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

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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
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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).

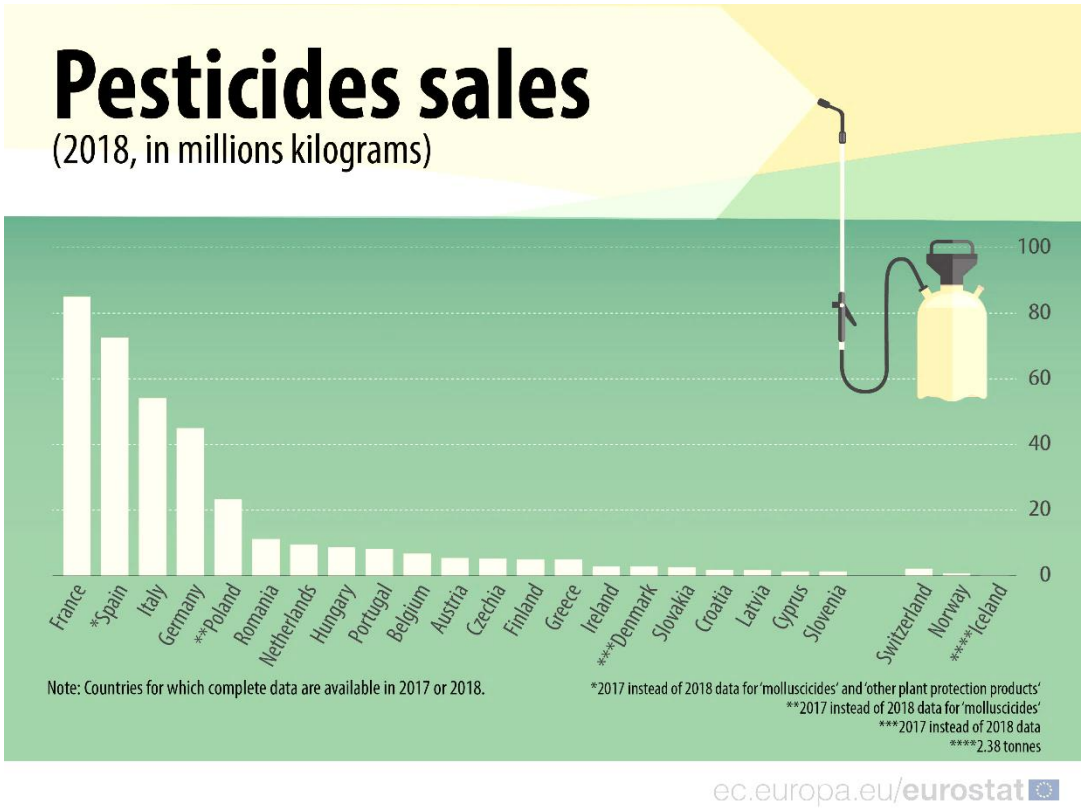

Figure 1 Consumption of pesticides in the member states of EU (source: https://ec.europa.eu/eurostat/statistics-explained/index.php?title=Agri-environmental_indicators)

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).

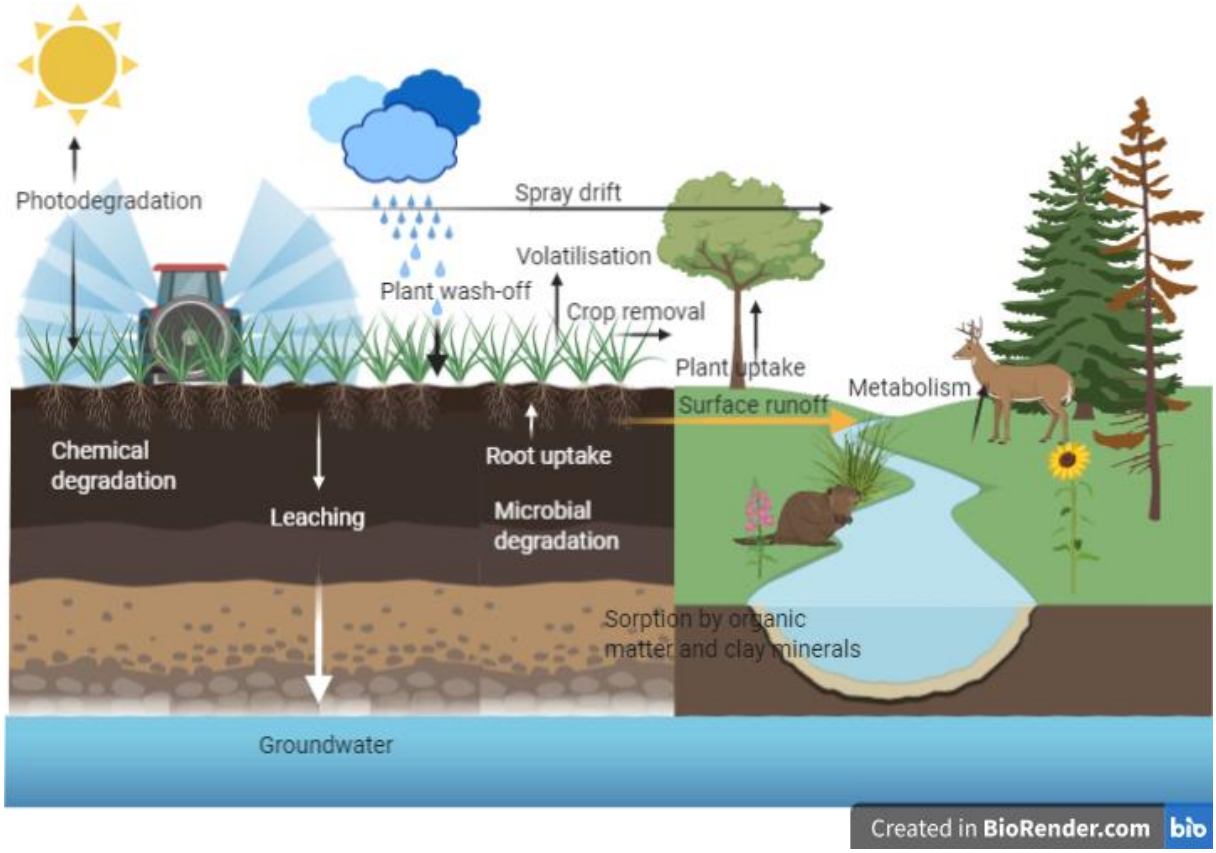

Figure 2 Fate of pesticides and their transfer in waters; Created with Biorender.com

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).

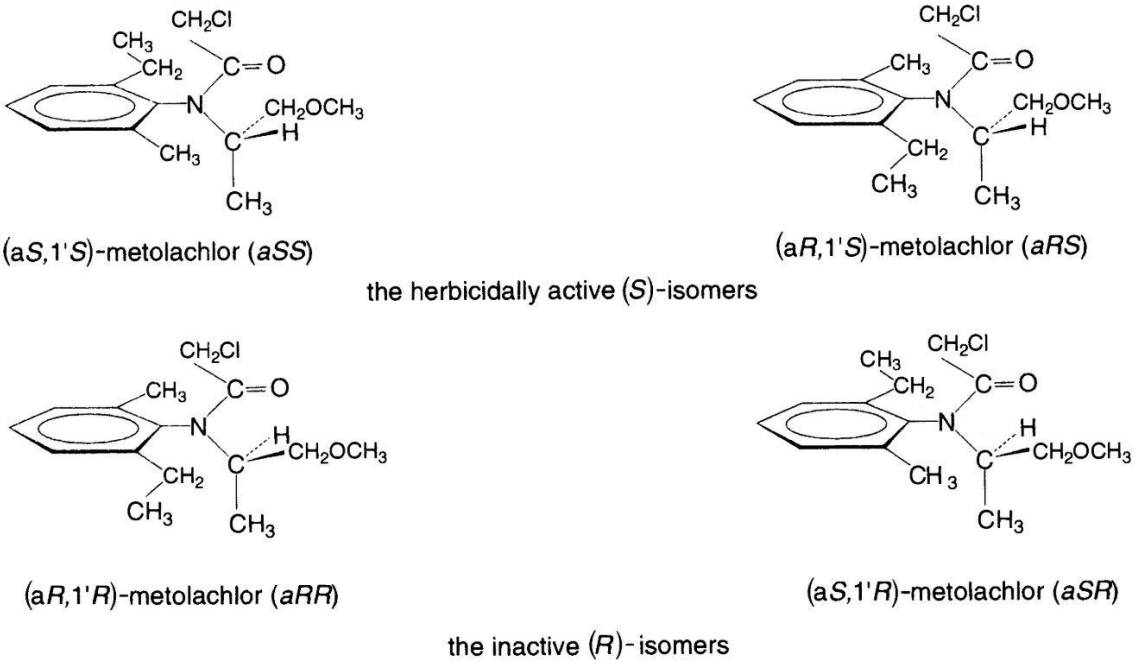

Figure 3 Chemical structure of the four stereoisomers of metolachlor. Modified from Poiger et al. (2002).

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).

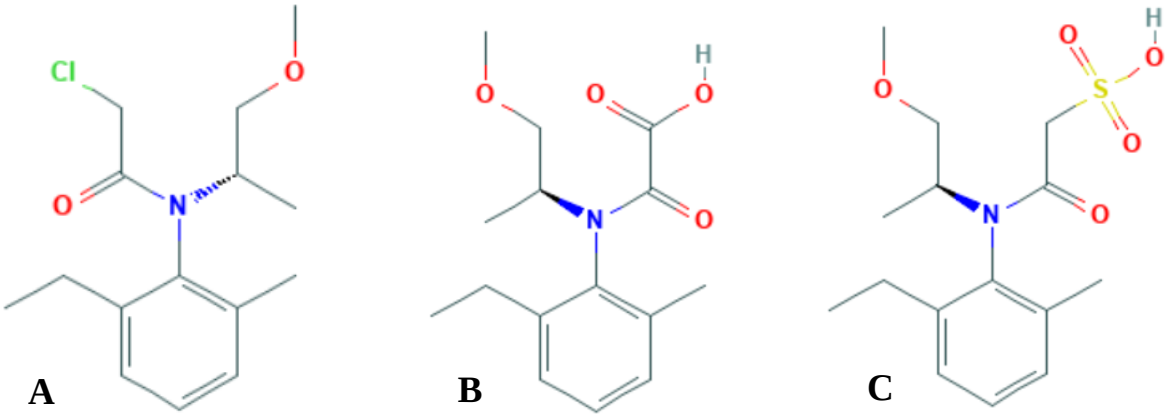

Figure 4 A Metolachlor ($C_{15}H_{22}ClNO_2$) with its two main degradation products: **B** MOA ($C_{15}H_{21}NO_4$) and **C** MESA ($C_{15}H_{23}NO_5S$). Source: https://pubchem.ncbi.nlm.nih.gov.

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).

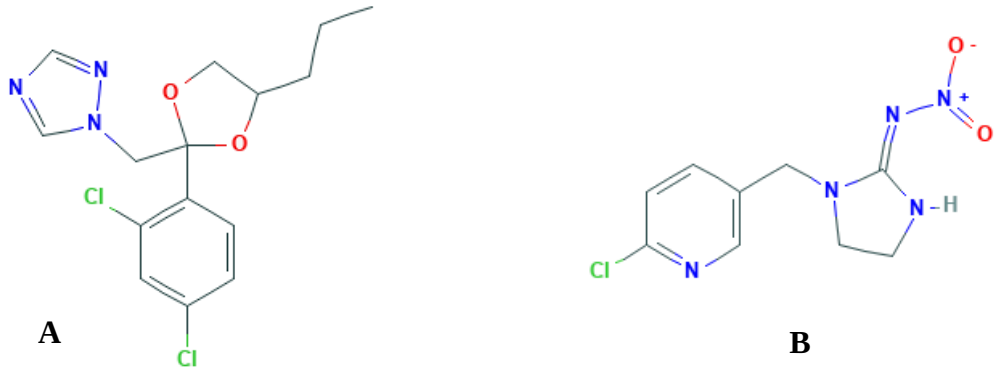

Figure 5 Structure of **A** propiconazole ($C_{15}H_{17}Cl_2N_3O_2$) **B** imidacloprid ($C_9H_{10}ClN_5O_2$). Source: https://pubchem.ncbi.nlm.nih.gov.

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).

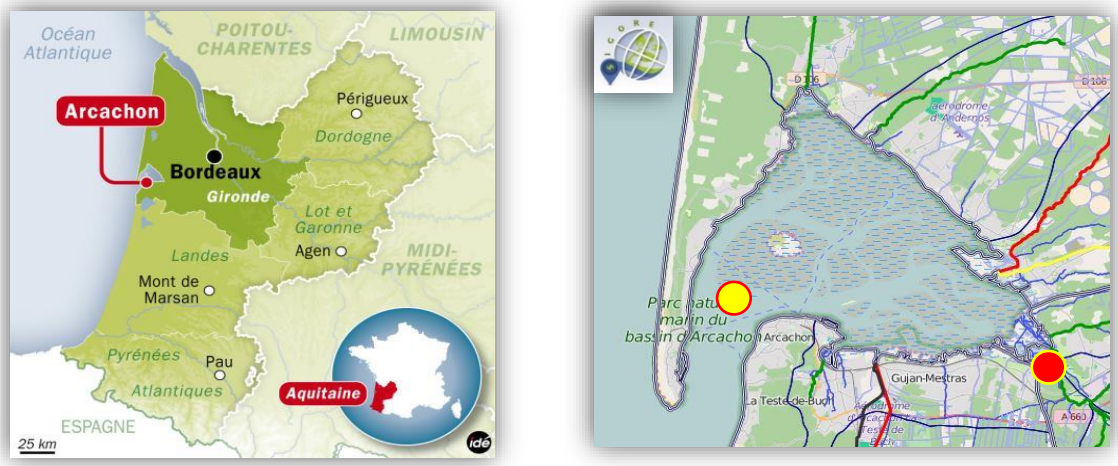

Figure 6 Location of Arcachon Bay in France (©Carte Idé) and its water system (rivers emphasized with colors; red circle: mouth of the river Leyre, yellow circle: Grand Banc) (©SIGORE Gironde)

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).

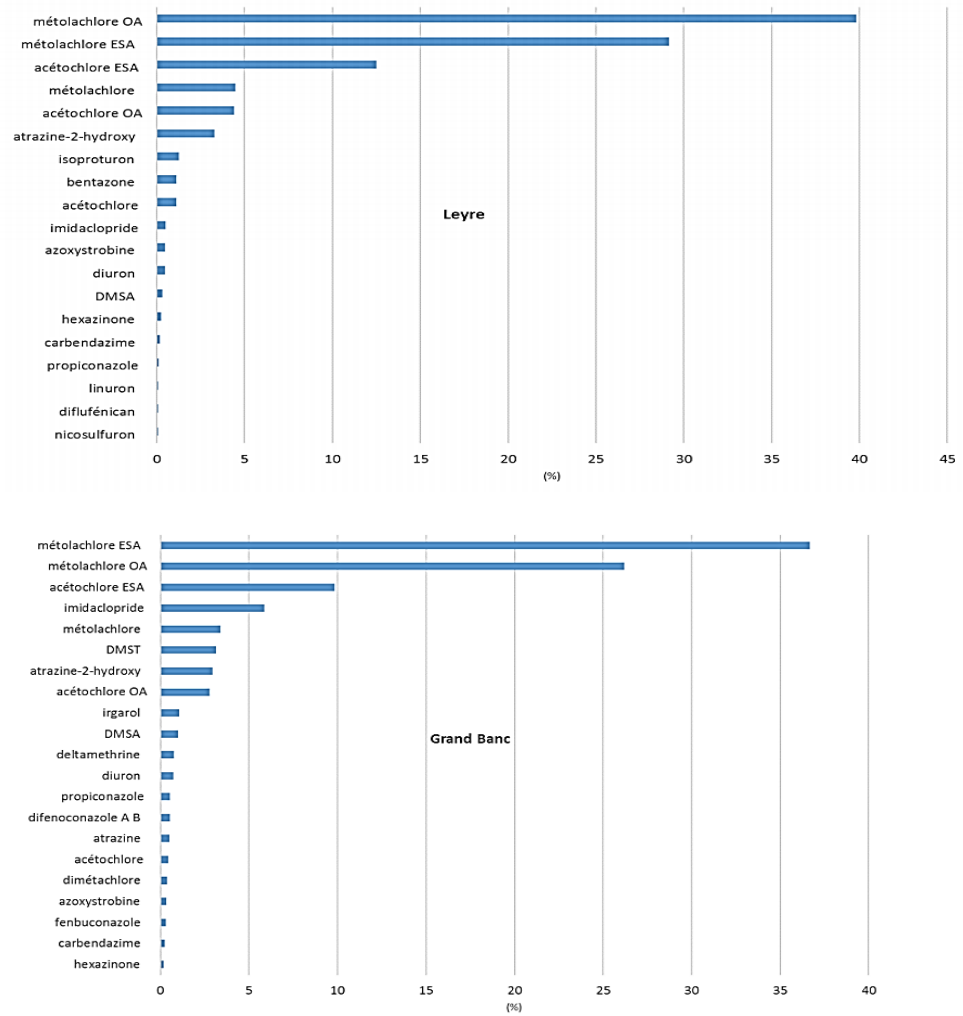

Figure 7 The most commonly detected pesticides (expressed as % of the total pesticide concentration) in the river Leyre, the biggest tributary of Arcachon Bay, and at Grand Banc near the mouth of the lagoon (for locations see Figure 6). Reprinted from Tapie et al. (2012).

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.

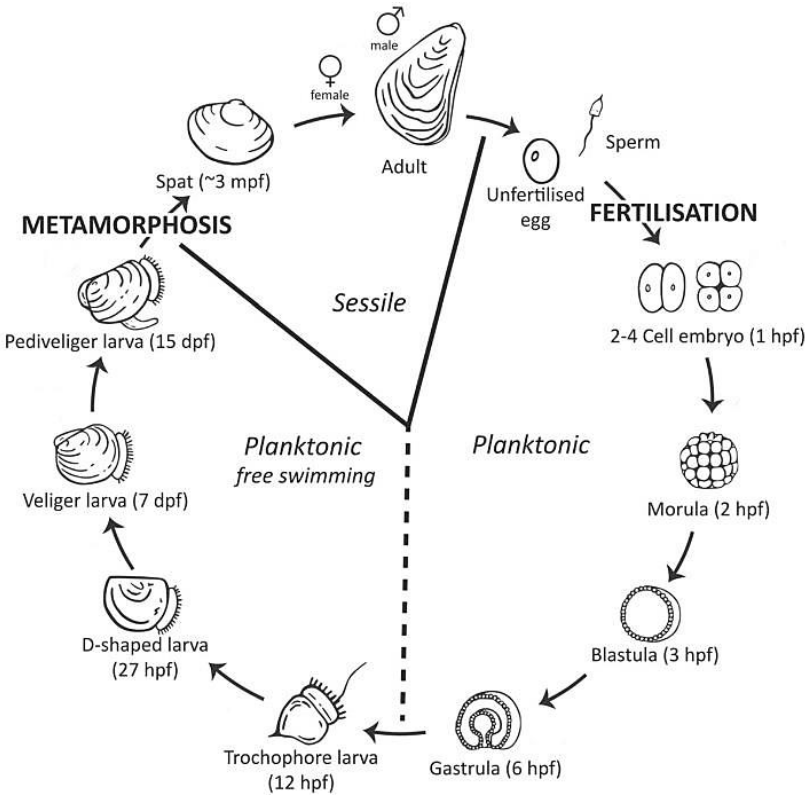

Figure 8 Life cycle of Pacific oyster (*Magallana gigas*) reprinted from (Vogeler et al., 2016).

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).

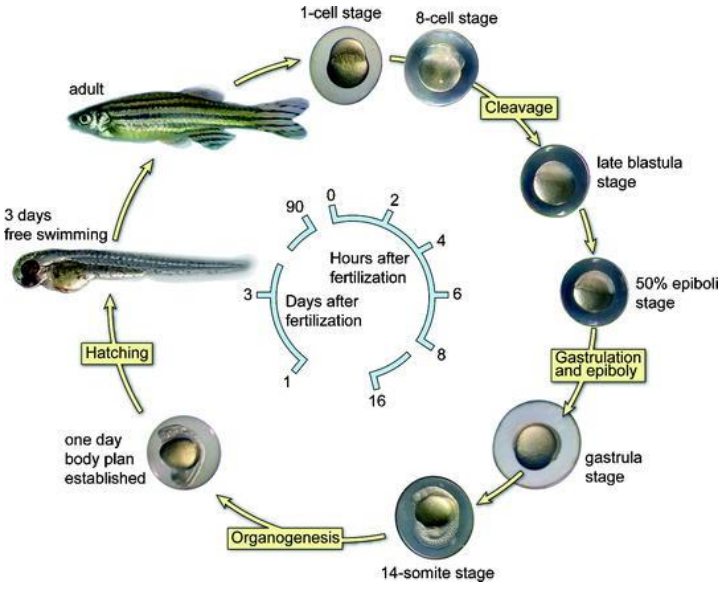

Figure 9 Life cycle of zebrafish (*Danio rerio*) reprinted from (Willemsen et al., 2011).

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.

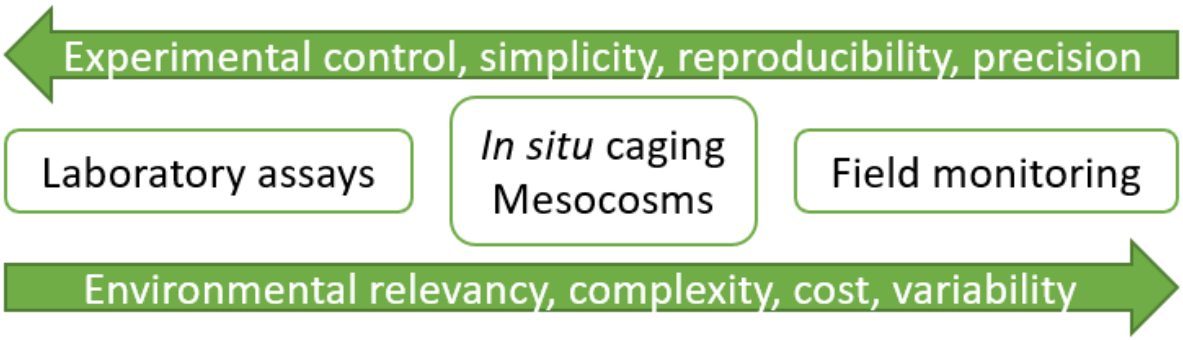

Figure 10 Relationship and properties between the main ecotoxicological approaches in toxicity assessment.

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).

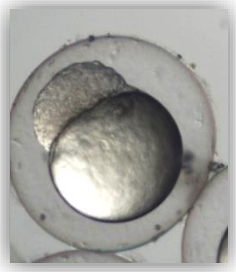

Figure 11 Zebrafish embryo at 3 hpf (original image by the author of the dissertation).

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.

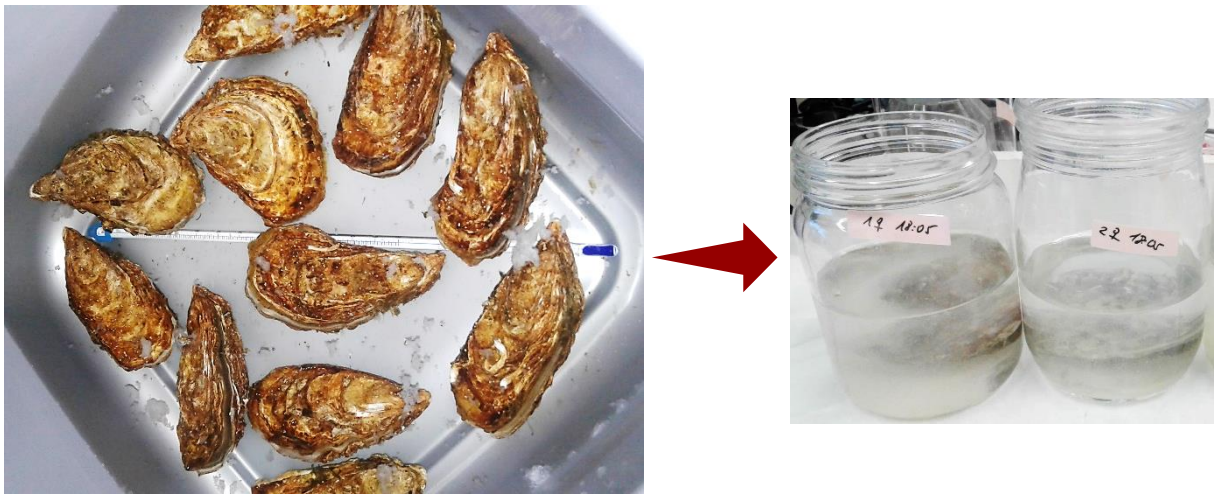

Figure 12 Oyster spawning induced by alternating thermal shocks (original images by the author of the dissertation).

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.

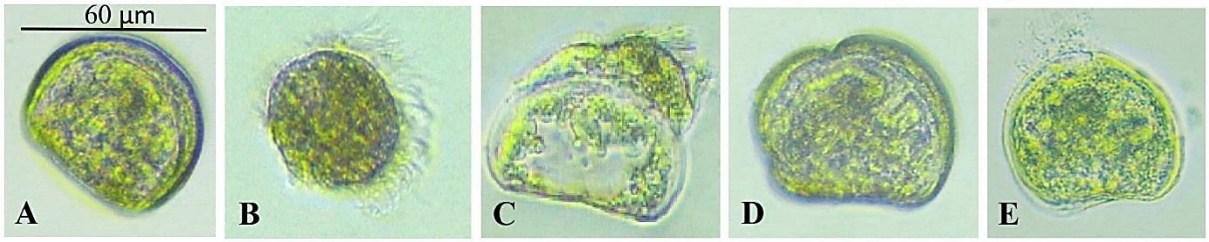

Figure 13 Different types of developmental malformations of oyster larvae (*Magallana gigas*) at 30 hpf: well-formed D-larva (A), developmental arrest (B), mantle malformation (C), (scalloped) shell malformation (D), (concave) shell malformation (E). (Original images by the author of the dissertation).

