Effects of early life exposure to environmental pesticides in wild and laboratory fishes

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Table of Contents

I. Abstract

Fishes worldwide spawn in rivers and streams that are polluted with complex mixtures of environmental contaminants including pesticides. In agricultural regions, pesticide concentrations in rivers and tributaries often peak in spring and early summer, a favored time for many fishes to spawn. Embryos and larvae developing under these conditions are at risk of being negatively impacted, with potential repercussions on fish population health and even conservation status. Contamination of spawning grounds may be of particular concern since early life stage (ELS) organisms are often more sensitive to contaminants than adults of the same species. Even though an increasing number of studies have focused on fish ELS and pesticide exposure, very few have assessed the effects of pesticide mixtures.

In this thesis, I investigate the effects of river water from an agricultural landscape on ELS fish. The focus is on the Richelieu River, a river in southeastern Quebec, which is home to over 80 species of fish including 9 listed species such as river (*Moxostoma carinatum*) and copper redhorse (*Moxostoma hubbsi*). More than 70% of the Richelieu River watershed is dedicated to agriculture. The only two known spawning grounds of the copper redhorse are in the Richelieu River, which means that river contamination could have direct repercussions on that listed species population. In this thesis I first establish the effects of river water exposure on copper and river redhorse ELS (Chapters 3). Exposure to river water caused premature hatching in both species and a decrease of survival in copper redhorse larvae. Changes in gene expression were also assessed in the copper redhorse following exposure and genes related to immune functions were dysregulated. Next, I focused on identifying components of river water that may be associated with the effects observed by analyzing and describing spatial and temporal pesticide contamination of the Richelieu River during the spawning season and identifying the components of the mixture (Chapter 4). A total of 69 compounds, including 31 pesticides, were detected in the river water. During the copper and river redhorse spawning season, river pesticide concentrations were demonstrated to peak following heavy rain events. Of the pesticides, only the neonicotinoids clothianidin and thiamethoxam were found at levels exceeding safety thresholds for aquatic life. In Chapter 5, I used a model organism, the zebrafish, to determine the full life cycle effects resulting from early life exposure to river water. Effects such as delayed hatching, reduced fertilization rate and

increased offspring deformities were reported in fish exposed as embryos and larvae then raised to adulthood in clean water. For hatching and fertilization rate, the range of effects followed the contamination gradient of the river. Finally in Chapter 6, I used laboratory experiments to attempt to link the previously observed effects to specific pesticides that were identified for prioritization in Chapters 3 and 4. Copper and river redhorse ELS were exposed in a laboratory setting to four single pesticides. No effects were observed at concentrations approximately 10 times higher than environmental levels. In summary, this thesis suggests that current levels of contaminants in the Richelieu River have immediate and lasting effects on fish ELS, which have the potential to be harmful to wild fish populations but which can't be traced to the individual pesticides that were tested. I have also provided the first toxicity data from two listed fish species, and the first transcriptome of the copper redhorse.

II. Résumé

Partout dans le monde, les poissons fraient dans des cours d'eau pollués par des mélanges complexes de contaminants, notamment des pesticides. Dans les régions agricoles, la concentration de pesticides culmine souvent au printemps et au début de l'été, période privilégiée pour le frai de nombreuses espèces de poissons. Les embryons et les larves qui se développent dans ces conditions risquent d'être impactés négativement, avec des répercussions potentielles sur la santé des populations de poissons. La contamination des frayères peut être particulièrement préoccupante puisque les organismes aux premiers stades du cycle de la vie (PSCV) sont souvent plus sensibles aux contaminants. Même si un nombre croissant d'études se concentre sur l'exposition des poissons durant les PSCV aux pesticides, très peu ont évalué les effets des mélanges de pesticides.

Dans cette thèse, j'étudie les effets de l'eau de rivière d'un paysage agricole sur les PSCV des poissons. L'accent est mis sur la rivière Richelieu, une rivière du sud-est du Québec qui abrite plus de 80 espèces de poissons, dont 11 espèces à statut comme le chevalier de rivière (Moxostoma carinatum) et le chevalier cuivré (Moxostoma hubbsi). Plus de 70 % du bassin versant du Richelieu est dédié à l'agriculture. Les deux seules frayères connues du chevalier cuivré se trouvent dans la rivière Richelieu, ce qui signifie que sa contamination pourrait avoir des répercussions directes sur la population de cette espèce. J'établis d'abord les effets de l'exposition à l'eau de la rivière sur les PSCV du chevalier cuivré et du chevalier de rivière (Chapitre 3). L'exposition à l'eau de la rivière a causé des éclosions prématurées chez les deux espèces et une diminution de la survie des larves de chevalier cuivré. Les changements dans l'expression des gènes ont également été évalués chez le chevalier cuivré et les gènes liés aux fonctions immunitaires ont été dérégulés suite à l'exposition. Ensuite, je me suis concentré sur l'identification des composantes de l'eau de la rivière pouvant être associées aux effets observés en analysant la contamination spatiale et temporelle par les pesticides de la rivière Richelieu pendant la saison de frai (Chapitre 4). Au total, 69 composés, dont 31 pesticides, ont été détectés dans l'eau de la rivière. Il a été démontré que les concentrations de pesticides dans les rivières culminaient à la suite de fortes pluies. Parmi les pesticides, seuls les néonicotinoïdes clothianidine et thiaméthoxame ont été trouvés à des niveaux dépassant les seuils de sécurité pour la vie aquatique. Au Chapitre 5, j'ai utilisé un organisme modèle, le poisson zèbre,

pour déterminer les effets sur le cycle de vie complet d'une exposition durant les PSCV à un mélange environnemental. Des effets tels qu'une éclosion retardée, un taux de fécondation réduit et une augmentation des malformations de la progéniture ont été signalés chez des poissons durant les PSCV puis élevés jusqu'à l'âge adulte dans de l'eau propre. Pour le taux d'éclosion et de fécondation, la gamme des effets a suivi le gradient de contamination de la rivière. Enfin, au Chapitre 6, j'ai utilisé des expériences en laboratoire pour tenter de lier les effets précédemment observés à des pesticides spécifiques identifiés dans les Chapitres 3 et 4. Les PSCV des chevaliers cuivrés et de rivière ont été exposés en laboratoire à quatre pesticides uniques. Aucun effet n'a été observé à des concentrations environ 10 fois plus élevées que les niveaux environnementaux. En résumé, cette thèse suggère que les niveaux actuels de contaminants dans la rivière Richelieu ont des effets immédiats et durables sur les PSCV des poissons, qui ont le potentiel d'être nocifs pour les populations de poissons sauvages, mais qui ne peuvent être attribués aux pesticides individuels qui ont été testés. J'ai également fourni les premières données de toxicité de deux espèces de poissons à statut, et le premier transcriptome du chevalier cuivré.

III. Acknowledgements

The works presented herein would not have been possible without the help I have received along the way from friends and colleagues. First and foremost, I would like to thank my supervisor Dr Jessica Head for her most appreciated mentorship, patience, trust and support. Past and present members of the Head and of the Basu labs for their advice, constant encouragement and great attitude. A big thank you to all the students who spent summers helping at Saint-Ours. I will forever have fond memories of our time spent at B17. And a special thanks to Emily Boulanger and Jenny Eng for their invaluable help, knowledge and expertise of the technical world.

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Finally, but not the least, I would like to thank my family, especially my parents Danielle Champagne and Richard Marchand for their support and encouragement and to my partner Pascale Dupuis, who lovingly stood by my side through the hardships of graduate school. I owe a special

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IV. Preface and Contribution to original knowledge

This thesis is written in the manuscript format, in compliance with McGill's "Guidelines for Thesis Preparation". Chapter 1 is a general introduction which includes the rationale and objectives of the research. Chapter 2 is a literature review that pertains to the entirety of the work presented. Chapters 3 to 6 are manuscripts published or to be submitted for publication in peer reviewed journals. Chapter 7 is a summary and discussion that pertains to the entirety of the work presented. The manuscripts presented in chapters 3 to 6 were first authored by the candidate. Chapter 3 is already published in a peer reviewed journal. Chapters 4 to 6 will be submitted shortly.

This thesis fills important knowledge gaps and contributes to the advancement of knowledge as follows:

In Chapter 3, we investigated the effects of river water on river and copper redhorse ELS. We provided evidence that environmental levels of pesticides have deleterious effects on fish. To the best of our knowledge, this was the first exposure study performed using river or copper redhorse embryos. We gained knowledge about the artificial rearing of listed fish and provided the first copper redhorse transcriptome. The importance of this study lies in the fact that it shows that environmental levels of contamination may be impeding the recovery of an endangered species.

In Chapter 4, we provided, to the best of our knowledge, the finest temporal resolution of pesticide contamination data for the copper redhorse spawning grounds. We were able to demonstrate that heavy rain events during river and copper redhorse spawning seasons cause pesticide concentrations to peak in the river. We also provided evidence that pesticide concentrations are higher in the tributaries than in the main river, which could affect other wild fish spawning in those areas. We identified two neonicotinoid insecticides with concentrations exceeding the safety threshold for aquatic life in the Richelieu River and its tributaries. We also showed that pesticides that were banned decades ago for their persistence in the environment can still be detected in river water.

In Chapter 5, we confirmed that ELS exposure to river water can also have an effect on the zebrafish, a model organism. Using the zebrafish, we were able to assess effects of ELS exposure to an environmental mixture over the complete life cycle. We observed a decrease in fertilization rate following the contamination gradient in the exposed fish, and an increase of the rate of deformities in their offspring. These persistent effects could affect the population of endangered fish such as the copper redhorse as annual recruitment for the entire species is from the Richelieu River.

In Chapter 6; we evaluated the single toxicity for river and copper redhorse of four pesticides that were omnipresent in the Richelieu River. We showed that alone, these four compounds could not explain the decline of the copper redhorse. We provided the first toxicity data of 4 active compounds for both copper and river redhorse. Our results also increase awareness of the potential mixture effects of environmental contaminants in surface water.

Overall, my thesis is a case study for understanding the potential impacts of pesticides on endangered fish. My results suggest that current levels of contaminants in the Richelieu River have immediate and lasting effects on fish ELS which have the potential to be harmful to wild fish populations but which can't be traced to the individual pesticides that were tested.

V. Contribution of Authors

Chapter 3: This chapter was authored by the candidate and co-authored by Dr Benjamin Barst, Emily Boulanger, Nathalie Vachon, Dr Magali Houde, Dr Jeff Xia, Dr Peng Liu, Dr Jessica Ewald, Dr Stephane Bayen, Dr Lan Liu and the candidate's supervisor Dr Jessica Head. The proposal for funding was developed by Dr Benjamin Barst, Nathalie Vachon, Dr Magali Houde, and Dr Jessica Head. Study design and implementation of the experimental methodology was led by the candidate with support from the rest of the project team. The candidate collaborated and coordinated with Nathalie Vachon to provide her field team with help and to obtain wild river and copper redhorse embryos. Data collection was led by the candidate with help from Dr Benjamin Barst, and Emily Boulanger. Water chemistry analysis was performed by Dr Lan Liu (Stephane Bayen). The candidate led the analysis and interpretation of the organismal data with the help of Dr Benjamin Barst, of the water chemistry data with the help of Dr Lan Liu (Stephane Bayen) and of the transcriptomic data with the help of Dr Peng Liu and Dr Jessica Ewald (Jeff Xia). The candidate wrote the manuscript and obtained editorial input from all co-authors. The manuscript was published in Environmental Toxicology and Chemistry volume 41, issue 8 in August 2022 (pages 1950-1966). Copyright approval was obtained, see section 8 of this thesis.

Chapter 4: This chapter was authored by the candidate and co-authored by Dr Benjamin Barst, Emily Boulanger, Nathalie Vachon, Dr Magali Houde, Dr Lan Liu, Dr Stephane Bayen, and the candidate's supervisor Dr Jessica Head. The proposal for funding was developed by Dr Benjamin Barst, Nathalie Vachon, Dr Magali Houde, and Dr Jessica Head. Study design and implementation of the experimental methodology was led by the candidate with support from the rest of the project team. Data collection was led by the candidate with help from Dr Benjamin Barst, and Emily Boulanger. Water chemistry analysis was performed by Dr Lan Liu (Stephane Bayen). The candidate led the analysis and interpretation of the data with the help of Dr Benjamin Barst, and Dr Lan Liu (Stephane Bayen). The candidate wrote the manuscript and obtained editorial input from all co-authors. The manuscript is in preparation and will be submitted shortly to a peer-reviewed journal.

Chapter 5: This chapter was authored by the candidate and co-authored by Dr Benjamin Barst, Emily Boulanger, Nathalie Vachon, Dr Magali Houde, Dr Xu Ke, Dr Saji George and the candidate's supervisor Dr Jessica Head. The proposal for funding was developed by Dr Benjamin Barst, Nathalie Vachon, Dr Magali Houde, and Dr Jessica Head. Study design and implementation of the experimental methodology was led by the candidate with support from the rest of the project team. Data collection was led by the candidate with help from Dr Benjamin Barst, Dr Xu Ke (Saji George) and Emily Boulanger. The candidate was responsible for the analysis and interpretation of the data. The candidate wrote the manuscript and obtained editorial input from all co-authors. The manuscript is in preparation and will be submitted shortly to a peer-reviewed journal.

Chapter 6: This chapter was authored by the candidate and co-authored by Dr Benjamin Barst, Emily Boulanger, Nathalie Vachon, Dr Magali Houde, and the candidate supervisor Dr Jessica Head. The proposal for funding was developed by Dr Benjamin Barst, Nathalie Vachon, Dr Magali Houde, and Dr Jessica Head. Study design and implementation of the experimental methodology was led by the candidate with support from the rest of the project team. The candidate collaborated and coordinated with Nathalie Vachon to provide her field team with help and to obtain wild river and copper redhorse embryos. Data collection was led by the candidate with help from Emily Boulanger. The candidate was responsible for the analysis and interpretation of the data. The candidate wrote the manuscript and obtained editorial input from all co-authors. The manuscript is in preparation and will be submitted shortly to a peer-reviewed journal.

VI. List of Figures

VII. List of Tables

VIII. List of Abbreviations

AMPA: Aminomethylphosphonic acid

CCME: Canadian Council of Ministers of the Environment

CDD: Cumulative degree days

CTD: Comparative toxicology database

DEGs: Differentially expressed genes

DPF: Day post-fertilization

DPH: Day post-hatch

ELS: Early life stage

FC: Fold change

FDR: False discovery rate

GC: Gas Chromatography

HLPC: High-performance liquid chromatography

HRGC: High-resolution gas chromatography

HRMS: High-resolution mass spectrometry

KEGG: Kyoto Encyclopedia of Genes and Genomes

KOs: KEGG orthologs

LC50: Lethal concentration 50

MDL: Method detection limit

MFFP: Ministère des Forêts, de la Faune et des Parcs du Québec

MS: Mass spectrometry

ND: Not detected

PAHs: Polycyclic aromatic hydrocarbons

PCBEs: Polybrominated diphenyl ethers

POCIS: Polar organic chemical integrative sampler

PPCPs: Pharmaceutical and personal care products

RNA: Ribonucleic acid

RNAseq: Ribonucleic acid sequencing

QC: Quebec

Chapter 1

1. Introduction

It is well known that early life exposure to environmental contaminants can have profound effects on the embryonic development of biological organisms (Li et al. 2011, Falisse et al. 2017). Early life is a crucial developmental period during which organisms are particularly sensitive to environmental stressors. In fish, developmental stages (embryo and larval) before complete resorption of the yolk sac (fry stage) are considered to be early life stage (ELS). The ELS fish may be particularly vulnerable, as their development may occur while directly in contact with contaminated water and sediments (Li et al. 2011). Even though there is a consensus that ELS fish are more vulnerable than adults, they are not all equally sensitive. There is an agreement in the literature that the larval stage is more vulnerable than embryonic and juvenile stages (Oliva et al. 2008; Lazhar et al. 2012; Mhadhbi & Beiras 2012; Velki et al. 2017, Wang et al. 2017). This could be explained by the fact that the larval stage lacks the embryo's chorion, which acts as a protective barrier, and has under- or undeveloped biological defenses or means of detoxification that are present in the juvenile stage (Velki et al. 2017). In comparison with embryo and juvenile stages, larvae can be sensitive to pesticide doses 10 to 100x lower in concentration and should not be overlooked when identifying potential effects on fish breeding in contaminated waters (Oliva et al. 2008).

Contamination of waterways by multiple pesticides is an increasing concern. Pesticides are almost always detected in mixtures since many are applied together or in succession as part of integrated pest management (Kogan 1998; Ahmad et al. 2008). This means that fish and ELS fish are seldom exposed to individual pesticides but rather to a mixture of pesticides and other contaminants. For example, 4 southern Quebec rivers monitored for pesticides by the provincial government contained between 18 and 34 pesticides when sampled between 2015 and 2017 (Giroux 2019). These pesticide mixtures may have synergistic effects, which worsens their impacts on fish. For example, atrazine and chlorpyrifos have been known to have such a synergistic effect; when detected together, their toxicity potential is increased compared to what would be predicted by the simple principle of addition (Perez et al. 2013). This is also true of chlorpyrifos and betacypermethrin (Zhang et al. 2017) and phoxim and atrazine (Wang et al. 2017). Interactions

among pesticides and contaminants from wastewater effluents have also been documented, which even further complicates the effects of mixtures on fish ELS. It is also worth mentioning that pesticide use is not static and varies according to location and culture, but also time as new "better" products with varying properties are engineered to replace previous ones.

Pesticide exposure during ELS may cause overt toxicological effects such as deformities and mortality which have often been reported, but more subtle effects may also occur (Görge & Nagel 1990, Mhadhbi & Beiras 2012, Shahjahan et al. 2017). For example, memory loss, neurological disorders and reduced foraging and escape capacities have been observed in fish in response to early life contaminant exposure (Knecht et al. 2007, Gao et al. 2017). A growing body of research suggests that early life exposure to contaminants such as pesticides may also cause persistent negative effects in many organisms, including fish. These effects can sometimes be observed in subsequent generations in the absence of re-exposure (Weinhold 2012; Bhanndari et al. 2015; Singh et al. 2016, Carvan et al. 2017).

1.1 Objectives, Hypotheses & Specific Aims

Overall objectives: Determine the molecular and organismal effects of early life exposure to environmental mixtures dominated by pesticides in fish.

Overall hypothesis: Early-life exposure to pesticides will lead to dysregulation of gene expression. In addition, early life exposure to contaminants will increase deformities, and decrease growth, survival, and reproductive output of wild and laboratory fishes.

This hypothesis will be addressed in 5 chapters as follows:

Chapter 2: Literature review: A Canadian perspective on the effects of current use pesticides of concern on freshwater fish early life stage

Aim: Describe the known effects of current use environmental pesticides in ELS fish.

Chapter 3: Exposure to Contaminated River Water is Associated with Early Hatching and Dysregulation of Gene Expression in Early Life Stages of the Endangered Copper Redhorse (*Moxostoma hubbsi*)

Aims & hypotheses:

1. Determine whether ELS exposure to Richelieu River water is detrimental to the copper redhorse.

I hypothesize that ELS of the copper/river redhorse exposed to contaminated water will have a lower hatch rate while mortality and deformities will be higher compared to the control group. Gene expression will be dysregulated following exposure to contaminated water.

2. Determine if the copper redhorse is more sensitive to contaminants present in the water than the river redhorse.

I hypothesize that copper redhorse will exhibit earlier hatching, lower survival and increased number of deformities than river redhorse following river water exposure.

Chapter 4: Pesticide concentrations in the Richelieu River and its tributaries during the spawning period of threatened and endangered fishes.

Aims & hypotheses:

1. Identify how tributaries (Huron and Acadie rivers) contribute to the contamination of copper redhorse spawning grounds.

I hypothesize that the influx of pesticides from tributaries will result in higher pesticide concentrations at the spawning ground downstream of the tributaries (Saint-Ours) and will have no effect on the spawning ground upstream of the tributaries (Chambly).

2. Determine which contaminants are present in the Richelieu River and explore their concentrations throughout the copper redhorse breeding period.

I hypothesize that pesticides, pharmaceuticals and personal care products will be present in the Richelieu River. The concentrations of contaminants will peak following rain events. Pesticides leaching into the river will peak during the copper redhorse breeding period.

Chapter 5: Decrease in fertilization rate of zebrafish exposed during early life stage to Richelieu River (QC, Canada) water

1. Determine the effects of ELS exposure to pesticide in zebrafish.

I hypothesize that fish ELS exposure will have decreased hatching success and survival, an increase in the number of deformities and will hatch prematurely. I also expect increased tail coiling activity.

2. Determine the later life effects of ELS exposure to pesticides in zebrafish.

I hypothesize that zebrafish ELS exposure to pesticides will exhibit decreased growth rate, body condition factor and reproductive output. I also expect the sex ratio to be skewed towards female.

3. Determine the multi-generational effects of ELS exposure to pesticide in zebrafish.

I hypothesize that zebrafish offspring from parents previously exposed to pesticides will exhibit a decrease in hatching success and survival, an increase in the number of deformities and will hatch prematurely. I also expect decreased growth rate, body condition factor and reproductive output.

Chapter 6: Assessment of 4 current use pesticides in early life stage of two listed *Moxostoma* species: the river and copper redhorse

<u>Aims & hypotheses:</u>

1. Determine the effects of laboratory ELS exposure to atrazine, clothianidin, glyphosate and metolachlor on hatch rate, mortality, deformities, mRNA expression and DNA methylation in copper redhorse and river redhorse ELS.

I hypothesize that ELS of the copper redhorse and river redhorse exposed to pesticides will hatch prematurely compared to their control groups. Mortality and deformities will be higher following pesticide exposure. Genes related to xenobiotic mechanisms and oxidative stress are expected to be dysregulated and show a higher expression level.

2. Identify and confirm differences in sensitivity between fish species to atrazine, clothianidin, glyphosate and metolachlor exposure.

I hypothesize that river redhorse, which is listed as special concern, will be less sensitive than copper redhorse, which is listed as endangered. Copper redhorse will be the species most sensitive to pesticide exposure in terms of premature hatching and survival.

Chapter 2

2. Literature review: A Canadian perspective on the effects of current use pesticides of concern on freshwater fish early life stage

Fish are important bioindicators for contamination of aquatic environments (Naigaga et al. 2011). Current environmental levels of contaminants are generally below concentrations expected to cause mortality but can alter behavior and physio- and biochemical parameters, which can have repercussions on wild fish populations (Lopes et al. 2022). Testing is often performed on later life stages but there is an increasing awareness that early life stage (ELS) is very important due to increased sensitivity but also ease of maintenance. Even there, limited information is still available on the effects of current use chemicals such as pesticides on ELS fish. Here we identify current use pesticides of concern in a Canadian context and reviewed the literature that describes their effects in ELS fish.

2.1 Scope of the Review

The first step in establishing the scope of the review was to select which pesticides of concern to include. I focussed on pesticides that are detected in the environment and that are currently registered for use in Canada. Twelve chemicals were selected among pesticides that are omnipresent in Canadian surface waters (Sultana et al. 2018, Lalonde & Garron 2020, Giroux 2022) including six that were previously identified as of concern (Anderson et al. 2021). Three compounds overlapped between the two groups: atrazine, clothianidin and metolachlor. Chlorpyrifos, which was identified as a contaminant of concern was not included here due to the already extensive number of reviews on its toxicity to fish and fish ELS (Wang et al. 2011, Jin et al. 2015, Sunanda et al. 2016, Farkhondeh et al. 2021). The twelve selected chemicals included five herbicides (atrazine, glyphosate, metolachlor, diquat, imazethapyr), one herbicide degradation product (Aminomethylphosphonic acid (AMPA)), two insecticides (permethrin and chlorantraniliprole), three neonicotinoid insecticides (imidacloprid, thiamethoxam, clothianidin) and one fungicide (chlorothalonil). The research was then narrowed down to studies using fish early life exposure using the key terms “pesticide (active molecule name) fish early life”. Since freshwater habitats are currently the first reached by pesticides, only freshwater fish were

considered. The focus was set on single pesticide waterborne exposure to the active molecule; if a research used single pesticide and mixture or single pesticide and formulation, only the information from the single active compound was reported. The only non-active compound assessed here was the glyphosate degradation product AMPA since it is detected as often or even more than its parent compound. Most of the studies were conducted in a laboratory setting since we focused on the effects of single active compounds, which are seldom present singly in the environment. Recent studies have started to evaluate the potential effects of pesticide metabolites and formulants. We acknowledge that these avenues should be investigated and that often formulants or metabolites have been found to be more toxic than the parent compounds, but formulations were not used due to the lack of information on formulants, the numerous variations across brands and because it often contains a mixture of pesticides which makes it hard to narrow down effects to a specific compound. Literature that did not meet all the previous criteria was rejected.

2.2 Herbicides

Herbicides are used for the control of vegetation and represent the highest proportion of agricultural pesticides (Salomon et al. 2013). The use of herbicides has increased with the rising reliance to genetically modified crops (Salomon et al. 2013). Herbicides work by targeting plant-specific elements such as the cell membrane and the photosynthesis apparatus. This means that they are less likely to affect non-plant organisms, but high concentrations can lead to toxicity in untargeted species such as fish (Salomon et al. 2013). Herbicides are ubiquitous in surface water (Salomon et al. 2013. Giroux 2022) and there is concern about chronic exposure and sublethal effects.

2.2.1 Atrazine

Atrazine is a pre-emergent triazine herbicide, used to control broadleaf weeds by disrupting the photosynthesis mechanisms (Gammon et al. 2005). It is one of the most commonly used herbicides in the world, with concentrations in surface water across the globe usually measured in µg/L range (Gammon et al. 2005, Liu et al. 2016). Atrazine can have a wide range of effects in fish ELS such as alteration of hatching and swimming behavior, increased rate of deformity and oxidative stress, endocrine disruption and neurotoxicity.

