MÉTHODES MOLÉCULAIRES POUR ÉTUDIER LES EFFETS SOUS-LÉTAUX DE CONTAMINANTS D'INTÉRÊT SUR LE MODÈLE *DAPHNIA MAGNA*

par

Hugo Alarie

Thèse présentée au Département de chimie en vue
de l'obtention du grade de docteur ès sciences (Ph.D.)

FACULTÉ DES SCIENCES
UNIVERSITÉ DE SHERBROOKE

Sherbrooke, Québec, Canada, décembre 2023

Le 14 décembre 2023

le jury a accepté la thèse de Monsieur Hugo Alarie
dans sa version finale.

Membres du jury

Professeur Pedro Alejandro Segura
Directeur de recherche
Département de chimie

Dr Magali Houde
Codirectrice de recherche
Environnement et Changement climatique Canada

Professeur Patrice Couture
Évaluateur externe
Institut national de la recherche scientifique

Professeur Jean-Philippe Bellenger
Évaluateur interne
Département de chimie

Professeur Pierre Harvey
Président-rapporteur
Département de chimie

Professeur Hubert Cabana
Évaluateur interne
Département de génie

SOMMAIRE

Un grand nombre de produits chimiques d'origine naturelle et anthropogénique sont synthétisés, exploités, transformés et utilisés par les êtres humains pour accommoder leur mode de vie et leurs activités quotidiennes. Certains composés organiques sont appliqués ou relâchés volontairement dans l'environnement, d'autres s'y retrouveront involontairement, par relâche accidentelle ou lorsque disposés en fin d'utilisation. Les différentes méthodes de traitement pour les eaux usées ou autres types d'émission sont souvent insuffisantes pour les éliminer, puisqu'elles n'ont pas toujours été conçues à cette fin. Plusieurs substances naturelles ou synthétiques ont été quantifiées dans les écosystèmes aquatiques ou sont suspectées d'être présentes dans l'environnement. On nomme ces composés organiques contaminants d'intérêt émergent (CIE). Certains s'élimineront naturellement sans causer de problèmes alors que d'autres seront persistants et susceptibles d'altérer la biologie des organismes qui y seront exposés[1]. Ce phénomène est répandu globalement et implique différentes catégories de contaminants, dont les produits pharmaceutiques, les pesticides, les produits pétroliers, les métaux, etc.[2]

Afin d'évaluer les effets de l'exposition aux composés chimiques, il est nécessaire de faire des études écotoxicologiques à l'aide d'espèces situées à différents niveaux trophiques[2]. La période d'évaluation de ces tests couvre rarement la durée de vie totale des organismes étudiés et ne reflète donc pas parfaitement l'exposition continue retrouvée dans l'environnement. Plusieurs générations peuvent ainsi être affectées, mais les effets intergénérationnels sont peu étudiés lors des tests d'écotoxicologie classiques. Il est donc nécessaire de développer de nouvelles méthodes afin d'évaluer les effets sous-létaux des expositions environnementales. Ces effets peuvent non seulement nous fournir davantage d'informations sur les risques encourus liés à une molécule et à son exposition, mais également permettre de prédire ces risques et même d'extrapoler les résultats pour d'autres molécules d'une même famille ou ayant un mode d'action similaire. Ces informations permettront aux autorités gouvernementales de prendre des décisions éclairées quant à la législation des différents composés jugés risqués. Cette thèse explore l'ajout de paramètres biologiques chez *Daphnia magna*, comme le niveau d'ecdystéroïdes, la transcription des gènes associés à leur métabolisme ainsi que l'étude de leur profil lipidique de manière non ciblée qui pourraient se révélés plus sensibles que les paramètres traditionnellement utilisés.

La présente thèse comporte trois articles scientifiques dans lesquels des méthodes sont développées et utilisées afin d'évaluer les effets de différents types de contaminants sur le modèle *Daphnia magna*.

Le premier article vise à mieux comprendre les effets des CIEs (acide clofibrique, gemfibrozil et fénarimol) sur le métabolisme hormonal de *D. magna*. Pour ce faire des outils moléculaires et biochimiques ont été développés et utilisés afin d'évaluer les variations basales de quatre gènes et une hormone, impliqués dans le métabolisme des ecdystéroïdes, une voie importante dans la croissance et reproduction des crustacés tel que *D. magna*. Peu de variation a été observée pour le niveau de transcription des gènes, mais il a été remarqué que le taux de 20-hydroxyecdysone variait de manière pulsatoire selon des cycles de 48h, correspondant à la période de mue des daphnies. Par la suite, des tests d'exposition chronique ont été effectués en utilisant les paramètres développés. Une diminution du nombre de nouveau-nés était observée à certains moments, mais le niveau de transcription des gènes et le taux de 20-hydroxyecdysone ne changeaient pas comparativement au groupe contrôle, ce qui ne permettait pas d'expliquer cette diminution.

Le 2^{e} article porte sur l'évaluation de la toxicité des eaux de ruissellement d'une mine désaffectée où des parcelles de sol contaminé ont été traitées par phytoremédiation. Les outils biologiques développés dans le premier article ont été utilisés lors d'expositions chroniques aux eaux de ruissellement avec *D. magna*, en combinaison avec des analyses chimiques afin de voir si l'approche de remédiation pouvait causer des risques pour la faune aquatique. Les résultats indiquent que l'exposition à l'eau de ruissellement non traitée n'impactait pas la reproduction, la masse des daphnies ainsi que le taux de 20-hydroxyecdysone. La survie d'un groupe exposé à l'eau de ruissellement était légèrement inférieure comparée au contrôle lors de l'un des tests (eau provenant de la parcelle #1). Le cas inverse a aussi été observé où la survie du groupe exposé à l'eau de ruissellement (parcelle #3) était supérieure à celle du groupe contrôle. Ces différences étaient toutefois minimes et n'étaient pas en cohérence avec les autres paramètres mesurés. En général, aucune différence n'a été observée entre les groupes contrôles et exposés (à l'eau de ruissellement) suggérant que l'eau de ruissellement provenant des parcelles de sol contaminé ne présente pas de risque pour *D. magna.* Ce chapitre a également démontré l'efficacité de l'approche ''whole effluent toxicity'' (WET) afin d'étudier de manière représentative l'eau de ruissellement provenant des parcelles de sol contaminé.

Dans le 3e article, une méthode lipidomique non ciblée a été développée et utilisée afin de mieux comprendre les effets de l'exposition à des CIEs à l'échelle moléculaire (concentration en lipide semi-quantitatif) chez *D. magna*. Une période de récupération où le contaminant étudié (l'agent hypolipémiant gemfibrozil) est retiré du milieu de culture a aussi été ajoutée à la fin de l'exposition afin d'observer le possible rétablissement de *D. magna*. Les paramètres classiques ont été étudiés en plus du profil lipidique de manière non ciblée. Il a été observé que plusieurs lipides étaient impactés lors de l'exposition, et que le degré de dysrégulation augmentait avec le temps dans plusieurs cas. Cette dysrégulation coïncidait avec une augmentation de la fertilité suite à 7 jours d'exposition continue ce qui pourrait avoir des conséquences néfastes pour l'adaptation aux facteurs de stress dans le milieu naturel. Lors de l'arrêt de l'exposition pour le groupe récupération, un retour à la normale pour plusieurs des métabolites affectés a été remarqué, ce qui suggère une capacité de récupération relativement rapide (48 h) chez ce crustacé.

Le développement de méthodes moléculaires ciblées et non ciblées présenté dans cette thèse supporte l'évaluation écotoxicologique de différents types de contaminants d'intérêt émergent ou effluents et l'acquisition de connaissances pouvant aider la gestion de leur risque à l'aide de méthodes selon l'état de l'art actuel. Ces méthodes novatrices permettant l'étude de paramètres sous-létaux au niveau des gènes et des métabolites peuvent aussi supporter le travail des autorités gouvernementales chargées des programmes de surveillance de la qualité de l'eau.

Mots clés : Écotoxicologie, crustacés, contaminants d'intérêt émergent, ecdystéroïdes, lipidomique, transcription de gènes, spectrométrie de masse

REMERCIEMENTS

Je voudrais d'abord remercier mon directeur de recherche, Pedro Segura, pour m'avoir donné l'opportunité de faire mon doctorat dans son laboratoire de recherche, mais également pour ses conseils et son support. J'aimerais également remercier Magali Houde, ma co-directrice, pour ses nombreux suivies, ses nombreuses corrections et également pour son support. Viennent ensuite les autres membres de mon comité; les professeurs Jean-Philippe Bellenger, Hubert Cabana, Patrice Couture et Pierre Harvey. Merci de votre implication, merci pour les questions, et merci pour les réflexions. Merci également aux professeurs Luc Gaudreau et Francis Beaudry de m'avoir permis d'utiliser leurs instruments.

Ces 4 années n'auraient pas été les mêmes sans les membres du labo Younès, Nicolas, Mégane, Cassandra, Valérie, Emmanuel, FX et Lisa.

Les membres du personnel techniques, Philippe Venne, René Gagnon et Michel Trottier pour leur soutien lors de problèmes techniques.

Nadia pour ses conseils en biochimie.

Je tiens aussi à remercier l'entreprise Englobe de m'avoir laissé faire un stage avec eux, et Dany Landry mon superviseur de stage.

Je voudrais remercier mes stagiaires, Pavlina Plisson et Aurélie Bazin-Capet pour leur bon travail.

À mes amis de Sherbrooke, de Montréal et d'ailleurs pour les moments d'évasion.

À mes parents, Marc et Mylène, et grands-parents, pour leur support de près ou de loin.

À Ronny, pour les séances de visionnement et à ma sœur Chloé.

Finalement, merci aux organismes qui m'ont subventionné, Hydro-Québec et FONCER TEDGIER.

TABLE DES MATIÈRES

LISTE DES ABRÉVIATIONS

ACN	Acétonitrile
ADN	Acide désoxyribonucléique
AMPc	Adénosine monophosphate cyclique
ARN	Acide ribonucléique
CIE	Contaminants d'intérêt émergent
CYP	Cytochrome P450
EC50	Effective concentration 50%
EcR	Ecdysteroid receptor
ED50	Effective dose 50%
FA	Formic acid
FISH	Fluorescent in situ hybridization
GC	Gas Chromatography
HAP	Hydrocarbure aromatique polycyclique
HP	Hydrocarbure pétrolier
ICP	Inductively coupled plasma
ISO	International Organization for Standardization
LC	Liquid chromatography
LC50	Lethal concentration 50%
LOAEC	Lowest observed adverse effect concentration
MeOH	Méthanol
MRM	Multiple reaction monitoring
MS	Mass spectrometry
NOAEC	No observed adverse effect concentration
OECD	Organisation for Economic Cooperation and Development
PKA	Protéine kinase A
PRM	Parallel reaction monitoring
QTOF	Quadrupole time of flight
RMN	Résonnance magnétique nucléaire
RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction

SPE	Extraction en phase solide
US EPA	United State Environnemental Protection Agency
USP	Ultraspiracle
WET	Whole effluent testing
20E	20-Hydroxyecdysone

LISTE DES TABLEAUX

LISTE DES FIGURES

CHAPITRE 1. INTRODUCTION

1.1 Mise en contexte

Chaque année, un grand nombre de produits chimiques d'origines anthropogénique (plus de 500 millions de tonnes) et naturelle est utilisé dans les activités quotidiennes des êtres humains.[1] Ce nombre est croissant étant donné l'augmentation constante de la population, mais également l'amélioration de la qualité de vie apportée par l'utilisation de ces produits chimiques.[1] Plus de 350 000 composés chimiques ont été enregistrés en termes de production et d'utilisation.[2] De ce nombre, une certaine portion se retrouvera dans l'environnement à la suite de leur usage et de leur rejet des usines de traitement des eaux, des hôpitaux ou autres sites de production/utilisation. Certains de ces composés sont appelés contaminants d'intérêt émergent (CIEs) et sont définis comme des matériaux et produits chimiques naturels, manufacturés ou synthétiques qui ont été découverts ou sont suspectés présents dans divers compartiments environnementaux et dont la persistance ou la toxicité sont susceptibles d'altérer le métabolisme des êtres vivants.[3] Cette contamination est globalement répandue et plusieurs types de contaminants (ex : produits pharmaceutiques, pesticides, produits pétroliers, composés inorganiques, etc.) sont retrouvés dans les cours d'eau et autres milieux aquatiques un peu partout sur la planète.[4,5] Les cours d'eau sont particulièrement susceptibles de recueillir les contaminants; le ruissellement et l'érosion sont des phénomènes naturels pouvant contribuer au transport des contaminants, contribuant ainsi à leur distribution et accumulation dans les environnements aquatiques situés plus en aval.[6]

Cette problématique n'est pas apparue durant la période moderne de notre histoire. La pollution d'origine anthropogénique date de plus de deux mille ans suite à l'exploitation et la transformation des minerais lors de l'époque romaine.[7] Les récentes avancés des technologies utilisées pour la détection et l'analyse de ces contaminants permettent de mieux cerner l'ampleur actuelle de la problématique de la pollution aquatique.[8] L'accès à l'information et les différentes campagnes de sensibilisation ont contribué à la prise de conscience de cette problématique. Le livre *Silent Spring* (1962) dénonçait déjà il y a plusieurs décennies nos choix d'utilisation de certains de ces produits chimiques, tels que les pesticides, et expliquaient les effets néfastes que ces choix peuvent causer à l'environnement, mais également pour nous les êtres humains.[9] Il existe toujours un manque d'informations sur les effets que les contaminants environnementaux peuvent produire sur la faune et la flore exposées. Pour évaluer ces effets, des études

écotoxicologiques effectuées sur plusieurs espèces de différents niveaux trophiques sont nécessaires. Plusieurs tests standardisés existent incluant deux évaluations recommandées par l'Organisation de Coopération et de Développement Économique (OCDE) pour l'évaluation écotoxicologique sur le modèle *Daphnia magna*: un test d'exposition aigüe (48h), où la concentration létale où 50% des organismes meurent (LC_{50}) est rapidement déterminée, et un test d'exposition chronique (21 jours), où la croissance, la mortalité et la fécondité sont observées [10,11]. Ces conditions d'exposition ne sont cependant pas représentatives des conditions réelles auxquelles sont exposés les différents organismes dans la nature. Le problème avec ces paramètres se situe d'abord au niveau de la sensibilité. Il faut généralement des doses très élevées afin de pouvoir observer un changement au niveau de la fertilité, et encore plus pour la mortalité. Les concentrations observées dans l'environnement sont habituellement beaucoup plus faibles que les concentrations utilisées lors des tests de toxicité.[2] Par exemple, chez *D. magna*, les LC_{50} 48h des anti-inflammatoires diclofénac, acétaminophène et ibuprofène sont de 18.1, 40 et 23.5 mg/L, respectivement. Les concentrations auxquelles une diminution de fertilité est observée lors d'expositions chroniques se situent entre 2.16 et 32.4 mg/L.[12] Si l'on compare ces concentrations à celles retrouvées dans l'environnement pour les mêmes composés (ng/L à µg/L), on remarque plusieurs ordres de grandeur de différence. Ensuite, la durée d'exposition est beaucoup plus longue dans la nature que lors des tests chroniques. La période d'évaluation couvre rarement la durée de vie totale des organismes étudiés. Cela est sans compter que plusieurs générations pourraient être affectées. Une évaluation écotoxicologique devrait donc être plus longue, à plus faible concentration afin d'être plus représentative de la réalité. Cependant, les paramètres utilisés lors des tests classiques (mortalité, fécondité) se révèlent trop peu sensibles pour détecter quelconques changements lorsque de faibles concentrations sont utilisées. D'autres paramètres pourraient être observés suite à ces tests afin de pouvoir observer des effets sous-létaux. Ces paramètres peuvent être le comportement et les habitudes de l'organisme étudié, ou bien un biomarqueur pertinent pouvant être quantifié.[13,14,15] Il est même recommandé de modifier les lignes directrices d'une évaluation écotoxicologique lorsque jugé pertinent, par exemple en ajoutant les paramètres mentionnés précédemment, si les paramètres habituellement utilisés et recommandés se révèlent inadéquats.[16]

1.2 Écotoxicologie aquatique

1.2.1 Modèles et concepts utilisés en écotoxicologie aquatique

L'écotoxicologie est un domaine d'étude composé de l'écologie, qui étudie les liens entre les organismes et leur environnement, et de la toxicologie, qui étudie les effets néfastes causés par une source ou un produit chimique ou physique avec lequel un organisme est entré en contact.[17] L'écotoxicologie s'intéresse donc aux effets que les contaminants peuvent avoir dans la biosphère à tous les niveaux d'organisation biologique, allant des organelles d'une cellule jusqu'à un écosystème complet.[18] Elle est habituellement plus concentrée autour de l'étude des effets biomoléculaires et physiologiques au sein d'un même organisme, comme il est question dans la présente thèse. L'écotoxicologie s'intéresse aux différents compartiments de l'environnement, tels que l'eau, l'air et la terre. Pour chacun de ces compartiments, il existe différents modèles propres à chacun. Il est intéressant d'étudier des modèles de différents niveaux trophiques (algues, insectes, poissons, etc.). Les modèles les plus utilisés en écotoxicologie aquatique sont les algues et autres plantes aquatiques, la puce d'eau (*D. magna)*, le poisson-zèbre (*Danio rerio*), le méné à grosse tête (*Pimephales promelas*), la truite arc-en-ciel (*Oncorhynchus mykiss)*, les têtards de grenouilles (*Xenopus laevis*) ainsi que des lignées cellulaires de ces différents modèles et plusieurs autres[19,20,21,22,23] Ces modèles peuvent varier dépendamment des régions du monde afin d'inclure des modèles indigènes. Cette thèse s'intéresse au développement de méthode pour l'étude du modèle *D. magna* qui sera présenté dans une prochaine section.

1.2.1.1 Concepts théoriques de la toxicologie

Un concept fondamental en toxicologie est la relation dose-réponse, ou exposition-réponse. Généralement, plus la dose ou l'exposition est élevée, plus la réponse est élevée.[24] Ce concept peut servir à démontrer les preuves de concept d'efficacité et de toxicité, et aider à cibler les mécanismes d'action derrière les effets observés.[25] Le mécanisme d'action est l'explication du processus physiologique derrière la toxicité observée. Le mécanisme d'action peut être spécifique, c'est-à-dire qu'il n'affectera qu'un type de molécule ou de site, ou non spécifique, où une nécrose généralisée sera observée sans cible spécifique.[26]

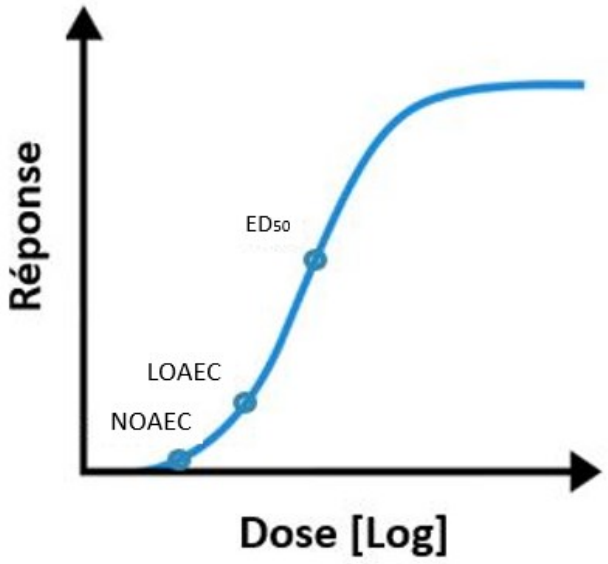


Figure 1. Courbe dose-réponse incluant différent niveau de réponse (NOAEC, LOEAC, ED_{50}).

Différents types d'expositions sont possibles. Lors des expositions aiguës, ou de courte durée, la LC_{50} (concentration létale), la « concentration sans effet observé » (NOAEC, de l'anglais *no observed adverse effect concentration*) ainsi que la « plus faible concentration où un effet néfaste est observé » (LOAEC de l'anglais *lowest observed adverse effect concentration*) peuvent être déterminées (Figure 1).[27] La LC_{50} représente la concentration ou la dose où 50% des individus exposés meurent. La NOAEC est la concentration ou la dose la plus élevée où aucun effet néfaste n'est observé. La LOAEC est la dose ou la concentration la plus faible où un effet néfaste est détecté. [28] Ces valeurs serviront à définir les limites d'exposition sécuritaires ainsi que les doses ou concentrations utilisées lors des études chroniques. Une exposition chronique est définie comme une exposition qui dure plus de 10% de la durée de vie de l'organisme exposé et où une l'administration du composé à l'étude est faite de manière répétée ou continue.[29] Lors des expositions chroniques, on cherche également à vérifier la manifestation d'effets néfastes plus ciblés, tel que la génotoxicité, la carcinogénicité, la neurotoxicité, des effets sur la reproduction, etc.[29] On peut alors parler de ED_{50} (dose efficace) EC_{50} (concentration efficace) ou pour un paramètre spécifique lorsque 50% des individus sont affectés selon ce paramètre à une certaine dose ou concentration. Les LC_{50}, NOAEC et LOAEC sont également déterminées de manière chronique.[30] Différentes méthodes peuvent être utilisées pour exposer l'organisme utilisé comme modèle au contaminant étudié. Il peut s'agir de gavage, d'injection, ou bien d'introduire le contaminant dans l'environnement immédiat de l'organisme, par exemple dans l'eau dans lequel il vit dépendamment du

type de contaminant et du type de modèle. Il est aussi possible d'évaluer un effluent complet avec ou sans traitement au préalable (whole effluent toxicity, WET).

1.2.1.2 Le modèle Daphnia magna

D. magna est une espèce de crustacés d'eau douce dite sentinelle utilisée lors d'études écotoxicologiques. La daphnie est un modèle animal présentant plusieurs avantages et elle est donc largement utilisée dans les laboratoires à travers le monde. En plus d'être retrouvées dans les écosystèmes lentiques de l'hémisphère nord, la culture des daphnies est simple et peu coûteuse à entretenir. La daphnie se reproduit de manière parthénogénétique, ce qui veut dire que la progéniture d'une femelle est génétiquement identique à sa génitrice.[31] Cette caractéristique fait en sorte que les variabilités génétiques interindividus sont grandement réduites comparativement à d'autres espèces.[32] Cette caractéristique est importante pour les études génomiques ou multigénérationnelles. La biologie de la daphnie est très bien connue étant étudiée dans les domaines de l'évolution, de l'écologie, de l'écotoxicologie et de la génomique depuis plusieurs décennies.[33,34]

Différents paramètres biologiques et biochimiques peuvent être mesurés chez la daphnie lors des tests de toxicité. Parmi ceux-ci, la mesure des concentrations de métabolites (i.e. une molécule formée durant le métabolisme ou nécessaire à celui-ci) tissulaires. Un exemple de type de métabolites sont les lipides, composés de différentes familles et classes ayant différents rôles physiologiques. Plusieurs classes de lipides agissent comme ligand et médiateur cellulaire et contrôle plusieurs aspects physiologiques tels que la croissance, la reproduction, l'inflammation et le métabolisme de l'énergie.[35,36] Les concentrations mesurées de ces métabolites peuvent parfois expliquer les effets observés au niveau physiologique. Lari *et al*. (2022) ont observé qu'un changement au niveau métabolique (augmentation de la synthèse protéique) menait à une croissance plus élevée chez les jeunes daphnies exposées à des concentrations sous-létales d'acétaminophène.[37] Si le mode d'action du composé à l'étude est connu, il sera possible de faire un lien avec celui-ci et les potentiels paramètres visés afin d'approfondir la compréhension des effets du composé étudié.

Les ecdystéroïdes sont chez *D. magna* une famille de lipides de type stéroïdiens. Ils sont responsables de la mue et de la reproduction de l'espèce.[38] Les ecdystéroïdes sont dérivés du cholestérol suite à une série

de modifications enzymatiques jusqu'au métabolite le plus actif, la 20-hydroxyecdysone (20E) (voir Figure 2).[39] Les enzymes régulant ce groupe d'hormones sont encodées par la famille de gènes appelée Halloween. Les gènes *Shade, Shadow* et *CYP18A1* font partie de cette famille comme on peut le voir sur la figure 2. Les ecdystéroïdes se lient au récepteur nucléaire de l'ecdysone (*EcR*), qui vient ensuite se dimériser avec ultraspiracle (*USP*) pour enfin se lier à l'élément de réponse de l'ecdysone sur les gènes cibles. Cette liaison enclenche la transcription du gène. Il se pourrait également que les ecdystéroïdes aient des actions non génomiques en se liant à des récepteurs membranaires.[40] Une fois son rôle rempli, la 20-hydroxyecdysone est inactivée soit par épimérisation (3β-ecdysteroides en 3α-ecdysteroïdes), soit par la formation de la 20,26-dihydroxyecdysone qui est ensuite excrétée sous la forme de l'acide 26-hydroxyecdysonoique ou soit par phosphorylation en C22.[41,42,43]

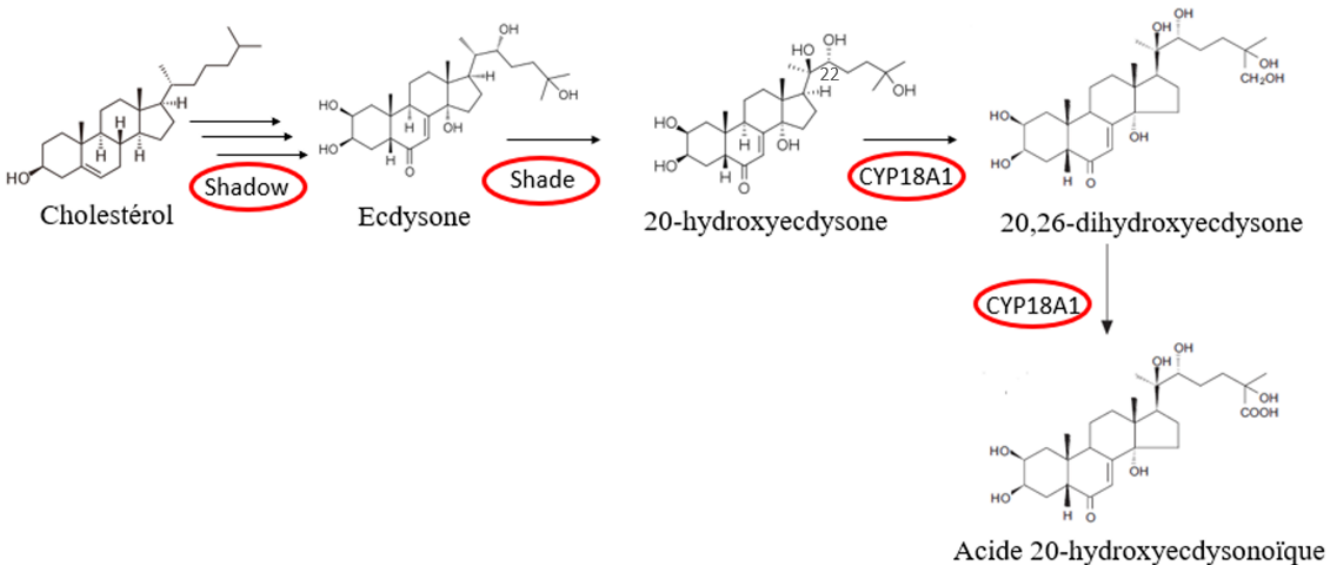


Figure 2. Biosynthèse et élimination de la 20-hydroxyecdysone à partir du cholestérol. Les gènes responsables des enzymes de la biosynthèse sont encerclés en rouge. Quelques étapes entre le cholestérol et l'ecdysone ont été omises.[44]

1.2.2 Les techniques et paramètres mesurés

Lors des tests d'exposition, différents paramètres sont mesurés afin d'observer les effets du ou des contaminants sur les organismes exposés. Il peut s'agir de la mortalité, de la fécondité, des habitudes comportementales, du poids, du taux de certains métabolites ou encore du niveau de transcription de gènes. Ces paramètres sont souvent limités par les techniques et appareils disponibles pour faire les mesures. Récemment, l'amélioration et le développement des méthodes de mesure ont permis de quantifier de nouveaux paramètres (grâce aux méthodes non ciblées par exemple) fournissant plus d'information que les paramètres classiquement utilisés. On appelle ces paramètres « sous-létaux », puisque des changements au niveau de ces mesures se manifestent avant la mort de l'organisme étudié ou ne causent pas celle-ci.[45] Ils peuvent permettre d'obtenir de nouvelles informations afin d'expliquer les effets plus facilement observables, mais pas nécessairement compris.

1.2.2.1 Mortalité et fécondité

Les paramètres de mortalité et de fécondité sont couramment utilisés en écotoxicologie. Ils ne requièrent pas d'appareils sophistiqués pour les mesures et ils sont parmi les plus utilisés auprès des autorités gouvernementales et l'application des réglementations puisque qu'ils sont objectifs et facilement standardisées.[46,47,48] Ces paramètres ne sont cependant pas spécifiques et ne fournissent pas d'information sur les mécanismes de toxicité observée.[49]

1.2.2.2 La transcription des gènes

Pour ce qui est de la mesure de la transcription des gènes, la première étape de l'expression de ceux-ci, la méthode par Reverse Transcriptase-quantitative Polymerase Chain Reaction (RT-qPCR) est couramment utilisée.[50] Une extraction de l'acide ribonucléique (ARN) doit d'abord être effectuée sur les échantillons. Les extraits d'ARN sont ensuite analysés à l'aide d'un appareil Nanodrop, un spectrophotomètre pouvant mesurer l'absorbance des échantillons à très petits volumes (1-2 µL), afin de déterminer la pureté ainsi que la concentration. Ceci assure une quantité identique d'ARN pour chaque échantillon et élimine les variations dues à la taille des daphnies. Les extraits d'ARN sont ensuite transformés en ADNc, ce qui leur permet ensuite d'être amplifiés par plusieurs cycles de PCR. Lorsque

cette détection survient, il est possible de déterminer la quantité de copies initiales de la séquence d'intérêt en fonction du nombre de cycles qui a été nécessaire pour atteindre ce seuil.

La transcription des gènes est très spécifique et peut être utilisée pour mieux comprendre les mécanismes d'action de la toxicité observée lorsque le rôle des gènes affectés est connu.[51,52,53] Par exemple, Poynton *et al*. (2011) ont pu observer une relation exposition-réponse en mesurant l'expression de gènes ciblés chez la daphnie exposée au cadmium à l'aide de cette technique.[54] D'autres méthodes comme le Northern blot et le FISH (*Fluorescent In Situ Hybridization*) sont utilisées de manière moins courante pour la quantification de gènes ciblés. Les gènes de la famille Halloween, responsables pour la biosynthèse des ecdystéroïdes chez les arthropodes, seront étudiés comme paramètre lors de cette thèse.[55] Peu de travaux ont étudié les gènes Halloween chez *D. magna* en contexte d'écotoxicité, mais ils ont déjà été remarqués lors d'études transcriptomiques.[56] Sumiya *et al*. (2014) ont effectué des travaux extensifs sur les gènes de la familles Halloween chez *D. magna* par RT-qPCR et ont pu caractériser le cycle de mue en fonction du niveau de transcription de ces gènes.[57,58]

D'autres types de gènes ont été explorés chez *D. magna* tels que ceux associés à l'hémoglobine ainsi qu'au stress oxydatif. Par exemple, la transcription de gènes ciblés a été quantifiée suite à des expositions du crustacé à différents composés chimiques (nonylphenol, bisphénol A, benzo[a]pyrène, chloropyriphos et plomb).[59] À la fin d'une exposition de 21 jours une augmentation significative de l'expression des gènes associés à l'hémoglobine fut observée de manière concentration dépendante pour la plupart des composés à l'étude. Le groupe de Sancho *et al*. (2022) a pu observer une différence au niveau de l'expression de certains gènes associés aux protéines de choc thermique à la suite d'une exposition de 21 jours au prochloraz à une concentration de 380 µg/L chez *D. magna*.[60]

1.2.2.3 L'analyse ciblée de métabolites

Le niveau d'un métabolite, c'est-à-dire une molécule endogène jouant un rôle biologique chez l'organisme étudié, peut servir de biomarqueur lors des tests de toxicologie et fournir de l'information sur le mécanisme de toxicité observé.[61] La métabolomique ciblée est la mesure de groupes définis de métabolites caractérisés chimiquement et biochimiquement. Grâce à l'utilisation d'étalons internes, l'analyse peut être faite de manière quantitative ou semi-quantitative.[62] La spectrométrie de masse (MS)

couplée à la chromatographie liquide (LC) est une méthode importante pour la détection et quantification ciblée. En raison des avantages significatifs de la grande sélectivité et sensibilité, la LC-MS en mode de suivi des réactions multiples (MRM) fournit d'excellents résultats pour la quantification de métabolites.[63]

Les premiers dosages d'ecdystéroïdes ont été effectués à l'aide des méthodes radio-immunologiques et immuno-enzymatiques. [64,65,66,67,68] Ces méthodes très sensibles pour l'époque (limite de quantification de l'ordre du pg/mL) sont encore largement utilisées de nos jours. Les désavantages de ces méthodes incluent un coût élevé ainsi qu'un manque de spécificité. En effet, les méthodes de quantification de type immunologique ont parfois des problèmes à discriminer deux molécules très semblables comme les stéréo-isomères.

La LC-MS est une technique n'ayant pas ce problème de spécificité, en plus d'être moins coûteuse en termes de réactifs utilisés. Certains groupes de recherche ont utilisé cette méthode afin de quantifier différents ecdystéroïdes chez d'autres espèces que la daphnie (drosophile, scorpion, ver à soie, etc.).[69,70] Récemment, notre groupe de recherche a développé une méthode de quantification LC-QqQMS (Triple quadrupole) pouvant quantifier la 20-hydroxyecdysone dans une daphnie entière.[71]

Pour ce qui est de l'étude du système ecdystéroïdien chez la daphnie, Leblanc *et al*. (2002) ont remarqué, par dosage immunologique, une diminution du taux d'ecdystéroïdes à la suite d'une exposition au fénarimol, un fongicide.[72] Ils ont aussi observé qu'une co-administration de 20-hydroxyecdysone annulait les effets produits par le fénarimol. Les expositions ont été effectuées sur des daphnies nouvellement nées à une concentration de 0.2 à 1.5 µM pendant 21 jours. Ils ont ainsi démontré l'action anti-ecdystéroïdienne du fénarimol. Bodar et.al (1990) se sont également intéressé au niveau des ecdystéroïdes chez la daphnie à la suite d'expositions au cadmium.[73] Le dosage était cependant effectué par dosage immunologique. Le profil entier des ecdystéroïdes présentement connus n'était donc pas mesuré.

Le groupe de Pamla *et al*. (2009) a suivi une approche similaire pour étudier le système ecdystéroïdien, mais avec les pesticides atrazine et sulfate d'endosulfan.[74] Dans le cas de l'atrazine, le taux d'ecdystéroïdes diminuait malgré la co-administration de 20-hydroxyecdysone, ce qui suggère un mécanisme d'action autre que la voie ecdystéroïdienne. Un autre paramètre qui peut être mesuré de

manière ciblée est le niveau de transcription de gènes spécifiques. La méthode généralement utilisée pour cette mesure est la RT-qPCR.[75,76] Une combinaison d'analyses au niveau des gènes et des métabolites peut être faite pour mieux comprendre les mécanismes d'action, bien que cette approche ne soit pas très souvent employée. Les liens entre une approche de type métabolique et son transcrit ne sont pas toujours directs. Cette approche permet cependant d'obtenir plus d'informations, et ce de manière plus sensible et spécifique.

1.2.2.4 L'analyse non-ciblée de métabolites (métabolomique)

Une approche très prometteuse pour l'évaluation toxicologique de molécules, l'identification de biomarqueurs ainsi que l'approfondissement des mécanismes de toxicité est l'utilisation d'analyses non-ciblées.[77] Les analyses métabolomiques s'intéressent aux métabolomes, définis comme l'ensemble des molécules de faible poids moléculaire (acides aminés, acides gras, vitamines, carbohydrates et lipides) produites par le métabolisme cellulaire d'un organisme.[78] Il est possible de cibler un groupe de métabolites, par exemple les lipides. On parle alors de lipidomique. À l'aide de ce type d'approche, les cibles affectées par un composé peuvent rapidement être identifiées lorsque comparées à un groupe contrôle.[79] Ces analyses sont habituellement faites par GC-MS, LC-MS ou par résonnance magnétique nucléaire (RMN).[80] Quelques travaux ont été effectués avec succès sur le modèle *D. magna* en utilisant la spectrométrie de masse.[81,82,83] Le groupe de Simpson *et al*. (2016) s'est intéressé au profil métabolique mesuré par résonnance magnétique ^{1}H des daphnies exposées à un mélange de contaminants (propranolol, carbamazépine et acide perfluorooctanesulfonique).[84] Ils ont également procédé à des expositions en paires des mêmes contaminants. Ils ont observé une hausse chez certains acides aminés et une baisse au niveau du glucose lorsque les profils métaboliques des groupes contrôles et exposés étaient comparés. Taylor *et al*. (2017) se sont intéressés au profil lipidique de *D. magna* à l'aide d'infusion directe dans un spectromètre de masse. Ils ont remarqué des perturbations du lipidome lors d'exposition au Cu^{2+} à des concentrations environnementales (20 µg/L).[85] Plusieurs lipides identifiés n'avaient jamais été reportés chez *D. magna*. Smith *et al*. (2022) ont utilisé la désorption-ionisation par électronébulisation afin de cartographier les perturbations moléculaires du lipidome dans des segments de tissue de daphnies lors d'exposition au bisphénol A.[86] La majorité des variations du lipidome ont été annotées en tant que triacylglycérides et phosphatidylcholine. Des profils distincts de lipides ont pu être observés entre les

différents types de tissus analysés. La lipidomique et métabolomique par RMN ont été combinées à l'analyse du niveau de transcription des gènes afin d'étudier les effets des retardateurs de flammes chez *D. magna*.[87] Des réponses distinctes ont pu être observées entre les différentes expositions aux composés ignifuges (pentabromophénoxy benzène, oxybistétrabromobenzène, bis(2-ethylhexyl) tetrabromophthalate et Firemaster BZ-54) et des mécanismes de toxicités ont donc pu être proposés en combinant les différentes informations recueillies.

1.2.2.5 Les méthodes d'identification de métabolites

Une fois les données MS acquises en haute résolution en mode balayage, les différents pics de chaque *m/z* peuvent être séparés par déconvolution ou MS différentielle (extraction d'un signal désiré à l'aide d'un algorithme afin de l'isoler).[88] Une fois les *m/z* intéressants repérés par analyses statistiques, il est ensuite possible de les identifier afin de leur attribuer une formule chimique et, idéalement, une structure. Vu la haute résolution des appareils (> 10 000) et l'excellente exactitude sur la masse (< 5 mDa) des spectromètres de masse d'aujourd'hui, il est possible d'attribuer une formule chimique potentielle en utilisant la masse exacte expérimentale. Le patron isotopique peut également être utilisé afin de confirmer les potentielles formules chimiques.[89] La fragmentation des ions précurseurs permettra d'obtenir des spectres des ions produits qui pourront nous renseigner sur la structure de la molécule. Ces spectres peuvent ensuite être comparés à des bases de données de spectrométrie de masse en tandem (MS^2) dans le but d'obtenir un match potentiel.

Plusieurs banques de données existent en fonction du type de molécules à identifier. Lipid Maps, Human metabolome database, Lipidblast, Swiss Lipids sont des exemples de banques de données de lipides. Les différentes étapes d'identification d'un signal jusqu'à une molécule sans ambiguïté correspondent à différents niveaux de confiance selon Schymanski *et al*. Le dernier niveau (5^e) est le *m/z* obtenu par spectrométrie de masse de haute résolution, sans avoir suffisamment d'informations pour lui attribuer une formule ou structure moléculaire. Le 4^e niveau correspond à l'attribution d'une formule moléculaire sans équivoque, mais sans pouvoir déterminer la structure. Le 3^e niveau représente des candidats possibles. Il est possible d'avoir une idée de la structure, sans en être certain. Un bon exemple pour ce niveau sont les isomères structurels. Le 2^e niveau est la structure probable. Suffisamment d'informations sont disponibles

pour déterminer la structure (patron de fragmentation MS^2), mais celle-ci n'est pas confirmée à l'aide d'un standard. Le premier niveau de confiance est une structure moléculaire validée par standard de référence.[90]

1.3 Occurrence et effets des contaminants d'intérêt émergent dans l'environnement aquatique

Les contaminants environnementaux peuvent parvenir dans l'environnement par différentes sources selon leur type, leur production et leur utilisation. Certains contaminants sont plus préoccupants que d'autres selon leur nature. Certains sont très persistants tels que les molécules perfluorées, d'autres, comme les composés pharmaceutiques, sont fabriqués expressément pour avoir une activité physiologique.[91] Certains seront présents dans l'environnement en fortes concentrations, mais se dégradent rapidement comme les pesticides organophosphorés [92], alors que d'autres sont retrouvés en concentrations traces comme les antibiotiques.[93] Si un contaminant est persistant, introduit régulièrement dans l'environnement, néfaste et retrouvé en grande concentration, il sera naturellement plus préoccupant qu'un composé inerte, à faible concentration et rapidement biodégradable.[94] Les produits de transformation ne sont cependant pas à négliger. Il existe plusieurs catégories de contaminants, les catégories étudiées dans cette thèse sont détaillées dans cette section.