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.

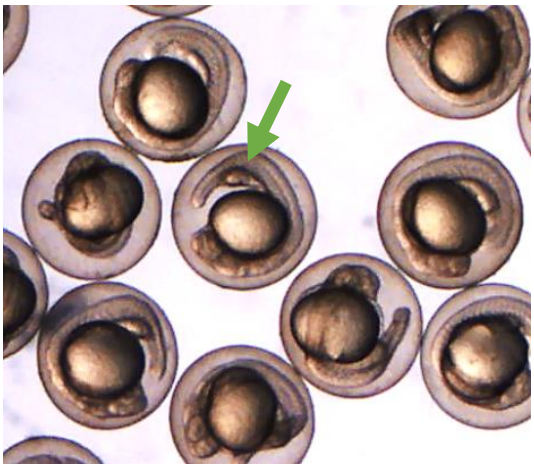

Figure 15 Zebrafish embryos at 22 hpf; the arrow is pointing at the tail. Assessment of the tail coiling (several times per minute: depending on the age of the embryo and environmental conditions such as the presence of a chemical) is used as the effect biomarker. (Original image by the author of the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

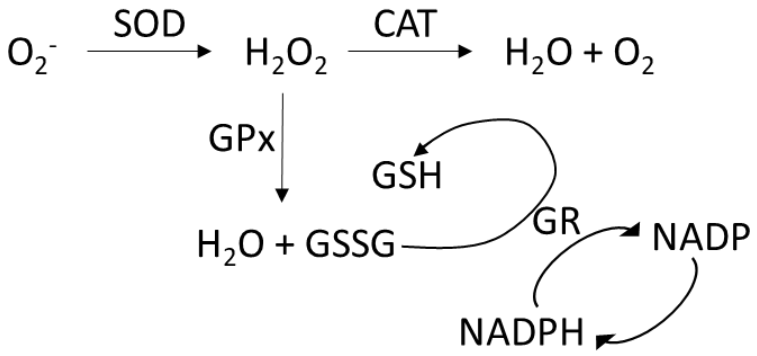

Figure 18 Schema of antioxidant system: CAT catalase; SOD superoxide dismutase; GPx glutathione peroxidase; GR glutathione reductase; GSH reduced glutathione. Adapted from Almroth (2008).

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).

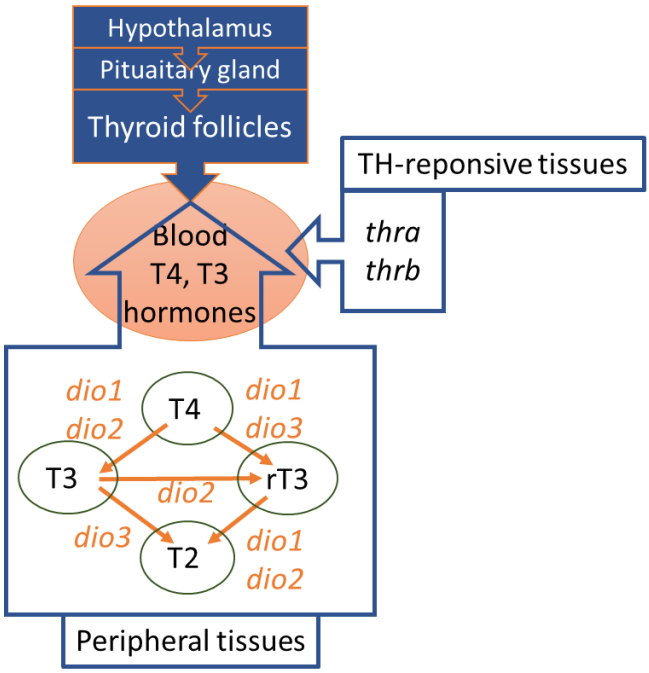

Figure 19 Overview of the elements of interest of the hypothalamus-pituitary-thyroid axis: hormones T4, T3; metabolites T2, rT3; genes coding for iodothyronine deiodinases *deio1-3* and thyroid receptors *tra, trb*. Modified from Jarque and Piña (2014) and Spaan et al. (2019).

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.

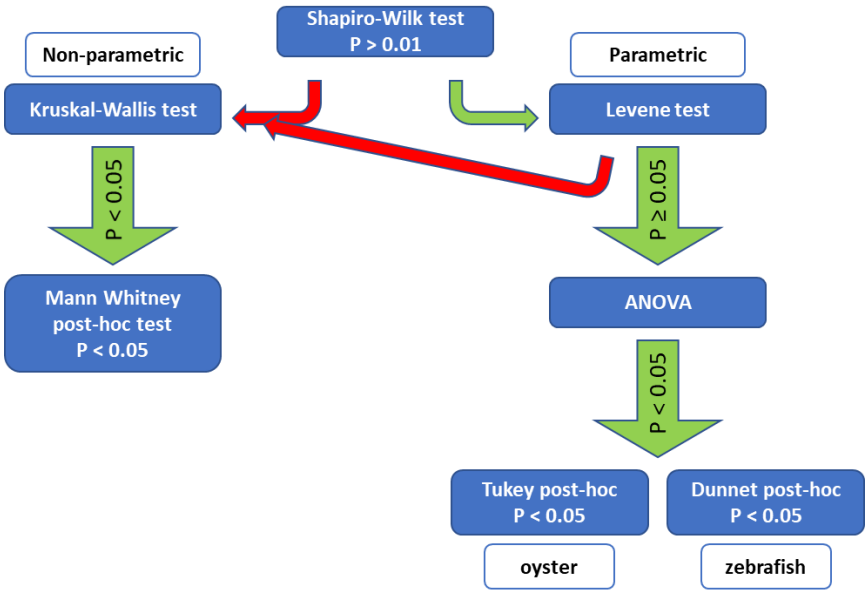

Figure 20 Scheme of statistical analyses used for the assessment of different endpoints in this thesis. Green arrows indicate the fulfillment of the condition, red arrows indicate alternative path.

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

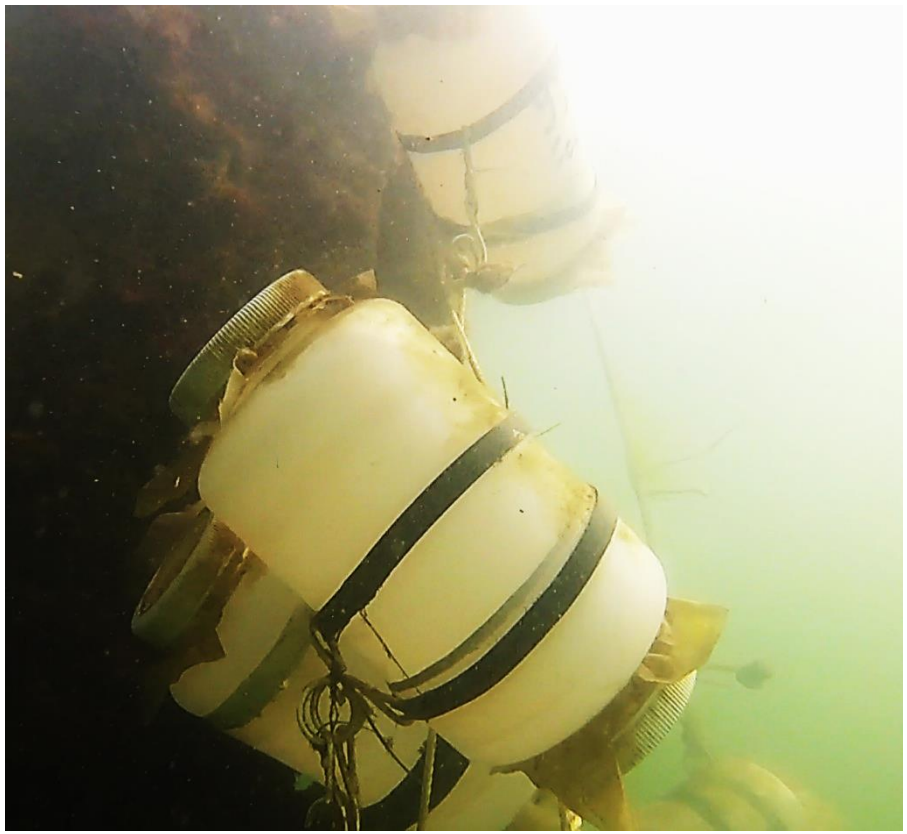

Figure 22 Deployment of the caging devices by sailor Stéphane Bujan. Four devices were attached to each of the three buoys

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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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.

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

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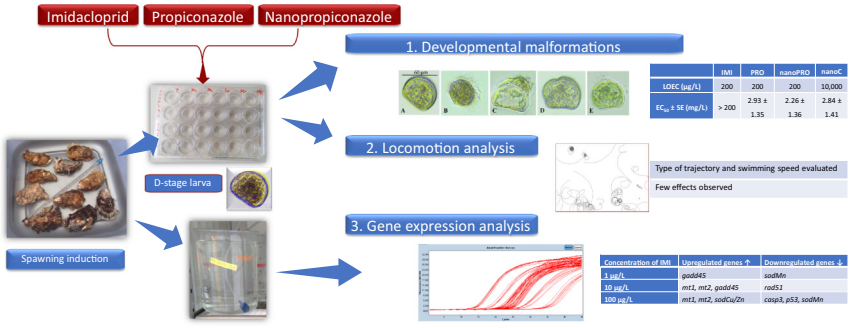
HIGHLIGHTS

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

GRAPHICAL ABSTRACT



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ABSTRACT

Coastal areas are final recipients of various contaminants including pesticides. The effects of pesticides on non-target organisms are often unclear, especially at environmentally relevant concentrations. This study investigated the impacts of insecticide imidacloprid (IMI) and fungicide propiconazole (PRO), some of the most detected pesticides in the Arcachon Bay in France. This work also included the research of propiconazole nanoformulation (nanoPRO). The effects were assessed studying the development of the early life stages of the Pacific oyster (*Magallana gigas*). Oyster embryos were exposed for 24, 30, and 42 h (depending on the endpoint) at 24 °C to environmentally relevant concentrations of the two pesticides as well as to nanoPRO. The research focused on sublethal endpoints such as the presence of developmental malformations, alterations of locomotion patterns, or changes in the gene expression levels. No developmental abnormalities were observed after exposure to environmental concentrations detected in the Arcachon Bay in recent years (maximal detected concentration of IMI and PRO were 174 ng/L and 29 ng/L, respectively). EC_{50} of PRO and nanoPRO were comparable, 2.93 ± 1.35 and 2.26 ± 1.36 mg/L, while EC_{50} of IMI exceeded 200 mg/L. IMI did not affect larval behavior. PRO affected larval movement trajectory and decreased average larvae swimming speed (2 μg/L), while nanoPRO increased the maximal larvae swimming speed (0.02 μg/L). PRO upregulated especially genes linked to reactive oxygen species (ROS) production and detoxification. NanoPRO effects on gene expression were less pronounced - half of the genes were altered in comparison with PRO. IMI induced a strong dose-response impact on the genes linked to the detoxification, ROS production, cell cycle, and apoptosis regulation. In conclusion, our results sug-

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gest that current pesticide concentrations detected in the Arcachon Bay are safe for the Pacific oyster early development, but they might have a small direct effect via altered gene expressions, whose longer-term impacts cannot be ruled out.

1. Introduction

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

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

Numerous hazardous substances are regularly detected in Arcachon Bay, such as pesticides monitored by survey network REPAR (https://www.siba-bassin-arcachon.fr/actions-environnementales/les-reseaux-de-surveillance-repar-et-rempar). Pesticides are known for causing adverse effects on non-target estuarine/marine aquatic organisms (Gutiérrez et al., 2019; Parsons et al., 2020; Vignet et al., 2019), at environmentally relevant concentrations (Bechmann et al., 2020; Behrens et al., 2016; Epelboin et al., 2015; Gamain et al., 2018; Mai et al., 2013, 2014). The REPAR monitoring network showed that the insecticide, fungicide, and herbicide with the highest detected average concentration (cf. Table 1) during years 2010–2014 in the Arcachon Bay were imidacloprid, propiconazole (as well as other fungicides like carbendazim and metabolites of dichlofluanid), and herbicide S-metolachlor (Tapie and Budzinski, 2018). The toxicity of S-metolachlor to oyster larvae has already been assessed (Gamain et al., 2016, 2017; Mai et al., 2013, 2014). Imidacloprid (IMI) is a neurotoxic insecticide of the neonicotinoid family which binds agonistically to the post-synaptic nicotinic acetylcholine receptors (nAChRs) and is highly selective for insects (Matsuda et al., 2001). Its use has been banned since 2018 by regulation of the EU Commission (European Commission, 2018) except the use in permanent greenhouses; due to the risks to honey bees and other pollinators. However, it is still widely used in other countries in the world (Butcherine et al., 2019). IMI is one of the most detected insecticides in waters usually in a range of hundreds of ng/L (Anderson et al., 2015; Morrissey et al., 2015), which is also the case of Arcachon Bay in France (Tapie and Budzinski, 2018). However, the peak concentrations of imidacloprid in waters might be high as 320 μg/L in the Netherlands (Van Dijk et al., 2013), 3.29 μg/L in California (Starner and Goh, 2012), or 0.26 μg/L in Canada (Main et al., 2014), Several studies reported toxicity to mollusks, but the effects were not evaluated at environmentally relevant conditions corresponding to the Arcachon Bay in France and only high concentrations were used (Dondero et al., 2010; Ewere et al., 2019a, 2019b, 2020; Prosser et al., 2016; Shan et al., 2020). Propiconazole (PRO), a triazole fungicide, stops the fungal growth as it inhibits the demethylation by fungal sterol 14α-demethylase and thus obstructs the biosynthesis of ergosterol, a component of fungal cell membranes (Zarn et al., 2003). Its occurrence in surface waters all around the world in a range of ng/L to μg/L is well documented (Elfikrie et al., 2020; Papadakis et al., 2018; Quintana et al., 2019; Toan et al., 2013; Van De Steene et al., 2010) with peak concentrations going up to 0.81 μg/L in China (Peng et al., 2018). Studies on the effects of propiconazole on mollusks are even scarcer (Bringolf et al., 2007; Gottardi et al., 2018).

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

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

Table 1
Concentrations of pesticides of interest detected at different sampling points in Arcachon Bay during the years 2010–2014. $N = 669$ for each pesticide. Concentrations were calculated according to the data of Tapie and Budzinski (2018).

	PRO	IMI
Limit of quantification (ng/L)	1	1
Samples with detected substance (%)	20.7	34.8
Average concentration in all samples (ng/L)	0.7	2.6
Average concentration in samples with detected substance (ng/L)	3.1	7.6
Maximal concentration (ng/L)	29.1	173.6

2. Materials and methods

2.1. Chemicals and reference seawater

Imidacloprid (IMI, CAS 138261-41-3, Pestanal, purity 100%), propiconazole (PRO, CAS 60207-90-1, Pestanal, purity 100%), and $CuSO_4$ were purchased from Sigma-Aldrich. The stock solution of PRO

(5 g/L) was prepared in DMSO and was stored at 5 °C. The stock solution of IMI (200 mg/L) was prepared directly in seawater and was used for testing immediately. The solution of Cu^{++} was used as a positive control and stored at 5 °C (stock solution in milliQ water at 100 mg/L). Exposure solutions were prepared by serial dilution in seawater. Seawater was collected at beach Petit Nice (approx. 44°33′40.3″N 1°14′27.1″W), serially filtered at 0.22 μm, and passed through UV light to eliminate debris and microorganisms. Filtered seawater (FSW) was stored at 5 °C in the dark and was used typically within two days (within 7 days at the latest). It was refiltered through 0.22 μm for solution preparation and oyster spawning. The presence of pesticides and copper in FSW was verified by LC-MS/MS (cf. Sections 2.7 and 3.1).

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

2.2. Nanopropiconazole

The nanopropiconazole formulation used in this work consisted of poly-ε-caprolactone nanocapsules loaded with the fungicide. The method used for its preparation was the interfacial deposition of a preformed polymer as described by Grillo et al. (2012) with one modification: Myritol 318, instead of Miglyoil 810, was used as the triglyceride oil. As a control, nanocapsules (nanoC) not containing the active ingredient (verified by LC-MS/MS; the used method was the same as for the nanoformulation mentioned below) were also prepared using the same method. The stock suspension of nanopropiconazole (nanoPRO) with a propiconazole concentration of 325 mg/L (determined by LC-MS/MS as described in Section 2.7 after diluting the sample in acetonitrile, centrifuging it through nylon filter – 6000 rpm/6 min, and freezing at −20 °C; more details in Section 2.7) was stored at ambient temperature in an amber glass vial. The average size (z-average diameter) and the zeta potential of nanoparticles was measured in the stock suspension in milliQ water (diluted 100×) by dynamic light scattering (DLS) using Malvern Instruments with software Zetasizer Ver. 6.20 with a detector at a fixed angle of 173° and the average size measurement was repeated by Cordouan Technologies SAS with software NanoQ V2.6.3.0 with a detector at a fixed angle of 135°. Cordouan Technologies SAS was also used to measure the average size of nanoparticles in seawater (only the highest exposure concentrations, 10 mg/L of loaded PRO, was measured due to the power of the laser). Both average size and zeta potential results are expressed as the means of three acquisitions.

The encapsulation efficiency characterizes the percentage of pesticide loaded into the nanocarrier in reference to the total amount of pesticide in the system. The total quantity of PRO was determined by diluting a sample suspension with acetonitrile as detailed above. The amount of fungicide associated with the nanocarriers was measured by the centrifugal ultrafiltration method: samples in triplicates were centrifuged (11,481 rpm, 30 min) using Microcon-30 kDa Centrifugal Filter Unit with Ultracel-30 membrane (Millipore). A solution of propiconazole (conventional formulation) was analyzed in parallel to measure a potential loss/adsorption of propiconazole on the filter. The filtrates were then diluted in 20% acetonitrile and measured by LC-MS/MS to determine the free amount of propiconazole, as described in Section 2.7. The encapsulation efficiency was calculated according to the formula

$$EE\ (\%) = \frac{W(associated)}{W(total)} = \frac{W(total) - W(free)}{W(total)}$$

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

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

2.3. Test organism

Pacific oyster (*Magallana gigas*, called also *Crassostrea gigas*) mature adults (5 couples for each test) were received from Guernesey Sea Farm hatchery (Guernesey, UK) and were used immediately, or kept in oxygenated FSW at 11 °C during 24 h. Oysters were placed in FSW (12 °C, 30 min) for the acclimatization and were then subjected to alternating thermal shock in FSW at 18 °C and 28 °C for 30 min. Female spawning was facilitated by adding frozen filtered oyster sperm which contains diantlin (Dupuy et al., 1977). Detailed spawning method is described by Gamain et al. (2016). The embryos were then transferred to experimental units and kept at 24 °C in the dark until they reach the developmental stage of D-larva.

2.4. Embryo-larval test

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

At 24 hpf (hours post-fertilization), the microplates were used for the locomotion analysis (cf. Section 2.5). After the video capture (approximately at 30 hpf), formaldehyde (25 μL at 37%) was added to every well (final volume 2025 μL), and the microplates were kept at 4 °C until the analysis of developmental malformations was carried out (within 14 days). Percentage of different developmental malformations (mantle and shell malformation), arrested development, or well-developed D-shaped larvae (Fig. 1) per 100 embryos per well was

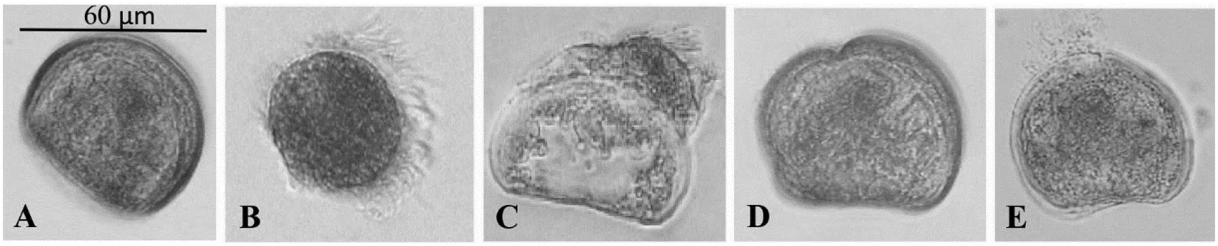

Fig. 1. Different types of developmental malformations of oyster larvae (*Magallana gigas*) at 30 hpf: well-formed D-larva (A), developmental arrest (B), mantle malformation (C), (scalloped) shell malformation (D), (concave) shell malformation (E).

determined using an inverted microscope (Nikon Eclipse TS100). For all tests, validity criteria were fulfilled as follows, malformation rate lower than 20% in the control, and EC_{50} for abnormal larvae exposed to positive control Cu^{++} between 6 and 16 µg/L (tested concentration range: 0–5–10–20–50 µg/L).

2.5. Locomotion analysis

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

Before adding the formaldehyde into the microplates (c.f. Section 2.4) to evaluate the morphologic abnormalities, videos of larvae locomotion were captured. Videos were taken after 24 h of incubation at the stage of the D-shaped larva. The temperature in the solutions/suspensions with larvae was maintained at 24 °C during the video capture (using room air conditioning). Two-minute video per well was captured at zoom 40× using an inverted microscope Nikon Eclipse TS100 equipped with camera Nikon DS-Fi2, and software NIS Element. The videos were then converted into 4 fps with software VirtualDub and analyzed using ImageJ to acquire the trajectory type, the average, and the maximal swimming speed of each tracked oyster larva. The ImageJ plugin and method are described in detail by Gamain et al. (2020). Three different trajectory paths were discriminated as follows: rectilinear, circular, and stationary (presented in Fig. 2). The results provided by ImageJ were manually checked for artifacts (larvae exiting field of view after too short trajectory; larvae collisions influencing the speed and trajectory; larvae passing too close to each other and exchanging their tracking identities etc.).

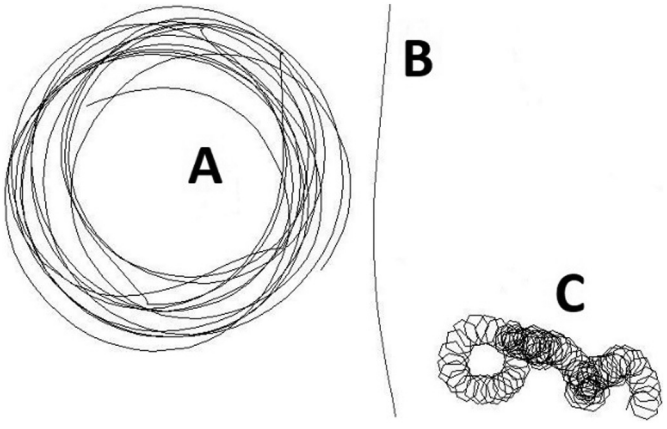

Fig. 2. Different types of trajectory paths of oyster larvae observed during locomotion experiments: circular (A), rectilinear (B), stationary (C).

2.6. Oyster exposures for analysis of gene expressions

To collect enough RNA for the analysis, 500,000 embryos (originated from one oyster couple) were incubated in three-liter glass beakers (exposure to PRO and nanoPRO) or plastic bottles (exposure to IMI) at 24 °C in the dark for 42 h. For oxygenation and for keeping embryos suspended in the water column, solutions were aerated with aquarium airstones. Dissolved oxygen was checked at the beginning and the end of the tests. The concentrations of the exposure solutions were the same as for the locomotion analysis: PRO and nanoPRO (20 ng/L, 200 ng/L, 2 µg/L), and IMI (1 µg/L, 10 µg/L, 100 µg/L). After 42 h, the larvae were collected on a 20 µm mesh (SEFAR NITEX®) using a vacuum pump, resuspended in 5 mL of exposure solution, and kept on ice. Their concentration was calculated immediately and five replicates, each containing 30,000 larvae, were collected in 1.5 mL polypropylene microtubes tubes. These replicates were centrifuged (2 min, 1000 rpm) and the larvae pellet was resuspended in 500 µL of RNA later. Samples were then kept at −80 °C until RNA extraction.

The total RNAs were extracted using the SV Total RNA Isolation System Kit (Promega). Samples were first homogenized using vortex and 200 µL of glass beads (0.10–0.11 mm, acid washed, B. Braun Biotech International) in 500 µL of RNA Lysis buffer, and centrifuged (7500 rpm, 1 min). Lysed samples were collected, 500 µL of phenol-chloroform-isoamyl alcohol (25-24-1) was added, and the tubes were vortexed. Centrifugation (13,500 rpm, 5 min) divided the samples into two phases and the upper (aqueous) was collected, mixed with 450 µL of 75% ethanol, vortexed, and transferred onto a spin column following manufacturer's instructions with few modifications as follows: RNA samples were treated with DNase I mixture for 15 min at 37 °C, and purified RNAs were collected in 50 µL of Nuclease-Free water. The concentration and purity of collected RNA samples were checked spectrophotometrically at 260/280 nm with software Gen5 (Biotek), using a spectrophotometer (Spectro Multivolume Epoch; BioTek). The purity of all samples was between 2.0 and 2.2. Reverse transcription was performed with the GoScript™ Reverse Transcription System kit (Promega) according to the manufacturer's instructions. Purified 1 µg of RNA was reversely transcribed to get the final volume of 20 µL of cDNA, which was stored at −20 °C until the quantitative PCR analysis was performed.