Survival & Hatching Time

Environmental concentrations of atrazine are generally lower than levels expected to be lethal to ELS fish, but environmental concentrations can have sublethal effects. In zebrafish (*Danio rerio*), lethal concentrations are in the mg/L range with median lethal concentrations (LC50s) of 34.19 and 15.63 mg/L for embryo and larvae respectively (Wang et al. 2017). Due to this, most studies on ELS fish focus on sub-lethal endpoints. For example, at environmental concentrations, atrazine had no effect on survival but caused premature hatching of both zebrafish (300 and 1000 µg/L) (Blahova et al. 2020) and sockeye salmon (250 µg/L) and early emergence of salmon (at 25 and 250 µg/L) (Du Gas et al. 2017). Decreased hatching rate was also observed in zebrafish exposed to 900 µg/L of atrazine (Walker et al. 2018). Overall, environmental levels of atrazine should have no effect on fish survival but could accelerate fish hatching time.

Deformities

The literature reports incidences of deformities in some fish species exposed during ELS to environmental concentrations of atrazine. For example, increased rate of deformities and edema were observed in zebrafish ELS exposed to 1300 µg/L of atrazine (Görge and Nagel. 1990). In another study, differences in craniofacial cartilage elements, delayed vertebrae mineralization and gross craniofacial deformities were reported in zebrafish exposed to 20, 200 and 900 µg/L of atrazine respectively (Walker et al. 2018). However, in common carp (*Cyprinus carpio*), 33 days ELS exposure to 0.3, 30, 100 and 300 µg/L of atrazine had no incidence on deformity rate, condition characteristics or histology (Chromcova et al. 2013). Common carp may be less sensitive than zebrafish but more research is needed to better understand species sensitivity to atrazine.

Behavior

Only one study has assessed the effects of atrazine on ELS fish behavior. Swimming speed and free-swimming distance were assessed in zebrafish exposed to 30, 100 and 300 µg/L of atrazine and both parameters were decreased at the two highest doses only (Liu et al. 2016). The concentrations assessed were environmentally relevant, but more studies are necessary to strengthen the conclusions.

Biochemical

Exposure to atrazine during ELS in fish has previously been associated with oxidative stress, endocrine disruption and neurotoxicity. For example, exposure of ELS common carp to 0.3, 30, 100 and 300 µg/L increased the activities of the oxidative stress markers glutathione peroxidase, gluthathione S-transferase, superoxide dismutase, and catalase at all tested doses (Chromcova et al. 2013). Estrogenic response in the developing gonads and disruption of star, cyp11a2, and cyp1a mRNA expression were observed in the highest tested concentration of largemouth bass (*Micropterus salmoides*) ELS exposed to 1, 10, 100 µg/L of atrazine (Leet et al. 2020). In zebrafish exposed to 30, 100 and 300 ug/L of atrazine, acetylcholinesterase activity and nervous system related gene expression were decreased in both 100 and 300 µg/L exposed fish. The chorion was not a good barrier and did not prevent atrazine uptake and accumulation inside the embryo (Wiegand et al. 2000). Overall atrazine, at environmental concentrations could affect numerous biochemical pathways of ELS fish.

Later in life

Transgenerational effects of atrazine exposure during ELS have also been reported. For example, in Japanese medaka (*Oryzias latipes*), exposure during ELS to 5 or 50 µg/L of atrazine, decreased the sperm count and motility in the F2 generation but not in the exposed F0 fish. No effects on fecundity were observed in the F0 through F2 generations, but decreased fertilization rate was observed in the F2. Transgenerational alterations of expression of genes involved in steroidogenesis and DNA methylation were also reported (Cleary et al. 2019). These results suggest that exposure during ELS could have detrimental transgenerational effects which can result in decreased reproductive potential.

As many others have previously pointed out, environmental concentrations, which are usually below 100 µg/L are unlikely to have significant effects on fish populations (Van der Kraak 2014, Hanson et al. 2019). However, as with many compounds, studies have mostly focused on laboratory organisms which may not be representative of wild fish species.

2.2.2 Glyphosate

Glyphosate is a non-selective organophosphate herbicide and is also one of the most widely applied herbicides in the world. It is often uses in conjunction with genetically engineered

herbicide-tolerant crops. Glyphosate inhibits the 5-enolpyruvyl-shikimate-3-phosphate-synthase enzyme, which is essential for the biosynthesis of 3 plant amino acids: phenylalanine, tryptophan and tyrosine (Schönbrunn et al. 2001). Non targeted animals such as fish lack this biochemical pathway which suggests limited effects (de Brito Rodrigues et al. 2017). However, effects of glyphosate such as alteration of hatching, development and larval activity, increased oxidative stress and neurotoxicity have all been reported in ELS fish. In the environment, concentrations of glyphosate in surface water are frequently measured in at the µg /L level (Tresnakova et al. 2021).

Survival

For most fish species, embryonic LC50s for glyphosate are in mg/L range (Golovanova & Aminov 2019), which is higher than most reported environmentally relevant concentrations. For example, in Java medaka (*Oryzias javanicus*) and zebrafish embryo, no mortality during ELS exposure was reported at concentrations up to 100 mg/L (Yusof et al. 2014) and 250 mg/L (Uren Webster et al. 2014) respectively.

Hatching

Many studies report alterations in hatching time of fish embryos raised in glyphosate-spiked solutions. Early hatching has been reported in zebrafish at concentrations of 0.7 mg/L (Liu et al. 2022), 1.69 mg/L (Schweizer et al. 2019), 5 mg/L (Fiorino et al. 2018) and 50 mg/L (Uren Webster et al. 2014). But not all zebrafish studies report effects on hatching. For example, Diaz-Martin et al. (2021) observed no effects on zebrafish hatching during ELS exposure to 1, 5, 10 or 50 mg/L of glyphosate. Similarly, no effects on hatching were reported in ELS zebrafish exposed to 0.017, 0.17 and 1.7 mg/L of glyphosate (Ivantsova et al. 2022). Also, the opposite effect was reported in common carp, at 5mg/L of glyphosate: hatch was retarded (Fiorino et al. 2018). Delays in development and hatching have also observed in zebrafish but only when the concentration of glyphosate was above 100 mg/L (Uren Webster et al. 2014).

Only one study on Java medaka (Yusof et al. 2014) and one on Japanese medaka (Smith et al. 2019) reported a decrease in hatching rate resulting from a ELS exposure to glyphosate. Hatching success in Java medaka was decreased with increasing concentrations when exposed to 100, 200, 300, 400 and 500 mg/L of glyphosate (Yusof et al. 2014). For Japanese medaka, decreased

hatching success was observed at 0.5 mg/L, the only dose assessed (Smith et al. 2019). Glyphosate seems to have species dependent effects on hatching time and success but more research is required to consolidate this conclusion.

Morphological

Many studies report morphological alterations in ELS fish raised in glyphosate-spiked water. Numerous deformities have been reported at different concentrations of glyphosate in ELS zebrafish. For example, ocular distance was decreased at 0.5 mg/L (Bridi et al. 2017), pericardial and yolk sac edema, swim bladder deficiency and shortened body length were reported at 7 mg/L, craniofacial (Diaz-Martin et al. 2021) and heart malformation (Roy et al. 2016) was seen at 50 mg/L. Spinal curvature was also observed in adult zebrafish which were exposed during ELS to 10 and 50 mg/L (Diaz-Martin et al. 2021). In zebrafish, exposure to 0.017, 0.17 and 1.7 mg/L had no effect on the frequency of deformities, but increased incidence of spinal curvature and increased severity of edema were reported for all doses (Ivantsova et al. 2022). Glyphosate appears to be teratogenic in ELS zebrafish but different deformities, with very little overlap between studies, were reported. Also, in one study no effect on deformities was reported for both zebrafish and common carp exposed to glyphosate concentrations up to 50 mg/L (Fiorino et al. 2018).

There is some evidence in the literature that rainbow trout is more sensitive to glyphosate than zebrafish as deformities were observed at lower dosages. For example, reduction in head size was reported by 2 different studies at concentrations of 1 µg/L (Du-Carrée et al. 2021) and 1 mg/L (Santos et al. 2019). But in both studies, no other deformity other than reduced head size was noted (Santos et al. 2019, Du-Carrée et al. 2021). It is worth mentioning that this endpoint was not measured in most of the previously cited studies. Various deformities were also observed in Japanese medaka exposed to 0.5 mg/L of glyphosate (Smith et al. 2019) and in Java medaka exposed to 100 mg/L and deformity rates increased with increasing concentrations 200, 300, 400 and 500 mg/L (Yusof et al. 2014).

Behavior

Only a few studies have assessed the effects of ELS exposure to glyphosate on behavior. The first one, using zebrafish, which were exposed to 0.01, 0.065 and 0.5 mg/L of glyphosate, reported

altered locomotion and aversive behavior only in the highest dose (Bridi et al. 2017). The second one, also using zebrafish, reported increased activity (hyperactivity) in zebrafish larvae exposed to 0.017 and 1.7 mg/L of glyphosate but not in the 0.17 mg/L exposed group. They also reported no change in anxiety-like behavior for all doses (Ivantsova et al. 2022). The last one assessed rainbow trout larval motility in 0.1 and 1 mg/L glyphosate solution, and motility was increased only in the 0.1 mg/L group. In the wild, increased and/or altered larval movement could lead to increased predation (Bridi et al. 2017, Santos et al. 2019). Current literature suggests that concentrations of glyphosate in the low mg/L range seem to have an effect on larval fish behavior but more studies assessing this endpoint are required.

Biochemical

Glyphosate has been associated with cardiac teratogenesis and has effects on ELS heart rate (Roy et al. 2016). Embryo exposure to 50 mg/L of glyphosate caused structural abnormalities in the heart atrium and ventricles, irregular heart looping of the developing heart, wrong organ positioning such as *situs inversus* and decreased heart rate (Roy et al. 2016). Reduction of cardiac rate was also observed in zebrafish exposed to 5, 10 and 50 mg/L of glyphosate but no change was reported in the 1mg/L exposed group. Interestingly, increased heart rate was also often reported. For example, in one study, elevated heart rate was observed in zebrafish exposed to 7 and 35 mg/L of glyphosate, with no change was observed in the 0.7 mg/L exposed fish (Liu et al. 2022). Increased heart rate was also reported at 17 mg/L of glyphosate in another study with zebrafish (Schweizer et al. 2019) and in Java medaka exposed to 100, 200, 300, 400 and 500 mg/L of glyphosate (Yusof et al. 2014). It was suggested that the increased heart rate was proof that ELS exposure to glyphosate was causing stress (Yusof et al. 2014). Even though different levels and direction of effects were reported, glyphosate seems to affect ELS heart development and heart rate.

Glyphosate exposure is also associated with oxidative stress in ELS fish. In a 120-hour ELS zebrafish exposure to 0.7, 7 and 35 mg/L of glyphosate, increased presence of reactive oxygen species and altered antioxidant defense, such as superoxide dismutase and catalase activities, were reported in the 7 and 35 mg/L exposed fish and endoplasmic reticulum stress was reported in all dosage groups. Decreases in antioxidant systems, including inhibition of catalase, glutathione

transferase and reductase and increased lipoperoxidation, were also observed in ELS silver catfish (Rhamdia quelen) exposed to 6.5 mg/L of glyphosate (Maylin Sobjak et al. 2017). Increased levels of thiobarbituric acid reactive substances, which are by-products of lipid peroxidation, were also observed in rainbow trout exposed to 1mg/L of glyphosate but not in the 0.1 mg/L exposed fish (Santos et al. 2019). Studies demonstrate that concentrations of glyphosate in the mg/L range can cause oxidative stress in ELS fish.

Glyphosate also affected a variety of other toxicity endpoints. For example, changes in spontaneous tail movement patterns were observed in zebrafish in the 50 mg/L glyphosate exposure group but not in the 5 and 10 mg/L (Diaz-Martin et al. 2021). Glyphosate also had a neurotoxic effect (inhibition of cholinesterase activity) in silver catfish (Rhamdia quelen) exposed to 6.5 mg/L glyphosate (Maylin Sobjak et al. 2017) and genotoxic effect (DNA strand breaks) in zebrafish exposed to glyphosate concentrations ranging from 1.7 to 100 mg/L (Rodrigues et al. 2019). Sex related gene expression was dysregulated in testes of adult Japanese medaka exposed for 15 days during ELS to 0.5 mg/L of glyphosate, but no change was observed in females (Smith et al. 2019).

Dysregulation of cat and cox1 genes expression was reported in rainbow trout exposed to 1 mg/L of glyphosate but not to 0.1 mg/L (Santos et al. 2019). Increased expression of Cox1 and Hadh was also reported in zebrafish exposed to 0.017, 0.17 and 1.7 mg/L of glyphosate (Ivantsova et al. 2022). Immunological effects such as inflammatory response and apoptosis were reported in zebrafish larvae exposed to 35 mg/L of glyphosate but not in those exposed to 0.7 or 7 mg/L (Liu et al. 2022). Overall, glyphosate toxicity can be expressed over numerous non-lethal endpoints.

No effect

Negative effects are less often published in literature, but some published studies indicate that even high concentrations of glyphosate do not always cause overt effects. For example, no effects on survival, heart rate, morphometrics or behavior were reported in the Australian Murray cod and Murray river rainbow fish at concentrations of glyphosate up to 10 mg/L (Raymond et al. 2006). Another study also found no effects on survival, deformities, morphometrics, behavior, oxidative stress markers, acetylcholine esterase activity and energy metabolism in ELS rainbow trout exposed to concentrations of glyphosate up to 2 µg/L (Du-Carrée et al. 2021). This concentration

is more environmentally relevant, but much lower than in most previous studies that did demonstrated effects.

Overall, global environmental concentrations of glyphosate alone should have minimal, if any, effects on ELS fish. As with other pesticides, glyphosate can be found in a number of different formulation in which other formulants may be toxic themselves or contribute to the toxicity of glyphosate.

2.2.3 AMPA

Aminomethlylphosphonic acid (AMPA) is the highest occurrence glyphosate by-product in surface water (Landry et al. 2005; Al-Rajab et al. 2008) and has greater persistence and environmental motility than its parent compound (Kolpin et al. 2006). Very little information is available regarding its potential toxicity to ELS fish. Based on survival, growth and development of fathead minnow (*Pimephales promelas*) the no-observed-adverse-effect concentration was set at 12 mg/L AMPA, the highest concentration tested (Levine et al. 2015). No acute toxicity was observed in zebrafish embryos exposed to concentrations ranging from 1.7 to 100 mg/L but just as with glyphosate, AMPA was genotoxic (DNA strand breaks) starting at 1.7 mg/L in a larval comet assay (Rodrigues et al. 2019).

A recent exposure study compared ELS zebrafish exposure between 0.017, 0.17 and 1.7 mg/L glyphosate and 0.019, 0.19 and 1.9 mg/L AMPA. Neither compound had an effect on survival, hatching success or frequency of deformities. But for both compounds, the deformed fish exhibited increased spinal curvature and more severe edema than in control for all doses. For both compounds, no induction of reactive oxygen species was noted but glyphosate increased superoxide dismutase 2 mRNA while AMPA increased catalase and superoxide dismutase mRNA in a concentration-specific manner. Contrary to glyphosate, AMPA was not associated with hyperactivity or anxiety-like behavior in zebrafish larvae (Ivantsova et al 2022).

Overall, AMPA does not appear to be more toxic than glyphosate to ELS fish, and as with its parent compound glyphosate, environmental concentrations of this compound alone are unlikely to cause an effect in ELS of wild fish.

2.2.4 Metolachlor

Metolachlor is a selective pre-emergent herbicide from the chloracetanilides family, that targets grass weeds. Metolachlor works by inhibiting the biosynthesis of very-long-chain fatty acids and interferes with gibberellin synthesis enzymes (Götz & Böger 2004, Heydens et al. 2010; Rose et al. 2016,). The racemic mixture of metolachlor was replaced by the single use of metoachlor S-enantiomer which has superior herbicidal properties (Muller et al. 2001; Poiger et al 2002). S-Metolachlor is one of the most commonly used herbicides (Atwood & Paisley-Jones, 2017) and agricultural runoff can easily contaminate surface waters (Zemolin et al. 2014). Metolachlor concentrations in the environment are often reported in µg/L range (Battaglin et al. 2000, Vryzas et al. 2011, Quintaneiro et al, 2017, Giroux 2022) but concentrations up to mg/L level have also been reported (Tapie & Budzinski 2018, Rozmankova et al. 2020). Previous studies in ELS zebrafish, have reported effects of exposure to metolachlor on survival rate, hatching success, deformity rate, behavior and physiochemical-biochemical parameters (Quintaneiro et al. 2017, Rozmankova et al. 2020; Yang et al. 2021a; Yi et al. 2022).

Survival

The effects of metolachlor on survival were only reported at concentrations above what is measured in the environment. For example, decrease of survival was only observed in ELS fish exposed to 358 mg/L of metolachlor, no effects on survival were reported for all lower concentrations tested (≤184 mg/L) (Yang et al. 2021a). Other studies established 96-hour exposure metolachlor LC50 to 19.11 (Yi et al. 2022) and 46.21 mg/L (Quintaneiro et al. 2017). No effect on survival was observed in zebrafish exposed to 1, 30, 100 and 300 µg/L of metolachlor (Rozmankova et al. 2020). Overall, at environmental concentrations, metolachlor does not have an effect on zebrafish survival.

Hatching

The effects of metolachlor on hatching were also only reported at concentrations above what is measured in the environment. In Quintaneiro et al. (2017), metolachlor lowest-observed-effect concentration for deformities was 29 mg/L. In the same study, metolachlor concentration of 70 mg/L completely inhibited hatching (Quintaneiro et al. 2017). Similar results were also observed

in another study which noted a 40-50% and 90% decrease in hatch rate of ELS zebrafish exposed to 14.20 and 358 mg/L of metolachlor respectively (Yang et al. 2021a). In another example, hatching rate was inhibited in the 3 highest concentrations tested in zebrafish exposed to 8, 12, 18, 27 and 40.5 mg/L of metolachlor (Yi et al. 2022). No effect on hatching success was observed in zebrafish exposed to environmentally relevant and higher concentrations (1, 30, 100 and 300 µg/L) of metolachlor (Rozmankova et al. 2020). Overall, at environmental concentrations, metolachlor does not have an effect on zebrafish hatching success, but concentrations one or two order of magnitude higher can decrease hatch rate.

Morphological

Exposure to metolachlor during ELS fish could lead to increased rate of deformities. In zebrafish exposed to 1, 30, 100 and 300 µg/L of metolachlor, adverse effects on the development of embryos were reported at the 2 highest doses (Rozmankova et al. 2020). For example, non-inflation of the swim bladder and malabsorption of the yolk sac were observed in the 100 and 300 µg/L exposed fish respectively (Rozmankova et al. 2020). In another study, increase in deformity rate was observed at 142 mg/L and concentrations above 184 mg/L resulted in 100% deformities. Reduced size of the swim bladder was also reported in fish exposed to 14.20 mg/L (Yang et al. 2021a). In zebrafish exposed to 8, 12, 18, 27 and 40.5 mg/L of metolachlor, increased incidence of spinal curvature was reported in the 8 and 18 mg/L while decrease in body length was observed in the 40.5 mg/L exposed fish (Yi et al. 2022). Quintaneiro et al. (2017) reported 29.4 mg/L as half-maximal-effective concentration for deformity and deformation rate of 100% was reported at 70 mg/L. The main deformities observed were pericardial and abdominal edema, tail deformation and again impairment of yolk sac absorption (Quintaneiro et al. 2017). The main deformities reported in the studies following exposure to metolachlor in zebrafish were reduced swim bladder and yolk sac malabsorption. Some effects on fish ELS deformity could be expected at environmental and higher concentrations.

Behavior

Few studies assessed and reported effects of metolachlor exposure in ELS zebrafish. No effect on swimming behavior was reported in zebrafish exposed to 1, 30, 100 and 300 µg/L of metolachlor (Rozmankova et al. 2020). Reduced frequency of embryo tail movement was observed but only in

the 1 µg/L exposed fish (Rozmankova et al. 2020). In another study, no anxiolytic effect (light/dark preference) was reported in zebrafish exposed to 141, 283, 709.45 µg/L and 1.42, 2.84, 7.10 and 14.20 mg/L of metolachlor but altered levels of activity were noted in the 2 highest doses (Yang et al. 2021a). So far there is no evidence that environmental concentrations of metolachlor have a meaningful effect on ELS zebrafish but more studies on this endpoint are required.

Biochemical

Other endpoints assessed in zebrafish exposed to metolachlor include heart rate, gene expression and oxidative stress markers. Zebrafish exposure to concentrations up to 300 µg/L had no effect on ELS zebrafish heart rate (Rozmankova et al. 2020), but the highest concentration tested (40.5 mg/L) in Yi et al. (2022) caused a decrease in heart rate. In concentrations up to 300 µg/, no effect on the expression of genes involved in thyroid system regulation gene (dio2, thra, thrb) or xenobiotic metabolism (Cyp26a1) was reported (Rozmankova et al. 2020). Alteration of expression of genes related to swim bladder formation and inflation (acta2, anxa5, pbxa1) was reported in zebrafish exposed to 7.10 and 14.20 mg/L (Yang et al. 2021a). In ELS zebrafish exposed to 0.5, 1.33, 3.54, 9.40, 25 mg/L of metolachlor, there was no effect on catalase activity at all concentration, cholinestera activity only decreased in the highest dose and gluthathione S-transferase was increased in a dose-response manner in the three highest doses. Lipid peroxidation was lesser in all exposed fish and lactate dehydrogenase activity was decreased only in the 3.54 and 9.40 mg/L groups (Quintaneiro et al. 2017). Overall, at environmental concentrations, metolachlor seems to have minimal effects on ELS fish. However, so far only effects on zebrafish were studied, more studies on different species are required to better assess the risk of metolachlor on wild fish.

2.2.5 Diquat

Diquat is a non-selective herbicide that inhibits plant photosynthesis. Diquat exhibits rapid dissipation in water (Ritter et al. 2009) and very little information is available on environmental concentrations. There is very little information on toxicity of diquat for fish and only one study assessed the effect of diquat (pure form) on ELS fish (Wang et al. 2018). In that study, ELS zebrafish exposure to 0.34, 3.4, 34 µg/L of diquat had no effect on survival, hatching, deformity rate and no indication of stress (Wang et al. 2018). However, diquat disrupted the mitochondrial

bioenergetics and behavior. For example, decreased oxidative phosphorylation, respiration and adenosine triphosphate production was observed in the highest dose assessed (34 µg/L). Also, starting at 3.4 µg/L, catalase activity was increased but no other change in oxidative stress or apoptosis markers was observed. Exposure to 3.4 and 34 µg/L of diquat also caused increased activity of the zebrafish larvae (Wang et al. 2018). Overall, diquat can induce molecular changes in ELS zebrafish, but more research is needed to better understand the potential effects of diquat exposure to ELS fish.

2.2.6 Imazethapyr

Imazethapyr is a broad spectrum pre- and post-emergent herbicide of the imidazolinones family (Battaglin et al. 2000). Imazethapyr works by suppressing the synthesis of the essential amino acids isoleucine, leucine and valine by inhibiting the enzyme acetolactate synthase (Qian et al. 2009). Imazethapyr has a high selectivity to target organisms (Battaglin et al. 2000) and is often used in combination with glyphosate to increase effectiveness and eliminate weed resistance (Costa et al. 2022). Only one study focused on the effects of imazethapyr on ELS fish but the study used a formulation rather than the active molecule (Costa et al. 2022), and therefore will not be discussed here. Imazethapyr is often detected in the environment (Giroux 2022) and more studies are needed to evaluate its potential toxicity to ELS fish.

2.3 Insecticides

Insecticides are used to control insect pests on crops and are designed to cause toxicity in organisms. Previous generations of synthetic insecticides targeted biochemical pathways that were often conserved amongst taxa, which means that they were also highly toxic to non-target organisms (Fulton et al. 2013). Newer generations of insecticides target insect-specific pathways, such as nicotinic acetylcholine receptors. In general, insecticide toxicity to fish is greater than herbicide, but they generally degrade quicker in aquatic environment (Fulton et al. 2013).

2.3.1 Permethrin

Permethrin is a previous generation synthetic pyrethroid used as an insect repellent and insecticide and works by disrupting sodium channels, leading to insect paralysis and death. It exhibits short persistence and low mammal toxicity but can be highly toxic to aquatic organisms (Coats et al.

1989) with fish LC50 often measured below 10 µg/L (Bradbury & Coats 1989). Environmental surface water concentrations of permethrin are often measured at 1 µg/L, but concentrations in sediment, to which it binds easily, can reach up to 20 µg/L (NORMAN EMPODAT database 2020). Over the years, many studies have focused on the effects of permethrin on ELS fish.

Survival

Many studies have assessed the lethality of permethrin on ELS fish and there is variability in species sensitivity. For example, the LC50 for ELS African mud catfish (*Clarias gariepinus*) was 80 µg/L (Zabbey et al. 2014) but no effects on mortality were reported in Japanese medaka or zebrafish at concentrations below 300 µg/L (Gonzalez-Doncel et al. 2003; Yang et al. 2014). Decreased survival was also reported in fathead minnows and sheepshead minnows exposed during ELS to 1.40 and 22 µg/L respectively. In zebrafish, decrease in survival was only reported at concentrations above 200 (Zhang et al. 2017) or 300 µg/L (Yang et al. 2014). Zebrafish survival past 300 µg/L decreased in a dose-response manner with increasing concentrations, up to 800 µg/L, the highest dose tested (Yang et al. 2014). Overall, lethality of permethrin varies from low µg/L level to hundreds of µg/L level, but in most cases lethal values are well above environmental values.

