fish larvae possess already all vital organs (some not yet fully developed) i.e. heart, brain, digestive system (mouth, liver, pancreas, gut, open anus), swimming necessities (fins, inflated swim bladder), eye, etc., and the yolk sac is almost resorbed (Kimmel et al., 1995; Strähle et al., 2012). Most of the

morphogenesis is completed at 3 dpf (Kimmel et al., 1995). Thus, the evaluation of developmental abnormalities is more complex than in oyster larvae. Examples of zebrafish malformations are shown in Figure 14. Poorly developed larvae (both oyster and zebrafish) are disadvantaged in nature; they are more likely to become prey. Moreover, their biological functions may be affected e.g. immune system defending the organism against the infection, swimming activity influenced by non-inflated swim bladder, feeding hindered because of a malformed digestive system, etc.

Figure 14 Examples of zebrafish malformations observed at 120 hpf during various bioassays of this dissertation thesis. Well-developed zebrafish larvae (A); Mild craniofacial malformation (B); Non-inflated swimming bladder and mild malabsorption of yolk sac (C); Severe craniofacial malformation, malabsorption of yolk sac, no swim bladder (D); Severe craniofacial malformation, malabsorption of yolk sac, no swim bladder, heart edema (E); Severe craniofacial malformation, malabsorption of yolk sac, non-inflated swim bladder, spinal and tail deformation (F). (Original images by the author of the dissertation).

Hatching

Hatching of the zebrafish embryos takes place asynchronously on the 3 dpf or later. The morphogenesis is progressing independently on the hatching stage of the embryo so embryos hatched earlier or later should not be disadvantaged (Kimmel et al., 1995). However, it seems that the time of hatching affects fish behavior, e.g. late-emerging salmonids were observed to have a more proactive style and be bolder (Andersson et al., 2013). Moreover, Leite-Ferreira et al. (2019) reported different effects of alcohol on late and early emerging zebrafish including higher sensitivity to the alcohol exposure. Fungicide difenoconazole was observed to inhibit

the hatching success of zebrafish embryos and this function was likely linked to yolk sac malabsorption (Mu et al., 2016).

Behavior analysis

Behavioral alterations are a sensitive biomarker affected already by exposure to low doses of contaminants, such as environmental concentrations of pesticides. The textbook cause of impaired behavior is inhibition of acetylcholine esterase, which can be affected e.g. by organophosphate and carbamate pesticides (Amiard-Triquet and Berthet, 2015). Monitoring of changes in behavior may be used for *in situ* evaluation of the ecological status of individuals and populations of different species (Weis et al., 2011). Neurotoxicity expressed as an effect on behavior may be evaluated in embryos and larvae by various means. Oyster larvae, already at 1 dpf, exhibit swim at different swimming speeds depending on environmental conditions and employ different swimming trajectories. At the stage of endotrophically feeding D-larva, a rectilinear trajectory is considered normal, however, when the larvae start to feed and hunt prey, helical swimming is the most advantageous trajectory for invertebrate larvae in general (Maciejewski et al., 2019). Swimming activity is routinely evaluated also in zebrafish, with a light-dark response locomotor test being often employed. Even embryos as young as 1 dpf exhibit their first movement by spontaneously coiling their tails (Figure 15), which is a suitable biomarker of the advancement of the nervous system development.

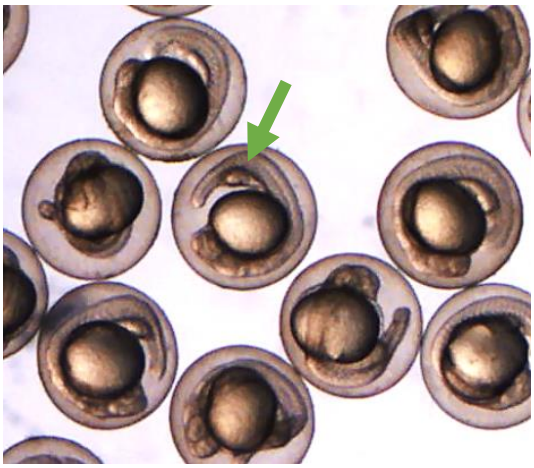

Figure 15 Zebrafish embryos at 22 hpf; the arrow is pointing at the tail. Assessment of the tail coiling (several times per minute: depending on the age of the embryo and environmental conditions such as the presence of a chemical) is used as the effect biomarker. (Original image by the author of the dissertation).

Heartbeat

Heart rate analysis is a non-invasive method used for measuring the effects on autonomic nervous system activity. Moreover, the human cardiotoxicity of various compounds may be predicted possibly in most cases (>80%) using the zebrafish model (Milan et al., 2003; Strähle et al., 2012). The heart of zebrafish during the measurement is shown in Figure 16.

Figure 16 Zebrafish larvae at 3 dpf; the arrow is pointing at the heart (original image by the author of the dissertation).

Gene expression analysis

Gene expression biomarkers reveal changes in the transcription levels of genes which could reflect the amount of proteins for which the genes are coding. Consequently, changes in the concentration of these proteins influence various other biochemical biomarkers. The technique used in this work, qPCR, involves normalizing the target gene expression to the reference's ones, i.e. genes which are expressed constitutively without any impact of the studied agent. This quantitative PCR analysis was focused on a subset of well-selected target genes. Another approach is e.g. the microarray and the next generation sequencing (NGS) techniques which can analyze the whole transcriptome at once (Gonzalez and Pierron, 2015; Piña et al., 2007). To gather sufficient amount of RNA for qPCR analysis, 20 pooled zebrafish larvae and 30,000 pooled oyster larvae are needed for one replicate. Oyster exposure beakers for the qPCR analysis are shown in Figure 17.

Figure 17 Glass or plastic three-liter beakers (depending on the hydrophobicity of the studied compound in the solution) used for the exposure of oyster larvae to pesticides in high yield. One beaker contains 500,000 larvae released by one mature oyster couple. The beakers are constantly oxygenated using aquarium airstones and kept in dark (due to the fast photodegradation of imidacloprid) in an incubator to maintain a constant temperature of 24 °C. (Original image by the author of the dissertation).

The present study used *β-actin*, *ef1α,* and *rpl7* as reference genes due to their stable expression in oyster analysis and *β-actin*, *ef1a,* and *rpl13* in zebrafish analysis. The studied target genes are summarized in Table 4 and individual biological functions briefly described below.

Table 4 Genes of interest in qPCR analyses with their respective functions

Gene	Function	Gene	Function
12S *cox*	Mitochondrial metabolism	*bax* *casp3*	Apoptosis
p53	Regulation of the cell cycle/apoptosis	*gadd45*	Growth arrest and DNA damage repair
cat	Oxidative stress defense	*rad51*	DNA repair
sodMn *sodCu/Zn* *gpx*		*dio1* *dio2* *dio3*	Iodothyronine deiodinases
mt1 *mt2*	Detoxification	*thra* *thrb*	Thyroid hormone nuclear receptors
cyp1a	Biotransformation	*cyp26a1*	Retinoic acid signaling pathway

Mitochondrial metabolism is crucial for the good functioning of the cell and the health of the organisms. Its principal role is to synthetize ATP via oxidative phosphorylation which includes five electron transport chains. The last 4th complex before the synthesis of ATP contains cytochrome c oxidase enzyme encoded by genes *cox*. The gene 12S is, on the other hand, reflecting the mitochondrial DNA quantity (Achard-Joris et al., 2006; Florane Le Bihanic, 2013; Gamain, 2016).

Apoptosis is programmed cell death and is essential e.g. during the development, cell turnover, or in elimination of cancerous or infected cells. Importantly, if the apoptosis cannot take place, it can lead to cancers and diseases. The beginning of apoptosis is initiated by the cleavage and thus activation of a cysteine protease caspase 3 (coded by gene *casp3*). Moreover, the apoptosis (as well as the cell cycle which may lead to cell growth arrest) may be regulated by tumor suppressor protein *p53* through multiple complex mechanisms. One of the mechanisms consists of regulation of the pro-apoptotic proteins such as *bax* (Elmore, 2007; Haupt et al., 2003).

Oxidative stress is a general nonspecific form of toxicity and takes place when there is an imbalance between the presence of reactive oxygen species (e.g. O_2^-, ·OH, H_2O_2) and the antioxidant defense systems (Figure 18). The most important genes selected for this study were genes *cat*, *sodMn*, *sodCu/Zn*, and *gpx* coding for enzymes catalase, cytoplasmic superoxide dismutase Cu/Zn and mitochondrial superoxide dismutase Mn, and glutathione peroxidase, respectively.

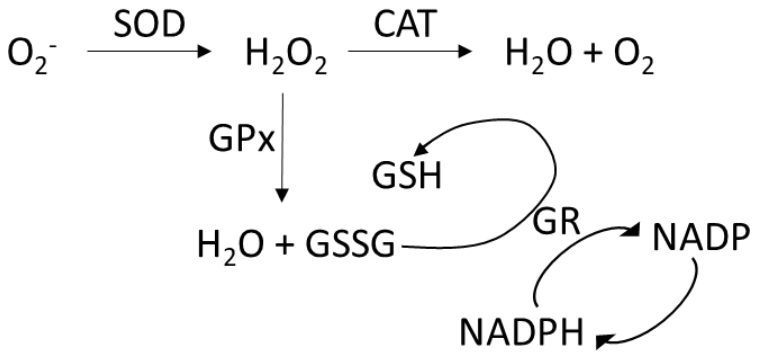

Figure 18 Schema of antioxidant system: CAT catalase; SOD superoxide dismutase; GPx glutathione peroxidase; GR glutathione reductase; GSH reduced glutathione. Adapted from Almroth (2008).

Metallothioneins are intracellular cysteine-rich proteins that can bind metals and have redox abilities which allow them to interfere with multiple biochemical processes. They protect against toxic metals, oxidative radical species, and inflammation (Coyle et al., 2002; Ruttkay-Nedecky et al., 2013). Two genes *mt1* and *mt2* coding for two metallothionein isoforms are assessed in this work.

Cyp1a enzyme of the cytochrome P450 superfamily is routinely used as a biomarker of aquatic pollution. It biotransforms various organic pollutants including polychlorinated biphenyls, dioxins, polycyclic aromatic hydrocarbons but also pesticides. The gene expression of *cyp1a* is induced by aromatic compounds, making thus this enzyme a biomarker of organic pollution (Uno et al., 2012).

The Growth Arrest and DNA Damage-inducible (GADD45) protein is a small ubiquitous protein with multiple important functions such as regulation of DNA repair mechanisms, cell cycle control, senescence, genotoxic stress, and apoptosis. *Gadd45* is induced after DNA damage and its malfunctioning may cause cancer induction and progression (E. Tamura et al., 2012).

DNA repair is necessary when the organism is exposed to various contaminating substances inducing DNA damage. Protein RAD51 (encoded by *rad51* gene) plays a major role in homologous recombination of DNA during double strand break repair creating a new homologous DNA sequence to the broken one and replace it (Laurini et al., 2020).

Thyroid metabolism significantly influences the development of early-life stages (Jarque and Piña, 2014) and its impairment may cause strong adverse effects on the organism. It is a complex system and thus to evaluate its functioning and disruption, multiple biomarkers need to be assessed including morphology (eye malformation, yolk sac malabsorption, unsuccessful hatching), behavior, hormone levels, or gene expression of genes involved in the hypothalamus-pituitary-thyroid axis (Spaan et al., 2019). Genes which were evaluated in this work (overview shown in Figure 19) were iodothyronine deiodinases *dio1*, *dio2*, *dio3*, that (de)activates thyroid hormones, and the thyroid nuclear receptors *thra*, *thrb* (ligand-dependent transcription factors), to which the thyroid hormones bind and thus demonstrate their biological effects (Marchand et al., 2001).

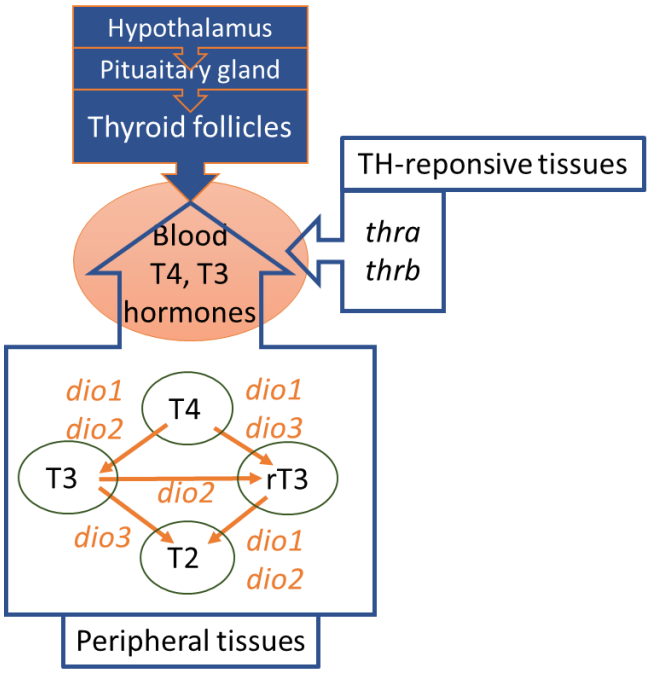

Figure 19 Overview of the elements of interest of the hypothalamus-pituitary-thyroid axis: hormones T4, T3; metabolites T2, rT3; genes coding for iodothyronine deiodinases *deio1-3* and thyroid receptors *tra, trb*. Modified from Jarque and Piña (2014) and Spaan et al. (2019).

The retinoic acid signaling pathway plays a crucial role during embryonic vertebrate nervous development. Retinoic acid (a metabolite of vitamin A and a known teratogen) is inactivated by cyp26 family enzymes (*cyp26a1*, *cyp26b1*, and *cyp26c1*). The retinoic acid deficiency hinders the establishment of the anterior-posterior pattern in the hindbrain. Retinoic acid then acts as a ligand for nuclear retinoic acid receptors and thus regulates gene transcription (Hernandez et al., 2007; Pípal et al., 2020; Uno et al., 2012).

Chemical analysis

Bioassays performed in this dissertation thesis were always complemented with chemical analysis of pesticides and metals (in the case of seawater) at the beginning and at the end of the tests. Although analyses of the zebrafish medium were carried out routinely with LC-MS/MS, the analyses of seawater needed optimization of the process. Indeed, it was necessary to remove the salts before the analyses. This was achieved in cooperation with Lucie Bláhová from RECETOX laboratories, where the pesticide analyses were carried out. To remove the salts, the seawater was first lyophilized, followed by sample dissolution in acetonitrile which precipitated the salts but at the same time extracted the analytes into the solvent phase.

Statistical analysis

For most assessed endpoints, the results were statistically analyzed following the scheme in Figure 20. Exceptions are specified in respective publications/manuscripts. Usually, results were normalized (e.g. log normalization of gene expression results) or transformed (e.g. arcsin transformation of oyster larvae malformation) before the statistical analysis. All details are always listed in corresponding publications/manuscripts. All statistical analyses were carried out using Statistica 13.3 (StatSoft, USA) or Graph Pad Prism 5 (Graph Pad Software, USA) for the EC_{50} determination.

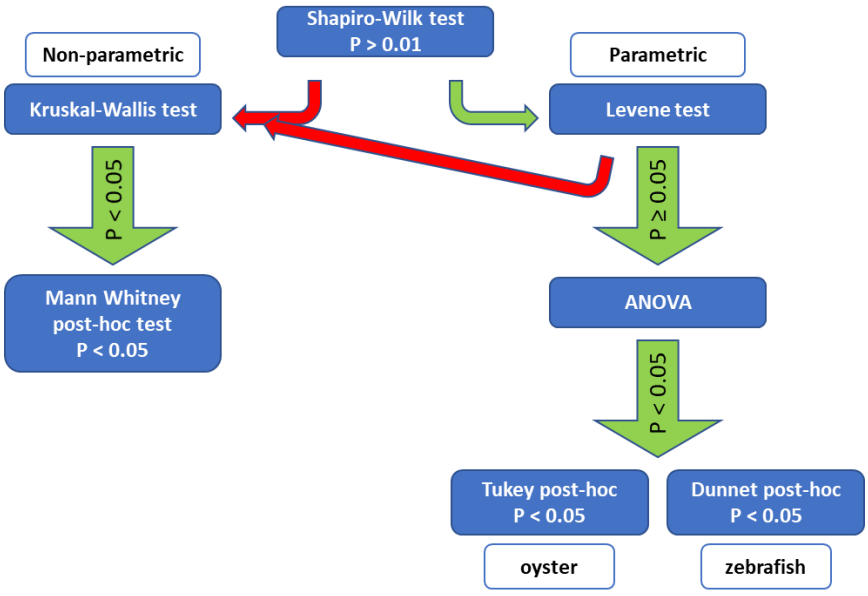

Figure 20 Scheme of statistical analyses used for the assessment of different endpoints in this thesis. Green arrows indicate the fulfillment of the condition, red arrows indicate alternative path.

RESULTS AND DISCUSSION

CHAPTER III.

WHAT ARE THE RISKS OF PESTICIDES TO PACIFIC OYSTER?

The Pacific oyster (*Magallana gigas*) is inhabiting brackish waters of coastal areas such as estuaries. The estuaries are however the final recipients of various (anthropogenic) pollutants brought in the estuary via tributaries which often traverse long distances and gather water of vast watersheds. Unfortunately, the oyster, as well as other indigenous organisms are exposed to a mixture of contaminants which may have adverse effects on its lifespan.

Accordingly, we focused our attention on a particular bay in France: Arcachon Bay, where oyster farms form the heart of the bay. We first identified the most detected insecticide, fungicide, and herbicide in Arcachon Bay and their concentrations based on regular analytic monitoring of the REPAR network. To confirm our hypothesis that these pesticide concentrations affect the development of oyster larvae, we exposed the embryo-larval stages of the Pacific oyster in the laboratory to the individual pesticides (**Publication I.**) and their mixture (**Publication II.**). A secondary prospective study also assessed effects of the nanoformulated pesticide form to oyster larvae (**Publication I.**). This approach was complemented with a field campaign, carried out in the summer of 2019 when the embryo-larval stages of oyster were deployed in caging devices on several, differently contaminated, sites of Arcachon Bay and exposed to the environmental conditions for two days (**Publication II.**).

In both laboratory and *in situ* approaches, the larvae were collected and subjected to developmental, behavioral, and molecular analysis.

Because the photographic documentation of the *in situ* campaign is not extendedly presented in the original manuscript (**Publication II.**), the following Figures (21-23) as well as Table 5 are used to illustrate the fieldwork.