QPCR was carried out with the GoTaq® qPCR Master Mix kit (Promega) on a LightCycler® 480 (Roche). QPCR mix was composed of 1 µL of cDNA, 2 µL of a mix containing each reverse and forward primer (2 µM), and 10 µL of 2× GoTaq master mix, which was completed with nuclease-free water to the final volume of 20 µL. Fourteen genes in total were selected to evaluate the effects of chosen pesticides on mitochondrial metabolism (*12S*, *cox1*), regulation of the cell cycle and apoptosis (*p53*), oxidative stress defense (*cat*, *sodMn*, *sodCu/Zn*, *gpx*), detoxification (*mt1*, *mt2*), apoptosis (*bax*, *casp3*), biotransformation (*cyp1a*), growth arrest and DNA damage (*gadd45*), and DNA repair (*rad51*). Three reference genes were used in the analysis (*β-actin*, *ef1α*, and *rpl7*). The genes were chosen to evaluate non-specific toxicity and general responses of oyster larvae to pollutant stress, and to correspond with the studies of Mai et al. and Gamain et al. referenced in this publication. Sequences, references, and accession numbers are

presented in Supplementary Table S1. Primers were purchased from Sigma proligo. Primer-pairs efficiencies for all genes were verified to be higher than 95%. The PCR procedure was as follows: the pre-incubation step lasted 2 min at 95 °C, then the amplification consisted of 50 cycles with each cycle at 95 °C for 15 s and 60 °C for 1 min. The melting curve continued at 95 °C for 30 s, at 60 °C for 2 min, and 95 °C until the next cycle.

Melting curves of every reaction were verified to assess reaction specificity. All data were normalized to the geometric mean of the Ct values of the three reference genes, *β-act*, *elf1a*, and *rpl7*, and treated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Results are shown as fold changes of the exposed group compared to the control group.

2.7. Chemical analysis and water quality

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

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

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

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

2.8. Data analysis

Relative abundance (%) of malformed larvae (sum of all types of abnormal development i.e. developmental arrest, mantle, and shell malformations) and the results (%) of trajectory analyses were first transformed using arcsine transformation $p' = \arcsin\sqrt{\frac{p}{100}}$ (Sokal and Rohlf, 2012), and checked 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 opposite case, non-parametric Kruskal-Wallis ($P < 0.05$) with Mann-Whitney post-hoc test was carried out. All analyses were carried out using Statistica 13.3 (StatSoft, USA). $EC_{50/20/30}$ were calculated from nonlinear logarithmic regression of the nominal concentration-response curves, using Graph Pad Prism 5 (Graph Pad Software, USA).

Raw data of larval swimming speed acquired by the imaging software were converted from pixel/s to μm/s (multiplication by 2.43; value corresponding to the microscope and zoom used during the capture of videos). Data were then normalized to average control swimming speed due to the high data variability of different test repetitions. Finally, normalized data were compared in Statistica 13.3 using the statistical tests described above (Shapiro-Wilk test; $P > 0.01$; Levene test; $P > 0.05$; ANOVA or Kruskal-Wallis; $P < 0.05$).

All data from the gene expression analysis were log-transformed before the analysis, treated as described above (Shapiro-Wilk test; $P > 0.01$; Levene test; $P > 0.05$), and tested for significant differences using ANOVA ($P < 0.05$) followed by Tukey post-hoc test. If normality or homoscedasticity were not confirmed, non-parametric Kruskal-Wallis ($P < 0.05$) test with Mann-Whitney post-hoc tests were performed.

3. Results

3.1. Exposure and chemical analysis

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

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

3.2. Nanopropiconazole and nanocarrier characterization

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

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

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

3.3. Embryo-larval development

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

Frequency (%) of developmental malformations and developmental arrests of oyster larvae exposed for 30 h to increasing concentrations of pesticides are shown in Fig. 3. Moreover, the sum of abnormal larvae is shown for every exposure condition. These abnormal larvae proportions served for the calculations of effective concentrations (EC_x), no observable effect concentration (NOEC), and the lowest observable effect concentration (LOEC), which are shown in Table 2. In general, IMI, PRO, and nanoPRO had comparable toxicity patterns since the developmental toxicity to oyster larvae occurred only at high concentrations (at and above 200 μg/L), whereas no effect was observed after exposure to environmental concentrations of IMI and PRO. The highest tested concentration (10 mg/L) of nanoPRO malformed or arrested development of all larvae and PRO affected 89.3 ± 4.3% of individuals. On the contrary, even the highest tested concentration of IMI (200 mg/L) affected only 33.1 ± 8.2% larvae. Conversely, nanoC did not cause developmental malformations irrespectively of concentration and only the highest tested concentration (10 mg/L) induced developmental arrests (Fig. 3), thus suggesting that whereas nanocarrier caused only developmental arrests, it was the propiconazole inside the capsules which was responsible for the developmental malformations.

IMI was the least toxic to oyster larvae, whereas PRO, nanoPRO, and nanoC had comparable EC_x values (Table 2). Interestingly, NOEC and LOEC were identical for the IMI, PRO, and nanoPRO (20 and 200 μg/L, respectively). These values were within the range of μg/L, i.e. higher than environmental concentrations in Arcachon Bay (Table 1). As expected, nanoC was not toxic with NOEC of 2 mg/L.

3.4. Locomotion analysis

The locomotion analysis consisted of trajectory type and maximal and average speed assessment (Fig. 4). The non-exposed larvae

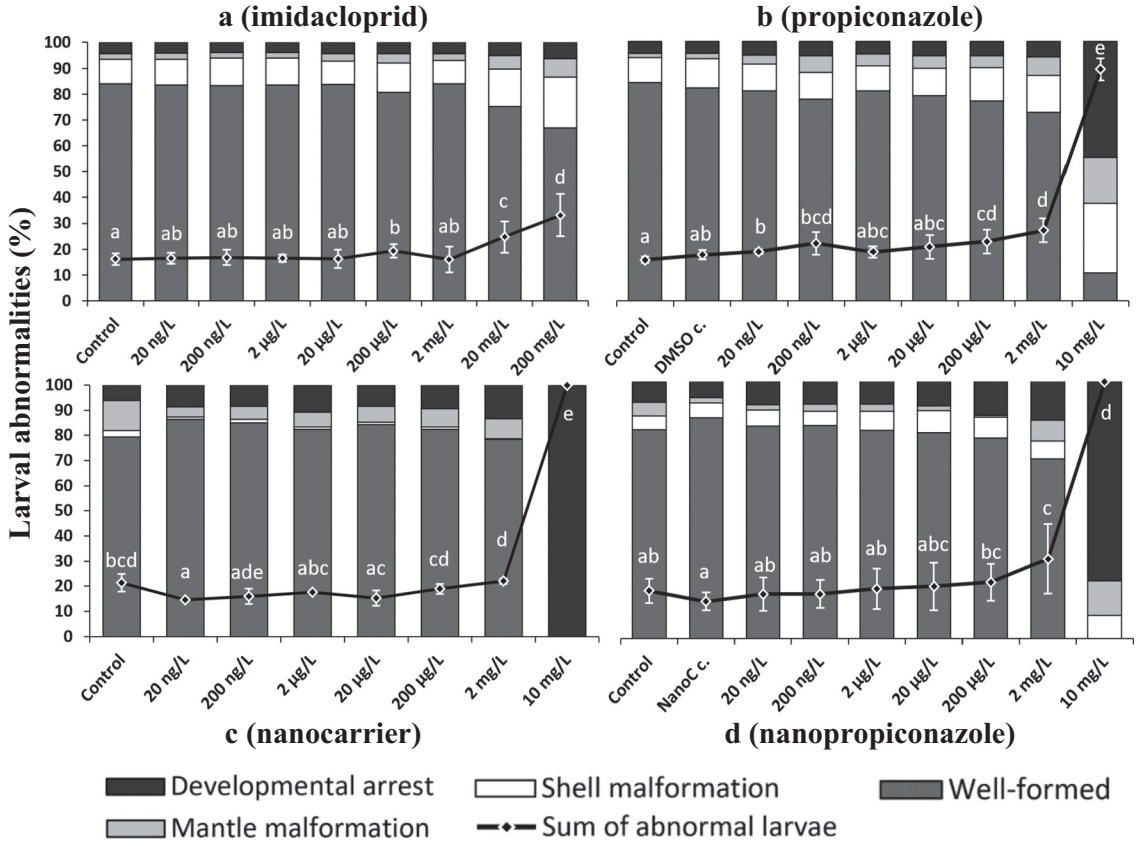

Fig. 3. Larval abnormalities and the sum of affected individuals of oyster larvae after 30 h of exposure to increasing concentrations of imidacloprid (a), propiconazole (b), nanocarrier (c), and nanopropiconazole (d). Different letters indicate statistical differences between variables ($P < 0.05$). Results are presented as the mean of 4 independent experiments ($n = 3$ in case of nanoC) ± SD. Solvent (DMSO) and nanocarrier (NanoC) controls are shown in panels b) and d).

Table 2
Effective concentrations (EC_{20}, EC_{30}, EC_{50}), no observable effect concentration (NOEC), and lowest observable effect concentration (LOEC) after 30 h-long exposure of oyster larvae to imidacloprid, propiconazole, nanopropiconazole, and nanocarrier. Imidacloprid did not reach EC_{50} up to the highest tested concentration (200 mg/L).

		IMI	PRO	nanoPRO	nanoC
μg/L	NOEC	20	20	20	2000
	LOEC	200	200	200	10,000
	$EC_{50} \pm SE$	>200	2.93 ± 1.35	2.26 ± 1.36	2.84 ± 1.41
mg/L	EC_{20}	6.43	0.73	0.56	0.71
	EC_{30}	70.50	1.26	0.97	1.22

generally displayed rectilinear swimming trajectories (72.3 ± 9.9%), less commonly the circular trajectory type (20 ± 11.7%), and rarely they stayed stationary (6.9 ± 2.8%). Maximum and average swimming speed employed by the non-exposed larvae were 309.3 ± 45.7 μm/s and 222.2 ± 29.1 μm/s, respectively, even though high variability was observed between the individuals. No statistically significant effects on swimming speed or trajectory were observed for IMI, but the frequency of rectilinear trajectories showed a decreasing tendency with increasing concentration (73.3–65.0–62.3–57.6%) and the increase of the circular type (22.8–31.4–27.4–34.8%). A statistically significant increase in stationary swimming patterns was observed in larvae exposed to 2 μg/L of PRO (17.3 ± 3.4%) when compared to other treatments. The same effect was also observed in larvae exposed to an environmentally relevant concentration of 0.02 μg/L (12.4 ± 5.1%) when compared to the DMSO control (5.2 ± 2.1%). Similarly, as for PRO, nanoPRO caused no effects on the frequency of rectilinear and circular trajectories. No effect on larvae swimming speed was observed after exposure to IMI. In contrast, PRO at 2 μg/L did cause a statistically significant decrease (205.1 ± 31.4 μm/s) in average swimming speed in comparison to the DMSO control (252.8 ± 46.0 μm/s). NanoPRO, unlike PRO, caused significant effects on both maximal as well as the average swimming speed. First, the maximal and average swimming speed of nanoC control (330.5 ± 35.4 μm/s and 246.8 ± 28.3 μm/s respectively) significantly differed from the non-exposed control (246.0 ± 43.4 μm/s and 182.9 ± 38.7 μm/s respectively). Furthermore, the low concentration of 0.02 μg/L of nanoPRO caused an even higher increase in maximal speed (374.2 ± 42.1 μm/s) statistically different from both relevant controls.

3.5. Gene expression analysis

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

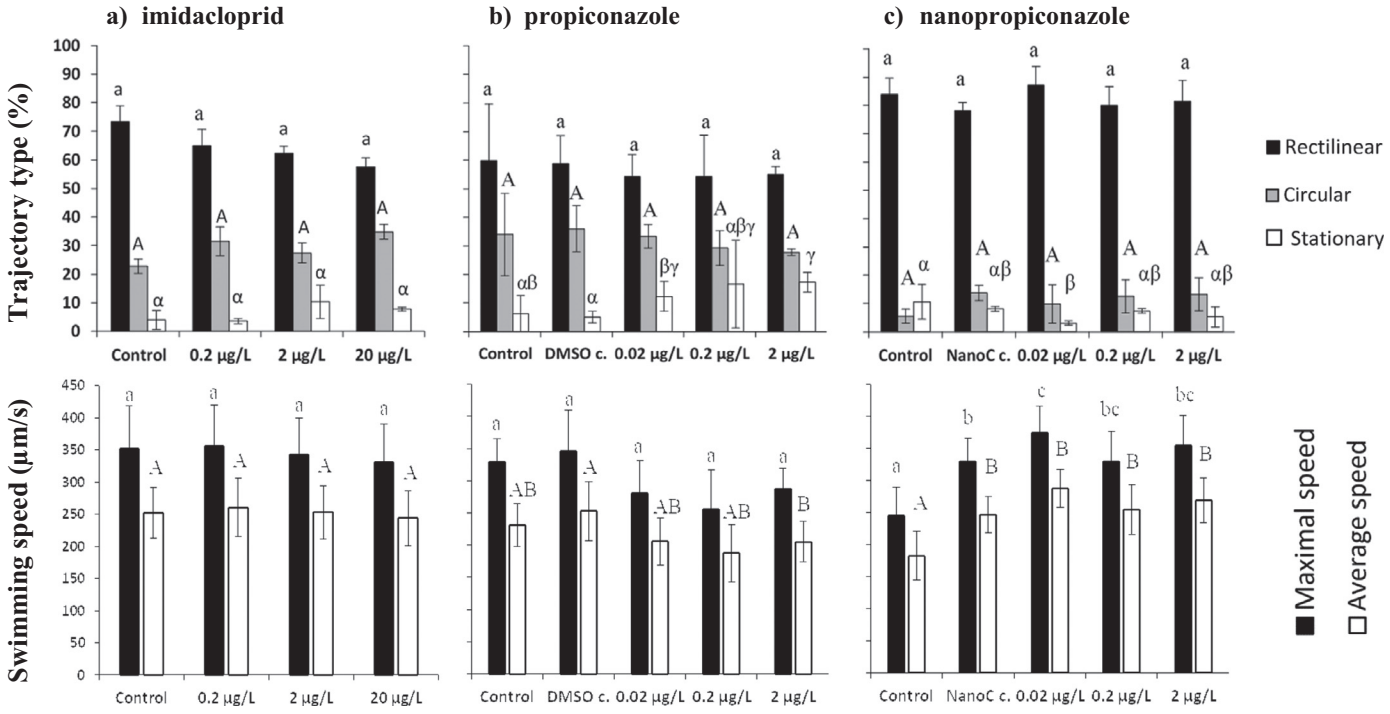

Fig. 4. Frequency of trajectory types and speed observed in the movement of oyster larvae after 24 h exposure to increasing concentrations of imidacloprid (a), propiconazole (b), and nanopropiconazole (c). Letters indicate statistical differences ($P < 0.05$). Results are presented as the mean of 3 values ($n = 3$) i.e. independent experiments ($n = 4$ in the case of PRO) ± SD. DMSO c. = DMSO control; NanoC c. = nanocarrier control.

Table 3
Expression of fourteen studied genes (relative to three housekeeping genes) involved in mitochondrial metabolism (*12S*, *cox*), regulation of the cell cycle/apoptosis (*p53*), oxidative stress defense (*cat*, *sodMn*, *sodCu/Zn*, *gpx*), detoxification (*mt1*, *mt2*), apoptosis regulation (*bax*, *casp3*), biotransformation (*cyp1a*), growth arrest and DNA damage (*gadd45*), and DNA reparation (*rad51*) in oyster larvae exposed for 72 h to different concentrations of imidacloprid, propiconazole, and nanopropiconazole. Pesticide treatments indicated by nominal concentrations; measured concentrations by LC-MS/MS shown in Supplementary Table S2. *P < 0.05; **P < 0.01; ***P < 0.001. Results are presented as the mean of 3 independent experiments ± SD. Downregulation: fold changes <1; upregulation: fold changes >1.

	Imidacloprid			Propiconazole			Nanopropiconazole		
	1 μg/L	10 μg/L	100 μg/L	20 ng/L	200 ng/L	2 μg/L	20 ng/L	200 ng/L	2 μg/L
12S	1.0 ± 0.1	1.2 ± 0.1	1.0 ± 0.3	1.1 ± 0.4	**0.7 ± 0.1****	**0.6 ± 0.1*****	1.1 ± 0.1	1.0 ± 0.4	1.0 ± 0.3
bax	0.8 ± 0.0	1.0 ± 0.3	1.1 ± 0.1	1.0 ± 0.2	0.9 ± 0.3	0.9 ± 0.1	0.9 ± 0.1	1.0 ± 0.2	1.2 ± 0.3
casp3	0.9 ± 0.1	1.0 ± 0.1	**0.8 ± 0.2****	1.1 ± 0.3	0.9 ± 0.1	0.8 ± 0.2	1.1 ± 0.1	0.9 ± 0.2	1.1 ± 0.2
cat	0.7 ± 0.1	1.3 ± 0.3	1.1 ± 0.4	1.0 ± 0.5	1.6 ± 1.3	2.7 ± 3.1	1.0 ± 0.5	1.0 ± 0.3	1.2 ± 0.7
cox	1.0 ± 0.0	1.0 ± 0.2	1.0 ± 0.2	1.2 ± 0.4	1.0 ± 0.3	1.0 ± 0.3	1.0 ± 0.2	1.1 ± 0.2	1.0 ± 0.1
cyp1a	1.7 ± 0.8	1.4 ± 0.6	1.3 ± 0.6	1.4 ± 0.6	1.8 ± 1.3	1.5 ± 0.6	1.0 ± 0.2	1.0 ± 0.1	1.3 ± 0.3
gpx	1.0 ± 0.1	1.1 ± 0.1	1.0 ± 0.2	1.1 ± 0.2	1.0 ± 0.2	0.9 ± 0.2	1.2 ± 0.2	1.2 ± 0.1	1.3 ± 0.4
mt1	1.4 ± 0.7	**2.6 ± 0.7*****	**2.0 ± 0.5*****	1.4 ± 0.6	**1.7 ± 0.3*****	1.6 ± 0.6	1.1 ± 0.2	1.2 ± 0.2	1.1 ± 0.3
mt2	1.4 ± 0.7	**2.5 ± 0.9*****	**2.1 ± 0.3*****	1.3 ± 0.4	1.3 ± 0.4	0.9 ± 0.2	1.2 ± 0.2	1.2 ± 0.0	1.3 ± 0.4
p53	1.0 ± 0.0	1.0 ± 0.0	**0.8 ± 0.1*****	1.0 ± 0.2	1.5 ± 1.0	1.4 ± 0.8	1.0 ± 0.1	1.0 ± 0.1	1.2 ± 0.3
sodCu/Zn	1.1 ± 0.2	1.1 ± 0.0	**1.3 ± 0.1*****	**1.5 ± 0.5****	**1.3 ± 0.2*****	1.4 ± 0.5	**1.6 ± 0.7***	1.3 ± 0.7	1.4 ± 0.4
sodMn	**0.9 ± 0.1***	1.0 ± 0.2	**0.8 ± 0.1****	1.0 ± 0.3	1.5 ± 1.0	1.4 ± 0.8	1.0 ± 0.1	**1.1 ± 0.1***	1.2 ± 0.2
gadd45	**1.6 ± 0.6***	**1.4 ± 0.2*****	1.7 ± 0.8	1.0 ± 0.4	1.0 ± 0.4	1.1 ± 0.5	**0.8 ± 0.2***	0.9 ± 0.2	1.0 ± 0.4
rad51	0.9 ± 0.1	**0.8 ± 0.0****	0.9 ± 0.1	1.1 ± 0.3	0.9 ± 0.1	**0.8 ± 0.0***	1.1 ± 0.1	1.1 ± 0.2	1.2 ± 0.4

4. Discussion

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

IMI had mild effects on the development of oyster larvae. The environmentally relevant concentrations detected in the Arcachon Bay in France did not cause developmental abnormalities, even though some mild toxic effects on development were observed at 200 μg/L or higher concentrations, which were detected for instance in the Netherlands as reported by Van Dijk et al. (2013). To the best of our knowledge, this is the first published study assessing IMI toxicity on the early-life stages of a marine bivalve species. Prosser et al. (2016) investigated IMI toxicity on early-life stages of freshwater mollusks and showed low sensitivity of the freshwater mussel *Lampsilis fasciola* (no effect on viability at 1 mg/L) and mild effect on the freshwater pulmonate gastropod *Planorbella pilsbryi* (LC_{10} about 800 μg/L). In our study, lethal effects are represented by developmental arrests, which, however, were not affected up to the highest tested IMI concentration of 200 mg/L. Few studies evaluated the IMI toxicity on adult bivalves. Shan et al. (2020) reported that chronic (30 d) exposure to 2 mg/L of IMI caused sublethal histological changes in adult freshwater clam (*Corbicula fluminea*), including degeneration of digestive tubules and contractions and adhesions in the hemolymphatic vessels. The concentration of 2 mg/L of IMI did not cause any mortality of adult Sydney rock oysters (*Saccostrea glomerate*) in a recent study of Ewere et al., 2019a.

In the present study, IMI did not cause any effect on the behavior (swimming speed and trajectory type) of oyster larvae at concentrations up to 20 μg/L. On the contrary, in another study 20 μg/L of IMI caused behavioral alterations in adult freshwater clams (*Corbicula fluminea*) with a decreased filtration rate and burrowing activity (Shan et al., 2020). Similarly, filtration activity of adult Sydney rock oysters (*S. glomerata*) was also decreased in the 4-day exposures to 0.5 and 1 mg/L of IMI (Ewere et al., 2019a). However, these concentrations were much higher than those used in the present study.

Gene expression was affected by IMI exposure, progressively with increasing concentration. The strongest effects (upregulation) were observed for metallothionein (*mt1*, *mt2*) expression, influencing thus the larvae's capacity to regulate metal content. Metallothionein proteins are originally known for binding and interacting with toxic metals (Coyle et al., 2002) but some studies reported their induction even after organic pesticide exposure (Erdoğan et al., 2011; Lim et al., 2015; Migliaccio et al., 2020). The direct link between IMI exposure and metallothionein induction was not studied in this work but it is clear that metallothioneins do not detoxify IMI since it is biotransformed by the CYP enzyme family (Wang et al., 2018). These ubiquitous proteins have diverse functions which may also include protective stress responses (Ruttkay-Nedecky et al., 2013). Özdemir et al. (2018) linked the metallothionein *mt1* gene induction after exposure of common carp (*Cyprinus carpio*) to IMI to the presence of reactive oxygen species (ROS). On the contrary, no measurable oxidative stress (no response at the molecular level using microarray and no accumulation of lipid peroxidation by-products) was observed in the adult marine mussel *Mytilus galloprovincialis* exposed for four days to IMI (1.8 mg/L), although the gene expressions of two metallothioneins (*mt10*, *mt20*) was also induced (Dondero et al., 2010). In the present study, ROS production was indirectly detected by the changed expression of genes encoding proteins involved in oxidative stress defense, with IMI inducing overexpression of copper/zinc superoxide dismutase as well as repression of manganese superoxide dismutase. These enzymes use different metals to transform the superoxide anion radical. *SodCu/Zn* was upregulated simultaneously with both metallothionein proteins *mt1* and *mt2* (100 μg/L of IMI), which seems interesting considering that metallothionein proteins also regulate copper and zinc metabolism (Krężel and Maret, 2017). While copper/zinc superoxide dismutase is cytosolic and extracellular, the manganese superoxide dismutase is found in mitochondria (Miller, 2012). Thus, suspected ROS production caused by imidacloprid exposure could have been limited to the cytoplasm, which might correspond to the known role of ROS in neonicotinoid toxicity as described in the recent review on neonicotinoid impact on oxidative stress (Wang et al., 2018). Corresponding to these findings, Ewere et al. (2020) detected signs of oxidative stress in adult Sydney rock oysters such as upregulation of proteins implicated in oxidative stress after exposure to 10 μg/L of IMI or elevated presence of glutathione-S-transferase (GST) in hemolymph after exposure to 100 μg/L of IMI (GST is an enzyme protecting against xenobiotics such as ROS).

Induction of the *gadd45* regulator gene might indicate both toxic effect of IMI (possibly caused by induced ROS) as well as a potential adaptive response – i.e. growth arrest that might minimize eventual cell damage (Crawford and Davies, 1994). Lastly, the highest tested concentration of IMI (100 μg/L) caused downregulation of the genes *casp3* and *p53* which suggests a modification of the regulation of the cell cycle and an anti-apoptotic effect. Downregulation of caspase-3 implies lesser

apoptotic activity, which, however, may hinder the development of the nervous system (D'Amelio et al., 2010).

In both treatments with nanoformulations (nanoPRO and nanoC), the average particle size did not change significantly over the three months that elapsed from their preparations to their use in different tests. The slight differences observed could also be attributed to the use of different instrumentations at the place of preparation (Masaryk University, Brno, Czech Republic) and use of the nanoformulation (University of Bordeaux, France). The z-average diameter of particles in the nanoformulations was slightly greater when the nanoformulation was diluted in FSW. However, this could be related to the presence of ions in the seawater that could also affect DLS analysis. Overall, both nanoformulations remained stable without the formation of aggregates over time. The zeta potential values were greater than ±30 mV, which means that the surface charge is high enough to produce a strong repulsive interaction between the particles and to avoid the forming of aggregates (Tamjidi et al., 2013). In addition, our nanoformulations contain a surfactant (PVA) that can be adsorbed on the surface of nanoparticles, preventing the aggregation of particles by steric effect (de Oliveira et al., 2015). In general, the contribution of the steric effect in the colloidal stabilization is more important than the electrostatic effect, therefore the stability of the nanoformulations of this work cannot be attributed to the surface electrostatic repulsion but to steric hindrance (Bhattacharjee, 2016; de Oliveira et al., 2015).