Hatching

No study has found an effect of permethrin exposure on fish hatching success or time. For example, in fathead minnows (Spehar et al. 1983) and sheepshead minnows (Hansen et al. 1983), no effect on hatching was reported at concentrations up to 0.66 and 43 µg/L respectively. The same was also true in zebrafish (Zhang et al. 2017) and Japanese medaka (Gonzalez-Doncel et al. 2003) in concentrations of permethrin up to 200 and 300 µg/L respectively. Overall permethrin does not seem to have any effect on hatching in fish.

Morphological

Permethrin can alter morphology and be teratogenic to ELS fish. No effect on length was reported in zebrafish (Tu et al. 2016) and sheepshead minnows (Hansen et al. 1983) exposed to concentrations of permethrin up to 4.30 µg/L and 42 µg/L respectively, but weight was reduced in zebrafish exposed to 4.30 µg/L (Tu et al. 2016). In Japanese medaka exposed to 25, 50, 100, 200,

300 µg/L of permethrin, increases in deformities such as non-inflation of the swim bladder, myoskeletal defects and enlargement of the gall bladder, were reported at all doses starting at 50 µg/L (Gonzalez-Doncel et al. 2003). In zebrafish exposed to permethrin, vascular malformation was observed at 196 µg/L and a dose-dependent increase of pericardial edema was observed in the two highest doses tested, 196 and 293 µg/L (Xu et al. 2018). In zebrafish, exposure to 100, 200, 300, 400, 600, 800 µg/L caused a dose response increase of spinal curvature, pericardial edema and non-inflation of the swim bladder starting at 300 µg/L (Yang et al. 2014). Overall, exposure during ELS fish often leads to an increase in deformity rate but at concentrations above levels found in the environment.

Behavior

Exposure to permethrin during ELS fish can influence activity levels. For example, exposure of African mud catfish to 0.5, 0.1, 0.15, 0.2, 0.25 mg/L of permethrin caused dose-dependent increased activity across all treatments (Zabbey et al. 2014). However, the opposite reaction was observed in Japanese medaka and zebrafish. Decreased hatchling activity and uncoordinated movements were reported in Japanese medaka exposed to concentrations of permethrin ranging from 50-300 µg/L (Gonzalez-Doncel et al. 2003). No change in zebrafish larvae was observed in concentrations up to 10 µg/L (Blanc et al. 2020) and 293 µg/L (Xu et al. 2018), but adults exposed to 1 and 10 µg/L during ELS exhibited decreased activity as adults (Blanc et al. 2021). Overall, permethrin can affect fish activity levels. The larval stage seems to only be affected by concentrations above environmental relevance, but persistent effects may occur at concentrations as low as 1 µg/L (Blanc et al. 2021).

Biochemical

Fish exposure to permethrin during ELS can cause alterations in neurodevelopment (Blanc et al. 2020, Blanc et al. 2021), as well as thyroid (Tu et al. 2016) and endocrine disruption (Zhang et al. 2017, Xu et al. 2018). Exposure of zebrafish during ELS to 1 and 10 µg/L was partially correlated to alterations of lysophosphatidylcholine levels, an important lipid for neurodevelopment (Blanc et al. 2020), glutamatergic synapse activity and brain methylated region (Blanc et al. 2021). This caused a neurodegenerative-like transgenerational reduction of larval activity in the two subsequent generations but not in the exposed one and normal activity levels were resumed in the

third generation (Blanc et al. 2020). Increased levels of thyroid hormones were also reported in zebrafish embryos exposed to 4.30 µg/L (highest test concentration) (Tu et al. 2016). Exposure of zebrafish to between 1 and 293 µg/L of permethrin also caused alterations of gene expression related to the hypothalamic-pituitary-adrenocortical axes, the hypothalamic-pituitary-thyroid, the hypothalamic-pituitary-adrenocortical axes and oxidative stress-related system in all tested doses (Zhang et al. 2017, Xu et al. 2018). Overall, permethrin caused neurotoxicity, thyroid, and endocrine disruption of ELS fish. Most of these effects were observed or made worse at concentrations above those measured in the environment. More studies are required to better understand the effects of permethrin exposure in ELS fish and differences in species sensitivity.

2.4 Neonicotinoid insecticides

Neonicotinoids are new generation broad-spectrum insecticides that block nicotinic acetylcholine receptors and disrupt ion transfer y-aminobutyric acid-regulated chloride channels. Neonicotinoids are highly selective for arthropod receptors over vertebrate's (Tomizawa & Casida 2005, Hladik et al. 2018). By the end of the 2000s, the three neonicotinoid nitroguanidine insecticides Imidacloprid, Thiamethoxam and Clothianidin dominated neonicotinoid sales (Jeschke et al. 2011) and accounted for 26% of global insecticide sales (Jeschke et al. 2011; Simon-Delso et al. 2014, Craddock et al. 2019). Neonicotinoid concentrations in the environment range from ng to µg/L range (Moschet et al. 2014; Anderson et al. 2015; Morrissey et al. 2015).

2.4.1 Imidacloprid

Survival & Hatching rate

Imidacloprid is one of the most used neonicotinoids (Jeschke et al. 2011; Craddock et al. 2019) and it can have effects on fish ELS survival, hatching, morphology and physiochemical-biochemical parameters. The ELS Common carp seem to be more sensitive than zebrafish to imidacloprid. For example, no effect was observed on survival and hatching in Japanese medaka or zebrafish over imidacloprid concentrations ranging from 0.2 µg/L to 2000 µg/L (Vignet et al. 2019) and 0.2 µg/L to 181.47 mg/L respectively (Scheil & Köhler 2009; Tisler et al. 2009; Crosby et al. 2015; Vignet et al. 2019; Könemann et al. 2022). In ELS common carp exposed to 7.8, 15.6, 23.4, 31.2 mg/L of imidacloprid, survival and hatching success decreased in a dose-response

manner. Survival decreased from 93.33% in control down to 61.33% in the highest dose. Early hatching was also recorded in the 31.2 mg/L exposed fish (Tyor & Harkrishan 2016). For lesser doses of imidacloprid (0, 10, 30, 300, 1000 µg/L), the same kind of dose-response relationship was observed in common carp hatching rate (Islam et al. 2019). The LC50 values reported for common carp by Islam et al. (2019) are 94, 22, 2 and 1 mg/L for 24, 48, 72 and 96 hours respectively, which are much lower than the doses used by Tyor & Harkrishan (2016) suggesting a difference in sensitivity across common carps. In zebrafish, LC50s for embryos and larvae were 121.6 mg/L and 128.9 mg/L respectively (Wu et al. 2018). These results suggest a strong difference in ELS fish species sensitivity to imidacloprid, with zebrafish unlikely to be affected by environmental concentrations and common carp more likely to be.

Morphological

Studies on the effects of aqueous exposure of ELS zebrafish to imidacloprid do not often report an effect on deformity rate except at very high concentrations but increases in deformity rates have been observed in Japanese medaka and common carp at environmentally relevant doses. Multiple studies reported no increased incidence of deformities in zebrafish exposed to concentrations of imidacloprid ranging from 1 to 181 mg/L (Scheil & Köhler 2009; Wu et al. 2018; Könemann et al. 2022). According to Tisler et al. 2009, the imidacloprid concentration at which 10% of zebrafish exhibited non-lethal and lethal deformities were 222 and 300 mg/L respectively. In common carp exposed to 0, 10, 30, 300, 1000 µg/L, increased deformity rate was observed only in the 300 and 1000 µg/L exposed fish (Islam et al 2019). In Vignet et al. 2019, both zebrafish and Japanese medaka were exposed to imidacloprid concentrations ranging from 0.2 to 2000 µg/L. Decreased growth and increased deformity rates were only observed in Japanese medaka with about 67% of individuals exhibiting deformities at 0.2 µg/L imidacloprid and > 80% at concentrations of 2 µg/L and higher. Spinal curvature, hemorrhage, and cranio-facial deformities appeared in a dose-response relationship in concentrations of 0.2 µg/L and above, yolk and bone edema and tail deformities at concentrations of 20 µg/L and over. Still only in Japanese medaka, disorganization of the retinal pigment epithelium was observed in all exposure concentrations and altered muscle (myomeric) structure in concentrations of 2 µg/L and over. In zebrafish, only a thickening of the muscle fibers was observed in the highest concentration (2000 µg/L) of imidacloprid assessed

(Vignet et al. 2019). Overall, environmental concentrations of imidacloprid increased the rate of deformity in Japanese medaka and common carp but not in zebrafish.

Behavior

Only two studies have investigated the behavioral effects of exposure to imidacloprid in ELS fish. In the first one, no effect on behavior was observed in larval zebrafish and Japanese medaka exposed to imidacloprid concentrations ranging from 0.2 to 2000 µg/L. In the second, ELS zebrafish were exposed to 11.5 and 15.3 mg/L of imidacloprid during ELS and a concentration-dependant decrease in larval swimming activity was observed in both doses (Crosby et al. 2015). Later life effects on behavior were also reported in the ELS exposed fish, such as decreased exploration and increased startle response (Crosby et al. 2015). More studies are required to understand the potential effects of imidacloprid on ELS fish behavior, and other more sensitive species should also be assessed.

Biochemical

Two studies have evaluated the effects of imidacloprid on biochemical parameters of ELS fish and both reported effects. Enzyme activity and gene expression were evaluated in ELS zebrafish exposed to 0.38, 1.52 or 6.08 mg/L of imidacloprid. All doses caused some level of alteration of enzyme activity related to detoxification, antioxidant and caspase apoptosis pathways and of gene expression related to oxidative stress, apoptosis, immune functions, and hypothalamic-pituitary-thyroid axis (Wu et al. 2018). Vignet et al. 2019, also reported metabolite alterations linked to energy metabolism, and cholinergic and adrenergic neurotransmission in Japanese medaka, but not in zebrafish, exposed during ELS to concentrations of imidacloprid ranging from 0.2 to 2000 µg/L. They also found that the chorion was a good barrier for imidacloprid uptake (Vignet et al. 2019), which suggests that larvae may be more sensitive than embryos. These results demonstrate that there can be some alterations in biochemical parameters at environmental concentrations of imidacloprid on the more sensitive fish species.

2.4.2 Thiamethoxam

Survival

By the end of the 2000s, thiamethoxam was the world second most used neonicotinoid insecticide (Jeschke et al. 2011) and many studies report toxicity to ELS fish. Lethal concentrations of Thiamethoxam appear to be in the mg/L range for ELS fish. For example, rare minnow (*Gobiocypris rarus*) larvae LC50 was 386 mg/L and 202 mg/L for 48- and 96-hour exposure respectively (Wang et al. 2020). For zebrafish, the LC50 was 246 mg/L for embryos and 194 mg/L for larvae. (Shen et al. 2021). Larvae of the rare minnow were also more sensitive than embryos (Yang et al. 2021b). The opposite was true for fathead minnows where exposure to 1.57 µg/L of thiamethoxam starting post-hatch had no effect while embryonic exposure to the same dose caused mortality (Victoria et al. 2022a). But other studies have seen effects on mortality in lower doses such as in the µg/L range. For example, ELS exposure of zebrafish to 0.016, 0.21, 1.29, 12.69 and 163 µg/L reduced survival by about 20% in the highest dose (Victoria et al. 2022b). In banded gourami, exposure to 0.02, 0.2, 2, 20, 200 mg/L concentrations of thiamethoxam caused an increase in mortality in both embryos and larvae at all doses following a dose-response relationship (Hasan et al. 2022). A similar relationship was observed in fathead minnows exposed to 0.02, 0.16, 1.57, 14.61 and 155 µg/L thiamethoxam, where mortality started at 1.57 µg/L and increased with increasing doses (Victoria et al. 2022a). But in ELS rainbow trout, no effects on survival were observed in all doses tested, which means concentrations up to 20 mg/L (Finnegan et al. 2017). At environmental concentrations, thiamethoxam could affect ELS fish survival but sensitivity seems to be different across species.

Hatching

Some studies report alterations in hatching success of fish embryos raised in thiamethoxam-spiked solutions. For example, hatching success decreased in a dose-dependence effects in banded gourami exposed to 0.02, 0.2, 2, 20, 200 mg/L of thiamethoxam. Hatching of zebrafish exposed to 0.016, 0.21, 1.29, 12.69 and 163 µg/L was impaired but the response was non-monotonic . Hatching was reduced by 75% in the embryos exposed to 0.21 µg/L of thiamethoxam but only 7% less in the 163 µg/L, with no effect in between (Victoria et al. 2022b). This may have been associated with a husbandry problem since no effect on hatching was reported in zebrafish exposed to concentrations up to 12.3 mg/L (Shen et al. 2021). No effect on hatching was reported in fathead minnow and rainbow trout for all doses tested with the highest being 155 µg/L (Victoria et al. 2022a) and 20 mg/L (Finnegan et al. 2017) respectively. Overall, some species seem to be sensitive

to thiamethoxam concentration 1 to 2 order of magnitude lesser than others, and effects on hatching also varied across species.

Morphological

Only a couple of studies have reported effects on morphological parameters of ELS exposure to thiamethoxam. Again, the banded gourami seems to be the most sensitive species to thiamethoxam. Increase in deformity rate was reported for all tested concentrations (0.02, 0.2, 2, 20, 200 mg/L) of thiamethoxam, following a dose-dependent relationship. Yolk sac edema was the most commonly reported deformity (Hasan et al. 2022). Decreased eye size was reported in zebrafish following exposure to 0.21 µg/L, but this is also the only the treatment (and not highest concentration) that exhibited a large (75%) decrease in hatching success (Victoria et al. 2022b). Other studies reported no effect on morphological deformities in rainbow trout (Finnegan et al. 2017), zebrafish (Shen et al. 2021) and fat head minnow (Victoria et al. 2022a). Thiamethoxam seems to induce mortality only in the most sensitive species.

Behavior

Only a couple of studies have assessed the effects of ELS exposure to thiamethoxam on behavior. The first one, focusing on zebrafish, found that exposure to 0.21, 12.69 and 163 µg/L of thiamethoxam altered predator escape response but no effect was observed in the 0.016 and 1.29 µg/L exposed fish (Victoria et al. 2022b). The second one, using fat head minnows showed that ELS exposure to concentrations of thiamethoxam ranging from 0.02 to 14.61 µg/L impaired larval foraging behavior and that exposure to 155 µg/L impaired predator escape response (Victoria et al. 2022a).

Other

Only one study, which used multiple endpoints, assessed molecular changes in ELS zebrafish following thiamethoxam exposure. Zebrafish were exposed to 0.77, 3 and 12.3 mg/L of thiamethoxam during ELS. Antioxidant related markers were increased only in the high dose group, no change in catalase was reported in any of the doses and total superoxide dismutase increased in a dose-dependent manner in all treatments. Peroxidase activity was decreased in the low and medium doses but increased in the high dose. Cytochrome P450 was inhibited, and

caspase 3 activity decreased in the low dose only while glutathione S-transferase was increased in the low and medium doses. Increased expression of apoptosis, antioxidation and immunity-related genes was observed in all three doses (Shen et al. 2021). Thiamethoxam exposure seems to cause oxidative stress and affect the immunological system of ELS fish.

Even though thiamethoxam has a low affinity with the vertebrate nicotinic acetylcholine receptors (Victoria et al. 2022b), it still poses a risk for ELS fish. However, more studies are required to really understand the effects of thiamethoxam on ELS fish.

2.4.3 Clothianidin

By the end of the 2000s, clothianidin was the world third most used neonicotinoid insecticide (Jeschke et al. 2011), which is the active degradation product of its precursor, thiamethoxam, and is also used and applied as an insecticide (Nauen et al. 2003). Only one study has focused on the effects of clothianidin on ELS fish. The ELS Sockeye salmon were exposed to 0.15, 1.5, 15 and 150 µg/L of clothianidin. The exposure had no effect on survival, hatching, growth nor deformities. An increase in 17B-estradiol levels in fry was observed in the fish exposed to the lowest dose (0.15 µg/L) and reduced liver glucocorticoid gene expression was reported in the fry exposed to the highest dose (150 µg/L) (Marlatt et al. 2019). More research is necessary to understand the potential effects of clothianidin exposure to ELS fish.

2.4.4 Chlorantraniliprole

Chlorantraniliprole is a new generation highly selective and persistent anthranilic diamide insecticide (Bentley et al. 2010; Rodrigues et al. 2015). Chlorantraniliprole works by upregulating the activation of insect's ryanodine receptor channels which depletes the internal calcium store impairing regulation of muscle contraction and leading to paralysis and death (Bentley et al. 2010). Anthranilic diamides have been suggested as a potential alternative to neonicotinoid pesticides (Schmidt-Jeffris & Nault 2016). Chlorantraniliprole exhibits excellent differential selectivity for insect ryanodine receptors over mammalian receptors. However little information is currently available for fish. Chlorantraniliprole has often been detected in surface water (Rodrigues et al. 2015, Giroux 2022, Sandstrom et al. 2022) and as of yet, no studies have focused on its effect of on ELS fish.

2.5. Fungicide

Agricultural fungicides are phytosanitary products used to protect crops from fungal diseases. Compared to herbicides and insecticides, much less information is available concerning the environmental fate of fungicides, but surface water concentrations have been measured at ng and µg/L level (Reilly et al. 2012).

2.5.1 Chlorothalonil

Chlorothalonil is a fungicide used to protect crops, grass turf, and tree surfaces from pathogens such as blight, leaf spots and mildew (Caux et al. 1996). It binds and depletes cellular glutathione and glyceraldehyde 3-phosphate dehydrogenase (glycolysis) (Caux et al. 1996). Environmental concentrations range from a few ng/L level (Caux et al. 1996) to hundreds of µg/L level (Van Scoy & Tjeerdema 2014). Only a few studies have focused on the effects of chlorothalonil on fish ELS.

Survival & hatching

Chlorothalonil concentrations in the µg/L range have been associated with a decrease of survival in 3 fish species and alterations in hatching time have been reported in sockeye salmon. Concentration as low as 5 µg/L decreased survival of ELS sockeye salmon by about 30%, but no effect was observed at 0.5 µg/L (Du Gas et al. 2017). In zebrafish however, exposure to 5, 10, 25 and 50 µg/L caused a decrease in survival of approximately 22% at the highest dose versus control (Zhang et al. 2016). In fat head minnow, the lowest-observed effect concentration for survival was 15 µg/L while LC50 was 22.6 µg/L Sherrard et al. 2003). Exposure of Japanese medaka to 0.06 µg/L had no effect on survival or hatching (Teather et al. 2015). Delayed hatch in ELS sockeye salmon exposed to 5 µg/L and early emergence in both tested concentrations (0.5 and 5 µg/L) were reported (Du Gas et al. 2017). It appears that concentrations in the µg/L range can be lethal and alter hatching of ELS fish.

Morphological & biochemical

Exposure to chlorothalonil during ELS fish seems to negatively impact fish growth. For example, exposure of fat head minnows to concentrations above 20 µg/L (20, 22.5, 25 and 30) caused a decrease in fry size (Sherrard et al. 2003). In sockeye salmon, reduced condition factors and

elevated levels of triglyceride in 0.5 and 5 µg/L chlorothalonil exposed fish were observed. (Du Gas et al. 2017).

Hypoactivity, skewed sex ratio, endocrine and energy metabolism disruption were reported in fish exposed during ELS to chlorothalonil. Exposure of Japanese medaka to 0.06 µg/L of chlorothalonil had no effect on foraging ability and size but reduced activity level in larvae and a skewed sex ratio toward female were reported (Teather et al. 2015). Chlorothalonil has been reported as a potential endocrine disruptor (Zhang et al. 2016) and may also alter glycolysis and amino acids metabolisms (Yang et al. 2021c) of ELS fish. Overall few studies, with limited endpoints have focused on the effects of chlorothalonil on ELS fish, but from the available results it is likely that this fungicide could have an effect on ELS of wild fish.

2.6 Summary

At environmental levels, most herbicides (AMPA, atrazine, glyphosate, metolachor) and the insecticide permethrin are unlikely to cause major effect to ELS fish. The compounds most likely to negatively impact fish at environmental levels were the three neonicotinoid insecticides (imidacloprid, thiamethoxam and clothianidin) and the fungicide chlorothalonil. For the other two herbicides (diquat and imazethapyr) and the insecticide chlorantraniliprole, there is still very limited information available on their potential toxicity to ELS fish. This is a major contrast to compounds such as atrazine and glyphosate which have been extensively studied. There is still much more to be done to fully understand the potential toxicity of these widely used compounds on wildlife.

Most of the species targeted in the studies included in this review were laboratory organisms, with zebrafish and Japanese medaka being the most common ones. When information on other species was available, it was clear that there were major discrepancies in species sensitivity. Yet, very few studies have assessed the effects of these chemicals on wild fish species. This is concerning since these pesticides are ubiquitous in the environment and it is of the utmost important to assess their potential toxicity on wild fish.

2.7 Knowledge gaps and significance

Even though an increasing number of studies have focused on ELS fish and pesticide exposure, we are still very far from understanding their effects on wild fish. In the environment, pesticides are always detected in mixtures and very few studies have assessed the effects of pesticide mixtures on ELS fish. My study design allows us to assess the effects of environmentally relevant pesticide mixtures on ELS fish. Pesticide concentrations vary across space and time. While mature fish can travel long distances, embryos and larvae are much less motile and are often restricted to areas surrounding the adults' spawning grounds. Fish, according to species, tend to have a very specific breeding period making contamination variable. Endangered species are probably the most impacted by contaminants and yet they are often the least studied. There is limited information available for endangered species with no economical or recreational values, which makes risk assessment harder. Here we will try to determine if the endangered status may be explained by greater species sensitivity to pesticides and how important pesticides are as stressors to ELS fish. Hence, in this project, the investigation over many species will be beneficial since species spawning in the same location may encounter different contamination gradients due to difference in spawning time. Many studies have focused on parental exposure but to examine solely the effects of potentially contaminated spawning grounds, we are isolating the effects of exposure during embryogenesis from exposure at other phases of the life cycle. Very few studies have reared fish to maturity to detect latent effects resulting from the initial exposure. Expanding on this, even less data are available for multigenerational effects. In this study, my goal is to specifically link molecular changes to organismal changes and to explore possible multigenerational effects resulting from ELS exposure to pesticides in fish. Our series of experiments will allow me to explore the effects resulting from ELS exposure to pesticides on all life stages of fish and to identify potential contaminants of concern.

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Preface to Chapter 3

From the literature review, it was clear that there are still many unknowns about pesticide toxicity to fish ELS. Most of the studies published have focused on laboratory fish species and limited information is available for wild species. There is very limited direct evidence of effects at environmental levels of pesticides on ELS fish and endangered species are likely the most affected.

For the next chapter, I had the opportunity to work with the copper redhorse (*Moxostoma hubbsi*), an endangered fish that spawns only in the Richelieu River. The main activity in the Richelieu watershed is agriculture, hence the hypothesis that pesticide contamination may have an impact on the recovery of the copper redhorse. To test this hypothesis, I have looked at the effects of the Richelieu River on the ELS of the threatened river redhorse (*Moxostoma carinatum*) and the endangered copper redhorse. Since the only spawning grounds of the copper redhorse are in the Richelieu, any effect on their ELS could have population level effects. We also assessed the effects of river water on river redhorse, which share the same genus and much of the copper redhorse biology and spawning grounds, but is currently faring better. This was the first step to understanding the effects of early life exposure to environmental pesticides in wild fish.

Chapter 3

3. Exposure to Contaminated River Water is Associated with Early Hatching and Dysregulation of Gene Expression in Early Life Stages of the Endangered Copper Redhorse (Moxostoma hubbsi)

3.1 Title page

Title of the manuscript

Exposure to contaminated river water is associated with early hatching and dysregulation of gene expression in early life stages of the endangered copper redhorse (*Moxostoma hubbsi*)

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Conflict of interest

The authors declare that there is no conflict of interest.

3.2 List of Figures

3.3 List of Tables

3.4 Abstract

The copper redhorse (*Moxostoma hubbsi*) is an endangered fish that spawns exclusively in the Richelieu River (Quebec, Canada). Tributaries of the Richelieu are contaminated with high levels of current-use pesticides, which may impact early–life stage (ELS) copper redhorse and other native fishes. We assessed the effects of exposure to contaminated river water on ELS copper redhorse and river redhorse (*Moxostoma carinatum*), a related fish that shares the copper redhorse's spawning grounds and nursery habitat. A riverside flow-through system was used to expose copper and river redhorse embryos (1000 each) to Richelieu River water or laboratory water as a control. Fish were maintained until 14 days post-hatch, and water samples were taken daily for chemical analysis. Following a heavy rain event, concentrations of two neonicotinoid pesticides, clothianidin and thiamethoxam, exceeded water quality guidelines for aquatic life (20 ng/L). Using nontargeted screening, we tentatively identified an additional 24 pharmaceutical and personal care products and 23 pesticides in river water. Effects of river water on ELS fish were observed in both species, but the copper redhorse appeared to be more sensitive. Fish exposed to river water hatched 10.7 (copper redhorse) and 2.4 (river redhorse) cumulative degree days earlier than controls. Copper redhorse survival was significantly lower in river water (73 ± 16%) compared to laboratory water (93 ± 3%), whereas river redhorse survival was similar between treatments (84 ± 6% and 89 ± 4%, respectively). Sequencing of copper redhorse larvae RNA revealed 18 differentially expressed genes (DEGs) following 14 days of exposure to river water. Eight up-regulated DEGs were linked to immune function and injury response, and seven down-regulated DEGs were involved with digestion and nutrient absorption. The present study provided valuable data on the effects of ELS exposure to a real-world mixture of contaminants in two fish species of concern.