Figure 21 Images illustrating the field work in Arcachon Bay. **A** Arrival to one of the selected buoys; **B** Boat filled with the devices full of water and larvae being transported to the laboratory at the end of exposure to be analyzed; **C** Preparation of devices to the sailor to attach them at the buoy; **D** Water sampling for the chemical analysis of pesticides

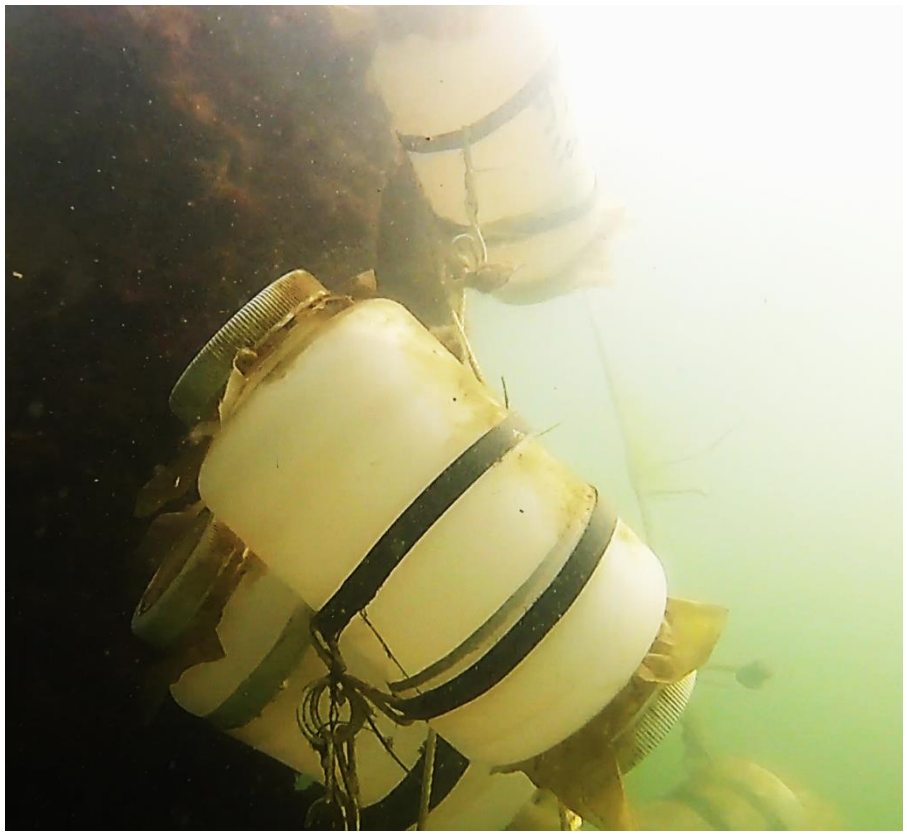

Figure 22 Deployment of the caging devices by sailor Stéphane Bujan. Four devices were attached to each of the three buoys

Figure 23 Localization of areas of interest in Arcachon Bay (source: googlemaps.com)

Table 5 GPS coordinates, localization, and number of used buoys of areas of interest

Site name	Grand Banc	Les Jacquets	Comprian
Buoy n°	Balise 1	Balise 9	Balise 16
GPS coordinates	44° 39.914' N 001° 13.076' W	44° 42.831' N 001° 11.235' W	44° 40.833' N 001° 07.096' W
Channel	Chenal du Teychan	Chenal de l'Île	Chenal du Teychan

PUBLICATION I.

COMPARISON OF IMIDACLOPRID, PROPICONAZOLE, AND NANOPROPICONAZOLE EFFECTS ON THE DEVELOPMENT, BEHAVIOR, AND GENE EXPRESSION BIOMARKERS OF THE PACIFIC OYSTER (*MAGALLANA GIGAS*)

Eliška Kuchovská, Bénédicte Morin, Rocío López-Cabeza, Mathilde Barré, Corentin Gouffier, Lucie Bláhová, Jérôme Cachot, Luděk Bláha, Patrice Gonzalez

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Supplementary Materials:

https://ars.els-cdn.com/content/image/1-s2.0-S0048969720364512-mmc1.docx

Main findings of Publication I.

- The environmental concentrations of the two studied pesticides: imidacloprid and propiconazole, detected in Arcachon Bay seem to be safe for the development of oyster embryo-larval stages (as evaluated by the presence of malformations). The maximum detected concentrations in Arcachon Bay (2010-2014) were 174 ng/L and 30 ng/L of imidacloprid and propiconazole, respectively. However, the observed LOECs were 200 µg/L for both pesticides.
- Oyster larvae were less sensitive to imidacloprid with the EC_{50} for malformations exceeding the highest tested concentration of 200 mg/L. EC_{50} for propiconazole was 2.9 ± 1.4 mg/L. Both these concentrations are higher than what is usually detected in the aquatic environment throughout the world. However, accidental spills or some unprecedented events of imidacloprid contamination may cause adverse effects because a) imidacloprid as an insecticide is used in bigger quantities than propiconazole b) its peak detected concentration may reach tens or rarely hundreds of µg/L (Van Dijk et al., 2013) reaching thus the LOEC, and c) although its use is banned in the EU, on other continents, it is still widely used and higher environmental concentrations may be expected.
- Imidacloprid caused no effect on the locomotion patterns of oyster larvae, unlike propiconazole which decreased the average swimming speed and increased the stationary trajectory type after exposure to 2 µg/L, a concentration higher than that found in Arcachon Bay.
- Imidacloprid altered the expression of several genes (linked to detoxification, oxidative stress, apoptosis and cell cycle regulation, DNA reparation, and DNA damage) in a dose-dependent manner.
- The prospective toxicity evaluation of nanoformulated propiconazole revealed comparable developmental toxicity with conventional propiconazole but a bigger impact on larvae behavior (increased both maximal and average swimming speed). The impact on the molecular level was not strong with only few genes impacted.

PUBLICATION II.

PESTICIDE MIXTURE TOXICITY ASSESSMENT THROUGH *IN SITU* AND LABORATORY APPROACHES USING EMBRYO-LARVAL STAGES OF THE PACIFIC (*MAGALLANA GIGAS*)

Eliška Kuchovská, Patrice Gonzalez, Lucie Bláhová, Mathilde Barré, Corentin Gouffier, Jérôme Cachot, Luděk Bláha, Bénédicte Morin

Manuscript prepared for the Science of the Total Environment

Supplementary Materials are attached in annexes

Main findings of Publication II.

Laboratory approach:

- The evaluation of a mixture of five pesticides revealed high developmental toxicity to embryo-larval stages of oyster from the lowest tested concentration (0.32 μg/L), which corresponded to concentration detected in Arcachon Bay. The EC_{50} was found to be 10.70 ± 1.32 mg/L. (Concentrations are presented as the sum of concentrations of the five pesticides in a ratio corresponding to the one observed in Arcachon Bay: imidacloprid: metolachlor OA: metolachlor ESA: propiconazole: S-metolachlor = 10:10:10:1:1).
- No effects of the pesticide mixture were observed on the trajectory patterns and swimming speed of oyster larvae.
- Few effects were observed on gene expression after exposure to an environmentally relevant concentration of 0.32 μg/L, such as downregulation of genes implicated in mitochondrial metabolism, biotransformation, growth arrest, and DNA damage. Higher concentration (8 μg/L) also downregulated genes linked to apoptosis and metallothionein function.

***In situ* approach:**

- The embryo-larval caging device was successfully used in the field and may be employed to monitor the water quality of coastal areas.
- Water quality in Arcachon Bay was sufficient for the successful development of caged oyster larvae.
- This study was the first to evaluate the locomotion of oyster larvae exposed *in situ*, however, no significant effect was found on trajectory patterns or larvae swimming speed.
- The molecular assessment revealed differences in gene expression profiles of larvae exposed on three different sites of Arcachon Bay. In comparison with the reference site Grand Banc:
    - larvae at Les Jacquets had upregulated two genes implicated in oxidative stress defense and mitochondrial metabolism, and downregulated genes linked to the metallothionein function, DNA repair, and one implicated in oxidative stress response
    - larvae at Comprian site had upregulated two genes linked to oxidative stress defense, and one linked to mitochondrial metabolism but also downregulated two metallothionein related genes and one gene implicated in oxidative stress response

Pesticide mixture toxicity assessment through *in situ* and laboratory approaches using embryo-larval stages of the Pacific oyster (*Magallana gigas*)

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KEYWORDS

Developmental effect; Embryotoxicity; *In situ*; Pacific oyster; Pesticide mixture; Swimming behavior

Abstract

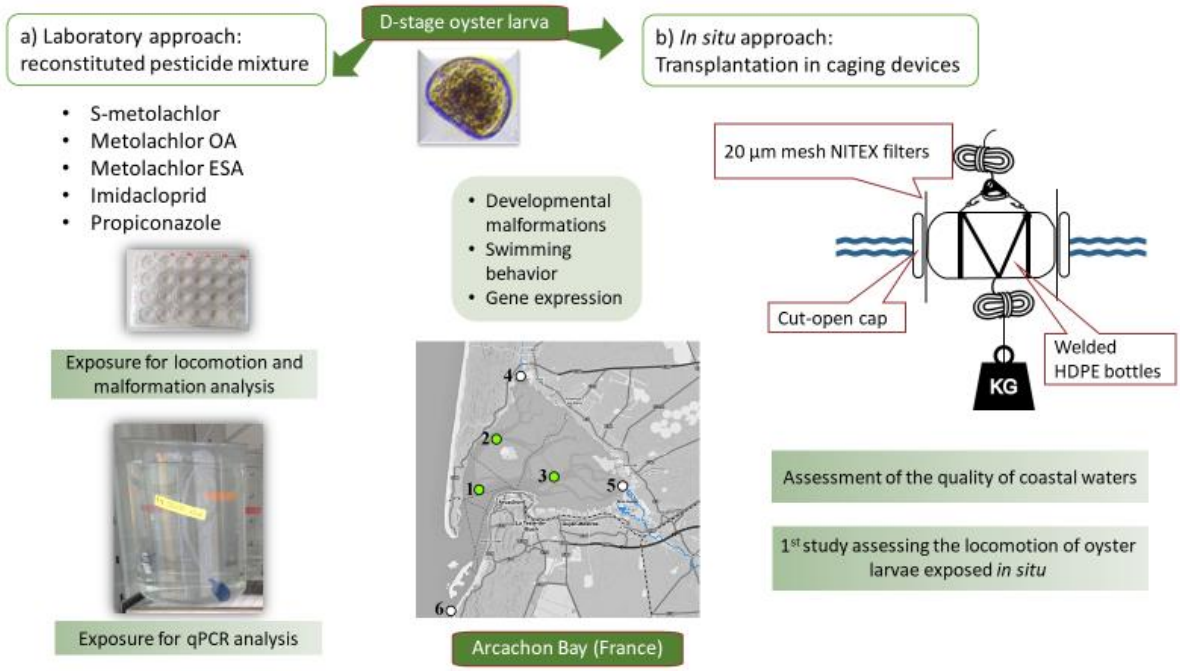
Arcachon Bay, a shallow marine lagoon on the French Atlantic coast is famous for its oyster farming. However, the worsened state of oysters and their spat in recent years demand an investigation of possible causes. Thus, this study aimed to evaluate the effects of an environmentally relevant mixture of common pesticides - herbicide S-metolachlor, its two metabolites, insecticide imidacloprid, and fungicide propiconazole - on the early-life stages of the Pacific oyster (*Magallana gigas*). Two complementary approaches, laboratory assays with artificial mixture and *in situ* transplantation in caging devices were used to investigate a series of sublethal endpoints such as developmental malformations, alterations of locomotion patterns, and gene expression levels. The laboratory exposure to the mixture revealed developmental toxicity at 0.32 µg/L (5 substances sum), which corresponds to the pesticide concentrations in Arcachon Bay. Downregulation of transcription was observed at

0.32 – 8 µg/L for genes involved in the mitochondrial metabolism, biotransformation, growth arrest, and DNA damage, and at 8 µg/L also for genes involved in apoptosis and metal regulation. A comparison of caging experiments at three sites in Arcachon Bay, each with different pollution level, revealed no difference in larvae development. To our knowledge, this study was the first to evaluate locomotion of oyster larvae exposed *in situ*. Compared to standard assay, larvae caged *in situ* (all sites) exhibited very low rectilinear (i.e. normal) trajectories, which were, however, not statistically different among sites. Interestingly, suspected poor water quality in the inner part of Arcachon Bay was reflected by impairment at the molecular level, especially with upregulated genes linked to oxidative stress defense, mitochondrial metabolism, and downregulated transcription of metallothionein genes. In conclusion, current concentrations of the tested pesticide mixture found in Arcachon Bay hinder larval development and affect several biological functions as revealed by combined laboratory and *in situ* caging experiments.

HIGHLIGHTS

- 1st study to evaluate locomotion of oyster larvae exposed in situ
- Pesticide mixture even at low concentration is not safe for oyster development
- Water quality in Arcachon Bay is sufficient for development of well-formed larvae
- Effects on the gene expression were observed at realistic concentrations
- Larvae caged at sites with different pollution had variable gene expressions

Graphical abstract



1. Introduction

Arcachon Bay is a semi-sheltered shallow lagoon (174 km^2) on the Atlantic coastline in the South-west of France (Bertrand, 2014). The Pacific oyster (*Magallana gigas*, also known as *Crassostrea gigas*), at first an invasive species on the European coast introduced from the northwest Pacific Ocean (Troost, 2010), has become an emblematic and farmed species in Arcachon Bay. For more than 20 years ago, Arcachon Bay started to face several issues such as the decline in oyster recruitment and spat collection (Auby et al., 2014), the decrease in the seagrass *Zostera* population (Auby et al., 2011; Cognat et al., 2018), and anomalies in the production of phytoplankton (Auby and Maurer, 2004). Possible causes of the oyster recruitment decline have been investigated, such as oyster herpes virus (Labbate et al., 2015) or climate change coupled with pesticide pollution (Gamain, 2016). However, the described issues have not been resolved and other causes are being searched for. Numerous hazardous substances are regularly detected in the lagoon by REMPAR, the survey network for monitoring of pesticides and micropollutants (https://www.siba-bassin-arcachon.fr/actions-environnementales/les-reseaux-de-surveillance-repar-et-rempar). Pesticides are brought into the Bay by lagoon tributaries, mainly by river Leyre (Tapie and Budzinski, 2018). While the impacts of individual pesticide compounds on non-target organisms are usually studied, we still lack information about the toxicity of environmentally relevant mixtures as also emphasized in the European Green Deal (European Commission, 2020). Mixtures can result in synergistic, antagonistic, or additive effects, as documented by Gustavsson et al. (2017) and Cedergreen (2014). For instance, neonicotinoids are known to exert addition or synergy effects when occurring in mixtures with some fungicides (Morrissey et al., 2015).

In Arcachon Bay, complex mixtures of tens of pesticides (out of a hundred of molecules screened) are often detected (Tapie and Budzinski, 2018). According to this monitoring study, some of the most commonly found molecules in the waters of the lagoon are insecticide

imidacloprid (IMI), fungicide propiconazole (PRO), and herbicide S-metolachlor (SM) with its two dominant metabolites metolachlor oxanilic acid (MOA) and metolachlor ethanesulfonic acid (MESA). IMI is a neurotoxic neonicotinoid banned in the EU, acting as agonist on theto the post-synaptic nicotinic acetylcholine receptors (Matsuda et al., 2001), PRO is a triazole hindering the synthesis of fungal cell membranes by inhibiting the ergosterol formation (Oliver and Hewitt, 2014), and SM is a chloroacetanilide suppressing the plant growth by impairing the biosynthesis of very-long-chain fatty acids (Götz and Böger, 2004). Effects of these individual compounds on the embryo-larval stages of the Pacific oyster were already assessed (Gamain et al., 2017, 2016; Kuchovská et al., 2020; Mai et al., 2014, 2013), and some of these were even present in a recent complex mixture of 14 pesticides toxicity evaluation (Mai et al., 2020).

One of the possibilities to assess the effects of environmental mixtures of pollutants are laboratory bioassays with a reconstituted mixture at relevant concentrations. More valuable insight is gained when laboratory bioassays are coupled with *in situ* caging experiments. Results obtained by this combined approach are ecologically relevant and reflecting realistic and complex site-specific conditions including not only the chemical exposure but also tidal movements, weather conditions, temperature changes, or natural presence of microorganisms, i.e. factors difficult to replicate in the laboratory (Ferrari et al., 2013). There is a long tradition in using bivalves (collected in the field or deployed via transplant caging) such as mussels or oysters in *in situ* coastal monitoring programs (Besse et al., 2012; Beyer et al., 2017). Moreover, an ASTM guideline for field bivalve testing exists (ASTM, 2013). However, these monitoring studies use transplanted adult (rarely juvenile) oysters (Cao and Wang, 2016; Clara Rebouças Do Amaral et al., 2005; Hédouin et al., 2011; Jenny et al., 2016; Lee and Birch, 2016) or mussels (Benedicto et al., 2011; Brooks et al., 2012; Cappello et al., 2015; Devier et al., 2005; Haynes et al., 1995; Lehtonen et al., 2016). One of the rare studies not empolying adult organisms was carried out with a six-week-old oyster spat in *in situ* microcosms (Stachowski-Haberkorn et al.,

2008). To the best of our knowledge, only two biomonitoring studies (Geffard et al., 2001; Quiniou et al., 2007) used the embryo-larval stages of bivalves. Early life stages of bivalves have a high sensitivity to contaminants, quick development (D-larvae is formed after 24 h), and convenient high-throughput screening format.

Accordingly, the present study aimed to investigate and compare the sublethal effects (developmental malformations, neurobehavioral locomotion patterns, and gene expression levels) on Pacific oyster larvae (*Magallana gigas*) in two complementary exposure conditions, i.e. i) laboratory exposure to environmentally relevant reconstituted mixture of pesticides and ii) *in situ* transplantation in caging devices for two days on three different sites in Arcachon Bay. This study is the first to combine *in situ* and laboratory approaches with embryo-larval stages of Pacific oyster.

2. Materials and methods

2.1 Chemicals and reference seawater handling

Imidacloprid (IMI, CAS 138261-41-3, Pestanal, purity 100 %), propiconazole (PRO, CAS 60207-90-1, Pestanal, purity 100 %), S-metolachlor (SM, CAS 87392-12-9, Pestanal, purity ≥ 98.0 %), metolachlor oxanilic acid (MOA, CAS 152019-73-3, Pestanal, purity ≥ 98.0 %), metolachlor ethanesulfonic acid (MESA, CAS 947601-85-6, Pestanal, purity ≥ 95.0 %), and $CuSO_4$ were purchased from Sigma-Aldrich. Concerning gene expression analysis, primers were purchased from Sigma proligo, RNA later buffer was purchased from Qiagen, and phenol and chloroform Rectapur ® were purchased from Sigma.

Copper solution (stock solution of 100 mg/L in milliQ water) was used as positive control in laboratory experiments and was stored at 5 °C, as well as PRO solution, which was prepared in DMSO (5 g/L) and other pesticide solutions (SM, IMI, MOA, MESA) which were prepared in milliQ water (50 mg/L). Dilutions in final exposure solutions were carried out using reference seawater collected at beach Petit Nice (approx. 44°33'40.3"N 1°14'27.1"W). Seawater was transported in 10 L plastic containers, filtered at 0.22 µm, and passed through UV light to eliminate microorganisms. Filtered seawater (FSW) was stored at 5 °C in the dark and was used usually within a few days. Few hours before the experimentations, FSW was filtered again at 0.22 µm. The background concentration of pesticides and copper in FSW was measured by LC-MS/MS (cf. section 2.6).