PRO and its nanoformulation induced more significant effects on the development of oyster larvae compared to IMI. Although LOECs were identical, PRO and nanoPRO caused greater effects at higher concentrations. Fractions of abnormal larvae increased only at the highest, yet not environmentally relevant concentrations measured in the Arcachon Bay in France and elsewhere in the world as referenced in the introduction. As non-target organisms, Pacific oyster larvae seem to be more sensitive to propiconazole toxicity than freshwater mussels. Bringolf et al. (2007) reported the EC_{50} of propiconazole around 20 mg/L for acute (24 and 48 h) toxicity test with glochidia of mussel *Lampsilis siliquoidea*, and EC_{50} of 10 mg/L for 96 h toxicity test with juveniles of the same species. The propiconazole toxicity observed in the present study is comparable to that of other early-life stages of non-target aquatic species e.g. LC_{50} of 20.4 mg/L for zebrafish (*Danio rerio*) in a 5-day-long test (Coors et al., 2012) and LC_{50} of 5 mg/L for water fleas *Daphnia magna* in a 48 h-long test (Kast-Hutcheson et al., 2001).

The swimming behavior of oyster larvae was slightly affected by PRO and nanoPRO. At lower concentrations, the PRO-only treatment did not seem to affect this endpoint. Whereas the nanoPRO enhanced the larvae swimming speed which might be explained as the combined effect of the active ingredient and the nanocapsules. To the best of our knowledge, no work studying the behavioral effects of PRO on mollusks has been reported so far. In a study of Souders et al. (2019), early-life stages of zebrafish (*Danio rerio*) exerted hypoactive swimming behavior after exposure to 10 μM (3.4 mg/L) of PRO but no effect was seen at 0.1 μM (34 μg/L), a concentration still 17-times higher than the highest tested in the present study (2 μg/L). Adult freshwater mussel (*Unio tumidus*) exposed to a high concentration of 10 mg/L of tebuconazole, a fungicide with a similar mode of action as PRO, manifested decreased shell opening rate and daily activity time (Chmist et al., 2019). Nevertheless, oyster larvae swimming behavior seems to be a sensitive endpoint revealing the effects at low ng/L environmental concentrations (Gamain et al., 2020). Therefore, it seems that the nervous system controlling the behavior function is probably not an important toxicity target of PRO at low concentrations (0.02–2 μg/L) as based on the assessment of larvae swimming behavior. However, it cannot be fully excluded before other behavioral biomarkers are assessed (larvae feeding, capture success, settlement behavior).

PRO showed a lesser effect on gene expression in comparison with IMI, and only a few genes were affected (*12S*, *mt1*, *sodCu/Zn*, *rad51*). Moreover, nanoPRO altered expression of only 3 genes (*sodCu/Zn*, *sodMn*, *gadd45*). This suggests that nanoformulations such as nanoPRO should be explored as an alternative with potentially lower toxic impact on oyster larvae. The most important impact of PRO was the induction of copper/zinc superoxide dismutase and repression of the gene coding for the mitochondrial small ribosomal unit (*12S*). As in the case of IMI, the induction of superoxide dismutase might be related to the production of ROS supported by the observed induction of *mt1*, a scavenger of ROS. PRO is indeed known to trigger ROS production as shown previously (Li et al., 2011; Nesnow et al., 2011). On the other hand, repression of *12S* suggests an impact on the mitochondrial metabolism and mitochondria count. However, according to the literature, environmental pollution by metals or pesticides is often accompanied by upregulated *12S* keeping the mitochondria quantity and maintaining the level of ATP necessary when facing chemical stress (Kim Tiam et al., 2012; Moisset et al., 2015).

The present study aimed to investigate the sublethal effects of exposure to IMI and PRO at low environmental concentrations (complemented with higher concentrations to establish EC_{50} for the developmental malformations). However, only rough estimations are possible for nanoPRO because it has not (yet) been used in the field. Prediction and impacts of any nanopesticide in aquatic environments depend on various factors such as the type of the polymer used for the encapsulation and environmental conditions, which affect its bioavailability, degradability, persistence, and bioaccumulation. Shakiba et al. (2020) in their review summarize various – often contradictory - studies addressing the role of encapsulation in nano-formulated pesticides transport from soils into water. Nevertheless, the present study showed that nanoPRO had a comparable impact on the development of the oyster larvae compared to the active ingredient alone, but it increased the swimming speed of the oyster larvae movement. NanoPRO was less toxic on the molecular level than PRO but kept some comparable toxicity patterns such as the induction of one of the ROS associated genes. As demonstrated by the release experiment, the suspension of nanoPRO is in reality a combination of encapsulated and free PRO (44% of released PRO in the 10 mg/L dilution in seawater). This might explain the lower direct toxicity of nanoPRO. On the other hand, the nanocapsules themselves may also have an effect – as seen by the increased swimming speed of oyster larvae. While lower toxicity on the molecular level of nanoPRO might indicate lower risk to non-target organisms, it must be noted that the polymeric nanocapsules may degrade over time releasing the fungicide in a delayed fashion. This might prolong the exposure of non-target organisms, and further research is needed to elucidate potentially associated chronic toxicity to non-target organisms.

5. Conclusion

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

Compliance with ethical standards

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

CRediT authorship contribution statement

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

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.142921.

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PUBLICATION II.

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

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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

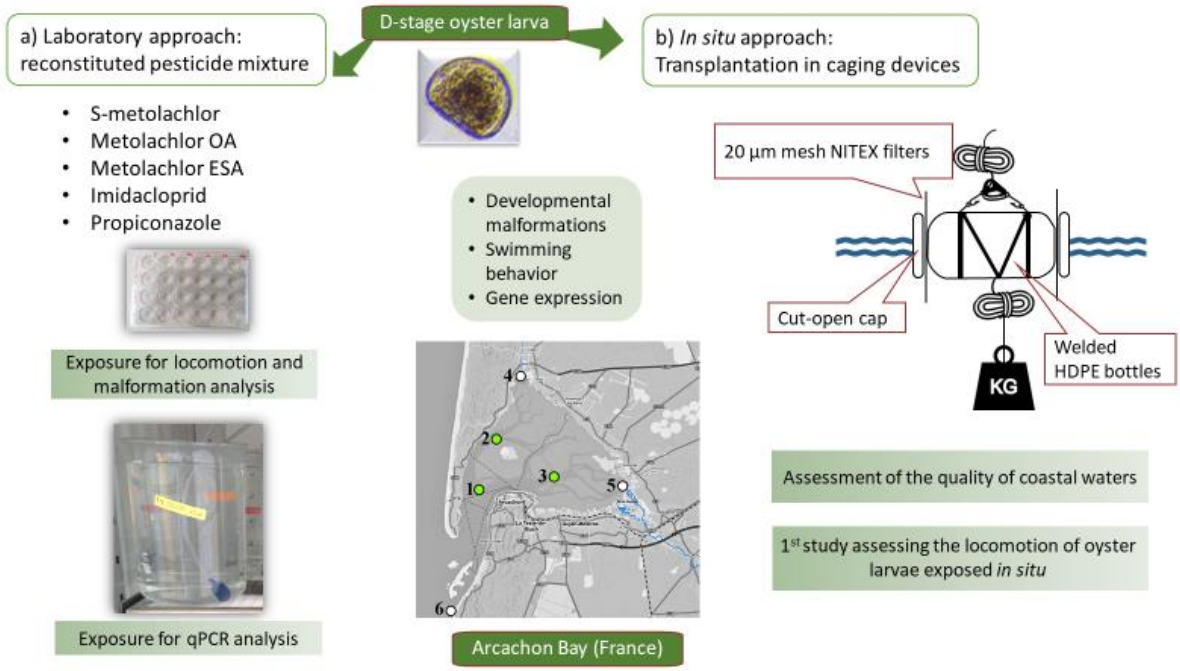
Arcachon Bay, a shallow marine lagoon on the French Atlantic coast is famous for its oyster farming. However, the worsened state of oysters and their spat in recent years demand an investigation of possible causes. Thus, this study aimed to evaluate the effects of an environmentally relevant mixture 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.

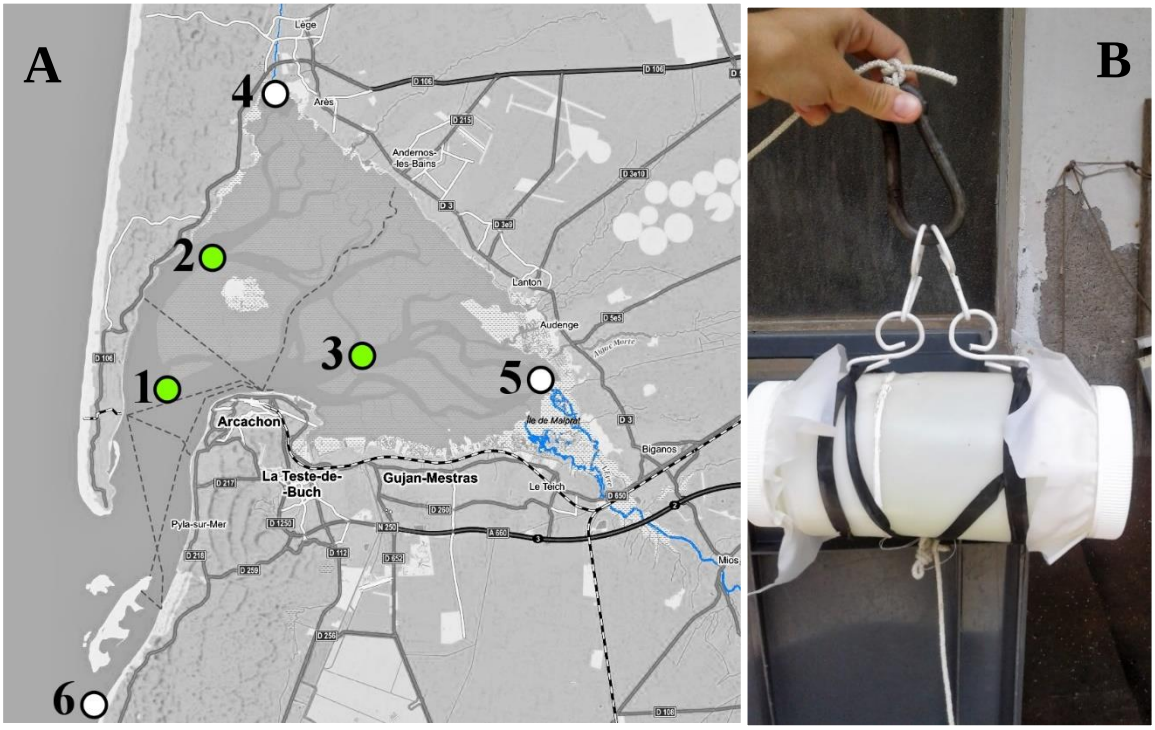


Figure 1 A Map of Arcachon Bay (France) with selected sites for the field transplantation experiment and other important points: 1 Reference site Grand Banc (GB); 2 Site Les Jacquets (J); 3 Site Comprian (C); 4 Mouth of the river Canal des Étangs; 5 Mouth of the river Leyre; 6

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.

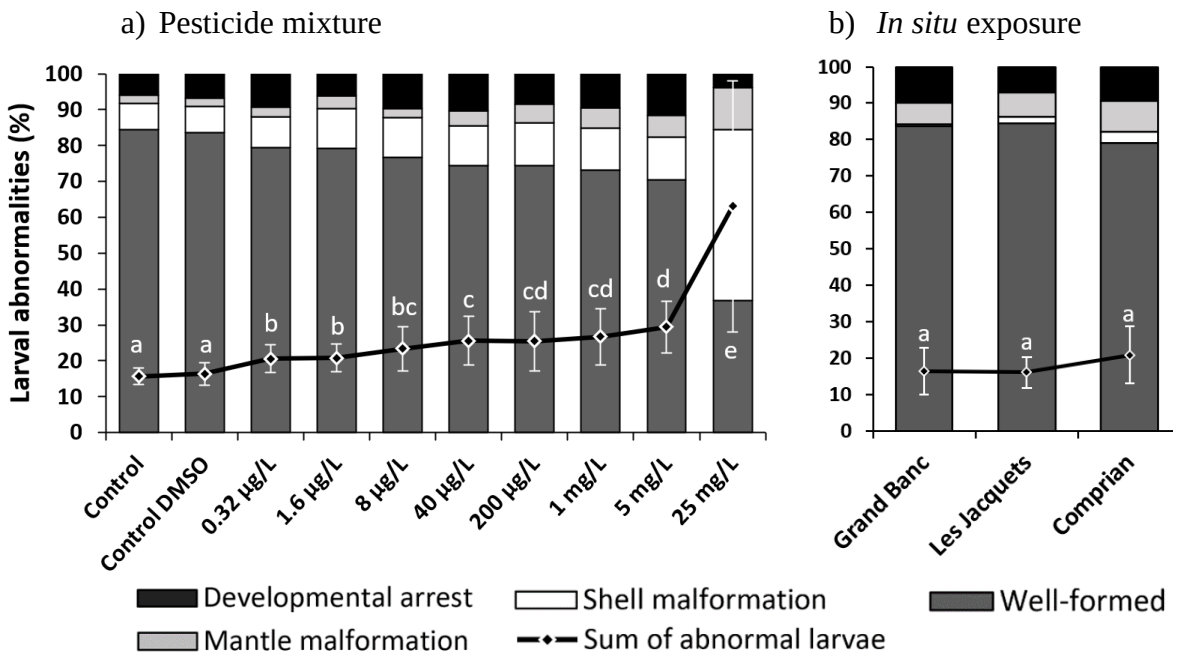


Figure 2 Larval abnormalities and the sum of affected oyster larvae a) after 30 hours of exposure to increasing concentrations of the MIX (individual concentrations of individual chemicals are shown in Table 1) and b) deployed in cages for two days *in situ* on three different sites of Arcachon Bay. 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.

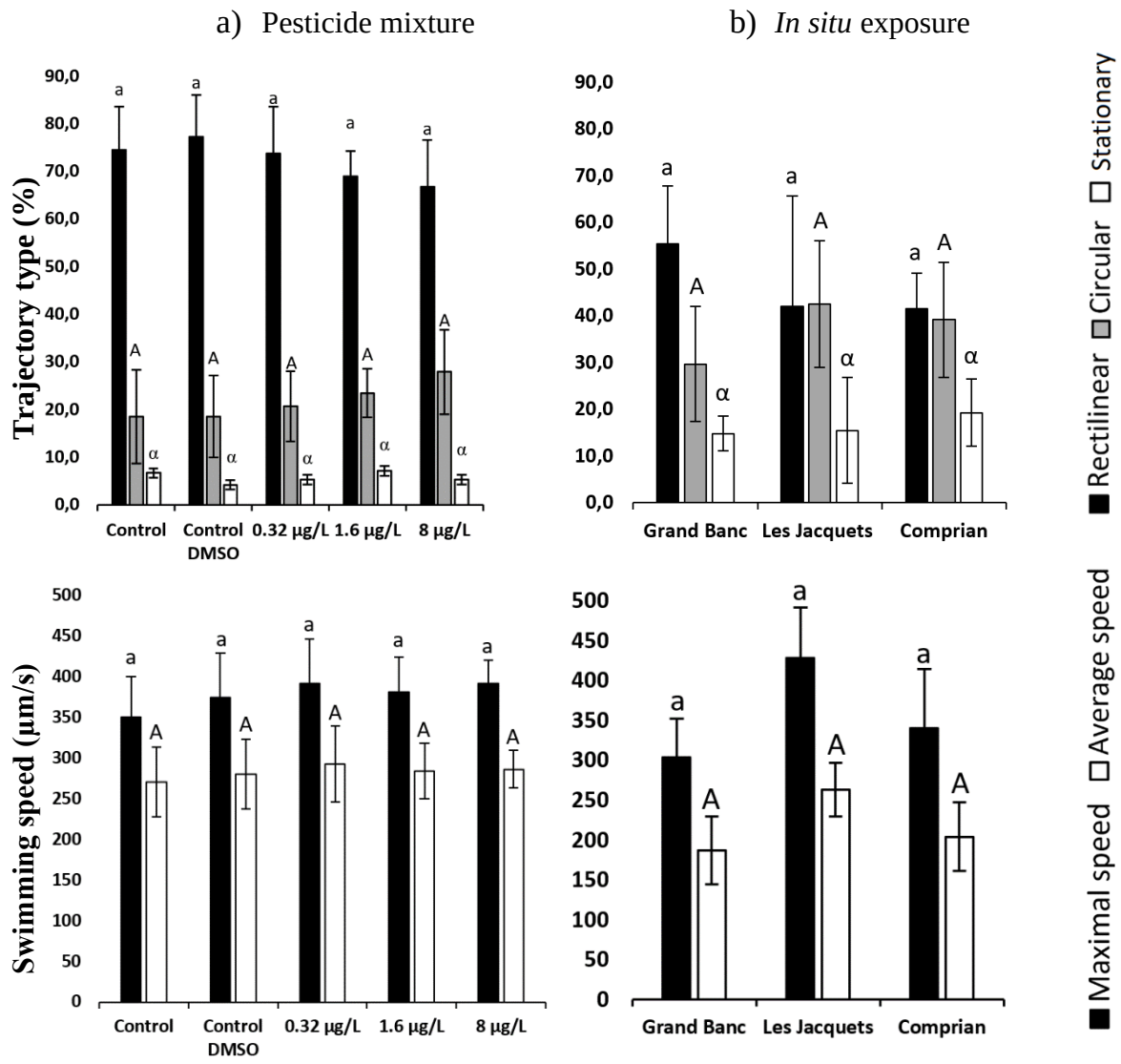


Figure 3 Trajectory types (%; upper graphs) and swimming speed (µm/s; lower graphs) observed in oyster larvae a) after 24 h exposure to three concentrations of the MIX (concentrations of individual chemicals are shown in Table 1) and b) after 2 days of transplantation at three different sites in Arcachon 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.

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Environmentally relevant mixture of S-metolachlor and its two metabolites affects thyroid metabolism in zebrafish embryos

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ABSTRACT

Herbicides and their metabolites are often detected in water bodies where they may cause adverse effects to non-target organisms. Their effects at environmentally relevant concentrations are often unclear, especially concerning mixtures of pesticides. This study thus investigated the impacts of one of the most used herbicides: S-metolachlor and its two metabolites, metolachlor oxanilic acid (MOA) and metolachlor ethanesulfonic acid (MESA) on the development of zebrafish embryos (*Danio rerio)*. Embryos were exposed to the individual substances and their environmentally relevant mixture until 120 hpf (hours post-fertilization). The focus was set on sublethal endpoints such as malformations, hatching success, length of fish larvae, spontaneous movements, heart rate and locomotion. Moreover, expression levels of eight genes linked to the thyroid system disruption, oxidative stress defense, mitochondrial metabolism, regulation of cell cycle and retinoic acid (RA) signaling pathway were analyzed. Exposure to S-metolachlor (1 µg/L) and the pesticide mixture (1 µg/L of each substance) significantly reduced spontaneous tail movements of 21 hpf embryos. Few rare developmental malformations were observed, but only in larvae exposed to more than 100 µg/L of individual substances (craniofacial deformation, non-inflated gas bladder, yolk sac malabsorption) and to 30 µg/L of each substance in the pesticide mixture (spine deformation). No effect on hatching success, length of larvae, heart rate or larvae locomotion were found. Strong responses were detected at the molecular level including induction of *p53* gene regulating the cell cycle (the pesticide mixture - 1 µg/L of each substance; MESA 30 µg/L; and MOA 100 µg/L), as induction of *cyp26a1* gene encoding cytochrome P450 (pesticide mixture - 1 µg/L of each substance). Genes implicated in the thyroid system regulation (*dio2*, *thra*, *thrb*) were all overexpressed by the environmentally relevant concentrations of the pesticide mixture (1 µg/L of each substance) and MESA metabolite (1 µg/L). Zebrafish thyroid system disruption was revealed by the overexpressed genes, as well as by some related developmental malformations (mainly gas bladder and yolk sac abnormalities), and reduced spontaneous tail movements. Thus, the thyroid system disruption represents a likely hypothesis behind the effects caused by the low environmental concentrations of S-metolachlor, its two metabolites and their mixture.

1. Introduction

S-metolachlor is one of the most commonly used pesticides in the world (Atwood and Paisley-Jones, 2017). This selective pre-emergent herbicide from the chloroacetanilides family was first registered in 1997 (Heydens et al., 2010). It is an S-enantiomer of 2-chloro-N-(2-ethyl-6-methylphenyl)-N-(1-methoxypropan-2-yl)acetamide. It replaced the racemic metolachlor mixture due to the higher herbicidal activity of S-metolachlor (Müller et al., 2001; Poiger et al., 2002), thus reducing the quantity of pesticide needed by 40 % (Blaser and Spindler, 1997). S-metolachlor inhibits the biosynthesis of very-long-chain fatty acids (Götz and Böger, 2004) and interferes with gibberellin synthesis enzymes (Rose et al., 2016), thereby inhibiting the growth of target plants such as grass weeds in cornfields (Heydens et al., 2010). However, S-metolachlor can easily contaminate surface water by agricultural runoff (Zemolin et al., 2014) and it is thus often detected in surface or coastal waters (Accinelli et al., 2002; De Liguoro et al., 2014; Fauvelle et al., 2018; Glinski et al., 2018; Kapsi et al., 2019; Meffe and de Bustamante, 2014; Ryberg et al., 2014). In soil, S-metolachlor is microbially degraded to two principal metabolites (Zemolin et al., 2014): metolachlor oxanilic acid

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(MOA) and metolachlor ethanesulfonic acid (MESA), which are mobile and persistent in the environment (Sidoli et al., 2016). As a result, these degradation products are often detected in higher concentrations than the parent compound itself in water samples (Elliott and VanderMeulen, 2017; Hladik et al., 2005). However, there is a general lack of information about the toxicity of individual pesticide metabolites and their mixtures occurring in the environment. Only recently, some studies highlighted the importance of aquatic toxicity of environmentally relevant pesticide mixtures (Gustavsson et al., 2017; Tian et al., 2018). Furthermore, the importance of evaluating mixture toxicity is emphasized by the European Chemicals Agency (2014).

The contamination of aquatic environments by herbicides may adversely affect non-target organisms such as fish. Fish are among the most sensitive organisms in aquatic ecosystems and are used as standard testing species in the hazard assessment of chemicals and their mixtures (Hayes et al., 2019). To the best of our knowledge, studies on the effects of S-metolachlor or its metabolites on fish are scarce. Most use high concentrations (mg/L) to obtain LC_{50} or EC_{50} values, and these are far from being environmentally relevant (Dobšíková et al., 2011; Munn et al., 2006; Quintaneiro et al., 2017). Unlike these studies, Jin et al. (2011) observed that lower concentrations (μg/L) of metolachlor (a mixture of both R- and S-enantiomers) altered the thyroid system in Japanese medaka. Thyroid system disruption was also caused by low concentrations of acetochlor (μg/L), another member of the chloroacetanilide family (Yang et al., 2016). In other non-target aquatic organisms, S-metolachlor and its metabolites MOA and MESA induced developmental abnormalities in Pacific oyster larvae and sperm at low environmental concentrations (Gamain et al., 2016; Mai et al., 2014). Moreover, the deleterious effects of environmental concentrations of S-metolachlor and MOA on crayfish were also reported (Velisek et al., 2019, 2018). Although several studies pointed out the toxicity of high concentrations of these substances on non-target aquatic organisms (Gutiérrez et al., 2019; Munn et al., 2006; Quintaneiro et al., 2018, 2017), comprehensive information on sublethal effects caused by environmental concentrations is still missing.

Early life stage zebrafish (*Danio rerio*) is widely used as sensitive and reliable alternative toxicity model with multiple advantages, including high-throughput screening, embryo transparency, quick breeding and development, a sequenced genome (Love et al., 2004), and are in compliance with the 3Rs Principle (Russell et al., 1959). Zebrafish development is highly influenced by its thyroid metabolism (Jarque and Piña, 2014). Moreover, the early development of zebrafish thyroid system is comparable to its development in humans (Alt et al., 2006), complete with a similar hypothalamus–pituitary–thyroid (HPT) axis (Porazzi et al., 2009). This similarity makes zebrafish a suitable model for evaluating thyroid gland disruption (Raldúa et al. 2012). In a recent review, Spaan et al. (2019) commented that the complexity of thyroid disruption assessment requires the evaluation of multiple endpoints. Morphological effects, transcription level of several genes interfering with the HPT axis, hatching, heart rate, behavior and swim bladder inflation were cited as the most common.

Although the toxicity of high concentrations of S-metolachlor on non-target aquatic organisms has been previously studied, the effects posed by low environmentally relevant concentrations of this compound and its metabolites have yet to be examined. The present study investigated the sublethal toxicity of environmentally relevant concentrations of S-metolachlor, its two metabolites MOA and MESA and their mixture on embryo-larval stages of *Danio rerio*. Apical endpoints of zebrafish embryos were evaluated, a neurobehavioral study, measuring swimming activity to assess potential neurotoxicity, was conducted, and biochemical responses were assessed by measuring the transcription changes of selected genes. This integrative approach allowed for a detailed examination of sublethal toxicity, including potential thyroid metabolism disruption.

2. Materials and methods

2.1. 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 %) were purchased from Sigma-Aldrich. Their stock solutions (50 mg/L) were prepared in Milli-Q water and were stored at 4 °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 spontaneous movements analysis 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.

2.2. Test organism

Zebrafish (*Danio rerio*) embryos were collected from an unspecified wild type zebrafish strain maintained at RECETOX, Masaryk University (Czech Republic). Adult fish were held in aquariums with tap water at 26 ± 1 °C, at 14:10 h light:dark photoperiod and fed 3 times a day with commercially available feeds (of which at least once with live brine shrimp *Artemia salina*). About 10 − 14 h before the experimentation, a box for collecting the embryos was placed in the aquarium, and the spawning was induced in the morning by turning the light on. The eggs were collected 30 min after, rinsed and transferred to the ISO medium (ISO, 1996). Only fertilized and normally developing eggs were picked for the tests using a stereomicroscope (OLYMPUS, Japan), and exposed at 3–4 hpf (hours post-fertilization), which correspond to blastula period as described by Kimmel et al. (1995). The embryos were then maintained at 26 °C, at 14:10 h light:dark photoperiod till 120 hpf without feeding or renewal of test solutions.