3.5 Introduction

Fishes worldwide spawn in rivers and streams that are polluted with complex mixtures of environmental contaminants including pesticides. In agricultural regions, pesticide concentrations in rivers and tributaries often peak in spring and early summer, a favored time for many fish species to spawn. Embryos and larvae developing under these conditions are at risk of being negatively impacted, with potential repercussions on fish population health and even conservation status. Contamination of spawning grounds may be of particular concern because early–life stage (ELS)

organisms are often more sensitive to contaminants than adults of the same species (Mohammed 2013).

Previous research has demonstrated that pesticides can be detrimental to ELS fish (Ullah et al. 2014). Laboratory studies have revealed effects of pesticides on a wide variety of endpoints including hatching success and timing (Du Gas et al. 2017; Fiorino et al. 2018), juvenile survival (Vignet et al. 2019), incidence of deformities (Sulukan et al. 2017), the immune system (Hong et al., 2018), and oxidative stress (Richterova et al. 2015). All of these studies focused on a single chemical, while natural waters can contain mixtures of thousands of chemicals which vary according to factors such as precipitation and season. Few studies have evaluated the effects of realistic environmental mixtures (Perez et al., 2013; Wang et al. 2017; Zhang et al. 2017), and even fewer have assessed effects of these mixtures on ELS fish (Bony et al. 2008; Marlatt et al. 2016).

Collectively this body of work suggests that negative impacts of pesticides on ELS fish can be observed at environmental concentrations. However, more studies are necessary to better understand the effects of real-world exposure to complex mixtures of pesticides in wild fish (Simonnet-Laprade et al. 2021).

The present study focuses on copper redhorse (*Moxostoma hubbsi*), an endangered species of fish that is endemic to Quebec and is only known to spawn at two sites in the Richelieu River, a waterway that runs from Lake Champlain (USA) to the St. Lawrence River (Canada). In response to declining numbers of copper redhorse, the Quebec government's Ministère des Forêts, de la Faune et des Parcs (MFFP) began an artificial breeding and monitoring program in 2004. Data from these efforts indicate that the copper redhorse population is shifting toward a higher weight distribution, which is a sign of population aging (Committee on the Status of Endangered Wildlife in Canada (COSEWIC) 2014). In fact, the number of 2- to 10-year-old subadults has been scarce over the past 30 years, but some capture of subadults and very young adults has occurred more frequently since 2016 (COSEWIC 2014; Vachon 2021a, 2021b). A closely related species that also spawns in the Richelieu River, the river redhorse (*Moxostoma carinatum*), shares the same habitat and spawning ground preferences as copper redhorse; but it is distributed more widely, with populations in Ontario and the United States (COSEWIC 2004). In contrast to the endangered status of the copper redhorse, the river redhorse is listed as a species of special concern (COSEWIC

2015). River redhorse spawn early to mid-June and copper redhorse, mid- to late June. Both species have unique and important ecological functions because they are some of the few freshwater fishes which feed extensively on mollusks (COSEWIC 2014, 2015).

The Richelieu watershed is extensively used for agricultural and urban land uses, and pesticides and other emerging contaminants have been frequently detected in the Richelieu River and its tributaries (Giroux 2015, 2019; Montiel-León et al. 2019). Corn and soy are the main crops cultivated in the Richelieu watershed (Comité de Concertation et de la Valorisation du Bassin de la Rivière Richelieu, 2015). Pesticides commonly used for these types of cultures (broad-spectrum herbicides and neonicotinoid insecticides) are among those with the highest detection levels and frequency in the Richelieu River tributaries which are fed by agricultural drainage (Giroux, 2015, 2019). Application of these pesticides often starts in May, and heavy rain in June mobilizes pesticides from fields to waters in the Richelieu region (Giroux 2019). Other compounds such as pharmaceuticals and personal care products (PPCPs), which lack established toxicity threshold values, have also been detected in surface water of the Richelieu River (Berryman et al. 2015; Giroux, 2015). The likely source of these contaminants is incomplete removal from municipal wastewater (Berryman et al. 2015) and overflowing of municipal wastewater-treatment plants into the river (Cliché & Saladzius 2018).

Recent water quality data demonstrated that the peak time for leaching from agricultural pesticide application coincides with the river and copper redhorse spawning period (COSEWIC 2014; Giroux 2015). Previous analyses indicated that concentrations of the chemicals atrazine, glyphosate, and neonicotinoids exceed the Canadian Council of Ministers of the Environment (2011) water quality guidelines for the protection of aquatic life in the Huron and Acadie rivers, two tributaries that join the Richelieu River near the spawning grounds. Although the presence of contaminants in these tributaries may have implications for copper redhorse spawning and early development stage, contaminants have not been measured specifically in the Richelieu River. A previous study focused on adult copper redhorse found that levels of legacy contaminants (such as polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs)) in a variety of tissues were below safety thresholds (De Lafontaine et al. 2002), but the potential impact of pesticides and other contaminants on ELS river and copper redhorses has yet to be studied.

Understanding the effects of pesticide mixtures is especially important for endangered species, which may be among the most affected but the least studied.

The reasons for the poor natural recruitment of copper redhorse are not well understood, but contamination of spawning grounds may be a contributing factor. To investigate this possibility, we conducted a riverside flow-through exposure, using Richelieu River water to assess the potential effects of pesticides and other contaminants on ELS copper redhorse. To broaden the scope of this research and to evaluate whether copper redhorse and the closely related river redhorse had differing contaminant sensitivities, we also exposed river redhorse in the same manner. Both redhorse species were exposed as newly fertilized embryos and maintained in the test system through hatching and the early larval stage. We used a combination of targeted and nontargeted chemical analyses to determine contaminant concentrations in river water. We assessed survival and sublethal endpoints, including timing of hatching, incidence of deformities for both redhorse species, and gene transcription in the hatched copper redhorse larvae using RNA sequencing. The data will improve our understanding of the potential impacts of exposure to environmentally relevant mixtures of contaminants in ELS fish and will contribute to a clearer picture of challenges to the recovery of the endangered copper redhorse.

3.6 Materials and Methods

Study site

Sixty-four municipalities border the Richelieu River, and 70% of its watershed is dedicated to agriculture (Simoneau & Thibault 2009). Although adult copper redhorse use various rivers in the St. Lawrence system, the only two known active spawning sites are in the Richelieu River within the 70-km section upstream of the St. Lawrence (**Figure 1**). One site is located in the Chambly rapids, and the other site is below the Saint-Ours dam. Both sites match the preferred characteristics for copper and river redhorse spawning: a coarse substrate consisting of medium pebbles in agitated water (COSEWIC 2004, 2014). The present experiment was conducted at the MFFP riverside seasonal research station used for artificial breeding activities located beside the river and the copper redhorse spawning site at the Saint-Ours dam.

Source of fish

All work with animals was approved under animal use protocols from MFFP (CPA-FAUNE 18-11) and McGill University (2018-7992). Research and collection permits were obtained from Parks Canada (CSO-2018-28364), Fisheries and Oceans Canada (SARA; MPO-LEP-QC-18-005), and MFFP (SEG; 2018- 04-03-2367-16-S-P). The MFFP artificial breeding program provided newly fertilized embryos for both river and copper redhorse. Wild parent fish were caught during their respective spawning season at the Vianney-Legendre fishway, a fish ladder located beside the Saint-Ours dam. Adult fish were kept in 2500-gallon basins in the MFFP riverside facility and released after spawning. Fish selected for breeding were declared matured and healthy following a visual inspection. Sexual readiness and final maturation were assessed and achieved using hormone injections. All males and females used in the present experiment were induced with an ovulating/spermiating agent (0.5 ml/kg; OvaprimTM; Syndel) and treated with an antibiotic (0.125 ml/kg; LiquamycinTM; Zoetis) according to the method described by Vachon et al. (2019). Eggs and milt were hand- stripped from individual fish. Milt was stored in Hanks' balanced salt solution with N-2-hydroxyethylpiperazine-N′-2- ethane-sulfonic acid buffer in a refrigerator. Before proceeding with fertilization, milt was assessed under a microscope for two measures of quality: percentage and duration of motility (Vachon et al., 2019). Milt was mixed with the oocytes and activated using 50%–100% of the oocytes' volume of room-temperature ActiFish solution (Syndel), prepared with one volume of Actifish for 29 volumes of reverse-osmosis water. For each species, approximately 40,000 eggs were harvested, of which 1000 were randomly selected for the experiment. The eggs of a single female were fertilized with the milt of a single male in a 500-ml container filled with reconstituted hard water (ultrapure water supplemented with sodium bicarbonate [96 mg/L], magnesium sulfate [60 mg/L], calcium chloride [39 mg/L], and potassium chloride [4 mg/L]; modified US Environmental Protection Agency [USEPA, 2002], hereinafter referred to as "laboratory water"). The remaining eggs, which were not used in the present study, were fertilized by milt from multiple males and sent to Baldwin-Coaticook government fish farm as part of the artificial breeding program. Freshly fertilized eggs were divided into two groups for each species and placed in 20-L containers filled with 10 L of laboratory water. Embryos were gently and continuously hand-mixed for the first 15 min post-fertilization and then once every 10–

15min (to prevent aggregation) for 2 h until the eggs expanded and the membranes hardened. River redhorse embryos were obtained on June 9, 2018, from a female captured on June 4, 2018, and a male captured on June 7, 2018. Copper redhorse embryos were obtained on June 15, 2018, from a female captured on June 10, 2018, and a male captured on June 6, 2018.

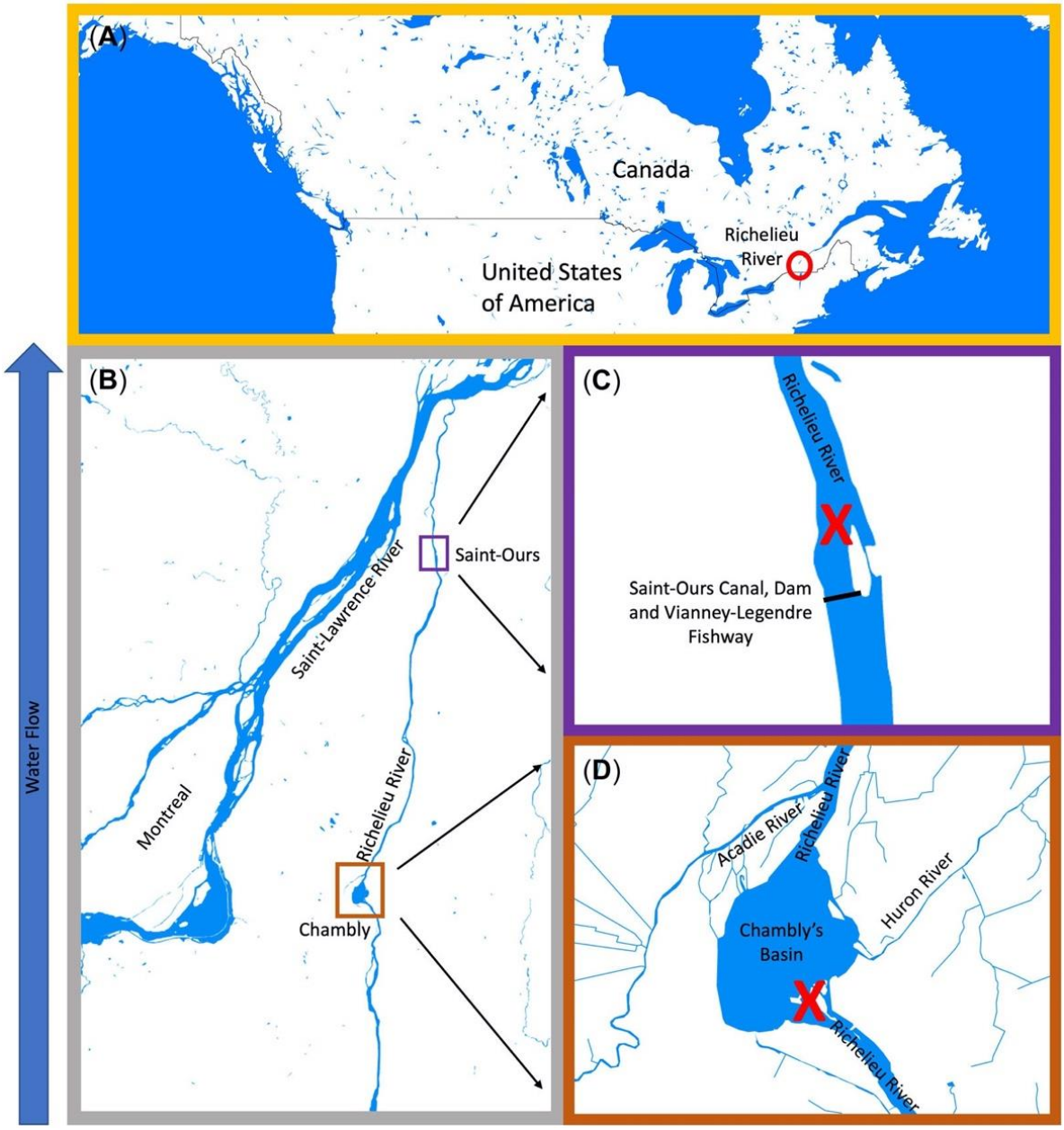

Figure 1: Map of the Richelieu River (Quebec, Canada) indicating the locations of the copper redhorse spawning grounds at Chambly and Saint- Ours. (A) The Richelieu River is located in southeastern Canada. (B) The Richelieu River is east of Montreal and flows from Lake Champlain to the St. Lawrence River. (C) Copper and river redhorse spawn in the rapids downstream of the Saint-Ours dam (marked with red "x"). The Vianney Legendre Fishway allows the fish to travel upstream of the Saint-Ours dam. The Quebec government's Ministère des Forêts, de la Faune et des Parcs operates a field laboratory near the fishway to conduct an artificial breeding program. This was the site for the riverside exposures. (D) Copper redhorse also spawn in the Chambly rapids within the Chambly basin (marked with red "x"). Many agricultural tributaries, including the L'Acadie and Huron rivers, join the Richelieu in between the two spawning grounds.

Experimental design and maintenance

River and copper redhorse embryos were divided equally into a control group ("laboratory water") and an exposed group ("river water"; **Figure 2**). For each treatment group, embryos were housed in nine replicate tumblers (Cobalt Aquatics) containing 55 embryos each. The tumblers were placed into three separate 38-L glass aquariums with three tumblers per species per aquarium. The river water group received a continuous flow of water from the river, while the laboratory water group received water that matched river water parameters for temperature, pH, conductivity, and hardness. Water from the river was pumped through the aquariums using a thrash pump (Multiquip ST2040T) through polyethylene and polymerized vinyl chloride piping. A plastic decantation basin limited sediment and debris that entered the pump from reaching the aquariums. The egg tumblers were equipped with a coarse sponge at the intake to prevent sediment from reaching the embryos and impeding the embryos' rocking motion. The large pore size of the sponge, which was needed in order to allow water to move through easily, suggests that it would not be expected to remove bioavailable contaminants from the river water. Given that the experiment was performed next to the river rather than in a laboratory facility, it was not possible to maintain flow-through conditions for the control group. Laboratory water for the control groups was therefore static with partial renewal every second day. River water flowed through an external aquarium to maintain the control group at river temperature (**Figure 2**).

Sponges from the egg tumblers in the river water group were cleaned daily in river water to prevent sediment from impeding the flow of water to the embryos. Every other day, the thin film of accumulated sediment was syphoned out of the bottom of the aquariums, and a third of the water from the laboratory water group was replaced with new laboratory water. Evaporated water from the laboratory water group was replaced daily. Water quality (pH, dissolved oxygen, temperature, conductivity, turbidity) was also measured daily in the aquariums and directly in the river, using a YSI probe.

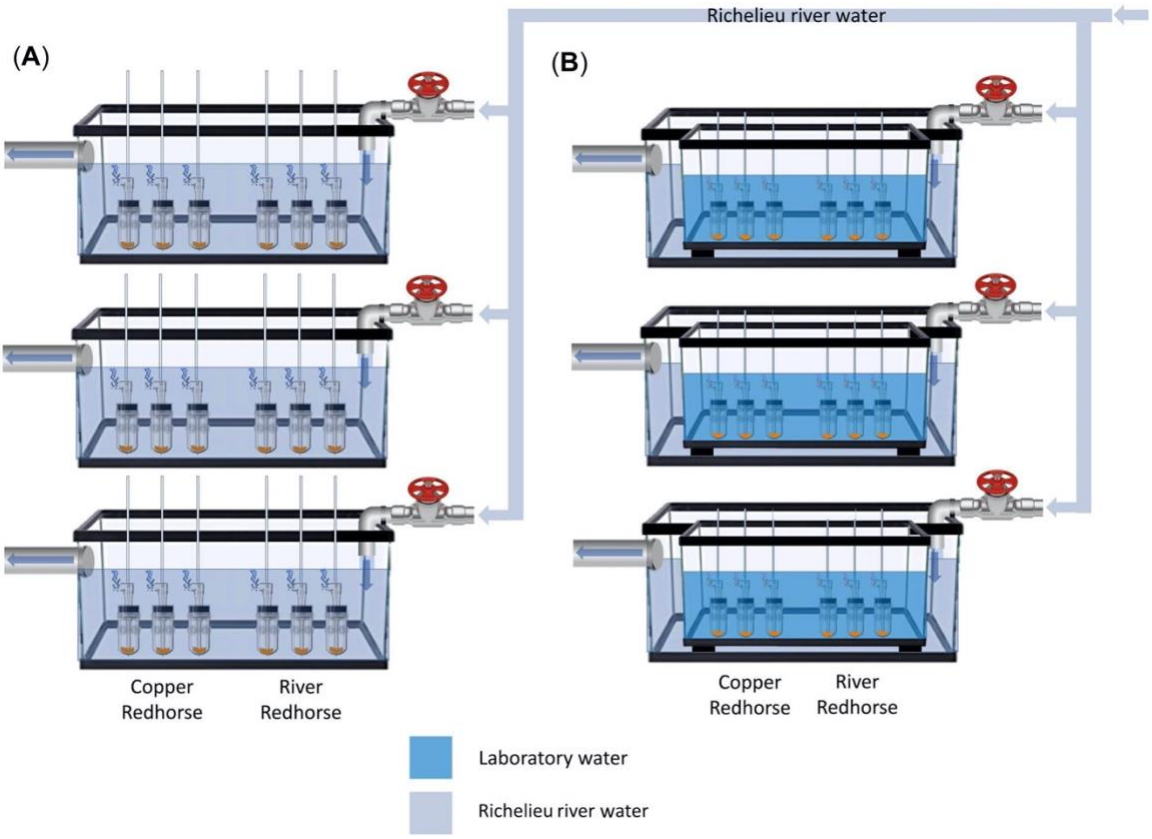

Figure 2: Experimental setup used to determine the effects of exposure to contaminated river water on early life stage copper and river redhorse. The experiment consisted of two groups: (A) the river water group, in which embryos were incubated in constantly flowing river water, and (B) the laboratory water group, in which embryos were incubated in static laboratory water with daily renewal. To maintain the same water temperature for both treatments, the laboratory water aquarium was placed inside a larger aquarium containing constantly flowing river water. Each treatment group consisted of three aquariums, each containing six egg tumblers (three for river redhorse and three for copper redhorse). Each tumbler was loaded with 55 eggs. Exposures lasted for 14 days post-fertilization. The river redhorse exposure started 6 days earlier than that for copper redhorse, to match natural spawning times in the Richelieu River.

Pesticide analysis

Water samples were collected from laboratory and river water aquariums daily over 19 days. Samples from replicate tanks were pooled each day. Water was also collected daily directly from the Richelieu River at Saint-Ours. All samples were frozen at −20°C until analysis. The samples were analyzed using targeted liquid chromatography/mass spectrometry (LC-MS) to quantify nine pesticides (aminomethylphosphonic acid (AMPA), atrazine, clothianidin, chlorantraniliprole, glyphosate, imazethapyr, imidacloprid, metolachlor, thiamethoxam). Details of standard solution preparation, sample preparation, and instrumental methods are given in the Supporting Information. Briefly, water samples were prepared by spiking a 10-µl internal standard mixture

and 100 µl methanol into 890 µl of river water, followed by filtration with a 0.22-µm polytetrafluoroethylene syringe filter (Canadian Life Science) before introduction into the LC-MS. Mass-labeled standards at constant concentration were used as internal standards to compensate for any systematic errors due to native compounds (as recommended in USEPA methods (e.g., USEPA, Method 1694, 2007)). Data were collected with an Agilent 1290 Infinity II LC system coupled to the 6545 quadrupole-time of flight–mass spectrometry (Q-TOF-MS; Agilent Technologies). The LC separation was performed on a CS-C18 column (2.1 µm × 150 mm; Agilent Technologies). Method performances were assessed in terms of linearity of the calibration response, method detection limits, method quantification limits, precision, relative recoveries, and matrix effects (Supporting Information, **Table 2**). The LC-Q-TOF-MS data obtained from targeted analysis were further screened for the presence of other contaminants in river water (suspect screening). Pooled samples from the river water aquariums and the river itself were compared to two control samples from the laboratory water aquarium. The samples from both river water aquariums and the river itself were collected on June 19, 2018, when contamination was expected to be maximal because of the heavy rain event on the previous day. The laboratory water samples used for comparison were from June 16 and June 19, 2018. Two controls were included to account for potential variation in the laboratory water tanks due to precipitation; however, no differences were observed. Suspect screening was performed using the software MassHunter Profinder B.10.00 from Agilent Technologies, where molecular features were aligned and extracted. These features were then statistically analyzed in Mass Profiler Professional by applying a fold change ≥2, followed by screening against the Environmental Water screening PCDL (Personal Compound Database and Library) and Pesticide PCDL. Molecular features were tentatively identified by comparing all ions and targeted MS/MS fragmentation with the MS/MS spectral library. For tentatively identified compounds, authentic analytical standards were used to confirm their identification.

Hatching, deformities, and survival

Fish embryos of each species were maintained in either laboratory water or river water, until 14 days post-fertilization (dpf). Timing of embryo hatching, incidence of deformities in embryos or larvae, and survival were assessed at the same time each day. Embryos were considered hatched when the larval body was fully extended and completely free of the egg membrane.

Embryo and larval size, shape, and developmental stage were observed. In larvae, deformities such as edema, craniofacial malformation, spinal curvature (lordosis and kyphosis), and yolk sac malformation were recorded as presence or absence. Photographs of deformed embryos were taken daily (Supporting Information, **Figure 7**).

Dead embryos and larvae were removed from tumblers as soon as they were noticed to reduce the risk of fungal spread. Embryos and larvae that were discolored or coagulated, showed shell damage, or were not showing movement and/or heartbeat were considered dead. A tally of surviving embryos was performed once daily, at the same time each day. Percentage of survival was calculated as the daily total number of live embryos and larvae over the initial number of embryos.

For each species, the experiment was terminated at 14 dpf, just before the complete resorption of the yolk sac. This corresponded to June 23, 2018, for the river redhorse and June 29, 2018, for the copper redhorse. Surviving larvae were pooled in groups of five in 2-ml cryotubes and euthanized by flash-freezing. Larvae were stored at −80 °C prior to RNA extraction.

RNA extraction and RNA sequencing

Sequencing of RNA was performed on the copper redhorse larvae only. Larval tissue from 18 (n=9/treatment) copper redhorse larvae pools (n = 5 larvae/pool) were homogenized in Buffer RLT using TissuLyser II (Qiagen). The RNA was extracted using a RNeasy Mini QIAcube kit according to the manufacturer's instructions, including an optional deoxyribonuclease I on-column digestion step (Qiagen). Extracted RNA was sent to the Genome Quebec Innovation Centre (Montreal, QC, Canada) for quality control screening, library preparation, and RNA sequencing (RNAseq), which included abundant transcript removal, fragmentation, reverse transcription, second-strand complementary DNA synthesis, end repair, dA-tailing, and adaptor ligation. Libraries were sequenced to produce paired- end 101 bp reads using Illumina HiSeq. 4000 (PE 100; 30M reads).

RNAseq data quantification using Seq. 2Fun

An all-in-one and ultrafast tool for RNAseq data analysis for organisms without reference genomes, such as many endangered species is Seq. 2Fun (Liu et al. 2021). Raw FASTQ read files

were submitted to Seq. 2Fun (Ver 1.0.0) Greedy mode with default parameters to conduct Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthologues (KOs) abundance quantification. The Animal protein database, consisting of 2,446,258 protein sequences and 12,984 KOs (of which 783,801 proteins and 10,510 KOs were from 39 fish species), was used. The KO abundance table from Seq. 2Fun was uploaded to NetworkAnalyst (Zhou et al. 2019; www.networkanalyst.ca) for differential expression analysis, overrepresentation analysis, and gene set enrichment analysis. The KOs were associated to gene names using the KEGG Orthology database (Mao et al. 2005). Differentially expressed genes (DEGs) between laboratory and river water groups were identified using the combination of fold-change (abs(log_2FC) >1) and adjusted p value (false discovery rate (FDR) < 0.05) based on edgeR (Ver 3.30.3; Robinson et al. 2010). Overrepresentation analysis was performed on the list of DEGs, and gene set enrichment analysis was performed on all genes ranked by absolute log_2FC, both with the KEGG pathway library. In both cases, significant pathways were identified based on an adjusted p value (FDR) <0.05. To associate DEGs and detected chemicals (tentatively identified and targeted), we referred to previously reported interactions from the Comparative Toxicogenomics Database (Davis et al. 2021; www.ctdbase.org).