2.2 Embryo-larval test: a laboratory approach

Five couples of mature pacific oysters (*Magallana gigas*, also called *Crassostrea gigas* (Bayne et al., 2017)) were obtained from Guernesey Sea Farm hatchery (Guernesey, UK). Oysters were immediately used on the day of the arrival or they were kept in oxygenated FSW at 11 °C for 24 h. A detailed version of the used method for oyster spawning (alternating thermal shocks) is described in Kuchovská et al. (2020) and Gamain et al. (2016) and follows the French guideline

(NF ISO 17244, 2015). Obtained embryos were then incubated in experimental units (type is depending on the measured endpoint) at 24 °C in the dark until they reached the developmental stage of D-larva. After the exposures, developmental malformations, locomotion (section 2.4), and gene expression analyses (section 2.5) were carried out. To assess the developmental malformations, an embryo-larval test was carried out according to the French guideline (NF ISO 17244, 2015) in 24-well microplates (Greiner Bio-One, Cellstar). Due to propiconazole hydrophobicity, the microplates were precoated 24 h before the test with the corresponding concentration of mixture solution (Kuchovská et al., 2020). Embryos from one oyster couple were considered as one independent experiment; each oyster couple formed 4 analytical replicates (wells) per concentration in the microplates with approximately 225 ± 10 % embryos per well. Seven oyster couples per condition were used for the assessment of developmental malformations. Negative control (FSW) and solvent control (0.016 % DMSO) were present on every microplate. The embryos were exposed to increasing concentrations of the pesticide mixture (MIX) composed of IMI, PRO, SM, MOA, and MESA (total concentration of five pesticides corresponding to 0.32 µg/L, 1.6 µg/L, 8 µg/L, 40 µg/L, 200 µg/L, 1 mg/L, 5 mg/L, 25 mg/L; individual concentrations are listed in Table 1).

Table 1 Concentrations of different pesticides in the reconstituted mixture used to expose embryos of oyster *Magallana gigas.*

Code	PRO	IMI	SM	MOA	MESA	Total concentration
C1	10 ng/L	100 ng/L	10 ng/L	100 ng/L	100 ng/L	0.32 µg/L
C2	50 ng/L	500 ng/L	50 ng/L	500 ng/L	500 ng/L	1.6 µg/L
C3	0.25 µg/L	2.5 µg/L	0.25 µg/L	2.5 µg/L	2.5 µg/L	8 µg/L
C4	1.25 µg/L	12.5 µg/L	1.25 µg/L	12.5 µg/L	12.5 µg/L	40 µg/L
C5	6.25 µg/L	62.5 µg/L	6.25 µg/L	62.5 µg/L	62.5 µg/L	200 µg/L
C6	31.25 µg/L	312.5 µg/L	31.25 µg/L	312.5 µg/L	312.5 µg/L	1 mg/L
C7	156.25 µg/L	1.563 mg/L	156.25 µg/L	1.563 mg/L	1.563 mg/L	5 mg/L
C8	781.25 µg/L	7.825 mg/L	781.25 µg/L	7.825 mg/L	7.825 mg/L	25 mg/L

The lowest used concentration (C1) is reflecting the usual concentrations often measured in Arcachon Bay in France (cf. Supplementary Table S1). Concentration C2 is corresponding approximately to the highest concentrations measured in Arcachon Bay. Concentration C3 and C4 may be considered as maximal environmental concentration measured in the surface waters in the world (Table 2). Higher concentrations (C5-C8) were used to allow for estimation of the EC_{50} or as a prediction of acute peak contamination periods.

Table 2 Detected concentrations of pesticides of interest in surface waters in various locations in the world.

Substance	**Detected concentration**	
Propiconazole	0.7 µg/L Vietnam (Toan et al., 2013)	0.81 µg/L China (Peng et al., 2018)
Imidacloprid	3.29 µg/L California (USA) (Starner and Goh, 2012)	320 µg/L Netherlands (Van Dijk et al., 2013)
Metolachlor	10.5 µg/L South Georgia (USA) (Glinski et al., 2018)	16.5 µg/L Italy (Meffe and de Bustamante, 2014)
MOA	1.21 µg/L Mississippi River Basin (Rebich et al., 2004)	5.3 µg/L Iowa (USA) (Kalkhoff et al., 2012)
MESA	2.51 µg/L Mississippi River Basin (Rebich et al., 2004)	10.3 µg/L Iowa (USA) (Kalkhoff et al., 2012)

After the incubation of embryos (24 h at 24 °C) exposed to the mentioned concentrations, the microplates were used for the non-invasive video capture (cf. 2.4 Locomotion analysis). After the video capture (at approximately 30 h), formaldehyde (25 µL at 37 %) was added to every well (final volume 2025 µL) to stabilize the larvae state for the developmental malformation analysis. The microplates were kept at 4 °C in the dark. An inverted microscope (Nikon Eclipse

TS100) was used to assess different forms of developmental malformations in 100 embryos per well, i.e. mantle malformation, scalloped shell malformation, concave shell malformation, developmental arrest. Photos of different malformations as well as well-formed larvae are published in Kuchovská et al. (2020) in Figure 1. According to the ISO norm (NF ISO 17244, 2015), the malformation rate in the negative control cannot exceed 20 % and the EC_{50} for abnormal larvae exposed to Cu^{++} should be between 6 and 16 µg/L.

2.3 *In situ* approach

Adult oysters producing embryos for the *in situ* caging approach were purchased from France Naissain (Bouin, France). The spawning of the mature oysters was carried out in the same manner as for the laboratory approach (cf. section 2.2). Obtained embryos were transported to the three selected sites (Figure 1A) in FSW and the caging devices were deployed at the site approximately at 2 hpf (hours post fertilization) using a quick small boat. The device was inspired by the caging device of Geffard et al. (2001) and was made of two welded 4 L HDPE bottles with open caps at each side (Figure 1B). Both caps were holding a 20 µm mesh filter (SEFAR NITEX®) to let the water freely circulate. Devices were held under the water surface using a weight and were attached to a buoy with a rope (approximately 2 meters long to keep the device underwater surface even at low tide). Four devices containing approximately 666,000 embryos from four mature oyster couples were deployed at each of the three sites. Before adding the larvae to the device, water from the site was slowly poured into the device through the 20 µm mesh filter to keep the water in the device clean from larger debris that would have corrupted the ulterior locomotion analysis. The transplantation was carried out on the 24th July 2019 between 11h and 13h15 local time (summertime is the period of oyster reproduction in Arcachon Bay). After being deployed two days (approx. 46 hours) in the field, the devices were transported in beakers into the laboratory and the larvae were gently collected on a 20 µm mesh and transferred into 24-well microplates in the approximate concentration of

225 embryos per well (4 replicates per caging device) to perform the video capture (section 2.4) followed by formaldehyde stabilization (as in the laboratory approach), and ulterior developmental malformation analysis. The rest of the larvae was collected in a polypropylene microtube for the gene expression analysis (section 2.5).

Three sites were chosen for the *in situ* study. The reference site "Grand Banc" (GB) is located in Teychan channel (44° 39, 914 N; 001° 13, 076 W) near the mouth of Arcachon Bay. The Passes is a narrow (2-3 km) but long (12 km) channel connecting Arcachon Bay with the Bay of Biscay. Therefore, water quality (concerning pesticides) at GB is in a relatively good state (Tapie and Budzinski, 2018). The two other sites are situated in the inner part of Arcachon Bay, thus undergoing higher anthropogenic pressure: "Les Jacquets" (J) and "Comprian" (C) are in channel Chenal de l'Île (44° 42, 831 N; 001° 11, 235 W) and Teychan channel (44° 40, 833 N; 001° 07, 096 W), respectively. The location of the sites is shown in Figure 1A.

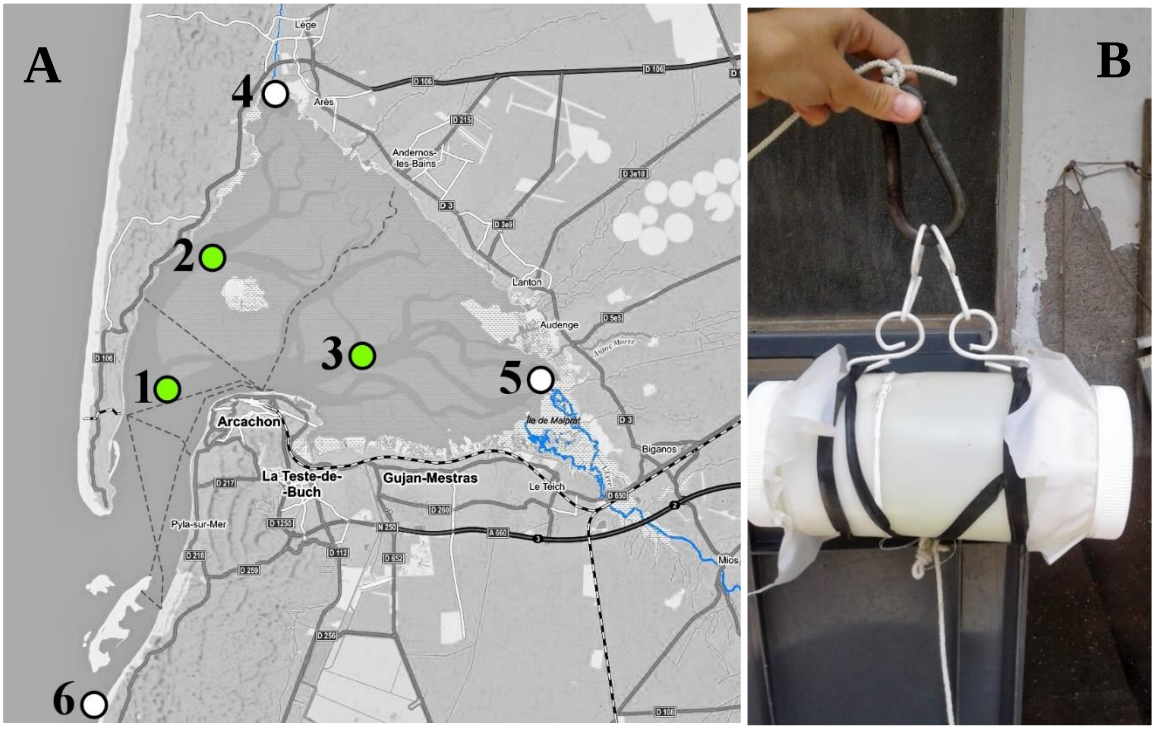


Figure 1 A Map of Arcachon Bay (France) with selected sites for the field transplantation experiment and other important points: 1 Reference site Grand Banc (GB); 2 Site Les Jacquets (J); 3 Site Comprian (C); 4 Mouth of the river Canal des Étangs; 5 Mouth of the river Leyre; 6

Beach Petit Nice – sampling point of the reference seawater. **B** Photo of the caging device. (Map modified from mapy.cz)

2.4 Locomotion analysis

In the laboratory approach, oyster embryos were exposed to three environmentally relevant concentrations of the pesticide mixture (total nominal concentrations of five pesticides 0.32 µg/L, 1.6 µg/L, and 8 µg/L; for individual concentrations cf. Table 1) for 24 h in the dark in 24-well microplates (Greiner Bio-One, Cellstar; 225 embryos per well). In the *in situ* approach, oyster larvae were gently collected at the end of the experiment (48 h) on a 20 µm mesh and transferred into 24-well microplates in the approximately same concentration of embryos per well (4 replicates per caging device). 2 min video of each well was taken at zoom 40x using an inverted microscope Nikon Eclipse TS100 equipped with camera Nikon DS-Fi2, and software NIS Element. The temperature of solutions in the microplates was kept at 24 °C all the time for both experimental approaches. The detailed method (including conversion of videos by VirtualDub and their analysis by ImageJ) is described in Gamain et al. (2019). At the end of the ImageJ analysis, the maximal and average swimming speed, and trajectory type (rectilinear, circular, and stationary) of each larva are determined. Graphical representation of different types of trajectory paths is published in Kuchovská et al. (2020) in Figure 2.

2.5 Gene expression analysis

Oyster embryo laboratory exposure for gene expression analysis is described in detail in Kuchovská et al. (2020). In brief, half of the million embryos issued from one oyster mature couple were incubated in three-liter glass beakers at 24 °C in the dark for 42 h with an oxygenation system (plastic tube with an aquarium air stone). The total pesticide exposure concentrations were 0.32 µg/L, 1.6 µg/L, and 8 µg/L (for concentrations of individual compounds cf. Table 1). Larvae samples from the laboratory exposure in glass beakers and the *in situ* caging devices were collected on a 20 µm mesh (SEFAR NITEX®) and distributed in

five replicates containing 30,000 larvae each. The samples were stored in RNA later at -80 °C until RNA extraction.

The RNA extraction, transcription, and qPCR analyses were carried out as described previously (Kuchovská et al., 2020). Briefly, SV Total RNA Isolation System Kit, Reverse Transcription System kit, and GoTaq® qPCR Master Mix kit (Promega) were used. The purity of all isolated RNA samples at 260/280 nm was between 2.14 and 2.18. Primer-pairs efficiency was checked beforehand (> 95 %). qPCR was carried out using the LightCycler480 (Roche). Results are expressed as fold changes of the exposed group compared to the control group (laboratory tests) or sampling site compared to the reference site Grand Banc (*in situ* tests).

Three reference genes (*β-actin*, *ef1α*, and *rpl7*) and fourteen genes of interest were used in the analysis. Genes of interest were implicated in mitochondrial metabolism (12S, *cox1*), regulation of the cell cycle/apoptosis (*p53*), oxidative stress defense (*cat*, *sodMn*, *sodCu/Zn*, *gpx*), metal regulation (*mt1*, *mt2*), apoptosis (*bax*, *casp3*), biotransformation (*cyp1a*), growth arrest and DNA damage repair (*gadd45, rad51*). Sequences, references, and accession numbers of all genes can be found in Kuchovská et al. (2020).

2.6 Chemical analysis and water quality

Dissolved oxygen, pH, and salinity were measured in the experimental units for gene expression (laboratory approach) at the beginning and the end of the laboratory experiments, using a probe Multi 340i (WTW). All checked parameters complied with the revised norm by Leverett and Thain (2013). In brief, oxygen saturation varied between 91.6 % and 98.2 % (on average 95.0 % and 93.8 % respectively at the beginning and the end of the test); pH values oscillated between 7.96 and 8.2; salinity ranged from 34.2 to 35.7 psu (in average 35.0 psu).

Salinity in the *in situ* samples varied greatly, depending on the location of the sampling site and tide level. Samples from GB had salinity 28.8 and 34.6 psu at the beginning and the end of the

experiments, respectively, samples from J site 22.4 and 23.5 psu, and samples from C site 17.1 and 18.7 psu. The tidal coefficient at the city of Arcachon was low: 50 on the first day and 41 on the last of the experiment.

LC-MS/MS was used to measure the concentrations of pesticides of interest. Samples of the *in situ* experiment were taken under the water surface (approx. 40 cm) at each sampling point at the beginning and the end of the *in situ* experiment and transferred in glass bottles using a sampling stick. Samples of the laboratory experiment were taken in the experimental units for gene expression at the beginning (30 min after the addition of the pesticide in the experimental unit with the aeration device) and the end of tests (at 42 h). Calibration solutions and all samples were spiked with 10 µL of the internal standard of tebuconazole D6 and imidacloprid D4 (both dissolved in 50 % methanol) and stored at -20°C. The samples (50 mL for the *in situ* experiment samples, 5 mL for the lowest used concentration of the mixture exposure, and 1.5 mL for the rest of the mixture exposure samples and calibration solutions) were lyophilized using a freeze dryer Alpha 2-4 LD Plus (Martin Christ Freeze Dryers). The samples were then dissolved in 1 mL of 100 % acetonitrile (except for the *in situ* samples which were dissolved in 3 mL of acetonitrile) and processed as described in Kuchovská et al. (2020). The quantification of analytes was based on the external calibration (0.01 – 50 µg/L in 20 % of acetonitrile) and normalized with internal deuterium-labeled standards (imidacloprid D4 and tebuconazole D6). The limits of quantification (LOQ; S/N>10) in the samples for LC-MS/MS for IMI, PRO, SM, MOA, and MESA were 0.05, 0.01, 0.01, 0.1, and 0.1 µg/L, respectively. However, the *in situ* samples were concentrated 33 times, thus the concentrations of *in situ* samples ultimately quantifiable (LOQ) were 1.5, 0.3, 0.3, 3.0, and 3.0 ng/L, for IMI, PRO, SM, MOA, and MESA respectively. The applied method is described in detail in Supplementary material S1.

Copper concentration was also checked in the reference seawater (copper concentration at the beginning of the laboratory tests i.e. after the filtration and transportation of the water;

ICP-MS), positive control spiked samples (ICP-OES), and in the *in situ* samples (ICP-MS). The *in situ* seawater samples (100 mL) were taken with a sampling stick with a plastic vial at the end of the exposure and carefully filtered through a filter into plastic falcons (all the material was beforehand cleaned with acid and was rinsed three times with seawater at the sampling point). The samples were then acidified with 5% final concentration of nitric acid in each sample and stored in the dark at 5 °C.

2.7 Data analysis

Total malformed larvae (p) i.e. the sum of mantle malformation, shell malformation, and developmental arrest; and type of larval trajectory (p) were transformed using the arcsine transformation p' = arcsin $\sqrt{\frac{p}{100}}$ (Sokal and Rohlf, 2012) before the statistical analysis. Because of the high variability of different test repetitions of larvae swimming speed data, the values (µm/sec) were normalized to the control before the statistical analysis. Gene expression results ($2^{-\Delta\Delta CT}$) were first log normalized. All data are shown with standard deviations (± SD) and corresponding numbers of independent values (N; indicated at each results presentation).

All pre-treated data were then compared using Statistica 13.3 (StatSoft, USA). Data were controlled for normality (Shapiro-Wilk test; $P > 0.01$) and homoscedasticity (Levene test; $P > 0.05$). If confirmed, ANOVA ($P < 0.05$) followed by Tukey post-hoc test was used. In the other case, a non-parametric Kruskal-Wallis ($P < 0.05$) with Mann-Whitney post-hoc test was carried out. EC_{50} was calculated using nonlinear logarithmic regression of the nominal concentration-response curves, using Graph Pad Prism 5 (Graph Pad Software, USA).

3. Results

3.1 Chemical analysis

Pesticide and copper concentrations in the *in situ* samples were measured (Table 3). All pesticide concentrations were found below a hundred ng/L except for SM at the Comprian site. Copper concentration at all sites was low and unlikely to cause any substantial developmental malformations.

Table 3 Measured concentrations of selected pesticides (and metabolites) and copper at three different sites of Arcachon Bay (GB: Grand Banc, J: Les Jacquets, C: Comprian) at the time of transplanting of the oyster embryos in the field and at the end of the exposure; LOD (MOA, MESA) = 1.5 ng/L

				ng/L			µg/L
Site	Sampling time (h)	**PRO**	**IMI**	**SM**	**MOA**	**MESA**	**Cu**
GB	0	6.30	26.96	72.59	<LOD	<LOD	0.59
	48	5.67	9.14	72.54	<LOD	37.17	0.54
J	0	12.69	14.63	97.20	<LOD	<LOD	0.42
	48	8.73	10.08	92.75	<LOD	54.54	0.79
C	0	9.27	10.89	119.79	<LOD	<LOD	0.65
	48	16.74	16.83	138.29	<LOD	26.01	0.49

The copper concentration in the FSW used for the laboratory approach was on average 2.4 µg/L (maximal value 3.3 µg/L), which is higher than the concentrations detected in the *in situ* samples, because the water used was collected at beach Petit Nice as described in section 2.1 (location of the collection site is shown in Figure 1A). Pesticide concentration was measured in the experimental units for gene expression and is shown in Supplementary Table S2. The concentrations varied largely from the expected ones and are discussed in the discussion part. Nominal values are used for tables and figures for practical reasons.

3.2 Embryo-larval development and observed malformations

Positive control (Cu^{2+}) in the embryo-larval test revealed oyster sensitivity with EC_{50} of 9.76 ± 1.58 µg/L which corresponds to the value range (6 - 16 µg/L) preconized by the norm (NF ISO 17244, 2015). Proportions of different larvae abnormalities caused by pesticide mixture in the laboratory approach and by water in Arcachon Bay in the *in situ* experiment are presented in Figure 2.