1.3.1 Les composés pharmaceutiques

Les composés pharmaceutiques sont une classe de contaminants d'origine anthropique. Bien qu'ils ne soient pas volontairement introduits dans l'environnement, ils s'y retrouvent souvent suite à leur utilisation en médecine humaine et vétérinaire.[95] Ils sont particulièrement préoccupants puisqu'ils ont été spécialement conçus pour avoir une activité biologique et pourraient produire des changements physiologiques sur les organismes exposés, mais non ciblés.[96] Leur occurrence est habituellement plus élevée dans l'eau de surface des pays et communautés qui ne possèdent pas les installations adéquates pour leur élimination dans les eaux usées avant d'être rejetées dans l'environnement.[5,97] Il existe cependant un manque d'information sur la distribution de ces composés, et ce principalement dans les pays en voie de développement.[98]

Vu leur grande consommation à travers le monde, les anti-inflammatoires non stéroïdiens tels que l'ibuprofène, le naproxène, le diclofénac et l'acétaminophène sont retrouvés à des concentrations allant du ng/L jusqu'au µg/L dans les eaux de surface à travers le monde.[99, 100,101,102] Ces concentrations sont parfois suffisamment élevées pour causer des problèmes aigus et chroniques aux diverses espèces aquatiques exposées.[4] Par exemple, des effets génotoxiques ont été observés chez le poisson-zèbre après des expositions de 28 jours à des concentrations aussi faibles que 0,092 µg/L d'ibuprofène.[103] De plus des effets chroniques sur la reproduction et la croissance chez plusieurs espèces de poissons telles que *Salmo trutta fario* et *Oryzias latipes* ont été observées à des concentrations de 0,1 à 100 µg/L de naproxène et diclofénac.[104,105] Des effets similaires ont également été observés chez d'autres espèces tels que *D. magna*. Lors d'une étude sur six générations exposées à 4 µg/L d'ibuprofène, une augmentation de déformations de génération en génération a été remarquée.[106] Michalaki *et al*. (2023) ont également observé des effets intergénérationnels chez *D. magna* étant de plus en plus marqués au fil des générations lors d'exposition à l'indométacine, l'ibuprofène ou un mélange des deux à 1 mg/L pendant 24h. L'activité des enzymes lipase, peptidase, β-galactosidase et glutathione-S-transférase fut significativement altérée. [107] De nombreux autres effets sous-létaux ont été observés chez *D. magna* tel que des dommages à l'ADN ainsi que l'augmentation du niveau de stress oxydatif lors que courte exposition (48h) au diclofénac, ibuprofène et naproxène à des concentrations de 9700 µg/L, 2900 µg/L et 17 µg/L respectivement.[108]

Une autre classe de pharmaceutiques très utilisée est celle des médicaments servant à prévenir et traiter les troubles du système cardiovasculaire comme les bêtabloquants, les statines ainsi que les fibrates. Ils ont été retrouvés dans les eaux de surface à des concentrations de l'ordre du ng/L au bas µg/L un peu partout au monde tel que dans des régions comme la Méditerranée, l'Amérique du Nord ainsi que l'Asie.[109,110,111,112] Leur fréquence de détection et les concentrations parfois importantes pourraient être expliquées par le nombre de prescriptions croissantes étant donné la population vieillissante ainsi que le taux d'obésité élevé de certains pays occidentaux. Ces composés peuvent également avoir divers effets sur des organismes non ciblés. Chez la moule méditerranéenne (*Mytilus galloprovincialis*), des altérations biochimiques et transcriptionnelles sont survenues lors d'une exposition de 7 jours au propranolol à des concentrations aussi faibles que 0,0003 µg/L. Celle-ci a entraîné une réduction significative des niveaux d'adénosine monophosphate cyclique (AMPc) et des activités de la protéine kinase A (PKA) dans la glande digestive, alors qu'ils ont été augmentés dans le manteau et les gonades.[113] Lors d'exposition

chronique au clofibrate 0,2 µg/L, le taux de triglycérides totaux a été réduit et celui des acides gras a augmenté chez la moule zébrée.[114] Une baisse de fécondité, diminution de croissance et altération du métabolisme ont été observées chez *D. magna* suite à une exposition de 9 jours à des concentrations de 110, 440 and 55 µg/L.[115] Lors d'exposition chronique de 21 jours, un changement au niveau de la nage des daphnies ainsi qu'une augmentation de l'expression de certains gènes reliés au stress (*Nrf2*, *Keap1*, *HO-1*, *GCLC*, *p53* and *PIG3*) en utilisant l'atorvastatine et le gemfibrozil à des concentrations de 5 µg/L, 50 µg/L, 500 µg/L et 5000 µg/L.[116]

Les antidépresseurs et les antibiotiques sont également présents dans les eaux de surface à des concentrations entre le ng/L et le µg/L.[117,118,119,120] Les antibiotiques, par leur bioactivité, peuvent avoir des effets préoccupants dans l'environnement. Chez *D. magna*, la survie a été diminuée lorsqu'elles étaient exposées chroniquement (21 jours) à 1 µg/L de tétracycline. Le microbiome intestinal de ces daphnies a également été affecté au niveau de la composition bactérienne.[121] L'antibiotique norfloxacine a eu des effets négatifs sur le rythme cardiaque ainsi que sur la vitesse de nage et d'alimentation des daphnies exposés à des concentrations de 25 000, 50 000 et 100 000 µg/L pendant 96h.[122] Pour ce qui est des antidépresseurs, ils ont le potentiel de causer des perturbations endocriniennes chez les espèces exposées.[123] Les effets des antidépresseurs peuvent se remarquer très rapidement chez les organismes exposés.[124] Demin *et al*. (2017) ont découvert que l'exposition à l'amitriptyline chez le poisson-zèbre entraînait une augmentation de la neurotransmission 5-HT dans le cerveau lorsqu'exposé 20 minutes à une concentration de 5 000 000 µg/L d'amitriptyline. Le comportement des poissons était également affecté (profondeur de nage).[125] Les moules zébrées peuvent être incitées à frayer de manière significative en quelques minutes après une exposition à la fluoxétine et à la fluvoxamine à des concentrations aussi faibles que 0,3 µg/L et 0,43 µg/L, respectivement.[126] Chez *D. magna ,* le nombre et la taille des nouveau-nés ont été affectés lors d'exposition à partir de la naissance à divers inhibiteurs de la recapture de la sérotonine tel que la fluoxétine et le 5,7-DHT à des concentrations de 3-40 µg/L pendant 8 jours.[127,128] Des effets similaires ont été observés lors d'exposition chronique de 21 jours à la sertraline ainsi que la venlafaxine à des concentrations de 0,3-100 µg/L.[129] Différents effets sous-létaux ont été observés chez *D. magna* lors d'expositions aux antidépresseurs. La vitesse d'alimentation des daphnies exposées 21 jours à une concentration de 195 µg/L de fluoxétine a été significativement augmentée. [130] Ding *et al*. (2017) ont observé des résultats similaires pour ce composé en plus de remarquer une inhibition de l'acétylcholinestérase lors d'expositions de 7 jours à des concentrations de 0,5 et 5 µg/L.[131] L'évaluation

de la toxicité des pharmaceutiques en utilisant des paramètres sous-létaux reste cependant limitée. Les tests d'immobilisation et de fertilité restent les plus utilisés.[132]

1.3.2 Les pesticides

Le groupe des pesticides est aussi un groupe très préoccupant puisqu'ils sont non seulement introduits volontairement dans l'environnement, mais aussi parce qu'ils sont utilisés dans le but d'éliminer les organismes nuisibles, sans être spécifiques à ceux-ci. Cela fait en sorte qu'un grand nombre d'espèces non visées peuvent être affectées par ces composés.[133] Les pesticides peuvent être séparés en trois classes. Les herbicides, généralement utilisés pour se débarrasser de plantes nuisibles comme les mauvaises herbes, sont considérés comme les moins risqués pour l'humain et l'environnement étant donné leurs modes d'action davantage ciblés pour les plantes tel que l'inhibition de la photosynthèse.[134] Viennent ensuite les fongicides, conçus pour éliminer les champignons contaminant principalement les plantes et les graines. Ces composés ont habituellement une toxicité de faible à moyenne (en termes de concentration ou dose nécessaire pour produire un effet néfaste), cependant certaines exceptions telles que les dérivés de l'acide carbamique et les benzimidazoles sont connus pour augmenter le risque de cancer et de problèmes de développement chez les organismes à cellule eucaryote.[135] La troisième et dernière classe de pesticides est les insecticides. Cette classe présente le plus grand risque pour l'environnement et les êtres humains étant donné leur large distribution dans l'environnement et le grand nombre de mécanismes d'actions existant et la non-spécificité de ceux-ci.[136] Par exemple, les herbicides atrazine, métolachlore, 2,4-D ont été retrouvés dans plusieurs régions du monde entre le ng/L et le bas µg/L.[137] Des insecticides tels que l'acéphate, le chlorpyrifos, l'heptachlore et l'imidaclopride ont également été retrouvés aux mêmes intervalles de concentrations dans les eaux de surface de différents pays incluant l'Espagne, l'Inde le Canada et le Chili. [138,139,140,141] Certains pesticides tels que le glyphosate ont été détectés à des concentrations élevé (µg/L) dans les eaux de surface de l'Amérique du Nord.[142] Étant donné le mode d'action de ces molécules, des effets tels que le développement de cancers et problèmes de reproduction peuvent subvenir pour les organismes exposés jusqu'à mener à un effondrement de la population et au débalancement de l'écosystème.[143] Ces concentrations peuvent aussi être létales pour des organismes tel que *D. magna*. En effet, les LC_{50} des composés pyrethroïdes, bifenthrine, cyfluthrine, cyhalothrine lambda et tralomethrine rapportées se situent entre 0,15 et 1.04 µg/L

après 48h d'exposition.[144] Fernandez-Casalderrey *et al*. (1994) ont remarqué qu'une exposition de 5h à des concentrations sous-létales (0,60 µg/L) de dianizon a réduit le taux d'ingestion d'algues de manière proportionnelle à la concentration utilisée lors de l'exposition.[145] L'insecticide fipronil a aussi affecté plusieurs comportements chez la daphnie tels que la vitesse de nage, la distance parcourue et l'activité des membres thoraciques lors d'expositions de 48h à des concentrations aussi faibles que 0,1 µg/L.[146]

1.3.3 Les hydrocarbures

Les hydrocarbures peuvent se retrouvent dans l'environnement suite à l'exploitation pétrolière et sont préoccupants dû à leur persistance et potentiel toxique.[147] Il existe différents types d'hydrocarbures pétroliers soit les paraffines (hydrocarbures saturés à chaînes droites ou ramifiées), les cycloparaffines (hydrocarbures saturés à un ou plusieurs cycles avec une ou plusieurs chaînes latérales de paraffine) et les aromatiques à un ou plusieurs cycles aromatiques (PAH) pouvant être liés à des cycles substitués et/ou chaînes latérales de paraffine).[148] Des concentrations de l'ordre du µg/L ont été observées dans différents cours d'eau de l'Amérique et de l'Afrique.[149,150,151,152,153] Des effets ont été rapportés chez la faune exposée, comme par exemple pour *Mytilus galloprovincialis*. Lorsqu'exposées 48h aux hydrocarbures polycycliques naphtalène (9,9 µg/L) et phénanthrène (37,6 µg/L), les embryons de moules ont vu leur développement ralentit.[154] Le benz[a]anthracène (BaA) et le 4-hydroxybenz[a]anthracène (4-OHBaA) à des concentrations de 10^{-8} et 10^{-7} M ont réduit le développement des spicules chez les oursins de mer (*Hemicentrotus pulcherrimus*) exposés à partir du stade embryonnaire. Le niveau de transcription des gènes associés au développement des spicules a également été réduit.[155] Carlson *et al*. (2002) ont démontré qu'une seule administration de 20 ou 200 µg/g de poids corporel de benzo(a) pyrene PC augmentait significativement la sensibilité du poisson médaka japonais à l'infection de la bactérie *Yersinia ruckeri*.[156] Les produits de photodégradation des PAHs se sont révélés toxiques pour *D. magna* à de faibles concentrations en nanomolaire causant une mortalité élevée lors des expositions.[157] Les hydrocarbures polyaromatiques N-hétérocycliques 1,10-phenanthroline (0,0625 µM) et benzo(h)quinoline (0,25 µM) ont aussi significativement réduit l'activité de l'enzyme glutathione peroxidase alors que l'acridine et le 1,10-phenanthroline (0,0625 µM) ont réduit celle de la glutathione S-transférase lors d'exposition de 96h chez *D. magna.*[158]

1.3.4 Les contaminants inorganiques

Les contaminants inorganiques tels que les métaux et anions inorganiques sont des composés naturels qui peuvent être relâchés ou concentrés en plus grande quantité dans l'environnement suite aux activités humaines. [159] Les sels sont facilement dégradés en d'autres composés, cependant, les métaux sont difficilement éliminables en plus d'être très toxiques, principalement pour le système nerveux. [160] Les métaux et métalloïdes les plus abondants sont le cadmium (Cd), le cuivre (Cu), le nickel (Ni), le plomb (Pb), le chrome (Cr), le zinc (Zn), l'arsenic (As) et le mercure (Hg). Parmi ces métaux, Cd et As sont extrêmement toxiques ; Hg, Pb, Ni sont modérément toxiques et Cu, Zn, Mn sont relativement moins toxiques.[161] Ces métaux ont été retrouvés à des concentrations allant jusqu'à 6,4 µg/L pour les métaux les plus toxiques (As, Cd, Hg, Pb, Ni) et jusqu'à plus de 1 g/L pour les moins toxiques (Cu, Zn, Mn) dans différents plans d'eau de l'Amérique du Nord, de l'Asie et de l'Afrique.[162,163,164,165] Les concentrations répertoriées étaient généralement beaucoup plus élevées dans les plans d'eau près de sites d'activités humaines, par exemple les mines et raffineries. Il est possible de voir une diminution des niveaux détectés depuis les années 90 en Amérique du Nord pour certains métaux étant donné la mise en place de programmes de suivi et de remédiation des sites, ainsi que l'amélioration des méthodes de traitement, mais les niveaux continuent cependant d'augmenter dans certains échantillons environnementaux dus à la croissance démographique et économique mondiale.[166] Bien qu'essentiels à la vie pour les fonctions de plusieurs enzymes, les métaux lourds ont également le potentiel de causer des effets néfastes lorsqu'en trop grandes concentrations dans l'organisme.[167,168] Le chrome(VI) s'est révélé génotoxique chez le poisson chat (*Heteropneustes fossilis*) lorsqu'exposé 96h à une concentration de 9000 µg/L.[169] Le plomb s'est également révélé neurotoxique pour le poisson chat africain (*Clarias gariepinus*) lorsqu'exposé 3 semaines à des concentrations de 6 µg/L.[170] Lorsqu'exposé à différente concentration de cadmium (5,0, 10,0 et 20,0 µg/L) pendant 8 jours, il a été remarqué que niveau d'ecdystéroïdes était augmenté chez *D. magna* et que cela affectait la mue des daphnies exposées.[171] Il a également été démontré que les expositions aux mélanges de métaux étaient toxiques à de plus faibles concentrations (27-63 fois plus faible) comparativement aux expositions à des métaux individuels. En effet, lors d'exposition à un mélange de Ni, Zn, Cu, et Cd à des concentrations de 0,5, 7,3, 3 et 16,5 µg/L, le taux de consommation de nourriture s'est vu réduit chez les daphnies exposées comparativement à celles exposées aux métaux aux mêmes concentrations, mais de manière individuelle.[172]

1.4 Objectifs du projet et définition des axes de recherche

Cette thèse est axée sur l'étude des effets au niveau moléculaire résultant de l'exposition chronique et sous-létale de *D. magna* à divers contaminants d'intérêt émergent. Les objectifs principaux de la thèse sont i) de développer des outils géniques et complémenter une méthode d'analyse des ecdystéroides afin d'évaluer les effets de contaminants d'intérêt et leur possibles modes d'action; ii) d'utiliser les outils métaboliques afin d'évaluer dans un contexte réel les effets d'un mélange de contaminants provenant d'eaux de ruissellement de sites miniers remédiés; et iii) de développer d'une méthode non ciblée afin d'étudier le profil lipidique de *D. magna* lors d'une période exposition au gemfibrozil, suivie par une période de récupération, ainsi que d'identifier des métabolites d'intérêt (bioindicateurs potentiels).

L'objectif du premier article a été d'observer les changements des niveaux d'ecdystéroïdes ainsi que la transcription des gènes liés à leur métabolisme en utilisant le fénarimol, un fongicide reconnu pour son activité anti-ecdysteroïdienne[173] ainsi que l'acide clofibrique et le gemfibrozil, deux composés hypolipémiants qui pourraient également affecter la synthèse des ecdystéroïdes. Ces paramètres pourraient être plus sensibles que la fertilité et la mortalité, et permettraient donc d'observer les effets d'une exposition plus rapidement.

Mon hypothèse pour le 1er article était qu'il est possible d'observer des effets sous-létaux (niveau d'ecdystéroïdes et transcription de gènes) à la suite d'une exposition au fénarimol chez les daphnies exposées lorsque comparé aux groupes contrôles. Ces approches moléculaires devraient permettre de détecter les effets d'exposition à des concentrations plus faibles que les méthodes traditionnelles. En utilisant des concentrations croissantes, en commençant par la concentration sans effet observé (0,113 mg/L), des changements pour le niveau d'ecdystéroïdes ainsi que le niveau d'expression des gènes associés à leur métabolisme devraient être de plus en plus marqués. La quantité et le niveau d'expression devraient diminuer avec l'augmentation de la dose de fénarimol. À un certain point, cette baisse entrainera une plus grande mortalité ainsi qu'une diminution du nombre de nouveau-nés comparativement aux groupes non exposés.

L'objectif du 2e article a été d'évaluer les effets de l'exposition de *D. magna* de manière aigüe et chronique à des eaux de ruissellement provenant de parcelles de sols contaminés aux hydrocarbures et

métaux servant à recouvrir des sites de résidus miniers. Une approche généralement utilisée pour les effluents de stations d'épuration (*whole effluent toxicity*, WET) a été appliquée et les paramètres classiques ainsi que la 20-hydroxyecdysone ont été mesurés en utilisant les mêmes techniques que dans le premier article afin d'évaluer le risque écotoxicologique sur le modèle *D. magna*.

Pour ce qui est de l'hypothèse pour ce 2e article, l'approche WET devrait permettre d'évaluer les eaux de ruissellement de manière réaliste et efficace. Tout dépendra des niveaux de métaux et d'hydrocarbures observés dans l'eau de ruissellement utilisée pour les tests d'exposition sur les daphnies. L'efficacité du traitement de phytoremédiation aura une grande importance sur ces niveaux. Si les niveaux de contaminant sont suffisamment élevés, des effets tels que la mortalité, diminution du nombre de nouveau-nés, ou changement au niveau de la concentration en 20E seront observés chez *D. magna.* Ces effets, si présents, augmenteront avec la durée de l'exposition. La mesure de la 20E pourrait permettre de déceler des changements autrement impossibles à déceler en utilisant les paramètres classiques de mortalité, fertilité et poids.

L'objectif du 3e article a été de développer une méthode permettant de quantifier le profil lipidique de manière non ciblée ainsi que l'identification des métabolites d'intérêt, afin d'évaluer les effets d'une exposition à un contaminant (gemfibrozil) chez *D. magna*. Peu d'études ont investigué le processus de récupération à la suite d'une exposition, spécialement lorsqu'il s'agit de mesures de paramètres non ciblées. Cette méthode a également été utilisée pour observer la capacité de récupération des daphnies après l'arrêt de l'exposition lorsque le contaminant a été retiré du milieu d'exposition. À la suite de l'identification de ratio *m/z* significativement différent entre les groupes exposés, contrôles et recouvrement, ces *m/z* ont ensuite été annotés afin de déterminer leur structure moléculaire et leur rôle physiologique.

Mon hypothèse pour le 3e article était que la lipidomique permettra d'observer des changements au niveau du profil lipidique suite à l'exposition au gemfibrozil. Étant donné la nature non ciblée de la méthode, plusieurs métabolites altérés devraient être identifiés, spécialement les lipides vu les méthodes et composés (hypolipémiants) utilisés. Pour certains, ce changement augmentera avec la durée de l'exposition, alors que pour d'autres, ils resteront stables dans le temps. Lors de l'arrêt de l'exposition,

certains de ces métabolites affectés devraient revenir au niveau initial. Il se pourrait cependant que la durée de la période de recouvrement soit trop courte, et que ce retour au niveau initial ne soit pas observé.

1.5 Bibliographie

[1] Khan S, Naushad Mu, Govarthanan M, Iqbal J, Alfadul SM. Emerging contaminants of high concern for the environment: Current trends and future research. Environmental Research. 2022 May 1;207:112609.

[2] Wang Z, Walker GW, Muir DCG, Nagatani-Yoshida K. Toward a Global Understanding of Chemical Pollution: A First Comprehensive Analysis of National and Regional Chemical Inventories. Environmental Science & Technology. 2020 Mar 3;54(5):2575-2584. doi: 10.1021/acs.est.9b06379.

[3] Sebastien Sauvé and Melanie Desrosiers : A review of what is an emerging contaminant. Chemistry Central Journal.2014; 8(1):15.

[4] Stephen R. Hughes, Paul Kay et Lee E. Brown : Global Synthesis and Critical Evaluation of Pharmaceutical Data Sets Collected from River Systems. Environmental Science & Technology. 2013; 47(2):661–677.

[5] Wilkinson JL, Boxall ABA, Kolpin DW, Leung KMY, Lai RWS, Galbán-Malagón C, et al. Pharmaceutical pollution of the world's rivers. Proceedings of the National Academy of Sciences. 2022 Feb 22;119(8):e2113947119.

[6] Essaid HI, Bekins BA, Cozzarelli IM. Organic contaminant transport and fate in the subsurface: Evolution of knowledge and understanding. Water Resources Research. 2015, 51, doi:10.1002/2015WR017121.

[8] Taheran M, Naghdi M, Brar SK, Verma M, Surampalli RY. Emerging contaminants: Here today, there tomorrow! Environmental Nanotechnology, Monitoring & Management. 2018 Dec 1;10:122–6.

[9] R. Carson. Silent Spring Houghton Mifflin, Boston, MA, USA (1962).

[10] Organisation for Economic Co-operation an Development: Test n°202: Daphnia sp., Acute Immobilisation Test. OECD Guidelines for the Testing of Chemicals, Section 2 : Effects on Botic System. 2004.

[11] Organisation for Economic Co-operation an Development: Test n°211 : *Daphnia magna* Reproduction Test. OECD Guidelines for the Testing of Chemicals, Section 2 : Effects on Botic System. 2008.

[12] Du J, Mei CF, Ying GG, Xu MY. Toxicity Thresholds for Diclofenac, Acetaminophen and Ibuprofen in the Water Flea *Daphnia magna*. Bulletin of Environmental Contamination and Toxicology. 2016 Jul;97(1):84-90.

[13] Mayer-Pinto M, Ledet J, Crowe TP, Johnston EL. Sublethal effects of contaminants on marine habitat-forming species: a review and meta-analysis. Biological reviews of the Cambridge Philosophical Society. 2020 Dec;95(6):1554-1573. doi: 10.1111/brv.12630.

[14] Fekete-Kertész I, László K, Terebesi C, Gyarmati BS, Farah S, Márton R, Molnár M. Ecotoxicity Assessment of Graphene Oxide by *Daphnia magna* through a Multimarker Approach from the Molecular to the Physiological Level including Behavioral Changes. Nanomaterials (Basel). 2020 Oct 16;10(10):2048. doi: 10.3390/nano10102048.

[15] Villarroel MJ, Sancho E, Ferrando MD, Andreu E. Acute, chronic and sublethal effects of the herbicide propanil on *Daphnia magna*. Chemosphere. 2003 Dec;53(8):857-64. doi: 10.1016/S0045-6535(03)00546-0.

[16] Host, G.E., R.R. Regal and C.E. Stephan. 1995. Analyses of acute and chronic data for aquatic life. U.S. Environmental Protection Agency, Office of Water, Office of Research and Development. Washington, D.C.

[17] Truhaut R. Ecotoxicology: Objectives, principles and perspectives. Ecotoxicology and Environmental Safety. 1977 Sep 1;1(2):151–73.

[18] Newman MC. Ecotoxicology: The History and Present Directions. In: Jørgensen SE, Fath BD, editors. Encyclopedia of Ecology [Internet]. Oxford: Academic Press; 2008. p. 1195–201. Available from: https://www.sciencedirect.com/science/article/pii/B9780080454054004316.

[19] Zhang C, Willett C, Fremgen T. Zebrafish: an animal model for toxicological studies. Current Protocols in Toxicology. 2003;Chapter 1:Unit1.7.

[20] Stadnicka-Michalak J, Schirmer K, Ashauer R. Toxicology across scales: Cell population growth in vitro predicts reduced fish growth. Science Advances. 2015 Aug 7;1(7):e1500302. doi: 10.1126/sciadv.1500302..

[21] Pollesch N, Etterson M, Flynn K, Kadlec S. Aquatic ecotoxicological population modeling using size-structured integral projection models. SETAC North America, Toronto, ON, CANADA, November 03 - 07, 2019.

[22] Ceschin S, Bellini A, Scalici M. Aquatic plants and ecotoxicological assessment in freshwater ecosystems: a review. Environmental Science and Pollution Research. 2021;28, 4975–4988.

[23] Langan LM, Harper GM, Owen SF, Purcell WM, Jackson SK, Jha AN. Application of the rainbow trout derived intestinal cell line (RTgutGC) for ecotoxicological studies: molecular and cellular responses following exposure to copper. Ecotoxicology. 2017 Oct;26(8):1117-1133.

[24] Piegorsch WW. Quantification of toxic response and the development of the median effective dose (ED50)—a historical perspective. Toxicology and Industrial Health. 1989;5(1):55–62.

[25] Franchetti Y, Anderson SJ, Sampson AR. An adaptive two-stage dose-response design method for establishing proof of concept. Journal of Biopharmaceutical Statistics. 2013;23(5):1124-54. doi: 10.1080/10543406.2013.813519.

[26] Gupta P. Chapter 3 - Mechanism of toxicity. In: Gupta P, editor. Illustrated Toxicology [Internet]. Academic Press; 2018. p. 107–29. Available from: https://www.sciencedirect.com/science/article/pii/B9780128132135000031.

[27] Alden CL, Beasley VR, Benjamin SA, Bertram TA, Black HE, Blakley PM, et al. Contributors. In: HASCHEK WM, ROUSSEAUX CG, WALLIG MA, editors. Handbook of Toxicologic Pathology (Second Edition) [Internet]. San Diego: Academic Press; 2002. p. xv–xviii. Available from: https://www.sciencedirect.com/science/article/pii/B9780123302151500004.

[28] Newman MC, Zhao Y. Ecotoxicology Nomenclature: LC, LD, LOC, LOEC, MAC. In: Jørgensen SE, Fath BD, editors. Encyclopedia of Ecology [Internet]. Oxford: Academic Press; 2008. p. 1187–93. Available from: https://www.sciencedirect.com/science/article/pii/B9780080454054004043.

[29] Waller WT, Allen HJ. Acute and Chronic Toxicity. In: Jørgensen SE, Fath BD, editors. Encyclopedia of Ecology [Internet]. Oxford: Academic Press; 2008. p. 32–43. Available from: https://www.sciencedirect.com/science/article/pii/B9780080454054003682.

[30] Combes RD. Progress in the Development, Validation and Regulatory Acceptance of In Vitro Methods for Toxicity Testing. 6th January 1948 – 13th March 2013. In: Reference Module in Chemistry, Molecular Sciences and Chemical Engineering [Internet]. Elsevier; 2013. Available from: https://www.sciencedirect.com/science/article/pii/B9780124095472024707.

[31] Zhang YN, Zhu XY, Wang WP, Wang Y, Wang L, Xu XX, Zhang K, Deng DG. Reproductive switching analysis of *Daphnia* similoides between sexual female and parthenogenetic female by transcriptome comparison. Scientific Reports. 2016 Sep 27;6:34241.

[32] Hebert PD, Ward RD. Inheritance during parthenogenesis in *Daphnia magna*. Genetics. 1972 Aug;71(4):639-42.

[33] Stollewerk A. The water flea *Daphnia*--a 'new' model system for ecology and evolution? Journal of Biological Chemistry. 2010;9(2):21.

[34] D. Ebert, Ecology, Epidemiology, and Evolution of Parasitism in *Daphnia*, National Library of Medicine (US), National Center for Biotechnology Information, Bethesda, Md, USA, 2005.

[35] Adhya D, Annuario E, Lancaster MA, Price J, Baron-Cohen S, Srivastava DP. Understanding the role of steroids in typical and atypical brain development: Advantages of using a "brain in a dish" approach. Journal of Neuroendocrinology. 2018 Feb;30(2):e12547.

[36] Hackney AC. Steroid Hormones. In: Mooren, F.C. (eds) Encyclopedia of Exercise Medicine in Health and Disease. Springer, Berlin, Heidelberg; 2012.

[37] Lari E, Jeong TY, Labine LM, Simpson MJ. Metabolomic analysis predicted changes in growth rate in *Daphnia magna* exposed to acetaminophen. Aquatic Toxicology. 2022 Aug;249:106233. doi: 10.1016/j.aquatox.2022.106233.

[38] Nijhout HF: Insect Hormones. Princeton University Press: Princeton; 1994.

[39] Nakagawa Y, Sonobe H. Subchapter 98A - 20-Hydroxyecdysone. In: Takei Y, Ando H, Tsutsui K, editors. Handbook of Hormones. San Diego: Academic Press; 2016. p. 560-e98A-2.

[40] Iga M, Sakurai S. Genomic and nongenomic actions of 20-hydroxyecdysone in programmed cell death. In: Smagghe G, ed. Ecdysone: Structures and Functions. New York, NY: Springer; 2009: 411-423.

[41] Takeuchi H, Rigden DJ, Ebrahimi B, et al. Regulation of ecdysteroid signalling during *Drosophila* development: identification, characterization and modelling of ecdysone oxidase, an enzyme involved in control of ligand concentration. Biochemical Journal. 2005;389:637-645.

[42] Lafont R, Dauphin-Villemant C, Warren JT, et al. Ecdysteroid chemistry and biochemistry. In: Gilbert LI, Iatrou K, Gill SS, eds. Comprehensive Molecular Insect Science. Amsterdam: Elsevier BV; 2005:125-195.

[43] Sonobe H, Ohira T, Ieki K, Ito Y, et al. Purification, kinetic characterization, and molecular cloning of a novel enzyme, ecdysteroid 22-kinase. Journal of Biological Chemistry. 2006;281:29513-24.

[44] Song Y, Villeneuve DL, Toyota K, Iguchi T, Tollefsen KE. Ecdysone Receptor Agonism Leading to Lethal Molting Disruption in Arthropods: Review and Adverse Outcome Pathway Development. Environmental Science & Technology. 2017 Apr 18;51(8):4142-4157.

[46] Ågerstrand M, Arnold K. Emerging investigator series: use of behavioural endpoints in the regulation of chemicals. Environmental Science: Processes & Impacts. 2020 Jan 1;22(1):49-65. doi: 10.1039/c9em00463g.

[47] European Chemicals Agency, Guidance on information requirements and chemical safety assessment. Part B: Hazard assessment, Version 2.1, 2011, http://echa.europa.eu/documents/10162/13643/ information_requirements_part_b_en.pdf.

[48] Chin R, Lee BY. Chapter 7 - Endpoints. In: Chin R, Lee BY, editors. Principles and Practice of Clinical Trial Medicine [Internet]. New York: Academic Press; 2008. p. 121–44. Available from: https://www.sciencedirect.com/science/article/pii/B9780123736956000077.

[49] Bownik A. Physiological endpoints in daphnid acute toxicity tests. Science of the Total Environment. 2020 Jan 15;700:134400. doi: 10.1016/j.scitotenv.2019.134400.

[50] Heid CA, Stevens J, Livak KJ, Williams PM. Real time quantitative PCR. Genome Research. 1996 Oct;6(10):986-94. doi: 10.1101/gr.6.10.986.

[51] Campos B, Fletcher D, Piña B, Tauler R, Barata C. Differential gene transcription across the life cycle in *Daphnia magna* using a new all genome custom-made microarray. BMC Genomics. 2018 May 18;19(1):370.

[52] Kim H, Koedrith P, Seo Y. Ecotoxicogenomic Approaches for Understanding Molecular Mechanisms of Environmental Chemical Toxicity Using Aquatic Invertebrate, *Daphnia* Model Organism. International Journal of Molecular Sciences 2015; 16: 12261–12287.

[53] Orsini L, Brown JB, Shams Solari O, Li D, He S, Podicheti R, Stoiber MH, Spanier KI, Gilbert D, Jansen M, Rusch DB, Pfrender ME, Colbourne JK, Frilander MJ, Kvist J, Decaestecker E, De Schamphelaere KAC, De Meester L. Early transcriptional response pathways in *Daphnia magna* are coordinated in networks of crustacean-specific genes. Molecular Ecology. 2018 Feb;27(4):886-897. doi: 10.1111/mec.14261.

[54] Poynton HC, Taylor NS, Hicks J, Colson K, Chan S, Clark C, Scanlan L, Loguinov AV, Vulpe C, Viant MR. Metabolomics of microliter hemolymph samples enables an improved understanding of the combined metabolic and transcriptional responses of *Daphnia magna* to cadmium. Environmental Science & Technology. 2011 Apr 15;45(8):3710-7. doi: 10.1021/es1037222.

[55] Gilbert LI, Rewitz KF. The Function and Evolution of the Halloween Genes: The Pathway to the Arthropod Molting Hormone. In: Smagghe G, editor. Ecdysone: Structures and Functions [Internet].

Dordrecht: Springer Netherlands; 2009. p. 231–69. Available from: https://doi.org/10.1007/978-1-4020-9112-4_9.

[56] Giraudo M, Douville M, Cottin G, Houde M. Transcriptomic, cellular and life-history responses of *Daphnia magna* chronically exposed to benzotriazoles: Endocrine-disrupting potential and molting effects. PLoS One. 2017 Feb 14;12(2):e0171763. doi: 10.1371/journal.pone.0171763.

[57] Sumiya, E., Ogino, Y., Miyakawa, H. et al. Roles of ecdysteroids for progression of reproductive cycle in the fresh water crustacean *Daphnia magna*. Frontiers in Zoology 11, 60 (2014). https://doi.org/10.1186/s12983-014-0060-2.

[58] Sumiya E, Ogino Y, Toyota K, Miyakawa H, Miyagawa S, Iguchi T. Neverland regulates embryonic moltings through the regulation of ecdysteroid synthesis in the water flea *Daphnia magna*, and may thus act as a target for chemical disruption of molting. Journal of Applied Toxicology. 2016 Nov;36(11):1476-85. doi: 10.1002/jat.3306.

[59] Ha MH, Choi J. Effects of environmental contaminants on hemoglobin gene expression in *Daphnia magna*: a potential biomarker for freshwater quality monitoring. Archives of environmental contamination and toxicology. 2009;57(2):330-7.

[60] Salesa B, Torres-Gavilá J, Ferrando-Rodrigo MD, Sancho E. Gene expression study alerted to possible impairment in *Daphnia magna* individuals as a consequence of exposure to sublethal concentrations of prochloraz. Chemosphere. 2022 Dec;308(Pt 1):136040. doi: 10.1016/j.chemosphere.2022.136040.

[61] Schnackenberg LK, Beger RD. The role of metabolic biomarkers in drug toxicity studies. Toxicol Mech Methods. 2008;18(4):301-11. doi: 10.1080/15376510701623193.

[62] Roberts LD, Souza AL, Gerszten RE, Clish CB. Targeted metabolomics. Current Protocols in Molecular Biology. 2012 Apr;Chapter 30:Unit 30.2.1-24.

[63] Gu H, Zhang P, Zhu J, Raftery D. Globally Optimized Targeted Mass Spectrometry: Reliable Metabolomics Analysis with Broad Coverage. Analytical Chemistry. 2015 Dec 15;87(24):12355-62.

[64] Borst DW, Bollenbacher WE, O'Connor JD, King DS, Fristrom JW. Ecdysone levels during metamorphosis of *Drosophila melanogaster*. Developmental Biology. 1974;39(1):308-16.

[65] Gharib B, de Reggi M. Changes in ecdysteroid and juvenile hormone levels in developing eggs of *Bombyx mori*. Journal of Insect Physiology. 1983;29(12):871-6.

[66] Porcheron P, Moriniere M, Grassi J, Pradelles P. Development of an enzyme immunoassay for ecdysteroids using acetylcholinesterase as label. Insect Biochemistry. 1989;19(2):117-22.

[67] Pascual N, Bellés X, Delbecque JP, Hua YJ, Koolman J. Quantification of ecdysteroids by immunoassay: comparison of enzyme immunoassay and radioimmunoassay. Z Naturforsch C. 1995 ; 11-12: 862-7.

[68] Gliscynski U, Delbecque J-P, Boecking D, Sedlmeier D, Dircksen H, Lafont R. Three new antisera with high-sensitivity to ecdysone, 3-dehydroecdysone and other a-ring derivatives: Production and characterization. European Journal of Entomology. 1995;92:75-9.

[69] Blais C, Blasco T, Maria A, Dauphin-Villemant C, Lafont R. Characterization of ecdysteroids in *Drosophila melanogaster* by enzyme immunoassay and nano-liquid chromatography-tandem mass spectrometry. Journal of chromatography B, Analytical technologies in the biomedical and life sciences. 2010;878(13-14):925-32.

[70] Miyashita M, Matsushita K, Nakamura S, Akahane S, Nakagawa Y, Miyagawa H. LC/MS/MS identification of 20-hydroxyecdysone in a scorpion (*Liocheles australasiae*) and its binding affinity to in vitro-translated molting hormone receptors. Insect biochemistry and molecular biology. 2011;41(12):932-7.

[71] Philippe Venne, Viviane Yargeau et Pedro A. Segura : Quantification of ecdysteroids
and retinoic acids in whole daphnids by liquid chromatography-triple quadrupole mass spectrometry. Journal of Chromatography A, 1438:57–64, mars 2016.

[72] Mu X, LeBlanc GA. Environmental antiecdysteroids alter embryo development in the crustacean *Daphnia magna*. The Journal of experimental zoology. 2002;292(3):287-92.

[73] Bodar CWM, Voogt PA, Zandee DI. Ecdysteroids in *Daphnia magna*: their role in moulting and reproduction and their levels upon exposure to cadmium. Aquatic Toxicology. 1990;17(4):339-50.

[74] Palma P, Palma VL, Matos C, Fernandes RM, Bohn A, Soares AM, et al. Effects of atrazine and endosulfan sulphate on the ecdysteroid system of *Daphnia magna*. Chemosphere. 2009;74(5):676-81.

[75] Giraudo M, Douville M, Cottin G, Houde M. Transcriptomic, cellular and life-history responses of *Daphnia magna* chronically exposed to benzotriazoles: Endocrine-disrupting potential and molting effects. PLOS ONE. 2017;12(2):e0171763.

[76] Schwarzenberger A, Courts C, von Elert E. Target gene approaches: Gene expression in *Daphnia magna* exposed to predator-borne kairomones or to microcystin-producing and microcystin-free Microcystis aeruginosa. BMC Genomics. 2009;10(1):527.

[77] Sobus JR, Wambaugh JF, Isaacs KK, Williams AJ, McEachran AD, Richard AM, et al. Integrating tools for non-targeted analysis research and chemical safety evaluations at the US EPA. Journal of Exposure Science & Environmental Epidemiology. 2018 Sep 1;28(5):411–26.

[78] Sun YV, Hu YJ. Chapter Three - Integrative Analysis of Multi-omics Data for Discovery and Functional Studies of Complex Human Diseases. In: Friedmann T, Dunlap JC, Goodwin SF, editors. Advances in Genetics [Internet]. Academic Press; 2016. p. 147–90. Available from: https://www.sciencedirect.com/science/article/pii/S0065266015000516.

[79] Ramirez T, Daneshian M, Kamp H, Bois FY, Clench MR, Coen M, Donley B, Fischer SM, Ekman DR, Fabian E, Guillou C, Heuer J, Hogberg HT, Jungnickel H, Keun HC, Krennrich G, Krupp E, Luch A, Noor F, Peter E, Riefke B, Seymour M, Skinner N, Smirnova L, Verheij E, Wagner S, Hartung T, van Ravenzwaay B, Leist M. Metabolomics in toxicology and preclinical research. ALTEX. 2013;30(2):209-25. doi: 10.14573/altex.2013.2.209.

[80] Beckonert O, Keun HC, Ebbels TM, Bundy J, Holmes E, Lindon JC, Nicholson JK. Metabolic profiling, metabolomic and metabonomic procedures for NMR spectroscopy of urine, plasma, serum and tissue extracts. Nature Protocol. 2007;2(11):2692-703. doi: 10.1038/nprot.2007.376.

[81] Taylor NS, Gavin A, Viant MR. Metabolomics Discovers Early-Response Metabolic Biomarkers that Can Predict Chronic Reproductive Fitness in Individual *Daphnia magna*. Metabolites. 2018 Jul 23;8(3):42. doi: 10.3390/metabo8030042.

[82] Garreta-Lara E, Gómez-Canela C, Campos B, Barata C, Tauler R, Lacorte S. Combined targeted/untargeted analytical and chemometric approaches in the characterization of *Daphnia magna* metabolomic changes under bisphenol A exposure. Microchemical Journal. 2021 Jun 1;165:106150.

[83] Smith MJ, Weber RJM, Viant MR. Spatially Mapping the Baseline and Bisphenol-A Exposed *Daphnia magna* Lipidome Using Desorption Electrospray Ionization—Mass Spectrometry. Metabolites. 2022; 12(1):33. https://doi.org/10.3390/metabo12010033.

[84] Kovacevic V, Simpson AJ, Simpson MJ. (1)H NMR-based metabolomics of *Daphnia magna* responses after sub-lethal exposure to triclosan, carbamazepine and ibuprofen. Comparative biochemistry and physiology Part D, Genomics & proteomics. 2016;19:199-210.

[85] Taylor NS, White TA, Viant MR. Defining the Baseline and Oxidant Perturbed Lipidomic Profiles of *Daphnia magna*. Metabolites. 2017 Mar 15;7(1):11 doi: 10.3390/metabo7010011.

[86] Smith MJ, Weber RJM, Viant MR. Spatially Mapping the Baseline and Bisphenol-A Exposed *Daphnia magna* Lipidome Using Desorption Electrospray Ionization-Mass Spectrometry. Metabolites. 2022 Jan 1;12(1):33. doi: 10.3390/metabo12010033.

[87] Scanlan LD, Loguinov AV, Teng Q, Antczak P, Dailey KP, Nowinski DT, Kornbluh J, Lin XX, Lachenauer E, Arai A, Douglas NK, Falciani F, Stapleton HM, Vulpe CD. Gene transcription, metabolite and lipid profiling in eco-indicator *daphnia magna* indicate diverse mechanisms of toxicity by legacy and emerging flame-retardants. Environmental Science & Technology. 2015 Jun 16;49(12):7400-10. doi: 10.1021/acs.est.5b00977. Epub 2015 Jun 1.

[88] Xu G, Stupak J, Yang L, Hu L, Guo B, Li J. Deconvolution in mass spectrometry based proteomics. Rapid Communications in Mass Spectrometry. 2018 May 30;32(10):763-774. doi: 10.1002/rcm.8103.

[89] Wang Y, Gu M. The Concept of Spectral Accuracy for MS. Analytical Chemistry. 2010 Sep 1;82(17):7055–62.

[90] Schymanski EL, Jeon J, Gulde R, Fenner K, Ruff M, Singer HP, et al. Identifying Small Molecules via High Resolution Mass Spectrometry: Communicating Confidence. Environmental Science & Technology. 2014 Feb 18;48(4):2097–8.

[91]Sungur Ş. Chapter 6 - Pharmaceutical and personal care products in the environment: occurrence and impact on the functioning of the ecosystem. In: Sarma H, Dominguez DC, Lee WY, editors. Emerging Contaminants in the Environment [Internet]. Elsevier; 2022. p. 137–57. Available from: https://www.sciencedirect.com/science/article/pii/B9780323851602000093.

[92] Ajiboye TO, Oladoye PO, Olanrewaju CA, Akinsola GO. Organophosphorus pesticides: Impacts, detection and removal strategies. Environmental Nanotechnology, Monitoring & Management. 2022 May 1;17.

[93] Dinh QT, Alliot F, Moreau-Guigon E, Eurin J, Chevreuil M, Labadie P. Measurement of trace levels of antibiotics in river water using on-line enrichment and triple-quadrupole LC-MS/MS. Talanta. 2011 Sep 15;85(3):1238-45. doi: 10.1016/j.talanta.2011.05.013.

[94] Pereira LC, de Souza AO, Franco Bernardes MF, Pazin M, Tasso MJ, Pereira PH, Dorta DJ. A perspective on the potential risks of emerging contaminants to human and environmental health. Environmental Science and Pollution Research. 2015 Sep;22(18):13800-23. doi: 10.1007/s11356-015-4896-6.

[95] Boxall AB, Rudd MA, Brooks BW. Pharmaceuticals and personal care products in the environment: what are the big questions?. Environmental Health Perspectives. 2012;120(9):1221-1229. doi:10.1289/ehp.1104477.

[96] Küster A, Adler N. Pharmaceuticals in the environment: scientific evidence of risks and its regulation. Philosophical Transactions of the Royal Society B. 2014 Nov 19;369(1656):20130587. doi: 10.1098/rstb.2013.0587.

[97] Wescoat, J. L.; White, G. F. Water for Life: Water Management and Environmental Policy; Cambridge University Press: Cambridge, U.K., 2003.

[98] Hughes SR, Kay P, Brown LE. Global Synthesis and Critical Evaluation of Pharmaceutical Data Sets Collected from River Systems. Environmental Science & Technology. 2013; 47 (2), 661-677.

[99] Hashim NH, Khan SJ. Enantioselective analysis of ibuprofen, ketoprofen and naproxen in wastewater and environmental water samples. J Chromatography A. 2011 Jul 22;1218(29):4746-54. doi: 10.1016/j.chroma.2011.05.046.

[100] Hanif H, Waseem A, Kali S, Qureshi NA, Majid M, Iqbal M, Ur-Rehman T, Tahir M, Yousaf S, Iqbal MM, Khan IA, Zafar MI. Environmental risk assessment of diclofenac residues in surface waters and wastewater: a hidden global threat to aquatic ecosystem. Environmental Monitoring and Assessment. 2020 Mar 2;192(4):204. doi: 10.1007/s10661-020-8151-3.

[101] Phong Vo HN, Le GK, Hong Nguyen TM, Bui XT, Nguyen KH, Rene ER, et al. Acetaminophen micropollutant: Historical and current occurrences, toxicity, removal strategies and transformation pathways in different environments. Chemosphere. 2019 Dec 1;236:124391.

[102] Wilkinson J, Hooda PS, Barker J, Barton S, Swinden J. Occurrence, fate and transformation of emerging contaminants in water: An overarching review of the field. Environmental Pollution. 2017 Dec 1;231:954–70.

[103] Rocco L, Frenzilli G, Fusco D, Peluso, Stingo V. Evaluation of zebrafish DNA integrity after exposure to pharmacological agents present in aquatic environments. Ecotoxicology and environmental safety. 2010; 73. 1530-6.

[104] Schwarz S, Schmieg H, Scheurer M, Köhler HR, Triebskorn R. Impact of the NSAID diclofenac on survival, development, behaviour and health of embryonic and juvenile stages of brown trout, *Salmo trutta f. fario*. Science of the Total Environment. 2017 Dec 31;607-608:1026-1036.

[105] Kwak K, Ji K, Kho Y, Kim P, Lee J, Ryu J, Choi K. Chronic toxicity and endocrine disruption of naproxen in freshwater waterfleas and fish, and steroidogenic alteration using H295R cell assay. Chemosphere. 2018 Aug;204:156-162.

[106] Grzesiuk M, Pijanowska J, Markowska M, Bednarska A. Morphological deformation of *Daphnia magna* embryos caused by prolonged exposure to ibuprofen. Environmental Pollution. 2020 Jun;261:114135.

[107] Michalaki A, Grintzalis K. Acute and Transgenerational Effects of Non-Steroidal Anti-Inflammatory Drugs on *Daphnia magna*. Toxics. 2023 Mar 29;11(4):320. doi: 10.3390/toxics11040320.

[108] Gómez-Oliván LM, Galar-Martínez M, García-Medina S, Valdés-Alanís A, Islas-Flores H, Neri-Cruz N. Genotoxic response and oxidative stress induced by diclofenac, ibuprofen and naproxen *in Daphnia magna*. Drug and Chemical Toxicology. 2014 Oct;37(4):391-9. doi: 10.3109/01480545.2013.870191.

[109] Desbiolles F, Malleret L, Tiliacos C, Wong-Wah-Chung P, Laffont-Schwob I. Occurrence and ecotoxicological assessment of pharmaceuticals: Is there a risk for the Mediterranean aquatic environment? Science of The Total Environment. 2018 Oct 15;639:1334–48.

[110] Zhang K, Zhao Y, Fent K. Cardiovascular drugs and lipid regulating agents in surface waters at global scale: Occurrence, ecotoxicity and risk assessment. Science of The Total Environment. 2020 Aug 10;729:138770.

[111] Patel M, Kumar R, Kishor K, Mlsna T, Pittman CUJr, Mohan D. Pharmaceuticals of Emerging Concern in Aquatic Systems: Chemistry, Occurrence, Effects, and Removal Methods. Chemical Reviews. 2019 Mar 27;119(6):3510–673.

[112] Deo RP. Pharmaceuticals in the Surface Water of the USA: A Review. Current Environmental Health Reports. 2014 Jun 1;1(2):113–22.

[113] Franzellitti S, Buratti S, Valbonesi P, Capuzzo A, Fabbri E. The β-blocker propranolol affects cAMP-dependent signaling and induces the stress response in Mediterranean mussels, *Mytilus galloprovincialis*. Aquatic Toxicology. 2011; 101, 299–308.

[114] Lazzara R, Fernandes D, Faria M, López JF, Tauler R, Porte C. Changes in lipid content and fatty acid composition along the reproductive cycle of the freshwater mussel *Dreissena polymorpha*: its modulation by clofibrate exposure. Science of the Total Environment. 2012; 432, 195–201.

[115] Dzialowski EM, Turner PK, Brooks BW. Physiological and reproductive effects of beta adrenergic receptor antagonists in *Daphnia magna*. Archives of Environmental Contamination and Toxicology. 2006; 50 (4), 503–510.

[116] Hu L, Ding R, Nie X. Comparison of toxic effects of atorvastatin and gemfibrozil on *Daphnia magna*. Comparative Biochemistry and Physiology C. 2022 Feb;252:109224. doi: 10.1016/j.cbpc.2021.109224.

[117] Loos R, Tavazzi S, Mariani G, Suurkuusk G, Paracchini B, Umlauf G. Analysis of emerging organic contaminants in water, fish and suspended particulate matter (SPM) in the Joint Danube Survey using solid-phase extraction followed by UHPLC-MS-MS and GC-MS analysis. Science of the Total Environment. 2017 Dec 31;607-608:1201-1212. doi: 10.1016/j.scitotenv.2017.07.039.

[118] Zuccato E, Castiglioni S, Fanelli R. Identification of the pharmaceuticals for human use contaminating the Italian aquatic environment. Journal of Hazardous Materials. 2005 Jul 15;122(3):205-9. doi: 10.1016/j.jhazmat.2005.03.001.

[119] Ben Y, Fu C, Hu M, Liu L, Wong MH, Zheng C. Human health risk assessment of antibiotic resistance associated with antibiotic residues in the environment: A review. Environmental Research. 2019 Feb;169:483-493. doi: 10.1016/j.envres.2018.11.040.

[120] Kovalakova P, Cizmas L, McDonald TJ, Marsalek B, Feng M, Sharma VK. Occurrence and toxicity of antibiotics in the aquatic environment: A review. Chemosphere. 2020 Jul;251:126351. doi: 10.1016/j.chemosphere.2020.126351.

[121] Akbar S, Gu L, Sun Y, Zhou Q, Zhang L, Lyu K, Huang Y, Yang Z. Changes in the life history traits of *Daphnia magna* are associated with the gut microbiota composition shaped by diet and antibiotics. Science of the Total Environment. 2020 Feb 25;705:135827.

[122] Pan Y, Yan SW, Li R. Lethal/sublethal responses of *Daphnia magna* to acute norfloxacin contamination and changes in phytoplankton-zooplankton interactions induced by this antibiotic. Scientific Reports. 2017, 7;40385. Doi: 10.1038/srep40385.

[123] Fong PP, Ford AT. The biological effects of antidepressants on the molluscs and crustaceans: a review. Aquatic Toxicology. 2014 Jun;151:4-13.

[124] Ford AT, Fong PP. The effects of antidepressants appear to be rapid and at environmentally relevant concentrations. Environmental Toxicology and Chemistry. 2016 Apr;35(4):794-8. doi: 10.1002/etc.3087.
[125] Demin KA, Kolesnikova TO, Khatsko SL, Meshalkina DA, Efimova EV, Morzherin YY, Kalueff AV. Acute effects of amitriptyline on adult zebrafish: Potential relevance to antidepressant drug screening and modeling human toxidromes. Neurotoxicology and Teratology. 2017 Jul;62:27-33.

[126] Fong PP. Zebra Mussel Spawning Is Induced in Low Concentrations of Putative Serotonin Reuptake Inhibitors. The Biological Bulletin. 1998 Apr;194(2):143-149.

[127] Campos B, Pina B, Fernandez-Sanjuan M, Lacorte S, Barata C. Enhanced offspring 408 production in *Daphnia magna* clones exposed to serotonin reuptake inhibitors and 4-nonylphenol. 409 Stage- and food-dependent effects. Aquatic Toxicology. 2012b, 109, 100-10.

[128] Campos B, Rivetti C. Antidepressant: Fluoxetine Affects Serotonin Neurons Causing Adverse Reproductive Responses in *Daphnia magna*. Environmental Science & Technology. 2016 50 ;11 : 6000-6007. DOI: 10.1021/acs.est.6b00826.

[129] Minguez L, Ballandonne C, Rakotomalala C, Dubreule C, Kientz-Bouchart V, Halm-Lemeille MP. Transgenerational effects of two antidepressants (sertraline and venlafaxine) on Daphnia magna life history traits. Environmental Science & Technology. 2015 Jan 20;49(2):1148-55. doi: 10.1021/es504808g.