Data analysis

Daily percentage hatching was calculated as a proportion of the total number of living embryos (hatched larvae/all alive) per day and per tumbler. Mean incidence of deformities and mean survival were calculated as a percentage of the initial number of embryos that were placed in each tumbler. All data residuals were tested for normality. Multivariate analysis of variance and a Bonferroni post hoc test were performed for daily percentage hatching, incidence of deformities, and survival between aquariums, species, and treatments using JMP statistical visualization software (SAS Institute). An $\alpha < 0.05$ was considered significant. Daily percentage hatching data were fitted to a sigmoidal four-parameter logistic regression, and the 50% hatch time was calculated using Prism 9 (GraphPad) in both days post-fertilization and cumulative degree days. The 50% hatch time values were compared using 95% confidence interval overlap.

Because previous data (Giroux 2015) demonstrated a correlation between heavy precipitation and high pesticide measurements in river water, hatching, survival, and contaminant concentrations

were visualized in relation to precipitation data obtained from the Government of Canada website (https://climate.weather.gc.ca) from the l'Assomption (QC, Canada) station, which is the closest (~21 km away) to our exposure site.

3.7 Results

Water physicochemical parameters

Water parameters in laboratory and river water aquariums and in the river itself were similar with respect to temperature, dissolved oxygen, and pH (Supporting Information, **Figure 8A–C**). As a result of human error, conductivity was approximately 100 μS/cm higher in the laboratory water group than in the river water group throughout the experiment (see Supporting Information for more information). Turbidity was negligible in the laboratory water aquariums throughout the experiment (0–1 formazin nephelometric units (FNU)) and was higher and more variable in the river water aquariums (5–32 FNU), which matched the river (Supporting Information, **Figure 8E**).

Analytical determination of pesticides in river water

Targeted analysis. Seven of the nine targeted pesticides were detected in the samples from the river water aquariums and the river itself (**Figure 3**). Imidacloprid and chlorantraniliprole were not detected in any samples. Levels of detected pesticides were consistently higher in the river water than in the laboratory water, particularly following the precipitation event on June 18 (**Figure 3**). Five pesticides—AMPA (glyphosate breakdown product), atrazine, glyphosate, metolachlor, and thiamethoxam (precursor of clothianidin)—were detected in the laboratory water aquariums. A background level of atrazine and metolachlor was present in all samples, while glyphosate and thiamethoxam were detected only once and AMPA thrice. The highest values detected in the laboratory water were 1441 ng/L AMPA (June 11), 57 ng/L atrazine (June 10), 156 ng/L glyphosate (June 24), 28 ng/L metolachlor (June 26), and 6 ng/L thiamethoxam (June 17; Supporting Information, **Table 3**). Background levels of atrazine and metolachlor were also detected in an ultrapure laboratory water sample at 3 and 6ng/L, respectively, and in all river water samples. These chemicals are routinely detected in Montreal's tap water (Montiel-León et al. 2019) from which the ultrapure water was made.

In river water aquariums, all of the compounds but glyphosate and AMPA peaked on June 19 following the June 18 heavy rain event (~30 mm) and then declined back to background levels over the next few days (**Figure 4**). Peak concentrations for the detected pesticides on June 19 in the river water aquariums were 1451 ng/L AMPA, 298 ng/L atrazine, 89 ng/L clothianidin, 244 ng/L imazethapyr, 1752 ng/L metolachlor, and 32 ng/L thiamethoxam. Temporal trends and absolute concentrations of targeted pesticides measured in the river water aquariums were generally well matched to levels measured in the Richelieu River at Saint-Ours (near the pump location; **Figure 3**). Some exceptions to this relationship were observed for clothianidin. On June 21 and June 22, clothianidin was detected in the river water aquarium at levels close to the method quantification limit (20 ng/L) but not in the river itself. In addition, glyphosate peaks in the river water aquariums did not correspond to the peaks from the river itself (**Figure 3**).

Nontargeted analysis. Suspect screening was performed on data from Richelieu River water samples collected at Saint-Ours on June 19 after heavy rain events and the control water. A total of 451 molecular features with abundance level at least two times higher in river water than in control water were extracted and screened against the Environmental Water screening PCDL and the Pesticide PCDL libraries. Suspected compounds from this initial screening included 24 PPCPs and 23 pesticides (**Figure 4**). For name-assigned molecular features with an MS/MS spectral library available, two suspected compounds with mass-to-charge ratios of 215.0963 and 229.0859 were tentatively identified as the pesticide metribuzin and the sunscreen additive oxybenzone, respectively, based on their multiple targeted MS/MS fragment ions well matched with those of the two compounds in PCDL library (Supporting Information, **Figure 9**). The correct identification of these two suspected compounds was further fully confirmed using the pure analytical standards based on matching retention times and fragment ions (Supporting Information, **Figure 9**). Three of the targeted pesticides (imazethapyr, metolachlor, and thiamethoxam) were also detected using suspect screening; for the other six targeted analytes, peak intensities were below the peak abundance threshold.

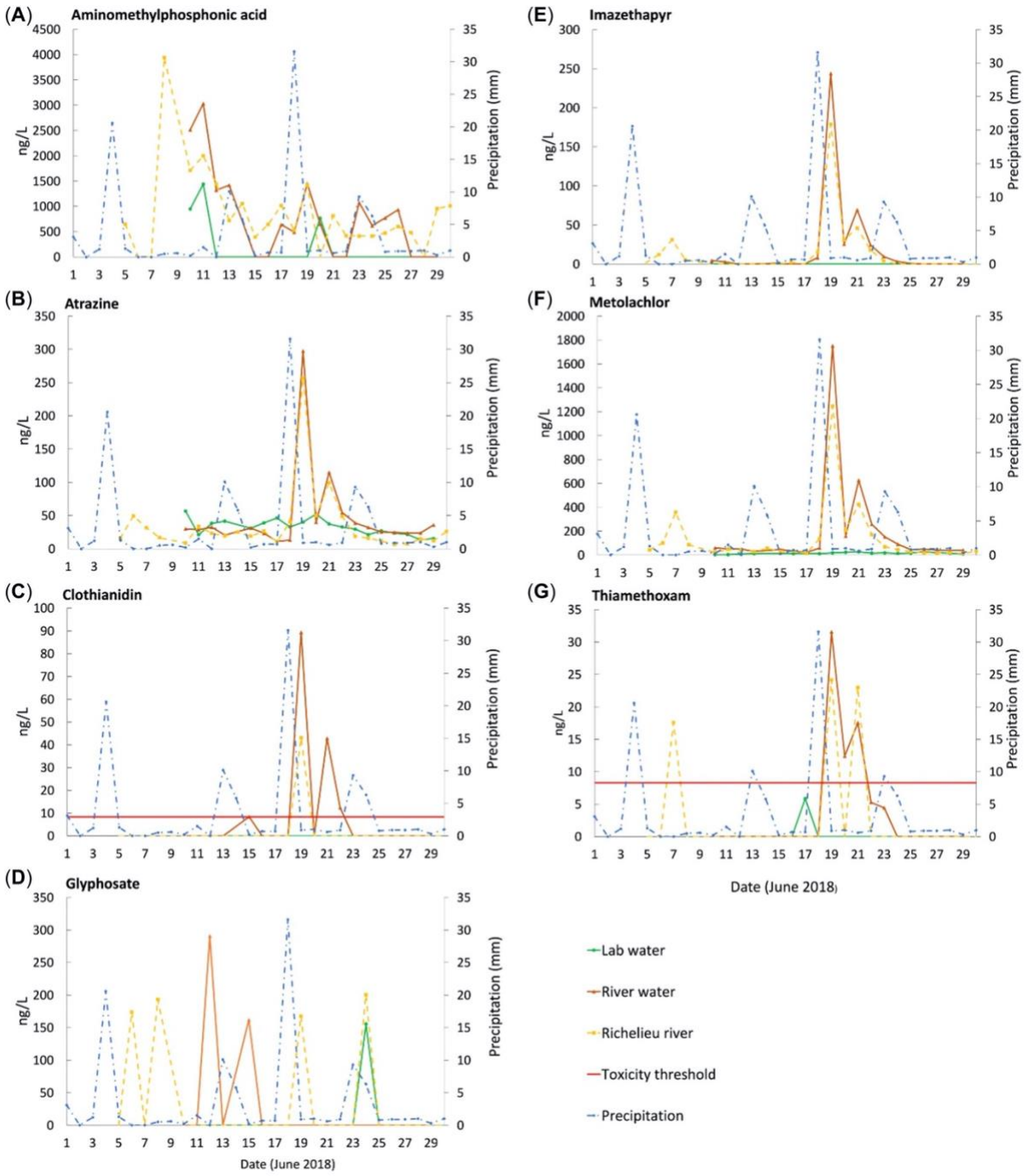

Figure 3: Daily pesticide concentrations measured in laboratory water (green) and river water (orange) aquariums and in the Richelieu River itself (yellow). Precipitation during sample collection is also presented. Water samples from the replicate laboratory and river aquariums were pooled before analysis. Richelieu River samples were collected near the pump intake feeding the aquariums. Two additional chemicals, chlorantraniliprole and imidacloprid, were also targeted but were not detected in any water samples. Note that aminomethylphosphonic acid (AMPA) is a degradation product of glyphosate and that thiamethoxam is the precursor of clothianidin. Canadian Council of Ministers of the Environment water quality guidelines for the protection of aquatic life are shown as solid red lines only for compounds in exceedance of the threshold (C, G). Thresholds for other compounds not shown on graphs are (A) AMPA, not available; (B) atrazine, 1800 ng/L; (D) glyphosate, 65,000 ng/L; (E) imazethapyr, 8100 ng/L; (F) metolachlor, 7800 ng/L.

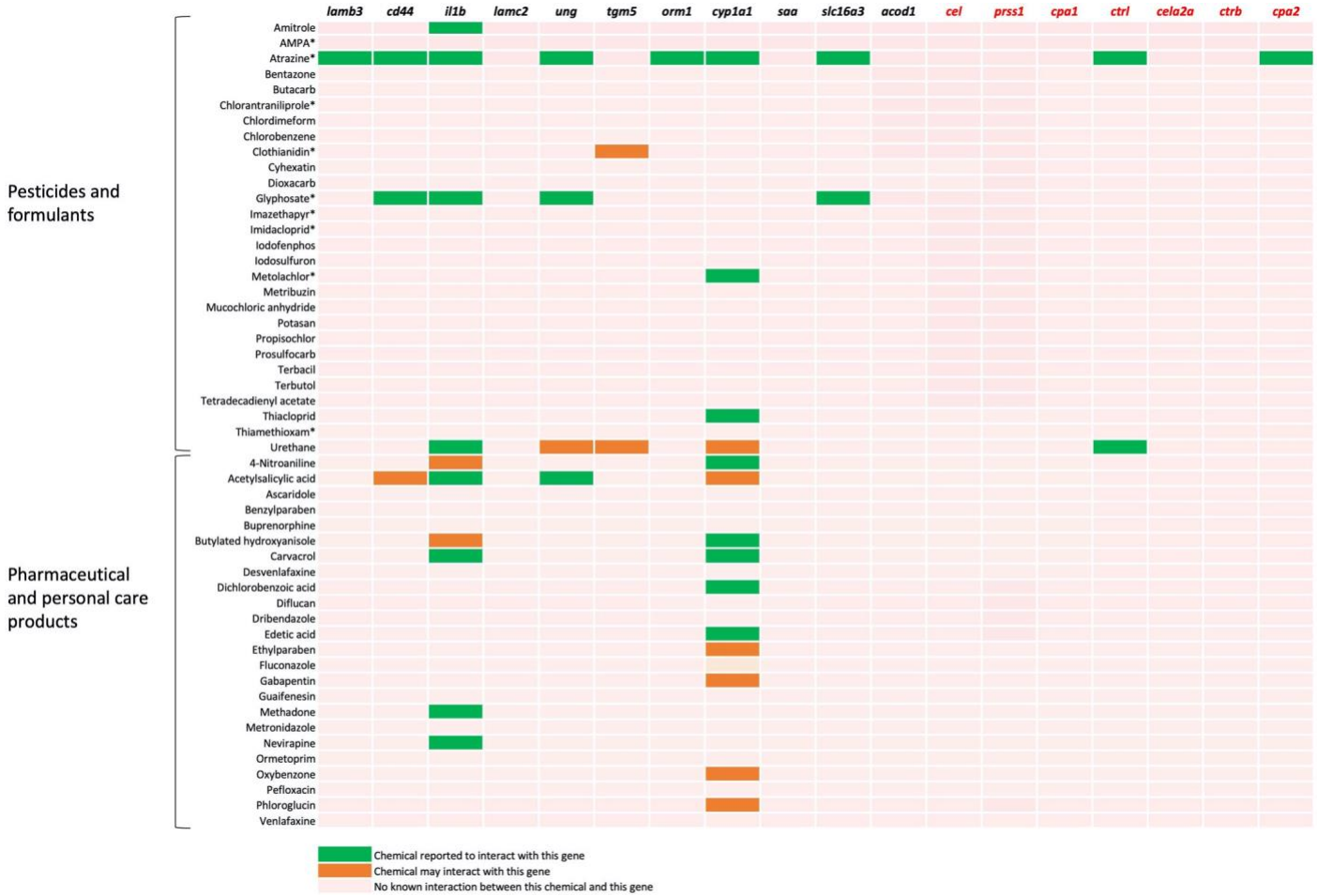

Figure 4: Previously reported gene interactions between differentially expressed genes in copper redhorse and chemicals detected in the present study. Interactions were identified from the Comparative Toxicogenomics Database. Detected chemicals include compounds that were quantified in the Richelieu River by targeted analysis (marked with asterisk) and tentatively identified with nontargeted analysis. Interactions marked in pink have not been reported or are not included in the database. Interactions marked in green were reported in the same direction (up- or down- regulated) as the changes observed in the copper redhorse. Interactions marked in orange either involve simultaneous exposure to multiple chemicals or involve changes in the opposite (or unknown) direction of those observed in the copper redhorse. The atrazine degradation product atrazine-desisopropyl and the pesticide esprocarb were detected in the river water but absent from the database. Genes in black were upregulated, while genes in red were down-regulated in copper redhorse. See Supporting Information, **Table 4** for the complete name of genes. AMPA = aminomethylphosphonic acid.

Survival

Overall survival for embryos of both species was high (>79%) throughout the experiment except for one of the nine river redhorse tumblers from the laboratory water group that malfunctioned, between days 11 and 12, trapping and killing larvae in the metal mesh blocking the exit. After that time point, data from this tumbler were excluded from the analysis. At 14 dpf, river redhorse survival was 89.0% (±4.5%) for the laboratory and 84.0% (±5.7%) for the river water group. No significant differences in survival between treatments were observed at any time point (**Figure 5**). For copper redhorse, survival was similar in the two treatment groups through the first 13 dpf. However, survival at 14 dpf was significantly higher in the laboratory water group (93.3%; ±3.1%) than in the river water group (79.2%; ±15.6%). The large standard deviation in the river water group at 14 dpf was driven by three tumblers (out of nine), one per aquarium with varying positions, which averaged 61.2% survival (Supporting Information, **Figure 10**). In the other six tumblers, survival was more comparable between treatments (88.2% vs. 93.3%). No significant batch effect from aquariums was detected. When comparing the laboratory water groups, there was a statistically significant difference in survival between the two species, with copper redhorse exhibiting higher survival than river redhorse at 14 dpf ($p = 0.023$; **Table 1**).

Timing of hatching

River redhorse in both treatments began hatching at 6 dpf, and hatching was complete at 9 dpf (**Figure 6A**). At 7 dpf, the percentage of hatched embryos was significantly higher in the river water group than in the laboratory water group. In the copper redhorse exposed to river water, hatching was first observed at 5 dpf, 24 h before it was observed in the laboratory water group. For both treatments, all embryos were hatched by 8 dpf. There was a significant difference in hatching between the laboratory and river water groups at 5, 6, and 7 dpf.

Hatching data were also expressed in terms of cumulative degree days, and fitted curves were used to determine the 50% hatch time (**Figure 6B**). For river redhorse, the time to 50% hatch was 2.4 cumulative degree days earlier for the river water group than for the laboratory group (**Table 1**). For copper redhorse, the time to 50% hatch was 10.7 cumulative degree days earlier for the river

water group than the laboratory water group. For both species, the difference in the 50% hatching time was statistically significant between the laboratory and river water groups.

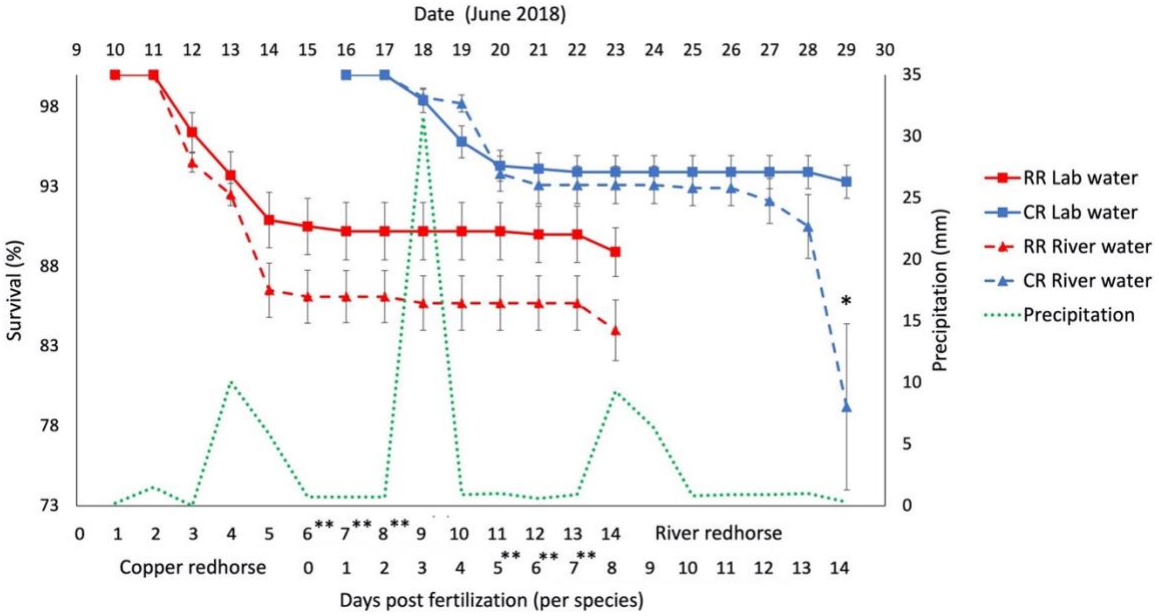

Figure 5: Effect of exposure to Richelieu River water on survival of copper redhorse and river redhorse embryos and larvae over the 14-day exposure period. Data are expressed as the percentage of live individuals over total embryos. Each data point represents the average of nine egg tumblers, and error bars represent standard error. Significant differences between treatments are marked with an asterisk ($p < 0.05$). Dual asterisks mark the days during which the embryos were hatching. Daily precipitation data were collected at the l'Assomption station (Quebec, Canada) by the Meteorological Service of Canada of Environment and Climate Change Canada (https://climate.weather.gc.ca). Given the strong relationship between precipitation and pesticide concentrations in the water column (Figure 4), these data may be used as surrogate data for the total pesticide concentration over time. See Supporting Information, Figure S2, for the individual copper redhorse incubator data from day 14 post-fertilization. RR = river redhorse; CR = copper redhorse; Lab = laboratory.

Deformities

The frequency of deformities was 4.0 ± 2.2% and 2.8 ± 2.6% in the laboratory and river water groups, respectively, for river redhorse and 9.3 ± 5.6% and 12.1 ± 3.7% for the laboratory and river water groups, respectively, for copper redhorse (**Table 1**). There was no statistically significant difference in deformity rates between laboratory and river water groups for either species. However, independent of treatment, river redhorse had a significantly lower incidence of deformities than copper redhorse. The most prevalent deformities were spinal curvature (kyphosis/lordosis), which represented 79.4% of the river redhorse deformities and 64.2% of the copper redhorse deformities (Supporting Information, **Table 4**).

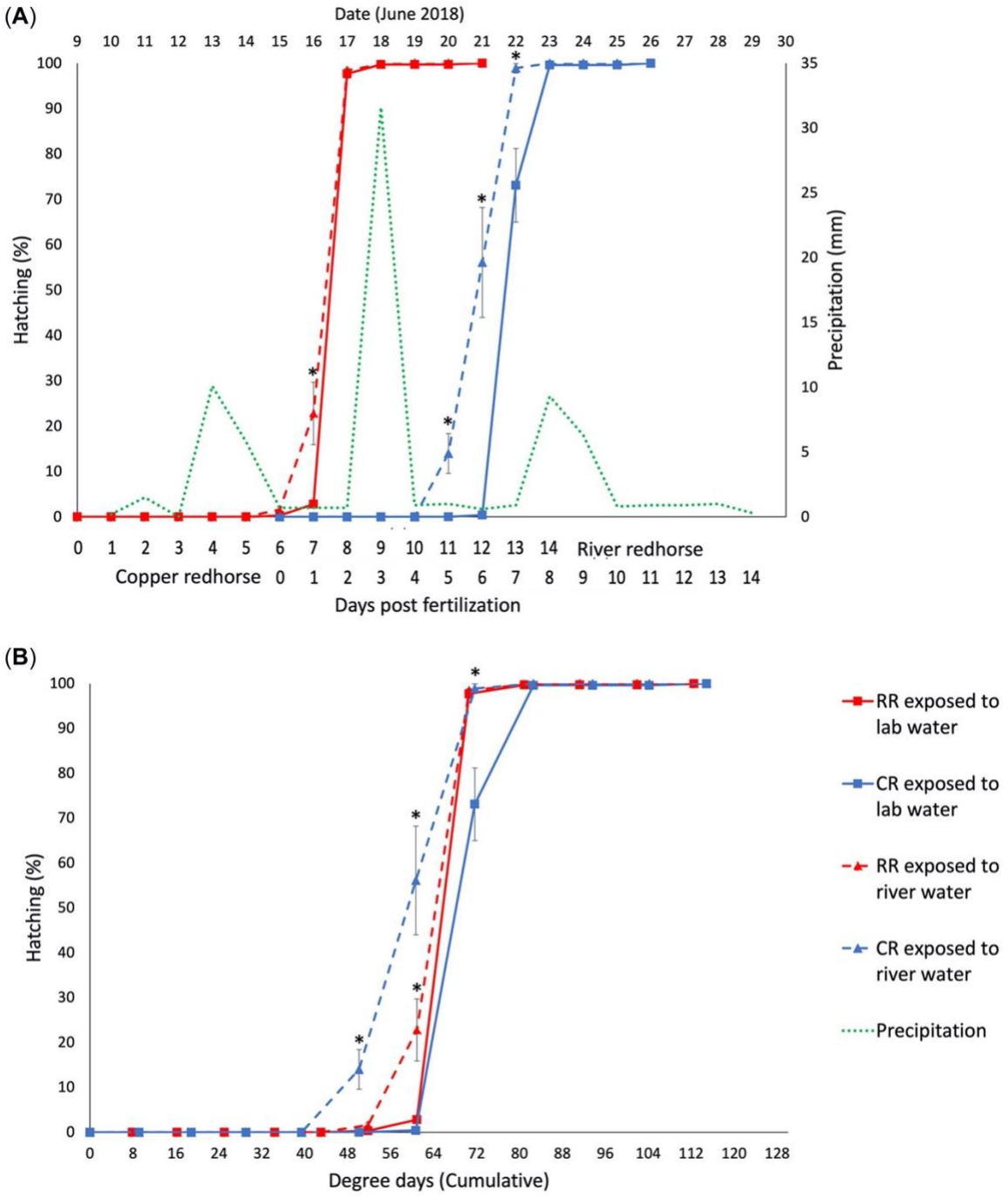

Figure 6: Effects of exposure to Richelieu River water on timing of hatching of copper redhorse and river redhorse embryos. Data are expressed as the percentage of live embryos that hatched on a given date. Each data point represents the average of nine egg tumblers per species, and error bars represent standard error. Significant differences between treatments are marked with an asterisk ($p < 0.05$). Data are plotted as a function of (A) days post-fertilization and (B) cumulative degree days, which takes into consideration the effect of water temperature on embryonic development. Daily precipitation data were collected at the l'Assomption station (Quebec, Canada) by the Meteorological Service of Canada of Environment and Climate Change Canada (https://climate.weather.gc.ca). RR = river redhorse; CR = copper redhorse; lab = laboratory.

Table 1. Summary of organismal level endpoints assessed in river and copper redhorse

Species	Treatment	**Frequency of Deformities (%)**	Std dev (%)	**Time to 50% hatch (CDD)**	95% Confidence interval	**Survival at 14 day post-fertilization (%)**	Std dev (%)
River redhorse	**Lab water**	4.0^{A}	*2.2*	63.8^{A}	*63.6 to 63.9*	89.0^{A,B}	*4.3*
	River water	2.8^{A}	*2.6*	61.5^{B}	*61.2 to 61.7*	84.0^{A}	*5.7*
Copper redhorse	**Lab water**	9.3^{B}	*5.6*	67.7^{C}	*67.5 to 68.0*	93.3^{B}	*3.1*
	River water	12.1^{B}	*3.7*	57.3^{D}	*56.1 to 58.4*	79.2^{C}	*15.6*

CDD: cumulative degree days
Percentage (%) are based on initial number of embryos per treatment, which was 495 larvae per species.
Different letters represent significative differences.