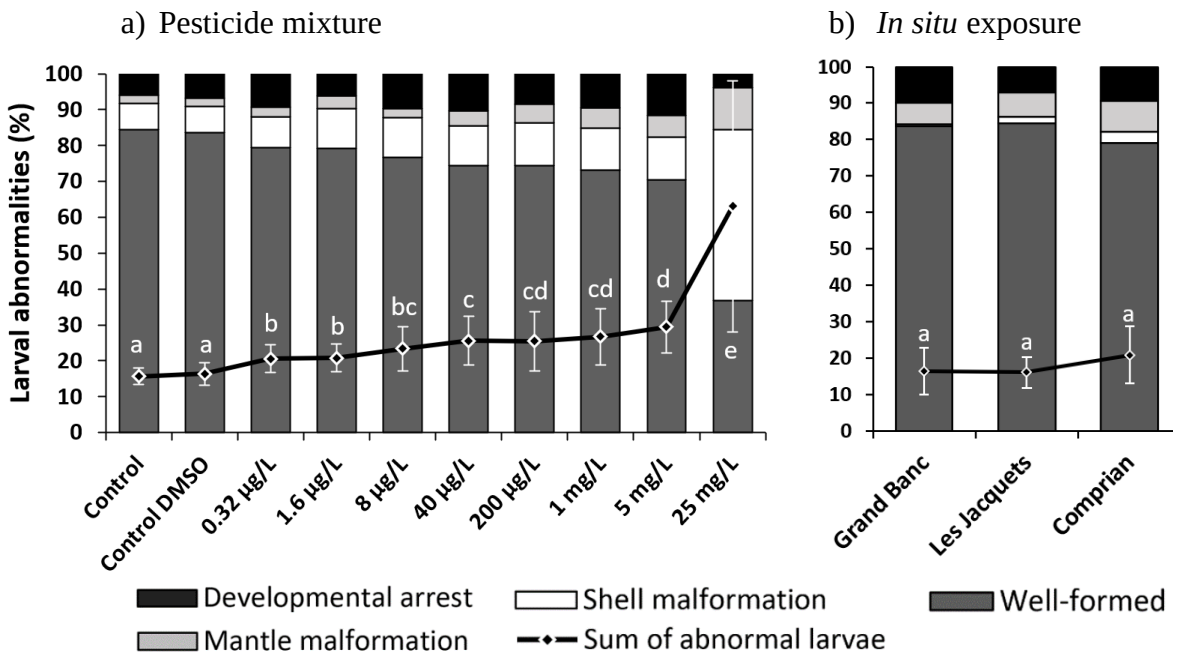


Figure 2 Larval abnormalities and the sum of affected oyster larvae a) after 30 hours of exposure to increasing concentrations of the MIX (individual concentrations of individual chemicals are shown in Table 1) and b) deployed in cages for two days *in situ* on three different sites of Arcachon Bay. Different letters indicate statistical differences between variables ($P < 0.05$). Results are presented as the mean (± SD) of 7 independent experiments in the case of pesticide mixture and 4 in the case of *in situ* caging.

High variability in the sum of abnormal larvae in the laboratory mixture experiment was possibly due to the fact that 4 experiments were carried out in 2018 and 3 in 2019. The sensitivity to the positive control (EC_{50}) was, however, in the correct value range for both years

(11.81 ± 1.18 µg/L and 8.80 ± 1.25 µg/L in 2018 and 2019, respectively). Sum of abnormal larvae served for the calculation of NOEC (< 0.32 µg/L), LOEC (0.32 µg/L), and EC_{50} (10.70 ± 1.32 mg/L) of the pesticide mixture (concentrations are expressed as the sum of nominal concentrations of the 5 pesticides). Even the lowest tested concentration of the pesticide mixture induced a significant increase of abnormal larvae (20.61 ± 3.84 %) from controls (15.66 ± 2.28 % and 16.38 ± 3.08 % of abnormal larvae in control FSW and DMSO control, respectively). On the other hand, no difference was observed between the three sites in Arcachon Bay.

3.3 Behavioral analysis

The behavioral assessment comprised different trajectory paths used by the oyster larvae and their maximal and average swimming speeds. The results are shown in Figure 3. No differences were observed after the laboratory exposure to the mixture of pesticides, neither between the different sites in Arcachon Bay. However, a general decrease of rectilinear trajectories may be observed at all *in situ* sites compared to the results obtained in the laboratory.

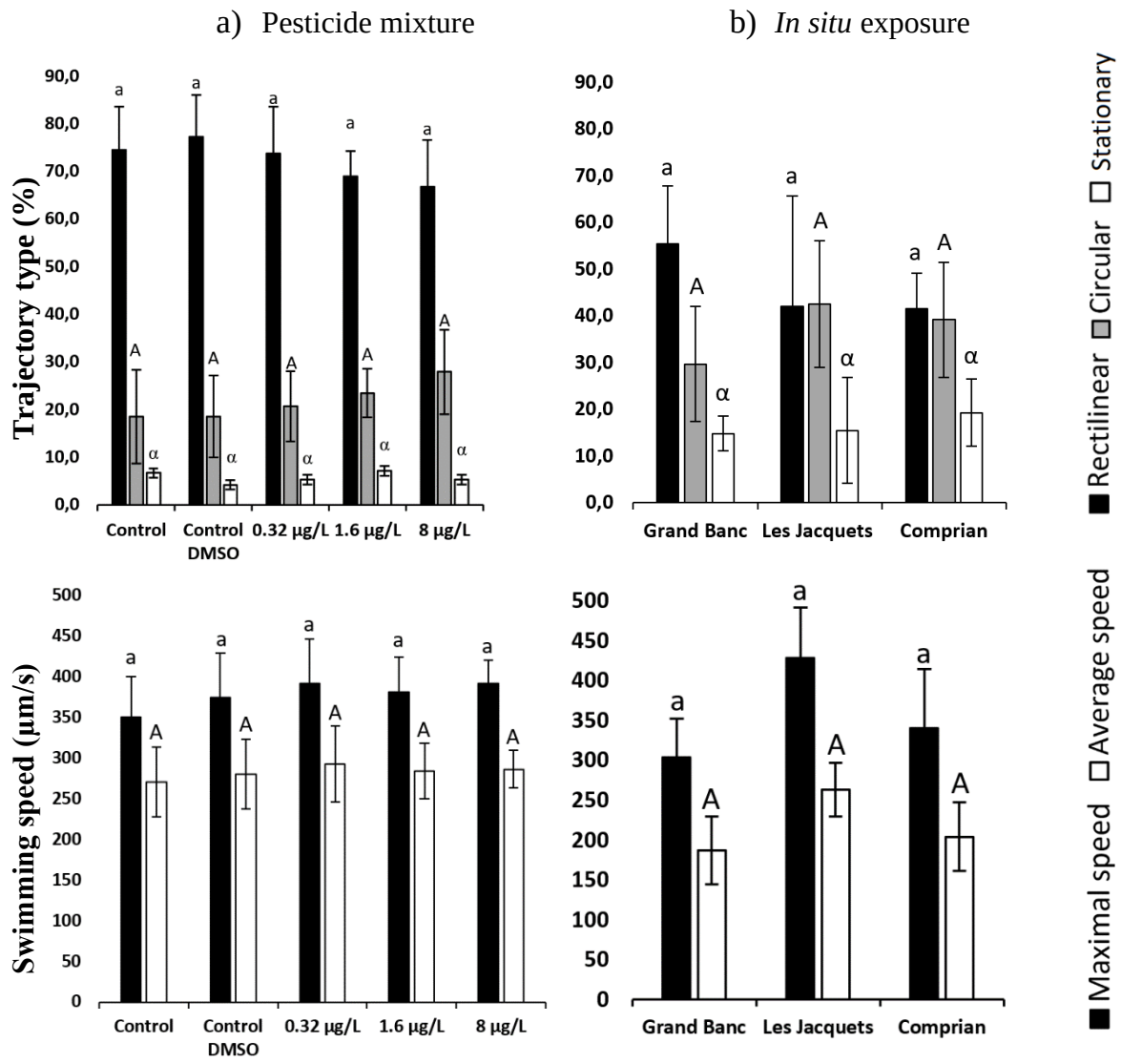


Figure 3 Trajectory types (%; upper graphs) and swimming speed (µm/s; lower graphs) observed in oyster larvae a) after 24 h exposure to three concentrations of the MIX (concentrations of individual chemicals are shown in Table 1) and b) after 2 days of transplantation at three different sites in Arcachon Bay. Different letters indicate statistical differences ($P < 0.05$). Results are presented as the mean (± SD) of 5 (pesticide mixture) and 4 (*in situ*) independent experiments.

3.4 Gene expression analysis

Transcriptions of 14 selected genes known to be involved in mitochondrial metabolism, detoxification, antioxidant defenses, biotransformation process, cycle cellular arrest and apoptosis, and DNA damage repair were investigated. The results of the gene expression level analysis are shown in Table 4. In the laboratory exposure study, only 5 genes were found to be repressed. Genes 12S, *cyp1a,* and *gadd45* showed comparable levels of repression in oyster larvae after exposure to the two lowest (environmental) concentrations of the MIX. In addition, one of the metallothionein genes (*mt2*) and *bax* gene were repressed in oyster larvae after exposure to 8 µg/L of the MIX. *In situ* exposure induced a stronger effect, thus revealing differences between the three sites. On the other hand, no differences between the sites were observed at the level of *bax, casp3, cyp1a, p53, sodCu*. Importantly, both C and J sites had highly induced *cat* and repressed *gpx* and *mt2* compared to the reference site GB.

Table 4 Gene expressions in the oyster larvae a) exposed in the laboratory for 42 h to three low, environmental concentrations of pesticide mixture (for individual concentrations cf. Table 1) and b) caged for two days at the three sampling sites. Results are shown as fold changes between target and housekeeping genes for a) MIX -laboratory approach or as the ratio between target and reference site for b) *In situ* approach. Results are presented as the mean (± SD) of 3 (pesticide mixture) and 4 (*in situ*) independent experiments. Statistically different results are highlighted in bold. *P < 0.05; **P < 0.01; ***P < 0.001. Downregulation: fold changes < 1; upregulation: fold changes > 1.

	a) MIX – laboratory approach			b) *In situ* approach	
	0.32 µg/L	1.6 µg/L	8 µg/L	J:GB	C:GB
12S	**0.7 ± 0.2****	**0.9 ± 0.1***	0.9 ± 0.2	1.2 ± 0.5	**1.3 ± 0.2****
bax	1.0 ± 0.1	1.0 ± 0.3	**0.8 ± 0.0****	1.0 ± 0.1	1.0 ± 0.1
casp3	0.9 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	0.9 ± 0.3	1.1 ± 0.1
cat	1.0 ± 0.5	0.7 ± 0.2	1.3 ± 0.5	**3.4 ± 1.1*****	**2.7 ± 1.2*****
cox1	1.5 ± 0.5	1.0 ± 0.3	1.1 ± 0.2	**1.3 ± 0.3****	1.2 ± 0.4
cyp1a	**0.7 ± 0.2*****	**0.8 ± 0.2*****	**0.9 ± 0.1***	0.9 ± 0.5	0.8 ± 0.2
gadd45	**0.8 ± 0.4***	**0.7 ± 0.1*****	**0.7 ± 0.2****	1.4 ± 0.8	1.1 ± 0.5
gpx	1.1 ± 0.1	0.8 ± 0.1	1.0 ± 0.2	**0.7 ± 0.1*****	**0.6 ± 0.1*****
mt1	1.6 ± 1.0	1.5 ± 0.7	1.5 ± 1.0	1.1 ± 0.7	**0.6 ± 0.5*****
mt2	0.8 ± 0.4	0.9 ± 0.4	**0.7 ± 0.3***	**0.8 ± 0.5***	**0.5 ± 0.3*****
p53	1.5 ± 1.1	1.8 ± 1.0	1.5 ± 0.9	1.0 ± 0.3	1.1 ± 0.2
rad51	0.9 ± 0.2	1.0 ± 0.2	0.8 ± 0.2	**0.8 ± 0.2***	1.0 ± 0.1
sodCu	1.5 ± 1.0	1.4 ± 0.9	1.6 ± 0.5	1.0 ± 0.4	1.1 ± 0.3
sodMn	1.4 ± 1.0	1.4 ± 0.8	1.3 ± 0.5	1.2 ± 0.2	**1.2 ± 0.2***

4. Discussion

GB site, as expected, presents the lowest pesticide concentrations in Arcachon Bay (except for IMI during the installation of the device). However, the differences in concentrations between the three sites were rather low (no more than 3 times higher at J or C sites in comparison with GB; cf. Table 3). Interestingly, unlike in previous years, concentrations of MOA and MESA were lower than concentrations of the parent SM or below the limit of detection. During 2010-2016, metolachlor with its metabolites was responsible for 79 % of total pesticide concentration on GB site, moreover, the parent compound represented only 5 % of the total concentration of these three molecules (Tapie and Budzinski, 2018). Variations throughout the year were observed with average concentrations between 500 and 1700 ng/L (sum of the three substances) with the lowest values recorded in autumn. Nevertheless, an average detected concentration in Julys during 2010-2016 was approx. 800 ng/L (sum of the three molecules). The differences may be explained by different sample processing (HDPE bottles used in 2010-2016 monitoring instead of glass vials used in this study, the concentration of samples using SPE instead of lyophilization method used in this study) and possibly also by variable period between sample collection and processing (analyses). Nevertheless, all these results represent only point measurements. We hypothesize that it would be best to use a passive sampling method (Tapie et al., 2011) to measure the concentration throughout all the experiments without being influenced by meteorological conditions (for instance heavy rain before the sampling) or the tide (after high tide the water is diluted by less contaminated oceanic water). The coupling of bivalve caging and passive samplers was already successfully used (Turja et al., 2015). Unfortunately, the passive samplers like POCIS usually need at least several days of sampling (Brooks et al., 2012), so their suitability for short embryo-larval oyster caging experiments may not be adequate. Although new types of passive samplers with shorter integration periods are

currently being developed such as PTFE-EC POCIS for neonicotinoids like imidacloprid sampling (Noro et al., 2020), they were not operational at the time of our study.

Methodological issues for the analysis of metabolites influenced probably also the results of chemical analysis of laboratory samples (cf. supplementary table S2) when most of the time the metabolites were below the limit of detection despite being correctly spiked in the experimental units. Few issues were also observed with propiconazole concentration in the laboratory experimental units which probably adsorbed on the walls of the experimental unit and plastic tubes used for aeration.

The effects of the pesticide mixture on the development of oyster embryo-larval stages were evaluated. Whereas no difference was observed between the three sites, even the lowest tested concentration of pesticide mixture (0.32 µg/L) in the laboratory approach led to a higher number of malformed larvae or larvae with arrested development. Interestingly, the number of abnormal larvae in laboratory non-exposed control was around 16 % (FSW control 15.66 ± 2.28 % and DMSO control 16.38 ± 3.08 %), similarly to our *in situ* exposure at the reference site GB (16.38 ± 6.49 %). However, the proportion of abnormal larvae exposed in the laboratory to the lowest mixture concentration (20.61 ± 3.84 %) is comparable with the number of abnormal larvae at the C site (20.81 ± 7.86 %). The difference between C and GB site is not significant, due to higher variability in the field data.

The LOEC for the mixture laboratory experiment was 0.32 µg/L (10 ng/L of PRO and SM and 100 ng/L of IMI, MOA, and MESA). Compared to results obtained for individual pesticide toxicity, the LOEC for PRO and IMI, evaluated in our previous study, are higher: 200 µg/L (Kuchovská et al., 2020) but the LOEC of SM was found to be 10 ng/L (Gamain et al., 2016) and LOEC of MOA and MESA was reported to be 100 ng/L (Mai et al., 2014). Data on SM, MOA, and MESA embryotoxicity has thus been previously published but additional experiment with these three substances was repeated in this study to compare toxicity with IMI, PRO, and

MIX within the same experiment: LOEC of SM, MOA, and MESA was calculated to be 100 ng/L for SM and MESA and 1000 ng/L for MOA based on additional experimentation (cf. Supplementary Figure S1). MOA, MESA, and SM are thus probably the main drivers behind the toxicity of the pesticide mixture. Because the EC_{50} was not achieved for some compounds, the comparison of EC_{30} indicates that the pesticides in the studied mixture acted rather through additive toxicity without synergistic or antagonistic interactions. Indeed, the EC_{30} of the mixture was calculated to be 1.44 mg/L of imidacloprid and the metabolites, and 335 µg/L of S-metolachlor and propiconazole (when divided proportionally between the compounds), while S-metolachlor, MOA, and MESA had EC_{30} around 10 ng/L, 100 ng/L, and between 10 and 100 ng/L, respectively, as visible from data of Mai et al. (2014). EC30 of imidacloprid and propiconazole were calculated to be 70.50 and 1.26 mg/L, respectively (Kuchovská et al., 2020). The effect of the mixture thus seemed to be slightly attenuated but insufficiently to be classified as antagonism, which is defined by a minimum two fold difference (Cedergreen, 2014)

No effects were found on the swimming speed or the trajectory paths in this study even though SM (10 and 1000 ng/L) decreased the rectilinear trajectories in the study of Gamain et al. (2020). The rectilinear trajectory is considered as the major one at this developmental stage of the oyster larva, unlike later stages, when helical swimming is the most useful for hunting prey, finding a settlement, or avoiding predators (Maciejewski et al., 2019). On the other hand, results from this study are in agreement with previous study reporting no effects on the same endpoints after exposure to IMI (up to 20 µg/L) and PRO (up to 2 µg/L) as (Kuchovská et al. 2020). To the best of our knowledge, no other behavioral studies with embryo-larval stages of bivalves exposed to pesticides were conducted. Concerning other embryo-larval stages, Rozmánková et al. (2020) evaluated the effects of SM and its metabolites on total distance swam in light/dark locomotor test of zebrafish (*Danio rerio*) larvae. This study reported no impact by individual

substances (up to 300 µg/L) and also by the mixture of SM, MOA, and MESA (up to 30 µg/L of each substance in the mixture). However, a decrease in spontaneous tail coiling in zebrafish embryo was observed after exposure to SM (1 µg/L) and the mixture (1 µg/L of each substance). The *in situ* study showed an increase in both maximal and average swimming speeds especially at J site (428 ± 63 and 263 ± 34 µm/s, respectively) in comparison with the reference site GB (304 ± 49 and 187 ± 43 µm/s, respectively), but these differences were not significantly different (summarizing table can be found in Supplemental Table S3). More field campaigns should be carried out to get completer and more representative picture. *In situ* results of trajectory paths showed a non-significant decrease in rectilinear paths and an increase in circular and stationary paths of larvae at J and C sites in comparison with larvae of the reference site GB. Interestingly, when comparing path results from the reference site GB (n=4) with laboratory non-treated controls from all the experimentations done during this project (n=16), clear difference is observed: GB: rectilinear – circular - motionless: 55.5 ± 12.3 % - 29.7 ± 12.4 % - 14.8 ± 3.77 %; laboratory controls: 74.5 ± 9.29 % - 18.5 ± 10.9 % - 6.91 ± 2.39 %, respectively. Thus, we may hypothesize that the locomotion patterns and swimming speed may be impacted by the pollution even at the "reference" site GB or that the observed *in situ* results were normal under the influence of the realistic environmental conditions, which were not considered in the laboratory experiments e.g. changes in the temperature, salinity variations, currents, etc. The difference could be also influenced by different age of larvae at time of the video captures in laboratory experiments (24-30 hpf) and in *in situ* experiments (47-50 hpf). Gamain et al. (2020) established a link between erratic larval behavior and the presence of developmental malformations. In the present study, the malformation rate for larvae exposed *in situ* was less than 20 % which is a validity threshold for the normalized embryo-larval biotest (NF ISO 17244, 2015). Therefore, we can hypothesize that the locomotion patterns of larvae *in situ* may result of a direct effect on the metabolism and

energetic reserves of the larvae or a direct effect of water quality of Arcachon Bay on the nervous system of oyster larvae. The nervous system is already developing at this stage of D-shaped (veliger) larva and is comprised of a compact apical organ with apical cells, dendrites, neurites (Yurchenko et al., 2018).