2.3. FET (Fish embryo toxicity) test

The experiments were conducted in accordance with the fish early-life stage toxicity guideline (OECD, 2013), with few modifications. Selected eggs were exposed to the chosen herbicide S-metolachlor and its two metabolites MOA and MESA. The experiments were done with individual compounds, and then with the mixture of the three. Chosen concentrations are based on environmental concentrations detected in the rivers of the Czech Republic and in the Arcachon Bay in France during 2010–2014 (Table 1).

The lowest used test concentration (1 μg/L) reflects the actual (high) environmental concentration, and three higher (non-lethal) concentrations were also tested (30, 100 and 300 μg/L). Corresponding concentrations in nmol/L are shown in Supplementary Table S2. The mixture exposure included two concentration levels: 1 μg/L of each

Table 1
Maximum concentrations of metolachlor and its metabolites MOA and MESA detected in Czech rivers (CHMU (Czech Hydrometeorological Institute), 2018) and Arcachon Bay in France (Tapie et al., 2018) during 2010–2014. Number of samples: N = 703 values for every substance in Arcachon Bay; N = 13 175, 7 503 and 7 502 for metolachlor, MOA and MESA respectively in surface water).

	Metolachlor	MOA	MESA	Location
Maximum concentration	5800	4200	4200	Surface water (CZ)
(ng/L)	1696	1609	1059	Arcachon Bay (FR)
% of values > 100 ng/L	2.5 %	4.1 %	19.1 %	Surface water (CZ)
	36.0 %	6.1 %	36.6 %	Arcachon Bay (FR)

substance and 30 µg/L of each substance. ISO medium was used as negative control. The tests were conducted in glass crystallization dishes containing 20 mL of media and 20 embryos in three replicates. Every test was repeated independently three times with eggs from different spawning. Mortality, hatching success and malformations were controlled daily using a stereomicroscope. Eventual dead embryos/larvae were immediately removed from dishes to prevent contamination. Length of larvae (without the caudal fin) was measured on the last (fifth) day of the experiment on photos of larvae mounted in a mixture of cellulose and anesthetics MS-222. Fifteen larvae per replicate in 3 independent tests were measured. Photos were analyzed using the software QuickPhoto Micro 2.3.

"Morphological score" endpoint was assessed in order to evaluate the global state of individual fish, using a slightly modified approach as previously published (Panzica-Kelly et al., 2010). Every fish embryo was classified using 0-3 point scores (description is in Supplementary Fig. S1).

2.4. Light: dark locomotor test

Embryos for the locomotor activity analysis were handled as described above (2.3). The evening before measurements (to allow for acclimation), one larva per well was transferred to transparent 96-well microplates (Gama, Czech Republic). The distribution was random; 32 fish per exposure condition were used. Only morphologically normal, i.e. non-malformed embryos were used. The experimentations were performed during the 5 dpf between 9 a.m. and 16 pm. Prior to the experiment, the microplates were kept for 1 h next to the ZEBRABOX (Viewpoint Life Sciences) at 26 °C for acclimation after the transport. Measurement done in the ZEBRABOX consisted of four 15 min alternating cycles (white light, i.e. 100 % stimulus / dark, i.e. no stimulus / white light / dark). Total distances swam (mm) by individual fish were measured with an integration period of 60 s and processed in real time using the software Zebralab (Viewpoint Life Sciences). The threshold between "inactive" fish and "active" fish was set on 0.3 cm/s. The sensitivity threshold of ZEBRABOX was set on 120.

2.5. Spontaneous movements and heartbeat

Embryos were handled as described above (2.2) and exposed to concentrations of 1 and 100 µg/L of substances alone and to mixtures at two concentration levels (1 and 30 µg/L of each substance in the mixture). These were maintained at 26 °C until 21 hpf when randomized capture of videos was realized until 23 hpf. Crystallization dishes containing 20 embryos per replicate were successively acclimatized under the stereomicroscope for 30 s and then 2 min video was captured. Positive control (1 % v/v ethanol) was used to enhance spontaneous movements (tail coils). Videos were then analyzed by DanioScope software (Noldus, Netherlands), and reported as spontaneous movements per min.

The same embryos were used for the heart rate activity measurement during the 3 dpf starting at 72 hpf. Hatched larvae were first put in a solution of 2.5 % methylcellulose containing 50 mg/L of MS-222 anesthetics at 26 °C and placed immediately in Petri dish. Larvae were acclimatized for 10 s under the stereomicroscope and 20 s videos were captured. Videos were analyzed using the DanioScope software, and reported as beat per min.

2.6. Zebrafish exposure for analysis of gene expressions

Embryos were handled as described above (2.3), and exposed to 1, 30 and 100 µg/L of pesticides assessed alone and two concentrations of the mixture of the three pesticides (1 and 30 µg/L, respectively of each compound). Hormone T3 (triiodothyronine) was used as positive control to assess the expression of thyroid-related genes (final concentration of methanol solvent was 0.1 % in the exposure solution). Dead or heavily malformed embryos/larvae were removed daily. Whole larvae were collected at 120 hpf into pools of 20 larvae per Eppendorf tube containing 500 µL of RNA*later* ® and stored at −20 °C upon RNA extraction.

2.6.1. RNA isolation

The total RNAs were extracted using the SV Total RNA Isolation System Kit (Promega). Whole larvae were homogenized in 500 µL of RNA Lysis Buffer using glass beads (710-1,180 µm; Sigma) in the FastPrep®-24 (M.P. Biomedicals, USA). Lysed samples were collected, centrifuged and mixed with 450 µL of 75 % v/v ethanol and transferred on the spin column. Handling with spin columns was performed according to the manufacturer's instructions. Before the DNase incubation step, an additional centrifuge step was added (13,500 rpm, 2 min) to dry the column. All RNA samples were treated with DNase I mixture for 15 min in a water bath at 37 °C. The rest of the isolation was performed according to the manufacturer's instructions, and purified RNAs were collected in 50 µL of Nuclease-Free water. The concentration and purity of collected RNA samples were verified spectrophotometrically at 260/280 and 260/230 nm with Nanodrop™. The purity of all samples was between 2.0–2.1 for the ratio 260/280 and 1.8–2.2 for the ratio 260/230.

2.6.2. Reverse transcription

Reverse transcription was performed with GoScript™ Reverse Transcription System kit (Promega) according to the manufacturer's instructions using TProfessional Thermocycler (Biometra, Analytik Jena). Purified RNA (1 µg) was reversely transcribed in order to get the final volume of 20 µL of cDNA, and it was stocked at −20 °C upon qPCR.

2.6.3. Quantitative PCR

8 genes were selected to evaluate the effects of chosen pesticides and their mixture on the thyroid hormone system, mitochondrial metabolism, regulation of the cell cycle and oxidative stress defense. Three reference zebrafish genes were used in the analysis (*β-actin*, *ef1a* and *rpl13*). Functioning and references of used primers are shown in Table 2. Sequences, efficiencies, and accession numbers are presented in Supplementary Table S1. Primers were purchased from Elisabeth Pharmacon (Czech Republic).

QPCR was carried out with Brillant III Ultra-fast SYBR Green QPCR Master Mix kit (Stratagene-Agilent) on the LightCycler® 480 (Roche). Each qPCR mix (20 µL) contained 5 µL of cDNA (10 times diluted), 2 µl of reverse and forward primer (2 µM), and 10 µL of SYBR from the kit and 3 µL of nuclease free water.

LightCycler was set according to the instructions of the SYBR kit manufacturer (Stratagene-Agilent). Briefly, the pre-incubation lasted 3 min at 95 °C, then the amplification consisted of 45 cycles with 1 cycle at 95 °C for 5 s and 60 °C for 10 s. The melting curve continued at 95 °C for 5 s and at 65 °C for 1 min. Finally, the cooling step was performed at 40 °C for 30 s. Melting curves of every reaction were analyzed to assess

Table 2
Primers used for qPCR analysis in zebrafish (*Danio rerio*) and their references.

Gene	Function	References
β-act	Reference gene	Dedeh et al., 2015
ef1a	Reference gene	Gentès et al., 2015
rpl13	Reference gene	Gentès et al., 2015
12S	Mitochondrial metabolism	Arini et al., 2015
p53	Regulation of the cell cycle/apoptose	Gentès et al., 2015
cat	Oxidative stress defense	Lerebours et al., 2009
sod1	Oxidative stress defense	Gentès et al., 2015
cyp26a1	RA (retinoic acid) signaling pathway	Oliveira et al., 2013
dio2	Thyroid hormone system	Yan et al., 2012
thra	Thyroid hormone system	Yan et al., 2012
thrb	Thyroid hormone system	Yan et al., 2012

Table 3
Frequencies (%) of different types of developmental malformations, length of larvae (μm) and morphological score of zebrafish after 120 h exposure to different concentrations of pesticide, metabolites and their mixtures. Each value represents mean ± standard deviation from 3 independent experiments each based on N = 60 embryos. *P < 0.05; **P < 0.01; [1] average of sum of 3 independent experiments; Measured concentrations by LC–MS/MS are shown in Supplementary Table S2.

Endpoint		Body length	Edemas	Craniofacial def.	Spine def.	Non-inflated gas bladder	Tail def.	Necrosis	Yolk sac malabsorption	Hemorrhage	Morphological score
Concentration μg/L		μm	%								Value[1]
SM	*Control*	3768 ± 103	1.1 ± 1.6	1.1 ± 1.6	0 ± 0	0.6 ± 0.8	0 ± 0	0.6 ± 0.8	1.1 ± 0.8	1.1 ± 1.6	0.9 ± 1.0
	1	3772 ± 118	0.6 ± 0.8	1.7 ± 1.4	1.1 ± 0.8	2.8 ± 2.8	0 ± 0	0.6 ± 0.8	3.9 ± 2.8	0 ± 0	1.6 ± 1.1
	30	3767 ± 131	1.7 ± 2.4	2.8 ± 3.9	1.1 ± 1.6	3.3 ± 1.4	0 ± 0	0 ± 0	3.3 ± 3.6	0 ± 0	1.8 ± 1.8
	100	3763 ± 123	1.7 ± 1.4	2.3 ± 3.2	0.6 ± 0.8	**4.6 ± 1.6***	0 ± 0	0.6 ± 0.8	4.0 ± 0.8	0 ± 0	**2.0 ± 1.1***
	300	3738 ± 131	1.7 ± 1.4	2.8 ± 1.6	1.7 ± 1.4	2.3 ± 0.8	0 ± 0	0.6 ± 0.8	**5.8 ± 2.2***	0 ± 0	**2.2 ± 0.8***
MOA	*Control*	3714 ± 136	1.1 ± 0.8	2.3 ± 2.1	0.6 ± 0.8	3.4 ± 2.4	0 ± 0	2.8 ± 4.0	2.3 ± 2.1	0 ± 0	1.9 ± 1.5
	1	3712 ± 130	2.3 ± 2.2	2.3 ± 2.2	1.8 ± 1.5	1.8 ± 2.5	0 ± 0	0.6 ± 0.8	1.8 ± 2.5	1.2 ± 1.7	1.8 ± 1.9
	30	3719 ± 160	1.1 ± 1.6	0.6 ± 0.8	0 ± 0	2.9 ± 1.6	0 ± 0	0.6 ± 0.8	1.7 ± 0	1.7 ± 2.4	1.3 ± 0.8
	100	3678 ± 169	3.5 ± 2.4	2.3 ± 1.6	2.9 ± 0.7	5.1 ± 2.4	0 ± 0	1.7 ± 2.4	2.8 ± 0.8	3.4 ± 4.8	3.0 ± 1.4
	300	3694 ± 163	1.1 ± 1.6	1.1 ± 1.6	1.7 ± 1.4	3.9 ± 0.7	0 ± 0	2.3 ± 3.2	2.9 ± 0.8	0.6 ± 0.8	2.1 ± 1.6
MESA	*Control*	3661 ± 140	0.6 ± 0.8	0.6 ± 0.8	1.1 ± 0.8	2.2 ± 2.1	0 ± 0	0 ± 0	1.7 ± 1.4	0 ± 0	0.7 ± 0.5
	1	3708 ± 112	0 ± 0	1.1 ± 1.6	0 ± 0	2.2 ± 2.1	0 ± 0	0 ± 0	0.6 ± 0.8	0 ± 0	0.6 ± 0.2
	30	3700 ± 146	0 ± 0	1.1 ± 1.6	0 ± 0	1.1 ± 1.6	0 ± 0	0.6 ± 0.8	0.6 ± 0.8	0 ± 0	0.6 ± 0.4
	100	3683 ± 118	1.1 ± 0.8	**4.4 ± 5.2***	1.7 ± 1.4	3.3 ± 1.4	0 ± 0	1.1 ± 1.6	2.8 ± 0.8	0 ± 0	**2.0 ± 1.7****
	300	3628 ± 127	0 ± 0	2.3 ± 2.1	0.6 ± 0.8	1.1 ± 0.8	0 ± 0	0 ± 0	1.7 ± 0	0 ± 0	0.9 ± 0.4
MIX	*Control*	3755 ± 149	0.7 ± 1.0	4.3 ± 2.6	0.6 ± 0.8	6.3 ± 4.4	0.6 ± 0.8	2.3 ± 1.7	6.9 ± 6.6	0.6 ± 0.8	3.2 ± 1.7
	1 (of each)	3763 ± 163	2.8 ± 0.8	2.9 ± 1.6	3.4 ± 2.8	4.5 ± 0.8	0.6 ± 0.8	1.1 ± 0.8	5.1 ± 2.4	0 ± 0	3.1 ± 1.1
	30 (of each)	3740 ± 193	3.1 ± 2.5	8.0 ± 1.0	**4.6 ± 3.5***	6.7 ± 5.2	0 ± 0	3.0 ± 2.3	12.2 ± 8.6	1.9 ± 1.6	**5.9 ± 2.0****

reaction specificity. The data were normalized to the mean of the Ct values for three reference genes, *β-act*, *elf1a* and *rpl13*, and analyzed using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). Results are shown as fold changes of exposed to the control group.

2.7. Chemical analysis and water quality

At the beginning, and at the end of the experiments, pH, dissolved oxygen and conductivity were controlled. Oxygen saturation was always higher than 98 %. pH varied between 7.6-7.9. Conductivity varied between 500 and 700 μS/cm, with no difference between ISO medium and pesticide solutions. The stability of substances during the five-day experiment was verified by measuring their concentrations by LC–MS/MS analysis, as described in detail in Supplementary Material S1. In brief, LC–MS/MS was performed with a Waters LC chromatograph (Waters, Manchester, U.K.), using Acquity BEH C18 column and gradient elution. Detection was performed on a Xevo TQ-S quadrupole mass spectrometer (Waters Manchester, U.K.) after ESI ionisation in a positive ion mode. The analytes were quantified using external calibration of S-metolachlor, MOA and MESA (0.1–500 μg/L in 20 % of methanol), and the limit of detection (LOD, signal to noise ratio S/N > 3), and quantification (LOQ, S/N > 10) were 0.05 and 0.1 μg/L, respectively for each analyte.

2.8. Data analysis

Length of the larvae was assessed with one-way ANOVA test. The observed malformations were summed in the individual treatments (N = 9), and compared with controls using Fisher's exact test in GraphPad Prism (Version 5, GraphPad Software). The morphological scores of individual fish were summed and compared with sums of controls using Fisher's exact test in GraphPad Prism (Version 5, GraphPad Software).

Independent locomotion tests (independent experiments) were statistically individually assessed because of high variability in controls. Final results are presented as a mean of fold changes with controls of the three independent experiments.

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, non-parametric Kruskal-Wallis test with Mann-Withney post-hoc test was used.

All data for the gene expression analysis were treated as described above (Shapiro-Wilk test; P > 0.01; Levene test; P > 0.05). Eventual values higher or smaller than 3 standard deviations were discarded. Data were log normalized prior to the analysis. ANOVA followed by Dunnet post-hoc test was used. If normality or homoscedasticity were not confirmed, non-parametric Kruskal-Wallis test with Mann-Whitney post-hoc tests were performed. All analyses were performed using software Statistica 13.3 (StatSoft, version 13.2, USA).

3. Results

3.1. Exposure and chemical analysis

Stability of all three compounds during the five-day experiment was verified. Nominal and measured concentrations, as well as the stability, are shown in Supplementary Table S2. The concentrations of compounds were stable with the maximum decline of 13.3 % at 30 μg/L of S-metolachlor. Measured values corresponded to nominal ones in the case of MOA and S-metolachlor, but were higher for MESA. To simplify the presentation, nominal concentrations are shown and discussed in the results and discussion sections.

3.2. Mortality, hatching success, length of fish and malformation results

No significant effects on mortality were observed in any treatement. Mortality in all controls was below 10 % as preconized by OECD guideline (OECD, 2013). Survival in all treatements was between 97.2 % and 100 %. Similarly, no significant effects on hatching success were found in all conditions tested. Hatching success rates were between 95 % and 100 % in all treatements.

Observed developmental abnormalities and length of the larvae are presented in Table 3. Generally, no patterns or dose-response effects were observed after exposure to S-metolachlor, its two metabolites MOA and MESA, or to the mixture. No effects on the length of larvae were observed in any condition tested. The most frequently observed malformations were of swim bladder (non-inflation) and yolk sac (malabsorption). No statistically significant effects were detected after

exposures to the lower concentrations (1 and 30 µg/L) of S-metolachlor, MOA and MESA, or to the mixture (1 µg/L of each substance). Following statistically significant effects were observed: S-metolachlor induced non-inflation of the swim bladder (100 µg/L) and malabsorption of the yolk sac (300 µg/L), and MESA (300 µg/L) induced craniofacial malformations. In addition, mixture of the three substances at concentration of 30 µg/L per each substance induced spinal deformations. Morphological score endpoint which allows assessment of the overall condition of the embryos in the individual treatments revealed the concentrations of 100 and 300 µg/L of S-metolachlor, 100 µg/L of MESA, and 30 µg/L of the mixture having significant adverse effect on the development of the embryos.

3.3. Light:dark locomotion test and heartbeat

None of the studied compounds or their mixtures caused any statistically significant effects on swimming behavior. Detailed results are shown in Supplementary Material S2. Similarly, there were no effects observed at the heart rate of zebrafish larvae, detailed results are shown in Supplementary Material S3.

3.4. Spontaneous movements

Observed spontaneous movements of the zebrafish embryos are presented in Fig. 1. Environmental concentrations of S-metolachlor (1 µg/L) and of the mixture of the three substances (1 µg/L of each substance) significantly ($P = 0.001$ and 0.022, respectively) reduced frequency of the tail movements. Solution of ethanol (0.5 %, v/v), used as a positive control, significantly enhanced tail movements ($P = 0.009$).

3.5. Analysis of gene expression

Gene expression of eight pre-selected genes was analyzed. Results are shown in Fig. 2 as fold changes of the ratio between selected genes and housekeeping genes. Exposures to T3 was used as a positive control for thyroid-related genes.

The expression of mitochondrial gene 12S RNA (*12S*) gene was not significantly upregulated or downregulated in comparison with the control for all the concentrations of all the pesticides tested (Fig. 2a). Transcription factor *p53,* gene related to the regulation of cell cycle and cell apoptosis was significantly upregulated at 100 µg/L of MOA (1.8f-old, $P = 0.005$) and 30 µg/L of MESA (2-fold, $P = 0.022$). The mixture of SM, MOA and MESA in concentration of 1 µg/L of each pesticide (2-fold, $P < 0.001$) induced significant upregulation of *p53* (Fig. 2b). Oxidative stress defense associated genes catalase - *cat* (Fig. 2c) and copper/zinc superoxide dismutase - *sod1* (Fig. 2d) were not affected by any of the pesticides tested.

The genes associated to RA (retinoic acid) signaling pathway were studied: gene coding for cytochrome P450, family 26, subfamily A, polypeptide 1 - *cyp26a1* (Fig. 2e) and genes associated to thyroid metabolism disruption: iodothyronine deiodinase 2 - *dio2* (Fig2f), thyroid hormone nuclear receptor αa - *thra* (Fig. 2g), and thyroid hormone nuclear receptor beta - *thrb* (Fig. 2h). None of the four genes were deregulated after exposure to S-metolachlor. On the contrary, the metabolites and the mixture affected gene transcriptions. MOA significantly increased the expression of *dio2* (3.5-fold, $P = 0.015$) and *thrb* (1.8-fold, $P = 0.034$) in the highest concentration tested 100 µg/L. MESA significantly increased the mRNA expression level of *dio2* (5.6-fold, $P = 0.002$), *thra* (2.1-fold, $P = 0.004$) and *thrb* (1.9-fold, $P = 0.003$) in the concentration of 1 µg/L. Upregulation of *thra* was also observed in MESA concentration of 30 µg/L (2-fold, $P = 0.027$). The mixture of the three substances (at low level - 1 µg/L of each substance) significantly upregulated the gene expression in all four genes - *cyp26a1* (2.3-fold, $P < 0.001$), *dio2* (2.5-fold,$P = 0.007$), *thra* (2.5-fold, $P = 0.001$), *thrb* (2.2-fold, $p = 0.001$). The T3 hormone significantly increased the expression of the mRNA transcription level of *cyp26a1* (4.2-fold, $p = 0.001$) and *thra* (4.7-fold, $P < 0.001$), as expected.

4. Discussion

S-metolachlor and its metabolites are among the most commonly reported herbicides occurring in relatively high concentrations in European water bodies (Accinelli et al., 2002; Cerejeira et al., 2003; Farlin et al., 2018; Kapsi et al., 2019; Meffe and de Bustamante, 2014; Vryzas et al., 2009). Despite the overall importance, the toxicity of S-metolachlor on fish was mostly evaluated at environmentally non-relevant conditions and high concentrations (Quintaneiro et al., 2017), or using only the racemic mixture of metolachlor (Jin et al., 2011). More recently, the toxicity of one of the metabolites - MOA, was assessed by Velisek et al. (2018a) on crayfish but the effects of S-metolachlor and its metabolites on fish remain poorly characterized. Our interest was mainly focused on thyroid disruption, based on findings of Jin et al. (2011), and Yang et al. (2016) who linked this type of disruption with some chloroacetanilide pesticides. In the current work, an integrative multi-endpoint approach was used to address the complexity of the thyroid system and its importance, especially during the early development of zebrafish.

4.1. Effects on embryo-larval development

Malformations were statistically significantly more frequent only at

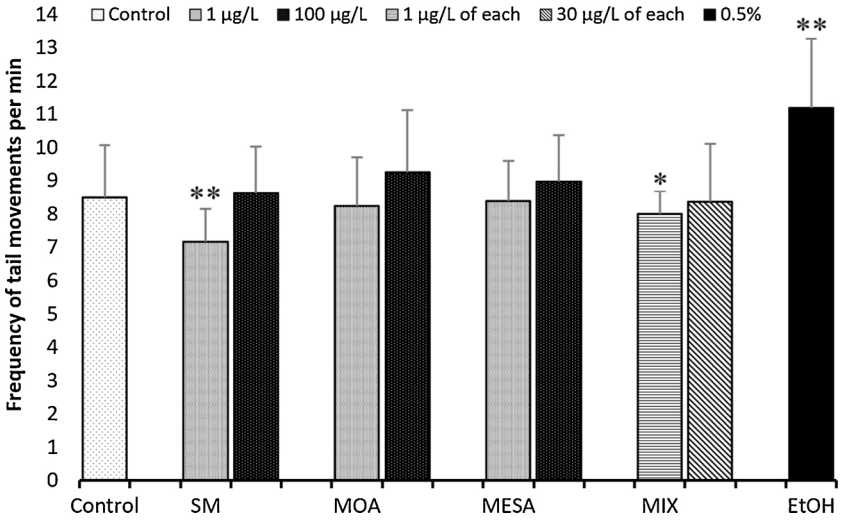

Fig. 1. Spontaneous tail movements frequency per min in zebrafish embryos exposed SM: S-metolachlor; MOA: Metolachlor oxanilic acid; MESA: Metolachlor ethanesulfonic acid; MIX: mixture of SM, MOA and MESA (concentration of 1 and 30 µg/L of each substance in the mixture); EtOH: ethanol; *$P < 0.05$, **$P < 0.01$; Measured concentrations by LC–MS/MS are shown in Supplementary Table S2.