RNAseq

A total of 9025 KOs were identified, 8903 of which were matched to unique gene-level (Entrez ID) expression. Of these, 18 genes were significantly differentially expressed (adjusted p value <0.05, and absolute $\log_2$FC >1) between the river water– and laboratory water–exposed copper redhorse (11 upregulated and seven down-regulated; Supporting Information, **Figure 11** and **Table 5**). Of the up-regulated DEGs, eight (cd44, il1b, lamb3, lamc2, tgm5, orm1, saa, acod1) were linked to immune function and injury response. The remaining were involved in xenobiotic metabolism (cyp1a1), mutagenesis prevention (ung), and membrane transport (slc16a3). All of the down-regulated DEGs (cpa2, ctrb, cela2a, ctrl, cpa1, prss1, cel) were involved with digestion and nutrient absorption. A single significant pathway was identified in the overrepresentation analysis (extracellular matrix–receptor interaction), and 60 were identified using gene set enrichment analysis (Supporting Information, **Table 6**). The significant enrichment of pathways showed 40 pathways with mainly up-regulated genes, many related to growth, development, and drug metabolism in copper redhorse raised in river water. The remaining 20, which showed mainly down-regulated genes, were mostly involved in various cellular and synapse signaling pathways.

The RNAseq profiles are publicly available through the National Center for Biotechnology Information's Gene Expression Omnibus database (GSE185175).

We used the Comparative Toxicogenomics Database to explore previously reported interactions between the 36 river water contaminants and the 18 DEGs identified in our study (**Figure 4**). The three genes affected by the highest number of compounds in the database were cyp1a1 (14 compounds), il1b (10), and ung (4). The compounds that had the most known interactions with the identified DEGs were atrazine (nine interactions), urethane (five), glyphosate (four), acetylsalicylic acid (four), 4-nitroaniline (two), butylated hydroxyanisole (two), and carvacrol (two).

3.8 Discussion

Our study used an ELS riverside exposure to evaluate the impacts of spawning site contamination on the threatened river redhorse and the endangered copper redhorse. The experimental design permitted continuous exposure of one treatment group to water pumped directly from the Richelieu River, while the other group was exposed to temperature- matched laboratory water. Targeted and nontargeted chemical analyses revealed the presence of complex mixtures of pesticides and other contaminants in the river water, whereas the laboratory water had lower background levels of contamination. The riverside system successfully maintained favorable conditions for embryo development, hatching, and early larval development; and water parameters were generally well matched between the laboratory and river water (Supporting Information, **Figure 8**).

Chemical analyses

Our investigation of contaminants in laboratory water, river water, and the river itself focused on nine current-use pesticides that were most frequently detected at higher concentrations in tributaries of the Richelieu River in the years preceding our study (Giroux, 2015, 2019). An initial broad screen of contaminants in Richelieu River water carried out in the winter previous to our experiments also indicated that pesticides were prevalent contaminants in this system (H. Marchand et al., unpublished data). Background levels of AMPA, atrazine, glyphosate, metolachlor, and thiamethoxam were detected in laboratory water, whereas higher levels and a larger variety of pesticides were detected in the river water (**Figure 3**). Most of the targeted

pesticides fluctuated in river water aquariums through the month of June with one small (June 7) and one large (June 19) spike following heavy rain events (**Figure 3**). This pattern is representative of many agricultural rivers, where pesticides tend to peak in the spring following heavy rain as pesticides and sediments are washed into the river (Giroux 2019). During the 3 days following the heavy rain event, peak concentrations of two pesticides, the neonicotinoid clothianidin (89 ng/L) and its precursor thiamethoxam (32 ng/L), exceeded Canadian water quality guidelines for the protection of aquatic life of 20ng/L (Health Canada 2018). Thiamethoxam is converted to clothianidin in plants and insects, but both compounds are also independently applied for pest control. Given the ubiquitous presence of contaminants in all environmental matrices, it is often difficult to identify a suitable control for ecotoxicological studies. We used reconstituted ultrapure laboratory water that matched river water in terms of temperature, pH, and hardness. Small differences in turbidity and conductivity were observed (see Supporting Information and **Figure 8).** In addition, our analysis revealed that the laboratory water was contaminated with low levels of pesticides. Previous drinking water surveys detected atrazine in 100% of water samples tested in the Montreal area (2015–2018; Montiel-León et al. 2019), where McGill University is located. During the experiment, the atrazine concentrations in laboratory water varied between 13 and 57 ng/L (Supporting Information, **Table 3**), which was at the lower end of the range (40–250 ng/L) reported by Montiel-León et al. (2019). Glyphosate and its metabolite AMPA have also been detected in drinking water in Montreal and surrounding areas (Montiel- León et al. 2019). In our study, laboratory water was contaminated with levels that were two (AMPA and glyphosate), six (atrazine and thiamethoxam), and almost 65 (metolachlor) times lower than the lowest level detected in the river water.

In addition to the targeted pesticides, other compounds such as PPCPs and additional pesticides were tentatively identified in the Richelieu River water through suspect screening (**Figure 4**). Local urban wastewater effluents are a likely source for PPCPs (Schreder & La Guardia 2014). Many of the wastewater-treatment plants along the Richelieu are known to overflow following heavy rain events (Cliché & Saladzius 2018). Oxybenzone, one of the two compounds that was tentatively identified through suspect screening and then validated using analytical standards, is an organic ultraviolet filter commonly used in sunscreen that has been detected in surface water worldwide (Schneider & Lim 2019). This compound is hard to remove from wastewaters and has

been identified in tissues of many fish species across Europe (Schneider & Lim 2019). In most cases, levels measured in surface water were two orders of magnitude below the predicted-no-effect concentrations (Burns et al. 2021). The second validated compound, metribuzin, is an herbicide which was detected below 1 μg/L each year between 2015 and 2017 in the Huron River (Giroux 2019). In a study with juvenile zebrafish, the no-observed-effect level of metribuzin was estimated to be 16 mg/L (four orders of magnitude higher than what was measured in the present study; Stepanova et al. 2012), suggesting that metribuzin is unlikely to pose a risk to copper and river redhorse.

Biological endpoints

Using a riverside system, artificial rearing of river and copper redhorse over a 2-week period was successful, with survival rates of 89% and 93%, respectively, in the laboratory water groups. Survival was similar in the laboratory and river water groups throughout the experiment until 14 dpf, when it sharply decreased for the copper redhorse exposed to river water (79.2% vs. 93.3%; **Table 1**). This decrease occurred when the fish were nearing the end of their larval stage, 10 days after the peak of the pesticides in the river water. Pesticides can be acutely toxic to ELS fish but typically only at much higher concentrations than were measured in our river water aquariums (Mhadhbi & Beiras 2012; Osterauer & Köhler 2008; Sulukan et al. 2017). This and the fact that the decrease in survival was not observed in all of the tumblers in each tank (Supporting Information, **Figure 10**) make it difficult to determine whether it was associated with the presence of contaminants. Some detoxification mechanisms in fish are energy-dependent and may reroute valuable energy from an already limited supply in developing embryos (Bains & Kennedy 2004). This could cause them to rely on an external food source earlier than the fish in laboratory water, which may explain why the die-off occurred only in the river water group.

Deformity rate is an important sublethal indicator of environmental pollutant exposure in ELS fish (Lin Sun et al. 2009). Deformity rates were low in both species exposed to laboratory water (river redhorse 4.0%, copper redhorse 9.3%) and river water (river redhorse 2.8%, copper redhorse 12.1%), with no indication that they were affected by the treatment (**Table 1**). Pesticides such as glyphosate (Sulukan et al. 2017), atrazine (Mhadhbi & Beiras 2012), diazinon, and thiacloprid (Osterauer & Köhler 2008) have been shown to be teratogenic but at much higher doses than

present concentrations that river and copper redhorses were exposed to in our study. Independent of treatment, copper redhorse had a significantly higher rate of deformities than river redhorse (9.3% vs. 4.0%). Little information is available from the literature on average rates of deformities in wild fish ELS, but they are likely to be highly variable. For example, the incidence of kyphosis deformities in a wild population of lagoonal sand smelts (Atherina lagunae) was 9.75% (Ayed et al. 2008), while spinal deformities reached 33% in wild haddock (Melanogrammus aeglefinus; Jawad et al. 2018).

During our experiment, both species hatched prematurely when exposed to river water. Many of the pesticides that we detected in the river water have previously been shown to individually alter hatching time in fish and other aquatic vertebrates, albeit at concentrations above those detected in our samples. For example, sockeye salmon (*Oncorhynchus nerka*) exposed to 25 and 250 µg/L of atrazine hatched prematurely only at the highest dose (Du Gas et al. 2017), which was almost three orders of magnitude higher than our highest measurement of 298 ng/L. Both diazinon (3 mg/L) and glyphosate (50mg/L) have been associated with premature hatching in zebrafish (Fiorino et al., 2018; Osterauer & Köhler 2008). However, the same dose of glyphosate (50 mg/L) had the opposite effect (hatch retardation) on common carp (*Cyprinus carpio*), which suggests that responses can differ among species (Fiorino et al. 2018). This dose of glyphosate was almost 35,000 times higher than the peak of 1451ng/L that we measured in the Richelieu River water. Previously reported effects on fish hatching time all occurred at higher concentrations than we observed, but these studies focused on a single compound, whereas our study involved a complex mixture of contaminants. Further research is needed to identify components of the mixture that may be responsible for the effects that we observed. The potential consequences of early hatching on copper and river redhorse populations are unclear, but Bowerman et al. (2014) reported that early emerging larvae were less developed, which could lead to lower survival. In our case, following a visual inspection, healthy larvae that hatched earlier could not be visually distinguished from those hatching later in time.

Factors other than contaminants may also influence fish hatching time. In many salmonids, the presence of fine sediment in the water can lead to premature hatching and emergence of the larvae (Bowerman et al. 2014). This may be explained by the localized hypoxia caused by fine sediment surrounding the embryos. Sediment may also prevent water flow, which would clean the embryo

surface of metabolic waste (Bowerman et al. 2014). In our study, it is possible that the fine sediment, which was only present in the river aquariums, influenced hatching time. However, the sponges at the entrance of the egg tumblers prevented most sediment from accumulating in the egg chambers, and constant agitation was maintained by the air pump. Given these considerations, it is unlikely that the presence of sediment was an important factor influencing hatching times in the river water groups.

Two neonicotinoid insecticides, clothianidin and thiamethoxam, were periodically detected in river water at levels that exceeded water quality thresholds for aquatic life (**Figure 3**). However, organism-level effects resulting from neonicotinoid exposure in ELS fish have only been previously reported at higher doses than these thresholds. In a 4-month study on ELS salmon exposed to clothianidin, no effects on hatching, growth, survival, or deformities were observed from concentrations of 0.15–150 μg/L (Marlatt et al. 2019). More subtle effects such as increases in whole-body 17β-estradiol were observed at 150 ng/L, the lowest tested dose (Marlatt et al. 2019), a concentration approximately two times higher than the highest concentration in our river water aquariums. In zebrafish, continuous exposure to thiamethoxam over 4 weeks caused an increase of reactive oxygen species, followed by an increased antioxidant response at concentrations of 300 μg/L (Yan et al. 2016). This is a dose more than 9000 times higher than what we measured in the Richelieu. No effects on hatching or survival were observed at this concentration. Concentrations of other individual pesticides measured were generally below the level where effects have previously been reported in ELS fish.

Some of the compounds tentatively identified through nontargeted screening may have sublethal effects on ELS fish. For example, zebrafish exposed to 5%, 10%, and 50% municipal wastewater effluent from Calgary (Canada) showed delayed hatching at 48 and 57h post-fertilization (Gauthier & Vijayan 2020). Known effects of PPCPs on aquatic organisms also include endocrine disruption, teratology, gene toxicity, and aberrant physiological processes (Wang et al., 2021). We were not able to quantify the concentrations of the nontargeted compounds in the present analysis, but this suggests that contaminants other than pesticides may also be involved in the observed changes to hatching time and gene expression.

The complexity of contaminant mixtures makes it difficult to link effects to specific chemicals, and more research is warranted in this area. In a notable recent example of this type of work, researchers attributed mortality observed in coho salmon (*Oncorhynchus kisutch*) exposed to stormwater runoff to a single chemical in the mixture: 6-p-phenylenediamine-quinone (Tian et al. 2020). In addition, contaminants within complex mixtures can have synergistic effects, which amplify their impacts on fish. For example, when atrazine and chlorpyrifos were present together, their toxicity potential was increased compared with what would be predicted by the simple principle of addition (Perez et al. 2013). This has also been demonstrated for chlorpyrifos and betacypermethrin (Zhang et al. 2017) as well as phoxim and atrazine (Wang et al. 2017).

In the present study the exposure to river water was associated with altered hatching time in both redhorse species and decreased larval survival in copper redhorse. These effects appeared to be most severe in the copper redhorse, pointing to a possible link between species sensitivity and conservation status; but other explanations are also possible. River and copper redhorse artificial breeding occurred a week apart; thus, the fish were exposed to pesticide peaks caused by rain events at different developmental stages (**Figures 5 and 6**). The major pesticide peak coincided with the advanced larval developmental stage of river redhorse (mesolarvae), whereas copper redhorse were exposed at the moment of hatching (protolarvae; which was premature). Even though there is a consensus that ELS are more vulnerable than adults, not all ELS are equally sensitive. The literature suggests that the larval stage is often more vulnerable than embryonic and juvenile stages of fish (Mhadhbi & Beiras 2012; Oliva et al., 2008; Velki et al. 2017; Wang et al. 2017). This could be explained by the fact that the early larval stage lacks the embryo's chorion, which acts as a protective barrier, and has under- or undeveloped biological defenses or means of detoxification that are present in the later larval and juvenile stage (Velki et al. 2017). In comparison with embryonic and juvenile stages, larvae can be sensitive to pesticide doses 10–100 times lower in concentration (Oliva et al. 2008). A second important consideration related to species sensitivity is that embryos for each species came from a single pair of fish. Siblings from a single cross cannot be considered to be representative of the species as a whole (Jager 2013). In the present study, using siblings was advantageous because it allowed for similar prenatal, genetic, and underlying conditions. Moreover, given that we were working with endangered species, sampling from multiple crosses was not possible. Overall, our results suggest that copper redhorse

were more sensitive than river redhorse, but further work is needed to confirm and explore the mechanism behind this observation.

RNAseq

We used RNAseq to identify genes and pathways that were differentially expressed in copper redhorse exposed to river water compared to control laboratory water. At 14dpf, 18 DEGs were identified (11 up- and 7 down-regulated; Supporting Information, **Table 4**). The low number of DEGs is likely reflective of the fact that copper redhorse larvae were exposed to relatively low levels of contaminants in the river water and that sampling occurred at 14 dpf rather than immediately after the peak of pesticides at 4 dpf (**Figure 5**). Interestingly, a large proportion (44%) of the 18 DEGs were involved in injury and immune response. For example, the gene with the highest fold change, lamb3 (2.4 $\log_2$FC), produces a protein that regulates cell growth and motility. This gene is important in the basement membrane of the skin but also plays an important role in wound healing (Miner & Yurchenco 2004). The gene with the second highest fold change, il1b, is a key mediator of the inflammatory response (Dinarello 1996). Six other DEGs have roles in injury healing (lamc2, tgm5, orm1, saa) or immune response (cd44, acod1). Pesticides, including neonicotinoids, which were the only measured and identified compounds above Canadian water quality guidelines for the protection of aquatic life, have been linked to immune suppression in fish (Gibbons et al., 2014). Of the 10 remaining overexpressed KOs, cyp1a1 was the most clearly linked to contaminant exposure. This Phase I metabolic enzyme can be induced by exposure to many contaminants including PAHs, PCBs, and some pesticides (Danielson 2002). A previous study, using opportunistically sampled adult copper redhorse, found PAHs and PCBs present in fish tissues but below toxic levels (De Lafontaine et al., 2002). Up-regulation of cyp1a1 was consistent with xenobiotic exposure and has often been used as a marker of pollution in fish (Goksoyr 1995). Among the targeted pesticides, atrazine (Fu et al. 2013), glyphosate (Wang et al. 2020), and metolachlor (Dierickx 1999) are known cyp1a1 inducers. For the other targeted pesticides, induction of cyp1a1 has not been documented.

In addition to the targeted pesticides, many other contaminants were tentatively identified in the river water that may have contributed to the observed dysregulation of gene expression. We explored potential associations between these contaminants and changes in gene expression by

probing the Comparative Toxicogenomics Database (**Figure 4**). This analysis revealed that the dysregulated genes had previously been associated with exposure to many of the contaminants detected in our river water samples. For example, atrazine has been linked to changes in messenger RNA expression of nine of the 18 DEGs (lamb3, cd44, il1b, ung, orm1, cyp1a1, slc16a3, ctrl, cpa2) and glyphosate to four (cd44, il1b, ung, slc16a3; **Figure 4**). Many contaminants alter levels of cyp1a1 expression; according to the Comparative Toxicogenomics Database, more than 6000 interactions between cyp1a1 and 1500 chemicals have been reported. Because of potential synergistic and antagonistic effects between compounds and the fact that the database is biased toward compounds that have been more extensively studied, it is difficult to decipher the specific role that a given contaminant plays in causing toxic effects within a complex mixture. However, this analysis suggests avenues for future research by identifying compounds of interest within the mixture.

Using gene set enrichment analysis, we identified 60 pathways in copper redhorse that were affected by the river water in comparison to laboratory water (Supporting Information, **Table 6**). It is notable that a much higher number of significant pathways than DEGs was detected. This is possible because gene set enrichment analysis is performed independently from differential expression analysis and captures more subtle changes in genes that are grouped together in pathways. However, these results should be interpreted with caution when working with Seq. 2Fun and KOs because the gene universe is restricted to the space covered by KOs rather than the complete transcriptome measured by RNAseq. In addition, the actual pathways present in the species under study are a subset of the total KEGG pathways. This reduced gene universe coupled with inflated pathway annotation will likely interfere with the analysis. The second pathway enrichment analysis that we used, overrepresentation analysis, is based on significant differential expression. In this case, the small number of DEGs explains why only a single significant pathway was detected. Many of the pathways identified by gene set enrichment analysis and the single pathway identified by overrepresentation analysis are related to metabolism, growth, and development. The increased metabolism that we observed in the river water group can be linked to increases in the energetic cost of detoxification (Bains & Kennedy 2004). Drug metabolism was also in the top 20 pathways that were affected by river water exposure. Because many of the DEGs

were related to immune or injury response, we expected that pathways related to immune functions would be affected.

Limitation of the present study and future research

Working with endangered wildlife brings extra challenges because of regulatory and practical considerations as well as ethics and the limited information available for these species. Focusing on ELS allowed us to work with copper redhorse, but the opportunities for a greater sample size and study replication were limited. In the present study, we had access to a single embryo collection from a single cross, which limited our ability to draw conclusions at the population level. Future studies should include laboratory studies to try to associate precise contaminants to observed effects and to evaluate species sensitivity of ELS redhorses to these contaminants.

In addition, the absence of an annotated genome hindered our ability to extract biologically meaningful information from the raw data. Even for fish species with annotated genomes, pathway enrichment analysis depends on databases that are biased toward mammals, leading to a more cautious interpretation of these results. This first publication of the copper redhorse transcriptome lays the groundwork for future molecular studies on this endangered fish.

3.9 Conclusion

Our study sheds light on the biological effects of exposure to a real-world mixture in important developmental stages of two fish species of concern. We provided evidence that the endangered copper redhorse is more sensitive than the threatened river redhorse, but more research is required to confirm this and to determine the potential consequences at the population level. Overall, multiple factors could be threatening the copper redhorse such as late age of maturity, the late season breeding, habitat loss and degradation including the reduced numbers of spawning grounds and nurseries, as well as ELS exposure to pesticides. Future work will be aimed at clarifying the individual effects of contaminants present in the Richelieu River on fish ELS. Laboratory research using zebrafish as a model and follow-up copper redhorse studies are already underway. The differential expression of genes related to the immune system suggests that an immune challenge of copper redhorse ELS is an interesting avenue for future research.

3.10 Supporting information

Chemical List and Purity

All the native standards (AMPA, atrazine, chlorantraniliprole, clothianidin, glyphosate, imazethapyr, imidacloprid and thiamethoxam) were purchase from Millipore Sigma (Sigma-Aldrich) with purity above 98%. All the isotope-labeled internal standards (atrazine-D3, chlorantraniliprole-D3, clothianidin-D3, imazethapyr-D3, thiamethoxam-D3) were purchased from Millipore Sigma (Sigma-Aldrich) except for glyphosate-D2, imidacloprid-D4, metolachlor-D6 which were from CDN Isotopes, all with purity above 98%. AMPA-$^{13}C_{15}ND_2$ was donated by Jean-Francois Roy from Agilent Technologies. All the solvents used were at HPLC grade (except for water at LC-MS grade) and were obtained from Fisher Scientific (Hampton, NH, United States).

LC-MS Analysis methods

Standards preparation. Stock solutions of individual native and mass-labeled compounds were prepared in methanol or LC-MS water at 500 and 100 µg/mL, respectively. Multiple sub-stock solutions of native compound mixtures were prepared in LC-MS water at a concentration range from 0.2 to 1000 ng.mL^{-1}. One solution of mixed mass-labeled analogs was prepared from primary stock solutions at 1 µg/mL in LC-MS water. Calibration standards solutions were prepared by adding 10 µL of the corresponding sub-stock solution of native analytes and 10 µL of mass labeled analog mixture into 880 µL of LC-MS water, or grab samples of river water matrix. 100 µL methanol was added to reach total volume of 1000 µL. The concentration of calibration standards ranged from 2 ng/L to 10000 ng/L.

Sample preparation

Grab samples of river water were prepared by spiking 10 µL internal standards mixture and 100 µL methanol into 890 µL river water sample, followed by filtration with 0.22 µm PTFE syringe filter (Canadian Life Science, Canada) before introduction into the LC-MS. All LC-MS samples were stored at -20°C in the dark.

Instrument analysis

Analysis were performed at McGill Bayen's Food Science Lab (Sainte-Anne-de-Bellevue, QC, Canada). Data were collected with an Agilent 1290 Infinity II LC system coupled to the 6545 Quadrupole-Time of Flight–Mass spectrometry (Q-TOF-MS) (Agilent Technologies, Santa Clara, USA). The LC separation was performed on a CS-MS C18 column (Agilent Technologies; 2.1 μm × 150 mm). The mobile phase A was LC-MS grade water with 0.1% formic acid and the mobile phase B was methanol with 0.1% formic acid. 5μM deactivator medronic acid (Agilent Technologies) was added into mobile phase A to reduce the possible interactions of polar analytes with stainless steel parts and improve the peak shape. The HPLC and MS parameters were optimized for positive (ESI+) modes based on the separation and MS responses of the nine targeted analytes. Samples were kept at 4°C in the multisampler compartment. HPLC parameters were as follows: the injection volume was 20 μL, the flow rate was 0.35 mL/min, the column temperature was 40°C. The elution gradient used was: 0.1% B (0 to 1.5 min), linear increase to 20% B (1.5 to 2 min), ramp to 40% B (2 to 4 min), ramp to 100% B (4 to 6 min). 100% B (6 to 8 min) and 0.1% B (8 to 8.5 min), and finally 0.1% B (8.5 to 10 min) with 3 min post-column run. Optimized MS conditions for ESI+ were: the drying gas temperature at 220 °C, drying gas flow rate at 11 L/min, sheath gas temperature at 300 °C, sheath gas flow rate at 11 L/min, nebulizer pressure at 30 psi, the capillary voltage at 3000 V, the fragmentor voltage at 100 V, the skimmer voltage at 65 V and the nozzle voltage t 1500 V. Full scan MS data were recorded at mass-to-charge ratio (*m/z*) range from 100 to 1100 with a scan rate of 2 spectra/s, and were collected at both centroid and profile mode. Two reference ions (*m/z* at 121.0508 and 922.0098) were used in ESI+ mode for automatic mass recalibration during data acquisition.

Targeted analysis of the compounds

Method validations were first conducted using standard solutions containing the 9 target compounds prepared in HPLC water. The quantification was performed using Quantitative Analysis 10.0 from Agilent MassHunter Workstation Software. For each target analyte, the most abundant isotope peak of either $[M+H]^+$ in ESI+ was selected as the quantifier ion. A mass extraction window (MEW) of ±20 ppm, was used for the present mass extraction and quantitative analysis.

For the quantification of each target analyte, a mass-labeled internal standard at constant concentration was used as internal standard to compensate any systematic errors subjected by native compounds. For each analyte, the relative response (RR) was calculated at each calibration point using the equations below:

$$RR=\frac{A_t/A_{is}}{C_t/C_{is}} \quad \text{(eq. 1)}$$

where A_t and A_{is} were the peak area of target analyte and corresponded mass-labeled internal standard respectively; and C_t and C_{is} were the concentration of target analyte and corresponded mass-labeled internal standard, respectively. The concentration of each target compound in the simulated matrix was calculated at the following equations:

$$C_t=\frac{A_t/A_{is}}{RR} \times C_{is} \quad \text{(eq. 2)}$$

where, mean RR values averaged from the whole calibration range were used for each target analyte.

Method performance was also assessed based on the linearity of the calibration response, method detection limits (MDLs; 3σ of the procedural blank signals), the method quantification limits (MQLs, 10σ of the procedural blank signals), precision, relative recoveries, and matrix effect. Recoveries of the target analytes were evaluated by spiking tests conducted at 3 levels (100, 1000 and 10000 ng L^{-1} ww, n=3 for each level) before filtration. Relative recovery (%) was calculated as the ratio of the peak area ratio for target analyte and its internal standard spiked into water prior to filtration, to the one for corresponded compounds spiked into the water without filtration. Matrix effects were assessed as the ratios of the slopes of matrix-matched calibration and the calibration in water.