Concerning the impacts on the molecular level, the MIX of pesticides changed expressions of several genes of oyster larvae exposed in the laboratory. Clear repression was observed for the gene 12S, coding for the small subunit ribosomal RNA in mitochondria which may result in a decreased ribosomal activity in synthesizing proteins needed for the mitochondrial membrane and thus decreased mitochondrial activity in creating ATP necessary for cell viability and defense against pollutants (De Silva et al., 2015). The repression of the 12S gene was also observed in oyster larvae after exposure to 100 ng/L of metolachlor (Mai et al., 2014) and 200 ng/L and 2 µg/L of propiconazole (Kuchovská et al., 2020). Both observations were made in higher concentrations of individual pesticides (10 and 20 times, respectively) than those used in the MIX (cf. Table 1) suggesting thus possible synergistic effect of the MIX on the expression of the 12S gene. Cytochrome P450 *cyp1a* was strongly repressed as well. On the contrary, exposure to individual pesticides from the MIX (up to 1 µg/L of metolachlor, MOA, and MESA, up to 2 µg/L of PRO, and up to 100 µg/L of IMI) did not induce any alterations in its expression (Kuchovská et al., 2020; Mai et al., 2014). Therefore, a possible interaction effects may have occurred among the components of the mixture. Interestingly, azoles compounds like propiconazole are known to inhibit cytochrome P450 enzymes (monooxygenases), which participate in the detoxification of xenobiotics (Gottardi et al., 2018), such as neonicotinoids like IMI or acetanilides as SM, where the main metabolic reaction is the displacement of the chlorine atom by glutathione (Roberts et al., 1998). This might be an important mechanism of PRO toxicity towards the Pacific oyster since the main mechanism of action of azoles is to inhibit the steroidogenesis and thus obstruct the creation of fungal cell membranes. However,

the Pacific oyster' sterols are likely mostly derived from its prey such as microalgae (Knauer et al., 1998).

Gene *gadd45*, coding for a stress response protein that regulates growth arrest and DNA damage repair, was repressed in oyster larvae in all laboratory conditions starting with exposure to 0.32 µg/L of MIX. Interestingly, this gene was reported to be upregulated after exposure to 1 µg/L of IMI contrary to the finding of this study, whilst exposure to PRO did not affect this gene (Kuchovská et al., 2020). To the best of our knowledge, the effect of metolachlor and its two metabolites on the expression of *gadd45* is not known, as well as any other chloroacetanilide herbicide. The observed downregulation of *gadd45* hinders correct regulation of the cell cycle and suggests possible effects on the DNA integrity (E. Tamura et al., 2012). Moreover, the highest tested concentration of MIX (8 µg/L) downregulated the expressions of *bax* and *mt2*. *Bax* downregulation corroborates the suspected anti-apoptotic effects of the MIX as suggested also by the downregulation of *gadd45*. The repression of *mt2*, a gene coding for a multifunctional protein regulating metal homeostasis but also ROS (reactive oxygen species) scavenging (Migliaccio et al., 2020), after exposure to the MIX is in concordance with repression of the same gene after exposure to 1 µg/L of MESA (Mai et al., 2014). However, the exposure to high concentrations (10 and 100 µg/L) of IMI caused the opposite effect (Kuchovská et al., 2020). Interestingly, no alteration of expression of genes linked to ROS content regulation was observed, whilst the effects of individual substances of the MIX caused contradictory results (Kuchovská et al., 2020; Mai et al., 2014).

It should be highlighted, that the MIX exposure in the laboratory was only a partial representation of the complex situation in Arcachon Bay. Although selected pesticides are the most representative ones of their respective classes (herbicides, insecticides, fungicides), the actual environmental pollution of Arcachon Bay is likely more complex including not only other pesticides but also other pollutants as pharmaceuticals, UV filters, trace metals,

organotins, etc. (Besse et al., 2019). Consequently, we will not discuss the situation *in situ* compared to the laboratory but only differences between the three sites. GB site was considered reference in the present study, but its contamination is naturally far from being ideal. The strongest effect was seen on the *cat* gene (coding for catalase), where both sites (J and C) highly induced its expression in comparison with the reference site GB, thus demonstrating a possible induction of defense mechanism against ROS (Gebicka and Krych-Madej, 2019). This finding agrees with the induction of *sodMn* (coding for manganese superoxide dismutase), another gene linked to the stress defense, at C site. On the other hand, repression of another ROS linked gene, *gpx* (coding for glutathione peroxidase), was observed at both sites. This suggests that whilst some antioxidant activities may protect cells against increased ROS production, the mitochondria may be more susceptible to the adverse effects along with the *gpx* suppression (Ighodaro and Akinloye, 2018). Metallothionein gene expression (*mt1*, *mt2*) was strongly downregulated at both sites (J and C) in comparison with GB site. The repressions of *mt1*, *mt2* (along with *gpx*) generally show lower scavenging and protective capacity of the cells in oysters exposed in sites J and C (Ruttkay-Nedecky et al., 2013; Takahashi, 2015). Genes *cox1* (upregulated at J site) and 12S (upregulated at C site) are both linked to mitochondrial metabolism. *Cox1*, coding for the enzyme cytochrome c oxidase subunit 1, upregulation suggests increased creation of ATP probably to effectively defend the organism against pollutants (Kadenbach, 2018), whilst upregulation of 12S indicates increased ribosomal activity in mitochondria or increased number of mitochondria which ultimately also leads to increased energy production (De Silva et al., 2015). Gene *rad51*, coding for a protein involved in DNA repair, was downregulated at J site, suggesting impairment of the DNA repair process (Laurini et al., 2020). Altogether, several lines of evidence from gene expression analyses indicate that both C and J sites showed a worse state of oyster larvae. This can be linked to their location in

the inner part of the bay and the proximity of the C site with river Leyre, which is the main entrance of the herbicides into Arcachon Bay.

5. Conclusion

The present work successfully demonstrated added value of joining two complementary approaches, i.e. laboratory and *in situ* to evaluate the sublethal impact of water pollution on Pacific oyster larvae (*Magallana gigas*). Early life stages were exposed either in the laboratory to environmentally relevant reconstituted pesticide mixture or transplanted *in situ* in caging devices and exposed in Arcachon Bay. Whereas the laboratory mixture, composed of 5 pesticides, caused developmental toxicity already at the environmental concentration of 0.32 µg/L (corresponding to current concentration in Arcachon Bay in France), it did not impair larval swimming. Several effects were found at the molecular level such as repression of genes linked to the mitochondrial metabolism, biotransformation, and growth arrest and DNA damage repair, and at higher concentration also apoptosis regulation and metal regulation. *In situ* approach allowed to compare three different sites in Arcachon bay: Grand Banc, Comprian, and Les Jacquets, each of them under different contamination pressure. No differences in larvae swimming or development were observed between sites, however, gene expression analysis revealed worsened state at both more contaminated sites Comprian and Les Jacquets. At these sites we have observed possible elevation of oxidative stress, repressed metallothionein function, and enhanced mitochondrial metabolism. This is the first study that successfully complemented laboratory tests with *in situ* approach, and for the first time it investigated swimming of oyster larvae after in situ caging exposures.

CRediT authorship contribution statement

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

Compliance with ethical standards

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

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CHAPTER IV.

WHAT ARE THE RISKS OF PESTICIDES TO FRESHWATER NON-TARGET ORGANISMS, ESPECIALLY TO ZEBRAFISH?

Freshwater non-target organisms are at risk of being affected by omnipresent anthropogenic pollution. Rivers, adjacent to agricultural fields, may receive high concentrations of pesticides, especially after rain events. Typically, early-life stages of organisms may be at greater risk, due to their not yet developed organ systems and less effective/immature detoxification mechanisms. The pesticides may ultimately hinder good development of the organisms.

The effects of the same pesticides as in Chapter 3, and in similar concentration ranges were evaluated also in this Chapter. We exposed the embryo-larval stages of zebrafish first to the herbicide S-metolachlor and its two metabolites, as well as to their mixture (**Publication III.**). Secondly, zebrafish embryos were exposed to insecticide imidacloprid, fungicide propiconazole, and the mixture of the five pesticides **(Publication IV.)** In both studies, developmental, behavioral, and molecular analyses were carried out with zebrafish embryos or larvae of different life stages depending on the evaluated endpoint. As an additional interest, the effects of pesticides on transcripts of selected thyroid system-related genes were evaluated because of existing scientific literature suggesting possible effects of selected pesticides on fish thyroid system. **Publication IV.** is presented in this dissertation thesis only in a form of a working manuscript with the majority of the results obtained.

As a secondary project, the effects of the insecticide imidacloprid on larval stages of a midge *Chironomus riparius* were also analyzed. The resulting co-authored **Publication V.** is attached in Annexes.

PUBLICATION III.

ENVIRONMENTALLY RELEVANT MIXTURE OF S-METOLACHLOR AND ITS TWO METABOLITES AFFECTS THYROID METABOLISM IN ZEBRAFISH EMBRYOS

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Main findings of Publication III.

- Studied pesticides (S-metolachlor and its two metabolites) up to 300 μg/L, as well as their mixture (up to 30 μg/L of each compound), caused no effects on survival of zebrafish larvae. No effects were also observed on sublethal endpoints such as hatching success, larvae body length, swimming activity (detected with light: dark locomotion test), and heartbeat.
- Only few infrequent developmental malformations were observed in larvae after exposure to high non-realistic concentrations. For example, S-metolachlor caused non-inflated gas bladder (100 μg/L) and yolk sac malabsorption (300 μg/L) and metolachlor ESA metabolite caused craniofacial deformations (100 μg/L). These malformations may be linked to thyroid disruption. Moreover, the mixture of the three compounds (30 μg/L of each substance) caused spine deformations, probably due to the concentration addition mixture effect. Nevertheless, these developmental abnormalities may be of minor biological importance because of their low frequency (<6%).
- On the other hand, low environmentally relevant concentrations of S-metolachlor (1 μg/L) and the mixture (1 μg/L of each compound) influenced the neurodevelopment of zebrafish embryos by decreasing their spontaneous tail movements.
- S-metolachlor did not influence gene expression levels of selected genes, unlike its metabolites or the environmental concentration of the mixture. The most pronounced effects (upregulation) were seen on the gene coding for the protein P53, genes related to the thyroid system (iodothyronine deiodinase 2 and nuclear receptors α and β), and a gene coding for cytochrome *cyp26a1* implicated in the retinoic acid inactivation.
- The concentration addition effects in the studied mixture were observed for most altered endpoints.
- Thyroid disruption by S-metolachlor and its metabolites is a likely hypothesis behind the observed effects.

PUBLICATION IV.

IMIDACLOPRID, PROPICONAZOLE, AND PESTICIDE MIXTURE TOXICITY ASSESSMENT USING EMBRYO-LARVAL STAGES OF ZEBRAFISH

- Draft working manuscript

Main findings of Publication IV.

- Imidacloprid, propiconazole, and a mixture of five pesticides i.e. imidacloprid, propiconazole, S-metolachlor, and its two metabolites (metolachlor oxanilic acid and ethanesulfonic acid) did not affect the survival and the hatching success of zebrafish larvae. Their NOECs for mortality and hatching success are thus higher than 312.5 µg/L for imidacloprid, 31.25 µg/L for propiconazole, and 1 mg/L of the sum of the five concentrations of the mixture.
- Only the highest concentrations tested caused developmental malformations, especially craniofacial malformations. Moreover, the mixture induced a higher frequency of yolk sac malabsorption and non-inflation of the gas bladder.
- Environmentally relevant concentrations (tens to hundreds ng/L) affected various sublethal biomarkers such as spontaneous tail coilings, heart rate, or larvae locomotion.
- qPCR analysis (ongoing experimentation not included in the thesis) shall bring new information and elucidate whether the pesticides of interest have an impact on the thyroid system of the zebrafish larvae

Introduction

The present study investigated the sublethal toxicity of two environmentally relevant concentrations, as well as two higher ones, of widely used insecticide imidacloprid, fungicide propiconazole, and a mixture of five pesticides: imidacloprid, propiconazole, and herbicide S-metolachlor with its two metabolites (MOA and MESA) on embryo-larval stages of zebrafish *Danio rerio*. This work is a follow-up of the **Publication III** of this dissertation thesis. The same pesticides in the same concentration range as in **Publications I., II., and III.** were used and the same endpoints were evaluated i.e. apical (developmental malformations), neurobehavioral (swimming activity, spontaneous tail coilings), cardiotoxic, and biochemical by measuring transcriptions of various genes implicated in non-specific toxicity as well as in thyroid disruption. Moreover, the measurement of thyroid hormone levels is planned in collected samples in cooperation with colleagues of the RECETOX laboratories.

Materials and methods

Chemicals

S-metolachlor (SM, CAS 87392-12-9, Pestanal, purity ≥ 98.0%), metolachlor oxanilic acid (MOA, CAS 152019-73-3, Pestanal, purity ≥ 98.0%), metolachlor ethanesulfonic acid (MESA, CAS 947601-85-6, Pestanal, purity ≥ 95.0%), imidacloprid (IMI, CAS 138261-41-3, purity ≥ 95.0%), and propiconazole (PRO, CAS 60207-90-1, Pestanal, purity ≥ 98.0 %) were purchased from Sigma-Aldrich. Stock solutions (10 g/L for PRO in DMSO and 50 mg/L for the other four in Milli-Q water) were stored at -20 °C. Working stock solutions (IMI 3.125 mg/L, PRO 312.5 mg/L, and the mixture (MIX) containing 3.125 mg/L of IMI, MOA, MESA, and 0.3125 mg/L of PRO and SM) were prepared in Milli-Q water and were stored at 5 °C. The hormone triiodothyronine (T3, dissolved in methanol, CAS 6893-02-3, purity ≥ 95%, purchased from Sigma-Aldrich, used immediately) was used as a positive control in the gene modulation experiments. Ethanol absolute (CAS 64-17-5, purity ≥ 99.8%, purchased from VWR Chemicals) was used as a positive control in the neurobehavioral analyses and for the PCR analysis. ISO medium (ISO, 1996) ($CaCl_2$*$2H_20$ (294 mg/L), $MgSO_4$*$7H_20$ (123.3 mg/L), $NaHCO_3$ (63 mg/L), KCl (5.5 mg/L) in Milli-Q water) was used to prepare the final dilutions for the tests. RNA*later* ® (Ambion) was purchased from Sigma-Aldrich.

Test organisms

Zebrafish (*Danio rerio*) embryos were collected from a wild type zebrafish strain AB, received as a gift from J. Legradi, Vrije Universiteit Amsterdam, and maintained at RECETOX, Masaryk University (Czech Republic) following appropriate guidelines (ISO, 2008; OECD, 2013b). Adult fish were kept as described earlier (Rozmánková et al., 2020).

FET (Fish embryotoxicity) test

The experiments were conducted following the fish early-life stage toxicity guideline (OECD, 2013a), with few modifications as described earlier (Rozmánková et al., 2020). Briefly, the embryos were exposed for 120 hpf without solution renewal at dark (because of the high photodegradation of imidacloprid). The tests were repeated independently three times with eggs from different spawning and conducted in glass crystallization dishes containing 20 embryos per 20 mL of solution in three replicates. The embryos were exposed to four increasing concentrations of imidacloprid 0.1 - 2.5 - 62.5 - 312.5 µg/L, propiconazole 0.01 - 0.25 - 6.25 - 31.25 µg/L, and a mixture of propiconazole, imidacloprid, S-metolachlor, metolachlor oxanilic acid, and metolachlor ethanesulfonic acid in concentrations as shown in Table 1. DMSO control of 0.0003% was used for propiconazole and mixture tests and the amount of DMSO was the same in all solutions.

Table 1 Nominal concentrations of different pesticides in the mixture used to expose embryos of zebrafish *Danio rerio.*

Code	PRO	IMI	SM	MOA	MESA	Total concentration
C1	10 ng/L	100 ng/L	10 ng/L	100 ng/L	100 ng/L	0.32 µg/L
C2	0.25 µg/L	2.5 µg/L	0.25 µg/L	2.5 µg/L	2.5 µg/L	8 µg/L
C3	6.25 µg/L	62.5 µg/L	6.25 µg/L	62.5 µg/L	62.5 µg/L	200 µg/L
C4	31.25 µg/L	312.5 µg/L	31.25 µg/L	312.5 µg/L	312.5 µg/L	1 mg/L

Sublethal analyses

The developmental malformations, spontaneous tail coilings, locomotor test, and heartbeat were assessed as described in Rozmánková et al., (2020) with few modifications as follows. The videos for the spontaneous movement analysis were recorded between 22 and 23 hpf and 0.5 % ethanol was used as a positive control. The 3 dpf larvae used for heartbeat analysis were captured when immobilized in a solution of 2.5% methylcellulose. The locomotor tests consisted of 4 alternating phases of 10 min (white light, i.e. 100% stimulus/dark, i.e. no stimulus /white light/dark) and were performed at 26 °C between 12:00 noon and 15:30 when the locomotion patterns should be stable (Colwill and Creton, 2011; MacPhail et al., 2009) in DanioVision Observation Chamber (Noldus). The used 96 microwell plates were precoated with respective concentrations of propiconazole or the mixture due to the adsorption of hydrophobic propiconazole on the walls of wells as discovered in our previous study (Kuchovská et al., 2020). Every condition was represented on the microplate by 19 fish larvae randomly distributed. The video capture was preceded by 10 min of dark (because the exposure was carried out in dark as well) to acclimatize the fish after the transfer in the Daniovision. The analysis was performed by software EthoVision XT (Noldus) with Maximum Distance Moved smoothing profile set at 2.1 mm to remove the erratic detection of objects within the arena.

Gene expression analyses

Gene expression analyses were performed as described earlier (Rozmánková et al., 2020). In brief, the total RNAs, reverse transcription, and quantitative PCR analysis was performed using the SV Total RNA Isolation System Kit (Promega), GoScript™ Reverse Transcription System kit (Promega), and GoTaq® qPCR Master Mix kit (Promega), using the LightCycler® 480 (Roche), respectively. Three reference genes for zebrafish (*β-actin*, *ef1α*, and *rpl13*) and nineteen genes of interest were used in the analysis. Genes of interest were implicated in mitochondrial metabolism (12S, *cox1*), regulation of the cell cycle/apoptosis (*p53*), oxidative stress defense (*cat*, *sodMn*, *sodCu/Zn*), metal regulation (*mt1*, *mt2*), apoptosis regulation (*bax*), retinoic acid signaling pathway (*cyp26a1*), and biotransformation (*cyp1a*). Moreover, levels of genes linked to the thyroid system (deiodinases *dio1*, *dio2*, *dio3,* and hormone receptors *tra*, *trb*) were analyzed in zebrafish larvae exposed to the two lowest environmental concentrations of the mixture (total concentration of five pesticides 0.32 and 8 µg/L). Sequences, references, and accession numbers of all genes will be shown in Supplemental materials.