[130] Stanley JK, Ramirez AJ, Chambliss CK, Brooks BW. Enantiospecific sublethal effects of the antidepressant fluoxetine to a model aquatic vertebrate and invertebrate. Chemosphere. 2007 Aug;69(1):9-16. doi: 10.1016/j.chemosphere.2007.04.080.

[131] Ding J, Zou H, Liu Q, Zhang S, Mamitiana Razanajatovo R. Bioconcentration of the antidepressant fluoxetine and its effects on the physiological and biochemical status in *Daphnia magna*. Ecotoxicology and Environmental Safety. 2017 Aug;142:102-109. doi: 10.1016/j.ecoenv.2017.03.042.

[132] Tkaczyk A, Bownik A, Dudka J, Kowal K, Ślaska B. Daphnia magna model in the toxicity assessment of pharmaceuticals: A review. Science of the Total Environment. 2021 Apr 1;763:143038. doi: 10.1016/j.scitotenv.2020.143038.

[133] de Albuquerque FP, de Oliveira JL, Moschini-Carlos V, Fraceto LF. An overview of the potential impacts of atrazine in aquatic environments: Perspectives for tailored solutions based on nanotechnology. Science of The Total Environment. 2020 Jan 15;700:134868.

[134] Duke SO. Overview of herbicide mechanisms of action. Environmental Health Perspectives. 1990 Jul;87:263-71. doi: 10.1289/ehp.9087263.

[135] Gupta KP. Chapter 45 - Toxicity of Fungicides. Veterinary Toxicology (Third Edition). Academic Press, 2018, Pages 569-580.

[137] de Souza RM, Seibert D, Quesada HB, de Jesus Bassetti F, Fagundes-Klen MR, Bergamasco R. Occurrence, impacts and general aspects of pesticides in surface water: A review. Process Safety and Environmental Protection. 2020 Mar 1;135:22–37.

[138] Climent MJ, Herrero-Hernández E, Sánchez-Martín MJ, Rodríguez-Cruz MS, Pedreros P, Urrutia R. Residues of pesticides and some metabolites in dissolved and particulate phase in surface stream water of Cachapoal River basin, central Chile. Environmental Pollution. 2019 Aug;251:90-101. doi: 10.1016/j.envpol.2019.04.117.

[139] Pascual Aguilar JA, Andreu V, Campo J, Picó Y, Masiá A. Pesticide occurrence in the waters of Júcar River, Spain from different farming landscapes. Science of The Total Environment. 2017 Dec 31;607-608:752-760. doi: 10.1016/j.scitotenv.2017.06.176.

[140] Lalonde B, Garron C. Temporal and Spatial Analysis of Surface Water Pesticide Occurrences in the Maritime Region of Canada. Archives of Environmental Contamination and Toxicology. 2020; 79, 12–22 .

[141] Khuman SN, Chakraborty P. Air-water exchange of pesticidal persistent organic pollutants in the lower stretch of the transboundary river Ganga, India. Chemosphere. 2019 Oct;233:966-974. doi: 10.1016/j.chemosphere.2019.05.223.

[142] Van Bruggen AHC, He MM, Shin K, Mai V, Jeong KC, Finckh MR, Morris JG Jr. Environmental and health effects of the herbicide glyphosate. Science of The Total Environment. 2018 Mar;616-617:255-268. doi: 10.1016/j.scitotenv.2017.10.309.

[143] Burns CJ, Juberg DR. Cancer and occupational exposure to pesticides: an umbrella review. International Archives of Occupational and Environmental Health. 2021 Jul;94(5):945-957. doi: 10.1007/s00420-020-01638-y.

[144] Mokry LE and Hoagland KD. Acute toxicities of five synthetic pyrethroid insecticides to *Daphnia magna* and *Ceriodaphnia dubia*. Environmental Toxicology and Chemistry, 1990; 9: 1045-1051. https://doi.org/10.1002/etc.5620090811.

[145] Fernández-Casalderrey A, Ferrando MD, Andreu-Moliner E. Effect of sublethal concentrations of pesticides on the feeding behavior of *Daphnia magna*. Ecotoxicology and Environmental Safety. 1994 Feb;27(1):82-9. doi: 10.1006/eesa.1994.

[146] Bownik A, Szabelak A. Short-term effects of pesticide fipronil on behavioral and physiological endpoints of *Daphnia magna*. Environmental Science and Pollution Research. 2021 Feb 26;28(25):33254–64. doi: 10.1007/s11356-021-13091-6.

[147] Ossai IC, Ahmed A, Hassan A, Hamid FS. Remediation of soil and water contaminated with petroleum hydrocarbon: A review. Environmental Technology & Innovation. 2020 Feb 1;17:100526.

[148] Logeshwaran P, Megharaj M, Chadalavada S, Bowman M, Naidu R. Petroleum hydrocarbons (PH) in groundwater aquifers: An overview of environmental fate, toxicity, microbial degradation and risk-based remediation approaches. Environmental Technology & Innovation. 2018 May 1;10:175–93.

[149] Bidleman TF, Castleberry AA, Foreman WT, Zaranski MT, Wall DW. Petroleum hydrocarbons in the surface water of two estuaries in the Southeastern united states. Estuarine, Coastal and Shelf Science. 1990 Jan 1;30(1):91–109.

[150] Ighariemu V, Belonwu DC, Wegwu MO. Level of Petroleum Hydrocarbons in Water and Sediments of Ikoli Creek Bayelsa State Nigeria. Toxicology and Environmental Health Sciences. 11, 114–119 (2019). https://doi.org/10.1007/s13530-019-0395-3.

[151] Naidu R, Nandy S, Megharaj M, Kumar RP, Chadalavada S, Chen Z, Bowman M. Monitored natural attenuation of a long-term petroleum hydrocarbon contaminated sites: a case study. Biodegradation. 2012 Nov;23(6):881-95. doi: 10.1007/s10532-012-9580-7.

[152] Asejeje GI, Ipeaiyeda AR, Onianwa PC. Occurrence of BTEX from petroleum hydrocarbons in surface water, sediment, and biota from Ubeji Creek of Delta State, Nigeria. Environmental Science and Pollution Research. 2021 Mar;28(12):15361-15379. doi: 10.1007/s11356-020-11196-y.

[153] Adeniji AO, Okoh OO, Okoh AI. Petroleum Hydrocarbon Profiles of Water and Sediment of Algoa Bay, Eastern Cape, South Africa. International Journal of Environmental Research and Public Health. 2017 Oct 20;14(10):1263. doi: 10.3390/ijerph14101263.

[154] Bellas J, Saco-Alvarez L, Nieto O, Beiras R. Ecotoxicological evaluation of polycyclic aromatic hydrocarbons using marine invertebrate embryo-larval bioassays. Marine Pollution Bulletin. 2008;57(6-12):493-502.

[155] Suzuki N, Ogiso S, Yachiguchi K, Kawabe K, Makino F, Toriba A, Kiyomoto M. Sekiguchi T, Tabuchi Y, Kondo T. Monohydroxylated polycyclic aromatic hydrocarbons influence spicule formation in the early development of sea urchins (*Hemicentrotus pulcherrimus*). Comparative Biochemistry and Physiology Part C. 2015;171:55–60.

[156] Carlson EA, Li Y, Zelikoff JT. Exposure of Japanese medaka (*Oryzias latipes*) to benzo[a]pyrene suppresses immune function and host resistance against bacterial challenge. Aquatic Toxicology. 2002 Mar;56(4):289-301.

[157] Lampi MA, Gurska J, McDonald KI, Xie F, Huang XD, Dixon DG, Greenberg BM. Photoinduced toxicity of polycyclic aromatic hydrocarbons to *Daphnia magna*: ultraviolet-mediated effects and the

toxicity of polycyclic aromatic hydrocarbon photoproducts. Environmental Toxicology and Chemistry. 2006 Apr;25(4):1079-87. doi: 10.1897/05-276r.1.

[158] Feldmannová M, Hilscherová K, Marsálek B, Bláha L. Effects of N-heterocyclic polyaromatic hydrocarbons on survival, reproduction, and biochemical parameters in *Daphnia magna*. Environmental Toxicology. 2006 Aug;21(4):425-31. doi: 10.1002/tox.20198.

[159] Goldscheider N. Chapter 8 - Delineation of spring protection zones. In: Kresic N, Stevanovic Z, editors. Groundwater Hydrology of Springs [Internet]. Boston: Butterworth-Heinemann; 2010. p. 305–38. Available from: https://www.sciencedirect.com/science/article/pii/B9781856175029000086.

[160] Tchounwou PB, Yedjou CG, Patlolla AK, Sutton DJ. Heavy metal toxicity and the environment. Experientia supplementum. 2012;101:133-64. doi: 10.1007/978-3-7643-8340-4_6.

[161] Gogoi L, Narzari R, Chutia RS, Borkotoki B, Gogoi N, Kataki R. Chapter Two - Remediation of heavy metal contaminated soil: Role of biochar. In: Sarmah AK, editor. Advances in Chemical Pollution, Environmental Management and Protection [Internet]. Elsevier; 2021. p. 39–63. Available from: https://www.sciencedirect.com/science/article/pii/S2468928921000022.

[162] Jiang J, Zhao G, Xu Y, Zhao J, Liu L, Liu C, et al. Occurrence and distribution characteristics of heavy metals in the surface water of Yongding River Basin, China. Environmental Science and Pollution Research. 2022 Mar 1;29(12):17821–31.

[163] Nguyen Duy H, Mendoza H, Nghiem D. Occurrence of Heavy Metals in Surface Water of Lam River. Conference: The 6th Vietnamese-Japanese Students' Scientific Exchange Meeting 2013.

[164] Dissanayke DMPNK, De Silva SNT, Pathmarajah S, Fernando CAN, Abeynayake NR, Kodagoda KADA, et al. Occurrence of heavy metals in surface water bodies in rice cultivation areas in Trincomalee district, Sri Lanka. Paddy and Water Environment [Internet]. 2022 Nov 4; Available from: https://doi.org/10.1007/s10333-022-00909-3.

[165] Akinnifesi, O. , Adesina, F. , Ogunwole, G. , Abiya, S. . Occurrence and Impact of Heavy Metals on Some Water, Land, Flora and Fauna Resources across Southwestern Nigeria. In: Nazal, M. K. , Zhao, H. , editors. Heavy Metals - Their Environmental Impacts and Mitigation [Internet]. London: IntechOpen; 2021.

[166] Zhou Q, Yang N, Li Y, Ren B, Ding X, Bian H, et al. Total concentrations and sources of heavy metal pollution in global river and lake water bodies from 1972 to 2017. Global Ecology and Conservation. 2020 Jun 1;22:e00925.

[167] WHO/FAO/IAEA. World Health Organization. Switzerland: Geneva; 1996. Trace Elements in Human Nutrition and Health.

[168] Harvey LJ, McArdle HJ. Biomarkers of copper status: a brief update. British Journal of Nutrition. 2008;99(S3):S10–S13.

[169] Ahmed MK, Kundu GK, Al-Mamun MH, Sarkar SK, Akter MS, Khan MS. Chromium (VI) induced acute toxicity and genotoxicity in freshwater stinging catfish, *Heteropneustes fossilis*. Ecotoxicology and Environmental Safety. 2013 Jun;92:64-70.

[170] Olojo E. Histopathology of the gill and liver tissues of the African catfish *Clarias gariepinus* exposed to lead. African Journal of Biotechnology, 2005; 4(1): 117-122.

[171] Bodar CWM, Voogt PA, Zandee DI. Ecdysteroids in *Daphnia magna*: their role in moulting and reproduction and their levels upon exposure to cadmium. Aquatic Toxicology, Volume 17, Issue 4, 1990, Pages 339-350,ISSN 0166-445X.

[172] Lari E, Gauthier P, Mohaddes E, Pyle GG. Interactive toxicity of Ni, Zn, Cu, and Cd on *Daphnia magna* at lethal and sub-lethal concentrations. Journal of Hazardous Materials. 2017 Jul 15;334:21-28. doi: 10.1016/j.jhazmat.2017.03.060.

[173] Mu X, Leblanc GA. Cross communication between signaling pathways: juvenoid hormones modulate ecdysteroid activity in a crustacean. The Journal of Experimental Zoology. 2004 Oct 1;301(10):793-801. doi: 10.1002/jez.a.104. PMID: 15449346.

CHAPITRE 2. LA 20-HYDROXYECDYSONE ET SES GÈNES ASSOCIÉS SONT-ILS DE POTENTIELS BIOMARQUEURS LORS D'EXPOSITIONS SOUS-LÉTHALES À DES CONTAMINANTS AFFECTANT LES LIPIDES?

Hugo Alarie [1], Nadia Côté [2], Luc Gaudreau [2], Magali Houde [3], Pedro A. Segura [1,*]

[1] Department of Chemistry, Université de Sherbrooke, Sherbrooke, QC, Canada, J1K 2R1

[2] Department of Biology, Université de Sherbrooke, Sherbrooke, QC, Canada, J1K 2R1

[3] Aquatic Contaminants Research Division, Environment and Climate Change Canada, Montreal, QC, Canada, H2Y 2E5

2.1. Notes préliminaires

Cet article a été accepté dans le journal *Environmental Science and Pollution Research* le 13 novembre 2023.

2.2. Contributions des auteurs

J'ai moi-même réalisé le développement et design de l'étude, l'entièreté des expériences, l'analyse de données, l'interprétation des résultats et la rédaction de la première version de l'article pour ce chapitre. Pedro A. Segura m'a également conseillé concernant le design de l'étude. Nadia Coté m'a aidée à développer les méthodes d'analyse des gènes de la famille Halloween qui ont été réalisés dans le laboratoire de Luc Gaudreau. Magali Houde et Pedro A. Segura ont participé à la correction et rédaction de la version finale de cet article.

Are 20-hydroxyecdysone and related genes potential biomarkers of sublethal exposure to lipid-altering contaminants?

2.3. Abstract

In *Daphnia magna*, 20-hydroecdysone (20E) is the main molting hormone and its metabolism is of interest to identify new biomarkers of exposure to contaminants. The present study aimed to: i) assess baseline levels of 20E and transcription levels of four related-genes (shade, neverland, ultraspiracle and ecdysteroid receptor); and ii) evaluate effects in *D. magna* after 21 days of exposure to fenarimol (anti-ecdysteroid) and a mixture of gemfibrozil and clofibric acid (lipid20 lowering drugs) at sublethal concentrations. Endpoints included transcription of the target genes and quantification of 20E, mortality and reproduction of daphnids.

Baseline results showed that average responses were relatively similar and did not vary more than 2-fold. However, intra-day variation was generally high and could be explained by sampling individuals with slightly different stages of their development.

Exposure tests indicated a significant decrease in daphnid reproduction following chronic exposure to a concentration of 565 µg/L of fenarimol. However, no difference was observed between the control and exposed groups for any of the investigated genes, nor for the levels of 20E after 21 days of exposure. Following exposition to gemfibrozil and clofibric acid at 1 µg /L, no changes were observed for the measured parameters. These results suggest that changes in transcription levels of the target genes and concentrations of 20E may not be sensitive endpoints that can be used as biomarkers of sublethal exposure to the target compounds in D. magna. Measuring multiple time points instead of a single measure as well as additional molecular endpoints obtained from transcriptomic and metabolomic studies could afford more insights on the changes occurring in exposed daphnids to lipid-altering compounds and identify efficient biomarkers of sublethal exposure.

Keywords: ecdysteroids; gene transcription; sublethal effects; crustaceans; ecotoxicology

2.4. Introduction

Currently, more than 350,000 chemicals and mixtures have been registered for production and use around the world (Wang *et al*. 2020). The high production volumes and mobility of compounds lead to their detection in surface waters worldwide at trace concentrations (ng/L to µg/L). Exposure and accumulation in aquatic species can also adversely impact ecosystems (Bradley *et al*. 2017, Hughes *et al*. 2013). Some of these compounds, such as pesticides, have been created to specifically affect the endocrine system of arthropods and thus are toxic to nontarget aquatic organisms such as crustaceans (Jansen *et al*. 2011, Mnif *et al*. 2011). Pharmaceuticals are also susceptible to causing subtle changes in nontarget species such as feminization and impacting the behavior of different aquatic species (Richmond *et al*. 2017).

Classic toxicity tests using endpoints such as survival, growth or reproduction are usually not sensitive enough to detect the effects of these compounds at environmental concentrations (Daughton &Ternes 1999). However, adverse effects such as changes in behavior, metabolic profile or gene transcription have been observed at sublethal levels (De Lange *et al*. 2006, Houde *et al*. 2013, Kovacevic *et al*. 2016, Wagner *et al*. 2017). While in-silico techniques based on molecular modeling and docking simulations (Hirano *et al*. 2020, Li *et al*. 2023) can be useful to predict interactions of contaminants with key enzymes, in-vivo studies are necessary to identify new biomarkers of sublethal effects in order to assess biological changes in aquatic organisms chronically exposed to low concentrations of contaminants. An interesting model to look for those biomarkers is *Daphnia magna.*

Daphnia is a genus of freshwater crustaceans widely used in ecotoxicology as model species to test the toxicity of chemicals an even wastewaters (Tonkes *et al*. 1999) because of its easy culture in the laboratory, small size as well as its parthenogenetic (clonal) reproduction (Dodson &Hanazato 1995). Daphnids occupy a key role in lentic ecosystems as filter feeders and prey of insects and small fishes (Miner *et al*. 2012). Reproduction and development in daphnids are regulated by ecdysteroids, a group of hormones derived from cholesterol. 20-hydroxyecdysone (20E) is the main molting hormone in crustaceans and other arthropods and is also involved in the reproduction process and embryonal development of daphnids (LeBlanc 2007). Levels of 20E increase and decrease between successive molts in a pulsative manner, inducing ecdysis through the activation of the ecdysone nuclear receptor (EcR) (Martin-Creuzburg *et al*. 2007, Song *et al*. 2017). 20E metabolism is regulated by a group of genes named

the Halloween family. These genes encode for cytochrome P450 (CYP450) enzymes that regulate the biosynthesis of ecdysteroids from cholesterol (Figure 3) (Rewitz &Gilbert 2008).

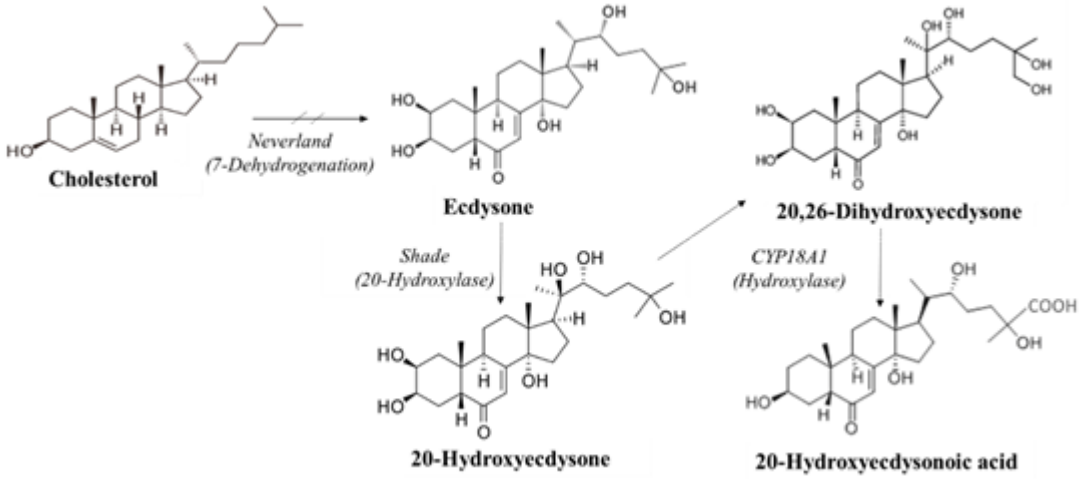


Figure 3. Biosynthesis of 20E from cholesterol in *D. magna*. Associated genes are presented in italics. Multiple steps have been omitted between cholesterol and ecdysone synthesis to simplify the figure. Image adapted from LaFont *et al*. (2012).

Given their importance for the survival and reproduction of *D. magna*, ecdysteroids are potential targets for biomarker studies. Only a few publications have investigated changes in concentrations of ecdysteroids in *D. magna* after exposure to contaminants. Bodar *et al*. (1990) reported an increase in ecdysteroid titers of 257% in adult females following exposure to 20 µg/L of cadmium for eight days and used enzyme immunoassay to quantify ecdysteroids as ecdysone equivalents. They speculated that the observed effect of cadmium on ecdysteroids was the product of cadmium interference with metallo-enzymes involved in the molting process. Mu and LeBlanc (2002) exposed neonates to 497 µg/L of fenarimol, a fungicide principally used on ornamental plants and vegetables, and observed a diminution of around 26 % in ecdysteroid levels in exposed neonates and an induced delay for the first and second molting in a dose-dependent manner. These effects were somewhat reverted with the co-administration of 20E, which demonstrated the anti-ecdysteroid activity of fenarimol.

Baseline assessment of both key metabolites and transcription of genes taking part in the synthesis of those metabolites and can help to differentiate natural fluctuations from responses induced by exposure

to exogenous compounds. Therefore, in order to understand the effects of contaminants on the metabolism of 20E it is important to determine first baseline levels of 20E as well as of genes associated with its synthesis and activity.

At the gene level, baseline information of Halloween genes transcription was evaluated during normal growth in daphnids by Sumiya *et al*. (2014). The transcription level of the evaluated genes fluctuated between 2 and 3-fold during an 80-hr period, equivalent to the duration of molting in adult daphnids.

At the metabolite level, two studies study have reported ecdysteroid baseline levels in adult *D. magna* during a single molt cycle (Martin-Creuzburg *et al*. 2007, Sumiya *et al*. 2016). In those studies, the authors used immunoassay-based techniques to quantify ecdysteroids, and they both observed an increase in basal levels of ecdysteroids between ≈ 30 to 50 h after ecdysis followed by return to basal levels ≈ 40 hours later. However, reported values by these studies diverge. Martin-Creuzburg *et al*. (2007) used a radioimmunoassay technique and observed that free ecdysteroids increased from ≈5-10 pg per individual at the end of the first molt to a maximum of ≈ 250 pg/individual 38-42 h later. Then ecdysteroids declined back to basal levels before the second molt. Sumiya *et al*. (2016) used enzyme-linked immunosorbent assay to measure ecdysteroids but maximum levels were only about ≈ 2.4 fg/ind. These differences may be due to the distinct techniques and sampling protocols employed. To the author's knowledge, the only study that has quantified 20E in *D. magna* is the work of Venne *et al*. (2016). The authors of that paper used liquid chromatography-triple quadrupole mass spectrometry (LC-QqQMS) to quantify 20E in adult daphnids (19 ± 8 pg/ind). However only one measure was performed. Therefore, up to now the baseline of 20E in multiple molt cycles of *D. magna* is unknown.

The working hypothesis of the present work was that changes in levels of 20E and/or changes in the transcription of 20E-related genes (shade, neverland, ultraspiracle and ecdysteroid receptor) would be observed in *D. magna* after exposure to low concentrations of lipid-altering organic contaminants. Fenarimol, a fungicide demethylation inhibitor and known ecdysteroid inhibitor, as well as a mixture of gemfibrozil and clofibric acid, two lipid-lowering molecules commonly found in environmental waters were chosen as target compounds. Thus, the present study aimed to: i) assess the baseline of 20E and transcription of the genes associated with 20E regulation in *D. magna* over a 21-day and ii) evaluate the effects following a 21-day exposure to the target compounds on the concentration of 20E, expression of target genes and life history parameters (i.e., fertility and mortality).

2.5. Materials and Methods

2.5.1. Reagents and chemicals

Standards of 20E (catalog number: SC-202407A, >98 % purity) and makisterone A (SC-202218A, >95 % purity) were obtained from Santa Cruz Biotech (Dallas, TX). Makisterone A is non-endogenous ecdysteroid with 28 carbon atoms that differs from all 27 carbon moulting hormones like ecdysone and 20E by having a methyl group at the C-24 position. This compound responds similarly to 20E during extraction and LC-QqQMS analysis; it was therefore used as an internal standard for 20E quantification. Additional purification of makisterone A to remove 20E and ecdysone impurities was done following the method described by Venne *et al*. (2016). Water, methanol (MeOH), acetonitrile (ACN), methyl *tert*-butyl ether (MTBE), formic acid (FA) and acetic acid (AA) were LC or LC-MS grade and were purchased from Thermo Fisher Scientific (Waltham, MA). The derivatization reagent hydroxylamine hydrochloride (159417-100G, >99%), fenarimol (45484-250MG, ≥99%), its internal standard nuarimol (31116, ≥99%), clofibric acid (90323-100MG, ≥99%) and gemfibrozil (91823-100MG, ≥98.5%) were purchased from Sigma Aldrich (St-Louis, MO). Deuterated standards, clofibric acid-d3 (D-6005, 98%) and gemfibrozil-d6 (D-6144, 99%) were purchased from CDN Isotopes (Pointe-Claire, QC, Canada). Stock solutions of 20E were prepared at 0.1 mg/mL in MeOH and working solutions, prepared in 1 % FA in MeOH and stored at -20 °C. The aqueous solution of hydroxylamine hydrochloride solution (100 mg/mL) was prepared before each derivatization. The main properties of the target compounds are found in Table S1 (Annexes)

2.5.2. *Daphnia magna* culture

D. magna parent stock originated from ephippia acquired from EBPI Canada (Burlington, ON) and maintained in the laboratory in synthetic Moderately Hard Reconstituted Water (Environment Canada 1990). Cultures were kept at 20 ± 1 °C with a 16-h light: 8-h dark photoperiod and were renewed every 2 months using neonates from 3rd to 5th broods. Daphnids were fed every second day with 2 mL of green algae *Raphidocellis subcapitata* (≈3.85 × 10^5 cells/mL). Microalgae were cultured in Bold Modified Basal Freshwater medium from Sigma-Aldrich under the same laboratory conditions described above for

D. magna. Algae were regularly harvested while still in the exponential growth phase and inoculated in fresh medium. All experiments were initiated with neonates (>24 h old), born between the 3rd and 5th broods, derived from a healthy parent stock.

2.5.3. Baseline levels of 20E and transcription of target genes

The first experiment aimed to evaluate the gene transcription of target genes and 20E levels over a 21-day period, the duration of standardized chronic toxicity tests for *D. magna*. During this experiment, daphnids were maintained under the conditions described above. Every second day, starting from day 9 (organisms at maturity, size needed for LC-QqQMS analysis) until day 21, three replicates of 15 daphnids were sampled for 20E quantification and stored in MeOH at -80℃ until analysis. Five replicates of one daphnid each were also collected for gene transcription analysis and stored in trizol at -80℃ until analysis.

2.5.4. Chronic exposure to fenarimol, gemfibrozil and clofibric acid

Three different exposure tests were performed to evaluate if changes in levels of 20E would be observed in D. magna after exposure to low concentrations of lipid-altering organic contaminants.

For test Nº 1, gemfibrozil and clofibric acid were used at a concentration of 1 µg/L each which is of the same order of magnitude as the maximum reported environmental concentrations in surface waters reported so far for these two compounds (Ebele *et al*. 2017). For tests Nº 2 and Nº 3, two concentrations of fenarimol (113 µg/L and 565 µg/L) corresponding to the no observed adverse effect concentration (NOAEC) and the lowest observed effect concentration (LOEC) for reduced fertility in *D. magna*, respectively, were employed. Thus, the employed exposure concentrations were selected 209 to reflect environmental levels (test Nº 1), as well as reported sublethal concentrations (test Nº 2 and Nº 3) that are1 representative of worst-case exposure scenarios such as contaminated discharges due to runoff events near agricultural fields (Lefrancq *et al*. 2017).

In all assays, neonates (<24 h) were exposed for 21 days to the contaminant following the OECD guidelines (OECD 2008). Tests were performed thrice using 10 replicate groups (5 control, 5 exposed)

of 25 daphnids each. Temperature was kept at 20 ± 1 °C using an incubator, light intensity was 2000 ± 70 lux and a 16-h light: 8-h dark photoperiod was maintained. On day 1 of the tests, neonates from the control groups were transferred in 2 L beakers filled with culture medium to which 40 µL of MeOH was added. Neonates from the exposed groups were transferred to 2 L beakers filled with culture medium containing 40 µL of MeOH containing the test compound. Medium was renewed 3 times a week. When performing these renewals, daphnids were sorted by size using a series of sieves, according to a standard protocol used by the Ministry of the Environment of Quebec, Canada (Centre d'expertise analytique environnementale du Québec 2011). Adults were collected on a 900 µm sieve, juveniles on a 560 µm sieve and neonates on a 300 µm sieve. Offspring (juveniles and neonates) were counted and then eliminated; only adults were transferred to the renewed solutions. At each media renewal, survival was determined by counting and averaging number of non-immobilized adults and reproduction was determined by counting and averaging the number of offspring per adult. Dormant eggs or males were never observed throughout the experiments, which indicated that experimental conditions were adequate to support a healthy population of *D. magna*. Daphnids were fed with 2 mL of a *Raphidocelis subcapitata* algae solution at every media renewal and the number of offspring and mortality was recorded. A maximum of 18.9 % of mortality was observed in the exposure experiment, thus respecting the sublethality criteria guidelines of the OECD (2008). Detailed mortality curves can be found in Figures S1 and S2 (Annexes). At the end of the exposure period, *D. magna* adults were collected in MeOH 1 % FA in MeOH or trizol for 20E levels and gene transcription analysis, respectively, and stored at -80℃ until analysis.

2.5.5. Quantification of 20E using liquid chromatography-triple quadrupole mass spectrometry

20E was extracted and quantified from whole daphnids (15-25 individuals) following the method developed by Venne *et al*. (2016) with minor modifications. Briefly, *D. magna* were sorted by size and adult individuals (>900 µm) were collected on a tissue strainer before being washed with deionized water (18 MΩ). Adult daphnids were homogenized with a mortar and a pestle and sonicated in an ultrasonic bath for 15 min in a volume of 1 mL of 1% FA in MeOH inside a 1.5 mL Eppendorf tube. Next, an aliquot of 800 µL was transferred in a glass tube with 50 µL of internal standard solution (makisterone A) and the solvent was evaporated under a gentle flow of $N_{2(g)}$. 20E and makisterone A were then derivatized to their oxime analogues with 1 mL of a hydroxylamine hydrochloride solution (100 mg/mL). A liquid-

liquid extraction with 2×1.5 mL of MTBE was carried out with a vortex mixer. The test tubes were then placed at -20 °C until the water froze, and the organic layer (unfrozen) containing the derivatized analyte and its internal standard was transferred to a test tube and evaporated to dryness. Finally, 250 µL of MeOH were added and the samples were transferred to vials for analysis.

The 20E concentration in daphnid extracts was quantified by LC-QqQMS using an Acquity UPLC system coupled to a Xevo TQ-S micro triple quadrupole mass spectrometer, both from Waters (Billerica, MA). The method was adapted from Venne *et al*. (2016). Briefly, the compounds were separated on a reversed-phase column Acquity UPLC Cortecs C18+ from Waters of dimensions 50×2.1 mm and 1.6 µm particle size. The mobile phase was composed of eluent A (H_2O containing 0.1% *v/v* of AA) and eluent B (mixture of MeOH-ACN 3:2 *v/v*, containing 0.1% *v/v* of AA). The elution gradient started with 5% of B, increasing to 55% in 7.9 min, rising immediately to 100% of B and hold for 2 min, then back to initial conditions for column re-equilibration (2.1 min). The sample injection volume was set to 10 µL. Electrospray in the positive mode was used as ionization source and the mass spectrometer was operated in the multiple reaction monitoring (MRM) mode. MRM transitions used were *m/z* 478.3 → *m/z* 316.3 for 20E oxime and *m/z* 492.2 → *m/z* 316.1 for makisterone A oxime. The concentration of 20E was reported as the average mass per adult individual. The total number of *D. magna* used and the number of pooled groups varied among the tests due to differences in the mortality of daphnids at the end of the 21-day period.

Quality control (QC) samples were used in order to determine the deviation percentage of the method and thereby verify the accuracy and precision of the measured concentrations. Three concentrations of QC samples were used: low (≈100 pg/mL), medium (≈300 pg/mL) and high (≈500 pg/mL). Matrix effects correction was done by using extracts of frozen daphnids at 50 mg/mL in 1% F.A. in MeOH. These extracts were used to prepare QC samples. Results were considered acceptable if the QC samples were within ± 20 % of the expected value.

2.5.6. Chemical stability

The chemical stability of fenarimol, gemfibrozil and clofibric acid was evaluated during the tests between two medium renewals. Nuarimol, gemfibrozil-d_6 and clofibric acid-d_4 were used as internal standards. Aliquots of 50 mL were sampled immediately after and before media renewal at three different moments

during the testing. Extraction was performed using Strata-X solid-phase extraction cartridges (polymeric reversed phase with a particle diameter of 33 µm, 200 mg of bed mass and 6 mL of volume) from Phenomenex (Torrance, CA). Quantification was carried out using the same LC-QqQMS system described previously. The entire protocol for the analysis of fenarimol is detailed in the Supporting Information.

2.5.7. RNA extraction

Total RNA was extracted from single *D. magna* using a Trizol-RNeasy Plus Mini Kit hybrid protocol. Briefly, daphnids were homogenized in 500 µL of trizol with a micro pestle before being sonicated for 5 min. A volume of 200 µL of chloroform was added and the tubes were centrifuged at 10,000 g for 18 min at 4°C. The top layer was then transferred on a RNeasy Plus Mini column from Qiagen Canada (Montreal, QC) and the manufacturer's instructions were followed from that point. Extractions were performed on 10 independent biological replicates for exposures to 113 µg/L and 15 replicates for the 565 µg/L treatment with fenarimol.

RNA was quantified with a NanoDrop ND-000 spectrophotometer from Thermo Fisher Scientific (Waltham, MA). All samples had a 260 nm/280 nm ratio > 1.8 and a concentration > 54 ng/µL. Chloroform (HPLC, 99%) and ethanol (98%) were purchased from Sigma-Aldrich Canada (Oakville, ON). Random hexamer 5'-NNN NNN-3' made for L. Gaudreau (IDT, lot 213632751) was used for reverse transcription in addition to dNTP mix (10mM) from KAPA Biosystems (Cape Town, South Africa), Moloney Murine Leukemia Virus (M-Mulv reverse transcriptase, 200,000 U/mL, lot 12R091118) and 10×M-MulV RT Buffer (lot 081618) from Qiagen and sterile water (Molecular grade) from Wisent (St-Bruno, QC). Advanced qPCR Mastermix (lot 800431) from Wisent, 96 well plates (Low profile, Clear) from Axygen (Union City, CA) and sealing tapes (optically clear) from Sarstead (Newton, NC) were used for qPCR reaction.

2.5.8. Real-time quantitative PCR (RT-qPCR)

RT-qPCR analyses were conducted on four selected transcripts of the target genes shade (*shd*), neverland (*nvd*), ecdysteroid receptor (*ecr*), and ultraspiracle (*usp*) and normalized with a combination of the most suitable reference genes cyclophilin (*cyc*), tubulin α (*tuba*), ubiquitin (*ubi*), elongation factor 2 (*eef2*), and glyceraldehyde 3-phosphate dehydrogenase (*gapdh*). Genes *ubi*, *eef2*, and *gapdh* were used for the baseline assessment experiment. Genes *ubi*, *tuba* and *eef2* were used for test N° 2 (exposure to 113 µg/L of fenarimol). Genes *ubi*, *tuba* and *gapdh* were used for test N° 3 (exposure to the 565 µg/L of fenarimol). Primer-specific efficiencies and sequences are listed in Table S2 (Annexes) along with sequences.

Total RNA (300 ng) was reverse transcribed using M-Mulv reverse transcriptase according to the manufacturer's instructions (Qiagen 2023). After dilution of the cDNA samples (1/8 dilution), analyses were then carried out on a CFX96 Connect real-time PCR detection system from Biorad (Hercules, CA) using Advanced qPCR Mastermix with a final concentration of 400 nM for each primer in a total reaction volume of 10 µL. The qPCR conditions were as follows: 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 15 s, and 68 °C for 15 s. Each reaction was run in technical triplicate and the mean of all independent biological replicates was calculated. All results were normalized using mRNA level of the reference genes recommended by geNorm depending on the exposition test. Relative expression values were calculated by the qBase relative quantification software (Hellemans *et al*. 2007). Microcapillary gel electrophoresis (Figure S3) and melt curve analysis were performed on amplicons to verify the specificity of the amplification using a 2100 Bioanalyzer from Agilent (Santa Clara, CA) and CFX96 Connect real-time PCR detection system.

2.5.9. Data analysis

Two-sample t-tests (α=0.05) using Microsoft Excel 365 were employed to evaluate significant differences between exposed and control samples for survival, number of offspring and 20E levels observed. F-test were also performed to compare variances between the two groups. For the baseline analysis of 20E levels and the transcription of target genes, analysis of variance (ANOVA) tests (α=0.05) were performed between time points using OriginPro version 2023. Before ANOVA tests, data normality and

homoscedasticity were verified using Kolmogorov- Smirnov's test and Levene's test, respectively. When one of these conditions were not respected, a Kruskall-Wallis ANOVA test was used instead. Tukey's and Dunn's post-hoc tests were used to determine which groups were different for ANOVA and Kruskall-Wallis tests, respectively. Graph Pad Prism version 6 was used to plot the results of all assays.

2.6. Results

2.6.1. Baseline of target genes and levels of 20E

Transcription of the target genes (*shd*, *nvd*, *ecr*, and *usp*) as well as 20E levels were evaluated starting from day 9 of the daphnids' life until day 21 to assess temporal variability during normal development. Most of the transcription of the targeted genes was relatively stable with little to no change over the duration of the observations (Figure 4). A slight but significant increase at day 19 compared to days 9, 11 and 15 was observed for the transcription levels of *ecr* (Tukey's test, $p < 0.05$) and at day 17 compared to day 15 for *usp* (Dunn's test, $p = 0.0084$). Transcripts of *shd* were the most stable over time with no significant difference ($p > 0.05$) at any day and *nvd* was the most active gene in terms of fluctuation. The relative transcription levels of the latter closely followed the concentrations of 20E (Figure 5). The gene *nvd* is responsible for the 7-dehydrogenase, the enzyme catalyzing the first step of ecdysteroid biosynthesis (Song *et al*. 2017). The transcription analysis results thus suggests that the retroaction in response to 20E levels controlling ecdysteroids production mainly affects this step (7-dehydrogenation of cholesterol).

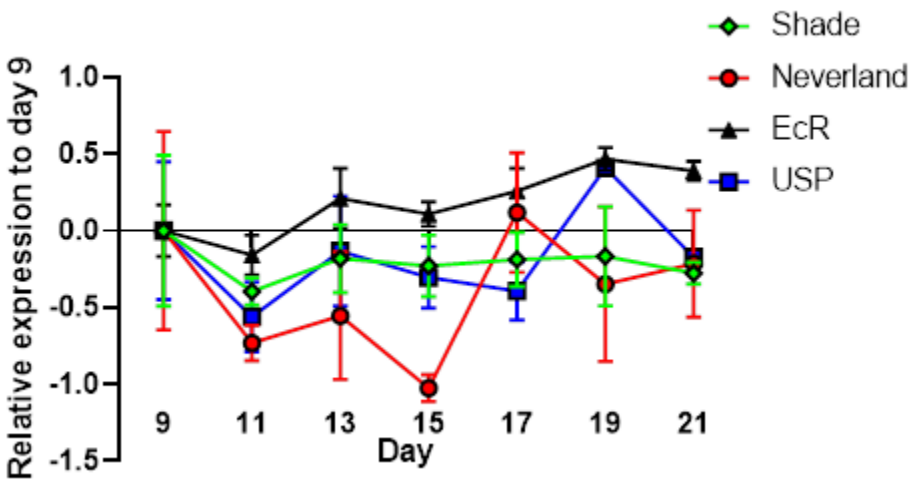


Figure 4. Relative gene transcription of Shade, Neverland, *EcR* and *USP* in daphnids for a 12-day period (*n*=5). Error bars represent 95% confidence interval limits. Data was normally distributed according to Kolmogorov-Smirnov's test (for all genes $p > 0.1$) and the variance was homogeneous according to Levene's test only for *EcR* ($p = 0.3952$).

Figure 5 shows the 20E levels in *D. magna* at different time points. Significantly different ($p < 0.05$) levels of 20E were seen depending on the age of the individuals and the concentration of 20E per daphnid oscillated about every 4 days since day 11 which seems to correspond to the molting cycle duration of *D. magna* in light and temperature conditions employed (Ebert 2005). However, contrary to previous reports (Martin-Creuzburg *et al*. 2007, Mu &Leblanc 2004), a clear ecdysteroid peak (up to ≈50 times the basal level) is absent in Figure 5.

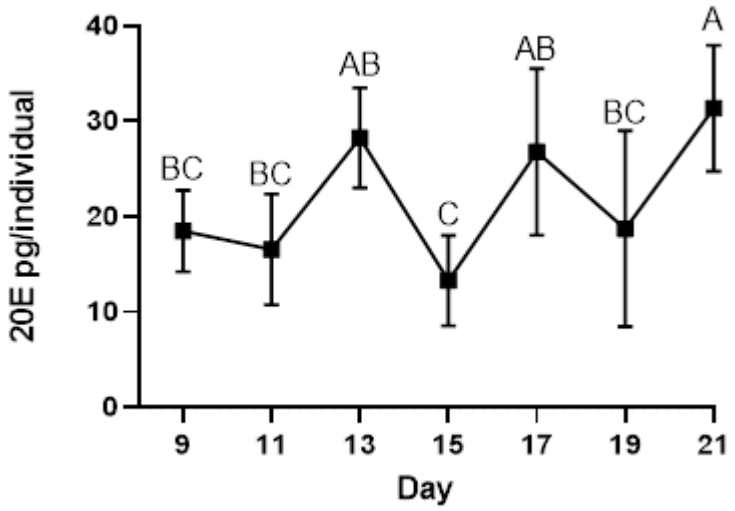


Figure 5. 20E baseline in daphnids over a 12-day period (*n*=3 per time point). Error bars represent ± 1 standard error. Means that do not share the same letter were significantly different ($p < 0.001$) according to Tukey's post-hoc test. Data was normally distributed according to Kolmogorov-Smirnov's test (p = 1.0) and the variance was homogeneous according to Levene's test (p =0.1477).

In the present study, the maximum difference between 20E concentrations measured was only 18 pg/ind and the relative standard deviation was between ± 20% and ± 50%. This observation could be explained by the desynchronization of the molting between sampled daphnids and differences in sampling frequency. Indeed, despite the authors' efforts to control culture experimental conditions (feeding, temperature, photoperiod, culture medium, etc.), molting cycles of daphnids could not be synchronized and it was hypothesized that small differences in their age (a few hours) could result in significant different levels of 20E at the time of sampling. According to Martin- Creuzburg *et al*. (2007), 20E concentrations could vary by a factor as high as 50x in less than 36 h. At this point, it cannot be ruled out that other uncontrolled experimental parameters affected the rhythm of the daphnids molting cycles which, like other rhythmic behaviors, could be influenced by numerous environmental cues (Häfker & Tessmar-Raible 2020).

2.6.2. Survival

Daphnids reached adulthood, defined in the present study as a size > 900 µm, between days 7 and 10. Survival was over 80% for all groups in all tests, thus respecting the sublethality criteria of the OECD. According to Figures S1 to S3 (Annexes), no significant difference ($p > 0.05$) in the survival was observed between exposed and control groups in test N° 1 (gemfibrozil and clofibric acid at 1 µg/L each), but a significant difference in survival was observed for tests N° 2 and N° 3 (fenarimol at NOAEC and LOEC, respectively) after 21 days. This was surprising for the lowest concentration of fenarimol, but the survival rate was still over 80%.

2.6.3. Reproduction

In test No 1 (gemfibrozil and clofibric acid each at 1 µg/L), only at day 10 a significant difference (p=0.0218) was observed on the number of offspring (Figure 6a). Results from daphnids exposed to fenarimol (test N° 2: 113 µg/L and N° 3: 565 µg/L) indicated that only the highest concentration used in latter test caused a consistent decrease in reproduction (Figures 6b and 6c). Indeed, in test No 3, a significant diminution in the number of offspring per adult was observed compared to controls in three consecutive days: 17 (p=0.0020), 19 (p=0.04955) and 21 (p =< 0.0013).

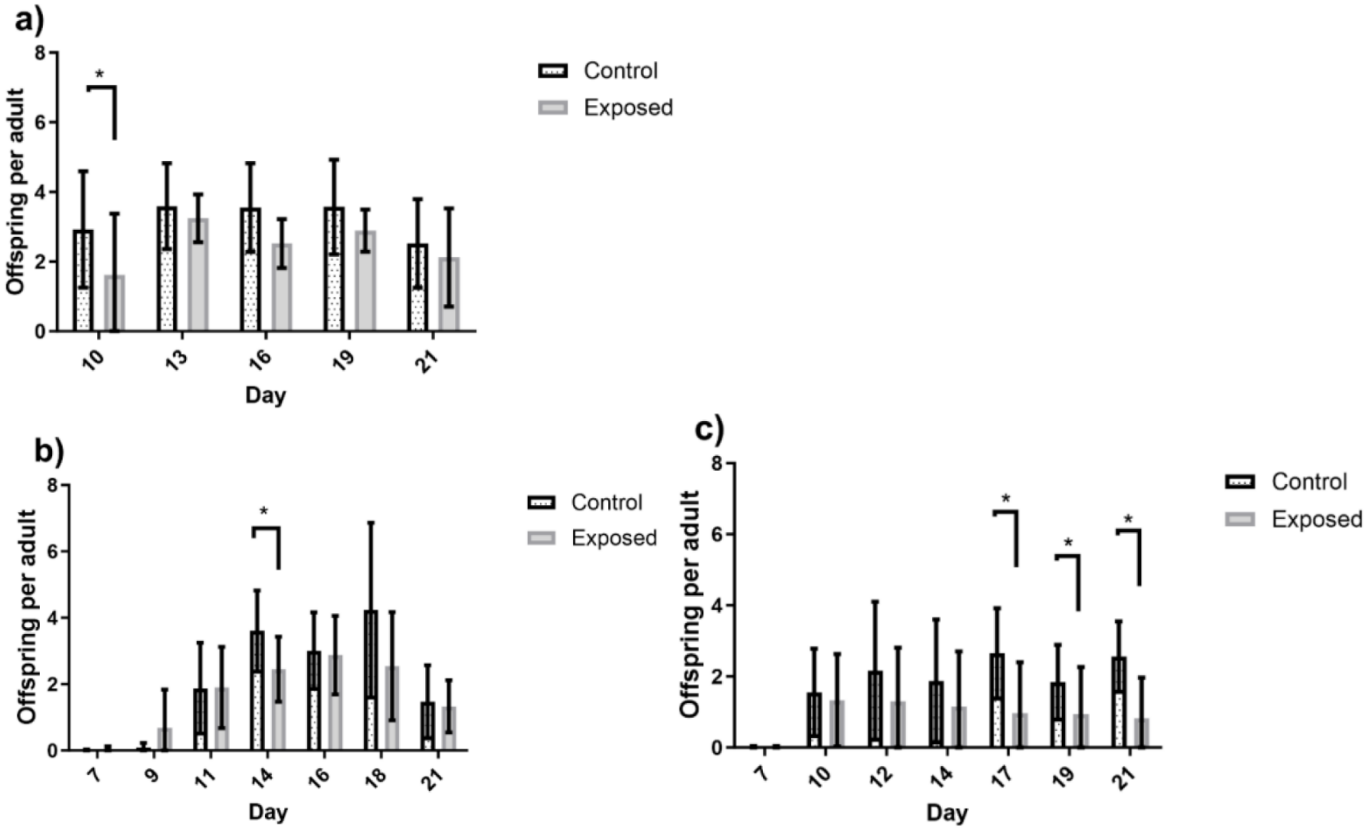


Figure 6. Offspring production per daphnid exposed for 21 days to a) 1 µg/L of gemfibrozil and clofibric acid (n=10), b) 113 µg/L (*n*=10) and c) 565 µg/L (*n*=15) of fenarimol. Error bars represent ± 1 standard. Asterisks (*) indicate statistically significant differences compared to controls ($p < 0.05$).