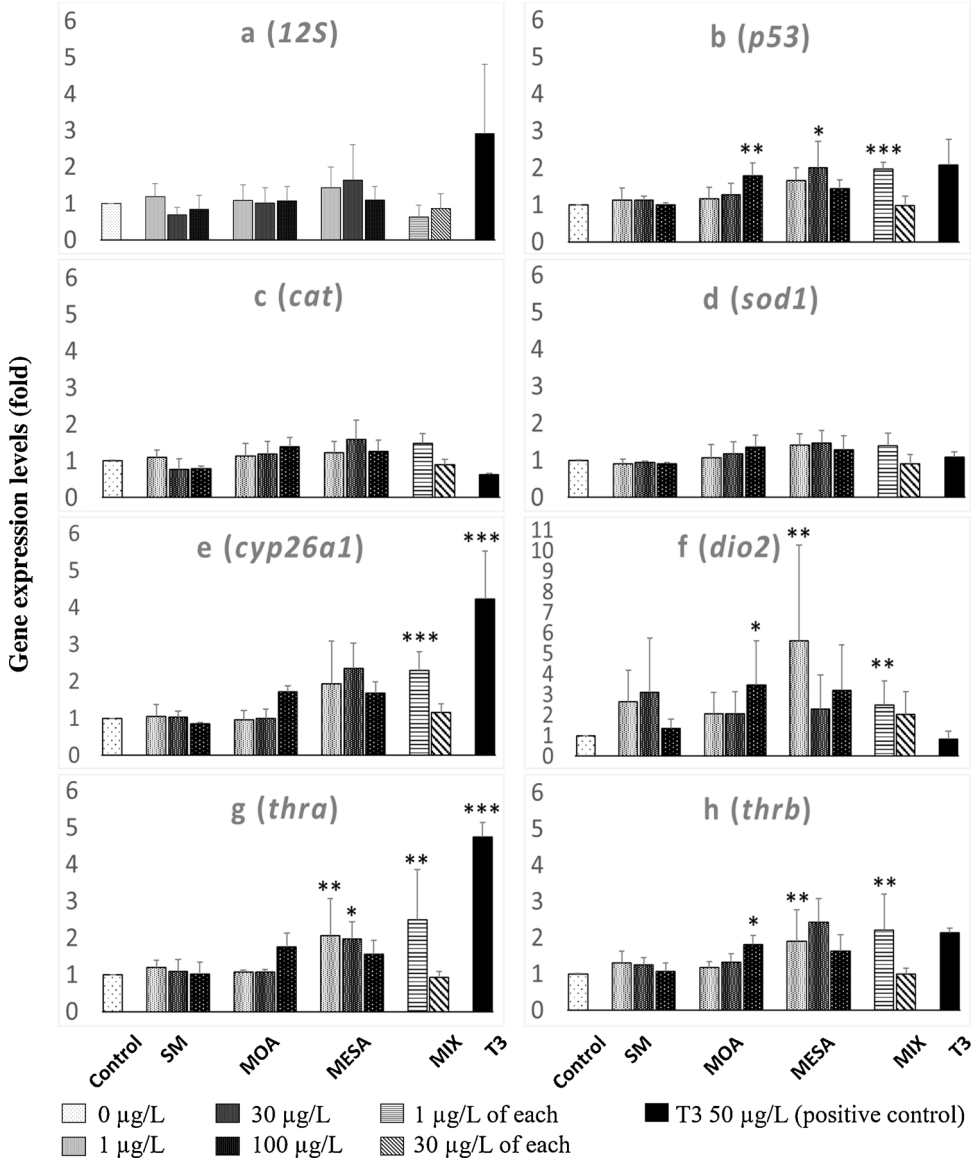

Fig. 2. Expression levels of selected genes involved in mitochondrial metabolism (a), regulation of cell cycle (b), oxidative stress defense (c, d), and thyroid metabolism disruption (e,f,g,h) associated genes in zebrafish larvae exposed for 5 days to SM: S-metolachlor; MOA: Metolachlor oxanilic acid; MESA: Metolachlor ethanesulfonic acid; MIX: mixture of SM, MOA and MESA; T3: triiodothyronine hormone; $P < 0.05$; **$P < 0.01$; ***$P < 0.001$; Measured concentrations by LC–MS/MS are shown in Supplementary Table S2.

the concentrations that were higher than those observed in the environment. Spine deformations were induced in fish exposed to mixture of substances (30 µg/L of each of the three compounds) but, interestingly, no such effects were observed at any of the substances alone up to 300 µg/L. It seems that concentration addition (CA) effects in the mixture of different compounds could be a major driver beyond this observation (European Chemicals Agency, 2014). Correspondingly, Lydy et al. (2004) discuss that, generally, pesticides of the same chemical class exert the CA effects. This is supported also by findings of Junghans et al. (2003) who reported the CA effects of the mixture of eight chloroacetanilide herbicides in the study with susceptible organism – alga *Scenedesmus vacuolatus*. It should be noted that despite the statistical significance, the frequency of spine malformation (4.6 %) is rather low and might be of minor biological significance. Nevertheless, further experiments could eventually further explore the mechanism behind this observation.

Other significant malformations (craniofacial deformations, non-inflated swim bladder, yolk sac malabsorption) were induced by individual substances at the two higher concentrations (100 or 300 µg/L). The observed malformations could be linked to the disruption of thyroid signaling. Liu and Chan (2002) showed that thyroid disruption lead to retarded inflation of the swim bladder, retarded absorption of the yolk sac and retarded maturation of the gastrointestinal system of zebrafish. Our observation of impaired development is in line with a previous study of Quintaneiro et al. (2017), where suppressed yolk absorption was observed. Although it was at higher concentration (45 mg/L), it shows possible link to thyroid disruption leading to hindered metabolization. Above all, Stinckens et al. (2018) linked the non-inflation of swim bladder with thyroid disruption via mainly iodothyronine deiodinase type 2, using the in chemico assays and zebrafish early life stage test based on the AOP (adverse outcome pathway) framework.

In this study, no statistically significant mortality was observed for tested concentrations of all substances or the mixture. According to U.S. EPA Ecotox database (US EPA 1992), NOEL of S-metolachlor for bluegill is 1.5 mg/L, and for rainbow trout 2.5 mg/L (measured at 96 hpf). Both concentrations are higher than our highest tested concentration of 300 µg/L. To our knowledge, no data are available for the effects of the

metabolites on fishes. However, Velisek et al. (2018a) recently reported no mortality of embryo-larval stages of crayfish exposed for 45 days up to 420 µg/L of MOA. Similarly, as for mortality, no effect was found on the length of larvae, or the hatching success of fish embryos exposed to tested substances and the mixture. This finding is in concordance with 100 times higher LOEC (29.0 mg/L) of S-metolachlor for zebrafish embryos (96 hpf) for hatching success established by Quintaneiro et al. (2017). Thus, as it was expected, low environmentally relevant concentrations used in this study elicited no significant effects on traditional apical endpoints such as mortality, hatching success and length of zebrafish larvae, and so other sublethal endpoints were further investigated.

4.2. Effects on behavior

Use of zebrafish larvae in behavioral studies has multiple advantages for the evaluation of neurotoxicity of pesticides, as discussed recently for example by Pittman (2017) and Legradi et al. (2018). To our knowledge, this is the first study investigating the effects of S-metolachlor and its metabolites on fish behavior, although some studies suggested S-metolachlor effects on neurobehavior. Quintaneiro et al. (2017) exposed zebrafish embryos for 4 days to high concentration of S-metolachlor (25 mg/L), and observed inhibited acetylcholinesterase activity. Villa et al. (2018) described decreased speed and distance travelled by *Chironomous* larvae upon metolachlor exposures (27.4 mg/L). Cook and Moore (2008) found that sublethal concentration of metolachlor (80 µg/L) altered the fighting behavior of crayfish. On the contrary, available study with MOA revealed no effect on behavior of crayfish (activity, distance, speed) exposed up to 420 µg/L (Velisek et al., 2018). Moreover, some pesticides of the same chemical class of chloroacetanilides were also reported to influence fish locomotion. Pretilachlor (1 mg/L) decreased feeding attempts and increased burst swimming reactions and buccal movements in adult fish *Clarias batrachus* (Soni and Verma, 2018). Acetochlor at 5 mg/L decreased spontaneous tail movements in zebrafish embryos (24 hpf), as well as total distance travelled, average speed and time of movement of larvae 6 dpf (Wang et al., 2019).

In the present study, S-metolachlor decreased spontaneous tail movements in zebrafish embryos, although this observation was recorded only in the lowest concentration (1 µg/L). Corresponding decrease was also observed after exposure to the lower concentration of mixture (all three compounds, 1 µg/L each). The observed effects on spontaneous movements may be caused by S-metolachlor interfering with maternal T4 stock in yolk sac or its signaling via an αVβ3 integrin, as shown by Yonkers and Ribera (2008). The thyroid follicles start to secrete T4 hormone at 72 hpf (Porazzi et al., 2009), and nongenomic mechanism with maternal T4 influencing the neurodevelopment of zebrafish was previously reported (Yonkers and Ribera, 2008).

The mixture effect on the spontaneous movements was most likely due to the CA effect, similarly as for the developmental abnormalities. Decreased tail movements were caused by S-metolachlor and the mixture. Because the effects were not enhanced or neutralized in the mixture, S-metolachlor is mostly likely the driver of the observed effect. On the contrary, our light:dark locomotion test did not reveal any abnormal behavior. High biological variability in larvae movement could be linked to a relatively wide time frame within the day (9 a.m. – 4 pm), when the measurements were carried out. This was discussed by some authors with recommendation of using more specific time frames like 11 a.m. – 3 pm (Colwill and Creton, 2011) or 1 pm – 3:30 pm (MacPhail et al., 2009).

4.3. Effects on gene expression

Exposures to the metabolites appeared to have more pronounced effects on zebrafish larvae than the exposure to the parent compound. The highest tested concentration (100 µg/L) of MOA caused overexpression of genes *dio2, thrb* and *p53*. MESA induced overexpression of *dio2, thra* and *thrb* in fish exposed to low 1 µg/L, and *thra* and *p53* in fish exposed to 30 µg/L. The induction of *p53* indicates activation of apoptotic processes or cell cycle arrest to protect the cells from additional detrimental effects (Ko and Prives, 1996). Genes *dio2, thra, thrb* were proposed by Jin et al. (2011) as suitable biomarkers for thyroid system disruption in fish. Spaan et al. (2019) described them as important genes (together with other deiodinases *dio1* and *dio3* and genes *hhex* and *NIS*) in the HPT axis. Deiodinase 2 is a selenoprotein that catalyzes the transformation of T4 to T3, thyroid hormones regulating neurodevelopment among other processes. It is the most important deiodinase in zebrafish embryonic development, as it controls the quantity of T3 hormone in the tissues (Walpita et al., 2009). Furthermore, deiodinases were found to affect the eye development of zebrafish (Houbrechts et al., 2016), and the overexpression of *dio2* has been linked with hypothyroidism (Orozco and Valverde-R, 2005). Deiodinases and thyroid hormone disruption are also largely discussed in the two already mentioned AOP pathways (Stinckens et al., 2018). Thyroid hormone receptors α and β act as transcription factors for thyroid hormones. According to Walter et al. (2019), mRNA levels of deiodinases and thyroid hormone receptors vary throughout the zebrafish development, with expression peak of *dio2* and *thrb* around 72-96 hpf and following decrease at 120 hpf. Expression of *thra* is stable from 24 hpf to 120 hpf. Furthermore, as described by Porazzi et al. (2009) and Raldúa et al. (2012), the thyroid system in zebrafish is fully developed at 5 dpf.

Although MESA and MOA affected expressions of several genes, no changes were observed after exposure to S-metolachlor. Contradictory observations for S-metolachlor were found by Jin et al. (2011), where 10 µg/L caused overexpression of *dio2*, *thra* and *thrb* in juvenile medaka (*Oryzias latipes*), only in females. These differences may be related to multiple factors such as exposure duration (14 days in Jin et al. (2011) in comparison to 5 days in our study), or use of racemic mixture of metolachlor by Jin et al. (2011), which has been found more toxic to non-target organisms than pure S-metolachlor (Liu et al., 2006; Ye et al., 2010). Use of different fish species in different life stage (1-month-old juveniles used by Jin et al. (2011) in comparison with embryos in the present study) is also of great relevance; on top of that, differences in sensitivity of medaka and zebrafish have already been documented (Perrichon et al., 2014; Vignet et al., 2019).

The mixture of S-metolachlor and its two metabolites appeared to be the most hazardous, and the low environmental concentration (1 µg/L of each substance) altered transcription of genes *p53, dio2*, *thra, thrb* and *cyp26a1*. Gene *cyp26a1,* encoding enzyme degrading retinoic acid, is very important in embryogenesis. Its overexpression can disturb the retinoic acid signaling pathway, since precise RA concentration is crucial in forming of e.g. body axis, craniofacial development, bone development or nervous system development (Dubey et al., 2018; Duester, 2008). Interference may lead to malformations (Hu et al., 2008) or hinder organ development especially early brain development (Emoto et al., 2005). However, the effects on gene expression observed at low concentration of the mixture were not confirmed at the high concentration. This mixture “low-dose effects” mechanism has been discussed in the field of the thyroid, and generally endocrine, system disruption, where non-monotonic responses and U-shaped dose-response curves have been documented (Melnick et al., 2002; Vandenberg et al., 2012).

The most well-known pesticides for exerting thyroid dysfunction are organochlorine pesticides (Calsolaro et al., 2017). However, several chloroacetanilides have also been shown to disrupt the thyroid system including the above-discussed metolachlor in adult and juvenile medaka (Jin et al., 2011), acetochlor (Jiang et al., 2015; Yang et al., 2016) and pretilachlor (Jiang et al., 2016) in zebrafish embryos, acetochlor in larval rare minnow (Li et al., 2009), or butachlor in adult rare minnow (Zhu et al., 2014). Similarly, as in our work, the above-mentioned studies documented changes in gene expressions of deiodinases and

thyroid nuclear receptors and used concentrations below hundreds of micrograms per liter. Comparatively, Li et al. (2009) and Yang et al., (2016) also mentioned a non-sigmoidal dose response curve.

The main goal of the present study was to evaluate the impact of S-metolachlor, its metabolites and their mixture at low environmentally relevant concentrations. Some patterns of the interaction mechanisms in the mixture could be distinguished. First, CA is likely to be a mechanism behind the inductions of *thra*, *thrb* and *dio2* at low concentration levels, where MESA was the main driver of toxicity. At higher concentration (30 µg/L of each chemical), weak antagonistic interaction on *p53* and *thra* expressions could be observed as MESA alone (30 µg/L) induced both genes but this effect was suppressed in the MIX sample at the same concentration level. However, natural variability cannot be excluded as well because the difference between the effects of MIX and MESA on the *thra* and *p53* expression was not significant $P = 0.054$ and $P = 0.0165$ respectively. On the other hand, the studied chemicals at low environmentally relevant concentrations 1 µg/L seem to exert a low dose synergistic effect on the *cyp26a1* and *p53* expression - only the MIX (1 µg/L of each chemical) significantly induced expression of these genes, while no effects were observed at S-metolachlor, MOA or MESA when tested individually at 1 µg/L.

No changes were observed in the expression of the next three genes: *12S*, *sod1* and *cat*. Gene *12S* is associated with mitochondrial metabolism thus suggesting no effect on metabolic homeostasis (Arini et al., 2015). *Sod1* and *cat* are important genes managing some parts of the chain of the reactive oxygen species (ROS) in cell, scavenging respectively the superoxide radicals and hydrogen peroxide (Seifried et al., 2007). From these findings it can be concluded that S-metolachlor, its two metabolites and their mixture did not induce any oxidative stress detectable by *sod1* and *cat* in the zebrafish embryos. Quintaneiro et al. (2017) did not found either an effect of S-metolachlor on the catalase activity in zebrafish embryos exposed up to 25 mg/L. In contrast, MOA seems to have an inhibiting effect on catalase activity of a water crustacean: crayfish exposed to low concentrations (4.2 µg/L) (Velisek et al., 2018).

5. Conclusion

Our work brings important findings about the effects of environmentally relevant mixture of one of the most used herbicides S-metolachlor and its two metabolites on early life stages of zebrafish. We have observed specific malformations that could be linked to thyroid disruption as well as decreased spontaneous movements of zebrafish larvae. Interestingly, metabolites were more harmful than S-metolachlor to zebrafish larvae at the level of gene expression with apparently strong effects on genes involved in cell cycle regulation and thyroid-related signaling. Concentration addition effects in mixture were observed for the majority of endpoints. Further detailed analyses are needed to fully elucidate the dose-response relationship. The present study brings new information highlighting the importance of metabolites and pesticides mixtures, as emphasized in the European Environmental Action Programme (European Parliament and Council, 2013).

Compliance with ethical standards

This work was done in compliance with the Publishing Ethics policy of Elsevier. The experimental work itself and the fish husbandry were carried out in accordance with the EU Directive 2010/63/EU. Fish embryos until 120 hpf, i.e. no independent feeding, were used.

CRediT authorship contribution statement

Eliška Rozmánková: Conceptualization, Investigation, Validation, Data curation, Formal analysis, Writing - original draft. **Marek Pípal:** Investigation, Methodology, Writing - review & editing. **Lucie Bláhová:** Investigation, Methodology, Validation, Writing - review & editing. **Naveen Njattuvetty Chandran:** Investigation. **Bénédicte Morin:** Conceptualization, Writing - review & editing. **Patrice Gonzalez:** Conceptualization, Writing - review & editing. **Luděk Bláha:** Supervision, Funding acquisition, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aquatox.2020.105444.

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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.

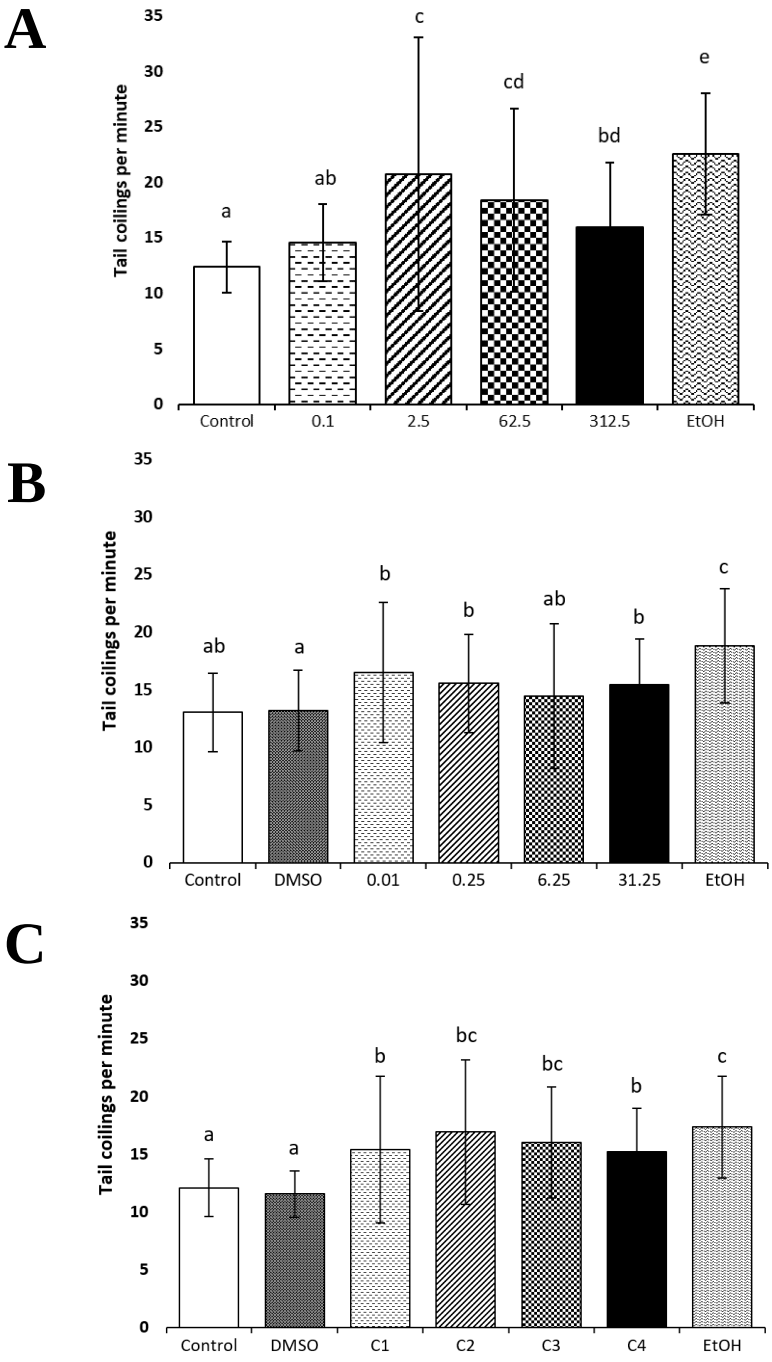

Figure 1 Frequency of tail coilings per minute measured in 22-23 hpf old zebrafish embryos exposed to imidacloprid (A), propiconazole (B), and the mixture (C) composed of the two mentioned compounds completed with S-metolachlor, metolachlor oxanilic acid, and metolachlor ethanesulfonic acid. Ethanol (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).

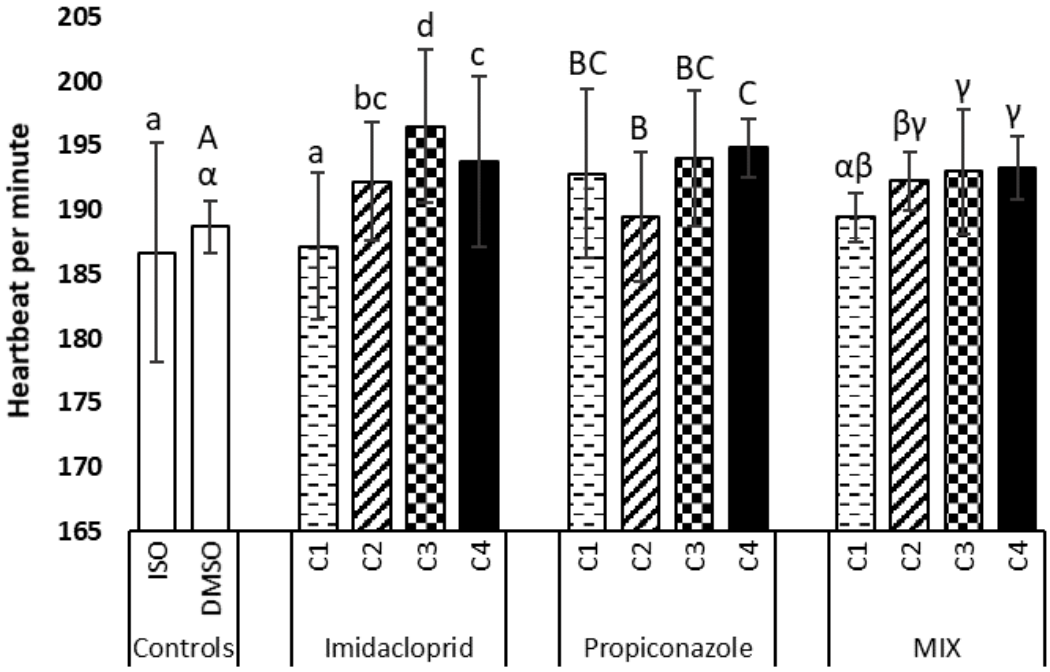

Figure 2 Heartbeat per minute in 3 dpf zebrafish larvae exposed to imidacloprid, propiconazole, and the mixture composed of the two mentioned completed with S-metolachlor, metolachlor oxanilic acid, and metolachlor ethanesulfonic acid. N=124-155 larvae per experimental variant.

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).

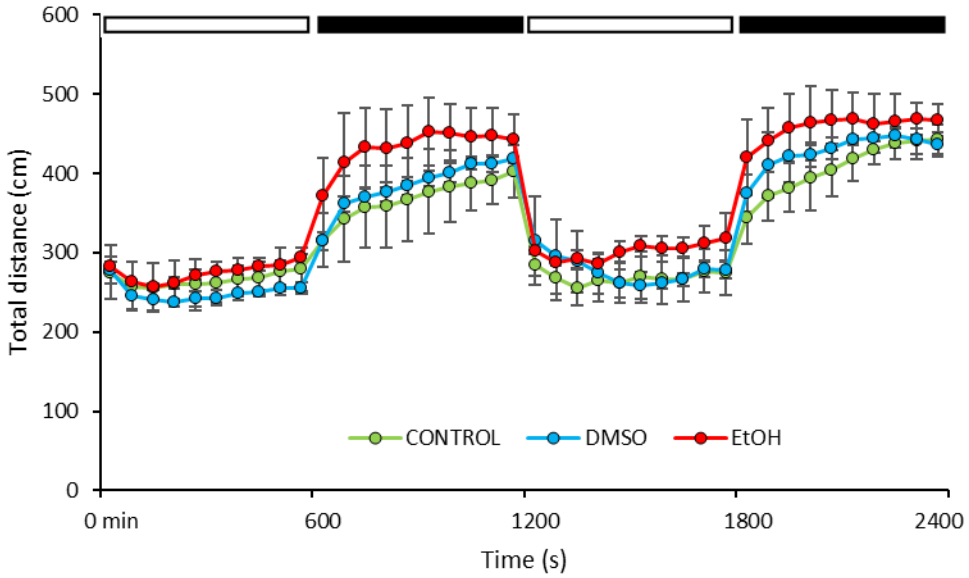

Figure 3 Average of 3 independent repetitions of the sum of total distance swam by each larva per min. N=24 per condition. Larvae were non-exposed (negative control in ISO medium, or solvent control with DMSO) and exposed to a 0.5% solution of ethanol as a positive control.

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.