Chemical analysis and water quality

The concentration stability of pesticides during the five-day experiment was measured by LC-MS/MS analysis, as described in detail in Supplementary Materials. The samples for the analysis were collected in Eppendorf plastic tubes (2 mL), centrifuged (15000 rpm, 15 min, 10 °C) to get rid of chorion debris, and 1.5 mL of supernatant was stored in glass vials at -20 °C upon analysis. Oxygen saturation on the 1st day was higher than 97.1% in all conditions, and on the 5th day it varied between 93.1 and 96.7%. Conductivity and pH ranged between 613 and 711 μS/cm, and 7.7 and 8.0, respectively.

Data analysis

Sums of observed malformations were compared with controls using Fisher's exact test in GraphPad Prism (Version 8, GraphPad Software). Data of locomotion tests, spontaneous movements, and heart rate were tested for normality (Shapiro-Wilk test; $P > 0.01$), and homoscedasticity (Levene test; $P > 0.05$), and if confirmed, ANOVA followed by Dunnet post-hoc test was used. In the other case, a non-parametric Kruskal-Wallis test with Mann-Whitney post-hoc test was used. Data for gene expression analysis were log normalized before the analysis, controlled for normality and homoscedasticity, and statistically compared as described above. All analyses were performed using software Statistica 13.3 (TIBCO Software Inc., version 13.5, USA).

Results

1. Compounds' stability

Stability of pesticides during the five day experiment is shown in Table 2. The measured concentrations corresponded to the nominal ones except for the lowest concentration of propiconazole (an increase of 80%) and the lowest concentration of propiconazole in the mixture (an increase of 60%). The compounds were stable during the 5-day test, except for the lowest concentration of imidacloprid which increased by 40%. Consequently, it can be considered that organisms were exposed to constant concentrations of pesticide during the five days of exposure.

Table 2 Nominal and measured concentrations, and percentage of the ratio of imidacloprid, propiconazole, S-metolachlor, and its two metabolites at the beginning (0 dpf) of the ZFET test and after five days of exposure. Stability (%) of pesticides during the five-day test is shown.

			Concentration (µg/L)				
	Compound		Nominal	Measured at 0 dpf	% measured/ nominal	Measured at 5 dpf	Stability (%)
	Negative control		0	0	-	0	-
	IMI	C1	0.1	0.091	91	0.128	141
		C2	2.5	2.71	109	2.84	105
		C3	62.5	63.4	101	64.4	102
		C4	312.5	314	100	317	101
	PRO	C1	0.01	0.018	180	0.016	88.9
		C2	0.25	0.260	104	0.237	91.2
		C3	6.25	6.41	103	6.16	96.1
		C4	31.25	31.9	102	31.8	99.7
Mixture	C1	IMI	0.1	0.114	114	0.122	107
		PRO	0.01	0.016	160	0.015	93.8
		SM	0.01	0.013	130	0.013	100
		MOA	0.1	0.095	95	0.112	118
		MESA	0.1	0.109	109	0.098	89.9
	C2	IMI	2.5	2.91	116	2.92	100
		PRO	0.25	0.263	105	0.248	94.3
		SM	0.25	0.290	116	0.291	100
		MOA	2.5	2.52	100	2.61	103
		MESA	2.5	2.43	97.1	2.89	119
	C3	IMI	62.5	69.2	110	68	98.3
		PRO	6.25	6.25	100	5.68	90.9
		SM	6.25	6.53	104	6.52	99.9
		MOA	62.5	61.2	97.9	62.5	102
		MESA	62.5	64.2	103	63.8	99.4
	C4	IMI	312.5	316.4	101	316	99.9
		PRO	31.25	29.7	95.1	28.9	97.2
		SM	31.25	31.1	99.4	29.7	95.7
		MOA	312.5	331	106	334	100.9
		MESA	312.5	321	103	312	96.9

2. Effects on mortality, hatching success, and malformations

Observed mortality was between 0.56 and 3.89% for all treatments including the controls as preconized (<10%) by OECD guideline (OECD, 2013). No effects were also noticed on the hatching success, which was higher than 99.44 % in all treatments. Observed developmental abnormalities are presented in Table 3. Generally, no patterns or dose-response effects were observed after exposure to imidacloprid, propiconazole, or the mixture. Only the highest tested concentration of imidacloprid (312.5 µg/L) and propiconazole (31.25 µg/L) caused non-severe (9.2 and 4.0%, respectively) statistically significant craniofacial deformations. The highest concentration of mixture (total concentration of five pesticides of 1 mg/L) caused the increased frequency of craniofacial malformations, non-inflated gas bladder, and yolk sac malabsorption.

Table 3 Frequencies (%) of different types of developmental malformations of zebrafish after 120 h exposure to different concentrations of imidacloprid, propiconazole, and a mixture of 5 compounds. Each value represents mean ± standard deviation from 3 independent experiments each based on N=60 embryos. *P < 0.05; **P < 0.01.

Endpoint		Edemas	Craniofacial def.	Spine def.	Non-inflated gas bladder	Tail def.	Yolk sac malabsorption
Concentration µg/L					%		
	Control	0.6 ± 0.8	2.8 ± 4.0	0.0 ± 0.0	1.1 ± 0.8	0.0 ± 0.0	19.3 ± 9.6
	C1	1.8 ± 1.5	4.8 ± 2.3	0.0 ± 0.0	3.1 ± 1.7	0.6 ± 0.8	21.0 ± 11.6
IMI	*C2*	0.0 ± 0.0	2.8 ± 2.1	0.6 ± 0.8	2.3 ± 1.6	0.6 ± 0.8	9.0 ± 6.3
	C3	1.1 ± 1.6	2.4 ± 0.9	0.6 ± 0.8	0.6 ± 0.8	0.6 ± 0.8	13.5 ± 8.2
	C4	3.5 ± 2.9	**9.2 ± 7.4***	1.1 ± 1.6	4.0 ± 2.9	0.6 ± 0.8	16.8 ± 4.6
	DMSO	0.0 ± 0.0	0.6 ± 0.8	1.1 ± 0.8	0.6 ± 0.8	0.0 ± 0.0	6.3 ± 5.0
	C1	0.6 ± 0.8	1.7 ± 1.4	0.6 ± 0.8	1.1 ± 0.8	0.0 ± 0.0	4.5 ± 3.2
PRO	*C2*	0.6 ± 0.8	1.3 ± 0.9	0.0 ± 0.0	1.1 ± 0.8	1.3 ± 0.9	5.6 ± 4.3
	C3	1.1 ± 0.8	3.5 ± 3.8	1.1 ± 0.8	0.6 ± 0.8	0.0 ± 0.0	7.0 ± 3.8
	C4	2.3 ± 2.1	**4.0 ± 0.7***	0.6 ± 0.9	2.9 ± 1.5	0.0 ± 0.0	5.4 ± 4.4
	DMSO	0.0 ± 0.0	1.7 ± 1.4	1.1 ± 0.8	0.6 ± 0.8	0.0 ± 0.0	11.4 ± 3.5
	C1	1.1 ± 0.8	2.8 ± 2.1	0.6 ± 0.8	2.8 ± 4.0	0.0 ± 0.0	13.5 ± 7.2
MIX	*C2*	0.0 ± 0.0	1.9 ± 1.4	0.0 ± 0.0	1.3 ± 1.8	0.0 ± 0.0	10.7 ± 8.1
	C3	0.6 ± 0.8	2.3 ± 1.6	0.0 ± 0.0	1.7 ± 2.4	0.0 ± 0.0	5.3 ± 2.7
	C4	2.3 ± 1.6	**7.9 ± 4.8****	0.0 ± 0.0	**6.3 ± 3.4****	0.0 ± 0.0	**20.3 ± 5.2***

3. Spontaneous movements of embryos

Observed spontaneous tail coilings of the zebrafish embryos are presented in Figure 1. The LOEC of imidacloprid was observed to be at 2.5 µg/L. Propiconazole increased the tail

movements from the lowest environmental concentration of 0.01 µg/L, however, one of the higher concentrations (6.25 µg/L) was not statistically different from the control. The mixture exerted the strongest effect and increased the tail coilings in all treatments including the environmental one.

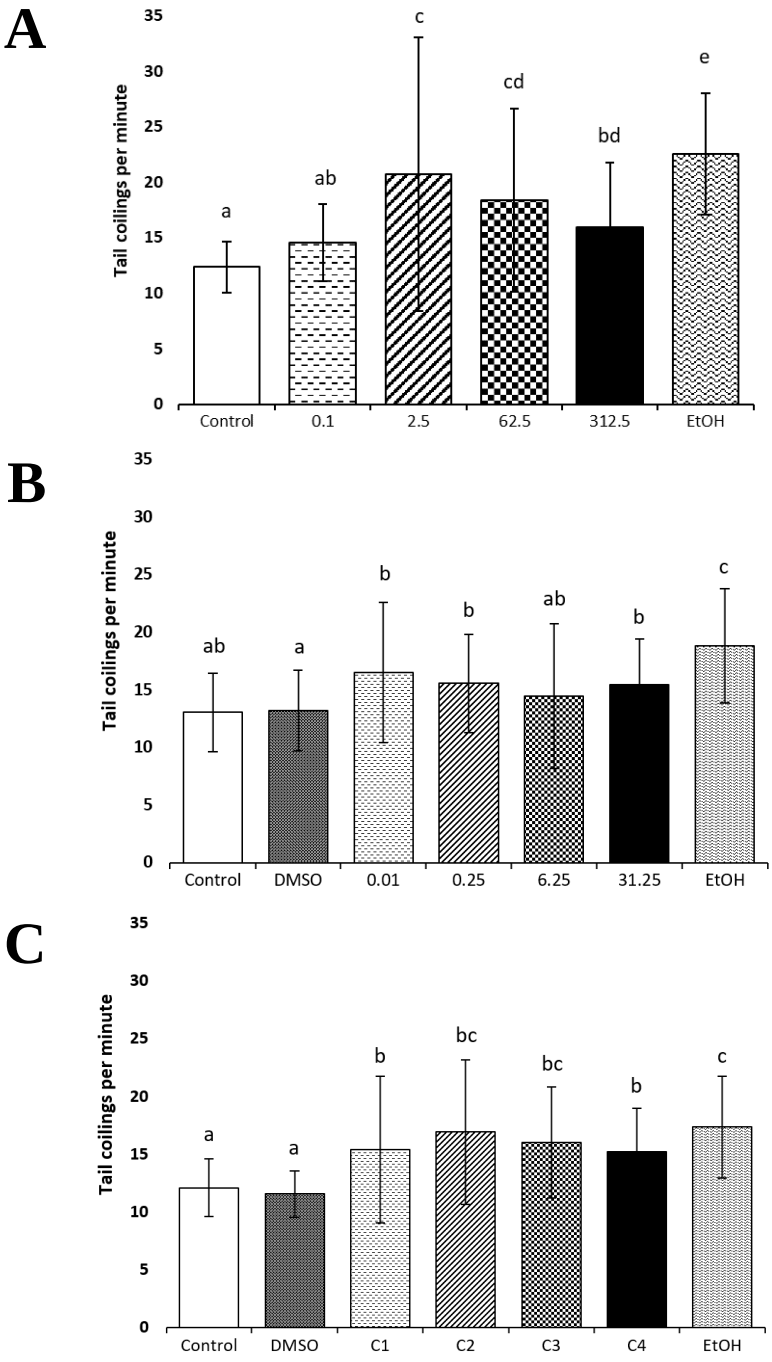

Figure 1 Frequency of tail coilings per minute measured in 22-23 hpf old zebrafish embryos exposed to imidacloprid (A), propiconazole (B), and the mixture (C) composed of the two mentioned compounds completed with S-metolachlor, metolachlor oxanilic acid, and metolachlor ethanesulfonic acid. Ethanol (0.5%) was used as a positive control. N=172-181 per condition of 3 pooled independent repetitions.

4. Heart rate of zebrafish larvae

Heart rate of 3 dpf zebrafish larvae exposed to imidacloprid, propiconazole, and the mixture, expressed as heartbeats per minute are shown in Figure 2. LOEC of imidacloprid and propiconazole were 2.5 and 0.01 µg/L, respectively. The mixture increased the larvae's heart rate starting at the total concentration of five pesticides of 8 µg/L (for detailed concentration see Table 1). The negative (ISO solution) and solvent (DMSO) control were not statistically different (P=0.595).

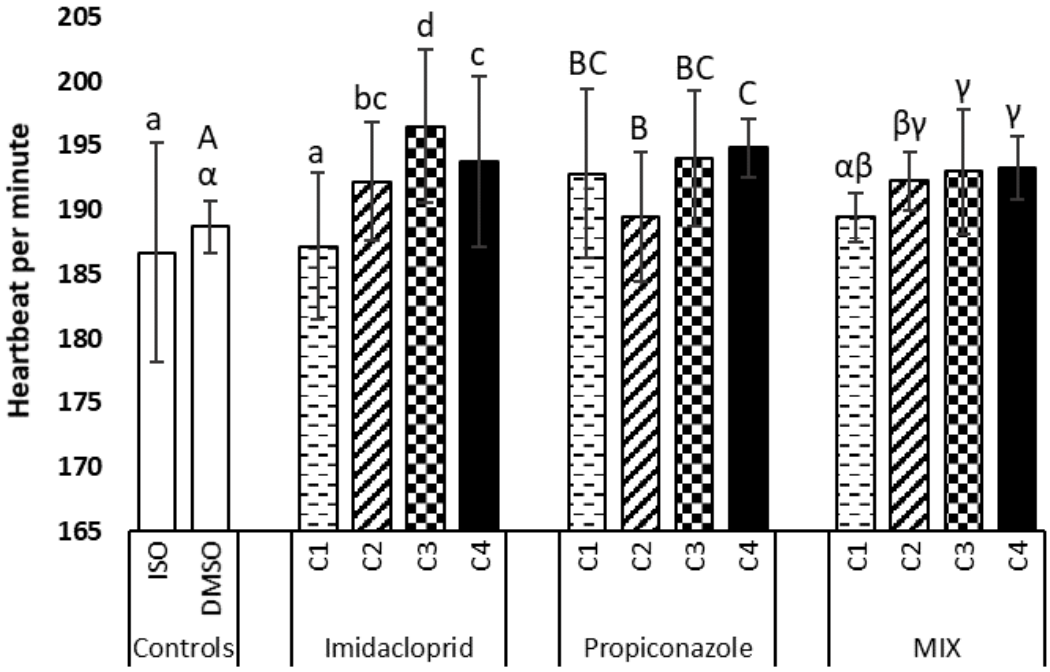

Figure 2 Heartbeat per minute in 3 dpf zebrafish larvae exposed to imidacloprid, propiconazole, and the mixture composed of the two mentioned completed with S-metolachlor, metolachlor oxanilic acid, and metolachlor ethanesulfonic acid. N=124-155 larvae per experimental variant.

5. Locomotion of zebrafish larvae

Locomotion of 5-day old larvae was measured as a distance moved in light and dark alternating phases. The typical locomotion pattern of control (negative and solvent) larvae, as well as of larvae exposed to the positive control of 0.5% ethanol solution is shown in Figure 3. Negative ISO control and solvent DMSO control exposed larvae were not exhibiting different behavior patterns (P=0.36 for the sum of light phases and P=0.16 for the sum of dark phases). On the other hand, larvae exposed to the positive control swam more (larger total distances), especially in dark phases, when zebrafish larvae are usually more active (P=0.0001 in comparison with ISO control and P=0.003 in comparison with DMSO control).

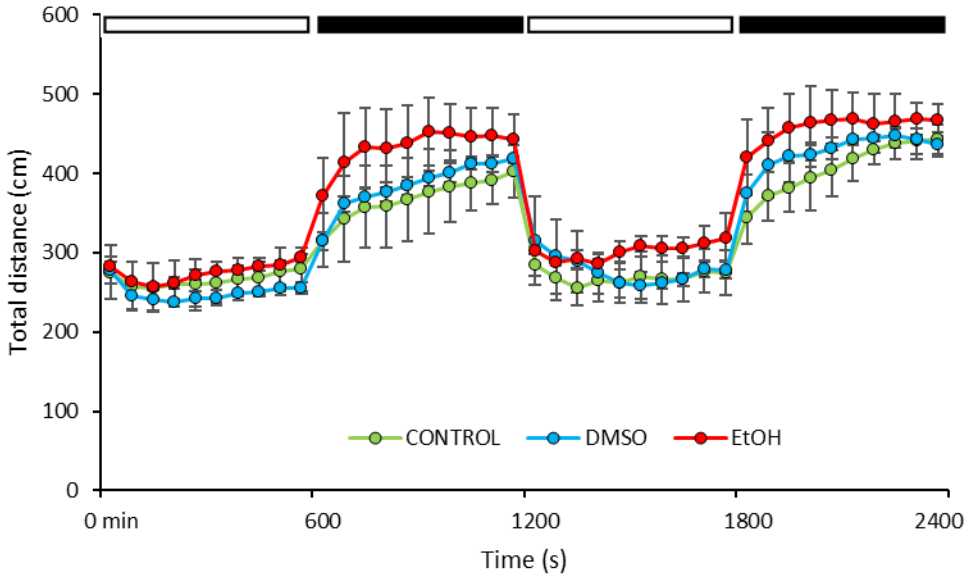

Figure 3 Average of 3 independent repetitions of the sum of total distance swam by each larva per min. N=24 per condition. Larvae were non-exposed (negative control in ISO medium, or solvent control with DMSO) and exposed to a 0.5% solution of ethanol as a positive control.

Results of larvae exposed to pesticides of interest are shown in Figure 4. All exposures significantly affected zebrafish behavior but only in the dark phases, no effects were seen in the light periods, when zebrafish larvae are usually less active. All concentrations of imidacloprid except for 2.5 µg/L decreased the total distance swam by zebrafish larvae. On the contrary, propiconazole increased the distance swam after larvae exposure to 6.25 µg/L. The mixture of pesticides at a total concentration of 8 µg/L (sum of the concentrations of the five pesticides) increased the distance swam by zebrafish larvae, however, this effect was not repeated in higher or lower concentrations.