2.6.4. Ecdysteroid levels

For all tests, experiments showed that the level of 20E did not differ significantly ($p > 0.05$) between controls and organisms exposed (Figure 7). For the gemfibrozil and clofibric acid exposure (Figure 7a), control and exposed groups had 20E mean levels of 21 ± 6.1 pg/ind and 16 ± 6.0 pg/ind, respectively. In test Nº 2 (Figure 7b), the values observed for control and exposed individuals were 20 ± 17 pg/ind and 9.8 ± 9.1 pg/ind, and for test No 3 (Figure 7c), 35 ±24 pg/ind and 24.0±9.7 pg/ind.

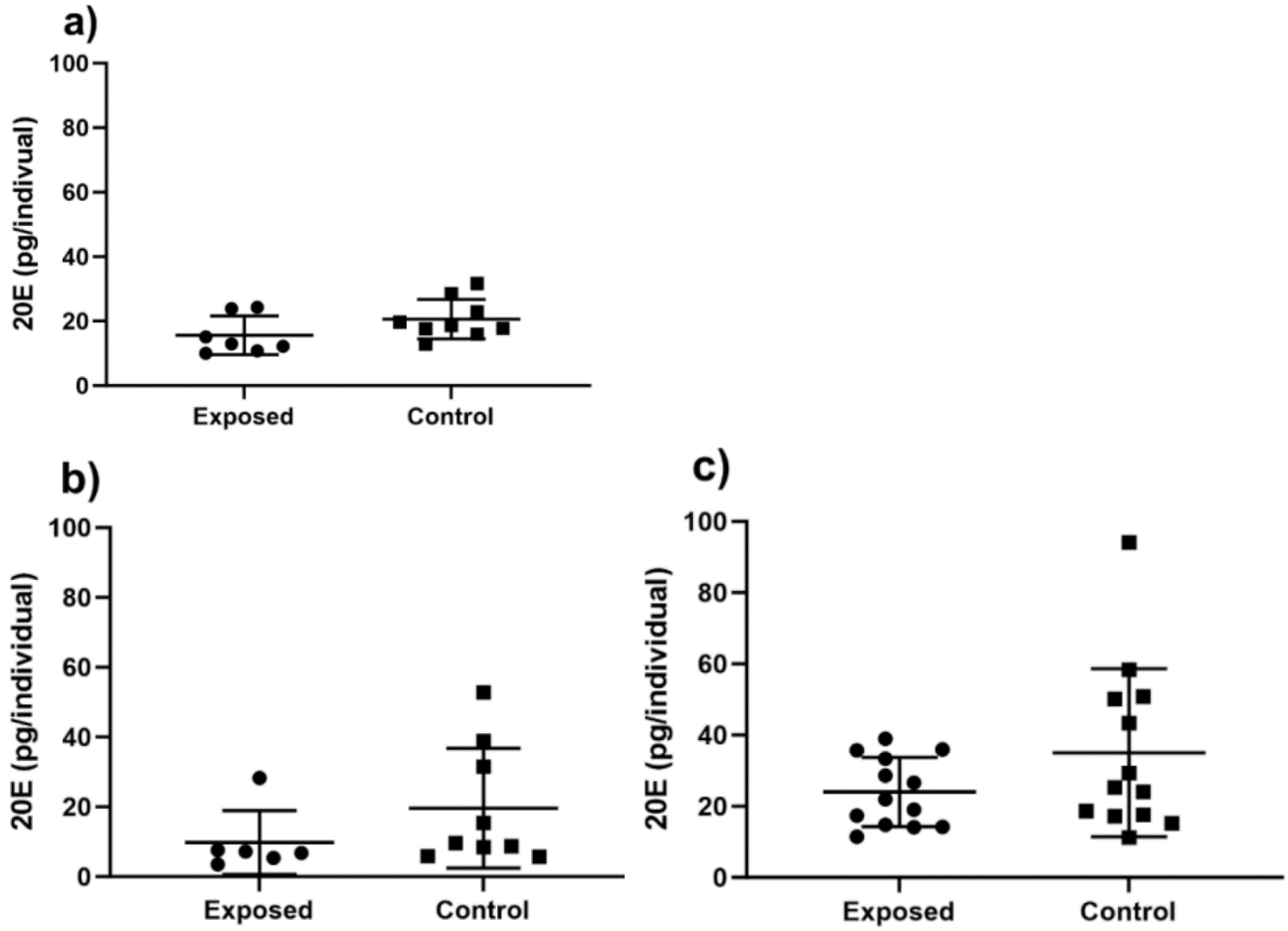


Figure 7. Concentration of 20E (pg/individual) in daphnids exposed for 21 days to a) 1 µg/L of gemfibrozil and clofibric acid (n=10), b) 113 µg/L (*n*=6 for exposed group and *n*=9 for control group) and c) 565 µg/L (*n*=13 for both group) of fenarimol. Error bars represent ± 1 standard. Asterisks (*) indicate statistically significant differences compared to controls ($p < 0.05$).

2.6.5. Transcription of targeted genes

Genes linked to ecdysteroid metabolism such as *shd*, *nvd*, *usp* and *ecr* (Goodman &Cusson 2012) were monitored to evaluate if exposure to fenarimol at sublethal levels (test Nº 2 and Nº 3) during 21 days could change their transcription levels. The results of these experiments are shown in Figure 8a and 8b.

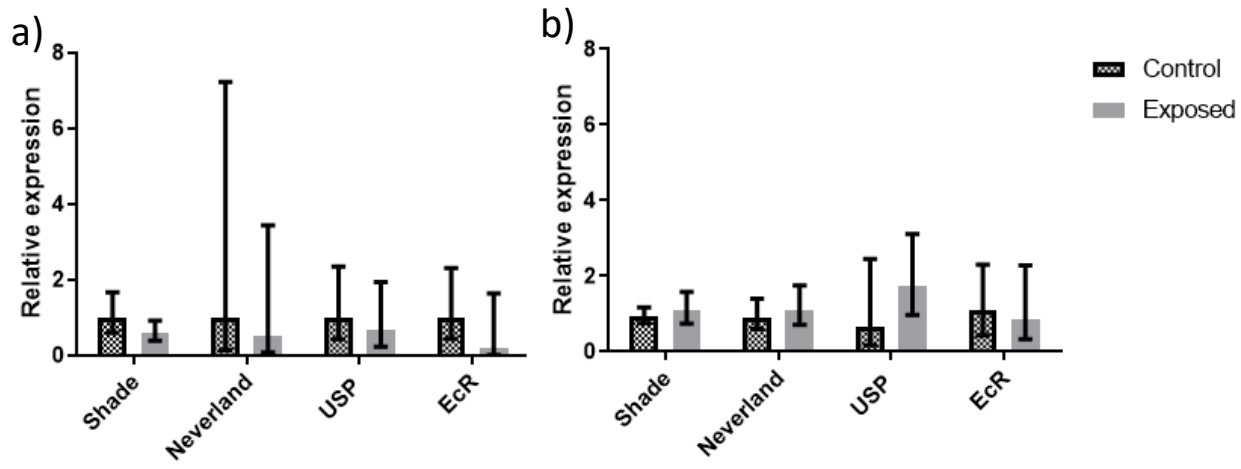


Figure 8. Relative gene transcription of Shade, Neverland, *EcR* and *USP* in daphnids exposed for 21 days to fenarimol a) 113 µg/L (n=8) and b) 565 µg/L (n=7). Error bars represent 95% confidence interval limits. Asterisks (*) indicate statistically significant differences compared to controls ($p < 0.05$).

As shown in Figures 8a and 8b, expression of any gene at the end of the experiments was not affected at the fenarimol NOAEC (113 µg/L) or LOEC (565 µg/L). These results agree with the 20E data presented in Figure 7. In Figure 8a we can see that *nvd* is the most variable gene in those observed. Since *nvd* regulates the first step of the ecdysteroids biosynthesis from cholesterol, it is possible that this gene is more up and down regulated, initializing and stopping the biosynthesis when needed, thus explaining these variations in transcription levels. However, it not yet clear why the such high variability it is only observed at the lowest concentration of fenarimol tested.

2.7. Discussion

2.7.1. Baseline of target genes and levels of 20E

Results reported by Sumiya *et al*. (2014) for *D. magna* were similar to the transcription levels measured in the present study for *shd*, *nvd*, *usp* and *ecr* across an intermolt sampling period, with variation ranging between 2 and 3 times the fold change depending on the time point. The relative stability observed here for gene transcription during the normal growth of daphnids could be due to the developmental stage of daphnids sampled and the selection of genes. Many other genes and their isoforms are responsible for the regulation of ecdysteroids. For example, besides *shd* and *nvd* other genes of the Halloween family (spook, spookier, disembodied and phantom) are implicated in the metabolic cascade that biosynthetizes 20E (Song *et al*. 2017). A time course transcriptomic analysis during the growth period would provide optimal information for the selection of the genes and periods to follow during exposures. When looking at the transcription levels of Halloween genes during normal development in other species, lower differences could be observed between time points. Indeed, for *shd* and *nvd*, two genes also measured in the present study, the relative transcription levels in the moth *Plutella xylostella* oscillated between 0.02 and 0.2 (Peng *et al*. 2019). The same study also observed differences in gene expression of Halloween genes in *P. xylostella* according to the developmental stage of individuals and the tissues sampled. These results indicate that even if very slight or non-significant changes in the transcription levels of these genes are observed, developmental changes (growth, reproduction) can still occur.

Finding the right moment for sampling gene transcription levels is crucial, especially for rapidly modulated transcripts. By following several genes of the same family throughout a time-course interval, a better picture of the metabolism regulated by those genes can be drawn. A pulsating pattern was observed during the normal growth of the daphnids, and the basal transcription levels were assessed. However, more genes should be studied, ideally following one another in the metabolic pathway of the studied way. As for the sampling interval, a much shorter time period could give a better picture of the transcription levels across time and will help in correlating these transcription levels with the corresponding metabolites such as ecdysteroids.

The results in Figure 5 were obtained with pooled individuals (15-25 daphnids). Therefore daphnids are no longer synchronized by day 9, and as a result, differences in 20E concentration as a function of time

are much more subtle and the variability of each measure is higher. Regarding sampling frequency, 7 measurements were done within a 12-day period, while Martin-Creuzburg *et al*. (2007) performed more than 20 measurements within a 3-day period. Finally, while it is not possible to compare the concentrations observed since previous studies used immunoassay-based techniques rather than mass spectrometry, the reported concentrations herein were about an order of magnitude lower than those reported by Martin-Creuzburg *et al*. (2007) but about 10000 times higher than those reported by Sumiya *et al*. (2016). Besides the techniques employed, those differences can be explained by the sampling of individuals at different stages of their molting cycles.

2.7.2. Reproduction

The results from tests N° 1 (gemfibrozil and clofibric acid each at 1 µg/L) (Figure 6a) are in agreement with a previous study that showed that chronic exposure (30 days) to 1 µg/L of clofibric acid did not affect the reproduction of *D. magna* (Flaherty &Dodson 2005). Steinkey *et al*. (2018) also reported that daphnids exposed to low concentrations of gemfibrozil (0.05 µg/L) produced broods at an earlier age and had larger broods than control individuals, but such effect was only observed when food availability was high. The authors also observed that organisms were larger and had higher lipid energy reserves for which mechanisms of action were unexplained by the authors.

Concerning tests Nº 2 (fenarimol 113 µg/L, Figure 6b) and Nº 3 (fenarimol 565 µg/L, Figure 6c), similar observations were also made by Mu and LeBlanc (2002) for *D. magna* exposed for 21 days to similar concentrations of fenarimol. A reduction in the number of offspring in aquatic invertebrates has been attributed to impairment of energy supply and demand or to endocrine disrupting effects (Barata *et al*. 2004). Under normal conditions, individuals use energy for their basal metabolism, growth, and reproduction. In the case of chemical exposure, a larger amount of the assimilated energy can be used to cope with the stressor and to maintain or compensate basal metabolism, leaving less energy available for growth and reproduction (Sokolova 2013). Therefore, to survive in test Nº 3, exposed daphnids had to diminish the production of their offspring.

2.7.3. Ecdysteroid levels

Little information is known on the impact of lipid-lowering molecules on the production of ecdysteroids in *D. magna*. The present results show that no effect is observed for this parameter in daphnids exposed for 21 days to a mixture of gemfibrozil and clofibric acid at a concentration of 1 µg/L (Figure 7a) as well as for the two fenarimol exposure assays (113 µg/L and 565 µg/L, Figures 7b and 7c, respectively). While the results of the exposure to 113 µg/L of fenarimol could be explained by a concentration too low to have an effect on the metabolism of ecdysteroids, the results for the exposition at 565 µg/L are contrary to those obtained by Mu and Leblanc (2004). In that study, the authors exposed *D. magna* neonates during the first intermolt period (approximatively during 25 h) to fenarimol at a similar concentration (497 µg/L) than the experiments presented here. This exposure caused a diminution of around 26% in ecdysteroid levels and induced a delay for the first and second molt. However, the discrepancies observed between their study and the present study can be explained by several factors such a difference in methods of quantification of ecdysteroids, the age of the daphnids used as well as the time of exposure. Another hypothesis explaining the similar 20E levels in exposed organisms is the transfer of 20E to embryos. Since ecdysteroids are transferred to neonates during embryogenesis (Subramoniam 2000), and that a lower number of neonates was produced in exposed daphnids, the total 20E present in the colony could have been lower in the exposed groups compared to controls if neonates had been included in the measurements. Unfortunately, the present method used was not sensitive enough to quantify 20E levels in neonates (Venne *et al*. 2016). When a lower number of neonates are produced, a lower quantity of ecdysteroids and overall resources are needed by the daphnids compared to daphnids producing many neonates. Finally, it could also be possible that cholesterol 7-dehydrogenation was indeed inhibited by exposure to fenarimol, but that the inhibition did not affect the end of the line concentrations of 20E as the organisms were able to cope by other mechanisms. Thus, cholesterol and 7-dehydrocholesterol levels should be assessed and compared between the control and exposed groups to verify this hypothesis.

2.7.4. Transcription of targeted genes

Since fenarimol targets the 7-dehydrogenation step in the biosynthesis of ecdysteroids (LeBlanc 2007), an increase in transcription of *nvd* would have been expected as this gene encodes for the 7-

dehydrogenase enzyme. It is important to note that a single time point (day 21) was measured in control and exposed groups. Therefore, it is possible that differences in transcription levels could have been occurring earlier in time. Soetaert *et al*. (2007) reported changes in gene expression (cuticula proteins, proteases related genes) in *D. magna* following exposure to fenarimol for 96 h at a concentration of 1 mg/L using cDNA microarray. When Soetaert, *et al.* (2007) used a concentration similar to the present study (500 µg/L), no changes were observed except for an unknown transcript. These results combined with the ones from the present study suggest that fenarimol may reduce fecundity in *D. magna* by first delaying molting and development as reported by Hassold and Backhaus (2009); but that *shd* and *nvd* genes are not involved in these changes.

Gene expression is a dynamic process where changes can occur within hours (Storey *et al*. 2005). When changes follow a transient manner, the expression of affected transcripts returns to pre-response levels (Bendjilali *et al*. 2017). Therefore, finding the right window for the measurements is essential. Only a few transcripts were evaluated in this study; it could be interesting to use techniques with a wider approach such as RNA-sequencing.

2.8. Conclusion

The present study aimed to assess the natural baseline of 20E and the transcription of four genes that regulate ecdysteroids in *D. magna* over a 21-day period, and to evaluate the chronic effects of fenarimol and a mixture of gemfibrozil and clofibric acid on multiple levels of biological responses.

Baseline measurement of 20E and transcriptions levels of *shd*, *nvd*, *usp* and *ecr* indicated concentration of 20E oscillating between 13 and 31 pg/individuals and transcription levels between 1 and -1 relative to day 9 (first measurement). The hypothesis of the present work that levels of 20E and the transcription of 20E-related genes would be affected by exposure to lipid-altering compounds could not be proved. Although a diminution in number of offspring was observed consistently in the exposition to 565 µg/L of fenarimol, no differences were observed for the molecular assays tested here, i.e., concentration of 20E and transcription levels of *shd*, *nvd*, *usp* and *ecr*. Other pathways regulating growth and reproduction, such as the juvenoid hormones (Goodman & Cusson 2012), might be affected and would need to be studied to understand what lies behind this observation. Results suggest that concentrations of 20E and gene transcription quantified in daphnids observed in this work might be too variable when using a single

punctual measure to discern effects due to exposure to sublethal concentrations of the target compounds. Therefore, at least for the compounds tested, changes in levels of 20E and/or changes in the transcription of 20E-related genes cannot be used as biomarkers of exposure. Nevertheless, it is not yet clear if the same outcome could be extrapolated to other inorganic or organic contaminants or by modifying the experimental conditions.

Identifying the molting stage of the daphnids is demanding and difficult to synchronize between individuals when exposed for several days. Therefore, another molecule, produced in a more stable way, could be used to normalize the ecdysteroids levels before statistical analysis. Measuring multiple time points instead of a single measure as well as additional molecular endpoints obtained from transcriptomic and metabolomic studies could also afford more insights on the changes occurring in exposed daphnids. Additionally, studying the role of neonates on 20E levels can help understand differences observed with previous studies. Indeed, measuring these parameters sooner in time could offer more insights on the adaptation of the daphnids to lipid-altering compounds on *D. magna*. In this way, it may be possible to obtain a more comprehensive view of the effects of stressors having common modes of action.

Additional experiments are required to help explain the lowered fertility observed in *D. magna* when exposed to an ecdysteroid synthesis inhibitor such as fenarimol. Future work could employ untargeted transcriptomic and lipidomic to evaluate changes in the lipid profile in whole daphnid extracts following exposure to other organic contaminants of interest. Such an approach could offer a better chance of identifying impacted metabolites and lead to a better understanding of mechanisms of toxicity. Moreover, it would be interesting to determine how daphnids adapt to exposure to different organic contaminants in the long term through their whole life cycle and multigenerational experiments since exposure periods of 21 days or less might be too short to clearly observe more gradual effects on survival or reproduction..

2.9. Author contribution

Hugo Alarie: Conceptualization, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – Original Draft, Writing – Review & Editing. **Nadia Côté:** Supervision. **Magali Houde:** Supervision, Resources, Writing – Review & Editing. **Luc R. Gaudreau:** Writing – Review &

Editing, Resources. **Pedro A. Segura:** Conceptualization, Funding Acquisition, Methodology, Resources, Supervision, Writing – Review & Editing, Project administration

2.10. Funding

This study was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) through a discovery grant awarded to P.A. Segura and by the Faculty of Sciences of the Université de Sherbrooke. We would like to thank Hydro-Québec, the Environmental Decontamination Technologies and Integrated Water and Wastewater Management (TEDGIEER) NSERC CREATE program and the Quebec Ecotoxicology Research Centre (EcotoQ) for scholarships granted to H. Alarie. Funding sources did not have any involvement in the design, experiments, data interpretation, writing, revision or submission of this study.

Availability of data and materials: Available upon reasonable request.

Ethical approval: Not applicable.

2.11. Declarations

Consent to participate: Not applicable.

Consent for publication: Not applicable.

Competing interests: The authors declare no competing interests.

2.12. References

Barata C, Porte C, Baird DJ (2004): Experimental designs to assess endocrine disrupting effects in invertebrates a review. Ecotoxicol. 13, 511-517

Bendjilali N, MacLeon S, Kalra G, Willis SD, Hossian AN, Avery E, Wojtowicz O, Hickman MJ (2017): Time-course analysis of gene expression during the Saccharomyces cerevisiae hypoxic response. G3: Genes, Genomes, Genetics 7, 221-231

Bodar C, Voogt P, Zandee D (1990): Ecdysteroids in Daphnia magna: their role in moulting and reproduction and their levels upon exposure to cadmium. Aquat. Toxicol. 17, 339-350

Bradley PM, Journey CA, Romanok KM, Barber LB, Buxton HT, Foreman WT, Furlong ET, Glassmeyer ST, Hladik ML, Iwanowicz LR (2017): Expanded Target-Chemical Analysis Reveals Extensive Mixed-Organic-Contaminant Exposure in US Streams. Environ. Sci. Technol. 51, 4792-4802

Centre d'expertise analytique environnementale du Québec (2011): Détermination de la toxicité létale CL50 48h Daphnia magna. MA. 500 D.mag 1.1, Rév. 1. Ministère du Développement durable, de l'Environnement et de la Lutlle contre les changements climatiques

Daughton CG, Ternes TA (1999): Pharmaceuticals and personal care products in the environment: agents of subtle change? Environ. Health Perspect. 107, 907-938

De Lange H, Noordoven W, Murk A, Lürling M, Peeters E (2006): Behavioural responses of Gammarus pulex (Crustacea, Amphipoda) to low concentrations of pharmaceuticals. Aquat. Toxicol. 78, 209-216

Dodson SI, Hanazato T (1995): Commentary on effects of anthropogenic and natural organic chemicals on development, swimming behavior, and reproduction of Daphnia, a key member of aquatic ecosystems. Environ. Health Perspect. 103, 7

Ebele AJ, Abdallah MA-E, Harrad S (2017): Pharmaceuticals and personal care products (PPCPs) in the freshwater aquatic environment. Emerg. Contam. 3, 1-16

Ebert D (2005): Ecology, Epidemiology, and Evolution of Parasitism in Daphnia. National Libray of Medicine, National Center for Biotechnology Information, Bethesda, MD, 98 pp

Environment Canada (1990): Biological test method. Acute Lethality Test Using Daphnia spp. In: Method Development and Applications Section (Hrsg.), Ottawa, ON, pp. 55

Flaherty CM, Dodson SI (2005): Effects of pharmaceuticals on Daphnia survival, growth, and reproduction. Chemosphere 61, 200-207

Goodman W, Cusson M (2012): The Juvenile Hormones. In: LI G (Editor), Insect Endocrinology Academic Press, San Diego, pp. 310-365

Hassold E, Backhaus T (2009): Chronic toxicity of five structurally diverse demethylase-inhibiting fungicides to the crustacean Daphnia magna: A comparative assessment. Environmental Toxicology and Chemistry: An International Journal 28, 1218-1226

Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesompele J (2007): qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome Biol. 8, 1-14

Hirano M, Toyota K, Ishibashi H, Tominaga N, Sato T, Tatarazako N, Iguchi T (2020): Molecular insights into structural and ligand binding features of methoprene-tolerant in daphnids. Chem. Res. Toxicol. 33, 2785-2792

Houde M, Carter B, Douville M (2013): Sublethal effects of the flame retardant intermediate hexachlorocyclopentadiene (HCCPD) on the gene transcription and protein activity of Daphnia magna. Aquat. Toxicol. 140, 213-219

Hughes SR, Kay P, Brown LE (2013): Global synthesis and critical evaluation of pharmaceutical data sets collected from river systems. Environ. Sci. Technol. 47, 661-677

Jansen M, Coors A, Stoks R, De Meester L (2011): Evolutionary ecotoxicology of pesticide resistance: a case study in Daphnia. Ecotoxicol. 20, 543-551

Kovacevic V, Simpson AJ, Simpson MJ (2016): 1H NMR-based metabolomics of Daphnia magna responses after sublethal exposure to triclosan, carbamazepine and ibuprofen. Comparative Biochemistry and Physiology Part D: Genomics and Proteomics 19, 199-210

LaFont R, Dauphin-Villemant C, Warren JT, Rees H (2012): Ecdysteroid Chemistry and Biochemistry. In: Gilbert LI (Editor), Insect Endocrinology. Academic Press, London, UK, pp. 106-176

LeBlanc GA (2007): Crustacean endocrine toxicology: a review. Ecotoxicol. 16, 61-81

Lefrancq M, Jadas-Hécart A, La Jeunesse I, Landry D, Payraudeau S (2017): High frequency monitoring of pesticides in runoff water to improve understanding of their transport and environmental impacts. Sci. Total Environ. 587, 75-86

Li JJ, Dai MJ, Xue JZ (2023): Investigation on toxicity and mechanism to Daphnia magna for 14 disinfection by-products: Enzyme activity and molecular docking. Sci. Total Environ. 905, 167059

Martin-Creuzburg D, Westerlund SA, Hoffmann KH (2007): Ecdysteroid levels in Daphnia magna during a molt cycle: determination by radioimmunoassay (RIA) and liquid chromatography-mass spectrometry (LC-MS). Gen. Comp. Endocr. 151, 66-71

Miner BE, De Meester L, Pfrender ME, Lampert W, Hairston Jr NG (2012): Linking genes to communities and ecosystems: Daphnia as an ecogenomic model. Proceedings of the Royal Society B: Biological Sciences 279, 1873-1882

Mnif W, Hassine AIH, Bouaziz A, Bartegi A, Thomas O, Roig B (2011): Effect of endocrine disruptor pesticides: a review. International journal of environmental research and public health 8, 2265-2303

Mu X, LeBlanc GA (2002): Environmental antiecdysteroids alter embryo development in the crustacean Daphnia magna. J. Exp. Zool. 292, 287-292

Mu X, Leblanc GA (2004): Synergistic interaction of endocrine-disrupting chemicals: Model development using an ecdysone receptor antagonist and a hormone synthesis inhibitor. Environ. Toxicol. Chem. 23, 1085-1091

OECD (2008): Test No. 211: Daphnia magna Reproduction Test, In: OECD Guidelines for the Testing of Chemicals, Section 2: Effects on Biotic Systems. OCDE Publishing

Peng L, Wang L, Zou M-M, Vasseur L, Chu L-N, Qin Y-D, Zhai Y-L, You M-S (2019): Identification of halloween genes and RNA interference-mediated functional characterization of a Halloween gene Shadow in Plutella xylostella. Frontiers in physiology 10, 1120

Ponton F, Chapuis M-P, Pernice M, Sword GA, Simpson SJ (2011): Evaluation of potential reference genes for reverse transcription-qPCR studies of physiological responses in Drosophila melanogaster. J Insect Physiol 57, 840-850

Qiagen (2023): M-MuLV Reverse Transcriptase. Qiagen, Ipswich, MA

Quiagen (2020): RNeasy Plus Mini Handbook (HB-0405-005). Quiagen, Hilden, Germany, pp. 47

Rewitz KF, Gilbert LI (2008): Daphnia Halloween genes that encode cytochrome P450s mediating the synthesis of the arthropod molting hormone: evolutionary implications. BMC Evolutionary Biology 8, 1-8

Richmond EK, Grace MR, Kelly JJ, Reisinger AJ, Rosi EJ, Walters DM (2017): Pharmaceuticals and personal care products (PPCPs) are ecological disrupting compounds (EcoDC). Elem. Sci. Anth. 5

Soetaert A, van der Ven K, Moens LN, Vandenbrouck T, van Remortel P, De Coen WM (2007): Daphnia magna and ecotoxicogenomics: gene expression profiles of the anti-ecdysteroidal fungicide fenarimol using energy-, molting-and life stage-related cDNA libraries. Chemosphere 67, 60-71

Sokolova IM (2013): Energy-limited tolerance to stress as a conceptual framework to integrate the effects of multiple stressors. Integrative and comparative biology 53, 597-608

Song Y, Villeneuve DL, Toyota K, Iguchi T, Tollefsen KE (2017): Ecdysone receptor agonism leading to lethal molting disruption in arthropods: review and adverse outcome pathway development. Environ. Sci. Technol. 51, 4142-4157

Steinkey D, Lari E, Woodman SG, Luong KH, Wong CS, Pyle GG (2018): Effects of gemfibrozil on the growth, reproduction, and energy stores of Daphnia magna in the presence of varying food concentrations. Chemosphere 192, 75-80

Storey JD, Xiao W, Leek JT, Tompkins RG, Davis RW (2005): Significance analysis of time course microarray experiments. P Natl Acad Sci USA 102, 12837-12842

Subramoniam T (2000): Crustacean ecdysteriods in reproduction and embryogenesis. Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology 125, 135-156

Sumiya E, Ogino Y, Miyakawa H, Hiruta C, Toyota K, Miyagawa S, Iguchi T (2014): Roles of ecdysteroids for progression of reproductive cycle in the fresh water crustacean Daphnia magna. Frontiers in zoology 11, 60

Sumiya E, Ogino Y, Toyota K, Miyakawa H, Miyagawa S, Iguchi T (2016): Neverland regulates embryonic moltings through the regulation of ecdysteroid synthesis in the water flea Daphnia magna, and may thus act as a target for chemical disruption of molting. Journal of Applied Toxicology 36, 1476-1485

Tonkes M, De Graaf PJ, Graansma J (1999): Assessment of complex 782 industrial effluents in the Netherlands using a whole effluent toxicity (or WET) approach. Water Sci. Technol. 39, 55-61

Venne P, Yargeau V, Segura PA (2016): Quantification of ecdysteroids and retinoic acids in whole daphnids by liquid chromatography-triple quadrupole mass spectrometry. J. Chromatogr. A 1438, 57-64

Wagner ND, Simpson AJ, Simpson MJ (2017): Metabolomic responses to sublethal contaminant exposure in neonate and adult Daphnia magna. Environ. Toxicol. Chem. 36, 938-946

Wang Z, Walker GW, Muir DC, Nagatani-Yoshida K (2020): Toward a global understanding of chemical pollution: a first comprehensive analysis of national and regional chemical inventories. Environ. Sci. Technol. 54, 2575-2584

CHAPITRE 3 L'ESSAI DE TOXICITÉ DES EFFLUENTS ENTIERS SUR *DAPHNIA MAGNA* N'INDIQUE AUCUN EFFET DES EAUX DE RUISSELLEMENT PROVENANT D'UN SITE MINIER REMÉDIÉ AVEC DES SOLS CONTAMINÉS

Hugo Alarie [1], Dany Landry [2], Magali Houde [3], Pedro A. Segura [1,*]

[1] Department of Chemistry, Université de Sherbrooke, Sherbrooke, QC, Canada J1K 2R1
[2] Englobe Corporation, Organic and Soil Treatment Centers, Québec, QC, Canada G1P 4S9
[3] Aquatic Contaminants Research Division, Environment and Climate Change Canada, Montreal, QC, Canada H2Y 2E7

3.1. Notes préliminaires

Cet article a été soumis le 9 août 2023 au journal *Environmental Science: Water Research & Technology.*

3.2. Contributions des auteurs

J'ai réalisé le développement et design de l'étude, l'entièreté des expériences, l'analyse de données, l'interprétation des résultats et la rédaction de la première version de l'article pour ce chapitre mis à part l'échantillonnage de l'eau qui a été fait par l'équipe d'Englobe ainsi que l'analyse des HAP, C_{10}-C_{50} dans l'eau qui a été faite par les laboratoires AGAT. Magali Houde, Dany Landry et Pedro A. Segura ont participé à la correction et/ou rédaction de la version finale de cet article.

***Daphnia magna* assay indicates no effect of leachate waters from mining sites remediated with contaminated soils**

3.3. Abstract

The remediation of mining sites is commonly done by adding soil and fertilizing material to the rock piles to allow the growth of vegetation. In a restoration project of a former mining site in Quebec (Canada), soils slightly to moderately contaminated by petroleum hydrocarbons (PHs), polycyclic aromatic hydrocarbons (PAHs), and metals were used for the revegetation process on four plots. Once in place, these soils were decontaminated and stabilised by phytoremediation. The objective of this study was to evaluate de ecotoxicological risk of this remediating method by using a whole effluent toxicity approach (exposing the daphnids to the leachate waters without pre-treatment) to evaluate the impact of leachate waters on the water flea *Daphnia magna*. During a 21-day period, mortality, reproduction, weight, and 20-hydroxyecdysone (20E) levels, an essential hormone for the growth and reproduction of *D. magna*, were assessed in exposed and control daphnids. No significant differences were observed in terms of time of first brood and number of neonates between the control and exposed groups. A significant ($p=0.0481$) yet only slightly higher mortality was observed in daphnids exposed to the leachate water of plot #1 and a significant ($p=0.004$) but marginally lower mortality was observed in daphnids exposed to the leachate waters from plot #3. These differences could be explained by the quantity of nutrients contained in the different leachate waters. No differences were observed for the humid mass and 20E concentration present in adult daphnids. These results suggest that the tested approach of using soils lightly to moderately contaminated with metals, PHs and PAHs does not produce leachate waters that could pose a risk to *D. magna* and suggest that the whole effluent toxicity (WET) approach may be an efficient way of evaluating the toxicity of leachate waters.

Keywords: ecotoxicology, *Daphnia magna*, phytoremediation, contaminated soils, trace metals, hydrocarbons

3.4. Introduction

Following the extraction of minerals from ores, large quantities of rock residues (waste rock piles) are deposited around mines creating fields of sterile ground due to their lack of organic matter to support life. Mine tailings (finely crushed ores) can also negatively impact nearby aquatic environments. These gravel mounds are often very rich in metals such as Fe, Cu, Ni and Mg and can affect the surrounding water quality by leaching [1, 2]. Changes in pH and suspended residues in the water can, in turn, affect living organisms such as algae, fish and invertebrates [3-5]. Salonen *et al.* [4] (2006) found that increased concentrations of Cu, Pb and Zn in sediments of water body near mine tailings would lead to the absence of practically all algae. Wong *et al.* [3] (1999) found that even after 50 years, mine tailings could still release trace metals that would impact the fish communities of downstream ecosystems. Surroundings terrestrial ecosystems can also be impacted due to soil erosion and acidification, loss of soil fertility, plant toxicity and food chain contaminations due to the numerous metals present in high concentrations in the mine tailings [6, 7].

In Quebec (Canada), regulations require that the company exploiting the mine returns the surface of the site to as near its original state as possible [8]. The mining company must also provide a plan and the proof of sufficient funds for the effective closing of the mine. In Canada, the specific regulations vary between provinces and territories, and even between mines. Once the mine exploitation is completed, a revitalization of the site must be carried out in order to bring it back to its original state.

Common restoration methods for mine tailings include covering the waste rock piles with soil and/or fertilizing residual materials and then sowing plants to stabilise the soil. This type of approach is known as physical remediation [9]. Chemical remediation is another alternative where chemical agents such a lignin sulfonate or resinous adhesives are used to cover the rock piles and prevent wind and water erosion [9]. Phytoremediation is a physical process that consist of planting vegetation on contaminated soil. These plants will remove, degrade, or stabilise contaminants on the site without having to move the contaminated soil. Several processes are involved in phytoremediation. Phytostabilisation has been proven effective for the stabilisation of heavy metals in the soils by retaining these metals in the root system of the plants and preventing their leaching [10]. Rhizodegradation can degrade organic contaminants, in this case petroleum aromatic hydrocarbons (PAHs) and petroleum hydrocarbons (PHs), directly in the soil by the means of bacteria or enzymes present in the plants root system [11].

Several approaches can be used to evaluate if remediation efforts on a site have been successful such as quantification of the contaminants in the remediated matrix (measuring the residual pollutants over time, changes in time and nature), evaluation of the soil characteristics (pH, structure, other properties), and ecological impact (phytotoxicity, animal toxicity, human risks) [12, 13]. Bioassays are particularly interesting since they provide meaningful information on the potential effects on living organisms and can be correlated with other parameters such as the levels of contaminants. Whole effluent toxicity (WET) is a type of bioassay that was originally developed to evaluate the toxicity of effluents from wastewater treatment plants. This type of experimental design can also be used to evaluate other effluents such as complex industrial effluents [14], leachate waters from e-waste [15] or wood waste piles [16]. The advantage of this approach is that it requires no pre-treatment following the sampling before using it in the toxicity

tests. It also represents a real-life scenario by using the leachate water as is, but in a controlled environment. In the present study, the water flea *Daphnia magna* was chosen as a model to investigate the effects of leachate waters originating from mine tailings covered by contaminated soils. *D. magna* is a small freshwater crustacean commonly found in the northern hemisphere and routinely used in ecotoxicology with an easy culture in the laboratory [17]. Daphnids also occupy a key role in the food chain as they are filter feeders and prey of insects and small fishes [18].

Organisms present in the nearby environment of the mine site such as *D. magna* could be impacted by the high concentration of metals contained in the mine tailings and waste rock piles. Although some metals are essential for several biological processes [19, 20], these are also known for their adverse effects. They can affect cellular organelles and components but also some essential function such as metabolism, detoxification, and damage repair [21-23]. Bodar *et al.* [24], found that metals such as cadmium at concentrations between 5.0 to 20.0 µg/L impacted ecdysteroids titer by increasing it and affected moulting of daphnids exposed for 8 days. Ecdysteroids are a group of hormones derived from cholesterol that regulate reproduction and development in daphnids. The main molting hormone in crustaceans and other arthropods, 20-hydroxyecdysone (20E), is also involved in the reproduction process and embryonal development of daphnids [25]. Bodar *et al.* [26] also found that the size of the daphnid neonates decreased with the increasing Cd concentration. Additionally, adult daphnids might be more susceptible than neonates to exposure to Cd, Zn, Cu, and Pb as it was observed that their mean lifetime decreased and their development time increased [27]. Therefore, chronic testing using *D. magna* as model species can be a good approach to assess risks of exposure to aquatic environments. With the increasing demands in transition metals for electronic appliances, it is expected to see an increase of these contaminants in the environment due to the disposal of the electric waste and increasing mining exploitation [28, 29].

PHs can also enter the environment following human exploitation where it can affect aquatic ecosystems [30-33]. Effects of PHs on *D. magna* have been reported such as reduced lifetime and fertility when exposed chronically to concentrations between 10 to 40 mg/L [34], but few sublethal parameters have been studied. This study aimed to assess the toxicity of leachate waters from soils slightly to moderately contaminated with transition metals, PAHs and PHs on *D. magna*. First, an acute evaluation was performed to assess the toxicity of leachate waters on the survival of crustaceans. Secondly, a 21-day chronic exposure assay was conducted and concentrations of 20E and life history parameters (i.e., weight, reproduction, and mortality) were assessed in *D. magna* using the WET assay. To our knowledge, this approach has never been used to test the chronic effects of leachate waters from contaminated soils.

3.5. Materials and methods

3.5.1. Reagents and chemicals

Water, methanol (MeOH), acetonitrile (ACN), methyl *tert*-butyl ether (MTBE), formic acid (FA), and acetic acid (AA) of LC or LC-MS grade were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Standards of 20E (>98 % purity) and makisterone A (>95 % purity) were purchased from Santa Cruz Biotech (Dallas, TX, USA). Makisterone A is an ecdysteroid that is not endogenously present in *D. magna* and that responds similarly to 20E during extraction and analysis by liquid chromatography-triple quadrupole mass spectrometry (LC-QqQMS). It was used as an internal standard for 20E quantification. Makisterone A was purified from 20E and ecdysone impurities following the method described by Venne *et al* [35]. Hydroxylamine hydrochloride (>99%), used for the derivatization of ecdysteroid, and potassium dichromate were obtained from Sigma Aldrich (St-Louis, MO, USA). 20E and Makisterone A were

dissolved at 0.1 mg/mL in MeOH and working solutions used in 20E quantification were prepared in MeOH containing 1 % formic acid (FA) and stored at -20 °C. The derivatization solution of hydroxylamine hydrochloride (100 mg/mL) was prepared before each analysis.

3.5.2. Site location and description

The site of interest is located in Thetford Mines, Quebec, Canada. This site has been in restoration since 2014; several phases were planned to consolidate the waste rock piles surrounding the site using revegetation. Slightly contaminated soils containing transition metals, PAHs and PHs were used to cover waste rock piles and selected plant species were sowed on the plots. This approach of using contaminated soil as a revegetation substrate for mine sites prevents the need to move the soils twice, as commonly done. Normally, soils are first moved from the original site to a decontamination site and then a second time to a valorisation site (i.e., over a mine tailing or a landfill site). With the proposed approach, the soils remain in place at the end of the treatment period and constitute the revegetation substrate, thus avoiding the transport of the treated soils to a third place for usage. On one hand, phytoremediation reduces the load of contaminants found in the environment and allows the recycling of the contaminated soils as a revegetation substrate. On the other hand, the restoration of the mining rock waste piles by revegetation makes it possible to secure the site while offering a new living environment where vegetation can grow naturally. Different soils with different degrees of contamination were used in this project.

Soils containing A-B levels of contaminants (see Table 1), which are considered adequate for residential usage [8], are generally used to cover mining rock piles. In this project, soils with mixed contamination

(organic and metal) in the B-C range [generally used for industrial purposes [8]] from Englobe's (Quebec, QC) contaminated soil treatment centers were imported to the site to cover three test plots. This approach could represent a potentially new viable option to valorize soils with B-C levels of contamination by metals and hydrocarbons. Considering that the site contains naturally high concentrations for certain metals and nonmetals such as Ca, Fe, Mg, K, and Na (>C range), the environmental impact of utilizing B-C soils with this type of contamination in a revegetation project for mining tailing piles appear negligible, or even positive. Although the current technology does not make it possible to treat metals, it aims to stabilize levels in the ground using phytostabilisation.

Four plots were studied on the site, a control plot made up of soil with A-B level of contamination and three experimental plots using soils contaminated in the B-C range. Each of the test plots were rectangular in shape, 20 m wide by 25 m long for an area of 500 m². Detailed contamination characteristics of each of the test plots are described in Table 1.

Table 1. Contaminant types and levels in soils used to cover the different tested plots.

Plot	Soil criteria *	Levels of contaminants in soils
Control plot (CP)	A-B for metals, PHs and PAHs	Less than 10 mg/kg for any PAHs Less than 700 mg/kg for total PHs
Plot #1 (P1)	A-B for metals B-C for PHs/PAHs	Less than 100 mg/kg for any PAHs Less than 3500 mg/kg for total PHs
Plot #2 (P2)	A-B for metals B-C for PHs/PAHs	Less than 100 mg/kg for any PAHs Less than 3500 mg/kg for total PHs
Plot #3 (P3)	B-C for metals B-C for PHs/PAHs	Less than 100 mg/kg for any PAHs Less than 3500 mg/kg for total PHs

* Values for metals can be found in Table S3 (Annexes)

Fifty centimeter in height of soil mixed with fertilizing residual materials (de-inking residues, gypsum sludge, biosolids and ashes) were deposited on the plots. The plots were separated by a buffer strip of at least 5 m inside which no soil was deposited (Fig. 9). This precaution made it possible to visually distinguish each of the test conditions in addition to preventing contact between soils with different levels of contamination. The installation of a sediment barrier surrounding each of the plots also prevented the spreading of the soil on the periphery which could be caused by the melting of snow in the spring or by heavy rains.

The soils should be treated from the C level of contamination down to the B level of contamination [8]. Once treated to the lower level of contamination, the soils will be left in place and used as a revegetation layer for the restoration of the mining tailing piles.

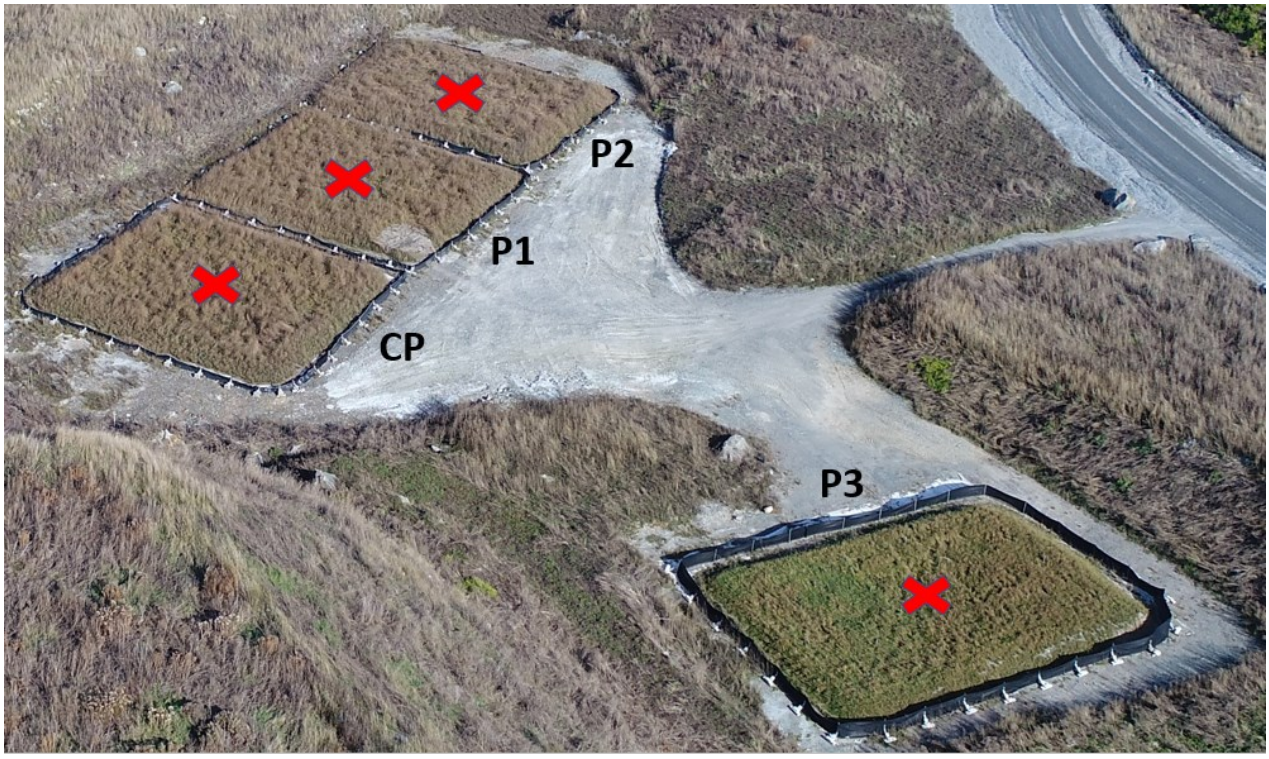


Figure 9. Aerial photograph of the restoration site, in Thetford Mines, QC, Canada. Lysimeters are represented by red crosses. CP: control plot, P1: plot #1, P2: plot #2 and P3: plot #3.

3.5.3. Phytostabilisation and rhizodegradation

The selection of plant species (i.e., Poaceae, Fabaceae) was done to favor the capacity to produce a strong root biomass favoring the establishment of a large microbial population in the soil. These species also limit the absorption of organic and inorganic contaminants at the root level and their transfer to the aerial parts [36]. This strategy promotes the biodegradation of organic contaminants at ground level and eventually limit the potential exposure of contamination to higher trophic level organisms during grazing [36].

The seeding of the test plots was done no later than one week following the placement of the soil (August 2020 for CP, P1, and P2 and August 2021 for P3). Six plant species, i.e., 4 grasses (Poaceae family) and 2 legumes (Fabaceae family) were selected for the project and used for all plots.

3.5.4. Leachate water sampling

Leachate water samples (60 L, *n*=1 per plot) used for the toxicity testing on *D. magna* were collected during the month of October 2021 using a lysimeter placed in the middle of each plot. The barrels of leachate water samples were kept refrigerated (4°C) and brought to room temperature before using them for testing. It is to be noted that the sampling of the leachate water was performed 14 months after sowing for CP, P1 and P2, which could affect the results of the WET test. For P3, which contains the highest levels of contaminants, the sampling of the leachate water was performed 2 months after the sowing. Other samples were also collected in 2020 (summer, fall), 2021 (spring) and 2022 (spring) to evaluate the levels of contaminants in the water.

3.5.5. Metals, PAHs, PHs and elemental analysis of leachate waters

The quantification of 17 metals, C_{10}-C_{50} PHs and 16 PAHs (Annexes) was performed in leachate water samples (*n*=1) according to methods from the Ministry of the Environment of Quebec (Canada) [37-39].

For analysis of metals, water samples were first filtered using 0.45 µm filters. Samples were then acidified to pH < 2 using nitric acid. The prepared samples were then analyzed by inductively coupled plasma-

mass spectrometry (ICP-MS) using daily prepared calibration curve for all metals. Quality control samples were also prepared and had to be within 15% of the expected value for the results to be accepted [37].