Non-targeted analysis of the compounds

Suspect screening was performed on data from Richelieu River sample collected at Saint-Ours (downstream) on June 19th after heavy rain events and the control water, using the software

MassHunter Profinder B.10.00 from Agilent Technologies. Data treatment was first conducted in the "Batch Recursive Feature Extraction" mode (mass window ± 20 ppm; RT window ± 0.15 min; peak filter (height) 300 counts), where 4189 molecular features were aligned and extracted. These features were then statistically analyzed in Mass Profiler Professional (MPP) by applying a fold change ≥ 2. In total, 451 molecular features with abundance level at least two-times higher than in control water were extracted, and further screened against *Environmental Water Screening PCDL* and pesticide *PCDL* in IDBrowser. For the molecular features with assigned compound names, manual checks were conducted in Qualitative Analysis (Agilent) in terms of 1) HPLC peak shape, 2) peaks present in both river water aquarium and river water on June 19, 2018, 3) signals in river water aquariums and river water on June 19, 2018 were at least two-times higher than in control for both June 16 and 19, 2018, 4) all ion and targeted MS/MS fragmentation by comparing with MS spectral library. For the two tentatively identified compounds (oxybenzone and metribuzin), authentic standards were used to confirm their identification.

Water physico-chemical parameters

Water parameters in lab water and river water aquariums were similar with respect to temperature, DO and pH (Figure 3 A-C). Over the course of the experiment, natural river water temperature, in and around the aquariums, varied from 18° C to 22° C, and generally increased over time. The average temperatures in the lab water aquariums, river water aquariums, and the river itself were within 1°C throughout the entire experimental period (Figure 3A). Dissolved oxygen was almost at saturation in lab and river water aquariums (96-98%), and in the river itself (94% -103%) (Figure 3B). The pH in both lab and river water aquariums was stable (8.2 to 8.6) and similar to river pH (8.0 to 8.6) (Figure 3C).

Conductivity varied widely (138-294 µS/cm) in the 3 lab water aquariums. The initial conductivities of the 3 aquariums differed due to a human error in preparing the reconstituted hard water. This error was corrected after the initial loading of the aquariums and variations among lab water aquariums diminished as the experiment went on and water was replaced. Conductivity in the lab water aquariums increased over time, likely because evaporated water from the aquariums was replaced with reconstituted hard water rather than ultrapure water. Conductivity in the river water aquariums matched the actual river conductivity (178 to 218 µS/cm) (Figure 3D). Overall

conductivity was higher in the lab water group than in the river water group throughout the experiment. Changes in water conductivity can have an effect on fish due to increased energy expenditure relating to osmoregulation. Little information is available for fish early life stages but conductivity below 3700 µS/cm had no negative impact on sensitive aquatic fauna (Kennedy et al. 2003), which suggests that the variation between 150 and 300 us/cm observed in our study is unlikely to have affected the results.

Turbidity was negligible in the lab water aquariums throughout the experiment (0-1 Formazin Nephelometric Units (FNU)) and was higher and more variable in the river water aquariums (5 to 32 FNU). Overall, the river water aquariums matched the turbidity in the actual river (Figure 3E). The coarse sponge at the entrance of the waterflow likely filtered out larger particles, but some turbidity in the tumbler water was visible. Turbidity peaked in the river and the river water aquariums following the large rain event on June 18. High turbidity or fine sediment accumulation on fish embryos can cause entombment leading to asphyxia (Bowerman et al. 2014), but this was not observed in our experiment. Since turbidity accumulated in the river water aquariums (where it was measured) rather than in the tumblers which were blocked by a coarse sponge, turbidity was almost certainly lower in the tumblers. Additionally, the controlled waterflow prevented asphyxiation of the embryos, limiting any potential negative effects of turbidity on the endpoints measured.

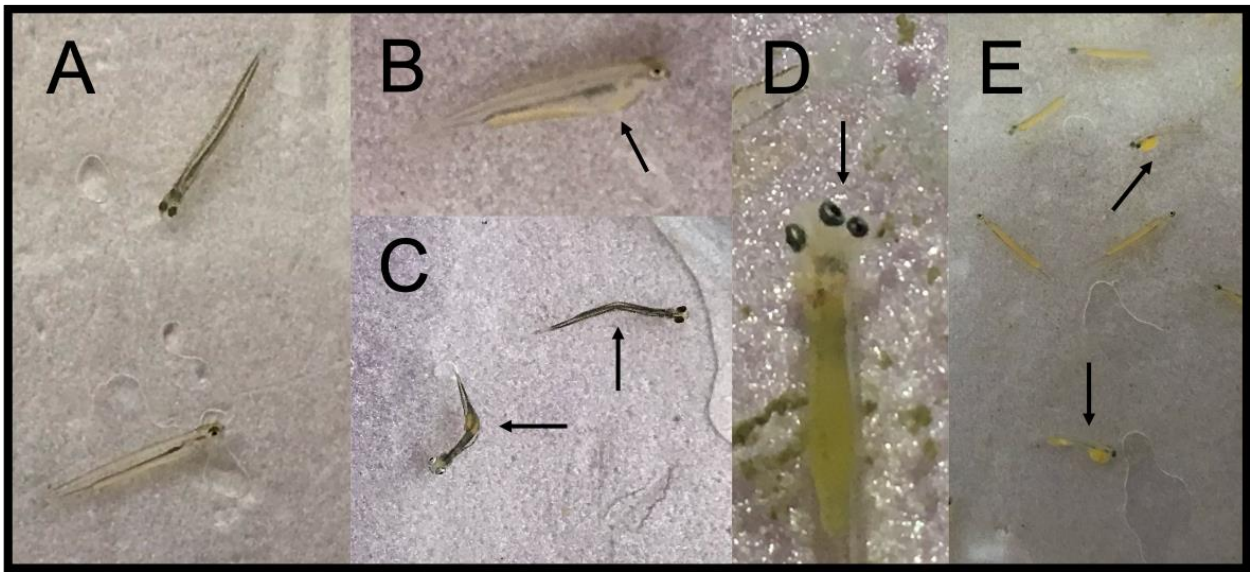

Figure 7: Examples of copper redhorse deformities observed. A) Normal larvae. B) Larva with edema in the thoracic region (identified by arrow). C) Larvae exhibiting different extents of spinal curvature (kyphosis/lordosis; arrows). D) Two-headed larva (arrow). E) Larvae with pinched yolk sac (and yolk sac edema; arrows).

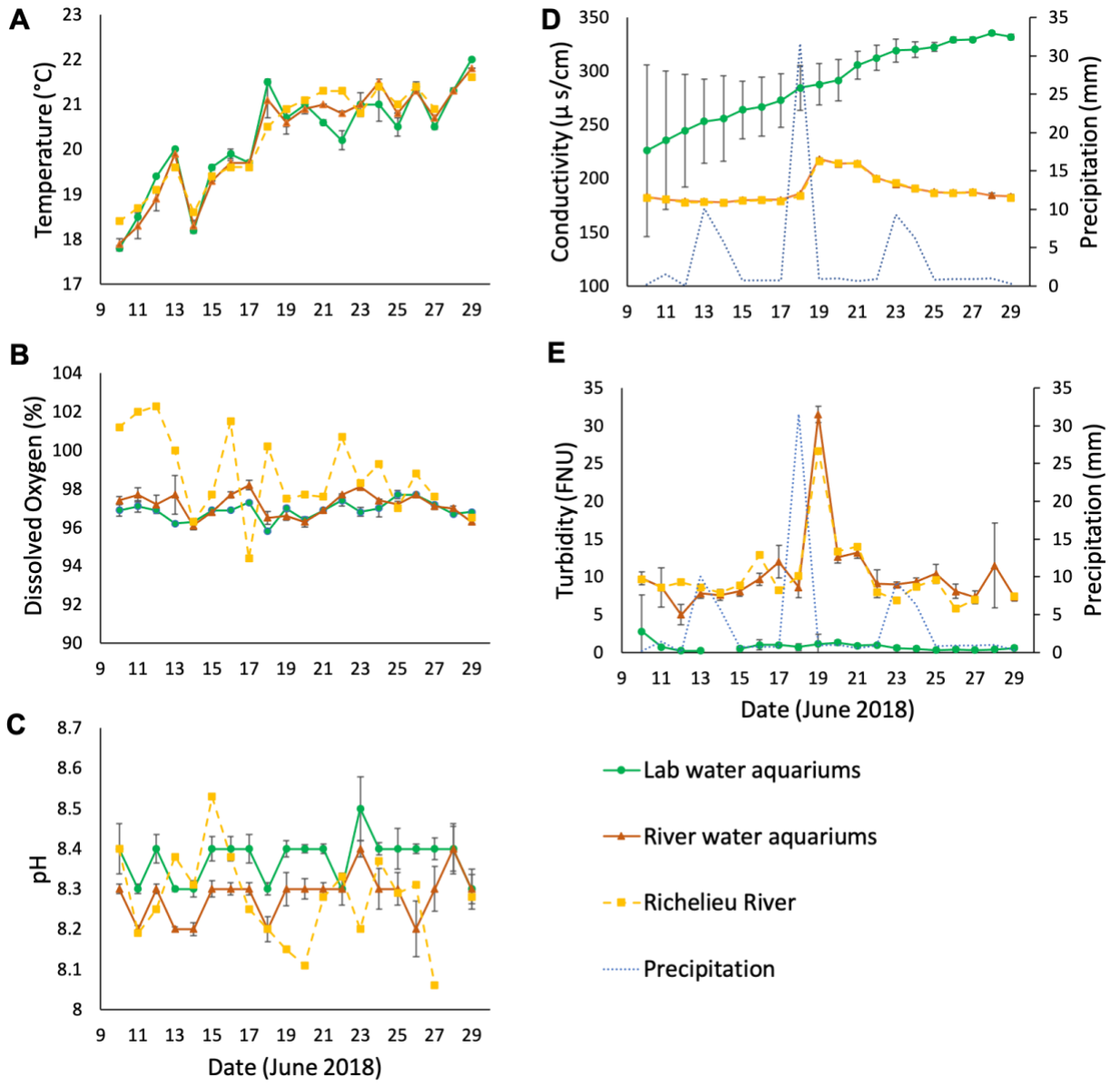

Figure 8: Water parameters, A) temperature, B) dissolved oxygen, C) pH, D) conductivity and E) turbidity, during the river and copper redhorse riverside exposure in June 2018. Parameters are reported as the average value measured in 3 laboratory water aquariums (control) (green solid line), 3 river water aquariums (red solid line) and in the Richelieu River itself (yellow dashed line). Error bars represent standard deviations amongst aquariums. For river water, a single value was taken each day. Daily precipitation data were collected at the l'Assomption station (QC), about 21 km away, by the Meteorological Service of Canada of Environment and Climate Change Canada (https://climate.weather.gc.ca).

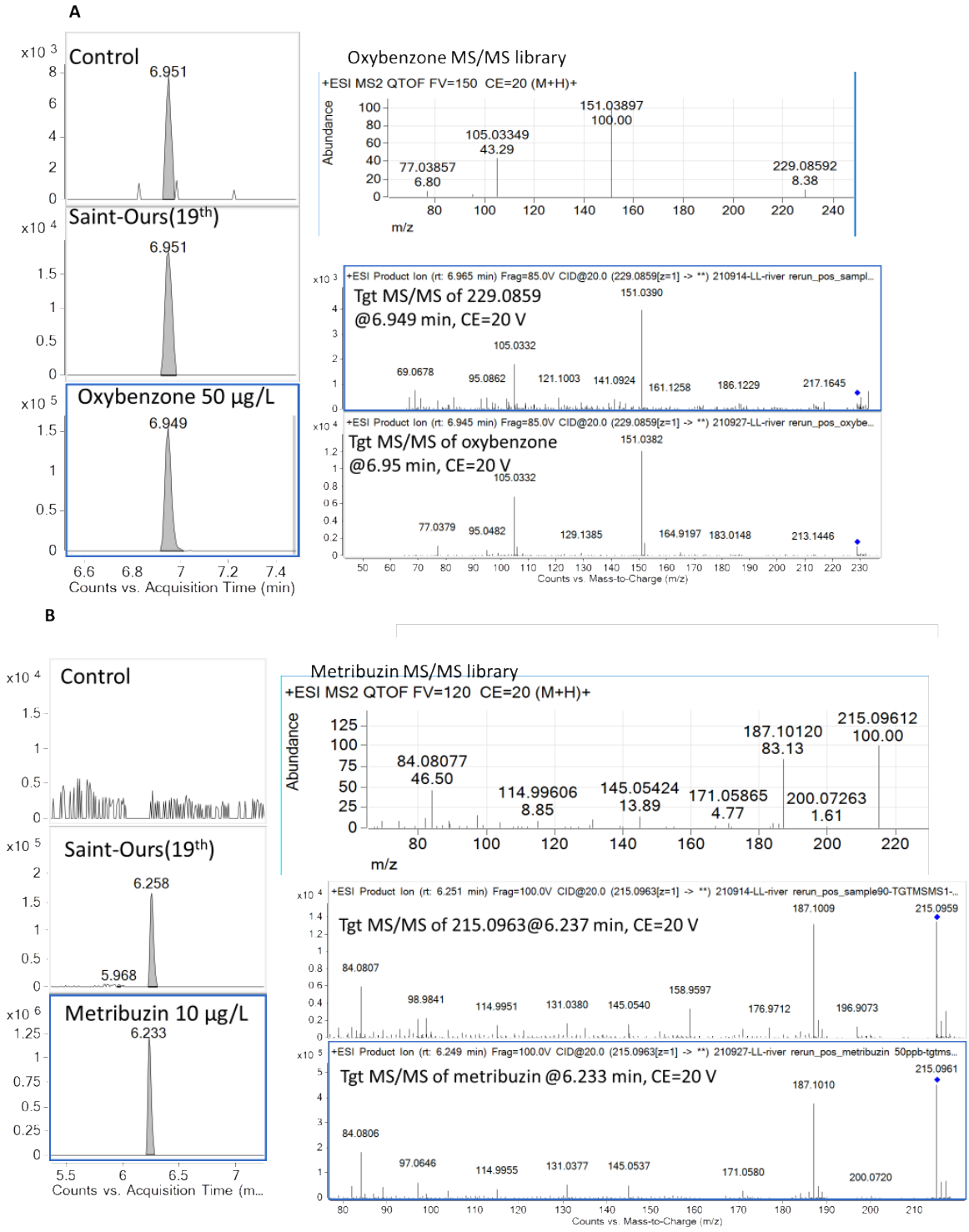

Figure 9: A includes the HPLC peak and MS/MS fragmentation information for the molecular feature m/z 229.0859 at 7.0 min and oxybenzone standard; B includes the HPLC peak and MS/MS fragmentation information for the molecular feature m/z 215.0963 at 6.2 min and metribuzin standard.

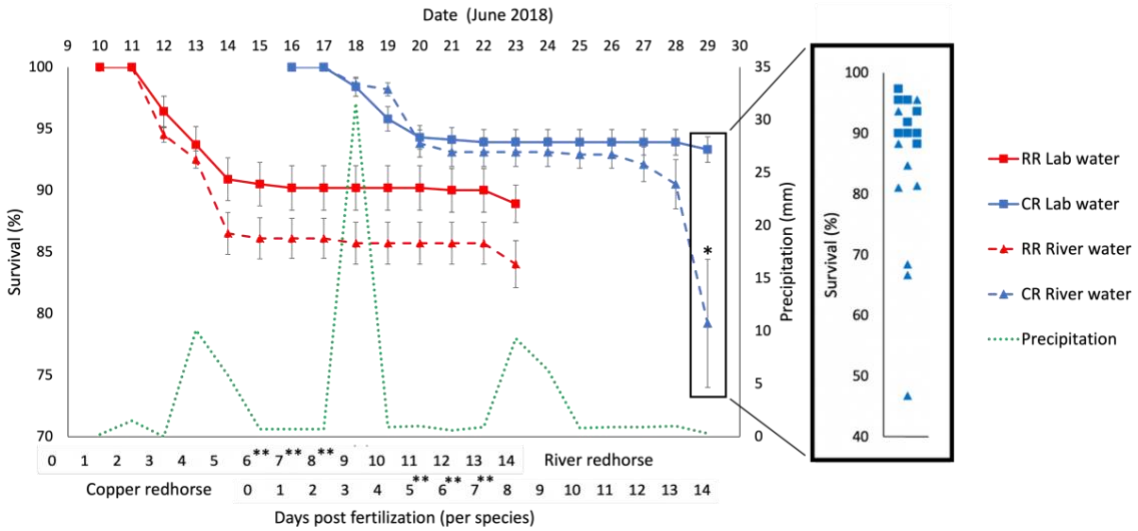

Figure 10: Effects of exposure to Richelieu River water on survival of copper redhorse (CR) and river redhorse (RR) embryos and larvae over the 14-day exposure period. Data are expressed as the percentage of live individuals over total embryos. Each data point represents the average of 9 egg tumblers, and error bars represent standard error. Significant differences between treatments are marked with an asterisk ($p < 0.05$). The dual asterisks mark the days during which the embryos were hatching. Daily precipitation data were collected at the l'Assomption station (QC) by the Meteorological Service of Canada of Environment and Climate Change Canada (https://climate.weather.gc.ca). Given the strong relationship between precipitation and pesticide concentrations in the water column (Figure 4), these data are a surrogate for the total pesticide concentration over time. On the right side panel are the survival data on day 14 post-fertilization for the individual copper redhorse incubators.

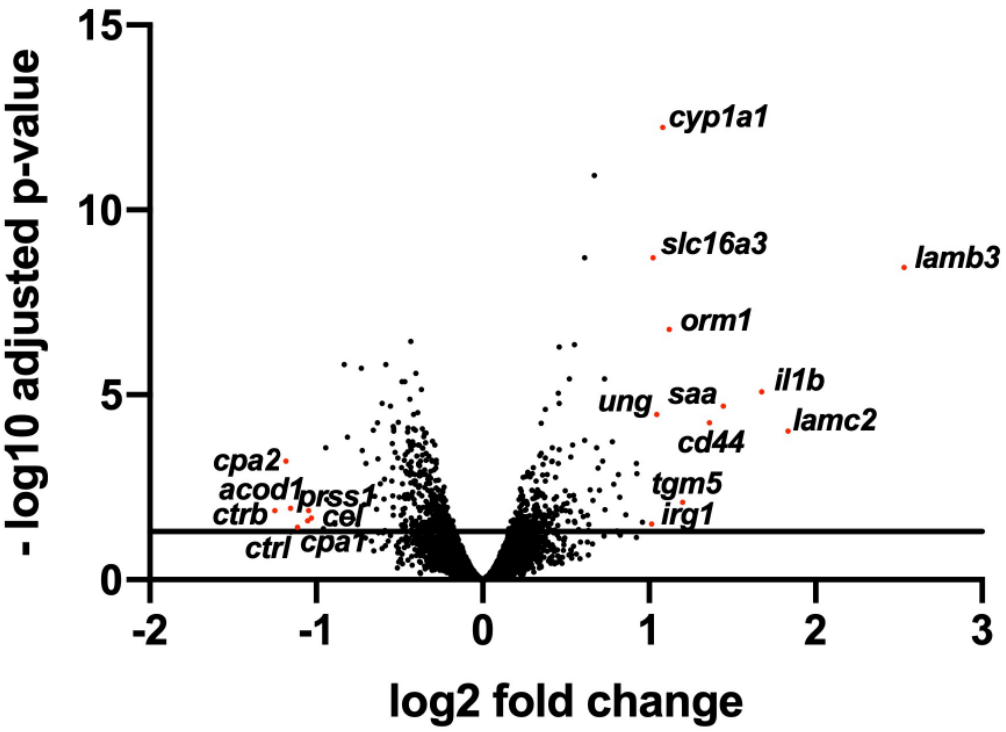

Figure 11: Volcano plot of differentially expressed genes (DEGs) from copper redhorse exposed to river water for 14 days post-fertilization. The labelled Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthologues (KOs) in red were significantly differentially expressed (adjusted p value < 0.05 and absolute log2 fold change > 1) when compared to the copper redhorse exposed to lab water.

Table 2. Targeted LC/MS method detection limits, method quantification limits, instrument detection limits, relative recoveries, and matrix effects and precision of chemicals analyzed.

Compounds	Mass to charge ratio(m/z, $[M+H]^+$)	Retention time RT (min)	Method Detection Limit MDLs (ng/L)	Method Quantification Limit MQLs (ng/L)	Instrument Detection Limits IDLs (pg injected)	Mean relative recovery (%, n=9)	Matrix effect (%)	Intraday Precision (RSD%)
Atrazine	216.101	6.955	0.97	3.23	0.31	87±9%	14%	5%
Metolachlor	284.1412	7.245	3.18	10.60	0.14	100±12%	8%	1%
Imazethapyr	290.1499	6.607	2.33	7.77	0.09	102±11%	-11%	9%
Clothianidin	250.016	5.886	6.26	20.87	0.72	127±11%	-35%	3%
Thiamethoxam	292.0266	5.091	0.24	0.79	0.39	83±8%	9%	6%
Imidacloprid	256.0596	5.729	0.19	0.63	0.18	92±9%	8%	6%
Chlorantraniliprole	481.9786	6.988	21.00	70.00	1.45	90±7%	-7%	5%
Glyphosate	170.0213	2.306	124.18	413.93	15.04	88±11%	5%	16%
AMPA	112.0158	1.022	321.53	1071.77	22.51	99±15%	-18%	33%
Atrazine-D_5	221.1324	6.938						
Metolachlor-D_6	290.1788	7.237						
Clothianidin-D3	253.0348	5.886						
Imidacloprid-D_4	260.0847	5.704						
AMPA-$^{13}C_{15}ND_2$	116.0287	0.7742						
Glyphosate-D_2	172.0344	2..298						

AMPA: Aminomethylphosphonic acid

Table 3. Summary table of means and ranges of the pesticide concentrations detected in the laboratory water aquariums, river water aquariums and the Richelieu River itself in June 2018. Chlorantraniliprole and imidacloprid were also targeted but were not detected in any samples.

Compounds	**Atrazine**		**Clothianidin**		**Glyphosate**		**AMPA***		**Imazethapyr**		**Metolachlor**		**Thiamethoxam**	
Treatments	Mean (ng/L)	Range (ng/L)	Mean (ng/L)	Range (ng/L)	Mean (ng/L)	Range (ng/L)	Mean (ng/L)	Range (ng/L)	Mean (ng/L)	Range (ng/L)	Mean (ng/L)	Range (ng/L)	Mean (ng/L)	Range (ng/L)
Lab water aquarium	33	13-57	-	-	8	0-155	166	0-1441	-	-	15	3-28	<1	0-6
River water aquarium	47	11-298	8	0-89	24	0-291	785	0-3032	21	0-244	189	20-1752	4	0-32
River water	36	7-257	2	0-43	29	0-201	844	0-3940	14	0-179	131	13-1247	3	0-24

AMPA: Aminomethylphosphonic acid

Table 4. Summary table of the deformities observed in river and copper redhorse larvae exposed to either river water or laboratory water matching river water physio-chemical parameters. Each treatment started with 495 larvae per species.

Species *	Treatment	Kyphosis/ lordosis Light to moderate (N=)	Kyphosis/ lordosis High (N=)	Edema (N=)	Shortened tail (N=)	Craniofacial malformation (N=)	Pinched yolk sac (N=)	Total number (N=)
River Redhorse	Lab water	18	0	1	0	1	0	20
	River water	9	0	2	2	0	1	14
Copper Redhorse	Lab water	28	5	1	8	0	3	46
	River water	32	3	6	3	2	14	60

Table 5. Differentially expressed Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthologues (KOs) from copper redhorse exposed to river water.

Gene name	Gene abbreviation	KO#	Log_2 FC
Laminin beta 3	*lamb3*	K06244	2.532
CD 44 antigen	*cd44*	K06256	1.836
Interleukin 1 beta	*il1B*	K04519	1.677
Laminin gamma 2	*lamc2*	K06246	1.445
Uracil-DNA glycosylase 2	*ung*	K10861	1.363
Transglutaminase 5	*tgm55*	K05622	1.201
Alpha-1-acid glycoprotein	*orm1*	K17308	1.121
Cytochrome P450 family 1 subfamily A1	*cyp1a1*	K07408	1.082
Serum amyloid A protein	*saa*	K17310	1.047
Solute carrier family 16 member 3	*slc16a3*	K08180	1.024
Aconitate decarboxylase	*acod1*	K17724	1.015
Bile salt-stimulated lipase	*cel*	K12298	-1.030
Trypsin	*prss1*	K01312	-1.046
Carboxypeptidase	*cpa1*	K08779	-1.050
Chymotrypsin-like protease	*ctrl*	K09632	-1.112
Pancreatic elastase II	*cela2a*	K01346	-1.153
Chymotrypsin	*ctrb*	K01310	-1.249
Carboxypeptidase A2	*cpa2*	K01298	-1.812

Table 6. Gene Set Enrichment Analysis (GSEA) for differentially expressed Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthologues (KOs) from copper redhorse exposed to river water. Genes with positive enrichment score were over expressed and gene with negative enrichment score (grey shading) were under expressed.