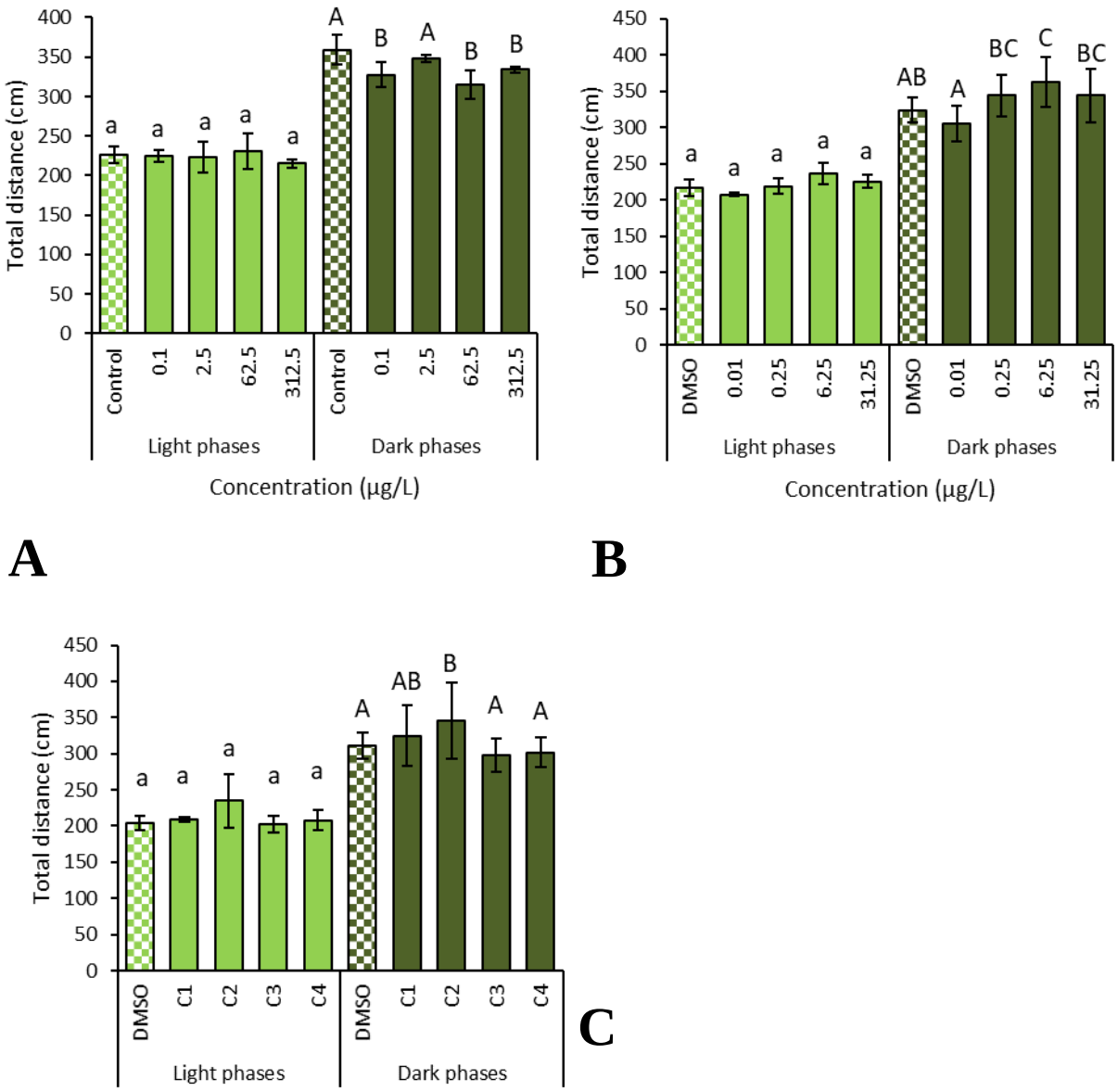

Figure 4 Total distance (cm) swam by one zebrafish larva during 30 min of both light or both dark phases after 5 days of exposure to imidacloprid (A), propiconazole (B), and the mixture (C) and subjected to light, dark transition locomotion test. N=57 larvae per condition of 3 pooled independent repetitions.

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

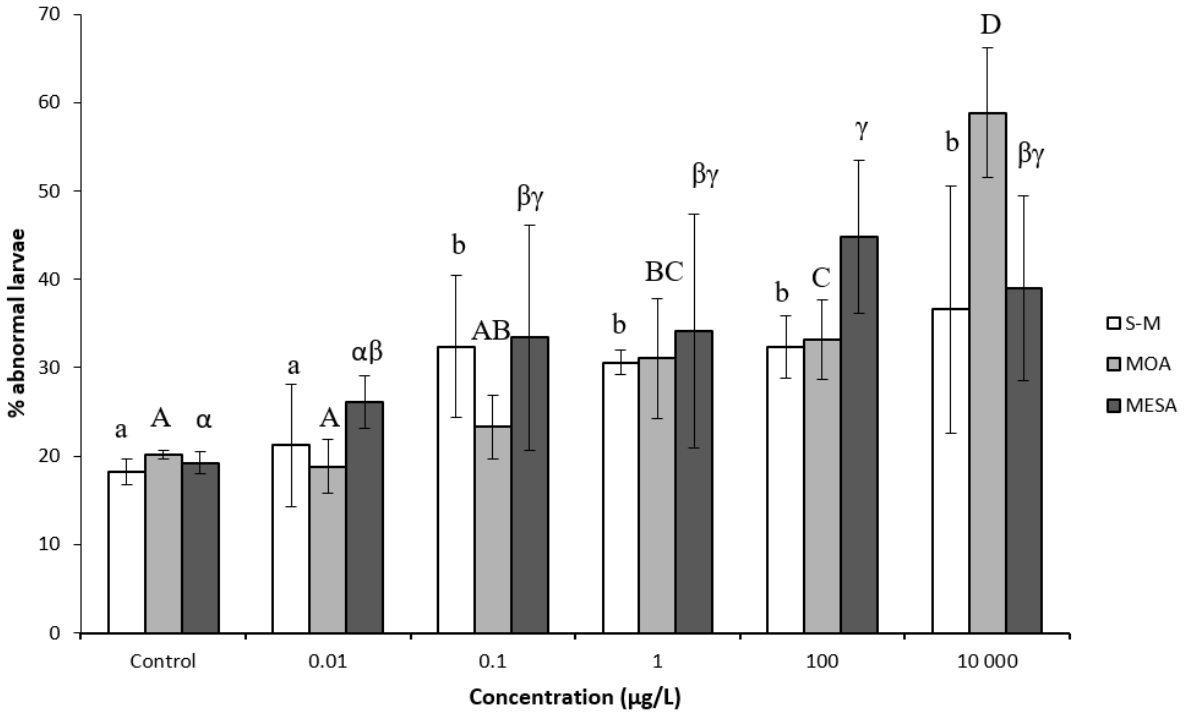
The analysis was performed with a Waters LC chromatograph (Waters, Manchester, U.K.) and the chromatographic separation was achieved using a column Acquity BEH C18 of 100 x 2.1 mm ID and 1.7 μm particle size. A gradient elution method was set with phase A (0.1 % formic acid in water) and phase B (0.1 % 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.

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Acute and (sub)chronic toxicity of the neonicotinoid imidacloprid on *Chironomus riparius*

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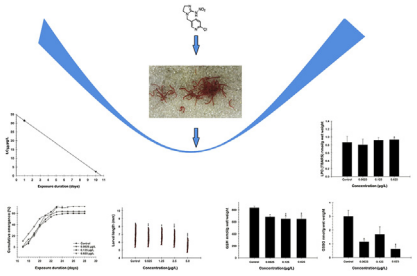
HIGHLIGHTS

- Detailed assessment of acute and chronic toxicity in *Chironomus riparius.*
- Imidacloprid inhibited larval development and affected emergence.
- Detected oxidative stress and effects on reduced and oxidized glutathione.

GRAPHICAL ABSTRACT



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ABSTRACT

Impacts of neonicotinoids on non-target insects, including aquatic species, may significantly influence ecosystem structure and functioning. The present study investigated the sensitivity of *Chironomus riparius* to imidacloprid exposures during 24-h, 10- and 28-days by assessing larval survival, growth, emergence and oxidative stress-related parameters. *C. riparius* exhibited high sensitivity compared to other model aquatic species with acute 24-h LC_{50} being 31.5 μg/L and 10-days LOEC (growth) 0.625 μg/L. A 28-days partial life cycle test demonstrated imidacloprid effects on the emergence of *C. riparius*. Exposure to sublethal concentrations during 10-days caused an imbalance in the reduced and oxidized glutathione (GSH and GSSG), and slightly induced lipid peroxidation (increased malondialdehyde, MDA). Our results indicate that oxidative stress may be a relevant mechanism in the neonicotinoid toxicity, reflected in the insect development and life cycle parameters.

1. Introduction

Imidacloprid, N-[1-[(6-chloropyridin-3-yl)methyl]-4,5-dihydroimidazol-2-yl]nitramide is a neurotoxic insecticide of the neonicotinoid family class (Kagabu, 2010). Since its introduction to the market in 1992, the use of imidacloprid has increased yearly and is now ranked one of the best-selling insecticides worldwide (Jeschke and Nauen, 2008; Simon-Delso et al., 2015). Imidacloprid has an outstanding toxic potency to the piercing and sucking pests, soil insects, termites, and some chewing insects on crops (Jeschke and Nauen, 2008; Tomizawa and Casida, 2005). Use of imidacloprid and other neonicotinoids has come under inspection during

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past years, because of concerns related to colony collapse disorder in honey bees, decline of other pollinators and broader effects on ecosystem functioning (Fairbrother et al., 2014; Van der Sluijs et al., 2013, 2015; Whitehorn et al., 2012). Several countries have banned the use of imidacloprid (or other neonicotinoids) on the crops attractive to bees (European Union, 2013; Simon-Delso et al., 2015; Van der Sluijs et al., 2013). Currently, European Commission and US Environmental Protection Agency are re-evaluating the safety of imidacloprid and other neonicotinoids; and are providing an opportunity to examine the impact of imidacloprid on nontarget animals in the aquatic system (USEPA, 2015; http://europa.eu/rapid/press-release_MEX-18-3583_en.htm). Regulatory guidelines have proposed acceptable imidacloprid concentrations in surface water for the protection of aquatic organisms, ranging between 0.0083 (Smit, 2014), 0.23 (CCME, 2007), and 1.05 μg/L (USEPA, 2014). The low octanol-water partitioning coefficient (Log Kow = 0.57 at 20 °C), high water solubility (0.61 g/L at 20 °C) and low soil adsorption coefficient (Log Koc = 2.19–2.90) promote the movement of imidacloprid to water bodies through surface runoff and leaching (Morrissey et al., 2015; Sanchez-Bayo et al., 2013). Additionally, it may enter water bodies from spray drift or accidental spills. As a consequence, elevated concentrations of imidacloprid may pose a risk to aquatic biota (Sánchez-Bayo et al., 2016). Recent reports indicate a decline of non-target animal species in imidacloprid contaminated surface waters and demonstrate cascading effects in ecosystems (Hallmann et al., 2014; Sánchez-Bayo, 2011; Sánchez-Bayo et al., 2016). Experimental evidence from mesocosm studies has shown detrimental effects of imidacloprid on ostracods, mayflies, snails, dragonflies, damselflies, chironomids, caddisflies and stoneflies (Daam et al., 2013; Pestana et al., 2009b; Sánchez-Bayo et al., 2007, 2016; Van Dijk et al., 2013). Consequently, vertebrates that depend on aquatic invertebrates as their primary food resource may also be affected as suggested, e.g. by a reported correlation between declines of the local bird populations in the Netherlands and elevated imidacloprid contamination in surface water (Hallmann et al., 2014).

Imidacloprid binds irreversibly to the nicotinic acetylcholine receptors (nAChRs) in the insect nervous system, and their activation ultimately leads to the death of the neuron (Casida and Durkin, 2013). Imidacloprid toxicity increases upon the molecule's cumulative binding to nAChRs, and the toxic effects can be reinforced even at low-dose exposure over extended periods of time (Tennekes and Sanchez-Bayo, 2011). Interestingly, most of the research concerning this insecticide was focused on acute toxicity studies, while the effects of low-dose and chronic exposure are less explored (Morrissey et al., 2015). According to the ECOTOX database of the US Environmental Protection Agency, aquatic insects are the most sensitive species to imidacloprid, followed by mussels, tubificid worms, shrimps, ostracods, amphipod crustaceans and fish (ECOTOX database https://cfpub.epa.gov/ecotox/). Among aquatic model organisms, chironomids have extensively been used in the acute and chronic toxicity tests with insecticides (Gourmelon and Ahtiainen, 2007; Taenzler et al., 2007). Chironomids are widely distributed and abundant macroinvertebrates, they play a significant role in detritus consumption and serve as a food source for birds and fish (Armitage, 1995). Likewise, chironomids have a short life cycle, and their larval stages live in close contact with sediments (Armitage, 1995).

Many agrochemicals including neonicotinoids induce oxidative stress (Ge et al., 2015; Özdemir et al., 2017; Qi et al., 2018; Vieira et al., 2018; Wang et al., 2016). Reactive oxygen species (ROS) cause damage to biological molecules like DNA, proteins and membrane lipids through lipid peroxidation (LPO), and few recent studies with invertebrates suggested the importance of LPO in neonicotinoid toxicity (Saraiva et al., 2017; Wang et al., 2016). To prevent damage caused by ROS, multiple scavenging and antioxidant mechanisms exist in biological systems. Among these, glutathione (GSH) plays a central role, and participates not only in detoxification but also in other cellular functions including modulation of the neurotransmitter receptors activity (Oja et al., 2000). Mitochondria are the primary intracellular source of ROS, and energy demanding tissues such as nervous system, heart and others, are especially prone to oxidative damage. Few studies related imidacloprid with inflammation and oxidative damage in nervous system in mammals (Duzguner and Erdogan, 2010, 2012) and, new evidences show imidacloprid-induced oxidative stress also in nontarget invertebrates such as *Daphnia magna* (Qi et al., 2018) or earthworm *Eisenia fetida* (Wang et al., 2016). However, understanding the role of oxidative stress in neonicotinoid-induced effects is far from being complete, and to our knowledge, no attempts have been made to evaluate oxidative stress markers in *Chironomus riparius* following the sublethal exposures to imidacloprid.

The objective of the present study was to compare lethal and sub-lethal effects of imidacloprid in *C. riparius* focusing on mortality, growth, and development as organismal endpoints. Also, we evaluated the impact of subchronic exposure of imidacloprid on biochemical markers of toxicity including the levels of lipid peroxidation (LPO) and concentrations of reduced and oxidized glutathione (GSH, GSSG). The present study provides further evidence for a realistic assessment of the potential effects of imidacloprid in aquatic ecosystems.

2. Materials and methods

2.1. Chironomus riparius

Stock cultures of *C. riparius* were obtained from in-house laboratory cultures established at the Research Centre for Toxic Compounds in the Environment, Masaryk University, Brno, Czech Republic. The culture was maintained in aquaria containing fine quartz sand and dechlorinated tap water under constant aeration. The culture was fed two times a week with a suspension of finely ground fish flakes TetraMin® (Tetra werke, Melle, Germany). The egg masses, which were attached to the aquarium wall, were collected every morning and used for experiments. Stockbreeding and all experiments were conducted in a climatized chamber at 20.0 ± 0.5 °C, with a 16:8 h Light:Dark photoperiod.

2.2. Imidacloprid

Imidacloprid (99%; Analytical grade, CAS No. 138261-41-3) was purchased from Sigma-Aldrich (Germany). A stock solution (100 μg/mL) was prepared by diluting imidacloprid in reverse osmosis water (Barnstead DiamondTM NANOpure, 18.2 MV/cm). The prepared stock solutions were then diluted to the final concentrations in the standard test medium. A freshly prepared stock solution of imidacloprid was used in every dosing experiment.

2.3. Water quality

Routine physicochemical parameters of water like pH, temperature, conductivity and dissolved oxygen were measured during each exposure experiment using multimeter Multi9420 (WTW GmbH, Weilheim, Germany). During subchronic and chronic test, water changes were conducted on every third day to maintain constant test concentrations of imidacloprid. Water quality was analysed before and after in the acute test. In the subchronic and chronic analysis, water quality parameters were examined before and after each partial water change.

2.4. Imidacloprid analysis

Imidacloprid concentrations in test media during the experiments were verified using LC-MS/MS analysis. Collected water samples were transferred into 2 mL amber glass vials containing 200 μL of glass insert and stored at −20 °C until examined. Analysis was performed with a Waters LC chromatograph (Waters, Manchester, U.K.) consisting of a vacuum degasser, a binary pump, a temperature controlled autosampler, and a column compartment. The chromatographic separation was achieved using a column Acquity BEH C18 of 100 × 2.1 mm ID and 1.7 μm particle size. A gradient elution method was set with a phase A (0.1% formic acid in water) and phase B (methanol). The gradient elution started with 20% B, increased to 90% B over 9 min, held (90% B) for 11 min, then decreased to 20% B in 11 min 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 at 5 μL. The column and sample temperatures were set at 35 and 10 °C, respectively.

Detection was performed on a Xevo TQ-S quadrupole mass spectrometer (Waters Manchester, U.K.) equipped with electrospray ionisation (ESI) source. Analytes after ESI ionisation were detected in positive ion mode using tandem mass spectrometry with multiple reaction monitoring (MRM) mode. The following *m/z* transitions of imidacloprid were monitored: *m/z* 256.1 > 175.0 ([M + H−NO2−Cl]+, quant.: cone voltage 25 V; collision energy 20 V) and *m/z* 256.1 > 209.0 ([M + H−NO2]+; qual.: cone voltage 25 V; collision energy 12 V). The average ratio of quantifier (*m/z* 175.0) ions to the qualifier (*m/z* 209.0) was 1.3. Internal standard imidacloprid-D_4 was detected by transitions: *m/z* 260.1 > 213.0 (collision energy 12 V). The dwell time and capillary voltage were set at 0.033 s and 3 kV, respectively. 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 and 400 °C, respectively. During LC-MS/MS analysis, internal standard (imidacloprid-D_4) was directly spiked into each sample. Data were processed by MassLynxTM software (Manchester, U.K.). The concentration of imidacloprid in calibration standards and samples was determined by correcting the response of imidacloprid in the internal standard (imidacloprid-D_4). The limit of detection (LOD, signal to noise ratio S/N > 3) and quantification (LOQ, S/N > 10) were 0.02 and 0.05 μg/L, respectively.

2.5. Acute 24-h toxicity test

Acute toxicity was assessed following the OECD guideline 235 (OECD, 2011). Less than 24-h old larvae, 100 mL glass beakers (Height: 7 cm, inner diameter: 4.5 cm) and 10 mL of appropriate volume (2 mL per each larva) of test solution (dechlorinated water) were used. Determination of test concentrations, larvae selection, and feeding procedure were carried out as recommended by OECD guideline 235 (OECD, 2011).

A 24-h static acute toxicity test was initiated by introducing groups of five larvae into glass beakers containing the imidacloprid test solutions (0.625, 1.25, 2.5, 5.0, 10, 20, 40 and 80 μg/L) and the control. All test vessels were sealed with the loosely covered glass lid to avoid evaporation and entry of dust into the test solutions. The controls and all treatments were run in five replicates. The replicates were randomly placed in a culture chamber at 20.0 ± 0.5 °C, with a 16:8 h Light:Dark photoperiod. After 24-h, mortality was determined by mechanical stimulation with animals failing to show any response considered dead. This was confirmed under a dissecting microscope. Decomposed and missing larvae were counted as dead. The experiment was performed 3-times independently.

2.6. Subchronic 10-day and partial life cycle 28-day tests

To determine the effects of imidacloprid on *C. riparius* growth and emergence, a 10-day subchronic and 28-day partial life-cycle tests were performed according to OECD guideline 219 (OECD, 2004). The test was conducted in 600 mL glass beakers (height 15 cm, inner diameter 8 cm). Each test vessel bottom was covered with a 1.5 cm layers of fine inorganic sediment (>1 mm silica sand), and 380 mL of the test solutions. Three days old first instar larvae were used as test animals and exposure period was continued up to 10 days. Throughout the test period, test solutions were continuously aerated to maintain adequate oxygen saturation (>80%). A 75% (285 mL) of test solution was changed every third day to assure constant test concentrations of imidacloprid. Determination of test concentrations, larvae selection, feeding, and aeration procedure was carried out as recommended by OECD guideline 219 (OECD, 2004). The control and all treatments were run in five replicates. The replicates were randomly placed in a culture chamber at 20.0 ± 0.5 °C, with a 16:8 h Light:Dark photoperiod. All test vessels were loosely covered with the glass lid to avoid evaporation and entry of the dust into the test solutions.

The growth rate of *C. riparius* was analysed using 10-day subchronic test. The test was initiated by introducing a group of 20 larvae into beakers containing imidacloprid (0.625, 1.25, 2.5, 5.0, and 10 μg/L; concentration range set based on the results of the acute toxicity test). On the day 10, survived chironomids larvae were collected from the sediment, counted and preserved in 70% ethanol. The total larval length (mm) was measured under a stereoscopic microscope with an ocular micrometer. Similarly to the acute toxicity test, criteria for death were immobility or lack of reaction to a mechanical stimulus, confirmed under a dissecting microscope. Larvae not recovered were counted as dead.

A 28-day partial life-cycle test was used to assess larval development to adult midge. The partial life cycle test was initiated by transferring groups of 20 individuals of three days old first instar larvae into glass beakers containing three imidacloprid treatment solutions (0.0625, 0.125, and 0.625 μg/L; concentration range set based on the results of the subchronic toxicity test). All test vessels were covered with the emergent trap to avoid unwanted escape of newly emerged adults. Emerging adults were collected daily from emergent traps with the aid of an aspirator and preserved in 70% ethanol for the assessment of following parameters such as cumulative emergence, emergence time, male (♂) and female (♀) population density, and sex ratio. Animals were considered to have emerged successfully when the adult completely dissociated from its pupal exuvia and exited the water (Benoit et al., 1997).

2.7. Subchronic exposures for biomarker determination

The test design for the biomarker determination was the same as that for the subchronic 10-days tests (OECD, 2004). Twenty individuals of three days old first instar larvae were introduced into glass beakers containing imidacloprid (0.0625, 0.125, and 0.625 μg/L; same concentrations as used in the 28-day life cycle test). All control and treatments were run at least six replicates. After 10-days exposure larvae were collected, quickly dried on a filter paper, weighed, and stored at −80 °C until further analyses of biomarkers.

2.8. GSH and GSSG assay

The GSH and GSSG were extracted as described previously (Blahova et al., 2014). Briefly, an aliquot of the frozen tissue (10 mg fresh weight) from replicates of each treatment was homogenised in an ice-cold solution (1:10 w/v) of potassium chloride (1.2% w/v)

and EDTA (0.03% w/v). A 20 μL of homogenised samples were mixed with 10 μL of the internal standard solution of glutathione glycine $^{13}C_2$,^{15}N (40 μg/mL), and 300 μL of cold 0.1 M Na_2HPO_4/KH_2PO_4 buffer (pH 8). Before the analysis, the samples were derivatised by mixing with 10 μL of 5,5′-dithiobis (2-nitrobenzoic acid) solution (20 mM in methanol), vortexed briefly and incubated at room temperature for 15 min. The reaction was stopped by the addition of 60 μL of the cold sulfosalicylic acid solution (20% w/v), proteins were then precipitated by incubating for 25 min at 4 °C and then removed by centrifugation at 20,800 × g for 15 min at 4 °C. The supernatant was immediately transferred to a glass vial insert and stored at −80 °C until analyses. The GSH and GSSG were analysed by LC-MS/MS using Waters Acquity LC chromatograph (Waters, Manchester, U.K.) and Xevo TQ-S quadrupole mass spectrometer (Waters Manchester, U.K.). During LC-MS/MS analysis, second internal standard (glutathione disulfide-$^{13}C_4^{15}N_2$) was directly spiked into each sample. Data were processed by MassLynxTM software (Manchester, U.K.). Concentrations of GSH and GSSG were determined by correcting their responses in the mass detector for the response of internal standards. Results were reported as nmol GSH or nmol GSSG per gram of wet tissue.

2.9. Lipid peroxidation

Lipid peroxidation was determined by the quantification of thiobarbituric acid reactive substances (TBARs) for example malondialdehyde (MDA), a specific end-product of the oxidative degradation of lipids. Thiobarbituric acid (TBA) was used as the reactive substance to quantify the MDA content. Chironomus individuals (wet mass 20 mg) from the replicates of each treatment were homogenised in cold phosphate buffer solution (1:10 w/v) (13.69 mM NaCl, 2.68 mM KCl, 8.10 mM Na_2HPO_4. $12H_2O$, and 1.47 mM KH_2PO_4; pH 7.2). Homogenate of 250 μL was mixed with 75 μL of 20% trichloroacetic acid, vortexed briefly and centrifuged at 4000 × g for 20 min at 4 °C. The clear supernatant was used to prepare TBARs conjugate using the method of Bastos et al. (2012) with slight modification. A volume of 250 μL of the samples was added to the 50 μL of 0.02% butylated hydroxytoluene (BHT) and 200 μL of 1.44% thiobarbituric acid (TBA). The mixture was vortexed briefly and incubated at 90 °C for 45 min. Following the conjugation, the TBARs complex was extracted with 200 μL of n-butanol, and the upper organic layer was used for HPLC-DAD analysis. Standard MDA solution was prepared by the acid hydrolysis of 1,1,3,3-tetraethoxypropane (Bastos et al., 2012), and the standard solutions were processed simultaneously with the sample using the same protocol as for the samples. Determinations of TBARs were performed on an Agilent 1100 Series Liquid Chromatograph equipped with Diode Array Detector (DAD). Chromatographic separation and detection of MDA conjugate were performed following a protocol adapted from Lebedová et al. (2016). Briefly, the separations of analyte were performed on a 250 × 4.6 mm C18 SUPELCOSIL LC-ABZ column (particle size 5 μm) using the mobile phase A (50 mM phosphate buffer, pH 7) and B (methanol) by isocratic elution (25% of B). The flow rate and temperature were set at 1 mL/min and 30 °C, respectively. The Diode array detector was set at 532 nm for detection of the MDA (TBARs) obtained from the reaction and chromatogram was evaluated by HP Agilent Chemstation software 1100. The TBARs concentration was determined from the external calibration curve, and results were reported as nmol TBARs per gram of wet tissue.

2.10. Data analysis

Statistical analysis was conducted using SPSS version 19.0. (SPSS Inc., Chicago, Illinois, USA). Lethal concentration (LC) values and effective concentration values (EC) were estimated by the Finney's probit analysis model (Finney, 1947) with PoloPlus software version 2.0 (LeOra Software, California, United States) and MedCalc software version 17.9.7 (MedCalc Software, Ostend, Belgium) respectively. Life history traits and biochemical data were analysed by analysis of variance (ANOVA) with multiple comparisons examined by Dunnett's post hoc test. Whenever data had non-symmetric distribution, Kruskal-Wallis ANOVA was performed, followed by Dunn's method. Chi-square test was used to assess the significance of sex ratio. Spearman correlation analysis was performed to compare times to effect versus LC_{50}. All p-values are two-sided unless otherwise indicated. One-sided p-values were used in cases where the means or correlations were anticipated to follow a prechosen trend. P-values less than 0.05 were considered significant.