C_{10}-C_{50} PHs were analyzed by gas chromatography coupled with a flame ionization detector (GC-FID). Water samples (*n*=1) containing PHs were acidified to pH<2 using sulfuric acid 50%. PHs were extracted from water by liquid-liquid extraction using hexane (50 mL, then 30 mL). Samples were then analysed with the following parameters: injection volume of 1 µl, vector gas; helium with a 5,0 ml/min flow, DB-1 type capillary chromatography column (dimensions; 15 m × 0.53 mm, 0.15 µm), initial temperature of the oven was 40°C, up to 340 °C. QC samples were prepared with diesel fuel no. 2 from Restek (Bellefonte, PA) had to be within 20% of the expected value for the results to be accepted [38].

PAHs were analyzed by gas chromatography-mass spectrometry. Water samples (*n*=1) were extracted using 100 mL of dichloromethane (DCM). DCM was evaporated and samples were reconstituted in 1mL of hexane. Samples were analysed using a DB-EUPAH type capillary chromatography column (30 m, 0.25 mm, 0.25 µm) and a gas flow of 1.4 mL/min. Initial oven temperature was 80 °C, and temperature was ramped to 320 °C at a rate of 35°C/min then to 335°C at a rate of 3 °C/min. The volume of injection was 1 µl, splitless, and single ion monitoring mode (SIM) was utilised. QC samples had to be within 25% of the expected for the results to be accepted [39].

Elemental analysis of carbon (C), nitrogen (N) and phosphorus (P) were performed to assess any difference between the leachate waters from the different plot as well as the control (International Organization for Standardization (ISO) water) (see Table S4 for detailed composition). Carbon was

analysed using a Sievers M9 Total Organic Carbon (TOC) Analyzer. TOC was oxidized by ammonium persulfate under a UV lamp to produce CO_2 measured by a conductivity cell. The instrument is calibrated using a potassium phthalate solution. Nitrogen was analysed using a using a Lachat 8500 auto-analyzer. The nitrogen was oxidized to nitrate by the action of potassium persulfate at 121℃ for 1h 15 min in an autoclave. The digestate was analyzed by reduction of nitrates to nitrites by a cadmium column and formation of a colored complex with sulfanilamide and *N*-(1-naphthyl)-ethylenediamine dihydrochloride (N-NED) [40]. The instrument was calibrated using potassium nitrate solutions. Digestion efficiency was checked by analysis of ammonium chloride solution. For phosphorus analysis, an Astoria 2 analyzer (Astoria Pacific, Oregon, USA) was used. Phosphorus was oxidized to phosphate by the action of potassium persulfate at 121℃ for 1h 15 min in an autoclave. The digestate was analyzed using the Astoria 2 auto-analyzer by formation of a blue colored complex by the reaction between ammonium molybdate and potassium antimony tartrate, followed by reduction with ascorbic acid [41]. The instrument was calibrated using potassium phosphate solutions.

3.5.6. *D. magna* culture

D. magna culture originated from ephippia acquired from EBPI Canada (Burlington, ON) and hatched in laboratory conditions using synthetic Moderately Hard Reconstituted Water (ISO water) [42]. A 16-h light: 8-h dark photoperiod was used to raise the daphnids. Cultures were kept at 20 ± 1 °C and renewed every 2 months using neonates from 3rd to 5th broods. Two mL of green algae *Raphidocellis subcapitata* (about 3.85×10^5 cells/mL) were fed to daphnids after media renewals (every second day). Algae were cultured using Bold Modified Basal Freshwater medium from Sigma-Aldrich under the same conditions described above for *D. magna*. Microalgae were harvested during the exponential growth phase and inoculated in

fresh medium. Exposure tests of leachate waters were initiated with neonates (<24 h old), born between the 3rd and 5th broods of the main culture.

3.5.7. Acute exposure to leachate water

Acute toxicity testing of the leachate water was first performed to evaluate the need of a dilution for the chronic exposures. The U.S. Environmental Protection Agency guidelines were followed [43]. Briefly, neonates (< 24 h) are deposited in the receptacles (5 daphnids per receptacle, 5 receptacles per concentration) containing the control medium [moderately hard synthetic water prepared using Milli-Q water from a Millipore filtration system [44]] or different dilutions of leachate water diluted with the control medium (100%, 50%, 25%, 12.5% and 6.25% of leachate water). Leachate water was coarsely filtered (2 mm mesh) to remove debris before the dilution. After 48 h, the mortality of the daphnids was evaluated. If a daphnid remained motionless for 15 s after slightly shaking the container, it was considered dead. Potassium dichromate ($K_2Cr_2O_7$) at 5 different levels (0.32 mg/L, 0.56 mg/L, 1 mg/L, 1.8 mg/L and 3.2 mg/L) was used as the reference toxicant during this test.

3.5.8. Chronic exposure to leachate water

Four different exposure tests were performed to observe the effects of the leachate water from the 4 plots. In all assays, neonates (< 24 h) were exposed for 21 days to the leachate waters of the different plots. following the U.S. EPA guideline [45]. Tests were performed using 10 replicates of 25 daphnids each separated in 2 groups (5 control, 5 exposed) in a static-renewal manner. Temperature was kept at 20 ± 1

°C using an incubator, light intensity was 2000 ± 70 lux and a 16-h light: 8-h dark photoperiod was maintained. On day 1 of the tests, neonates from the control groups were transferred in 2 L beakers filled with culture medium. Neonates from the exposed groups were transferred to 2 L beakers filled with filtered leachate water from the different plots. Culture medium was renewed 3 times a week. Following a standard protocol used by the Ministry of the Environment of Quebec (Canada), *D. magna* were sorted by size using a series of sieves during the media renewals [42]. Adults were collected on a 900 µm sieve, juveniles on a 560 µm sieve and neonates on a 300 µm sieve. After being counted, offspring (juveniles and neonates) were removed. Only adults were transferred to the renewed beakers. Males or dormant eggs were never observed across the experiments. Daphnids were fed with 2 mL of a *Raphidocelis subcapitata* algae solution after every media renewal. On day 21 of the exposure period, *D. magna* adults were sampled, washed with deionized water and weighed, then placed in MeOH 1 % FA and stored at -80°C until analysis.

3.5.9. Quantification of 20E using liquid chromatography-triple quadrupole mass spectrometry

Using whole adult daphnids (15-25 individuals), 20E was extracted and quantified following the method developed by Venne *et al.* [35] with minor modifications. After being collected and washed, daphnids were placed in a centrifuge tube containing 1 mL of 1% FA in methanol, homogenized using an plastic pestle and sonicated in an ultrasonic bath for 15 min. An aliquot of 800 µL was withdrawn from the tube and transferred in a glass tube and 50 µL of internal standard solution (makisterone A) was added. The solvent was evaporated under a gentle flow of $N_{2(g)}$. A volume of 1 mL of hydroxylamine hydrochloride solution (100 mg/mL) was added to the tubes and 20E and makisterone A were then derivatized to their oxime analogues for 90 minutes. with. 20E and makisterone were retrieved using a liquid-liquid extraction with

2×1.5 mL of MTBE. The test tubes were then placed at -20 °C until the water froze between each extraction, and the organic layer (unfrozen) containing the derivatized analyte and its internal standard was collected and evaporated to dryness. Samples were reconstituted in, 250 µL of and transferred to vials for analysis.

A liquid chromatography-triple quadrupole mass spectrometer (LC-QqQMS) system composed of an Acquity UPLC system coupled to a Xevo TQ-S micro, both from Waters (Billerica, MA) was used to quantify 20E in daphnid extracts. A reversed-phase column Acquity UPLC Cortecs C_{18+} from Waters of dimensions 50×2.1 mm and 1.6 µm particle size was used for chromatographic separation. Mobile phase was composed of solvent A (H_2O containing 0.1% *v/v* of AA) and solvent B (mixture of MeOH and ACN 3:2 *v/v*, containing 0.1% *v/v* of AA). The elution gradient started with 5% of B, increasing to 55% in 7.9 min, rising immediately to 100% of B and hold for 2 min, then back to initial conditions for column re-equilibration (2.1 min). The sample injection volume was set to 10 µL. Electrospray in the positive mode was used as ionization source and the mass spectrometer was operated in the multiple reaction monitoring (MRM) mode. MRM transitions used were *m/z* 478.3 → *m/z* 316.3 for 20E oxime and *m/z* 492.2 → *m/z* 316.1 for makisterone A oxime. Concentration of 20E was reported as average mass per adult daphnid wet mass. The total number of *D. magna* used varied among the samples due to differences in the mortality of daphnids at the end of the 21-day period.

Quality control (QC) samples were used in order to determine the deviation percentage of the method and thereby verify the accuracy and precision of measured concentrations. Three concentrations of QC samples were used: low (≈100 pg mL^{-1}), medium (≈300 pg mL^{-1}) and high (≈500 pg mL^{-1}). A solution of frozen daphnids (50 mg mL^{-1} in 1% F.A. in MeOH) was used to correct the matrix effects. This solution

was used to prepare QC samples. The value of the QC samples was within ± 20 % of the expected value for the results to be considered acceptable.

3.5.10. Data analysis

Two-sample t-tests (α=0.05) using Microsoft Excel 365 were employed to evaluate significant differences between exposed and control samples (control medium, not control plot leachate water) for survival, weight, number of neonates and 20E levels. F-test were also performed to compare variances between the two groups. Graph Pad Prism 6 (San Jose, CA) software was used to produce graphs for the results of all assays.

3.6. Results and discussion

3.6.1. Metals, PAHs, PHs and elemental analyses of leachate waters

The results of the quantification of metals are presented in Fig. 10 and those of PHs and PAHs in Table S6 and S7 respectively. Detailed metals concentrations are also reported in Table S5.

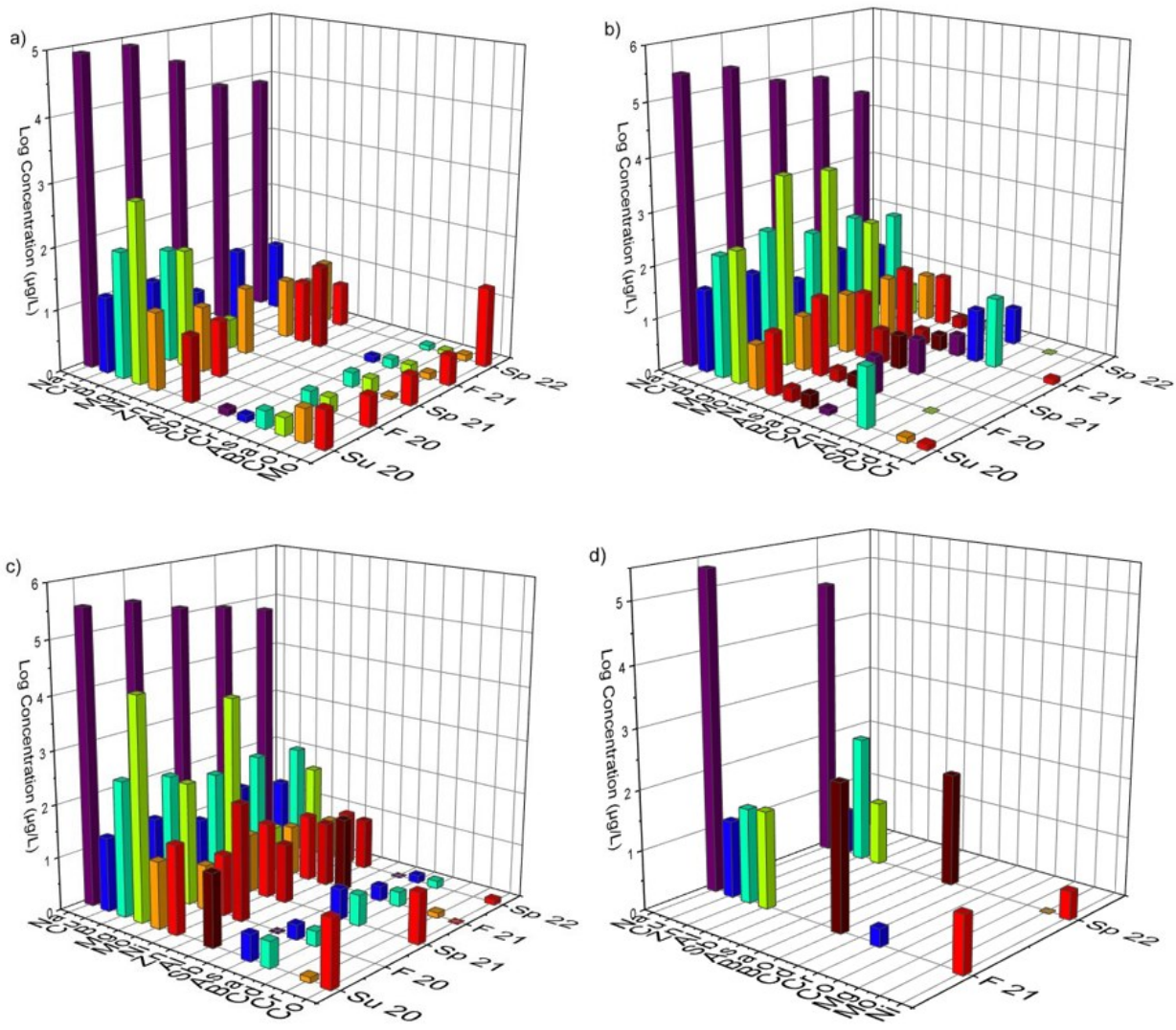


Figure 10. Metal concentrations (µg/L) in leachate water collected using lysimeters (n=1) from **a)** control plot CP, **b)** plot P1, **c)** plot P2 and **d)** plot P3. The absence of values indicates a concentration below the limit of detection (variable between analytes). Su 20: Summer 2020, F 20: Fall 2020, Sp 21: Spring 2021, F 21: Fall 2021 and Sp 22: Spring 22. Values for P3 start after the summer of 2021 (installation date) could not be collected since P3 was placed during the summer of 2021.

There was no clear difference in concentrations of metals among leachate water samples, except for Na and Ba. The Na and Ba concentrations in water decreased over time for all plots. These two metals could

form salts which would increase their solubility in water and therefore wash them off the plots over time [46]. Organic matter and plants with a dense and wide root system can retain transition metals and prevent their leeching [47].

A different trend was observed for the organic contaminants. The initial concentration of C_{10}-C_{50} PHs for CP, P1 and P2 during summer 2020 was of 995 µg/L, 409 µg/L and 849 µg/L respectively. After only a few months, the initial concentrations of PHs were diminished by 69% for the CP, 38% for P1 and 53% for P2. Following spring 2021, the concentration in PHs C10-C50 of all of these plots was under the limit of detection (5-100 µg/L), showing a reduction over time of the PHs in the leachate water. In the case of P3, the initial concentration was under the limit of detection, as were all samples of the following time points. This was expected as organic contaminants are often sequestered by soil or degraded by soil microorganisms [48]. All concentrations of the PHs can be found in Table S6 of the supplementary material.

Only the concentration benzo[a]pyrene was reported as all other measured PAHs were under the limit of detection (<0.1 µg/L). For CP and P1, benzo[a]pyrene concentration was under the limit at all sampling time except for the spring 2021 where a concentration of 0.1 µg/L was reported. This value was at the limit of detection of the method, and changes on benzo[a]pyrene concentration over time could be impacted by nearby sources such as biomass and coal combustion as well as the exhaust of vehicles [49] or by the mobilisation of what is adsorbed by the soil. For P2, the reported concentration was of 0.5 µg/L during fall 2020, 0.1 µg/L during spring 2021 and was then under the limit of detection for fall 2021 and spring 2022. The initial concentration of P3 (fall of 2021) was of 0.1 µg/L. It was then under the limit of detection. No PAHs were observed in the leachate water of any plots during spring 2022. Detailed concentrations of benzo[a]pyrene can be found in Table S7 of the supplementary material.

Results of the total nitrogen, total carbon and total phosphorus analyses are shown in Table 2. The leachate waters contain much more nitrogen, organic carbon and phosphorus than the control water. This was expected as they derive from the natural environment and contained a certain quantity of sediments and other organic mater. In streams and rivers in the USA, it was found that natural background concentrations varied between 20 and 500 µg/L for total nitrogen (TN) and between 6 and 80 µg/L for total phosphorus (TP) [50]. In Canada, TN and TP values in sampled streams ranged from 10 to 27400 µg/L and 1 to 6050 µg/L, respectively [51]. According to Chambers *et al*. [51] to protect aquatic ecosystems in Quebec and Ontario total nitrogen and total phosphorus thresholds are 1100 µg/L and 30 µg/L, respectively. The values found in the leachate waters are all higher than these values. Therefore, potential effects on aquatic biota caused by excess nutrients in the runoff cannot be disregarded.

Table 2. Elemental analysis from leachate waters and control medium. TN: total nitrogen, TOC: total organic carbon and TP: total phosphorus.

Water sample	TN (µg L^{-1})	TP (µg L^{-1})	TOC (mg L^{-1})
Reconstituted (ISO) water	26.3	4.6	0.4
CP	15395	81.5	22.6
P1	3353	86.1	77.1
P2	1870	51.2	21.6
P3	2246	72.5	25.5

3.6.2. Acute exposure to leachate water

No *D. magna* mortality was observed for any leachate sample, therefore no EC_{50} could be calculated, and the leachate water without dilution was used for the following chronic exposures. Validity of these tests was confirmed by the EC_{50} obtained with the positive control ($K_2Cr_2O_7$) which was 0.82 ± 0.108 mg/L

(See Fig. S5). This value is within the accepted range for the validity of the acute exposure conditions [52]. The control group survival percentage was of 100%.

3.6.3. Effects of leachate on survival over a 21-day period

Daphnids reached adulthood and produced their first brood between day 7 and 10. Survival was over 80% for all tests. For the survival criterion, no difference was observed (p>0.05) between the control group and the exposed group for the CP and P2 plots (Fig. 11).

The number of daphnids surviving in the water of plot P1 was significantly lower (p= 0.0481) than for the control group, but this difference was very small (<10%, or 2 daphnids). Moreover, the survival rate for the exposed group was superior to the test threshold of 80%, since natural mortality of less than 20% is generally expected in daphnids. According to analysis of the water samples, this plot has higher concentrations than the other plots for dissolved cobalt (2.5 µg/L), dissolved manganese (154 µg/L), dissolved molybdenum (14 µg/L), dissolved nickel (26 µg/L) as well as for uranium (5.7 µg/L). However, these values are much lower than the LC_{50} reported by various studies [53, 54] and therefore do not explain this difference in mortality. A possibility would be a cocktail effect, where a synergic effect could be seen due to the mixture of different metals in the water [55, 56]. The LC_{50} of metals can decrease by a 10-fold factor when in mixture [57]. However, the measured concentration in leachate waters were still lower than the reported LC_{50}. As for the PAHs concentration, they were under the limit of detection (<0.1 µg/L) for all plots. C_{10} –C_{50} PHs values were also under the limit of detection (<100 µg/L). Therefore, the lower survival rate than the control did not suggest that the leachate water from the plot P1 was toxic to daphnids.

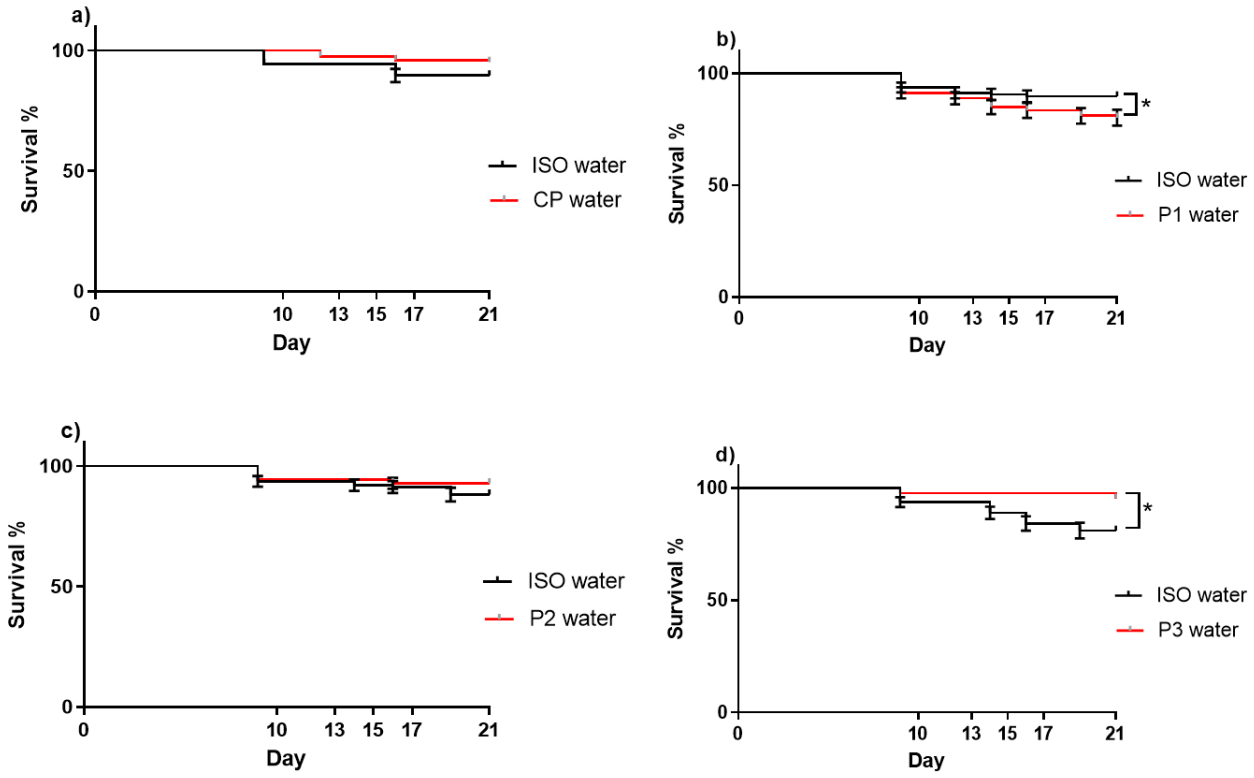


Figure 11. Survival curves of daphnids exposed 21 days to leachate water from **a)** control plot CP (*n*=5), **b)** plot P1 (*n*=5), **c)** plot P2 (*n*=5) and **d)** plot P3 (*n*=5). Error bars represent ± 1 standard error of the mean.

As for plot P3, less mortality was observed in the exposed group compared to the control group (p=0.004). In absolute terms, this represents a difference of less than 20%, or 4 daphnids. This could be explained by a greater amount of nutrients (TN, TP, TOC) present in the leachate water compared to the reconstituted water of the control group as shown in Table 2. Daphnids that have a better supply of algae and nutrients have a better survival rate, as shown in a study comparing different culture media [58].

3.6.4. Reproduction

For all tests on water from the different plots, no significant difference ($p > 0.05$) was observed between the control group and the exposed group in terms of the number of neonates produced by adult daphnids (Fig. 12). Metals can affect fertility in many organisms such as *D. magna* [59]. This wasn't the case with the concentrations present in the leachate waters and results suggest that the chronic exposure to these waters presented no risk for *D. magna*.

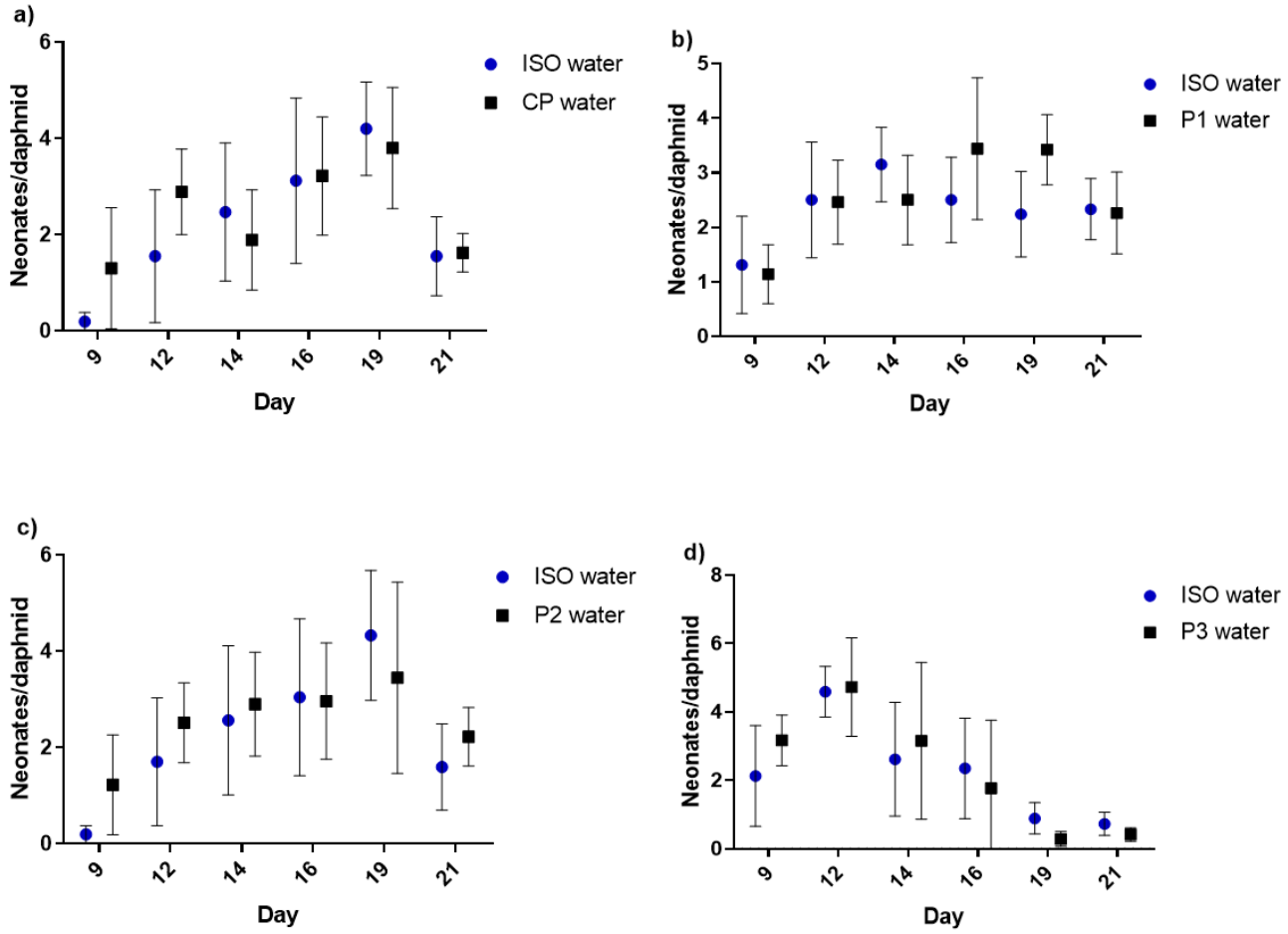


Figure 12. Offspring production per daphnid exposed for 21 days to leachate water from **a)** control plot CP (n=5), **b)** plot P1 (n=5), **c)** plot P2 (n=5) and **d)** plot P3 (n=5). Error bars represent ± 1 standard error of the mean.

3.6.5. Effects on weight

The weight of the daphnids was also measured on the 21st day. A lower weight could indicate stunted growth and therefore adverse sublethal effects. For all tests, no significant difference was observed between the control group and the exposed group regarding the weight of the adult daphnids (Fig. 12). Metals have induced reduced weight and growth during chronic exposure to Na(I), Ca(II), Mg(II), K(I), Sr(II), Ba(II), Fe(III), Mn(II), As(V), Sn(II), Cr(III), Al(III), Zn(II), Au(III), Ni(II), Pb(II), Cu(II), Pt(IV), Co(II), Hg(II) and Cd(II) in *D. magna*, but at higher concentrations (range: 3.4 to 680 000 mg/L) than those measured in the tested leachate waters [60, 61]. The present results support those of the previous sections concerning the absence of apparent toxicity.

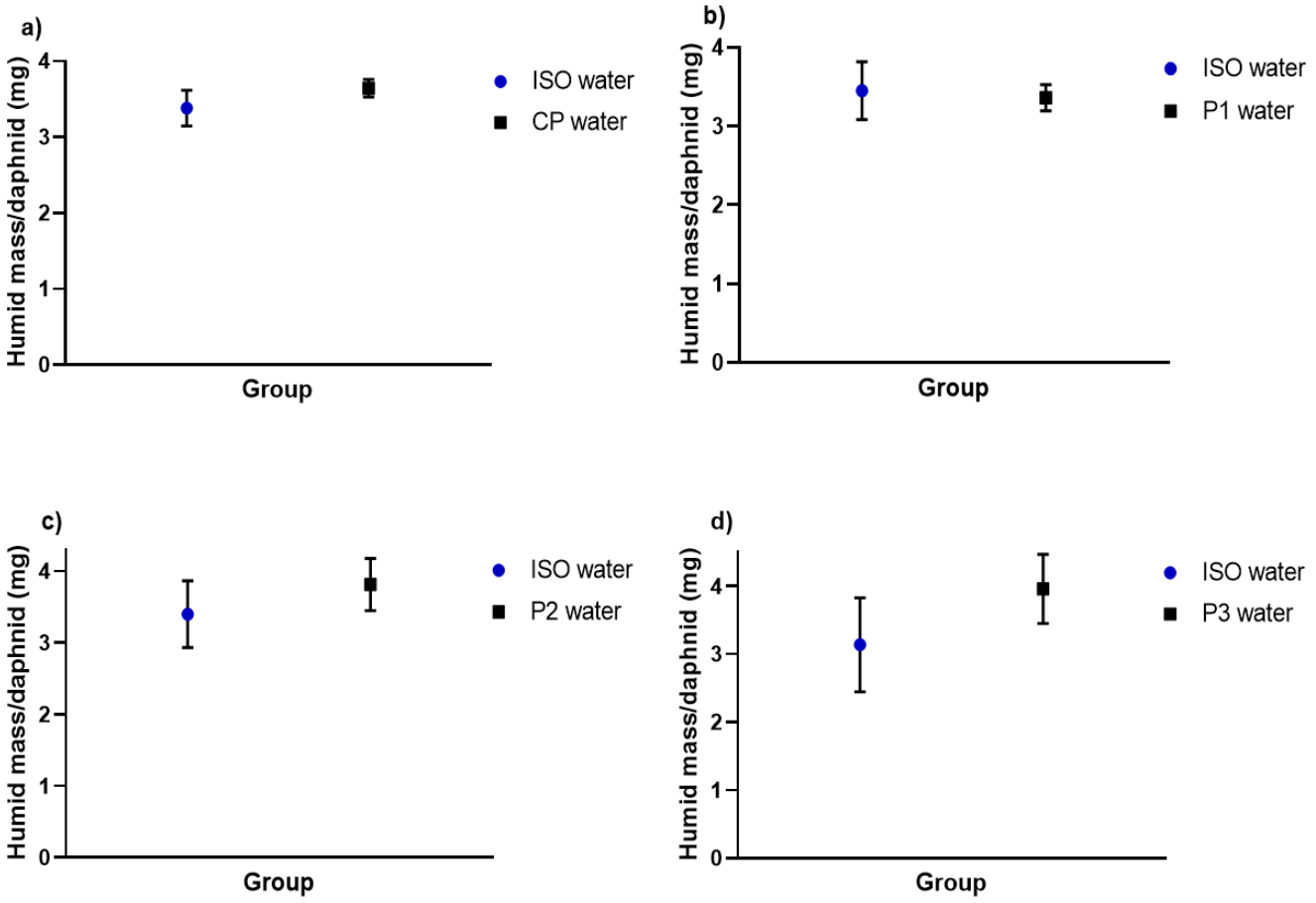


Figure 13. Weight of daphnids exposed for 21 days to leachate water from **a)** control plot PT (*n*=5), **b)** plot P1 (*n*=5), **c)** plot P2 (*n*=5) and **d)** plot P3 (*n*=5). Error bars represent ± 1 standard error of the mean.

3.6.6. Ecdysteroid levels

The last endpoint measured was the level of 20E (Table 3). 20E is an essential hormone for the growth and reproduction of *D. magna*. A change in this parameter could indicate a problem with its metabolism which would eventually lead to fertility problems or even death. The 20E concentration is reported per wet mass and compared between the control group and the group exposed to leaching water from the different plots. No significant difference was observed between the control group and the exposed group for all the tests regarding the concentrations of 20E in adult daphnids.

Table 3. 20E quantity per humid mass of daphnids exposed 21 days to leachate water (n=5 per plot).

Water sample	ISO water (pg/mg±S.D.)	Leachate water (pg/mg±S.D.)
CP	5.69 ± 2.26	4.21 ± 1.61
P1	4.52 ± 2.26	5.39 ± 2.25
P2	5.06 ± 2.19	3.80 ± 1.46
P3	2.34 ± 1.74	3.23 ± 0.80

20E levels fluctuates over time following an ecdysis cycle, which corresponds to the molting of the daphnids, as shown in previous works [62]. Therefore, the ecdysis cycle synchronization of daphnids is essential for the measure of such metabolites. After 21 days of life, it is possible that daphnids of the same age are no longer synchronized in their ecdysis cycle and that differences between groups are compromised by this phenomenon. Indeed, if some individuals are at the beginning of the cycle and others are in the middle, the inter-individual variability for 20E concentration is much higher than with synchronised individuals [63]. It could also be that the metals concentration in the leachate waters were not high enough to affect the daphnids in that regard.

3.7. Conclusion

The present work aimed to evaluate the acute and chronic effects of leachate waters from mine tailings covered with metals, PHs, and PAHs contaminated soil on *D. magna*. Life history parameters and the 20E level in daphnids were assessed over a 21-day period. Results showed that, the leachate water from the various plots of contaminated soil undergoing phytoremediation did not cause toxicity on *D. magna*. Indeed, except for the survival of daphnids reared in water from plot P1, no difference was observed between the control and exposed groups for all the criteria measured through the different tests. Levels

of PHs and PAHs rapidly decreased over time (2 years period) before reaching undetectable levels in the leachate waters in the last year of the measurements.

It would have been interesting to carry out sampling and testing during the first months following the spreading of the contaminated soils for all sites when the plots usually have the highest content of metals, PHs C_{10}-C_{50} and PAHs; this was the case only for plot P3, which was the most contaminated. This decrease in concentrations before the ecotoxicological evaluation could impact results of ecotoxicity tests. It is thus important to carry these evaluations early in the process to assess the worst-case scenario and conditions.

When implementing new environmental remediation strategies, it is important to assess all possible impacts on the environment. The WET approach allowed to evaluate the leachate waters on *D. magna* as close to a real-life scenario as possible with minimal pretreatment before testing. It was found to be efficient to assess the possible toxicity of leachate waters from soils used during remediation.

3.8. Author contributions

Hugo Alarie: Conceptualization, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – Original Draft

Dany Landry: Conceptualization, Writing – Review & Editing

Magali Houde: Supervision, Writing – Review & Editing,

Pedro A. Segura: Conceptualization, Funding Acquisition, Methodology, Resources, Supervision, Writing – Review & Editing

3.9. Conflicts of interest

Dany Landry is an employee of Englobe Corp.

3.10. Acknowledgements

We acknowledge the support from NSERC CREATE program TEDGIEER (*Technologies environnementales décontamination et gestion intégrée des eaux et effluents résiduaires*) and Hydro-Québec for the scholarships granted to H. Alarie. This study was possible thanks to funding from the Natural Sciences and Engineering Research Council of Canada (NSERC) through a Discovery grant awarded to P.A. Segura and by the Faculty of Sciences of the Université de Sherbrooke. Funding sources did not have any involvement in the design, experiments, data interpretation, writing, revision or submission of this study.

3.11. References

1. R. Tiwary, Environmental impact of coal mining on water regime and its management, *Water, Air, and Soil Pollution*, 2001, **132**, 185-199.

2. M. Prasanna, S. Praveena, S. Chidambaram, R. Nagarajan and A. Elayaraja, Evaluation of water quality pollution indices for heavy metal contamination monitoring: a case study from Curtin Lake, Miri City, East Malaysia, *Environmental Earth Sciences*, 2012, **67**, 1987-2001.

3. H. Wong, A. Gauthier and J. Nriagu, Dispersion and toxicity of metals from abandoned gold mine tailings at Goldenville, Nova Scotia, Canada, *Sci. Total Environ.*, 1999, **228**, 35-47.

4. V.-P. Salonen, N. Tuovinen and S. Valpola, History of mine drainage impact on Lake Orijärvi algal communities, SW Finland, *Journal of Paleolimnology*, 2006, **35**, 289-303.

5. S. Gheorghe, C. Stoica, G. G. Vasile, M. Nita-Lazar, E. Stanescu and I. E. Lucaciu, Metals toxic effects in aquatic ecosystems: modulators of water quality, *Water quality*, 2017, **87**, 59-89.

6. S. Dudka and D. C. Adriano, Environmental impacts of metal ore mining and processing: a review, *J. Environ. Qual.*, 1997, **26**, 590-602.

7. D.-M. Xu, C.-L. Zhan, H.-X. Liu and H.-Z. Lin, A critical review on environmental implications, recycling strategies, and ecological remediation for mine tailings, *Environ. Sci. Poll. Res.*, 2019, **26**, 35657-35669.

8. M. Beaulieu, *Guide d'intervention - Protection des sols et réhabilitation des terrains contaminés*, Ministère de l'Environnement et de la Lutte contre les changements climatiques, Québec, QC, 2021.

9. A. Dybowska, M. Farago, E. Valsami-Jones and I. Thornton, Remediation strategies for historical mining and smelting sites, *Science Progress*, 2006, **89**, 71-138.

10. A. Yan, Y. Wang, S. N. Tan, M. L. Mohd Yusof, S. Ghosh and Z. Chen, Phytoremediation: a promising approach for revegetation of heavy metal-polluted land, *Frontiers in Plant Science*, 2020, **11**, 359.

11. S. Ouvrard, P. Leglize and J.-L. Morel, PAH phytoremediation: rhizodegradation or rhizoattenuation?, *International Journal of Phytoremediation*, 2014, **16**, 46-61.

12. S. Li, L. He, B. Zhang, Y. Yan, W. Jiao and N. Ding, A comprehensive evaluation method for soil remediation technology selection: Case study of ex situ thermal desorption, *International Journal of Environmental Research and Public Health*, 2022, **19**, 3304.

13. B. Song, G. Zeng, J. Gong, J. Liang, P. Xu, Z. Liu, Y. Zhang, C. Zhang, M. Cheng and Y. Liu, Evaluation methods for assessing effectiveness of in situ remediation of soil and sediment contaminated with organic pollutants and heavy metals, *Environ. Int.*, 2017, **105**, 43-55.

14. M. Tonkes, P. J. De Graaf and J. Graansma, Assessment of complex industrial effluents in the Netherlands using a whole effluent toxicity (or WET) approach, *Water Sci. Technol.*, 1999, **39**, 55-61.

15. S. Bao, B. Pan, L. Wang, Z. Cheng, X. Liu, Z. Zhou and X. Nie, Adverse effects in Daphnia magna exposed to e-waste leachate: Assessment based on life trait changes and responses of detoxification-related genes, *Environmental Research*, 2020, **188**, 109821.

16. L. N. Sackey and V. Kočí, Assessing the effects of tropical wood leachate to Desmodesmus subspicatus, Lemna minor and Daphnia magna, *Heliyon*, 2020, **6**.

17. S. I. Dodson and T. Hanazato, Commentary on effects of anthropogenic and natural organic chemicals on development, swimming behavior, and reproduction of Daphnia, a key member of aquatic ecosystems, *Environ. Health Perspect.*, 1995, **103**, 7.

18. B. E. Miner, L. De Meester, M. E. Pfrender, W. Lampert and N. G. Hairston Jr, Linking genes to communities and ecosystems: Daphnia as an ecogenomic model, *Proceedings of the Royal Society B: Biological Sciences*, 2012, **279**, 1873-1882.

19. World Health Organization, International Atomic Energy Agency and Food and Agriculture Organization of the United Nations, *Trace Elements in Human Nutrition and Health*, WHO Press, Geneva, Switzerland, 1996. Available at: https://www.who.int/water_sanitation_health/publications/2012/pharmaceuticals/en/.

20. L. J. Harvey and H. J. McArdle, Biomarkers of copper status: a brief update, *Brit. J. Nutr.*, 2008, **99**, S10-S13.

21. S. Wang and X. Shi, Molecular mechanisms of metal toxicity and carcinogenesis, *Molecular and cellular biochemistry*, 2001, **222**, 3-9.

22. L. W. Chang, L. Magos and T. Suzuki, *Toxicology of metals*, CRC Boca Raton, FL, 1996.

23. D. Beyersmann and A. Hartwig, Carcinogenic metal compounds: recent insight into molecular and cellular mechanisms, *Archives of toxicology*, 2008, **82**, 493-512.

24. C. Bodar, P. Voogt and D. Zandee, Ecdysteroids in Daphnia magna: their role in moulting and reproduction and their levels upon exposure to cadmium, *Aquat. Toxicol.*, 1990, **17**, 339-350.

25. G. A. LeBlanc, Crustacean endocrine toxicology: a review, *Ecotoxicol.*, 2007, **16**, 61-81.

26. C. Bodar, C. Van Leeuwen, P. Voogt and D. Zandee, Effect of cadmium on the reproduction strategy of Daphnia magna, *Aquat. Toxicol.*, 1988, **12**, 301-309.

27. C. Bodar, A. Zee, P. Voogt, H. Wynne and D. Zandee, Toxicity of heavy metals to early life stages of Daphnia magna, *Ecotoxicol. Environ. Saf.*, 1989, **17**, 333-338.

28. M. Heacock, C. B. Kelly, K. A. Asante, L. S. Birnbaum, Å. L. Bergman, M.-N. Bruné, I. Buka, D. O. Carpenter, A. Chen and X. Huo, E-waste and harm to vulnerable populations: a growing global problem, *Environ. Health Perspect.*, 2016, **124**, 550-555.

29. N. R. Haddaway, S. J. Cooke, P. Lesser, B. Macura, A. E. Nilsson, J. J. Taylor and K. Raito, Evidence of the impacts of metal mining and the effectiveness of mining mitigation measures on social–ecological systems in Arctic and boreal regions: a systematic map protocol, *Environmental Evidence*, 2019, **8**, 1-11.

30. Z. Yang, K. Shah, S. Laforest, B. P. Hollebone, J. Situ, C. Crevier, P. Lambert, C. E. Brown and C. Yang, Occurrence and weathering of petroleum hydrocarbons deposited on the shoreline of the North Saskatchewan River from the 2016 Husky oil spill, *Environ. Poll.*, 2020, **258**, 113769.

31. J. S. Latimer and J. Zheng, The sources, transport, and fate of PAHs in the marine environment, *PAHs: an ecotoxicological perspective*, 2003, 7-33.

32. F. Ahmed and A. Fakhruddin, A review on environmental contamination of petroleum hydrocarbons and its biodegradation, *International Journal of Environmental Sciences & Natural Resources*, 2018, **11**, 1-7.

33. D. Connell, G. Miller and J. Farrington, Petroleum hydrocarbons in aquatic ecosystems—behavior and effects of sublethal concentrations: Part 1, *Crit. Rev. Environ. Sci. Tecnol.*, 1980, **11**, 37-104.

34. A. A. Ratushnyak, M. G. Andreeva, V. Z. Latypova, R. R. Shagidullin and M. V. Trushin, Toxicity of oil and products of its refinement to Daphnia magna: time and temperature dependences, *Am.-Euras. J. Agric. Environ. Sci*, 2009, **5**, 545-549.

35. P. Venne, V. Yargeau and P. A. Segura, Quantification of ecdysteroids and retinoic acids in whole daphnids by liquid chromatography-triple quadrupole mass spectrometry, *J. Chromatogr. A*, 2016, **1438**, 57-64.

36. J. Hall, Cellular mechanisms for heavy metal detoxification and tolerance, *Journal of experimental botany*, 2002, **53**, 1-11.

37. Centre d'Expertise en Analyse Environnemetale du Québec, *Détermination des métaux : méthode par spectrométrie de masse à source ionisante au plasma d'argon (MA. 200 – Mét. 1.2, révision 7)*, Ministère de l'Environnement et de la Lutte contre les changements climatiques, Québec, QC, 2020.

38. Centre d'Expertise en Analyse Environnemetale du Québec, *Détermination des hydrocarbures pétroliers (C10 à C50) : dosage par chromatographie en phase gazeuse couplée à un détecteur à ionisation de flamme (MA. 400 – HYD. 1.1, Rév. 3)*, Ministère de l'Environnement et de la Lutte contre les changements climatiques, Québec, QC, 2016.

39. Centre d'Expertise en Analyse Environnemetale du Québec, *Détermination des hydrocarbures aromatiques polycycliques : dosage par chromatographie en phase gazeuse couplée à un spectromètre de masse (MA. 400 – HAP 1.1, Rév. 4)*, Ministère de l'Environnement et de la Lutte contre les changements climatiques, Québec, QC, 2016.

40. U.S. Environmental Protection Agency, *Method 353.2, Revision 2.0: Determination of Nitrate-Nitrite Nitrogen by Automated Colorimetry*, U.S. Environmental Protection Agency, Cincinnati, OH, 1993.

41. U.S. Environmental Protection Agency, *Method 365.3: Phosphorous, All Forms (Colorimetric, Ascorbic Acid, Two Reagent)*, U.S. Environmental Protection Agency, 1978.

42. Environment Canada, *Biological test method. Acute Lethality Test Using Daphnia spp.*, Ottawa, ON, 1990.

43. U.S. Environmental Protection Agency, *Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms*, Office of Water, Washington, DC, 5th edn., 2002.

44. International Organization for Standardization, ISO 5667-16:2017(en) Water Quality-Sampling-Part 16: Guidance on Biotesting of Samples, https://www.iso.org/obp/ui/#iso:std:iso:5667:-16:ed-2:v1:en, (accessed 2023-08-01).

45. U.S. Environmental Protection Agency, *National Whole Effluent Toxicity (WET) Implementation Guidance under the NPDES Program* 2004.

46. J. Burgess, *Metal ions in solution*, Ellis Horwood ; Distributed by Halsted Press, Chichester, NY, 1978.

47. A. Shackira and J. T. Puthur, Phytostabilization of heavy metals: Understanding of principles and practices, *Plant-metal interactions*, 2019, 263-282.

48. Z. Li, H. Cabana, J. Lecka, S. K. Brar, R. Galvez and J.-P. Bellenger, Efficiencies of selected biotreatments for the remediation of PAH in diluted bitumen contaminated soil microcosms, *Biodegradation*, 2021, **32**, 563-576.

49. K. Liao and J. Z. Yu, Abundance and sources of benzo [a] pyrene and other PAHs in ambient air in Hong Kong: A review of 20-year measurements (1997–2016), *Chemosphere*, 2020, **259**, 127518.

50. R. A. Smith, R. B. Alexander and G. E. Schwarz, Natural background concentrations of nutrients in streams and rivers of the conterminous United States, *Environ. Sci. Technol.*, 2003, **37**, 3039-3047.

51. P. A. Chambers, D. J. McGoldrick, R. B. Brua, C. Vis, J. M. Culp and G. A. Benoy, Development of environmental thresholds for nitrogen and phosphorus in streams, *J. Environ. Qual.*, 2012, **41**, 7-20.

52. International Organization for Standardization, ISO 6341:2012(en) Water quality — Determination of the inhibition of the mobility of Daphnia magna Straus (Cladocera, Crustacea) — Acute toxicity test, https://www.iso.org/obp/ui/en/#iso:std:iso:6341:ed-4:v1:en, (accessed 2023-08-01).

53. A. Okamoto, M. Yamamuro and N. Tatarazako, Acute toxicity of 50 metals to Daphnia magna, *Journal of Applied Toxicology*, 2015, **35**, 824-830.

54. T. Poston, R. Hanf and M. Simmons, Toxicity of uranium to Daphnia magna, *Water, air, and soil pollution*, 1984, **22**, 289-298.

55. N. Cedergreen, Quantifying synergy: a systematic review of mixture toxicity studies within environmental toxicology, *PloS one*, 2014, **9**, e96580.

56. C. Nys, L. Versieren, K. I. Cordery, R. Blust, E. Smolders and K. A. De Schamphelaere, Systematic evaluation of chronic metal-mixture toxicity to three species and implications for risk assessment, *Environ. Sci. Technol.*, 2017, **51**, 4615-4623.