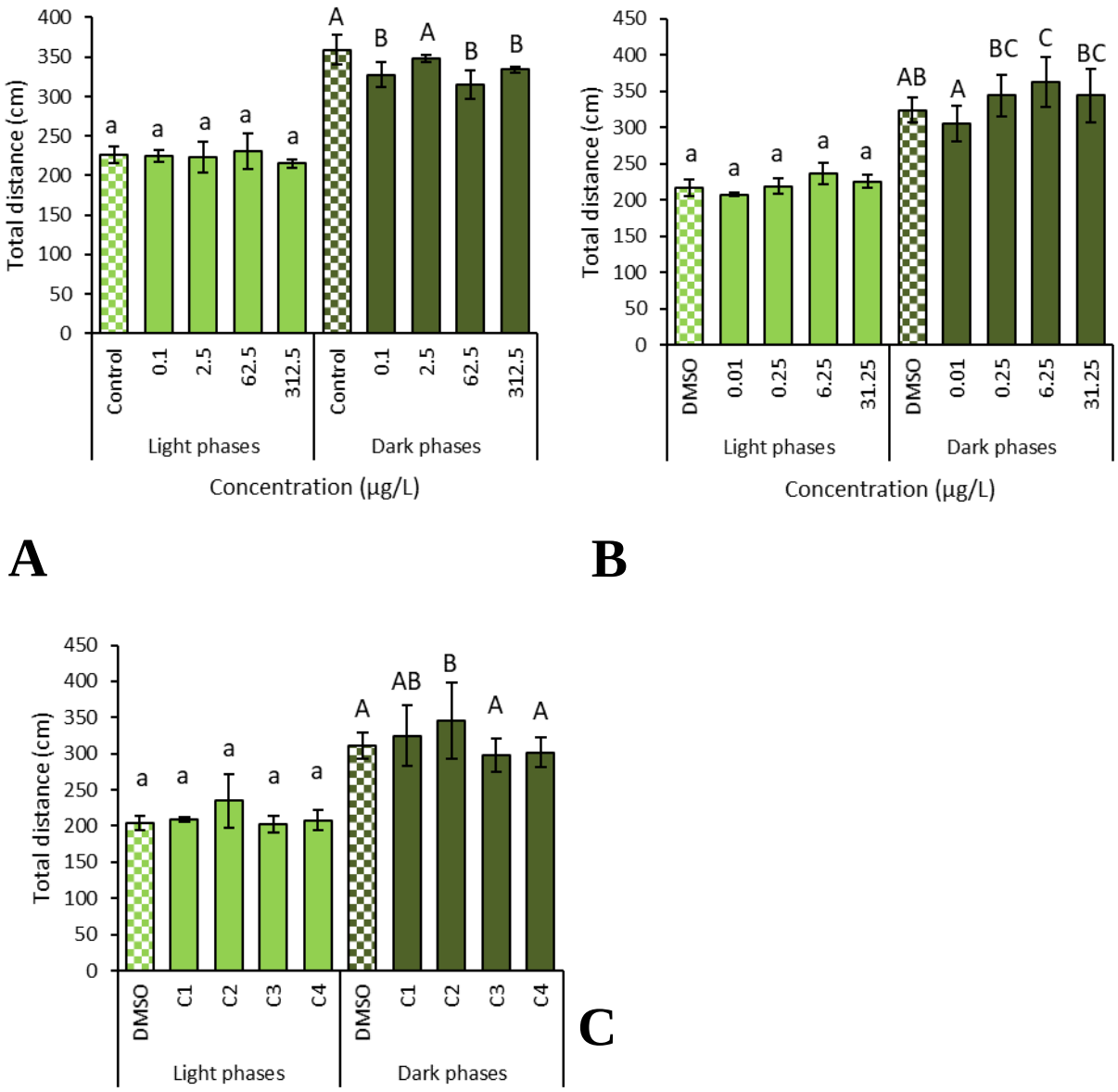

Figure 4 Total distance (cm) swam by one zebrafish larva during 30 min of both light or both dark phases after 5 days of exposure to imidacloprid (A), propiconazole (B), and the mixture (C) and subjected to light, dark transition locomotion test. N=57 larvae per condition of 3 pooled independent repetitions.

6. Gene expression analysis

The samples of zebrafish larvae for gene expression were transcribed in cDNA and are waiting for analysis by qPCR. The results cannot be presented at the time of the thesis submission.

Conclusion

Pesticides of interest affected various biological functions in zebrafish larvae, sometimes even at environmentally relevant concentrations. Developmental malformations were observed only after high, environmentally non-relevant concentrations. Spontaneous tail coilings of zebrafish embryos revealed itself as a sensitive analysis capable of detecting effects even after exposure to low concentrations of tens of nanograms per liter. Heart rate of zebrafish larvae was increased in comparison with the control after exposure to all conditions, except for the lowest concentrations of imidacloprid and the mixture. Ambiguous effects were detected at the level of zebrafish locomotion, where only some concentrations, not in a dose-response manner influenced the distance swam by zebrafish larvae. Results of gene expression analysis will elucidate and complete obtained results enabling thus a proper manuscript discussion and a final conclusion.

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PUBLICATION V.

ACUTE AND (SUB)CHRONIC TOXICITY OF THE NEONICOTINOID IMIDACLOPRID ON *CHIRONOMUS RIPARIUS*

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Supplementary Materials:

https://ars.els-cdn.com/content/image/1-s2.0-S0045653518311779-mmc1.docx

Publication is attached in Annexes

Main findings of Publication V.

- Acute (1 day) and subchronic (10 and 28 days) imidacloprid toxicity tests were carried out with midge *Chironomus riparius* acute one-day-old C. riparius LC50 31.5 µg/L, NOEC 5 µg/L, LOEC 10 µg/L
- 10-day LC50 2.33 µg/L, NOEC 0.625 µg/L, LOEC 1.25 µg/L
- 28-day test NOEC 0.125 µg/L, LOEC 0.625 µg/L
- Larval growth was hindered after 10 days of exposure to 0.625 µg/L of imidacloprid which may ultimately influence reproduction and population dynamics.
- The low environmental concentration of 0.0625 µg/L of imidacloprid caused a content reduction of reduced and oxidized glutathione biomarkers, moreover, a weak effect on lipid peroxidation (elevated concentrations of TBARs) was observed after exposure to 0.625 µg/L.
- Oxidative stress may be a relevant mechanism in the imidacloprid-induced toxicity in *Chironomus riparius*.
- *Chironomus riparius* was observed to be amongst the most sensitive aquatic insect species to acute imidacloprid exposure.

CHAPTER V.

OVERALL DISCUSSION, HIGHLIGHTS, AND FUTURE PERSPECTIVES

This dissertation thesis aimed to elucidate what are the risks to non-target aquatic organisms caused by currently used pesticides and their mixtures using two different approaches: laboratory and *in situ*. First, some of the most used pesticides and the concentrations in which they appear in European water bodies, more precisely in those of the Czech Republic and in Arcachon Bay in France, were identified. Secondly, two non-target organisms were selected for ecotoxicological investigation: a freshwater model organism (zebrafish) and a euryhaline invertebrate species local to Arcachon Bay (Pacific oyster). The effects of selected pesticides were studied on their embryo-larval stages, which are very sensitive to pollution and allow to spare unnecessary distress of great numbers of adult animals who would be otherwise needed.

Before answering the key questions about the risks of pesticides to non-target aquatic species, I would like to take a small detour into the used **methodology and its future perspectives**. In addition to well established normalized biotests with embryos of oyster or zebrafish, one of the used techniques is quite new, has the potential to be further optimized and automatized, and thus deserves more attention. The discussion thus aims to pass the information on to the readers of this dissertation thesis and its possible future users with special focus on the analysis of oyster larval locomotion patterns. This technique consists of capture of videos of larvae, and their software assessment, i.e. ImageJ plugin, which was developed by bioinformatician Alicia Romero Ramirez (EPOC laboratory, University of Bordeaux). Because of limitations in the recognition of individual larvae by the software, each of them are manually followed on videos to determine their trajectory. Future effort should be focused on the automatization of this analysis. Indeed, the mentioned plugin is programmed to assess multiple parameters like larvae average and maximal swimming speed, but also length of the larva's trajectory and the distance – i.e. different between its initial and terminal location. Three types of trajectories are defined: rectilinear (which is the predominant type in non-exposed larvae), circular, and stationary. While it is evident that rectilinear trajectories' length and distance parameters are ideally equal, for the circular ones it is valid that the length is multiple times greater than the distance. The stationary trajectories have low values of both distance and length. In practical use, these straightforward calculations have limitations when multiple larvae cross their paths on the video. The software confounds individual organisms and manual corrections are needed. Unfortunately, manual assessments are commonly the primary source of mistakes, hence there is a need to finalize this sensitive and excellent tool for assessing swimming behavior. Moreover, the development of a machine with a quality camera capable of differentiating small (60 μm) organisms while capturing the whole microwell plate at once would be excellent

progress when compared to current demanding process that takes several hours of capturing well per well. Behavior is a sensitive endpoint, and it is necessary to keep the same parameters during its measurement including the time of measurement.

Concerning zebrafish methodology, a part of this work consisted of optimization of several methods (heart rate, spontaneous tail coilings, and locomotion assessment) thanks to Danioscope and Ethovision software available at RECETOX laboratories. For instance, in comparison with the above-mentioned oyster larval locomotion, zebrafish spontaneous tail coilings need to be measured in the shortest time possible because of their frequency change during the first hours of the zebrafish development. Even small one-hour difference in video capture means the opposite effect of a positive control. As a result, standard operating procedures of these methods were newly created during the thesis for the laboratory allowing their further future use.

Were all these methods sensitive enough to measure the effects of low concentrations of pesticides? What pesticide had the greatest impact on the aquatic early-life life-stages?

First, this work was focused on the key question of whether **low, environmentally relevant concentrations of individual pesticides induced detectable effects especially at biochemical/physiological levels on the embryo-larval stages** of the Pacific oyster and the zebrafish. A summary of all observed effects during this thesis that meet the condition of exposure to a low, environmentally relevant concentration of selected pesticides is shown in Table 6. As low concentrations we considered the concentrations in the range up to 1 μg/L of S-metolachlor, metolachlor oxanilic acid, metolachlor ethanesulfonic acid, imidacloprid, and up to 0.25 μg/L of propiconazole. These concentrations are based on concentrations shown or calculated in Tables 1 and 2 in Chapter I. For the effects caused by higher than the environmental concentrations, the reader is invited to read the respective publications.

As expected, no direct mortality was observed after exposure to these low concentrations. In general, the herbicide S-metolachlor and its metabolites at these low concentrations were not toxic to the zebrafish larvae, except for a decreasing effect of S-metolachlor on spontaneous tail movements of zebrafish embryos and on thyroid-related genes (upregulation of the iodothyronine deiodinases and both thyroid nuclear receptors) caused by one of the metabolites,

metolachlor ethanesulfonic acid (MESA). On the other hand, the low concentrations of these substances had severe impact on the development of oyster larvae, causing an increase in developmental malformations. Correspondingly to our studies, Mai et al. (2014) revealed numerous effects on the gene expression of oyster larvae after exposure to low concentrations of S-metolachlor and its metabolites, especially on the genes linked to the defense against oxidative stress and mitochondrial metabolism. Moreover, Gamain et al. (2020) observed an impact on the larvae locomotion after exposure to S-metolachlor which decreased the proportions of normal swimming trajectories.

The most toxic pesticide on the development of zebrafish early-life stages in this low concentration range was the fungicide propiconazole. Propiconazole was already confirmed as a source of various toxic effects on zebrafish larvae (Souders et al., 2019b; Teng et al., 2020, 2019), and its low concentrations (0.1 µg/L) caused endocrine disruption in 5-day old zebrafish larvae (Teng et al., 2020). Concentrations, which we may be found commonly in European water bodies (0.01 µg/L) increased zebrafish heart rate and spontaneous tail coilings, and 0.25 µg/L increased distance swam by larvae demonstrating thus a neurotoxic effect. A complete propiconazole (and imidacloprid) effects profile on gene expressions is not known yet, and the experiments are ongoing. A stronger effect of propiconazole than imidacloprid is expected, with propiconazole possibly altering the levels of thyroid hormone-related genes. Some other conazole pesticides were indeed marked as thyroid disrupting compounds, although at higher concentrations (Liang et al., 2015; Yu et al., 2013).

Propiconazole exerted several adverse effects on oyster larvae (increase in stationary swimming patterns, upregulation of an oxidative stress defense linked gene, a metallothionein gene, and downregulation of gene coding for mitochondrial small subunit) showing thus that its concentrations commonly observed in European aquatic environment are not harmless.

Table 6 Summary of observed effects (statistically different in comparison with the non-exposed organism) on zebrafish and oyster embryos and larvae after exposure to low, environmentally relevant concentrations of imidacloprid, propiconazole, S-metolachlor, metolachlor oxanilic acid, and metolachlor ethanesulfonic acid up to 1 µg/L (0.25 µg/L for propiconazole). All concentrations in the table are in µg/L. Dev. malf. = developmental malformations. Traj. = trajectories. TBA = To be assessed. NA = Not available. All results were obtained during this work, except when stated otherwise.

	Zebrafish					**Pacific oyster**		
	Dev. malf.	Tail coilings	Heart rate	Locomotion	Gene expression	Dev. malf.	Locomotion	Gene expression
IMI	-	-	-	0.1 ↓	TBA	-	-	1 ↓ *sodMn*, ↑ *gadd45*
PRO	-	0.01 ↑	0.01 ↑	0.25 ↑	TBA	-	0.02 ↑ stationary traj.	0.02 ↑ *SodCu/Zn*, 0.2 ↓ 12S, ↑*mt1*, *sodCu/Zn*
SM	-	1 ↓	-	-	-	0.1↑	0.01 ↓ rectilinear traj. (Gamain et al., 2020)	0.001-0.01 ↑*sodMt* 0.1-1↓ *sodMt*, 0.01-1 ↑ *cat*, 0.1 ↑ *p53*, ↓12S (Mai et al., 2014)
MOA	-	-	-	-	-	1 ↑	NA	0.01 ↓*cox*, 1↓*cox* (Mai et al., 2014)
MESA	-	-	-	-	1 ↑ (*thra, thrb, dio2*)	0.1↑	NA	0.01-1 ↓*cat*, 0.01 ↓*cox*, 1 ↓*mt2*, ↑*gst* (Mai et al., 2014)

To keep pace with the rapid advancements of the agrochemical industry searching for novel efficient pesticide possibilities, an ecotoxicity comparison of propiconazole and its nanoformulation was carried out. Neither propiconazole nor its nanoformulation affected larval development (as assessed by the presence of developmental malformations). The nanoformulation increased larval swimming speed, unlike propiconazole, but this effect was probably linked to the capsules enclosing the nanoformulation.

The effects of low concentrations of insecticide imidacloprid on zebrafish larvae were scarce with only decreased swam distance by zebrafish larvae in comparison with non-exposed fish. Interestingly, imidacloprid was not toxic to oyster larvae. In comparison with zebrafish larvae, no effect on locomotion patterns was observed (even in higher non-environmental concentrations). However, sensitive qPCR analysis revealed some effects on the gene expression (downregulation of oxidative stress defense linked gene and upregulation of gene implicated in the growth arrest and DNA damage).

As well as the zebrafish, the Pacific oyster was not sensitive to low exposure of imidacloprid except for few effects on the gene expression. Imidacloprid altered several other genes, but only in higher non-environmental concentrations, which are not discussed in this Discussion chapter but are fully described in Publication I. Other effects caused by higher concentrations of pesticides of interest may be also viewed in respective publications of this dissertation thesis.

On the contrary to the impact on oyster and zebrafish larvae, the midge *Chironomus riparius*, a non-target aquatic insect species, was very sensitive to imidacloprid at low concentrations. The concentration of 0.0625 μg/L had an inhibiting effect on glutathione biomarkers, and 0.625 μg/L hindered larval growth. Imidacloprid low concentrations probably affected the midge via induction of oxidative stress, a mechanism of action which was also indirectly detected via gene expression alterations of oyster larvae.

In conclusion, low environmental concentrations of insecticide imidacloprid were the least toxic on oyster and zebrafish embryo-larval stages development. Different effects were seen with the herbicide S-metolachlor and its two metabolites which hindered the development and altered sublethal biomarkers of oyster larvae but made almost no impact on zebrafish larvae. Finally, both zebrafish and oyster early-life stages were sensitive to low concentrations of fungicide propiconazole.

Second, we hypothesized that **the effects of mixtures of the studied compounds were more pronounced and may have led to the effects that could not have been predicted from individual chemicals**. Although the response is complex and depends on assessed biomarker and organism, we may say that generally, the impact of the mixture of the five compounds was greater than the impact of individual compounds for both oyster and zebrafish larvae. Starting with the less sensitive endpoint – the presence of the developmental malformations in zebrafish larvae, the mixture indeed induced more frequent and the most malformation types (craniofacial malformations, non-inflation of the gas bladder, and malabsorption of the yolk sac) at the highest tested concentration (containing 312.5 µg/l of each imidacloprid and the metabolites, and 31.25 µg/l of S-metolachlor, and propiconazole) whereas individual compounds in the same concentration range had no effects or did induce - for instance - only one of the malformation types. However, these effects did not cause high malformations frequency, making thus impossible to calculate the EC_{50}, and subsequently, mathematically evaluate the mixture effect. Briefly, the effects of mixtures can be explained by concentration addition concept, synergistic concept (ratio between predicted and observed effect higher than 2), and antagonistic concept (ratio between predicted and observed effect lower than 0.5) as described in Cedergreen (2014), The mixture investigated in the present study exerted with the highest probability a concentration addition effect on the zebrafish development.

On the other hand, the mixture hindered oyster development at 0.32 µg/L (100 ng/L of imidacloprid and the metabolites, and 10 ng/L of propiconazole and S-metolachlor). In comparison, the LOECs of individual compounds were 200 µg/L for imidacloprid and propiconazole. The LOECs of S-metolachlor, metolachlor oxanilic acid, and metolachlor ethanesulfonic acid were previously reported as 0.01, 0.1, and 0.1 µg/L, respectively (Mai et al., 2014). However repeated experimentations during this work established LOECs at 0.1, 1, and 0.1 µg/L for S-metolachlor, metolachlor oxanilic acid, and metolachlor ethanesulfonic acid, respectively. In this view, the main drivers behind the mixture toxicity on oyster larvae are probably S-metolachlor with its metabolites. The EC_{50} could not have been obtained for some of the compounds (due to weak effects). However, when comparing concentrations causing lower effects (such as EC_{30}), we may notice, that EC_{30} of the mixture is approximately 1.44 mg/L of imidacloprid and the metabolites, each, and 335 µg/L of S-metolachlor and propiconazole (when divided proportionally between the compounds). Whereas individually, S-metolachlor EC_{30} was around 10 ng/L, metolachlor oxanilic acid around 100 ng/L, and metolachlor ethanesulfonic acid between 0.1 and 1 µg/L as visible from the paper of Mai et al.

(2014). Thus, it is possible that the effects were attenuated by the mixture. However, available data are not sufficient to classify the effects as antagonistic (the difference in abnormal larvae count would need to be less than two-fold for the antagonism by definition).

Locomotion assessment of oyster larvae revealed no effects of the pesticide mixture, even though Gamain et al. (2020) reported a strong effect on larvae trajectories after exposure to 10, 100, and 1000 ng/L of S-metolachlor. This would indicate possible antagonistic effect of the pesticide mixture. However, erratic behavior is often linked to the presence of developmental malformations. The above-mentioned study observed 60% of abnormal larvae after exposure to 1 µg/L of S-metolachlor, whereas in the present thesis, the concentration of 10 mg/L caused only 36.5% of abnormal larvae. These results thus do not seem directly comparable and so it is unsure whether an antagonistic effect is really behind the absence of impact on the larvae locomotion.

At the molecular level, a synergistic effect in the mixture was possibly observed. Indeed, oyster larvae exposed to the mixture presented repressed gene 12S, as well as after exposure to higher (10 and 20 times, respectively) concentrations of S-metolachlor and propiconazole, suggesting possible synergistic effect. The same effect may have occurred for repression of the gene *cyp1a*, which was not affected by individual compounds. On the contrary, the effect on genes implicated in the defense against reactive oxygen species was observed in larvae exposed to individual compounds, but not to the mixture.

Interesting is the sublethal assessment of zebrafish tail coilings. Whereas S-metolachlor (1 µg/L) and a mixture of S-metolachlor and its two metabolites (1 µg/L of each) decreased the spontaneous movements, the mixture of the 5 compounds (total concentration 0.32-8-200-1000 µg/L), as well as imidacloprid and propiconazole individually, increased the tail movements. Moreover, no effects of herbicide and its metabolites were seen on the heart rate and distance swam of zebrafish larvae, unlike imidacloprid, propiconazole, and the mixture. This endorses the hypothesis of the higher sensitivity of zebrafish larvae to propiconazole, and in higher concentrations to imidacloprid as well. Interestingly, propiconazole and imidacloprid had opposite (enhancing and inhibiting, respectively) effects on zebrafish larvae locomotion, resulting in no effect in larvae exposed to the mixture (except for mild effects in one concentration).