3. Results

3.1. Water parameters

Supplementary materials, Tables S1-S4, list the results of the physicochemical measurements of water during experiments. In the acute and (sub)chronic tests, the mean pH, temperature, and dissolved oxygen did not significantly change over the experimental period. The conductivity has been reduced during the exposures by 7–13% and 7–24% for the acute and (sub)chronic test, respectively.

3.2. Imidacloprid exposure

Results of the analytical measurement of imidacloprid concentrations for the acute and chronic tests are presented in supplemental data (Tables S5–S7). All measured concentrations in negative control were below the LOD (<0.02 μg/L). During the acute test, most of the imidacloprid concentrations were similar to the nominal values. In 10 and 28 days tests, all mean concentrations of imidacloprid ranged 80–120% (±20%) of nominal concentrations that were therefore used to express effective concentrations as recommended by OECD (OECD, 2004).

3.3. Acute lethal toxicity of imidacloprid

The results of the imidacloprid toxicity to *C. riparius* are summarised in Table 1. The calculated 24 h LC_{50} with 95% confidence intervals (CI) for one-day-old *C. riparius* was 31.5 μg/L (15.1–75.9 μg/L). Analysis of the acute toxicity data showed a monotonic increase of mortality with increasing concentration of imidacloprid ($F_{(7, 32)} = 6.86$; $p = 0.0001$). Comparison to the control treatment by Dunnett's test identified the no observed effect concentration (NOEC) being 5 μg/L imidacloprid. The lowest observed effect concentration (LOEC) was 10 μg/L imidacloprid, which resulted in a mortality of 40% (SE = ± 5.40). Observed survival in controls was 93% (SE = ± 4.52%), meeting thus the validity criteria for the acute test (OECD, 2011).

After ten days exposure to imidacloprid, *C. riparius* larval survival in controls was above 90%. The calculated 10-day LC_{50} (95% CI) was 2.33 μg/L (1.30–4.41 μg/L). Analysis of subchronic data showed a monotonic increase in the mortality with increasing concentration of imidacloprid ($F_{(5, 18)} = 65.91$; $p = 0.0001$). The NOEC was 0.625 μg/L imidacloprid, and the LOEC was 1.25 μg/L, which resulted in a mortality of 26.7% (SE = ± 8.81). Supplementary Fig. S1 shows the full concentration-response curves of imidacloprid during acute and subchronic 10-day exposures.

Table 1
Calculated lethal or effective concentration (LC or EC with 95% CI), no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) of imidacloprid in the experiments with *C. riparius*. All concentrations are in μg/L.

Test	NOEC*	LOEC*	LC_{10} or EC_{10} (CI 95%)	LC_{20} or EC_{20} (CI 95%)	LC_{25} or EC_{25} (CI 95%)	LC_{50} or EC_{50} (CI 95%)
Acute test for larval survival/mortality (24-h static)	5	10	1.62 (0.096–4.73)	4.48 (0.658–10.2)	6.6 (1.33–13.9)	31.5 (15.1–75.9)
Sub-chronic test larval survival/mortality (10-day renewal exposure)	0.625	1.25	0.83 (0.144–1.44)	1.18 (0.336–1.93)	1.35 (0.456–2.19)	2.33 (1.30–4.41)
Sub-chronic test for larval length (10-day renewal exposure)	<0.625	0.625	1.64 (1.38–1.95)	2.41 (2.03–2.87)	2.79 (2.34–3.32)	5.03 (4.23–6.00)
Partial life cycle test - EmT_{50} (pooled sex) (28-day renewal exposure)	0.125	0.625	Na	Na	Na	Na

* NOEC and LOEC were analysed by analysis of variance (ANOVA) with multiple comparisons of Dunnett's post hoc test ($p < 0.05$); Na: Not applicable.

3.4. Effects of imidacloprid on larval growth, adult emergence, and sex ratio

The sublethal endpoints of the larval development (total length) and emergence rate were assessed in the subchronic 10-day and chronic 28-day tests. A 10 days exposure to imidacloprid significantly affected larval growth ($F_{(4, 277)} = 11.3$; $p = 0.0001$). The total larval length was reduced by 26% upon exposure to 5 μg/L imidacloprid (Fig. 1). The calculated EC_{10} and LOEC for *C. riparius* were 1.64, and 0.625 μg/L of imidacloprid, respectively (Table 1). No animals survived on the day 10 at the highest concentration 10 μg/L, and the larval length could not be determined.

After the 28 days of exposure, emergence rate in the control treatment reached 74%, meeting the validity criteria according to the OECD guidelines (OECD, 2004). Non-significant >10% reduction in *C. riparius* emergence (survival) was observed in 0.125 and 0.625 μg/L imidacloprid treatments (Fig. 2a; Kruskal-Wallis test: $H = 3.736$, $df = 3$, $p = 0.291$). Emergence started on day 17 for all imidacloprid concentrations, whereas emergence of control animals started later on day eighteen. The mean emergence time (EmT_{50} - the time needed for 50% that had emerged successfully, as compared to negative controls) was significantly accelerated (LOEC) at 0.625 μg/L (pooled sex) (Table 1 and Fig. 2b; Kruskal-Wallis test: $H = 9.728$, $df = 1$, $p = 0.002$). Imidacloprid had no significant effect on ♂ and ♀ emergence times (Fig. 2c and d; Kruskal-Wallis test: for the EmT_{50} of ♂: $H = 6.81$, $df = 3$, $p = 0.078$; for the EmT_{50} of ♀: $H = 6.43$, $df = 3$, $p = 0.092$). The number of male emergences (Supplementary Table S10) was higher than the number of female ones (Supplementary Table S11) for all the treatments as well as controls. No significant imbalance in the ratio of male to female sex was observed across the imidacloprid exposures when compared to control (χ^2, all $p > 0.05$). Supplementary Tables S8-S14 list the results of the influence of imidacloprid on development and emergence of larvae *C. riparius*.

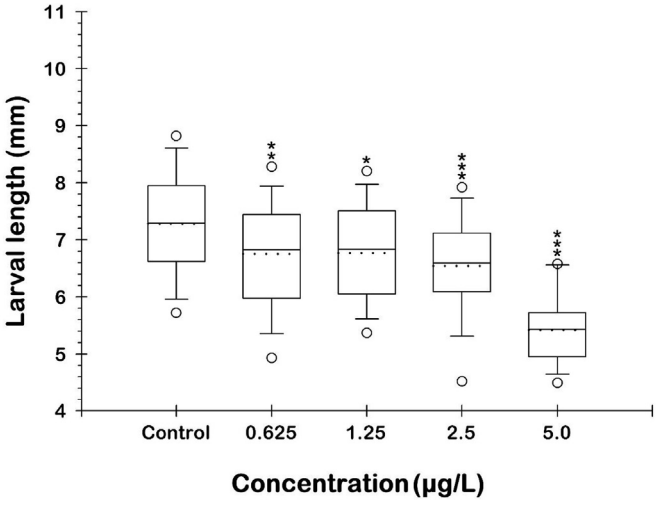

Fig. 1. Effect of different concentrations of imidacloprid on larval growth of C. riparius (total larval length, mm) after 10 days of exposure. The tops and bottoms of the rectangles indicate the 75th and 25th percentiles, respectively. The whiskers define the 90th and 10th percentiles, respectively. The open dots above and below the whiskers define the 95th and 5th percentiles, respectively. The horizontal lines and dotted lines within the boxes show the median and mean values, respectively. Asterisks indicate significant difference from the control (ANOVA with Dunnett's post hoc test, ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$).

3.5. Levels of GSH, GSSG and lipid peroxidation

After 10 days of exposure all imidacloprid-exposed groups exhibited significantly decreased levels of both reduced GSH ($F_{(3,23)} = 5.85$, $p = 0.004$) and oxidized GSSG ($F_{(3,23)} = 5.75$, $p = 0.004$) when compared to control group (Fig. 3a–b). Imidacloprid exposed groups had GSH levels between 77 and 82% of controls, whereas GSSG levels ranged between 21 and 54% of the control value. Because GSSG levels were more significantly affected, the GSH/GSSG ratio for the imidacloprid-exposed groups was higher than in controls (Fig. 3c; $F_{(3,23)} = 8.01$, $p = 0.001$). The LOEC for the reduction of GSH and GSSG was 0.0625 μg/L imidacloprid. Supplementary Table S15 lists the results of the imidacloprid effects on GSH and GSSG content. Lipid peroxidation (TBARs) slightly increased under exposure to imidacloprid (Fig. 3d; $n = 15$, Spearman's $\rho = -0.76$, $p = 0.0015$; LOEC = 0.625 μg/L) but the effect was only weak with probable minor biological significance at the concentration used. Supplementary Table S16 lists the results of the imidacloprid effects on TBARs content.

4. Discussion

In recent years, eco-toxicity and toxic mechanisms of neonicotinoids on nontarget organisms have attracted a lot of attention (Sánchez-Bayo et al., 2016; Van der Sluijs et al., 2013). Our study showed that imidacloprid is highly toxic to *C. riparius* causing lethality and modulating sublethal and biochemical endpoints at low concentrations.

The 24-h LC_{50} of 31.5 μg/L observed in the present study is close to the 48-h $LC_{50} = 19.9$ μg/L and 96-h $LC_{50} = 12.9$ μg/L derived in the studies of Azevedo-Pereira et al. (2011) and Pestana et al. (2009a), respectively, and it is lower than the reported regulatory value 24-h $LC_{50} = 55.2$ μg/L (Germany, 2005). In comparison to other insect test species used in aquatic toxicology, *C. riparius* is among the most sensitive to acute imidacloprid exposure. In our study, the 24-h LC_{50} of *C. riparius* was at least 5.8 fold lower than the mean 24-h LC_{50} of the mayfly *C. dipterum*. (Roessink et al., 2013; Van den Brink et al., 2016). However, it should be noted (see the discussion below) that the ephemeropteran species *C. dipterum* have estimated 28days LC_{50} values of 0.195 μg/L, which was considerably lower than the

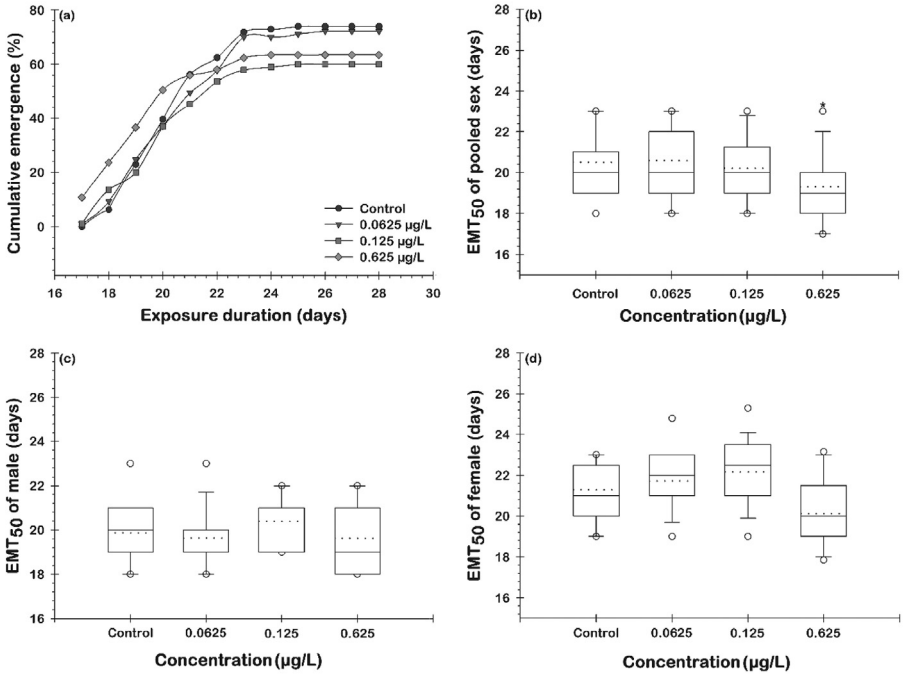

Fig. 2. Effect of different concentrations of imidacloprid on the emergence of C. riparius after 28 days of exposure. (a) Cumulative percentage of emergence; (b) Emergence time of pooled sex; (c) Emergence time of male; (d) Emergence time of female. Symbols in presented box-and-whisker plots are the same as in Fig. 1. Asterisks indicate significant difference from the control (Kruskal-Wallis ANOVA with Dunn's post hoc test, ***p < 0.001; **p < 0.01; *p < 0.05).

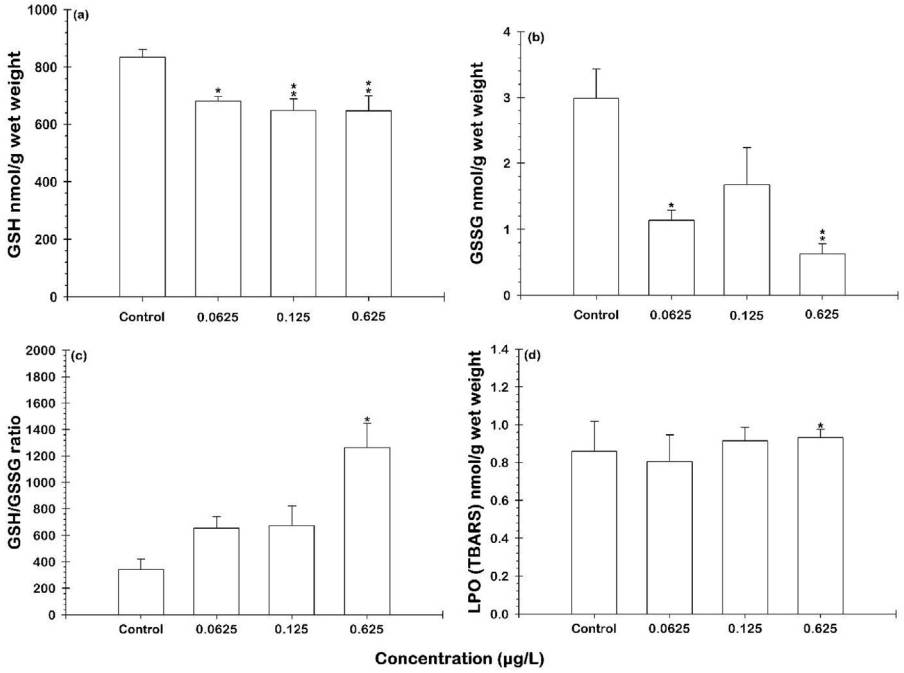

Fig. 3. Biomarker responses in C. riparius exposed to imidacloprid for 10 days; (a) Glutathione (GSH); (b) Glutathione oxidized (GSSG); (c); GSH/GSSG ratio; (d) Lipid peroxidation-TBARs (MDA) content. The error bars represented standard error (SE). Asterisks indicate significant difference from the control (ANOVA with Dunnett's post hoc test, ***p < 0.001; **p < 0.01; *p < 0.05).

$NOEC_{(emergence)}$ observed in the present study (Roessink et al., 2013). On the other hand, Alexander et al. (2007) reported higher susceptibility of the early stages of another mayfly (*Ephemeroptera*) species *Epeorus longimanus* to imidacloprid showing 24-h LC_{50} 2.1 µg/L, which is about 15-fold lower compared to our observations with *C. riparius*. Other dipteran taxa phylogenetically related to *Chironomidae* include *Aedes* and *Culex* were relatively tolerant to imidacloprid (Fig. 4a and Supplementary Table S17).

The most notable mode of action of neonicotinoid insecticides is their high binding affinity to the nAChR, which leads to lethal

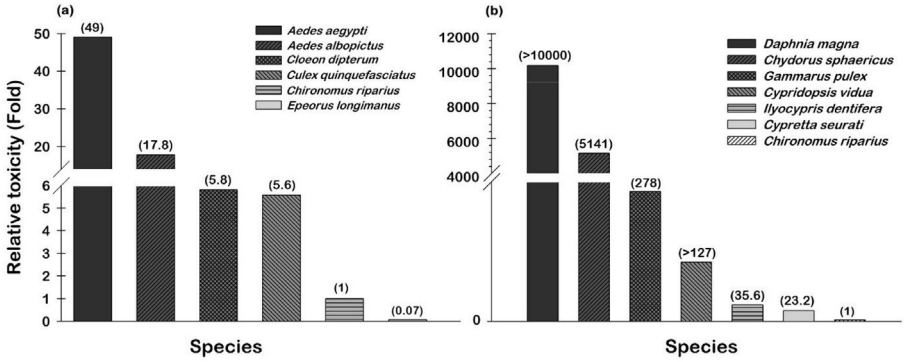

Fig. 4. Relative acute toxicity of imidacloprid. (a) ratio of 24-h LC_{50} of other aquatic insects species/24-h LC_{50} of *C. riparius*; (b) ratio of 24-h LC_{50} of aquatic crustaceans/24-h LC_{50} of *C. riparius*. The LC_{50} values with references can be found in the Supplementary Table S17.

effects even at low-doses and extended exposure periods (Morrissey et al., 2015). Related sublethal effects include impairment of flight, navigation or foraging ability, as well as effects on the growth and emergence (Morrissey et al., 2015; Van der Sluijs et al., 2015). Nevertheless, published studies with neonicotinoids predominantly focused on acute toxicity, whereas long-term effects are less known. Our study shows that the 10-day LC_{50} values of imidacloprid in *C. riparius* were 13.5-times lower than 24-h LC_{50} providing thus further evidence on fortified effects during extended exposure times (Tennekes and Sanchez-Bayo, 2011). Correspondingly, 7-day mean LC_{50} of imidacloprid (2.1 μg/L) for of *Cloeon dipterum* was approximately 68 times lower than the mean 24-h $LC_{50} = 143.5$ μg/L (Van den Brink et al., 2016), and similar trend was observed also in *Hyalella azteca* (Stoughton et al., 2008).

The present 10-day LC_{50} (2.33 μg/L) falls within the range of previously reported 7-day LC_{50} of 1.7 and 2.5 μg/L imidacloprid for *C. dipterum* (Roessink et al., 2013; Van den Brink et al., 2016), *C. tentans* (10-day $LC_{25} = 3.12$ μg/L) or *C. dilutus* (14-day $LC_{50} = 1.52$ μg/L) (Cavallaro et al., 2017; Stoughton et al., 2008). The 10-day NOEC 0.625 μg/L observed in the present study was 5.7 times lower than the 10-day NOEC of 3.57 μg/L reported in *C. tentans* (Stoughton et al., 2008), and our NOEC is similar to the concentrations affecting behavioural endpoints as ventilation and distance moved in the same species (Azevedo-Pereira et al., 2011). For comparison, the sensitivity of *D. magna* is several orders of magnitude lower (15 days $EC_{50\ (mortality)}$ ranges from 28350 to 35140 μg/L (Ieromina et al., 2014).

In agreement with previous reports, our study provides further evidence that crustaceans like *D. magna* and *Chydorus sphaericus* are less sensitive to acute neonicotinoid exposures (Sánchez-Bayo and Goka, 2006). Fig. 4b also shows a lower sensitivity of other crustaceans like amphipod *Gammarus pulex* or ostracods *Cypridopsis vidua*, *Ilyocypris dentifera*, and *Cypretta seurati* (Ashauer et al., 2010; Sánchez-Bayo and Goka, 2006). As shown in Fig. 5 and Supplementary Table S17 there is a wide range of toxicity values for imidacloprid in various aquatic non-target insects. The values from different studies and different organisms may not be directly comparable due to the differences in the exposure times and the endpoints. It highlights the need for testing across different aquatic invertebrate orders, especially using non-prototypical model species.

The most important immature stages of growth and development of the insects are instars from second to the fourth (Pinder, 1995), and all toxicity tests in the present study started with the first instar larvae. Sublethal 10-day exposures reduced the growth of the *C. riparius* larvae, which is in agreement with observations

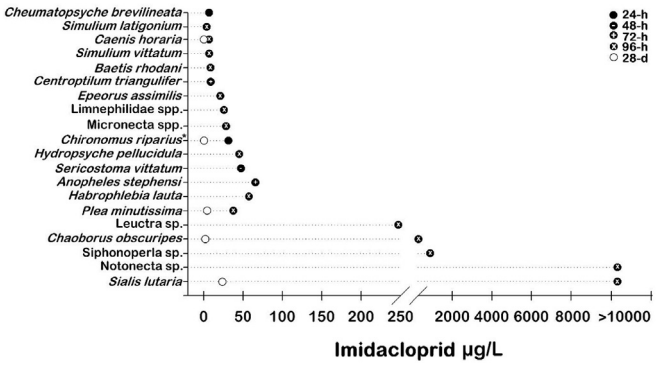

Fig. 5. Comparison of acute and sub-lethal toxicity of imidacloprid for standard and additional aquatic insect test species. Asterisk (*) shows the result obtained in the present study. The toxicity values collected from the literature along with the references are summarised in the Supplementary Table S17.

with other chironomids like *C. tentans* and *C. riparius*, where effective concentrations were within the same low μg/L range (Azevedo-Pereira et al., 2011; Stoughton et al., 2008). Larval growth in dipterans is a fitness-related trait since it is directly linked to the flying performance, pupation, emergence, adult female size and number of eggs per female (Liber et al., 1996; Sibley et al., 1997, 2001). Thus, larval growth inhibitions observed in the present study might indicate potential effects of imidacloprid on reproduction and population dynamics of aquatic insects.

Previous studies have shown that neonicotinoids influence the emergence of aquatic insect across several taxonomic groups (Cavallaro et al., 2017; Sánchez-Bayo et al., 2016). We found that imidacloprid treatments were reducing the emergence rate with increasing concentration (Fig. 2a). This observation is consistent with previous reports (Dorgerloh and Sommer, 2001; Germany, 2005) that showed that imidacloprid reduces the emergence of *C. riparius* by 10% at 2.09 (EC_{10}) and 50% at 3.11 μg/L (EC_{50}) during a 28-days toxicity test. Differences in EC values – compared to the present study – may be related previously used artificial sediment and constant exposure treatments, while the present study used inorganic sediment material (>1 mm silica sand) and renewal exposure. Further, Pestana et al. (2009a) also revealed a significant reduction in emergence after imidacloprid exposure and reported NOEC and LOEC for emergence ratio being 0.4 and 1.2 μg/L, respectively. Higher concentrations thus might cause more pronounced effects on emergence but these environmentally less

relevant levels were not investigated in the present study. Similar to the present study, Cavallaro et al. (2017) found 50% reduction of emergence *C. dilutus* when exposed to 0.39 μg/L of imidacloprid for 40 days. Also, Stoughton et al. (2008) found a significant decrease in the emergence of *C. tentans* after 28-days of constant exposures to imidacloprid with LC_{25} of 0.59 μg/L.

Our results also indicate that the emergence meantime (Pooled sex) was accelerated by imidacloprid (Fig. 2b). This observation is consistent with findings from full life-cycle toxicity tests using DDT and neonicotinoid clothianidin (Cavallaro et al., 2017; Rakotondravelo et al., 2006). Accelerated EMT_{50} can be related to the reduction of competition for food and space among relatively fewer surviving midges in the imidacloprid treatment as compared with the control. Further, chironomid females require more time to develop than males and have greater physiological demands during the transition from pupae to adult (Pinder, 1995). Insecticide exposure including neonicotinoids has been shown to shift sex ratios towards male-dominant populations (Cavallaro et al., 2017), which has also been confirmed in the present study where more males have emerged in the early days of imidacloprid treatments (Table S10).

As discussed previously (Nareshkumar et al., 2018; Nicodemo et al., 2014), the toxic effects of imidacloprid in insects may be - in addition to interaction with AChR - related to mitochondrial dysfunction and oxidative stress. For example, in honeybee, the imidacloprid toxicity has interfered with production ATP, mitochondrial bioenergetics and redox homeostasis (LaLone et al., 2017; Nicodemo et al., 2014). Similar events have been reported in the cotton bollworm, *Helicoverpa armigera* and bumblebee, *Bombus terrestris* (Moffat et al., 2015; Nareshkumar et al., 2018). The present study provides further evidence on the role of oxidative stress by showing a decreased content of both GSH and GSSG after 10-day exposures to imidacloprid, and slightly elevated concentrations of TBARs (LPO). These findings are in agreement with previous investigations (Özdemir et al., 2017; Qi et al., 2018; Vieira et al., 2018), and might indicate poor scavenging of ROS (Ge et al., 2015; Zhang et al., 2014). In addition, oxidative damage caused by imidacloprid has also been reported in other organisms like earthworm *Eisenia fetida* (Wang et al., 2016) and various fish species (Ge et al., 2015; Vieira et al., 2018). The present study thus shows that exposures to imidacloprid might affect the redox balance in *C. riparius,* which is reflected by changes in sensitive GSH and GSSG biomarkers.

5. Summary and conclusions

The present study highlights the sensitivity of *C. riparius* to imidacloprid in comparison to other investigated species in aquatic systems. Derived acute and sublethal toxicity values add to the growing body of literature used for environmental risk assessment related to neonicotinoids. We demonstrate sublethal effects on growth, an important limit for following successful emergence and reproduction. Our study also suggests that oxidative stress (observed reduced GSH and GSSG and slight induction of TBARs) may be a relevant mechanism in the imidacloprid-induced toxicity in *C. riparius*.

Conflicts of interest

The authors have declared that there are no conflicts of interest.

Author contributions

NCN. performed biological experiments, evaluated results and prepared the manuscript. DF. contributed and provided advice on biological experiments. LucBl. validated and run analyses for pesticides and biomarkers; contributed to the writing of the manuscript. ER. contributed to the planning of the experiments, and drafting of the manuscript. LudBl. designed and coordinated the study, contributed to interpretation of the results and writing of the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.chemosphere.2018.06.102.

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