57. Q. Meng, X. Li, Q. Feng and Z. Cao, 2008.

58. B.-P. Elendt and W.-R. Bias, Trace nutrient deficiency in Daphnia magna cultured in standard medium for toxicity testing. Effects of the optimization of culture conditions on life history parameters of D. magna, *Water Res.*, 1990, **24**, 1157-1167.

59. A. Münzinger and F. Monicelli, A comparison of the sensitivity of three Daphnia magna populations under chronic heavy metal stress, *Ecotoxicol. Environ. Saf.*, 1991, **22**, 24-31.

60. E. Pérez and T. C. Hoang, Responses of Daphnia magna to chronic exposure of cadmium and nickel mixtures, *Chemosphere*, 2018, **208**, 991-1001.

61. K. E. Biesinger and G. M. Christensen, Effects of various metals on survival, growth, reproduction, and metabolism of Daphnia magna, *Journal of the Fisheries Board of Canada*, 1972, **29**, 1691-1700.

62. D. Martin-Creuzburg, S. A. Westerlund and K. H. Hoffmann, Ecdysteroid levels in Daphnia magna during a molt cycle: determination by radioimmunoassay (RIA) and liquid chromatography-mass spectrometry (LC-MS), *Gen. Comp. Endocr.*, 2007, **151**, 66-71.

63. H. Alarie, N. Côté, L. Gaudreau, M. Houde and S. P. A., Are 20-hydroxyecdysone and related genes potential biomarkers of sublethal exposure to lipid-altering contaminants?, *Environ. Sci. Poll. Res.*, 2023.

CHAPITRE 4. LIPIDOMIQUE NON CIBLÉE POUR ÉTUDIER LES EFFETS SUBLÉTAUX DU GEMFIBROZIL SUR *DAPHNIA MAGNA*

Hugo Alarie[1], Francis Beaudry [2,3], Magali Houde [4], Pedro A. Segura [1*]

[1] Department of Chemistry, Université de Sherbrooke, Sherbrooke, QC, Canada,

[2] Département de Biomédecine Vétérinaire, Faculté de Médecine Vétérinaire, Université de Montréal, Saint-Hyacinthe, Québec, Canada

[3] Centre de recherche sur le cerveau et l'apprentissage (CIRCA), Université de Montréal, Montréal, Québec, Canada

[4] Aquatic Contaminants Research Division, Environment and Climate Change Canada, Montreal, QC, Canada

4.1. Notes préliminaires

Cet article a été soumis le 12 juillet 2023 au journal *Environmental Science: Processes & Impacts*.

4.2. Contributions des auteurs

J'ai moi-même réalisé le développement et design de l'étude, l'entièreté des expériences, l'analyse de données, l'interprétation des résultats et la rédaction de la première version de l'article pour ce chapitre. Francis Beaudry m'a aidé et supervisé lors de l'utilisation de son spectromètre de masse nécessaire à l'une des expériences de ce chapitre. Magali Houde et Pedro A. Segura ont participé à la correction et rédaction de la version finale de cet article.

Untargeted lipidomics to study sublethal effects of gemfibrozil on *Daphnia magna*

4.3. Abstract

Sublethal effects of pharmaceuticals are seldom studied in non-targeted species that can be exposed further down the line once these pharmaceuticals reach the environment. The objective of this project was to perform an untargeted lipidomic study of *Daphnia magna* exposed to gemfibrozil, a lipid lowering molecule frequently found in surface waters, to detect possible changes induced by this compound. Daphnids were exposed for 168 h to 1µg/L of gemfibrozil; a group of organisms was also subjected to 96 h of exposure followed by 72 h in clean media to evaluate the recuperation capacity of the organisms. Life history parameters (mortality, fertility and weight) as well as lipid profiles were assessed throughout the exposure and recuperation periods. A MTBE liquid-liquid extraction was used for extracting lipids from whole *D. magna* individuals and extracts were analyzed by liquid chromatography-quadrupole-time-of-flight mass spectrometry (LC-QTOF). Using the metabolomics platform XCMS online, features were identified and compared between control and exposed groups. Over 8000 features in positive mode and over 4000 features in the negative mode were observed. Significantly different features ($p<0.05$) were then identified by exact mass and tandem mass (MS^2) spectra using the lipid MAPS database. Results at the population level indicated that *D. magna* exposed to gemfibrozil had significantly more neonates after 168 h of continuous exposure. At the molecular level, in total, four lipids from two different classes (fatty acyls and glycerophosphocholines) were affected (lowered concentration) by gemfibrozil exposure. Several of the identified lipids [PC(34:6), PC(33:4), PC(17:2/18:4) or PC(18:4/17:2)] are involved in inflammation and cell death and should be studied as potential biomarkers of exposure to lipid lowering compounds since they are triglycerides intermediates affected by drugs such as gemfibrozil. Most of the significantly different identified features between exposed and control individuals returned to a normal level after the 72 h period of recuperation but remained significantly different in the exposed group after 168 h of exposure. These observations suggest a possibility of recuperation in exposed daphnids when gemfibrozil is removed from their environment. In summary, continuous exposure to environmental concentrations of gemfibrozil disrupt daphnids lipid metabolism and could impair their capacity to respond to environmental stressors.

Keywords: lipids, chronic effects, crustaceans, emerging contaminants, environmental toxicity

4.4. Introduction

Some trace organic contaminants, such as pharmaceuticals, are known to disrupt the endocrine system of aquatic organisms [1]. This includes the invertebrate *Daphnia magna*, a freshwater crustacean widely used in ecotoxicology studies because of its ubiquity and key role in aquatic ecosystems, easy culture maintenance in the laboratory as well as its sensitivity to organic contaminants exposure [2]. However, very few toxicity studies have investigated the sublethal effects of pharmaceuticals in *D. magna* using untargeted approaches [3,4]. Mass spectrometry-based lipidomics is of special interest to investigate subtle effects of such contaminants at environmental concentrations since lipids are involved in multiple metabolic processes [5]. For example, phospholipids and sphingolipids are involved in natural physiological processes such as inflammation and apoptosis [6]. Disruption of lipid homeostasis is now recognized to be involved in numerous pathologies such as energy metabolism deregulation, neurodegenerative disorders, endocrine perturbations or chronic inflammation in many organisms such as crustaceans [7-10].

Thus, using untargeted lipidomics, it is possible to quickly observe changes of several lipids concentration in complex biological samples. A few groups have already shown promising results when used in ecotoxicological context. Fuertes *et al.* (2020) [11] analyzed lipid profiles of *D. magna* chronically exposed (72 hours) to carbamazepine, diazepam, fluoxetine and propranolol at environmental concentrations (0.1-1 µg/L) and an increased fertility and changes in lipid profiles. Garreta-Lara *et al.* (2021) [12] found mechanistic insights of the toxicity induced by bisphenol A in exposed daphnids (24h) by using both untargeted and targeted approaches. Therefore, *Daphnia*

magna was proposed as a model to better understand the effects of lipid-lowering molecules using an untargeted lipidomic approach.

Many pharmaceuticals such as atorvastatin, lovastatin and fenofibrate are designed to reduce lipid level in blood. Among these pharmaceuticals that could potentially affect the lipid metabolism of *D. magna* is the lipid lowering gemfibrozil, used to help reducing cardiac events [13]. Gemfibrozil activates the peroxisome proliferator-activated receptor-α (PPARα), which once activated regulates the expression of multiple genes that control lipid metabolic processes such as fatty acid oxidation and lipogenesis [14]. Gemfibrozil has been frequently detected at concentrations up to 6.86 µg/L in rivers and groundwater [15-18]. This compound could have the potential to cause lipid metabolism disruption in *D. magna*, but also other crustaceans, and further effects such as growth defects, impacted reproduction or increased mortality by affecting the synthesis of ecdysteroids. Therefore, gemfibrozil was chosen as a model molecule to evaluate the capacity of untargeted lipidomics to measure changes on the lipidome of daphnids.

A few ecotoxicological studies have been conducted on *D. magna* to assess the effects of gemfibrozil. Most of these studies are of short duration (acute testing) and measure classical endpoints such as mortality [19, 20], growth, reproduction, and behaviour [21-23]. Other selected parameters such as gene expression, energy storage, stress markers and targeted metabolites have also previously been studied [20-22]. *D. magna* has shown to be sensitive to gemfibrozil, and therefore seems like a suitable model to further study the effect of this compound. Untargeted lipidomics may help better understand the perturbations of metabolic pathways. Moreover, exposure of aquatic organisms to trace organic contaminants can vary with time and can be continuous or punctual, as reported in previous studies [24-27]. Therefore, it is of interest to study the capacity of

organisms to recover following an exposure event. For example, Hayashi *et al.* (2008) [28] found that daphnids produced fewer offspring following an exposition to ibuprofen and that *D. magna* could recover and have a reproduction similar to control groups after a recuperation period of 10 days.

The aim of the present study was to develop and apply an untargeted lipidomics method to assess changes of the lipid metabolism in *D. magna* chronically exposed to the pharmaceutical gemfibrozil. Gemfibrozil stability and bioaccumulation in daphnids were also assessed during exposure. The proposed approach could be used in ecotoxicological assays to screen the subtle effects caused by exposure to regulated or emerging contaminants and help in the identification of potential biomarkers to better evaluate the risks of these contaminants.

4.5. Material and methods

4.5.1. Reagents and chemicals

Standards of prostaglandin E2 ethanolamide (PGE2-A2-d_4,>98 % purity), sphingosine-d_7, sphingomyelin (d_{18}:1/12:0) and C16 ceramide (d_{18}:1-d_7/16:0) were obtained from Avanti Polar Lipids (Alabaster, AL, USA). These standards were used to evaluate extraction and chromatographic separation quality and reproducibility as well as the stability of the mass spectrometry analysis. Water, methanol (MeOH), acetonitrile (ACN), methyl *tert*-butyl ether (MTBE), isopropanol (IPA), formic acid (FA) and acetic acid (AA) were LC or LC-MS grade and were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Gemfibrozil (≥99%) was purchased from Sigma Aldrich (Saint Louis, MO, USA) and its internal standard, gemfibrozil-d_6, from CDN isotopes (Pointe-Claire, QC, Canada). Stock solutions of standards were prepared at 100 μg mL^{-1} in MeOH and stored at -80 °C).

4.5.2. *Daphnia magna* culture

Ephippia of *D. magna* were purchased from Environmental Bio-detection Products Inc. (Mississauga, ON, Canada), hatched in the laboratory and maintained in synthetic Moderately Hard Reconstituted Water [29]. Cultures were kept at 20 ± 1 °C with a 16 h light: 8 h dark and were regularly renewed using neonates from 3rd to 5th broods. Daphnids were fed every second day with 2 mL of green algae *Raphidocellis subcapitata* (≈3.85 × 10^5 cells/mL). These microalgae were cultured in Bold Modified Basal Freshwater medium (Sigma-Aldrich) under the same laboratory conditions described above for *D. magna*. Algae were regularly harvested while still in the exponential growth phase and inoculated in fresh medium. All experiments were initiated with neonates (>24 h old), born between the 3rd and 5th broods, derived from a healthy parent stock. Frozen daphnids from Hikari Bio-Pure (Hayward, CA, USA) were purchased from a local store and were used as a matrix blank to prepare quality control samples.

4.5.3. Chronic exposure to gemfibrozil and recuperation

Gemfibrozil at a concentration of 1µg/L was used for testing. This concentration is in the same order of magnitude than maximal environmental concentrations reported in surface waters and groundwater for this compound [15-18].

Chronic exposure experiments were based on a modified protocol from the OECD guidelines [30]. One treatment exposed adult daphnids (9 days old) to gemfibrozil for 168 h and another one exposed them to gemfibrozil for 96 h followed by a 72 h of recuperation period (Figure 14). Briefly, chronic exposure experiments were performed using 3 groups (control, exposed, and recuperation) composed of 6 replicates of 45 daphnids each for each treatment. For all experiments, temperature was kept at 20 ± 1 °C using an incubator, light intensity was 2000 ± 70 lux and a photoperiod of 16 h of light and 8 h of dark was maintained. Medium was renewed 3 times a week. Daphnids were fed with 2 mL of a *R. subcapitata* algae solution (≈3.85 × 10^5 cells mL^{-1}) at every media renewal. On day 1 (0 h of exposure) of the tests, adult daphnids (9 days old) were transferred in 2 L beakers filled with the culture medium and were separated in control, exposed, and

recuperation groups. Gemfibrozil was added to the exposed and the recuperation groups (50 µl of methanol containing 20 µg mL^{-1} of gemfibrozil, final concentration: 1µg/L) and methanol (50 µL) was added to the control group to mitigate the solvent effect. After 96 h, gemfibrozil was only added to the exposed group (1µg/L) following the media renewal and methanol (50 µL) was added to the recuperation and control groups (see Figure 1). When performing these renewals, *D. magna* were sorted by size using a series of sieves, according to a standard protocol used by the Ministry of the Environment of Quebec [29]. Adults were collected on a 900 µm sieve, juveniles on a 560 µm sieve and neonates on a 300 µm sieve. Offspring (juveniles and neonates) were counted and then eliminated; only adults were transferred to the renewed solutions. Dormant eggs or males were never observed throughout the experiments, which indicated that experimental conditions were adequate to support a healthy population of *D. magna* throughout the experiments. The number of offspring and mortality were recorded at every medium renewal. Around 9 to 12 *D. magna* adults per beaker were collected at time 0 h, 48 h, 96 h and 168 h and stored at -80°C until analysis by liquid chromatography-quadrupole-time-of-flight mass spectrometry (LC-QTOF).

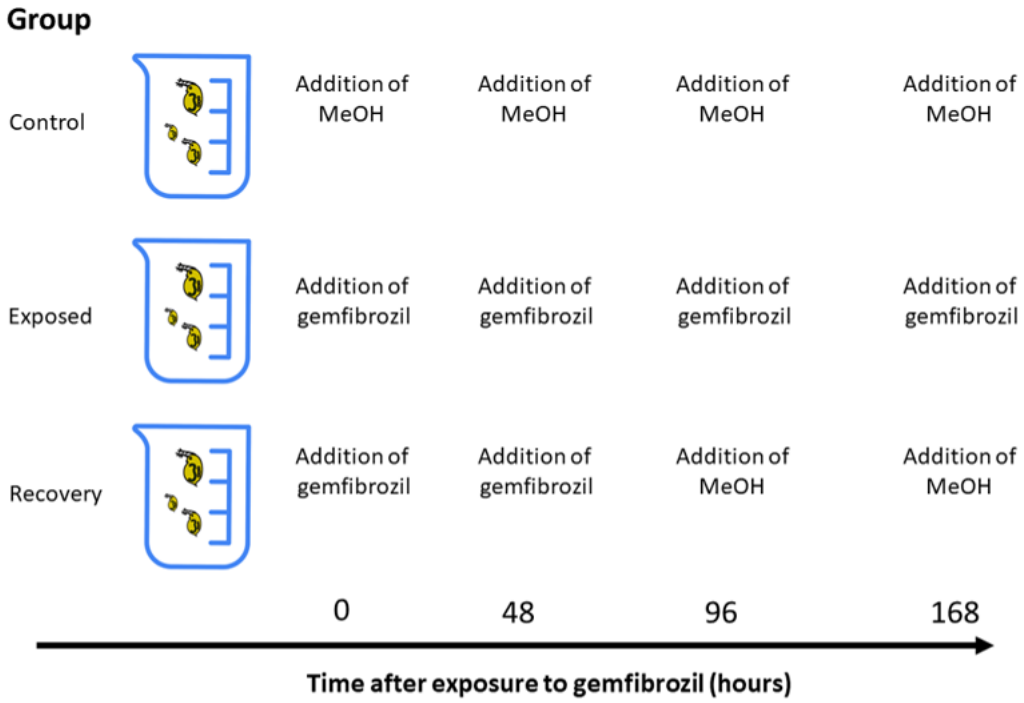


Figure 14. Experimental design of *D. magna* exposure to gemfibrozil 1µg/L. Daphnids were sampled at time of exposure 0, 48, 96 and 168 h during media renewal (n=6, 9 to 12 daphnids per replicate).

4.5.4. Chemical stability

Chemical stability of gemfibrozil during testing was evaluated. Gemfibrozil-d_6, was used as an internal standard. Aliquots (volume: 50 mL, *n*=3) were sampled immediately before and after media renewal at 48, 96 and 168 h of the exposure tests. Extraction was performed using Strata-X solid-phase extraction cartridges (polymeric reversed phase with particle diameter 33 µm, 200 mg of bed mass and 6 mL of volume) from Phenomenex (Torrance, California, USA). Quantification was carried out using a liquid chromatography-triple quadrupole mass spectrometry (LC-QqQ) system comprising an Acquity ultra-performance liquid chromatograph coupled to a Xevo TQ-S micro triple quadrupole mass spectrometer, both from Waters (Billerica, MA, USA). The entire protocol for the analysis of gemfibrozil is detailed in the Electronic Supplementary Information.

4.5.5. Lipids extraction and chromatographic separation

Whole daphnids (9 to 12 individuals) were extracted by liquid-liquid extraction method using MTBE [31]. Briefly, *D. magna* were sorted by size and adult individuals (>900 µm) were collected on a tissue strainer before being washed with deionized water (18 MΩ). Adult daphnids were then homogenized with a mortar and a pestle and sonicated in an ultrasonic bath for 15 min in 1 mL of 1% FA (*v/v)* in MeOH. Next, an aliquot of 900 µL was transferred in a glass tube and 5 mL of MTBE was added. A volume of 1.5 mL of water was added to induce phase separation and the organic (upper) phase was collected. Another 2 mL of MTBE was added to the sample and the organic phase was again collected and combined with the previous collection. This extraction protocol extracts all main lipid classes with high recoveries, specifically phosphatidylcholines (PC), sphingomyelins (SM), phosphatidylethanolamines (PE), lysophosphatidylcholines (LPC), ceramides (Cer), cholesteryl esters (CholE), and triacylglycerols (TG) [31].

Samples were then evaporated to dryness sing a gentle N_2 flow. Finally, 250 µL of MeOH were added and the samples were transferred to vials containing inserts (250 µL) for analysis. Following the analysis of the lipids, gemfibrozil was also quantified in these samples using the same LC-QqQ method used for the chemical stability evaluation.

Four commercial lipid standards [PGE2-EA, sphingosine-d_7, sphingomyelin (d_{18}:1/12:0) and C16 ceramide (d_{18}:1-d_7/16:0)] (Avanti lipids, Alabaster, AL, USA), were used to evaluate the quality of the chromatographic separation, the percentage of extraction recovery of the different lipid classes as well as the reproducibility of the method. The percentage of extraction recovery was evaluated by comparing extracted lipid standards' peak areas to the standards' peak areas in solvent (MeOH). Daphnid extracts were also evaluated for the presence of standards used for the lipid recovery evaluation, but no signal was observed.

A quality control (QC) sample (300 pg mL^{-1} of the lipid standard mix) was used in order to determine the stability of injection sequences and the carry-over effects. QCs were prepared in an

extract of 50 mg mL^{-1} of frozen daphnids in 1% FA (*v/v)* in MeOH. No signal from the lipid standards was observed in blank daphnid extracts.

For the gemfibrozil quantification in medium and daphnids, QC samples were used in order to verify the trueness and precision of the measured concentrations. Three levels of QC samples were used: low (≈100 pg mL^{-1}), medium (≈300 pg mL^{-1}) and high (≈500 pg mL^{-1}). Correction of matrix effects was done by using extracts of frozen daphnids of 50 mg mL^{-1} in 1% FA (*v/v)* in MeOH. These extracts were used to prepare QC samples. Results were considered acceptable if the relative bias of QC samples was within ± 20 % of the expected value.

4.5.6. Lipid profiling using liquid chromatography-high resolution mass spectrometry

The daphnid extracts were analyzed by liquid chromatography-quadrupole time of flight mass spectrometry (LC-QTOF) using a Shimadzu (Kyoto, Japan) UPLC system composed of a Nexera LC-30AD pump module, a SIL-30AC autosampler and a CTO-30A column oven module coupled to Maxis QTOF mass spectrometer made by Bruker (Billerica, MA, USA). Briefly, the compounds were separated on a reversed-phase column Acquity UPLC HSS T3 C_{18} from Waters of dimensions 50×2.1 mm and 1.8 µm particle size. Mobile phase was composed of eluent A (H_2O: ACN, 80:20 containing 0.1% FA *v/v*) and eluent B (ACN: IPA, 20:80 containing 0.1% FA *v/v*). The elution gradient started with 30% of B until 1 minute, increasing to 70% between minute 1 and 2.5, then to 80% (2.5-4 min), stayed at 80% for one minute, increased to 90% (5-6.5 min) then 100% until minute 8. The composition went back to 30% of B (8.1-11 min) with a minute of equilibration at the end. Flow rate was set to 0.25 mL/min and the sample injection volume was set to 10 µL. The autosampler rack temperature was set to 4°C and the column temperature was set to 40°C. ESI in the positive and negative mode was used as ionization source. The full spectrum (MS^1) conditions were the following: mass range: *m/z* 50-1200, dry gas: 10 L/min, capillary voltage: 3.5 kV for positive polarity and 4.5 kV for negative polarity. The QTOF system was mass calibrated via Data Analysis with a solution of sodium formate in high-precision calibration (HPC) mode after waiting 20 min for the system to stabilize before each sequence of acquisitions. The lipid profile was measured from pooled individuals from each treatment group. The total number of daphnids used in pooled groups for each sampling time varied due to differences in the mortality

of daphnids during the tests, however the humid mass of each sample was similar (± 0.1 mg or ≈ 5%).

Due to sensitivity issues, another liquid chromatography-high resolution tandem mass spectrometry system was used for fragmentation of the identified features. A Thermo Scientific Q Exactive Plus Orbitrap (San Jose, CA, USA) was interfaced with a Thermo Scientific Vanquish FLEX UHPLC system (San Jose, CA, USA) and a pneumatic assisted heated electrospray ion source. Mass spectrometric detection was performed in the positive and negative ion mode using parallel reaction monitoring (PRM) for the significantly different features between control and exposed groups detected priorly on the first injections using the QTOF. Acquisition in each DDA cycle entailed two MS^1 survey scans (*m/z* 50-750 and *m/z* 100–1500) acquired at R_{FWHM} of 140,000. Precursor ions were isolated using the quadrupole (2 Da isolation width) and activated by higher-energy collision dissociation using stepped normalized energy (30, 35 and 45 eV) and fragment ions were detected in the Orbitrap at R_{FWHM} = 17,500. Resolution parameters were selected to maximize the frequency of acquisition. The instrument calibration was performed prior to all analyses and mass accuracy was notably below 1 ppm using the Thermo Pierce calibration solution and the automated instrument protocol. Source parameters were the following: capillary temperature was 300 °C; sheath gas was 50 L/h ; auxiliary gas was 20 L/h ; spray voltage was 4000 V. The same LC method as the one described above for the QTOF system was used.

4.5.7. Identification and annotation of significant features

The lipidomics workflow is illustrated in Figure 15. Following the acquisition of the data, files were exported in mzXML format. MS^1 spectra from each group were combined and imported to XCMS online. Features were deconvoluted, aligned and integrated based on the generic pairwise UPLC/ Bruker QTOF method in both polarities of XCMS online. The parameters were the following: 10 ppm for the maximal tolerated *m/z* deviation in consecutive scans, Signal/Noise threshold of 6, minimum difference in *m/z* for peaks with overlapping retention times of 0.01, integration method 1 and obiwarp method for time retention correction. The recuperation and exposed group were individually compared to evaluate the differences between groups for all peaks. Blank subtraction and Student's t-tests ($p<0.05$) were used to compare the area of features

between different treatment groups at each time point. Features that were significantly different at all time points between treatment groups were selected for annotation.

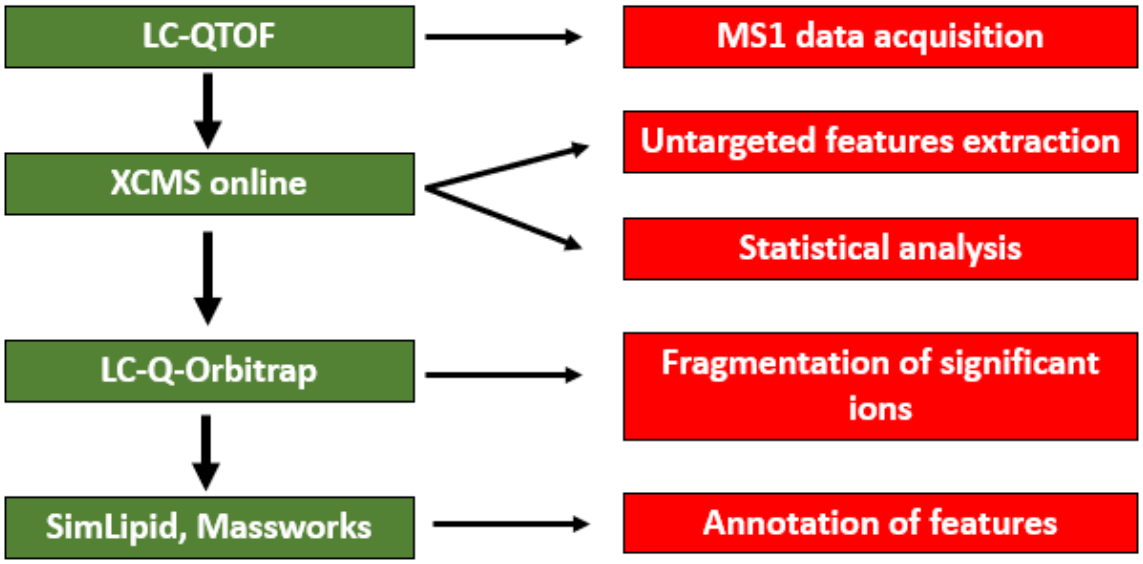


Figure 15. Processing organigram for data collected from the QTOF analysis of the daphnid extracts.

Unknown lipids were then identified based on their exact mass measurements, spectral accuracy, tandem mass (MS^2) spectra, as well as their t_R values. This approach was used to perform a lipidomic analysis on *D. magna* samples.

Massworks version 4.0 (Cerno Bioscience, Las Vegas, NV, USA) software was used for spectral accuracy evaluation. Allowed elements in the formulas generated were those commonly found in endogenous biological molecules (C, H, N, O, P, S). Minimal and maximal number of each element was set accordingly to the Seven Golden Rules for filtering molecular formulas [32]. Top 10 candidates were retained and compared with the molecular formulas given by the exact mass and MS^2 fragmentation. Detailed Massworks reports are included in the Electronic Supplementary Information.

SimLipid version 6.06 (Premier Biosoft, San Francisco, CA, USA) software was used for comparison of experimentally acquired MS^2 spectra to in silico spectrums from LipidMaps library. Spectra around the elution window (± 1 min of retention time) were analyzed and the ones with the highest ion intensities were used for the MS^2 search from precursor. A 10% relative intensity,

S/N>3 and ± 0.05 Da threshold was set for the *m/z* to be included in the search. Only fragments and candidates present in all samples were considered.

To support lipids annotation, four commercial standards [PGE2-A2-d_4, sphingosine-d_7, sphingomyelin (d_{18}:1/12:0) and C16 ceramide (d_{18}:1-d_7/16:0)] were used to define the relationship between lipid structure and retention time (t_R) and the potential candidate's structure and retention time were compared to evaluate the plausibility of the annotation.

4.5.8. Bioaccumulation of gemfibrozil in *D. magna*

Following lipid analysis, 50 µL of gemfibrozil-d_6 was added to the extract and gemfibrozil was quantified in samples (n=6 per treatment group) by liquid chromatography-triple quadrupole mass spectrometry (LC-QqQMS) using an Acquity UPLC system coupled to a Xevo TQ-S micro triple quadrupole mass spectrometer, both from Waters Corp. The method is detailed in the Annexes.

4.5.9. Data analysis

For the survival, number of neonates and humid mass comparisons, two-sample Student's t-tests (α=0.05) using Microsoft Excel 365 were employed to evaluate significant differences between exposed and recuperation groups compared control groups at each time point. When three conditions were compared, ANOVA tests were used. Normality and homoscedasticity were assessed priorly. Survival curves were traced with Graph Pad Prism 6 software (San Diego, USA).

4.6. Results and discussion

4.6.1. Gemfibrozil stability and bioaccumulation

Gemfibrozil concentration in the exposure media remained stable for the duration of the tests. Detailed results are presented in the Annexes. Previous studies have demonstrated that the bioconcentration of pharmaceuticals such as diclofenac and fluoxetine in *D. magna* tissues were linked to adverse effects [33, 34]. Therefore, it was hypothesized in the present study that gemfibrozil

bioconcentrations would increase during the exposure period, thus causing changes in the lipidome of daphnids, and then decrease during the recuperation period. Results indicated that concentrations of gemfibrozil in tissues were below the limit of detection (5 ng L^{-1}) in *D. magna* for all time periods. Elimination of gemfibrozil by detoxification enzymes (cytochromes P450, glutathione S-transferases, carboxylesterases, etc.) [35, 36] could explain those results, since the formation of conjugation and oxidation metabolites of several pharmaceuticals were reported after water exposure tests with *D. magna* [36]. In another study, 96-hour exposure tests with goldfish (*Carassius auratus*) resulted in plasma concentrations >75,000 µg L^{-1} [37], which demonstrated gemfibrozil uptake from water. However, much higher concentrations of gemfibrozil (1,500 or 10,000 µg L^{-1}) were used in that experiment compared to this study. A higher number of organisms and concentrations would be needed to assess efficiently the bioaccumulation of gemfibrozil in *D. magna*.

4.6.2. *Daphnia magna* life-history parameters

Following exposure of *D. magna* to 1µg/L of gemfibrozil for up to 7 days, less than 20 % of mortality was observed for all treatments, respecting the sublethality criteria of the OECD guidelines [30]. Mortality curves can be found in Figure S6 (Annexes).

Results showed that daphnids reached adulthood between day 7 and day 10 and that the number of offspring per adult in control and exposed individuals was similar at time 0, 48 and 96 h of exposure (Figure 3). After 168 h (7 days), the exposed group had significantly more neonates than the control and recuperation groups (p=0.013). However, no difference between treatments and timepoint was observed when comparing humid mass of adult organisms, therefore an increase in the size of the adults cannot explain this increase in the number of neonates for the exposed group (Figure S7). Hence, results suggest that an exposure to 1 µg/L of gemfibrozil for a 168 h period modified biomass allocation of adult daphnids by increasing their brood size. Such effect was also observed previously by Coors*, et al.* [38] using dispersant formulation (Dispersogen A) at a concentration about 10 times lower that the median effective concentration for chronic exposure. In another previous study by Steinkey*, et al.* [23], it was also reported that daphnids exposed for 21

days to gemfibrozil (5 to 50 ng mL^{-1}) produced broods at an earlier age and had larger broods than control individuals, but such effect was only observed when food availability was high, about 50 times higher than in the present study. Reproductive biomass (the number and size of neonates) allocation is an adaptational response of daphnids to environmental stress, therefore its disruption by gemfibrozil could reduce the ability of entire populations to survive [38, 39].

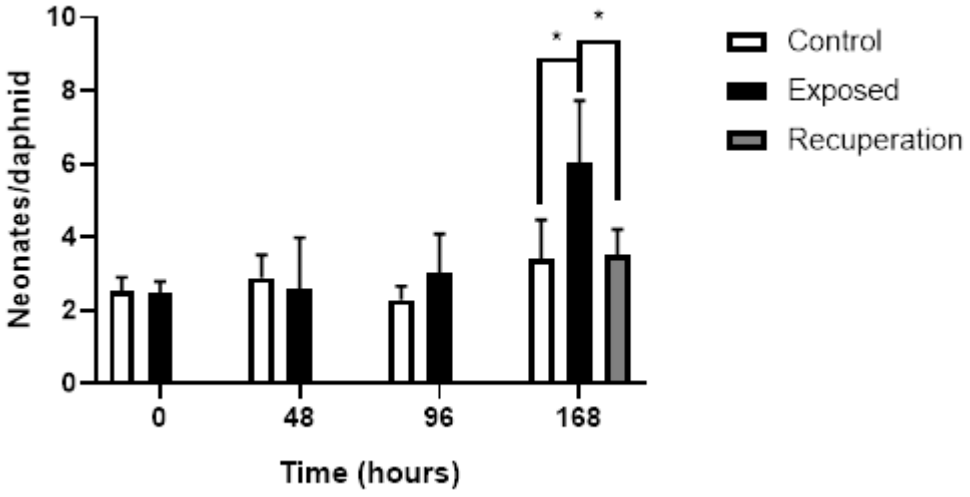
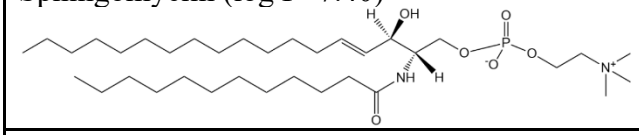
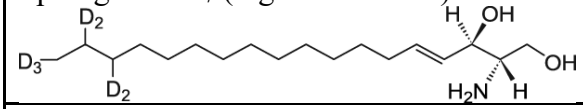
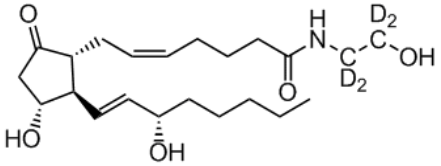
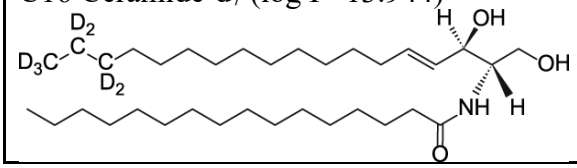


Figure 16. Average number of neonates produced per *D. magna* exposed to 1µg/L of gemfibrozil during after 0, 48, 96 and 168 h of exposure (n=6). Error bars represent ± 1 standard error of the mean. Asterisks indicate statistically significant differences compared to controls (ANOVA test, $p < 0.05$).

4.6.3. Lipids extraction and chromatographic separation

Recovery and reproducibility of the lipid standards extraction were evaluated after the liquid-liquid MTBE extraction. All standards had linear responses (R^2>0.99). Two concentrations (low QC: 55.5 ng mL^{-1} and high QC: 278 ng mL^{-1}) spiked in solvent or blank *D. magna* matrix were compared to the unextracted analytes in solvent to assess the recovery and matrix effects (Table 4). No major matrix effects were observed.

Table 4. Extraction recovery of lipid standards.

Lipid Standard	Extraction recovery	
	Low QC (%±SD)	**High QC (%±SD)**
Sphingomyelin (log P=7.40) 	28.0 ± 1.1	92.0 ± 6.5
Sphingosine-d_7 (log P=4.57-6.02) 	31.0 ± 4.0	101.0 ± 6.8
PGE2-A2-d_4 (log P=2.79) 	36.0 ± 2.3	111.0 ± 2.2
C16 Ceramide-d_7 (log P=13.944) 	20.0 ± 2.8	65.0 ± 11.3

Recovery was lower for the low concentration, but reproducibility was adequate, with a standard deviation < 4%. For the high concentration, the recovery was generally very close to 100%, except for the ceramide standard. This was unexpected as this compound is very nonpolar (logP=13.9) and a higher recovery was expected. The temperature of the solvents could have an impact on the extraction as this method is sometimes carried out using cold solvent [31]. However, a better reproducibility was obtained using solvents at room temperature. Therefore, the method was deemed suitable for the analysis of daphnid extracts.

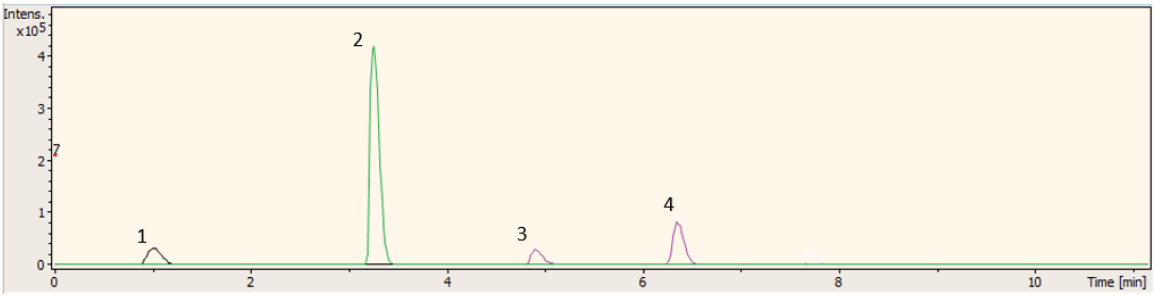


Figure 17. Chromatographic separation of lipid standards. 1: PGE2-EA-d_4, 2: sphingosine-d_7, 3: sphingomyelin (d_{18}:1/12:0), 4: C16 ceramide-d_7 (d_{18}:1-d_7/16:0).

Separation by liquid chromatography was adequate and reproducible as seen on Figure 17. PGE2-EA-d_4, sphingosine-d_7, sphingomyelin (d_{18}:1/12:0), C16 ceramide-d_7 (d_{18}:1-d_7/16:0) had retention time of 1.5 min, 4.3 min, 5.8 min and 7.4 min, respectively. Retention times were reproducible, peaks were sharp and had an asymmetry factor < 1.5.

4.6.4. Lipid profiling using LC-QTOFMS

Following features deconvolution, peaks alignment and integration by XCMS online, t-tests were performed to identify features with significant fold levels between control and the exposed and recuperation groups.

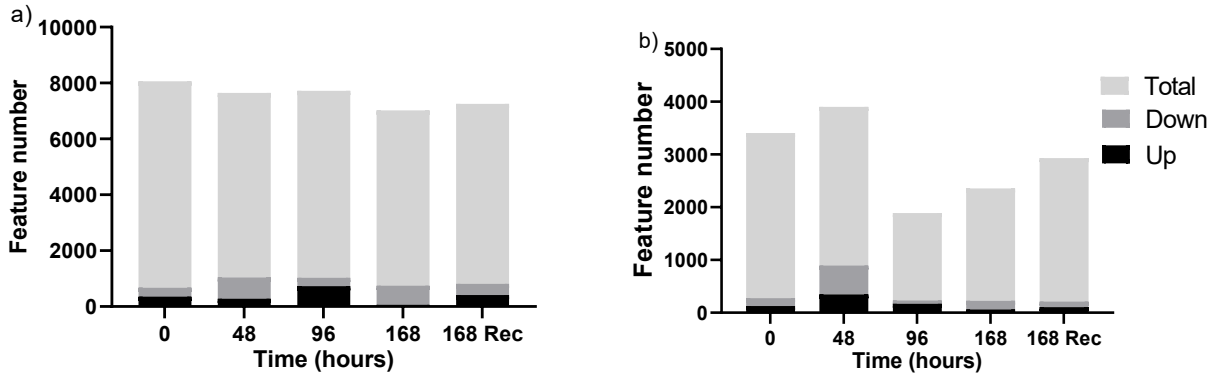


Figure 18. Number of features detected in MS^1 (full mass spectrum) in a) positive mode and b) negative mode. Significant up and down regulated features compared to the control group are shown in black and dark grey, respectively.

As shown in Figure 18, between 7000 and 8000 total features were registered in positive mode and between 2000 and 4000 in negative mode. From these, 730 were increased and 677 were decreased significantly ($p < 0.05$, fold change > 2) in the positive mode. In the negative mode, 349 were increased and 543 were decreased significantly ($p < 0.05$). Even if the accurate quantification of all these signals is not possible yet, drastic changes can still be observed and could lead to a better comprehension of toxicity mechanisms following the identification of the significantly affected features. Of all the significantly affected features, only the ones that were repeatedly and increasingly impacted over time were selected for the annotation by MS^2 spectra and spectral accuracy.

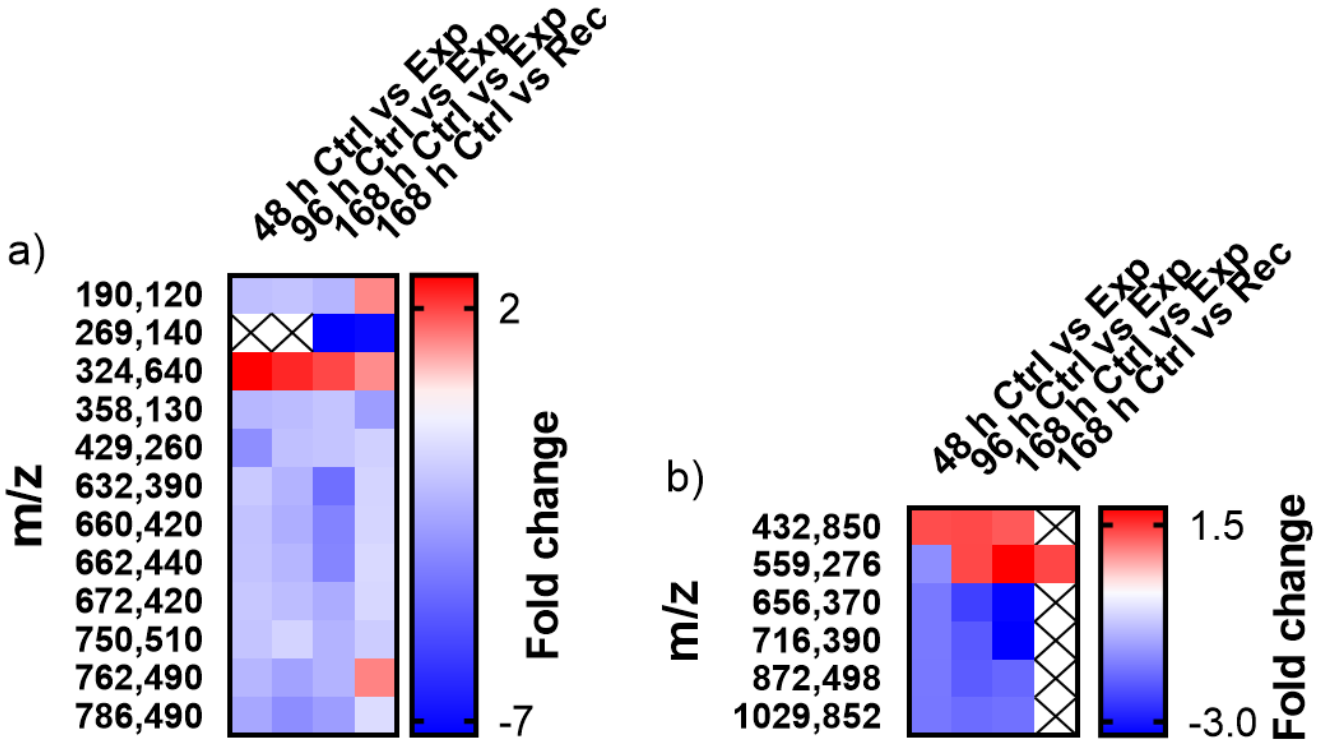


Figure 19. Fold change of m/z (detected ions) as a function of exposure time and treatments of significantly different features ($p < 0.05$) and detected in *D. magna* exposed for up to 168 h to gemfibrozil (1µg/L). a) Results of the analysis in positive mode (n=6). b) Results in negative mode (n=6). Boxes with crosses indicate that not significant changes between the control and exposed group were observed. A blue color indicates a negative fold change while a red color indicates a positive fold change.

Results indicated that the fold change for most of the significantly different features observed between the control and exposed group increases over time (Figure 19), thus suggesting that the concentrations of these molecules significantly varied (increase in red or decrease in blue) in the lipidome of daphnids. These observations were expected as gemfibrozil is known for its lipid-lowering activity in humans as well as daphnids [21]. The most important difference in the signal for most features between control, exposed and recuperation groups also coincided with the higher number of produced neonates after 168 h of exposure for the exposed group. Therefore, it was hypothesized that these lipidomic changes were most probably related to the higher number of neonates observed in the exposed group. Figure 19 also shows that most of the feature levels returned toward the values of the control group after the recuperation period. Therefore, these results show that altered lipid levels in *D. magna* exposed to gemfibrozil may be able to return to normal levels after a period of 72 hours.

4.6.5. Molecular formula confirmation and lipid annotation

Samples were reanalyzed in a LC-Q-Orbitrap instrument using the list of significant features obtained by XCMS online in order to obtain their MS^2 spectra. While XCMS online includes an annotation step and suggests potential molecular formulas for a given feature, it was decided to employ two additional tools, Massworks and SimLipid, to confirm molecular formulas and a dedicated lipid spectral database for matching acquired MS^2 spectra to theoretical lipid spectra.

MassWorks measures the spectral accuracy of the experimental isotopic pattern to evaluate the elemental composition of ions of interest [40, 41]. Instead of employing only the accurate mass of the monoisotopic peak to assign possible formulas, Massworks ranks possible formulas according to their match with the whole experimental isotopic pattern. This technique has been employed to confirm the presence of trace environmental contaminants in surface waters and has been useful to improve quality of formula assignment based on high resolution MS^1 spectra [42]. Therefore, by comparing the experimental and theoretical isotopic patterns, it is possible to add another filter and eliminate possible candidates for a given *m/z*. SimLipid uses MS full scan and MS^2 spectra to match product ions against in silico-generated databases [43]. It is an efficient software able to compare automatically a given spectra to theoretical ones available from the chosen database in a short amount of time. It allows reproducible results without any bias coming from the subjective opinion of a user comparing the spectra manually. It also supports many formats from different MS software [43]. Table 5 presents the lipid candidates obtained for each feature across the different annotation methods. The detailed SimLipid and Massworks analyses report are presented in the Annexes.

The confidence levels proposed by Schymanski*, et al.* [44] were used to describe the quality of the identification. According to Schymanski's scheme, level 5 indicates the lowest confidence level, an exact mass obtained by HRMS. Level 4: unequivocal molecular formula (MS isotope/adduct), can be obtained by spectral accuracy. Level 3: tentative candidate(s) of a molecular structure (MS, MS^2, Experimental data). Level 2: probable structure (MS^2 library match between experimental and theoretical spectrum) and level 1: confirmed structure (MS, MS^2, t_R and reference standard).

Table 5. Identification of significant features according to MassWorks and SimLipid.

Experimental mass (*m/z*)	t_R (min)	Precursor ion (*m/z*)	Δm (mDa)	Spectral accuracy (%)	Formula Rank [1]	Product ions (*m/z*)	Fragment [2]	Lipid name (ID)	Lipid Class [3]	Confidence level
750.5097	4.9	$[C_{42}H_{72}NO_8P+H]^+$	+2.87	96.13	1	86.0971 104.1075 184.0730	$[C_5H_{13}N]^+$ $[M-C_{37}H_{59}O_7P]^+$ $[HG+H]^+$	Isomers of PC(34:6) (LMGP01010447/ LMGP01011402/ LMGP01011929/ LMGP01012097)	GP0101	2
786.4954	5.2	$[C_{43}H_{74}NO_8P+Na]^+$	-9.05	<80%	N.A.	184.0733 510.3021	$[HG+H]^+$ $[M-18:4-H_2O+Na]^+$	PC(17:2/18:4) (LMGP01011566) or PC(18:4/17:2) (LMGP01011710)	GP0101	2
762.5105	4.9	$[C_{41}H_{74}NO_8P+Na]^+$	+6.1	<80%	N.A.	184.0733	$[HG+H]^+$	Isomers of PC(33:4) (LMGP01011356/ LMGP01011418/ LMGP01011445/ LMGP01011446/ LMGP01011645/ LMGP01011676/ LMGP01011704/ LMGP01011898)	GP0101	2
559.2750	4.0	$[C_{25}H_{42}N_2O_7S+HCOO]^-$	+5.5	<80%	N.A.	152.9936 223.0987	$[C_9H_{13}O_2]$ $[C_{13}H_{19}O_3]$	14,15-HxA3-D(11S) (LMFA03090009)	FA0309	2

[1] Formula rank according to MassWorks; [2] Fragment according to SimLipid [3] Lipid class: GP101: diacylglycerolphosphocholine, FA0309: Hepoxilins

Out of the 18 features of interests, only four could be matched with known molecules based on the spectral accuracy and MS^2 spectrum: isomers of PC(34:6), PC(17:2/18:4) or PC(18:4/17:2), PS(12:0/13:0) or PS(13:0/12:0), isomers of PC(33:4) and 14,15-HxA3-D(11S). In most cases, the spectral accuracy was low, probably due to the low intensity of the ions or because of the interference on the isotopic patron caused by the other ions with close *m/z*. When the spectral accuracy was < 80%, it was not considered in the annotation of the ions.