In conclusion, the effects caused by the mixture on zebrafish and oyster early-life stages were predominantly characterized by the concentration addition and could have been thus

approximately predicted from the effects of individual chemicals. However, effects on several sublethal endpoints were less predictable: a) oyster larval trajectories, where the toxic effect of S-metolachlor was possibly antagonized by the other compounds, and b) gene expression of some genes of oyster larvae, whose levels were possibly synergistically altered (summary shown in Table 7).

To fully understand the mixture impact on fish gene expression, including the thyroid-related genes, additional insight could be obtained by the qPCR results of zebrafish larvae which are currently being carried out. Moreover, to compare the obtained gene expression results, a trout gill fish cell line RT Gill W1 was exposed to the mixture and its samples are currently being analyzed. Furthermore, an additional LC-MS/MS analysis of thyroid hormones and their metabolites in whole zebrafish larvae is planned to be included in the final version of Publication IV. to complete information on the eventual thyroid disruption of the mixture. New pieces of knowledge on the mixture mechanisms of action will be acquired in the planned co-authored publication where analysis of multiple biomarkers is being carried out with zebrafish larvae and also in fish cell line model. Concerning deeper knowledge of mixture effects on oyster larvae, eventual future assessment of epigenetic effects may be carried out by colleagues at EPOC laboratory since oyster larvae samples dedicated for this analysis were collected throughout the work on this dissertation thesis.

Table 7 Summary of observed mixture effects on zebrafish and oyster embryos and larvae. CA effect = Concentration addition effect. TBA = To be assessed. NA = Not applicable.

	Development	Spontaneous movements	Heart rate	Locomotion	Gene expression
Zebrafish	CA effect	CA effect	CA effect	CA effect	TBA
Pacific oyster	CA effect	NA	NA	Possible antagonistic effect	Possible synergistic effect (2 genes)

The third and last hypothesis presumed that the **effects on oyster larvae observed in the laboratory could be extrapolated to field *in situ* observations and that the pesticide contamination in the inner part of Arcachon Bay might be responsible for the worsened**

state of oyster development observed in recent years. A well-designed exposure device is the key to a high-quality assessment of the *in situ* impact on oyster larvae. The device used in this study was welded in the workshop of Marine Research Station of Arcachon and operated several times before the real *in situ* campaign to ensure that it worked properly. The pilot assessment were performed in an aquarium in the laboratory or in a port of Arcachon attached to a wharf. Several *in situ* campaigns were planned, however, for various reasons (e.g. dangerous storm which made impossible to collect the devices deployed in Arcachon Bay) only one campaign was carried out. Thus, although robust (4 devices per site, each containing embryos of a different oyster couple) these results should be considered as preliminary and other campaigns might help to confirm the following up-to-date findings.

First, the water quality on the three sites of interest in Arcachon Bay is safe for oyster development as assessed by the low percentage of malformed oyster larvae. For comparison, the natural rate of larvae malformations is considered to be up to 20% in the normalized embryo-larval oyster toxicity test (NF ISO 17244, 2015). The reference site Grand Banc presented only 16.38 ± 6.49% of abnormal larvae, site Les Jacquets 15.97 ± 4.29%, and site Comprian 20,81 ± 7.86%. As hypothesized, the malformation rate in larvae exposed in the laboratory to the environmental concentration of the mixture (20.61 ± 3.84%) indeed corresponded to the malformation rate observed in larvae exposed at the site Comprian, which is the most influenced site by anthropogenic pollution. Nevertheless, on the contrary to laboratory experiments, where a statistical difference was observed between the non-exposed larvae and those exposed to the mixture, no statistical difference was seen when comparing the reference site Grand Banc and site Comprian. The source of this difference may lay in the robustness of each experiment (several laboratory experiments composed of embryos of 7 oyster couples versus one *in situ* campaign composed of embryos of 4 oyster couples) and may be resolved by conducting another *in situ* campaign, as already mentioned above.

No differences in larvae swimming speed or used trajectories were seen between the three locations. However, generally, the proportions of the rectilinear (=normal) trajectory were lower in the field regardless of the site in comparison with laboratory exposure. This may be caused by multiple factors (longer exposure in the field than in the laboratory, changes in the temperature or salinity in the field, etc.). The pollution in the inner part of Arcachon Bay thus did not seem to affect larvae locomotion when compared with the reference site (whose water quality is also burdened by pesticides and other anthropogenic compounds, but in lower quantities thanks to its location near the ocean entry).

On the contrary to the previous approaches, gene expression analysis by qPCR revealed several differences between the two sites and the reference site of Arcachon Bay. Induction of genes linked to defense against oxidative species was detected at both sites of interest Les Jacquets and Comprian, as well as downregulation of metallothionein genes implying lower scavenging and protective capacity of oyster cells. Finally, genes linked to the mitochondrial metabolism were upregulated at Les Jacquets and Comprian sites as well. All these alterations, which may have longer-term consequences on the life of oyster larvae, indicated a worsened state of oyster larvae in the inner part of Arcachon Bay.

In conclusion, the observed effects on oyster larvae at the three sites were milder than expected before the beginning of this study and the absence of high malformation rates of oyster larvae brings rather reassuring message to everyone worried about the water quality of Arcachon Bay. Longer *in situ* exposures may elucidate the impact of gene expression alterations observed on oyster larvae exposed in the inner part of Arcachon Bay.

CONCLUSION

This dissertation thesis aimed to evaluate the effects of currently used pesticides and their mixtures on non-target aquatic organisms. Two approaches were used in this work: laboratory and *in situ* and the results obtained were summarized in five publications, of which three were already published in peer-reviewed journals. The core of this work was focused on early-life stages of zebrafish (*Danio rerio*) and Pacific oyster (*Magallana gigas*) that were exposed to environmentally relevant concentrations (as well few higher ones) of pesticides of interest which are currently commonly used throughout the world: herbicide S-metolachlor and its two metabolites metolachlor oxanilic acid, and metolachlor ethanesulfonic acid, insecticide imidacloprid, and fungicide propiconazole. The used pesticide concentrations were based on concentrations detected in European water bodies, especially in Czech rivers, and French lagoon on the Atlantic coastline, Arcachon Bay.

First, the studies with zebrafish revealed the vulnerability of its embryo-larval stages to selected pesticides from the least toxic with almost no effects caused by S-metolachlor + its metabolites to imidacloprid with effects observed in higher concentrations, and finally to propiconazole with toxicity in low concentrations (tested up to tens of μg/L of propiconazole and hundreds of μg/L of other compounds). Indeed, sensitive sublethal techniques showed that the zebrafish was affected even by low environmentally relevant concentrations of propiconazole implying that the development of freshwater fish may be at risk with current agricultural practice. Moreover, an ongoing qPCR analysis will help elucidate its role in thyroid metabolism disruption, as well as that of a mixture of these five compounds. Generally, the mixture effect on zebrafish early-life stages was defined by the concentration addition concept.

Second, work with Pacific oyster embryos and larvae allowed to establish relatively high lowest observed effect concentration to cause developmental malformations for imidacloprid and propiconazole (200 μg/L). Considerably higher toxicity of S-metolachlor and its metabolites was observed in the range of 0.1-1 μg/L, making thus the herbicide with its metabolites the main driver behind the mixture toxicity as well. Gene expression alterations revealed possible long-term impacts of exposure to imidacloprid and propiconazole low concentrations as well, especially non-specific toxicity related to the presence of reactive oxidative species was observed. The eventual agricultural solution to propiconazole toxicity to non-target species, a novel effective nano formulated pesticide with a slow release of propiconazole did not convince, due to its relatively comparable ecotoxicity with the conventional molecule. Furthermore, contrary to a few observed effects of imidacloprid on the development of Pacific

oyster, the freshwater midge *Chironomus riparius* was sensitive to even low concentrations of this insecticide.

The pesticide mixture caused an ambiguous impact on the larvae of Pacific oysters. Whereas its capacity to induce developmental malformations was very high (even at the lowest tested, environmentally relevant concentration) and driven by concentration addition effect, there is a possible explanation of an antagonistic activity on larvae swimming trajectories. Moreover, some gene alterations were possibly synergistically enhanced by the present mixture.

The up-to-date knowledge of the ecotoxicity of the reconstituted pesticide mixture on oyster embryo-larval stages was used as a proxy of the water quality in Arcachon Bay, however, the realistic mixture in the lagoon presents considerably higher complexity (pesticides, metals, drugs, personal care products, hydrocarbons…). Thus, an *in situ* campaign consisting of transplanting oyster embryos in caging devices on three different sites of Arcachon Bay, each of different pollution background, was carried out. Surprisingly, the water quality at all studied sites of Arcachon Bay was sufficient for the successful development of oyster larvae with no differences in locomotion patterns between the sites. Nevertheless, gene expression larvae profiles revealed a worsened state of oyster larvae in the inner, higher polluted part of Arcachon Bay. These various pieces of knowledge suggest that the worsened state of oysters in Arcachon Bay may be caused by alterations in older organisms (linked or not to possible long-term impacts of observed gene expression alterations) and are asking for future prolonged *in situ* exposures or exposures with older larvae.

In conclusion, this work helped to understand the impact of environmentally relevant concentrations of currently used pesticides and their mixtures. These low concentrations are often considered safe and thus neglected in the ecotoxicological assessment. This work also illustrated the usefulness and ecological relevance of studies complemented with field research. At last, but not least, this study demonstrated the sensitivity and usefulness of embryo-larval stages thus proving the replaceability of adult organisms along with their distress and unnecessary sacrifices in the (eco)toxicology research.

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ANNEXES

Publication II. Supplementary Materials

Pesticide mixture toxicity assessment through *in situ* and laboratory approaches using embryo-larval stages of the Pacific oyster (*Magallana gigas*)

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Supplementary Table S1:

Concentrations of pesticides of interest detected on multiple sites in Arcachon Bay in the years 2010-2014. N=669 for each pesticide. Concentrations were calculated according to the data of Tapie et al., (2018).

	PRO	IMI	Metolachlor	MOA	MESA
Limit of quantification (ng/L)	1	1	0.5	3	2
Samples with detected substance (%)	20.7	34.8	90.3	87.6	88.0
Average concentration in all samples (ng/L)	0.7	2.6	31.9	163.5	117.6
Average concentration in samples with detected substance (ng/L)	3.1	7.6	35.4	186.9	133.8
Maximal concentration (ng/L)	**29.1**	**173.6**	**1695.9**	**1609.9**	**1059.2**

Supplementary Table S2:

The concentration of the selected pesticides in the 3-liter glass beakers used in the experiments investigating the gene expression analysis in oyster larvae. In total, 3 independent repetitions of the experiment were carried out. The first two repetitions were done during EXPERIMENT 1 but only one sample series was collected for the chemical analyses, the third repetition was carried out during EXPERIMENT 2.

		EXPERIMENT 1		EXPERIMENT 2	
Pesticide	*Nominal (µg/L)*	Measured (0h) (µg/L)	Measured (42h) (µg/L)	Measured (0h) (µg/L)	Measured (42h) (µg/L)
IMI	*0.1*	0.061	0.109	<LOD	0.118
	0.5	0.535	0.520	0.226	0.518
	2.5	2.475	2.519	<LOD	2.534
PRO	*0.01*	0.012	<LOD	0.010	NQ
	0.05	0.041	<LOD	0.167	<LOD
	0.25	0.341	0.014	0.924	NQ
SM	*0.01*	0.165	0.041	0.138	0.036
	0.05	0.496	<LOD	0.168	<LOD
	0.25	3.33	0.237	<LOD	0.632
MOA	*0.1*	<LOD	<LOD	<LOD	<LOD
	0.5	<LOD	<LOD	<LOD	<LOD
	2.5	<LOD	<LOD	<LOD	<LOD
MESA	*0.1*	<LOD	<LOD	<LOD	<LOD
	0.5	<LOD	<LOD	<LOD	0.582
	2.5	0.462	0.444	<LOD	0.592

Supplementary material S1:

LC-MS/MS analysis of pesticides

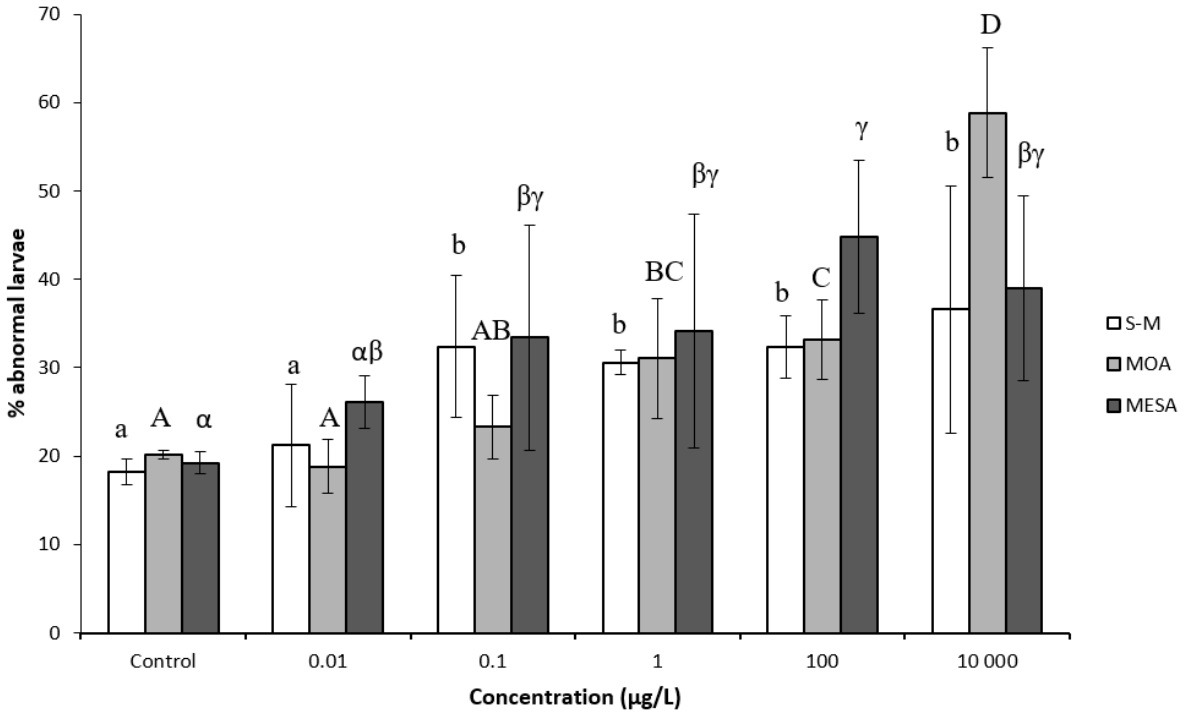
The analysis was performed with a Waters LC chromatograph (Waters, Manchester, U.K.) and the chromatographic separation was achieved using a column Acquity BEH C18 of 100 x 2.1 mm ID and 1.7 μm particle size. A gradient elution method was set with phase A (0.1 % formic acid in water) and phase B (0.1 % formic acid in acetonitrile). The gradient elution started with 20 % B, increased to 90 % B over 9 min, followed by 3 min of washing (100 % B, then decreased to 20 % B and held for 4 min to equilibrate the system before the next injection. The flow rate was set at 0.3 mL/min and the injection volume of samples in 20 % of acetonitrile at 8 μL. The column and sample temperatures were set at 35°C and 10°C, respectively.

Detection was performed on a Xevo TQ-S quadrupole mass spectrometer (Waters Manchester, U.K.) and analytes after ESI ionisation were detected in positive ion mode using tandem mass spectrometry. The following *m/z* transitions were monitored, for imidacloprid: *m/z* 256.1 > 175.0, (quant.: cone voltage 25V; collision energy 20V) and *m/z* 256.1 > 209.0 (qual.: cone voltage 25V; collision energy 12 V); for propiconazole: *m/z* 342.1 > 159.0 (quant.: cone voltage 42 V; collision energy 30 V) and *m/z* 342.1 > 69.0 (qual.: cone voltage 42 V; collision energy 15 V); for imidacloprid D4: *m/z* 260.1 > 213.0 (quant.: cone voltage 25 V; collision energy 12 V); for tebuconazole D6: *m/z* 314.3 > 72.1 (quant.: cone voltage 35 V; collision energy 20 V); for S-metolachlor: *m/z* 284.1 > 176.1, (quant.: cone voltage 30V; collision energy 25V) and *m/z* 284.1 > 252.1 (qual.: cone voltage 30V; collision energy 15 V); for metolachlor OA: *m/z* 280.3 > 146.2, (quant.: cone voltage 20V; collision energy 25V); *m/z* 280.3 > 119.0 (qual.: cone voltage 20V; collision energy 30 V) and *m/z* 280.3 > 131.0 (qual.: cone voltage 20V; collision energy 30 V); for metolachlor ESA: *m/z* 330.4 > 160.2, (quant.: cone voltage 25V; collision energy 30V); *m/z* 330.4 > 132.1 (qual.: cone voltage 25V; collision energy 50 V)); *m/z* 330.4 > 145.5 (qual.: cone voltage 25V; collision energy 50 V) and *m/z* 330.4 > 174.1 (qual.: cone

voltage 25V; collision energy 35 V). The capillary voltage was set at 3 kV and the cone, desolvation and collision gas flows were set at 150 (L/h), 700 (L/h) and 0.15 (mL/min), respectively. The source and desolvation temperature were set at 150°C and 400°C, respectively. Data were processed by MassLynxTM software (Manchester, U.K.). The limit of detection (LOD, signal to noise ratio S/N>3) for imidacloprid, propiconazole, S-metolachlor, metolachlor OA, and metolachlor ESA were 0.02, 0.005, 0.005, 0.05, and 0.05 µg/L, respectively. The limit of quantification (LOQ, signal to noise ratio S/N>10) for imidacloprid, propiconazole, S-metolachlor, metolachlor OA, and metolachlor ESA were 0.05, 0.01, 0.01, 0.1 and 0.1 µg/L, respectively. The analytes were quantified using external calibration (0.01 – 50 µg/L in 20 % of acetonitrile) and normalized with internal deuterium labelled standards (imidacloprid D4 for imidacloprid, MOA and MESA and tebuconazole D6 for SM and propiconazole).

Supplementary Figure S1:

Sum of abnormal oyster larvae (arrested development and different malformation types) after 30 hours of exposure to increasing concentrations of SM, MOA, and MESA. Different letters indicate statistical differences between variables ($P < 0.05$). Results are presented as the mean of 3 independent experiments ± SD.



Supplementary Table S3:

Swimming speed (µm/s) observed in oyster larvae after 2 days of transplantation at three different sites in Arcachon Bay. Results are presented as the mean of 4 independent experiments ± SD.

	Speed (µm/s)	
Sampling site	***maximal***	***average***
Grand Banc	***304 ± 49***	***187 ± 43***
Les Jacquets	***428 ± 63***	***263 ± 34***
Comprian	***341 ± 74***	***204 ± 43***

Publication V.

Njattuvetty Chandran, N., Fojtova, D., Blahova, L., Rozmankova, E., Blaha, L., 2018. Acute and (sub)chronic toxicity of the neonicotinoid imidacloprid on *Chironomus riparius*. Chemosphere 209, 568–577.

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