The most confident annotation was for *m/z* 750.5097 which was identified as PC (34:6), a diacylglycerophosphocholine. The mass accuracy was +2.87 mDa and the experimental and theoretical MS^2 spectra coincide perfectly with an excellent spectral accuracy (Please consult the SimLipid report for *m/z* 750.5097in the Electronic Supplementary Information). The identified fragments did not give information on the chain composition other than the total number of unsaturations. Top candidates for annotation of other features (*m/z* 786.4954 and *m/z* 762.5105) were also diacylglycerophosphocholines, which are a subclass of glycerophospholipids consisting of a polar head group attached to a glycerol backbone and up to two fatty acyl chains (diacyl). Glycerophospholipids are considered the backbone in eukaryote cellular membrane providing stability, fluidity, and permeability [45]. They can also act as second messenger implicated in the function of membrane protein such as ion channels or receptors. Glycerophospholipids are also precursors of physiologically active molecules such as arachidonic acid, which is implicated in inflammation [46, 47].

The last feature to be annotated (*m/z* 559.2750) was found to be an hepoxilin. 14,15-HxA3-D(11S) is derived from arachidonic acid [48]. It was found to be implicated in processes such as cellular calcium level regulation and inflammation in human neutrophils [49, 50]. This compound can also potentiate vasoconstriction [51].

Another study found similar results for lipids (e.g., cholesteryl ester, phosphatidic lysophosphatic acid) when exposing mice to fenofibrate (another PPARα activator) when analysing pancreatic samples using an untargeted approach; levels of the lipids were lowered for the exposed group when compared to the control group [52]. Gemfibrozil is usually taken by humans to increase plasma HDL and to decrease plasma TG, very low-density lipoprotein and low-density lipoprotein cholesterol [53]. By activating the PPARα receptor, it increases HDL, apo AI, apo AII, LPL and inhibits apo B synthesis and peripheral lipolysis [54].

The upregulation of the lipoprotein lipase leads to a reduction of the triglyceride levels. Glycerophospholipids are derived from phosphatidic acids, which are intermediates in the TG synthesis [47]. As gemfibrozil downregulates this process, lipids deriving from TG might be impacted as observed in the present study.

Glycerophospholipids appear to be the most affected of the observed lipid classes following exposure to gemfibrozil. This could be due to the molecular structure of these types of lipids. The highly polar head of these molecules proffers a high ionisation efficiency and improves their detection. It is to be noted that glycerophospholipids are a very abundant class of lipids in living organisms due to their structural implication in the cell membranes. Other molecules with lesser ionisation efficiency and insufficient concentration such as steroids were difficult to detect during the analysis.

It was observed during the 72 h recuperation period that most feature levels returned to normal (control values). Duquesne and Küster [55] found that following a 24 h recuperation period, the choline esterase enzyme activity in *D. magna* had recovered significantly, but not completely when compared to the control group. This was after a 24 h of exposure to paraoxon-methyl, an organophosphate pesticide, at a concentration of 1.0 and 1.5 ng L^{-1} where choline esterase activity was reduced by 70% or more following the exposure and before the recuperation period. The swimming behaviour and filtration activity were also affected during the exposure but came back to normal compared to the control group after the recuperation period [55].

4.7. Conclusion

The present study developed and applied an untargeted metabolomic approach to evaluate changes on the lipid profile of *D. magna* following exposure to gemfibrozil during a 168 h period. Results showed that a constant exposure of *D. magna* to environmental levels of gemfibrozil (1000 ng mL^{-1}) led to an increase in the production of neonates after 168 h, suggesting that environmental concentrations of gemfibrozil may affect reproductive biomass allocation, an adaptational response of daphnids to natural stressors. Following 96 h of exposure and 72 h of recovery, daphnids showed the ability to recover in terms of reproduction and the levels of most lipids returned to the values observed in the control group.

In total, the untargeted method detected over 8000 features and 18 of those were found to have concentrations significantly changed following exposure. Out of those 18 features, 4 of them could be identified as lipid species. These results demonstrated that exposure of daphnids to gemfibrozil caused homeostasis disruption of at least two lipid classes (e.g., fatty acyls and glycerophosphocholines). Some of the identified lipids [PC(34:6), PC(33:4), PC(17:2/18:4) or PC(18:4/17:2)] are involved in inflammation and cell death. Most of the identified features that were significantly different between exposed and control groups returned to normal levels after the 72 h period of recuperation but remained significantly different in the exposed group after 168 h of exposure. These observations suggest possible recuperation in exposed daphnids when gemfibrozil is removed from their environment.

These types of experimental designs can provide useful information on the possible recuperation of the organisms following an exposure and could be included in the risk assessment of new compounds. A wider range of concentration and compounds should be evaluated and longer period of exposition and recuperation could also be studied in addition to other endpoints (e.g., transcriptomics or behaviour).

Untargeted analysis such as the one presented here can lead to the identification of sensible biomarkers that can be used for the ecotoxicity studies of lipid altering compounds. Also, metabolomics studies could be performed on the polar fraction to widen the number of potential biomarkers. Future studies should investigate if the observed effects on reproductive biomass allocation are also detected outside laboratory-controlled conditions.

4.8. Author contributions

Hugo Alarie: Conceptualization, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – Original Draft
Francis Beaudry: Resources, Writing – Review & Editing
Magali Houde: Supervision, Writing – Review & Editing,
Pedro A. Segura: Conceptualization, Funding Acquisition, Methodology, Resources, Supervision, Writing – Review & Editing

4.9. Conflicts of interest

There are no conflicts of interest to declare.

4.10. Acknowledgements

This study was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) through a discovery grant awarded to P.A. Segura and by the Faculty of Sciences of the Université de Sherbrooke. We would like to thank Hydro-Québec and the NSERC CREATE program TEDGIEER (*Technologies environnementales décontamination et gestion intégrée des eaux et effluents résiduaires*) for the scholarships granted to H. Alarie. Funding sources did not have any involvement in the design, experiments, data interpretation, writing, revision or submission of this study.

4.11. References

1. C. Pironti, M. Ricciardi, A. Proto, P. M. Bianco, L. Montano and O. Motta, Endocrine-disrupting compounds: An overview on their occurrence in the aquatic environment and human exposure, *Water*, 2021, **13**, 1347.

2. S. I. Dodson and T. Hanazato, Commentary on effects of anthropogenic and natural organic chemicals on development, swimming behavior, and reproduction of Daphnia, a key member of aquatic ecosystems, *Environ. Health Perspect.*, 1995, **103**, 7.

3. M. N. Kariuki, E. G. Nagato, B. P. Lankadurai, A. J. Simpson and M. J. Simpson, Analysis of sub-lethal toxicity of perfluorooctane sulfonate (PFOS) to Daphnia magna using 1H nuclear magnetic resonance-based metabolomics, *Metabolites*, 2017, **7**, 15.

4. N. D. Wagner, A. J. Simpson and M. J. Simpson, Metabolomic responses to sublethal contaminant exposure in neonate and adult Daphnia magna, *Environ. Toxicol. Chem.*, 2017, **36**, 938-946.

5. K. Yang and X. Han, Lipidomics: techniques, applications, and outcomes related to biomedical sciences, *Trends Biochem. Sci.*, 2016, **41**, 954-969.

6. E. Agmon and B. R. Stockwell, Lipid homeostasis and regulated cell death, *Curr. Opin, Chem. Biol.*, 2017, **39**, 83-89.

7. S. Cisa-Wieczorek and M. I. Hernández-Alvarez, Deregulation of lipid homeostasis: a fa (c) t in the development of metabolic diseases, *Cells*, 2020, **9**, 2605.

8. R. Jordão, J. Casas, G. Fabrias, B. Campos, B. Piña, M. F. Lemos, A. M. Soares, R. Tauler and C. Barata, Obesogens beyond vertebrates: lipid perturbation by tributyltin in the crustacean Daphnia magna, *Environ. Health Perspect.*, 2015, **123**, 813-819.

9. M. Vendruscolo, Lipid homeostasis and its links with protein Misfolding diseases, *Front. Mol. Neurosci.*, 2022, 46.

10. R. N. Helsley and C. Zhou, Epigenetic impact of endocrine disrupting chemicals on lipid homeostasis and atherosclerosis: a pregnane X receptor-centric view, *Environ. Epigen.*, 2017, **3**, dvx017.

11. Fuertes I, Piña B, Barata C. Changes in lipid profiles in Daphnia magna individuals exposed to low environmental levels of neuroactive pharmaceuticals. Sci Total Environ. 2020 Sep 1;733:139029.

12. E. Garreta-Lara, C. Gómez-Canela, B. Campos, C. Barata, R. Tauler and S. Lacorte, Combined targeted/untargeted analytical and chemometric approaches in the characterization of Daphnia magna metabolomic changes under bisphenol A exposure, *Microchem J*, 2021, **165**, 106150.

13. A. Roy and K. Pahan, Gemfibrozil, stretching arms beyond lipid lowering, *Immunopharmacology and immunotoxicology*, 2009, **31**, 339-351.

14. S. Mandard, M. Müller and S. Kersten, Peroxisome proliferator-activated receptor α target genes, *Cellular and Molecular Life Sciences CMLS*, 2004, **61**, 393-416.

15. M. J. Wells, K. Y. Bell, K. A. Traexler, M. L. Pellegrin and A. Morse, Emerging pollutants, *Water Environ. Res.*, 2010, **82**, 2095-2170.

16. Y. Fang, A. Karnjanapiboonwong, D. A. Chase, J. Wang, A. N. Morse and T. A. Anderson, Occurrence, fate, and persistence of gemfibrozil in water and soil, *Environ. Toxicol. Chem.*, 2012, **31**, 550-555.

17. V. S. Tete, H. Nyoni, B. B. Mamba and T. A. Msagati, Occurrence and spatial distribution of statins, fibrates and their metabolites in aquatic environments, *Arab. J. Chem.*, 2020, **13**, 4358-4373.

18. A. J. Ebele, M. A.-E. Abdallah and S. Harrad, Pharmaceuticals and personal care products (PPCPs) in the freshwater aquatic environment, *Emerg. Contam.*, 2017, **3**, 1-16.

19. J. L. Zurita, G. Repetto, Á. Jos, M. Salguero, M. López-Artíguez and A. M. Cameán, Toxicological effects of the lipid regulator gemfibrozil in four aquatic systems, *Aquat. Toxicol.*, 2007, **81**, 106-115.

20. B. Quinn, W. Schmidt, K. O'Rourke and R. Hernan, Effects of the pharmaceuticals gemfibrozil and diclofenac on biomarker expression in the zebra mussel (Dreissena polymorpha) and their comparison with standardised toxicity tests, *Chemosphere*, 2011, **84**, 657-663.
21. B. Salesa, M. D. Ferrando, M. J. Villarroel and E. Sancho, Effect of the lipid regulator Gemfibrozil in the Cladocera Daphnia magna at different temperatures, *J. Environ. Sci. Heal. A*, 2017, **52**, 228-234.

22. L. Hu, R. Ding and X. Nie, Comparison of toxic effects of atorvastatin and gemfibrozil on Daphnia magna, *Comp. Biochem. Physiol., Part C: Toxicol. Pharmacol.*, 2022, **252**, 109224.

23. D. Steinkey, E. Lari, S. G. Woodman, K. H. Luong, C. S. Wong and G. G. Pyle, Effects of gemfibrozil on the growth, reproduction, and energy stores of Daphnia magna in the presence of varying food concentrations, *Chemosphere*, 2018, **192**, 75-80.

24. S. Comber, M. Gardner and B. Ellor, Seasonal variation of contaminant concentrations in wastewater treatment works effluents and river waters, *Environ. Technol.*, 2020, **41**, 2716-2730.

25. T. Zubala and M. Patro, Time and spatial variability in concentrations of selected pollutants in the new bypass rainwater harvesting system, *Water Air Soil Poll.*, 2021, **232**, 211.

26. I. C. Vasilachi, D. M. Asiminicesei, D. I. Fertu and M. Gavrilescu, Occurrence and fate of emerging pollutants in water environment and options for their removal, *Water*, 2021, **13**, 181.

27. S. Yang, M. Liang, Z. Qin, Y. Qian, M. Li and Y. Cao, A novel assessment considering spatial and temporal variations of water quality to identify pollution sources in urban rivers, *Sci. Rep.*, 2021, **11**, 8714.

28. Y. Hayashi, L.-H. Heckmann, A. Callaghan and R. M. Sibly, Reproduction recovery of the crustacean Daphnia magna after chronic exposure to ibuprofen, *Ecotoxicol.*, 2008, **17**, 246-251.

29. Environment Canada, *Biological test method. Acute Lethality Test Using Daphnia spp.*, Ottawa, ON, 1990.

30. OECD, *Test No. 211: Daphnia magna Reproduction Test, In: OECD Guidelines for the Testing of Chemicals, Section 2: Effects on Biotic Systems*, OCDE Publishing, 2008.

31. T. Cajka and O. Fiehn, LC–MS-based lipidomics and automated identification of lipids using the LipidBlast in-silico MS/MS library. In *Lipidomics: methods and protocols*, ed. S. K. Bhattacharya, Humana, New York, NY, 2017, pp. 149-170.

32. T. Kind and O. Fiehn, Seven golden rules for heuristic filtering of molecular formulas obtained by accurate mass spectrometry, *BMC bioinformatics*, 2007, **8**, 1.

33. M. Nkoom, G. Lu, J. Liu, H. Dong and H. Yang, Bioconcentration, behavioral, and biochemical effects of the non-steroidal anti-inflammatory drug diclofenac in Daphnia magna, *Environ. Sci. Poll. Res.*, 2019, **26**, 5704-5712.

34. J. Ding, H. Zou, Q. Liu, S. Zhang and R. M. Razanajatovo, Bioconcentration of the antidepressant fluoxetine and its effects on the physiological and biochemical status in Daphnia magna, *Ecotoxicol. Environ. Saf.*, 2017, **142**, 102-109.
35. A. Tkaczyk, A. Bownik, J. Dudka, K. Kowal and B. Ślaska, Daphnia magna model in the toxicity assessment of pharmaceuticals: A review, *Sci. Total Environ.*, 2021, **763**, 143038.

36. J. Jeon, D. Kurth and J. Hollender, Biotransformation pathways of biocides and pharmaceuticals in freshwater crustaceans based on structure elucidation of metabolites using high resolution mass spectrometry, *Chem. Res. Toxicol.*, 2013, **26**, 313-324.

37. C. Mimeault, A. Woodhouse, X.-S. Miao, C. Metcalfe, T. Moon and V. Trudeau, The human lipid regulator, gemfibrozil bioconcentrates and reduces testosterone in the goldfish, Carassius auratus, *Aquat. Toxicol.*, 2005, **73**, 44-54.

38. A. Coors, M. Hammers-Wirtz and H. T. Ratte, Adaptation to environmental stress in Daphnia magna simultaneously exposed to a xenobiotic, *Chemosphere*, 2004, **56**, 395-404.

39. A. Gergs, A. Zenker, V. Grimm and T. G. Preuss, Chemical and natural stressors combined: from cryptic effects to population extinction, *Sci. Rep.*, 2013, **3**, 2036.

40. Y. Wang and M. Gu, The concept of spectral accuracy for MS, *Anal. Chem.*, 2010, **82**, 7055-7062.

41. E. Eysseric, F. Beaudry, C. Gagnon and P. A. Segura, Non-targeted screening of trace organic contaminants in surface waters by a multi-tool approach based on combinatorial analysis of tandem mass spectra and open access databases, *Talanta*, 2021, **230**, 122293.

42. E. Eysseric, K. Barry, F. Beaudry, M. Houde, C. Gagnon and P. A. Segura, Application of spectral accuracy to improve the identification of organic compounds in environmental analysis, *Anal. Chem.*, 2017, **89**, 9805–9813.

43. N. S. Meitei and V. Shulaev, Bioinformatics in Lipidomics: Automating Large-Scale LC-MS-Based Untargeted Lipidomics Profiling with SimLipid Software. In *Plant Metabolic Engineering: Methods and Protocols*, ed. V. Shulaev, Humana New York, NY, 2022, pp. 197-214.

44. E. L. Schymanski, J. Jeon, R. Gulde, K. Fenner, M. Ruff, H. P. Singer and J. Hollender, Identifying small molecules via high resolution mass spectrometry: communicating confidence, *Environ. Sci. Technol.*, 2014, **48**, 2097-2098.

45. D. Hishikawa, T. Hashidate, T. Shimizu and H. Shindou, Diversity and function of membrane glycerophospholipids generated by the remodeling pathway in mammalian cells, *J. Lipid Res.*, 2014, **55**, 799-807.

46. H. Tallima and R. El Ridi, Arachidonic acid: Physiological roles and potential health benefits–A review, *J. Adv. Res.*, 2018, **11**, 33-41.

47. A. Blanco and G. Blanco, *Medical biochemistry*, Academic Press, 2017.

48. C. Pace-Asciak, D. Reynaud and P. M. Demin, Hepoxilins: a review on their enzymatic formation, metabolism and chemical synthesis, *Lipids*, 1995, **30**, 107-114.

49. S. Dho, S. Grinstein, E. Corey, W. Su and C. Pace-Asciak, Hepoxilin A3 induces changes in cytosolic calcium, intracellular pH and membrane potential in human neutrophils, *Biochem. J*, 1990, **266**, 63-68.

50. M. Sutherland, T. Schewe and S. Nigam, Biological actions of the free acid of hepoxilin A3 on human neutrophils, *Biochem Pharmacol*, 2000, **59**, 435-440.

51. O. Laneuville, R. Couture and C. R. Pace-Asciak, Hepoxilins sensitize blood vessels to noradrenaline—stereospecificity of action, *British journal of pharmacology*, 1992, **105**, 297-304.

52. L. J. Holm, M. Haupt-Jorgensen, J. D. Giacobini, J. P. Hasselby, M. Bilgin and K. Buschard, Fenofibrate increases very-long-chain sphingolipids and improves blood glucose homeostasis in NOD mice, *Diabetologia*, 2019, **62**, 2262-2272.

53. K. Saku, P. S. Gartside, B. A. Hynd and M. L. Kashyap, Mechanism of action of gemfibrozil on lipoprotein metabolism, *The Journal of clinical investigation*, 1985, **75**, 1702-1712.

54. B. S. Quintanilla Rodriguez and R. Correa, *Gemfibrozil*, StatPearls Publishing, Treasure Island, FL, 2023. Available at: https://www.ncbi.nlm.nih.gov/books/NBK545266/.

55. S. Duquesne and E. Küster, Biochemical, metabolic, and behavioural responses and recovery of Daphnia magna after exposure to an organophosphate, *Ecotoxicol. Environ. Saf.*, 2010, **73**, 353-359.

CHAPITRE 5. CONCLUSION GÉNÉRALE

Les chapitres de la présente thèse sont regroupés autour du développement de méthodes et de l'application de ces méthodes afin d'étudier les effets sous-létaux et chroniques des CIEs et mélanges de CIEs sur le modèle *D. magna*.

Les objectifs de la thèse étaient d'utiliser une méthode ciblée de quantification des ecdystéroïdes et de la complémenter de la mesure de la transcription des gènes associés à leur métabolisme en plus de développer une méthode non-ciblée pour l'analyse des lipides. Ces méthodes pourraient ensuite être utilisées pour quantifier les effets de différents contaminants lors d'études sur une ou plusieurs générations de daphnies.

Lors du 2^{e} chapitre, des études d'exposition ont donc été effectuées en utilisant le fénarimol, un composé connu pour affecter la concentration en 20E chez *D. magna*, en plus de deux agents hypolipémiants également susceptibles de diminuer la concentration de 20E. Le niveau basal des paramètres étudiés (concentration de 20E et niveau de transcription des gènes associés) a d'abord été évalué afin d'établir la variation temporelle naturelle avant de procéder aux expositions. Les résultats ont indiqué que ces marqueurs sont moins sensibles que le paramètre classique de fertilité. En effet, aucun effet n'a été observé lors des conditions testées, autant au niveau de la 20E que pour la transcription des gènes mesurés malgré l'excellente sensibilité et précision des méthodes développées. Cela démontre les difficultés et réalités d'utiliser des méthodes de quantification sur des modèles vivants et la limitation des analyses ciblées. Il se pourrait que la désynchronisation du cycle hormonal des daphnies soit une faiblesse du design expérimental et ait empêché de voir les changements s'il y a eu lieu. Cependant, cela démontre que l'utilisation de paramètres dont la valeur fluctue de manière trop importante tel que le niveau de transcription des gènes de la famille Halloween ne semble pas adéquat pour la mesure d'effets sous-léthaux lors d'expériences d'exposition chronique standardisées. La variation naturelle de la concentration de 20E a été mesurée sous forme de ligne de base en la comparant à la transcription des gènes régulant le métabolisme des ecdystéroïdes et ce, pour la première fois. Ces valeurs pourraient servir de référence à d'autres chercheurs étudiant les changements dans le métabolisme des ecdystéroïdes chez les daphnies. Ces données ont aussi démontré la variation possible de ces paramètres au sein de daphnies du même âge.

Un biomarqueur aurait pu permettre de déterminer le stade du cycle d'exuviation afin d'ajuster les valeurs des paramètres mesurés, ou de grouper les individus au même stade, cependant ce type de biomarqueur n'est pas encore connu ou utilisé dans ce contexte et aurait nécessité le développement d'une méthode supplémentaire afin de pouvoir utiliser les méthodes déjà développées. Le développement et la validation de paramètres ciblés restent un défi, mais leur découverte par les méthodes non ciblées pourrait faciliter leur sélection.

Lors du 3e chapitre, des essais écotoxicologiques en contexte réel ont été effectués. L'eau de ruissellement écoulée de parcelle contaminée en métaux et hydrocarbures pétroliers a été testée en utilisant l'approche WET afin de déterminer l'impact que ces eaux pourraient avoir sur la faune aquatique. La quantification de la 20E a également été utilisée comme paramètre, mais sans changement apparent entre les groupes testés. Aucun effet néfaste n'a été observé à la suite de l'exposition des daphnies aux eaux de ruissellement, démontrant que l'approche testée semble adéquate d'un point de vue environnementale. L'approche WET a également été très efficace et représentative du scénario environnemental réel et pourrait davantage être employée en écotoxicologie afin d'évaluer les effets de différentes eaux de ruissellement.

Pour le 4e chapitre, une autre approche a été envisagée. Une méthode non ciblée a été développée et employée afin de mesurer le profil lipidique de daphnies exposées au gemfibrozil. Cette exposition a été plus courte que les 21 jours habituels, mais une période d'observation avec le contaminant retiré du milieu de culture a été ajoutée à la fin de l'exposition afin d'étudier la récupération des daphnies post-exposition. Cette étude a permis de détecter un grand nombre de métabolites chez *D. magna* de manière non ciblée. Elle a également permis d'observer la résorption des effets causés par une exposition au gemfibrozil à la suite d'une période de récupération de quelques jours, démontrant ainsi la capacité des daphnies à revenir à leur état précédant l'exposition. L'utilisation de techniques d'identification modernes (bases de données et logiciels de traitement de données non ciblés) a permis d'identifier les métabolites les plus affectés, mais en quantité limitée. En effet, l'identification de manière efficace et confiante des signaux détectés lors d'étude non ciblée reste un énorme défi. Des approches automatisées ont été employées, mais elles requièrent encore tout de même une confirmation manuelle par l'utilisateur afin d'éviter les erreurs. Les métabolites les plus affectés qui ont été identifiés pourraient être étudiés de manière approfondie afin d'évaluer leur potentiel utilisation en tant que biomarqueurs pour les futurs tests de toxicologies.

Les approches utilisées lors de cette thèse démontrent bien l'accessibilité et le potentiel d'application des méthodes quantitatives contemporaines en contexte d'écotoxicologie. En utilisant les outils d'analyses puissants et novateurs d'aujourd'hui, il est maintenant possible de mesurer une panoplie d'effets sous-létaux de manière plus sensible et spécifique qu'il n'était pas possible d'observer auparavant, tel que des changements en concentration de métabolites non-ciblés. Ces paramètres peuvent non seulement fournir de l'information sur les mécanismes de toxicité observée, mais également aider à réduire le nombre et la durée des tests d'exposition. En effet, ces effets peuvent parfois être observés rapidement après le début de l'exposition, comme il fut le cas dans le chapitre 4 où des différences significatives au niveau de certaines molécules ont été mesurées après seulement quelques jours. Également, certaines molécules identifiées, tels que les glycérophospholipides, pourraient avoir un caractère dit « prédictif », permettant d'identifier des effets biologiques sous-létaux avant l'apparition d'effets au niveau physiologique. Un changement au niveau de ces biomarqueurs prédictifs peut indiquer une complication plus sévère à long terme, à condition que cela ait cela ait été démontré et validé de manière expérimentale. Des expositions de manière plus réalistes tels qu'en utilisant l'approche WET, des études sur plusieurs générations, ou bien avec un schéma d'exposition de type exposition-récupération sont définitivement à envisager afin d'améliorer les études de toxicités et combler le manque de connaissance dans ce domaine.

Une standardisation de ces différents schémas d'exposition ainsi que des différentes méthodes servant à la mesure des paramètres mesurés est cependant nécessaire, puisque les façons de faire des différents groupes de recherche sont très variées et leurs méthodes d'analyses le sont tout autant. Des lignes directrices internationales pourraient être établit une fois les méthodes validées. Les études chroniques sont longues et couteuses, et ces raisons font en sorte qu'elles soient parfois négligées. L'utilisation de méthode non ciblée pourrait donc nous permettre d'identifier de plus de biomarqueurs, sensibles, spécifiques et, idéalement, prédictifs. Ces biomarqueurs, une fois validés, pourraient ensuite être intégrés à une liste qui serait utilisée lors des études de toxicologie. Cela permettrait de réduire en temps et en coût ces études, tout en améliorant leur efficacité à détecter les potentiels effets sous-létaux causés par une exposition à basse concentration, mais de manière chronique.

Le traitement de données demeure une étape limitante lors d'études non ciblées. L'amélioration des logiciels d'analyse semble essentielle afin d'améliorer leur automatisation et ainsi accélérer le processus

tout en diminuant les erreurs humaines. De vastes banques de données existent à ce jour, mais la plupart d'entre elles sont dédiées à certains organismes bien spécifiques ou bien ne sont pas disponibles en accès libre. L'amélioration à l'accès de ces bases de données ainsi que le partage de ces informations permettraient une grande amélioration des informations obtenues à la suite de l'analyse des données obtenues par des études non ciblées en écotoxicologie. Ces études sont essentielles afin de permettre aux autorités gouvernementales de poser des limites et des recommandations plus éclairées pour l'utilisation des différentes molécules produites. Vu la croissance incessante du nombre de nouvelles molécules produites par l'homme et la difficulté de suivre le rythme pour l'évaluation écotoxicologique de celles-ci, l'amélioration des études de toxicité parait primordiale.

6.1. Annexes du chapitre 2

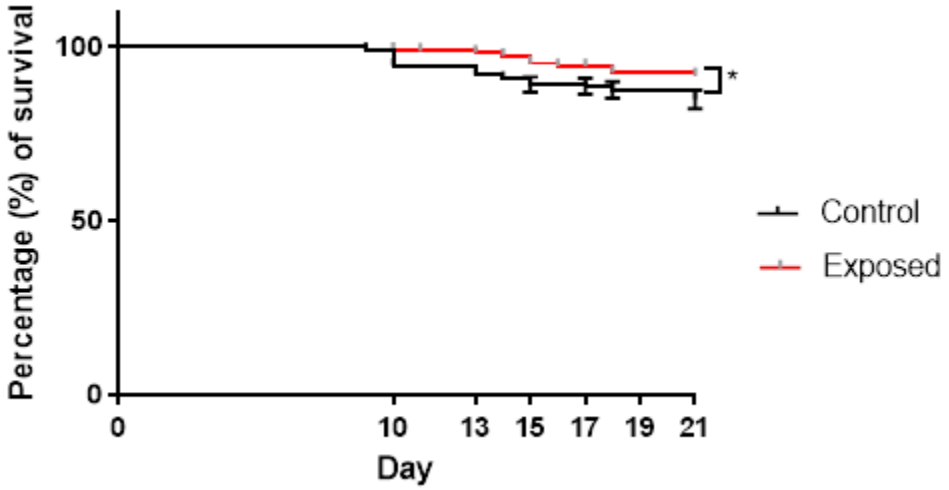


Figure S1. Survival curve (%) of *Daphnia magna* exposed to 0.113 mg/L of fenarimol for 21 days.

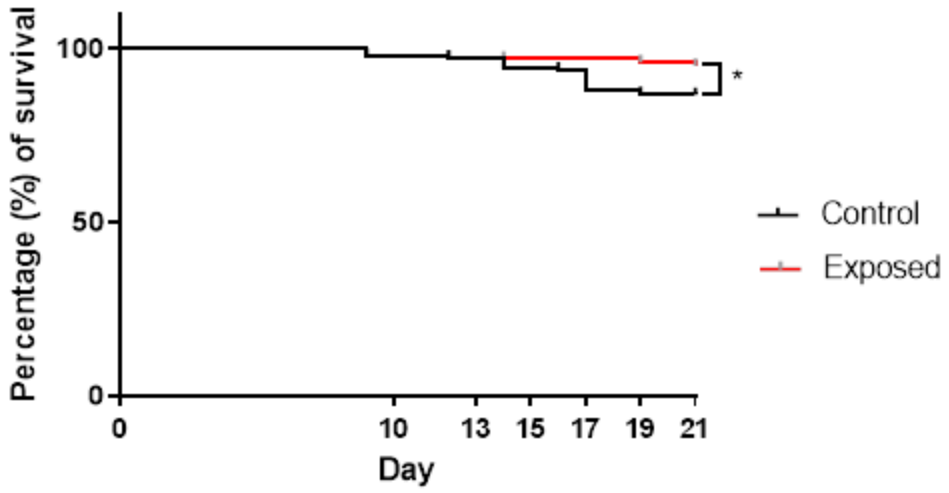


Figure S2. Survival curve (%) of *Daphnia magna* exposed to 0.565 mg/L of fenarimol for 21 days.

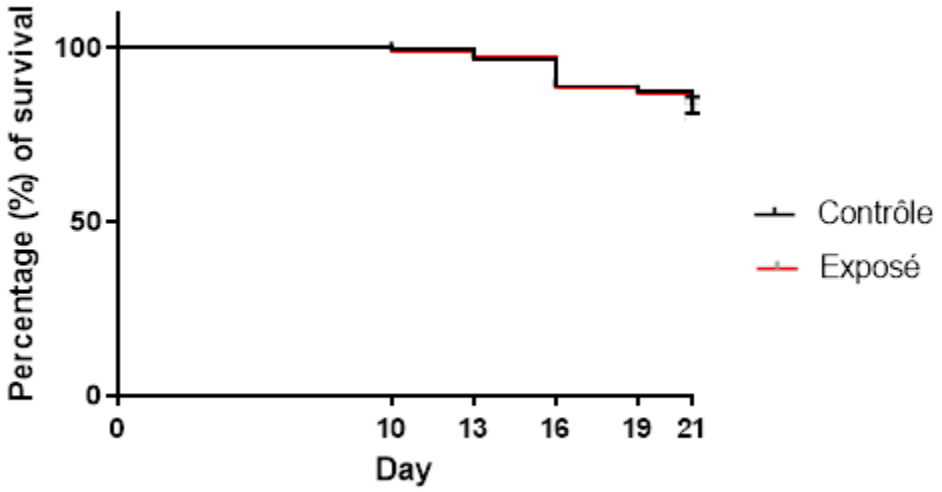


Figure S3. Survival curve (%) of *Daphnia magna* exposed to 1 µg/L of gemfibrozil and clofibric acid for 21 days.

Table S1. Properties of target compounds

Compound	CAS number	Solubility in water at 25 °C (mg/L)	Log P	Predicted LC50 for *D. magna* (48 h) (mg/L)
Clofibric acid	882-09-7	612.23	2.6	56.46
Fenarimol	60168-88-9	14.00	3.6	10.54
Gemfibrozil	25812-30-0	10.76	3.8	8.38

Note: Solubility in water and LC50 values were obtained from T.E.S.T software developed by the US EPA (v. 4.2.1). Log P values were obtained from PubChem as XLogP3 values (https://pubchem.ncbi.nlm.nih.gov/)

Table S2. Name, symbol and oligonucleotide primers used for RT-PCR analyses.

Gene name	Symbol	Primer sequence 5'-3'	Amplicon length (bp)	Efficiency (%)	References
20E-dependent and molting genes					
Ecdysone receptor	*EcR*	F- GAGGCGCTGCAGGCTTAC R- GAGTTTGGCAAACTCCGTCATC		108	Sumiya et al. (2014)
Ultraspiracle	*Usp*	F- GTTGGAGTCAAGGATGGTATCGT R- AGCCGAGTTCCGGTGGAT		99	Sumiya et al. (2014)
Neverland	*Nv1*	F- AGCACAAGGCGGGAAGAGT R- GCTTCCCATTTCACCTTCCA		99	Sumiya et al. (2014)
Shade	*Shd*	F- GACTGCTGAAGGCGTTGACA R- CGGCTGCCACTAGGTCGATA	62	100	Leblanc et al. (2007)
Reference genes					
Elongation Factor α	*ElongF*	F- GCACTGGTATGAAAGCCCGCA R- ACGCTGGATTGCCTTCTCGT	83	99	Environment Canada
Glyceraldehyde-3-phosphate dehydrogenase	*Gapdh*	F- ACGAGACCCGAAAAACATTCC R- CAATGTGAGCATGGGCCTTT	174	103	Environment Canada
Ubiquitin	*Ubi*	F- ACCACACGCATCTATCATCCCAA R- TGGGTCGCATAGCAGAGAACA	132	92	Giraudo et al. (2017)
Tubulin α	*Tub*	F- GCAAGGAAGATGCCGCTAATAAC R- CCAGTGCCACCGAAAGAGT	154	99	Giraudo et al. (2017)
Cyclophilin	*Cyc*	F- GACTTTCCACCAGTGCCATT R- AACTTTCCATCGCATCATCC		94	Sumiya et al. (2014)

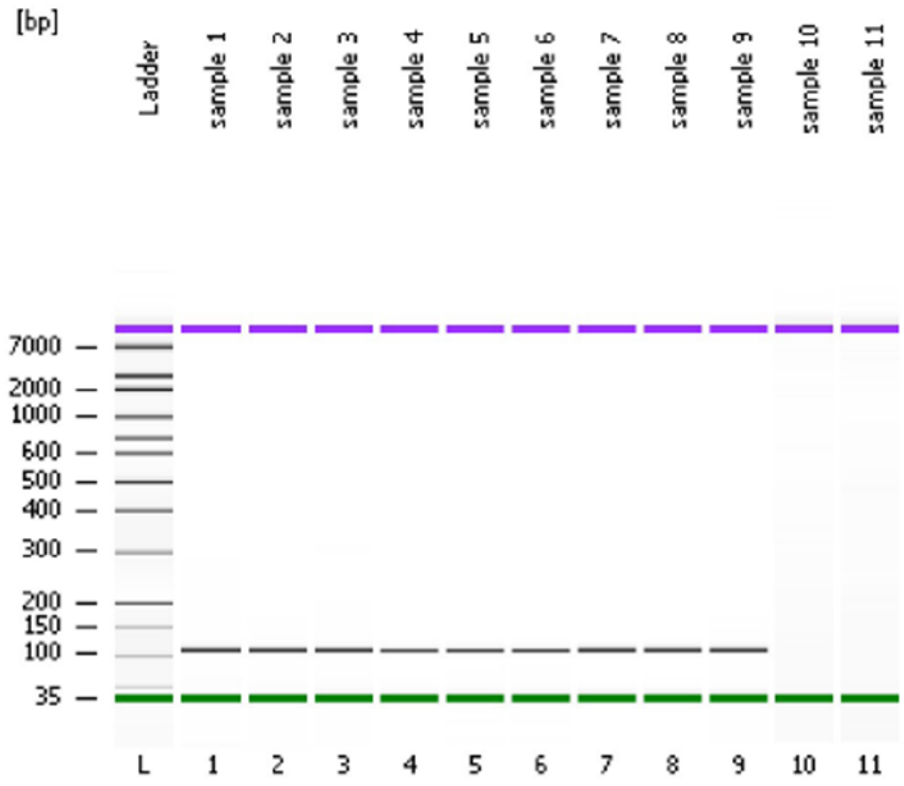


Figure S4. Capillary electrophoresis of Shade amplicon on bioanalyzer 2100.

6.1.1 Chemical stability of fenarimol

The chemical stability of fenarimol was assessed during testing to ensure that both concentrations remained constant during the whole duration of the experiment. 50 mL of medium were sampled before and after the renewal and frozen at -80°C. An internal standard (nuarimol) was added before SPE extraction in a volumetric flask (10 mL).

Quality control (QC) samples were used to determine the deviation percentage of the method and thereby verify the accuracy and precision of measured concentrations. Three concentrations of QC samples were used: low (75% of nominal concentration), medium (100% of nominal concentration) and high (200% of

nominal concentration). Matrix effects correction was done by using the culture medium in which daphnids lived for a week; this water was used to prepare QC samples (*n*=3). Results were considered acceptable if the QC samples were within ± 20 % of the expected value.

SPE cartridges were conditioned with 1 mL of phosphate buffer in H_2O (pH 7.5). A volume of 1 mL of the sampled water or calibration point containing internal standards was added to the cartridges. Then, a volume of 2 mL of MeOH:H_2O (20:80) was used to wash salts or other interfering compounds from the cartridges. Next, 2×2 mL of MeOH:ACN (1:1) were used to elute fenarimol and nuarimol. Samples were evaporated under N_2 before being reconstituted to 1 mL of MeOH:H_2O (1:1) for analysis.

Fenarimol and nuarimol were separated on a reversed-phase column Acquity UPLC HSS T3 C_{18+} from Waters of dimensions 2.1 mm × 50 mm and 1.8 µm particle size. Mobile phase was composed of eluent A (0.2% formic acid in H_2O) and eluent B (0.2% formic acid in ACN). The elution gradient started with 20% of B, increasing to 100% in 5 min, held for 1 min then back to initial conditions for column re-equilibration (3 min). The sample injection volume was set to 10 µL. A retention time of 2.68 min was obtained for fenarimol and 2.35 min for nuarimol. Electrospray in the positive mode was used as ionization source and the mass spectrometer was operated in the multiple reaction monitoring (MRM) mode. MRM transitions used were *m/z* 330.8 → *m/z* 267.23 for fenarimol and *m/z* 314.9 → *m/z* 251.9 for nuarimol.

The initial concentration was compared to the final concentration for each sampling points and no degradation was observed. All sampling points were within the 20% variation limit of the nominal concentration accordingly to the OECD guidelines (mean of 102.2±16% of target concentration). A reproductive recovery was obtained for fenarimol (mean of 96.6±9.4% of recovery) when comparing solvent area to the area of the spiked contaminant in the extracted matrix.

6.1.2. Chemical stability of gemfibrozil and clofibric acid

The chemical stability of gemfibrozil and clofibric acid was assessed during testing to ensure that both contaminants' concentration remained constant during the whole duration of the experiment. 50 mL of medium were sampled before and after the renewal and frozen at -80°C. Internal standards (gemfibrozil-d6 and clofibric acid-d4) were added before SPE extraction in a volumetric flask (10 mL).

Quality control (QC) samples were used to determine the deviation percentage of the method and thereby verify the accuracy and precision of measured concentrations. Three concentrations of QC samples were used: low (75% of nominal concentration), medium (100% of nominal concentration) and high (200% of nominal concentration). Matrix effects correction was done by using the culture medium in which daphnids lived for a week; this water was used to prepare QC samples (n=3). Results were considered acceptable if the QC samples were within ± 20 % of the expected value.

SPE cartridges were conditioned with 1 mL of phosphate buffer in H_2O (pH 7.5). 10 mL of the sampled water or calibration point containing internal standards were added to the cartridges. 2 mL of MeOH:H_2O (20:80) were used to wash salts or other interfering compounds from the cartridges. 2x2 mL of MeOH were used to elute gemfibrozil and clofibric acid and their internal standards. Samples were evaporated under N_2 before being reconstituted in 1 mL of MeOH:H_2O (pH 7.5) (1:1) for analysis.

Gemfibrozil, clofibric acid and their internal standards were separated on a reversed-phase column Acquity UPLC HSS T3 C_{18+} from Waters of dimensions 2.1 mm X 50 mm and 1.8 µm particle size. Mobile phase was composed of eluent A (H_2O 1 mM ammonium acetate) and eluent B (MeoH 1 mM ammonium acetate). The elution gradient started with 2% of B, increasing to 15% in 2 min, then up to 50% at 5 min, then 100% at 7 min before holding the same composition for 2 min and then back to initial conditions for column re-equilibration (3 min). The sample injection volume was set to 5 µL. Retention times of 4.8, 4.81, 6.61 and 6.63 min were obtained for clofibric acid-d4, clofibric acid, gemfibrozil-d6 and gemfibrozil respectively. Electrospray in the positive mode was used as ionization source and the mass spectrometer was operated in the multiple reaction monitoring (MRM) mode. MRM transitions used were m/z 217 → m/z 131 for clofibric acid-d4, m/z 213 → m/z 127 for clofibric acid, m/z 255.5 → m/z 121 for gemfibrozil-d6 and m/z 249.5 → m/z 121.1 for gemfibrozil. The initial concentration was compared to the final concentration for each sampling points and no degradation was observed. All sampling point were within the 20% variation limit of the nominal concentration accordingly to the OECD guidelines.

A reproductive recovery was obtained with a mean of 96% for clofibric acid and 92% for gemfibrozil when comparing solvent area to the area of the spiked contaminants in the extracted matrix.

6.1.3. References

1. LeBlanc GA. Crustacean endocrine toxicology: a review. *Ecotoxicol* 2007; 16:61-81.

2. Sumiya E, Ogino Y, Miyakawa H, Hiruta C, Toyota K, Miyagawa S, Iguchi T. Roles of ecdysteroids for progression of reproductive cycle in the fresh water crustacean Daphnia magna. *Frontiers in zoology* 2014; 11:60.

3. Giraudo M, Dubé M, Lépine M, Gagnon P, Douville M, Houde M. Multigenerational effects evaluation of the flame retardant tris(2-butoxyethyl) phosphate (TBOEP) using Daphnia magna. *Aquatic Toxicology* 2017; 190:142-149.

6.2. Annexes du chapitre 3

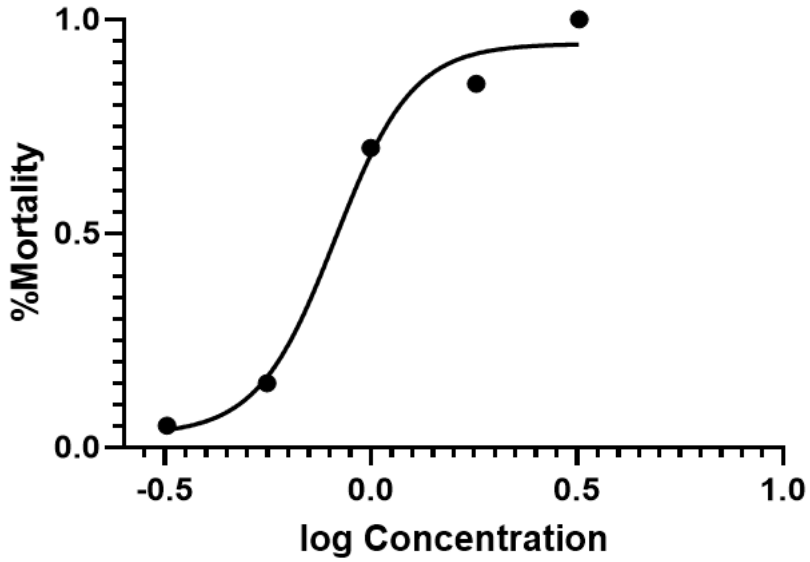


Figure S5. LC_{50} of potassium dichromate for *D. magna*. (*n*=5).

Table S3. Metal levels in soil type criteria according to the MEL.

	Soil criteria (mg/kg)		
Metal	A	B	C
Silver (Ag)	2	20	40
Arsenic (As)	6	30	50
Baryum (Ba)	340	500	2000
Cadmium (Cd)	1,5	5	20
Cobalt (Co)	25	50	300
Total Chrome (Cr)	100	250	800
Chrome VI (Cr VI)	2	6	10
Copper (Cu)	50	100	500
Tin (Sn)	5	50	300
Manganese (Mn)	1000	1000	2200
Mercury (Hg)	0,2	2	10
Molybdenum (Mo)	2	10	40

Nickel (Ni)	50	100	500
Lead (Pb)	50	500	1000
Selenium (Se)	1	3	10
Zinc (Zn)	140	500	1500

Table S4. Reconstituted water composition.

Salt	Mass per liter (g)
$CaCl_2 \cdot 2H_2O$	0.2940
$MgSO_4 \cdot 7H_2O$	0.1233
$NaHCO_3$	0.0648
KCl	0.0058

Table S5. Metals quantification from leachate waters. (n=1 per plot).

	Dissolved copper (µg/L)				
	Summer 2020	Fall 2020	Spring 2021	Fall 2021	Spring 2022
PT	16.6	16.3	6.1	17.2	13.1
PE1	36.7	37.7	13.7	28.7	17.7
PE2	24.8	27.6	12.8	30.6	20
PE3	-	-	-	19.5	6
	Dissolved zinc (µg/L)				
	Summer 2020	Fall 2020	Spring 2021	Fall 2021	Spring 2022
PT	9	8	<3	10	5
PE1	272	<15	<3	10	5
PE2	<3	157	13	16	9
PE3	-	-	-	39	139
	Dissolved aluminum (µg/L)				
	Summer 2020	Fall 2020	Spring 2021	Fall 2021	Spring 2022
PT	11	<10	<10	22	11
PE1	13	<50	<10	21	<10
PE2	23	<50	<10	24	<10
PE3	-	-	-	43	12
	Dissolved antimony (µg/L)				

	Summer 2020	Fall 2020	Spring 2021	Fall 2021	Spring 2022
PT	<1	<1	<1	<1	<1
PE1	1	<5	<1	1	<1
PE2	1	<5	<1	1	1
PE3	-	-	-	<1	<1
	Dissolved silver (µg/L)				
	Summer 2020	Fall 2020	Spring 2021	Fall 2021	Spring 2022
PT	<0,1	<0,1	<0,1	<0,1	<0,1
PE1	<0,1	<0,5	<0,1	<0,1	<0,1
PE2	<0,1	<0,5	<0,1	<0,1	<0,1
PE3	-	-	-	<0,1	<0,1
	Dissolved arsenic (µg/L)				
	Summer 2020	Fall 2020	Spring 2021	Fall 2021	Spring 2022
PT	1.9	1.8	1.7	1.4	1.2
PE1	1.8	1.7	4.4	2	1.6
PE2	3.1	1.9	3.8	1.9	1.4
PE3	-	-	-	0.8	<0,3
	Dissolved barium (µg/L)				
	Summer 2020	Fall 2020	Spring 2021	Fall 2021	Spring 2022
PT	92	90	39	24	10
PE1	111	101	32	30	12
PE2	168	109	50	48	31
PE3	-	-	-	191	35
	Dissolved boron (µg/L)				
	Summer 2020	Fall 2020	Spring 2021	Fall 2021	Spring 2022
PT	104	63	<40	<40	<40
PE1	201	306	149	162	96
PE2	350	226	129	155	119
PE3	-	-	-	275	80
	Dissolved cadmium (µg/L)				
	Summer 2020	Fall 2020	Spring 2021	Fall 2021	Spring 2022
PT	<0,1	<0,1	<0,1	<0,1	<0,1
PE1	0.1	<0,5	<0,1	<0,1	<0,1
PE2	0.2	<0,5	<0,1	<0,1	<0,1
PE3	-	-	-	0.3	0.2
	Dissolved chromium (µg/L)				
	Summer 2020	Fall 2020	Spring 2021	Fall 2021	Spring 2022
PT	1.8	2.5	<0,5	0.7	<0,5
PE1	0.7	<2,5	<0,5	0.7	<0,5

PE2	0.7	<2,5	<0,5	0.8	<0,5
PE3	-	-	-	2	1
	Dissolved cobalt (µg/L)				
	Summer 2020	Fall 2020	Spring 2021	Fall 2021	Spring 2022
PT	2.2	3.3	0.7	1.1	0.7
PE1	0.9	5	4.7	2.5	0.9
PE2	17.8	<2,5	9.2	1	0.6
PE3	-	-	-	<0,5	<0,5
	Dissolved manganese (µg/L)				
	Summer 2020	Fall 2020	Spring 2021	Fall 2021	Spring 2022
PT	736	733	74	3	<1
PE1	310	4040	2970	154	4
PE2	15200	202	4660	7	56
PE3	-	-	-	19	153
	Dissolved molybdenum (µg/L)				
	Summer 2020	Fall 2020	Spring 2021	Fall 2021	Spring 2022
PT	4	4	3	3	3
PE1	7	11	14	14	8
PE2	18	7	13	9	6
PE3	-	-	-	2	1
	Dissolved nickel (µg/L)				
	Summer 2020	Fall 2020	Spring 2021	Fall 2021	Spring 2022
PT	19	17	11	12	9
PE1	15	31	18	26	9
PE2	46	14	26	18	10
PE3	-	-	-	9	3
	Dissolved lead (µg/L)				
	Summer 2020	Fall 2020	Spring 2021	Fall 2021	Spring 2022
PT	<0,1	<0,1	<0,1	<1	<1
PE1	<0,1	<0,5	<0,1	1	<1
PE2	<0,1	<0,5	<0,1	<1	<1
PE3	-	-	-	<1	<1
	Dissolved sodium (µg/L)				
	Summer 2020	Fall 2020	Spring 2021	Fall 2021	Spring 2022
PT	83500	80600	33800	10100	8240
PE1	282000	247000	95500	73500	24600
PE2	342000	289000	151000	105000	64500
PE3	-	-	-	230000	53200
	Dissolved selenium (µg/L)				

	Summer 2020	Fall 2020	Spring 2021	Fall 2021	Spring 2022
PT	<1	1	<1	<1	<1
PE1	1	<5	<1	<1	<1
PE2	<1	<5	<1	<1	<1
PE3	-	-	-	<1	<1

Table S6. C_{10}-C_{50} PHs concentrations (µg/L) in leachate water from the studied plots (n=1) collected using lysimeters. The absence of values indicates a concentration below the limit of detection (<5-100 µg/L). Values for P3 before the summer of 2021 could not be collected since P3 was placed during the summer of 2021.

	C_{10}-C_{50} PHs concentrations (µg/L)				
	Summer 2020	Fall 2020	Spring 2021	Fall 2021	Spring 2022
PT	995	408	-	-	-
PE1	409	254	-	-	-
PE2	849	399	-	-	-
PE3	N\A	N\A	N\A	-	-

Table S7. Benzo[a]pyrene PAHs concentration (µg/L) in leachate water from different plots (n=1). When no values appear, the concentration was below the limit of detection (<0.1 µg/L). Values for P3 before the summer of 2021 could not be collected since P3 was placed during the summer of 2021.

	Benzo[a]pyrene PAHs concentration (µg/L)				
	Summer 2020	Fall 2020	Spring 2021	Fall 2021	Spring 2022
PT	<0.1	<0.1	0.1	<0.1	<0.1
PE1	<0.1	<0.1	0.1	<0.1	<0.1
PE2	<0.1	0.5	0.1	<0.1	<0.1
PE3	N\A	N\A	N\A	0.1	<0.1

6.3 Annexes du chapitre 4

6.3.1 Chemical stability of gemfibrozil

The chemical stability of gemfibrozil was assessed during exposure tests to ensure that the concentration remained constant during the whole duration of the experiments. A volume of 50 mL of medium was sampled before and after the renewal and frozen at -80°C until analysis.

Before analysis, samples were thawed at room temperature and an internal standard (gemfibrozil-d_6) was added before SPE extraction analysis in a volumetric flask (10 mL).

Quality control (QC) samples were used to measure the relative bias and relative standard deviation and thereby verify the accuracy and precision of measured concentrations. Three concentrations of QC samples were used: low (75% of nominal concentration), medium (100% of nominal concentration) and high (200% of nominal concentration). Matrix effects correction was done by using the culture medium in which daphnids lived for a week; this water was used to prepare QC samples (n=3). Results were considered acceptable if the QC samples were within ± 20 % of the expected value.

SPE cartridges were conditioned with 1 mL of phosphate buffer in H_2O (pH 7.5). A volume of 1 mL of the sampled water or calibration point containing internal standards was added to the cartridges. Then, a volume of 2 mL of MeOH:H_2O (20:80) was used to wash salts or other interfering compounds from the cartridges. Next, 2×2 mL of MeOH:ACN (1:1) were used to elute gemfibrozil and gemfibrozil-d_6. Samples were evaporated under N_2 before being reconstituted to 1 mL of MeOH:H_2O (1:1) for analysis.

Gemfibrozil and gemfibrozil-d6 were separated on a reversed-phase column Acquity UPLC HSS T3 C_{18+} from Waters of dimensions 2.1 mm × 50 mm and 1.8 µm particle size. Mobile phase was composed of eluent A (H_2O 5 mM ammonium acetate) and eluent B (MeOH 5 mM ammonium acetate). The elution gradient started with 2% of B, increasing to 15% in 2 min, then up to 50% at 5 min, then 100% at 7 min before holding the same composition for 2 min and then back to initial conditions for column re-equilibration (3 min). The sample injection volume was set to 5 µL. Retention times of 6.61 and 6.63 min were obtained for gemfibrozil-d_6 and gemfibrozil respectively. Electrospray in the positive mode was used as ionization source and the mass spectrometer was operated in the multiple reaction monitoring (MRM) mode. MRM transitions used were m/z 255.5 → m/z 121 for gemfibrozil-d_6 and m/z 249.5 → m/z 121.1 for gemfibrozil. The initial concentration was compared to the final concentration for each sampling

point and no degradation was observed. A reproductive recovery was obtained with a mean of 92% for gemfibrozil when comparing solvent area to the area of the spiked contaminants in the extracted matrix.

The initial concentration was compared to the final concentration for each sampling point and no degradation was observed. All sampling points were within the 20% variation limit of the nominal concentration accordingly to the OECD guidelines (mean of 90.2±10.0% of target concentration). The same LC-MS/MS method was used for the quantification of gemfibrozil in the MTBE extract of daphnids also used for lipidomic analysis.

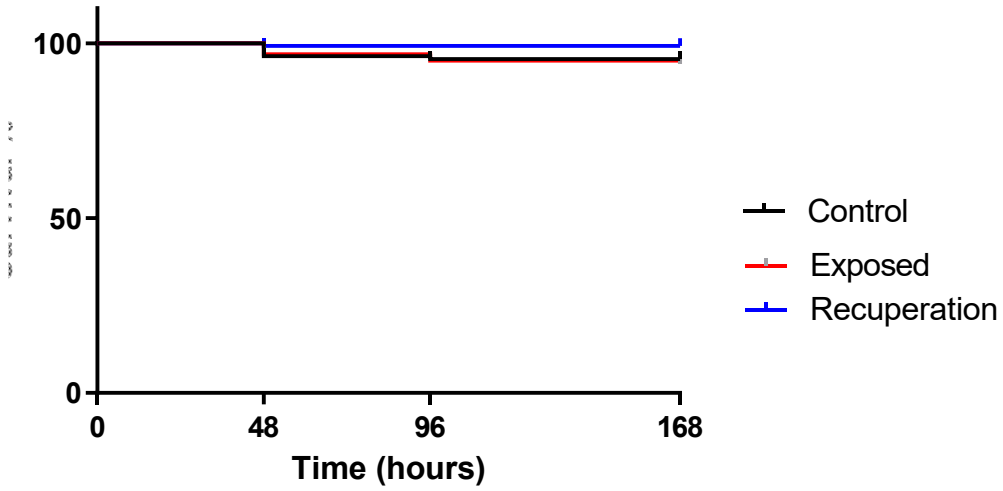


Figure S6. Survival curve (%) of *Daphnia magna* exposed to 1µg/L of gemfibrozil for 96 h or 168 h.

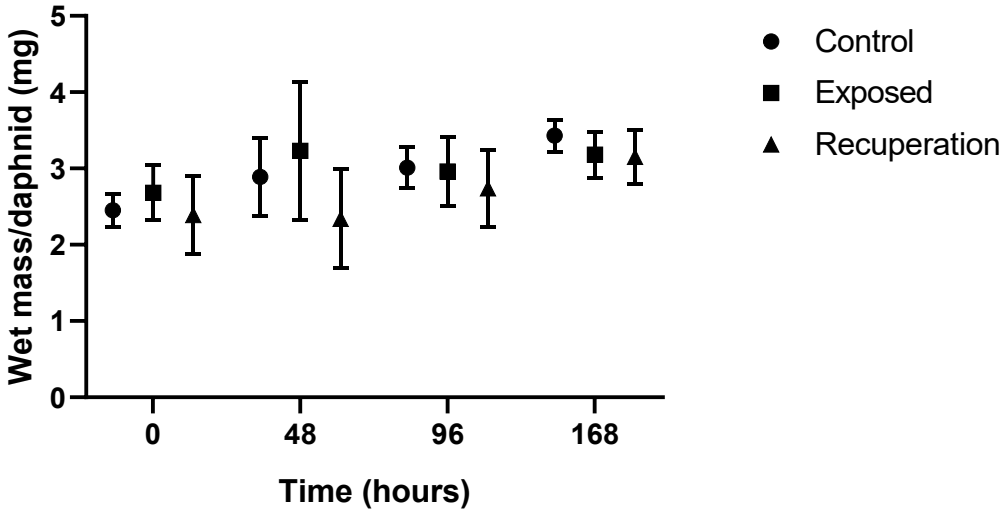


Figure S7. Wet mass of sampled daphnids (mg/individual) exposed to 1 µg/L of gemfibrozil during a 96 h or 168 h period (*n*=6). Error bars represent ± 1 standard error of the mean.

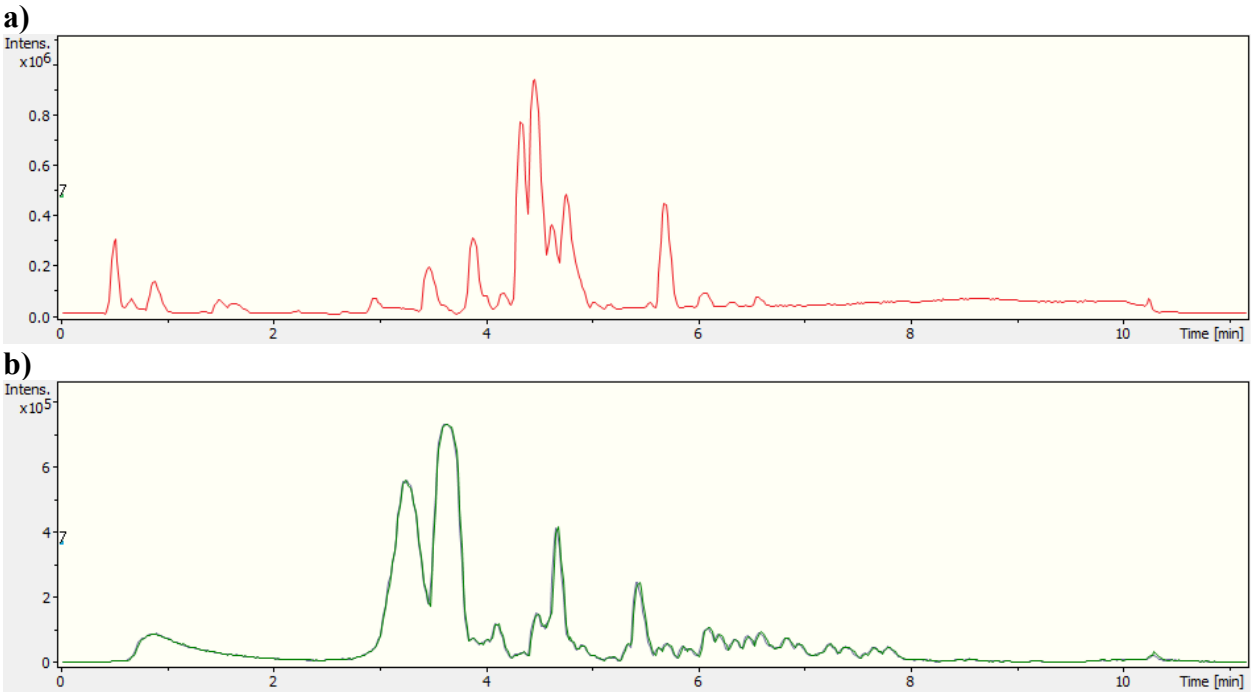


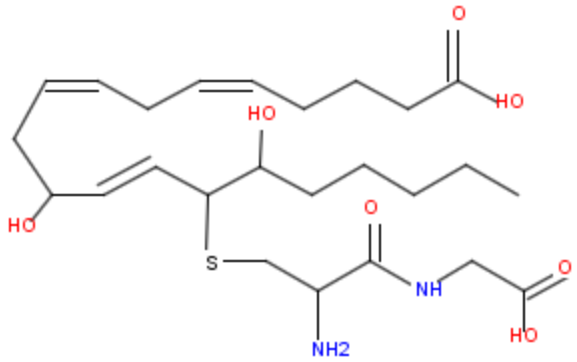
Figure S8. Total ion chromatogram of daphnids extract in a) positive mode and b) negative mode.

6.3.2. Annotation of significant features (in m/z order)

m/z 559.2750 retention time: 4.0 min

Table S8. SimLipid report for *m/z* 559.2750.

Lipid ID	Composition	Experimental *m/z*	Theoretical *m/z*	Delta mass (Da)	RI	Probability
LMFA03090009	[C25H42N2O7S+HCOO] 1-	559.2750	559.2695	0.0055	40.4358	0.262



LMFA03090009 14,15-HxA3-D(11S)

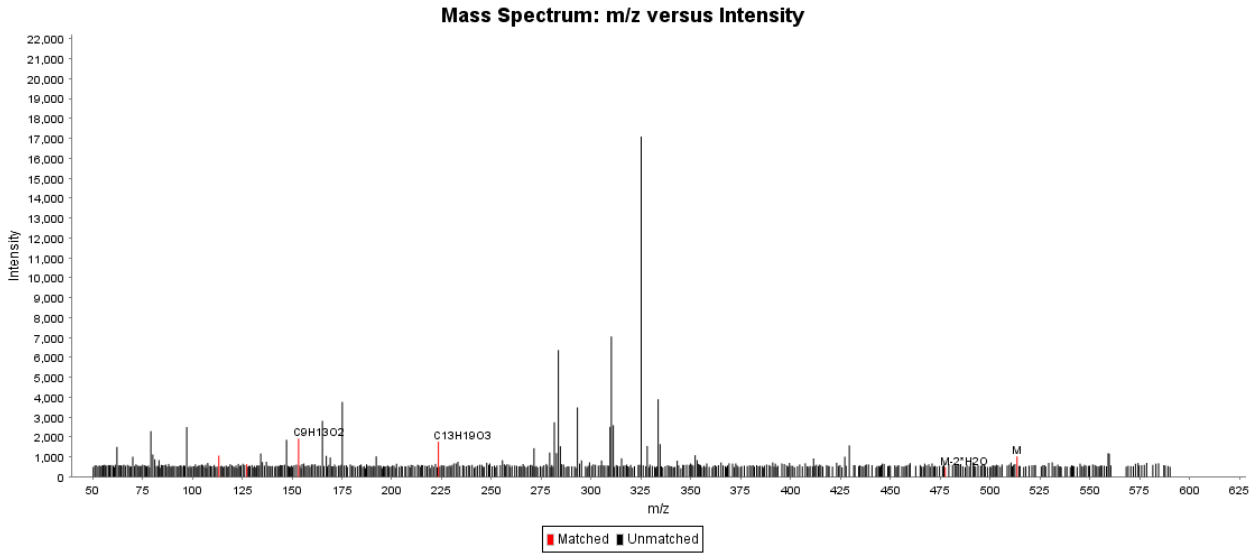
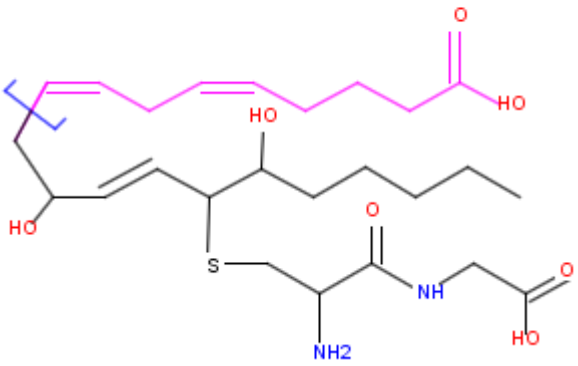


Figure S9. Compared experimental and theoretical spectrum following fragmentation of *m/z* 559.2750.

Table S9. Fragments information from SimLipid for *m/z* 559.2750.

m/z	Intensity	Fragment	Presence
113.0222	1065.5073	C6H9O2	false
127.0208	634.0584	C7H11O2	false
152.9936	1927.7598	C9H13O2	true
223.0987	1764.3081	C13H19O3	true

C9H13O2



C13H19O3

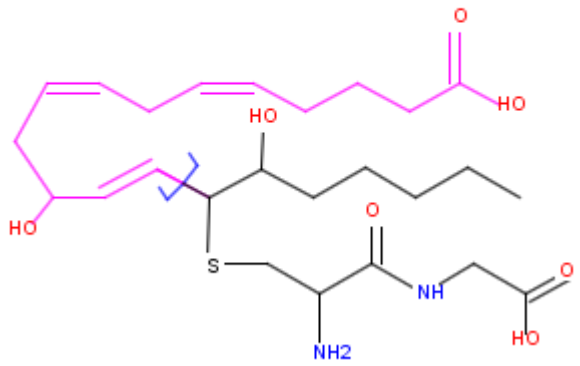


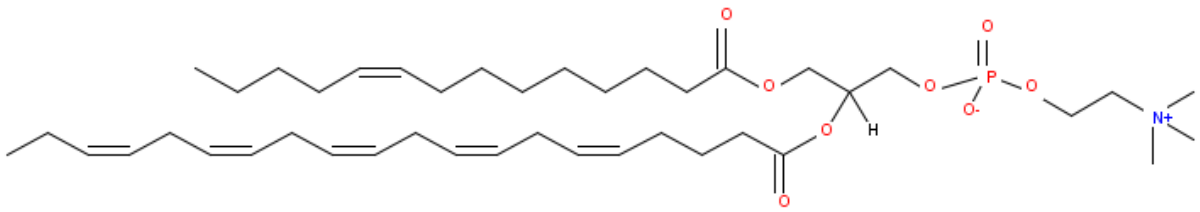
Table S10. Massworks report for *m/z* 559.2750.

	Formula	**Mono Isotope**	**Mass Error (mDa)**	**Mass Error (PPM)**	**Spectral Accuracy**	**RMSE**	**DBE**
1	"C34H41NO4S"	559.2762	-1.3781	-2.4640	35.5840	86	15.0
2	"C31H43O7S"	559.2735	1.3020	2.3280	35.3015	87	10.5
3	"C32H39N4O3S"	559.2748	-0.0354	-0.0633	35.2993	87	15.5
4	"C32H42NO4NaS"	559.2738	1.0272	1.8367	35.2669	87	12.0
5	"C33H42N3OPS"	559.2792	-4.3688	-7.8115	35.2668	87	15.0
6	"C33H38N5NaS"	559.2751	-0.3102	-0.5546	35.2545	87	17.0
7	"C33H45O2PNaS"	559.2781	-3.3061	-5.9115	35.2359	87	11.5
8	"C30H37N7O2S"	559.2735	1.3073	2.3374	35.0087	87	16.0
9	"C29H41N3O6S"	559.2722	2.6447	4.7287	35.0047	87	11.0
10	"C30H44N2O4PS"	559.2765	-1.6887	-3.0194	34.9784	87	10.5

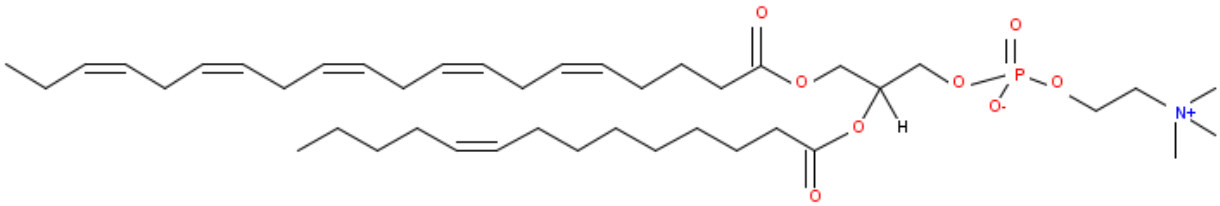
m/z 750.5097 retention time: 4.9 min

Table S11. SimLipid report for *m/z* 750.5097.

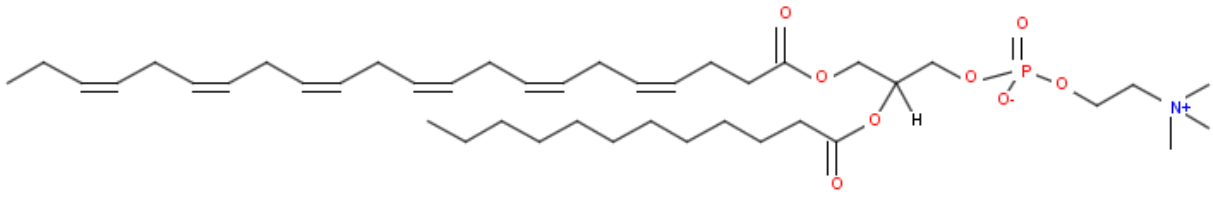
Lipid ID	Composition	Experimental *m/z*	Theoretical *m/z*	Delta mass (Da)	RI	Probability
LMGP01010447	$[C_{42}H_{72}NO_8P+H]^{1+}$	750.5097	750.5068	0.00029	123.2446	0.08
LMGP01011402	$[C_{42}H_{72}NO_8P+H]^{1+}$	750.5097	750.5068	0.00029	123.2446	0.08
LMGP01011929	$[C_{42}H_{72}NO_8P+H]^{1+}$	750.5097	750.5068	0.00029	123.2446	0.08
LMGP01012097	$[C_{42}H_{72}NO_8P+H]^{1+}$	750.5097	750.5068	0.00029	123.2446	0.08



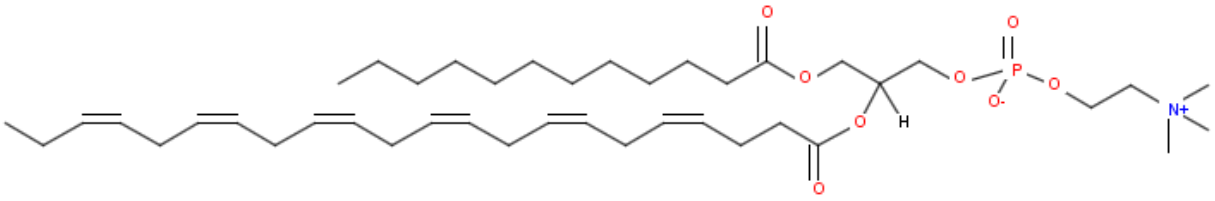
LMGP01011402 PC(14:1(9Z)/20:5(5Z,8Z,11Z,14Z,17Z))



LMGP01011929 PC(20:5(5Z,8Z,11Z,14Z,17Z)/14:1(9Z))



LMGP01012097 PC(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/12:0)



LMGP01010447 PC(12:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z))

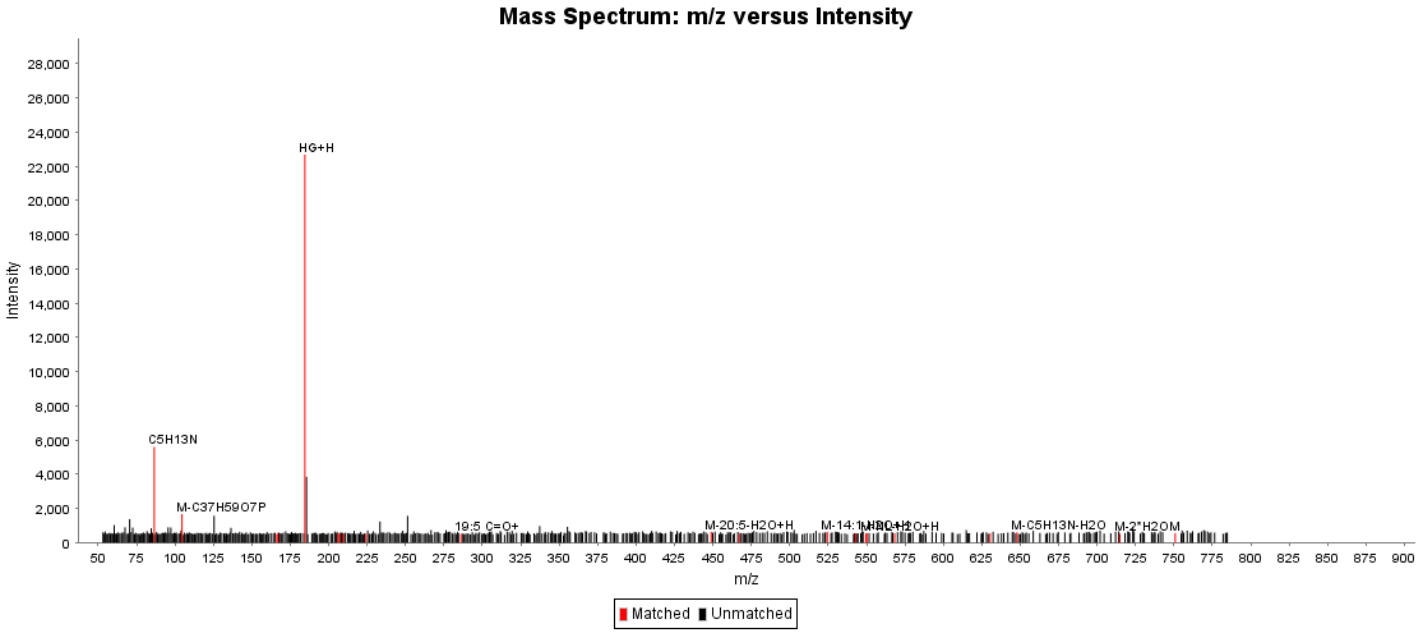
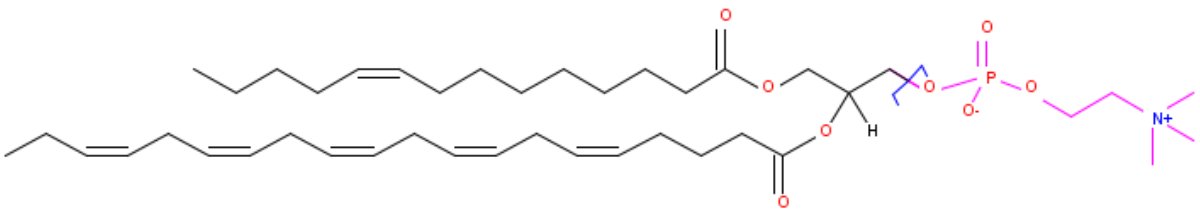


Figure S10. Compared experimental and theoretical spectrum following fragmentation of *m/z* 750.5097.

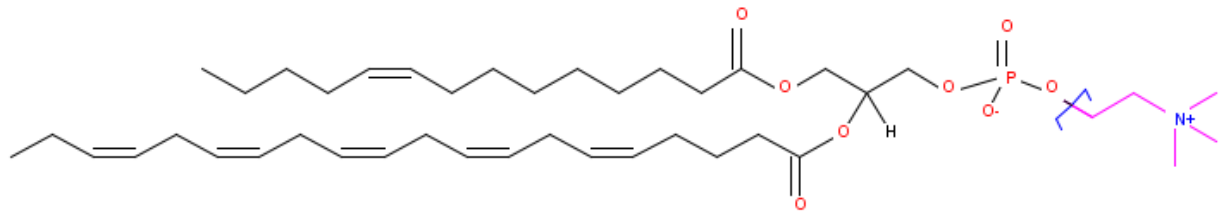
Table S12. Fragments information from SimLipid for *m/z* 750.5097.

m/z	Intensity	Fragment	Presence
86.0971	5602.9815	C5H13N	true
86.0971	5602.9815	M-C37H59O7P-H2O	false
104.1075	1679.7699	M-C37H59O7P	true
165.7786	498.0194	HG-H2O+H	false
165.7786	498.0194	C5H13NO3P	false
166.4881	577.7191	HG-H2O+H	false
166.4881	577.7191	C5H13NO3P	false
184.073	22680.6309	HG+H	true
205.731	554.8565	M-14:1-20:5-H2O	false
206.5417	523.4633	M-14:1-20:5-H2O	false
209.1166	531.2437	13:1 C=O+	false
223.7899	531.2715	M-14:1-20:5	false
285.7045	529.4487	19:5 C=O+	true
448.5041	637.5773	M-20:5-H2O+H	true
466.6522	528.1241	M-20:5+H	false
523.9949	635.7651	M-14:1-H2O+H	true
542.446	541.7045	M-14:1+H	false
549.034	534.4975	M-NL-H2O+H	false
549.8066	560.4616	M-NL-H2O+H	true
567.8266	559.4321	M-NL+H	false
567.8266	559.4321	M-C5H13NO3P-H2O	false
629.8802	552.2845	C37H59O7P-H2O	false
647.4432	606.2211	M-C5H13N-H2O	true
647.4432	606.2211	C37H59O7P	false
714.4091	528.5766	M-2*H2O	true
750.3924	565.4122	M	true

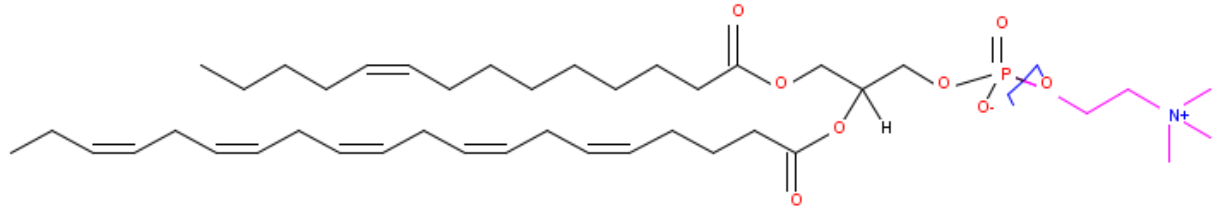
HG+H



C5H13N



M-C37H59O7P-H2O



M-C37H59O7P

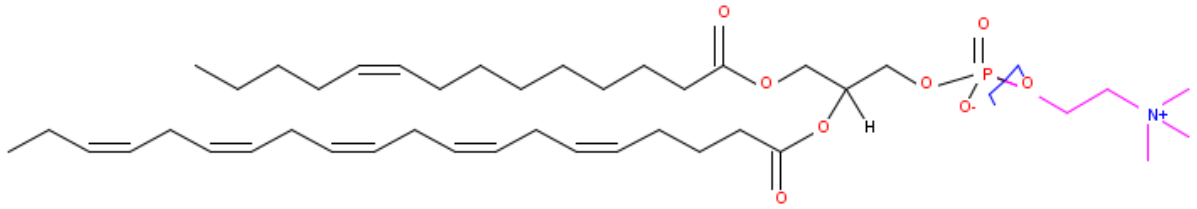


Table S13. Massworks report for *m/z* 750.5097.

	Formula	Mono Isotope	Mass Error (mDa)	Mass Error (PPM)	Spectral Accuracy	RMSE	DBE
1	"C42H73NO8P"	750.5068	2.8687	3.8224	96.1253	31	7.5
2	"C40H73NO10Na"	750.5127	-2.9687	-3.9555	96.0926	31	4.5
3	"C41H74N3O5P2"	750.5098	-0.1219	-0.1625	96.0665	32	7.5
4	"C41H69N5O6Na"	750.5140	-4.3061	-5.7375	96.0044	32	9.5
5	"C41H70N5O4PNa"	750.5058	3.9366	5.2453	95.9507	32	9.5
6	"C38H68N7O8"	750.5124	-2.6886	-3.5824	95.8825	33	8.5
7	"C39H64N11O4"	750.5137	-4.0260	-5.3644	95.8213	34	13.5
8	"C40H75N5O2P3"	750.5128	-3.1126	-4.1474	95.8196	34	7.5
9	"C40H71N7OP2Na"	750.5088	0.9460	1.2604	95.7550	34	9.5
10	"C39H65N11O2P"	750.5055	4.2167	5.6184	95.7243	34	13.5

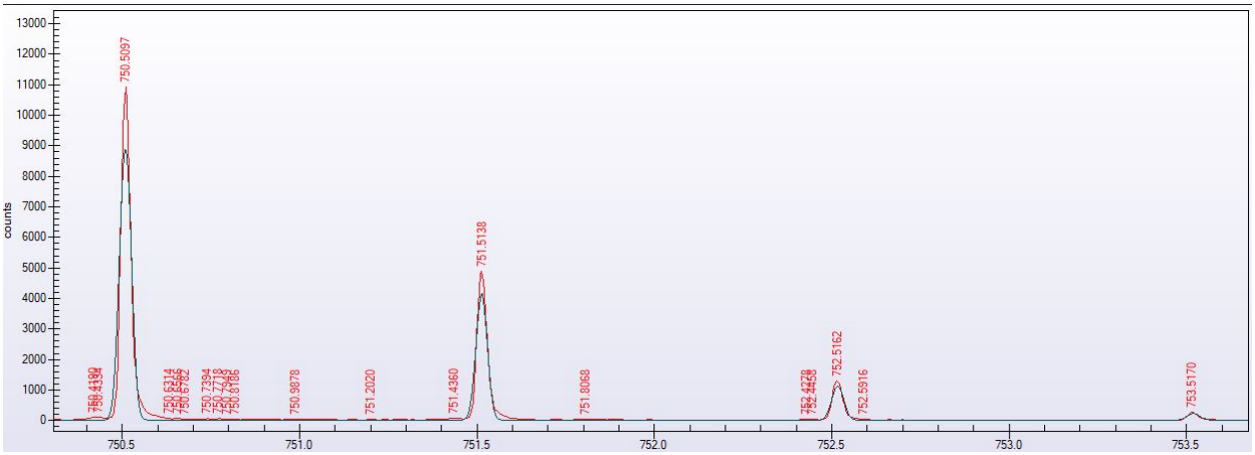


Figure S11. Spectral accuracy of *m/z* 750.5097.

m/z 762.5105 retention time: 4.9 min

Table S14. SimLipid report for *m/z* 762.5105

Lipid ID	Composition	Experimental *m/z*	Theoretical *m/z*	Delta mass (Da)	RI	Probability
LMGP01011356	$[C41H74NO8P+Na]^{1+}$	762.5105	762.5044	0.0006	100.0	0.028
LMGP01011418	$[C41H74NO8P+Na]^{1+}$	762.5105	762.5044	0.0006	100.0	0.028
LMGP01011445	$[C41H74NO8P+Na]^{1+}$	762.5105	762.5044	0.0006	100.0	0.028
LMGP01011446	$[C41H74NO8P+Na]^{1+}$	762.5105	762.5044	0.0006	100.0	0.028
LMGP01011645	[C41H74NO8P+Na] 1+	762.5105	762.5044	0.0006	100.0	0.028
LMGP01011676	[C41H74NO8P+Na] 1+	762.5105	762.5044	0.0006	100.0	0.028
LMGP01011704	[C41H74NO8P+Na] 1+	762.5105	762.5044	0.0006	100.0	0.028
LMGP01011898	[C41H74NO8P+Na] 1+	762.5105	762.5044	0.0006	100.0	0.028

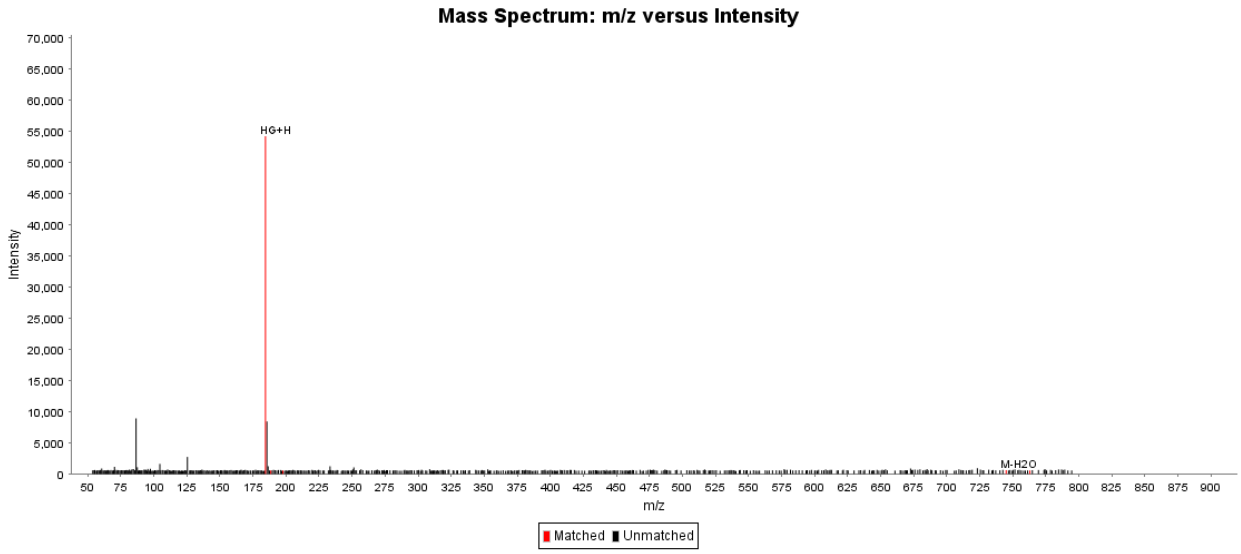


Figure S15. Compared experimental and theoretical spectrum following fragmentation of *m/z* 762.5105.

Table S15. Fragments information from SimLipid for *m/z* 762.5105.

m/z	Intensity	Fragment	Presence
184.0733	54219.8242	HG+H	true
188.007	559.5987	HG-H2O+Na	false
188.007	559.5987	C5H13NO3P	false
197.2254	577.9948	12:0 C=O+	false
744.4967	615.1807	M-H2O	true
762.4556	575.7409	M	false

HG+H

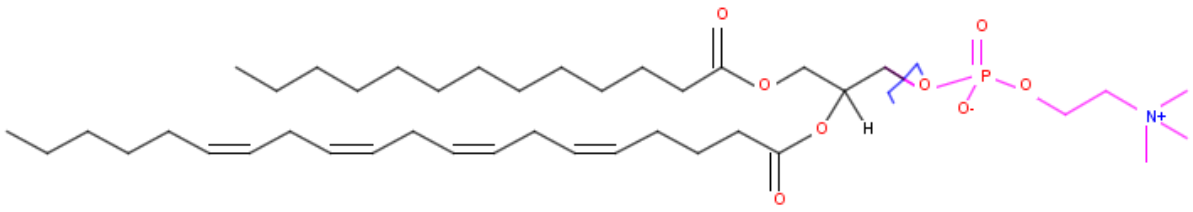


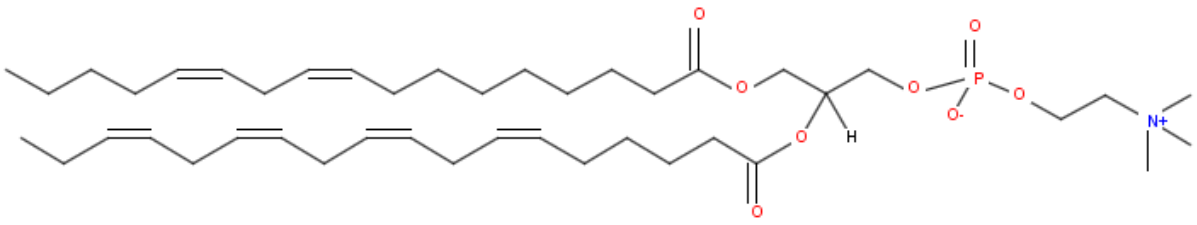
Table S16. Massworks report for *m/z* 762.5105

	Formula	Mono Isotope	Mass Error (mDa)	Mass Error (PPM)	Spectral Accuracy	RMSE	DBE
1	"C43H72NO10"	762.5151	-4.5740	-5.9986	91.9339	23	8.5
2	"C48H69NO5Na"	762.5068	3.7046	4.8584	91.7997	23	14.5
3	"C43H73NO8P"	762.5068	3.6687	4.8114	91.5725	24	8.5
4	"C46H64N7O3"	762.5065	3.9846	5.2257	91.4541	24	18.5
5	"C44H69N5O4P"	762.5082	2.3313	3.0575	91.4500	24	13.5
6	"C47H70N3O2PNa"	762.5098	0.7139	0.9362	91.3621	24	14.5
7	"C42H69N5O6Na"	762.5140	-3.5061	-4.5981	91.2627	24	10.5
8	"C49H69N3O2P"	762.5122	-1.6914	-2.2182	91.1683	25	17.5
9	"C43H65N9O2Na"	762.5153	-4.8435	-6.3520	91.1011	25	15.5
10	"C41H73NO10Na"	762.5127	-2.1687	-2.8441	91.0592	25	5.5

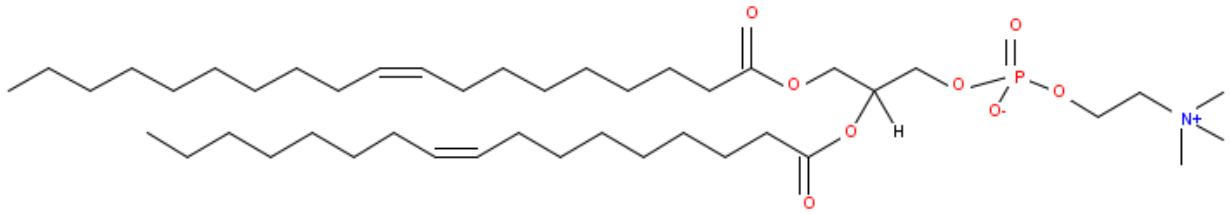
m/z 786.4954 retention time: 5.2 min

Table S17. SimLipid report for *m/z* 786.4954

Lipid ID	Composition	Experimental *m/z*	Theoretical *m/z*	Delta mass (Da)	RI	Probability
LMGP01011566	$[C43H74NO8P+Na]^{1+}$	786.4954	786.5044	0.009	101.7755	0.103
LMGP01011710	$[C43H74NO8P+Na]^{1+}$	786.4954	786.5044	0.009	101.7755	0.103



LMGP01011566 PC(17:2(9Z,12Z)/18:4(6Z,9Z,12Z,15Z))



LMGP01011710 PC(18:4(6Z,9Z,12Z,15Z)/17:2(9Z,12Z))

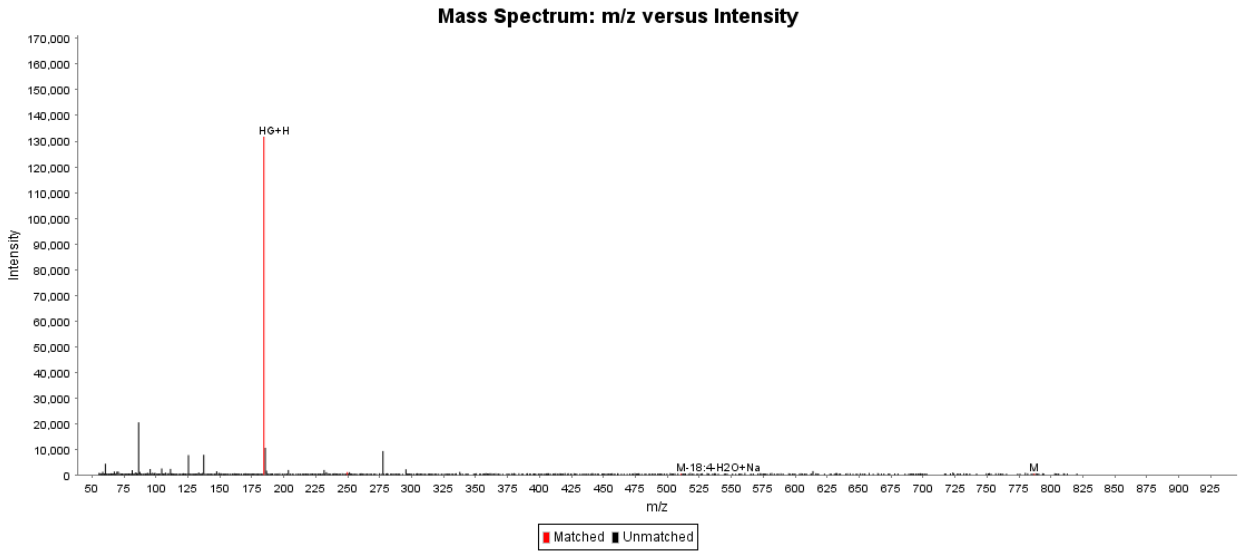


Figure S13. Compared experimental and theoretical spectrum following fragmentation of *m/z* 786.4954.

Table S18. Fragments information from SimLipid for *m/z* 786.4954.

m/z	Intensity	Fragment	Presence
184.0733	131731.2812	HG+H	true
249.1845	1168.9862	16:2 C=O+	false
510.3021	554.2866	M-18:4-H2O+Na	true
786.491	615.6309	M	true

HG+H

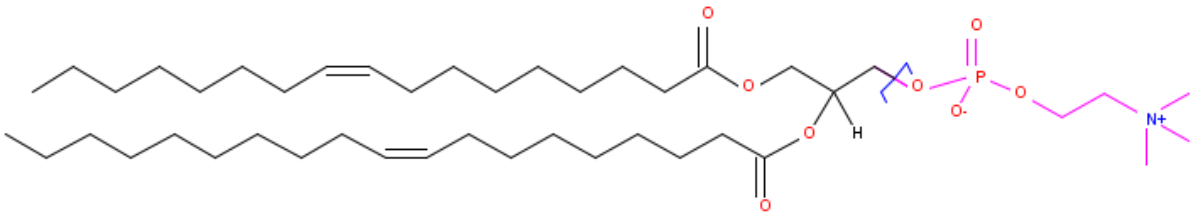


Table S19. Massworks report for *m/z* 786.4954.

	Formula	Mono Isotope	Mass Error (mDa)	Mass Error (PPM)	Spectral Accuracy	RMSE	DBE
1	"C48H68NO8"	786.4939	1.4554	1.8505	86.1707	8	15.5
2	"C46H69NO8Na"	786.4915	3.8607	4.9088	86.0852	8	12.5
3	"C47H69N3O5P"	786.4969	-1.5353	-1.9520	86.0324	8	15.5
4	"C47H65N5O4Na"	786.4929	2.5233	3.2083	85.9688	8	17.5
5	"C50H70NO3PNa"	786.4986	-3.1527	-4.0086	85.9117	8	16.5
6	"C44H64N7O6"	786.4913	4.1408	5.2648	85.8912	8	16.5
7	"C45H70N3O5PNa"	786.4945	0.8700	1.1062	85.8644	8	12.5
8	"C49H64N5O4"	786.4953	0.1180	0.1500	85.8536	8	20.5
9	"C46H66N7OPNa"	786.4959	-0.4674	-0.5943	85.7952	8	17.5
10	"C48H65N7OP"	786.4983	-2.8727	-3.6525	85.7629	8	20.5