	Name	Enrichment Score	Adjusted p value
1	DNA replication	0.882	0.002
2	Mismatch repair	0.818	0.002
3	One carbon pool by folate	0.792	0.002
4	Valine, leucine and isoleucine degradation	0.784	0.002
5	Citrate cycle (TCA cycle)	0.776	0.002
6	Fatty acid degradation	0.753	0.002
7	Nucleotide excision repair	0.751	0.002
8	Butanoate metabolism	0.722	0.002
9	Propanoate metabolism	0.713	0.002
10	Fat digestion and absorption	0.711	0.002
11	Base excision repair	0.706	0.002
12	Homologous recombination	0.689	0.002
13	Drug metabolism - other enzymes	0.671	0.002
14	Glyoxylate and dicarboxylate metabolism	0.651	0.002
15	Folate biosynthesis	0.638	0.007
16	Fanconi anemia pathway	0.635	0.002
17	Tyrosine metabolism	0.623	0.006
18	Fatty acid metabolism	0.622	0.002
19	Carbon metabolism	0.619	0.002
20	Pyrimidine metabolism	0.619	0.002
21	Fatty acid elongation	0.613	0.010
22	Glycine, serine and threonine metabolism	0.590	0.002
23	beta-Alanine metabolism	0.586	0.012
24	Cell cycle	0.579	0.002
25	Pyruvate metabolism	0.577	0.008
26	Protein digestion and absorption	0.575	0.002
27	Cysteine and methionine metabolism	0.572	0.003
28	Lysine degradation	0.565	0.002
29	Oxidative phosphorylation	0.533	0.002
30	Arginine and proline metabolism	0.520	0.014
31	Aminoacyl-tRNA biosynthesis	0.514	0.024

32	RNA polymerase	0.506	0.035
33	PPAR signaling pathway	0.498	0.008
34	Oocyte meiosis	0.474	0.006
35	Biosynthesis of amino acids	0.451	0.029
36	Cholesterol metabolism	0.449	0.022
37	Progesterone-mediated oocyte maturation	0.420	0.040
38	Purine metabolism	0.411	0.002
39	Thermogenesis	0.410	0.002
40	Ribosome	0.406	0.002
41	cAMP signaling pathway	-0.356	0.006
42	Axon guidance	-0.362	0.010
43	Autophagy - animal	-0.389	0.012
44	Neuroactive ligand-receptor interaction	-0.395	0.002
45	Oxytocin signaling pathway	-0.400	0.005
46	Chemokine signaling pathway	-0.403	0.005
47	Aldosterone synthesis and secretion	-0.404	0.031
48	Endocytosis	-0.415	0.002
49	Serotonergic synapse	-0.418	0.008
50	Circadian entrainment	-0.420	0.007
51	Estrogen signaling pathway	-0.421	0.008
52	Cell adhesion molecules (CAMs)	-0.422	0.006
53	GABAergic synapse	-0.463	0.003
54	Cholinergic synapse	-0.464	0.002
55	Long-term depression	-0.468	0.011
56	Dopaminergic synapse	-0.500	0.002
57	Taste transduction	-0.546	0.004
58	Glutamatergic synapse	-0.560	0.002
59	Long-term potentiation	-0.567	0.002
60	Synaptic vesicle cycle	-0.659	0.002

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Preface to Chapter 4

The previous chapter demonstrated that exposure to river water during ELS caused premature hatching of both river and copper redhorse and decreased the survival of the latter only. I have also shown that exposure to river water causes alteration in expression of genes related to the immune system and metabolism. I have also shown that pesticide concentrations peak at Saint-Ours along the Richelieu River following heavy rain events.

In this chapter I performed a more holistic, temporal and spatial analysis of the contamination of the Richelieu River and two of its tributaries to better understand the exposome of river and copper redhorse. Preliminary water samples were collected in winter, far from the agricultural season, to determine baseline contamination of the river. Then a complementary sampling approach was used in copper redhorse spawning grounds using passive samplers and daily grab samples during the river and copper redhorse spawning season. The data collected provided an overview of the river contamination, demonstrated peaks in pesticide concentrations following rain events during the fish spawning season. We also identified two insecticides that were above toxicity thresholds for aquatic life. Identifying the level of pesticide contamination in the river was the second step in understand the effects of early life exposure to environmental pesticides in wild fish.

Chapter 4

4. Pesticide concentrations in the Richelieu river and its tributaries during the spawning period of threatened and endangered fishes.

4.1 Title page

Title of the manuscript

Pesticide concentrations in the Richelieu river and its tributaries during the spawning period of threatened and endangered fishes.

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helped in the field. We thank Jean-François Roy (Agilent Technologies) for providing the CS-C18 column.

Conflict of interest

The authors declare that there is no conflict of interest.

4.2 List of Figures

4.3 List of Tables

4.4 Abstract

Many fishes spawn in the Richelieu River (Quebec, Canada) during the spring and summer months. This is also a period of intensive agricultural activity when pesticides are applied and leach from adjacent fields into the Richelieu and its tributaries. Here we provide a detailed temporal data describing concentrations of pesticides and other contaminants in the Richelieu River and two tributaries throughout the month of June, 2018. Our complementary and thorough approach based on targeted temporal trends, and integrative passive sampling, captures in detail what early life stage fishes may be exposed to during a sensitive developmental period. Several species of fish spawn in the Richelieu River during this month, including the threatened river redhorse (*Moxostoma carinatum*) and the endangered copper redhorse (*Moxostoma hubbsi*). We detected a total of 69 compounds in river water, of which 31 were pesticides (12 herbicides, 16 insecticides and 3 fungicides) including many that have been previously banned. The remaining compounds included 12 polybrominated diphenyl ethers (PBDEs) and 26 pharmaceutical and personal care products (PPCPs). Pesticide concentrations varied from ng/L to µg/L levels, and peaked following rainfall. Notably the 2 neonicotinoids, thiamethoxam and clothianidin, were detected at concentrations exceeding the Canadian freshwater chronic safety thresholds for aquatic life.

4.5 Introduction

Global increases in agricultural activities and pesticide use have occurred over the last half century (Fulton et al. 2013). This is concerning, in part, due to increasing contamination of surface water with pesticides and associated threats to aquatic biodiversity (Stehle & Schulz 2015). To increase crop yields, multiple pesticides are often applied to crops either together or in succession, which can lead to contamination of surface waters with a cocktail of compounds to which aquatic fauna are continuously exposed (de Souza et al. 2020). Moreover, in mixed use watersheds containing both urban areas and agricultural fields, pesticides in streams and rivers are also accompanied by other types of compounds sourced from urban runoff and municipal wastewater. Collectively these compounds may pose risks to various taxa, including algae (de Baat 2018), invertebrates (Morrissey 2015), crustaceans (Zaleska-Radziwill et al. 2011), and fishes (Zaleska-Radziwill et al. 2011).

The current study focuses on pesticides and other contaminants in surface water from the Richelieu River and two of its tributaries: the Acadie and Huron Tributaries. The Richelieu River originates from Lake Champlain (New York/Vermont) in the United States and flows northward to the Saint-Lawrence River (Quebec) in Canada (Figure 1). Up to 70% of the Richelieu River's watershed is dedicated to agriculture (Simoneau & Thibault 2009). Additionally, the river is bordered by 64 municipalities and 26 municipal wastewater treatment facilities. At least 80 species of fishes inhabit the Richelieu River's watershed (MFFP 2018), and it is a spawning location for many threatened species such as lake sturgeon (*Acipenser fulvescens*) (Thiem et al. 2013), the river redhorse (*Moxostoma carinatum*) (COSEWIC 2015), as well as the endangered copper redhorse (*Moxostoma hubbsi*) (COSEWIC 2014). Legacy organic contaminants such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and dioxins and furans have been monitored in Richelieu River water consistently since the early 2000s (Laliberté 2020). These compounds have all remained stable over the last 20 years and maximum concentrations are consistently below the provincial criteria for the prevention of aquatic organism contamination (CPCO).

Pesticide concentrations have been monitored in the Huron River, one of the Richelieu River tributaries, consistently since the early 1990s. Pesticide concentrations above the safety threshold for the protection of aquatic life have been reported for both the Acadie and Huron Tributaries (Giroux 2015 & 2019), as well as in the Richelieu River itself (Marchand et al. 2022). Between 2015 and 2017, 70% of the surface water samples collected from the Huron River had clothianidin and thiamethoxam concentrations exceeding the provincial surface water quality criteria for the protection of aquatic life (Giroux 2019). In the spring including during the copper redhorse spawning period, pesticide concentrations in river water peak following heavy rain events, a trend that has been consistently observed over many years of monitoring (Giroux 2015 & 2019). For example, baseline total pesticide concentrations in the water of tributaries of the Richelieu are typically in the ng/L range (Giroux 2015), but following heavy rain events, concentrations can be 10 times higher than the baseline levels. Contaminant monitoring in the Acadie and Huron Tributaries is part of a larger effort across Quebec's highly agricultural Saint-Lawrence Valley (Giroux 2015 & 2019). Though water quality monitoring has provided valuable data for contamination trends in the watershed since the early 1990s, due to the watershed's size monitoring efforts have provided an overview of pesticide concentrations at a large spatial scale and coarse

temporal resolution. In this critical habitat for fish, understanding the pattern of contamination at a finer temporal scale during critical periods of embryonic development for aquatic species is important. Precise daily variations of pesticide concentrations in small river systems of the region are poorly understood since previous data were collected over multiple day intervals.

Accordingly, the objective of the current study was to screen pesticides and other contaminants in surface waters of the Richelieu River, and two of its tributaries: the Acadie and Huron Tributaries. We specifically targeted two sites in the Richelieu River (Chambly and Saint-Ours) that are the only known spawning areas for the endangered copper redhorse. The two sites are also used for spawning by other native threatened fishes, including the river redhorse and both lake and Atlantic sturgeon. Daily grab samples of surface water and passive samplers were used as complementary methods to assess water contamination over broader and finer time scales. The collection of water from two major tributaries, helped us to understand how and when pesticides reach the Richelieu River. We focussed on a window of time that encompasses spawning and the first three to four weeks of development for many native fishes. Though this period is relatively short, it is a critical developmental period during which fishes are most sensitive to contaminants (Mohammed 2013). Our data will improve our understanding of which mixtures early life stage fishes and other aquatic fauna are exposed to in the environment and how these mixtures vary spatially and temporally in an ecologically important river system.

4.6 Materials and Methods

Study sites

We sampled water from four different sampling sites. The first two are spawning grounds, used by lake sturgeon, Atlantic sturgeon, river redhorse and copper redhorse, along the Richelieu River, in the Chambly basin (45.454787, -73.273872) and below the Saint-Ours dam (45.863251 -73.149889) (**Figure 12**). The two other sampling sites were at the mouth of two of the larger tributaries entering the Richelieu River: Acadie (45.480126, -73.280649) and Huron (45.458674, -73.258830) Tributaries (**Figure 12C**). The Chambly sampling site is upstream of where the two tributaries join the Richelieu River and the Saint-Ours sampling site is downstream (**Figure 12C**).

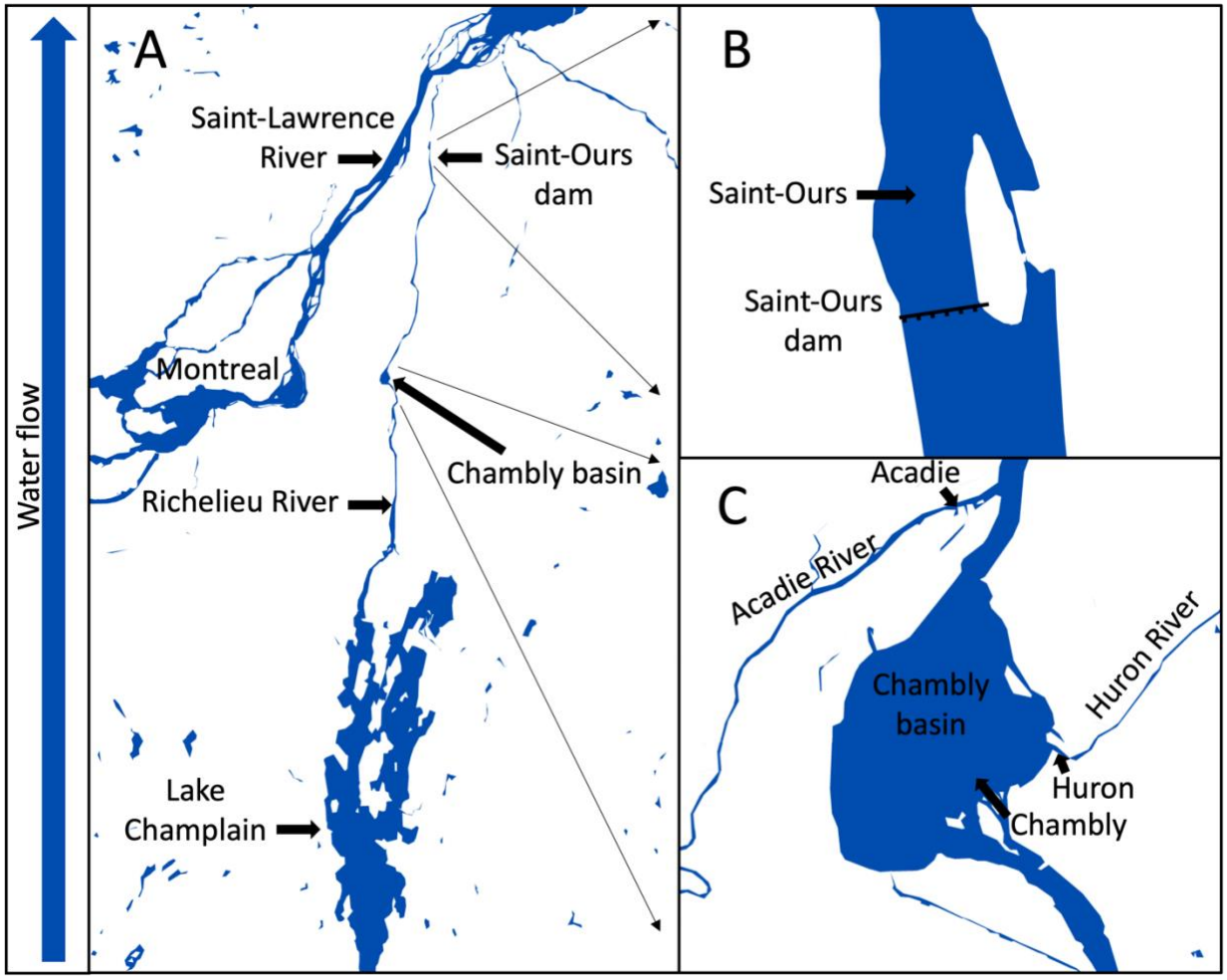

Figure 12: Map of the Richelieu River (Quebec, Canada) indicating the locations of water sampling. B) The Richelieu River is located in southeastern Canada. The Richelieu River is east of Montreal and flows from Lake Champlain (USA) to the St. Lawrence River (Canada). B) The Saint-Ours sampling site (indicated with the arrow) is downstream of the Saint-Ours Dam. This is the most downstream sampling location. Copper redhorse spawn in the rapids downstream of the Saint-Ours Dam. Passive samplers were also deployed at this location. C) The Acadie Tributary, Huron Tributary and Richelieu River Chambly sampling sites (indicated with the arrows) are in and around the Chambly basin. Chambly is the most upstream sampling location. Copper redhorse also spawn in the Chambly rapids within the Chambly basin. Passive samplers were also

deployed at the Richelieu River Chambly location. The two tributaries that pour into the Richelieu (Acadie and Huron) were sampled as close as possible to the Richelieu River.

Sampling

A set of preliminary surface water grab samples (n = 2 per location and 2 field blanks) were collected, outside of the agricultural season, in the Richelieu River at both Richelieu River (Chambly and Saint-Ours) locations on December 17, 2017. Then from June 5th to July 6th (2018), two complementary water sampling methods were used; daily grab samples and passive samplers. This period include both of river and copper redhorse spawning period which span from early to mid-June and from mid-June to early July respectively. Daily grab samples of water were collected on most days of the sampling period from the two locations in the Richelieu River and at the mouths of the two tributaries. For both the Richelieu River (Chambly and Saint-Ours sites), water was sampled daily below rapids where water was well mixed. For both Acadie and Huron Tributaries, surface water was sampled as close to the Richelieu River as possible. Daily grab samples were hand-collected following the Canadian Council of Ministers of the Environment (CCME) protocol (2011) using 50 mL polyethylene tubes. At each location four tubes filled to 40mL were collected. Once collected, samples were kept on ice packs in a cooler in the field before being kept frozen at -20°C until they were analyzed. The data from the Richelieu River Saint-Ours sampling location were previously presented in Marchand et al. 2022 in order to give context to results on the biological effects of river water exposure to early life stage river and copper redhorse at this site. Water quality measurements (pH, dissolved oxygen, temperature, conductivity, turbidity) were collected at each sampling location using a YSI ProDSS multiparameter water quality meter at the time of sampling.

Polar organic chemical integrative samplers (POCIS) (Environmental Sampling Technologies Inc, USA) were deployed to estimate bioavailable pesticides in the water over the month of June, which encompasses most of the river and copper redhorse breeding seasons. In total, four passive sampler units were deployed in the Richelieu River; two units in Chambly (45.452441; -73.278446 & 45.455628; -73.273586) and two units in Saint-Ours (45.864353; -73.148476 & 45.864175; -73.149934). Each unit consisted of 6 POCIS discs placed in a metal cage (total of 24 discs deployed). Passive sampler units were attached to chain-anchored buoys approximately 1 meter below the surface. Passive samplers were deployed near copper redhorse spawning grounds in areas with a high level of turbulence indicating that water was well mixed. Mixing of water was confirmed by water quality measurements (conductivity, dissolved oxygen, pH, temperature,

turbidity) at different depths using a YSI probe. The POCIS membrane were kept in sealed containers until deployment, when they were rapidly and carefully inserted in the protective unit using nitrile gloves. Passive samplers were deployed on June 8th, 2018 in Chambly and on June 9th, 2018 in Saint-Ours. All passive samplers were recovered on July 6th, 2018 (29 days in Chambly, 28 days in Saint-Ours). Once recovered, POCIS disks were kept in sealed containers in a cooler on ice packs and were sent overnight to SGS Axys (Sidney, British Columbia).

Preliminary off-season screening

The preliminary winter water samples were sent to SGS Axys (Sidney, British Columbia) for multi-residue pesticides (including glyphosate and aminomethylphosphonic acid (AMPA)), polybrominated diphenyl ethers (PBDEs) and pharmaceuticals and personal care products (PPCPs) analysis. Concentrations of pesticides in water were assessed following the US EPA method 1699 using high resolution gas chromatography and high resolution mass spectrometry (EPA 2007). Concentrations of PBDE and congeners in water was assessed following US EPA method 1614A using isotope dilution and high resolution gas chromatography and high resolution mass spectrometry (HRGC/HRMS) (EPA 2010). Concentrations of pharmaceuticals and personal care products in water was assessed following US EPA method 1694 using high performance liquid chromatography combined with mass spectrometry (HLPC/MS/MS) (EPA 2007). These results also helped to guide selection of 9 compounds for the targeted analysis.

Targeted analysis of daily grab samples

Nine pesticides were selected for targeted analysis, these pesticides were previously detected most often and/or with the highest concentrations in the Acadie and Huron Tributaries (Giroux 2015). Grab water samples were analyzed using targeted liquid chromatography/mass spectrometry (LC-MS) to identify 9 pesticides (AMPA, atrazine, clothianidin, chlorantraniliprole, glyphosate, imazethapyr, imidacloprid, metolachlor, thiamethoxam) and their concentrations. Details of standard solution preparation, sample preparation and instrumental methods are given in Marchand et al. 2022. Briefly, water samples were prepared by spiking a 10 µL internal standard mixture and 100 µL methanol into 890 µL of river water, followed by filtration with a 0.22 µm PTFE syringe filter (Canadian Life Science, Canada) before introduction into the LC-MS. Mass-labeled standards at constant concentration were used as an internal standard to compensate for any

systematic errors due to native compounds (as recommended in USEPA methods (e.g. Method 1694)). Data were collected with an Agilent 1290 Infinity II LC system coupled to the 6545 Quadrupole-Time of Flight–Mass Spectrometry (Q-TOF-MS) (Agilent Technologies, Santa Clara, USA). The LC separation was performed on a CS-C18 column (Agilent Technologies; 2.1 μm × 150 mm). Method performances were assessed in terms of linearity of the calibration response, method detection limits, the method quantification limits, precision, relative recoveries, and matrix effects.

Passive Samplers

At SGS Axys, each POCIS was individually extracted using methanol. Half of the six samplers extracted from each location were combined and then concentrated using rotary evaporation and nitrogen concentration. Extracts were transferred to analytical vials and laboratory blanks and mass-labeled spiked samples were prepared. Blanks, spiked samples, and POCIS extracts were analyzed for multi-residue pesticide (50 parent compounds and 25 degradation products) (Supporting information, **Table 11**) analysis by gas chromatography/mass spectrometry (GC-MS). Extracts from the remaining three samplers were combined, sealed in 1 mL amber glass ampullae and stored at -20°C.

Data analyses

Average concentrations per compounds were calculated from the two preliminary winter samples for the Richelieu River (Chambly and Saint-Ours locations). The averages per compound were then compared between the two locations and with the summer concentrations. For the targeted compounds, we used the daily values to determine the range of concentrations and the highest concentrations for each location. Concentration ranges and maximum concentrations were potted and visually compared between locations. We plotted precipitation data (obtained from the Government of Canada website (https://climate.weather.gc.ca) from the l'Assomption (QC) station which is the closest (~21 km from Richelieu River Saint-Ours site) to our sampling locations) with measured concentrations to establish a relationship. For the passive samplers, average water pesticide concentrations were obtained by dividing POCIS concentrations by individual sampling rates (Supporting information, **Table 12**) and time deployed in days (Alvarez et al. 2007). For each location, the average of the 2 deployed passive sampler units was calculated.

4.7 Results

Water Parameters

Water temperature was closely matched in the tributaries (Huron and Acadie) and the Richelieu River sites (St. Ours and Chambly) and increased throughout the month of June. On average, conductivity and turbidity were higher in the tributaries than in the Richelieu River, whereas dissolved oxygen and pH were higher in the Huron Tributary. All water parameters appeared to be affected by precipitation. In all the sampling locations, pH, percentage dissolved oxygen and temperature decreased following heavy rain events while turbidity increased. Conductivity decreased in the tributaries following heavy rain events while the opposite effect was observed in the Richelieu River (Supporting information**, Figure 14A-E**).

Preliminary off-season screening

Analysis of winter grab samples focussed on pesticides, PBDEs and PPCPs. In total, across the 2 sampling locations along the Richelieu, we detected 50 of the 291 analytes targeted in the water (**Table 7**). These included 12 pesticides, 12 PBDEs and 26 PPCPs (**Table 7**). Of the 50 analytes detected, 48 and 42 were detected in the Richelieu River sites (Chambly and Saint-Ours respectively). Two PBDEs (2,2',3,3',4,5,5',6,6'-NoBDE and 2,2',3,4,4',5',6-HpBDE) were only detected in the Richelieu River Saint-Ours site. Five PPCPs (10-hydroxy-amitriptyline, amphetamine, clarithromycin, Diphenhydramine and Oxazepam) and 3 pesticides (dieldrin, endrin and endrin ketone) were only detected in the Richelieu River Chambly site.

Table 7. Contaminant concentrations (ng/L) measured in winter samples (collected in December 2017) from the Richelieu River Chambly and Saint-Ours sampling locations. The concentration detected in each sample (n=2 per location) is presented. Canadian environmental water quality guidelines values for the protection of aquatic life (long term) are only available for atrazine (1,800 ng/L), glyphosate (800,000 ng/L), metolachlor (7800 ng/L) and simazine (10,000 ng/L). No concentrations exceeded these guidelines. Concentration preceded by "<" are below the detection limit, which is given as the value.

1		Concentrations ng/L	
Type	Compounds	Chambly	Saint-Ours
PBDEs	2,2',3,3',4,4',5,5',6,6'-DeBDE	<0.3, 0.6	<0.3, 0.4

	2,2',3,3',4,4',5,6,6'-NoBDE	<0.001, 0.089	<0.001, 0.005
	2,2',4,4',5-PeBDE	0.02, 0.02	0.024, 0.062
	2,2',4,4'-TeBDE	0.024, 0.024	<0.020, 0.026
	2,2',3,3',4,4',5,5',6-NoBDE	<0.020, 0.046	<0.020, 0.024
	2,2',3,3',4,5,5',6,6'-NoBDE	<0.001, <0.001	<0.001, 0.038
	2,2',4,4',6-PeBDE	<0.002, 0.005	0.005, 0.013
	2,2',3,4,4',5,5',6-OcBDE	0.002, 0.009	<0.001, 0.005
	2,2',4,4',5,6'-HxBDE	<0.002, 0.003	<0.002, 0.005
	2,2',4,4',5,5'-HxBDE	<0.002, 0.003	<0.002, 0.006
	2,2',3,4,4'-PeBDE	0.001, 0.002	0.002, 0.004
	2,2',3,4,4',5',6-HpBDE	<0.001, <0.001	<0.001, 0.003
PPCPs	Metformin	495, 497	497, 578
	Acetaminophen	346, 349	204, 258
	Caffeine	220, 238	195, 235
	Iopamidol	238, 258	178, 193
	Theophylline	145, 169	150, 178
	1,7-Dimethylxanthine	112, 125	123, 135
	Valsartan	24, 28	27, 27
	DEET	14, 15	8, 23
	Venlafaxine	9.4, 10.2	6.2, 6.6
	Benzoylecgonine	6.1, 6.5	7.1, 7.1
	Cotinine	6.7, 6.8	6.2, 6.8
	Atenolol	3.9, 4.3	4.0, 4.2
	Carbamazepine	3.4, 4.3	2.8, 4.3
	Ranitidine	2.8, 3.2	2.7, 2.7
	Sulfamethoxazole	2.4, 2.6	1.8, 1.9
	Diltiazem	1.4, 1.7	1.0, 1.1

	Oxazepam	<4.3, 4.4	<4.3, <4.3
	Cocaine	0.93, 0.95	0.66. 1.10
	Citalopram	1.1, 1.3	0.5, 0.8
	Sertraline	0.6, 0.7	0.6, 0.7
	Clarithromycin	<1.6, 2.3	<1.6, <1.6
	Triamterene	0.5, 0.6	0.4, 0.5
	Amphetamine	<1.6, 1.7	<1.6, <1.6
	Desmethyldiltiazem	0.4, 0.5	0.3, 0.3
	Diphenhydramine	<0.6, 0.7	<0.6, <0.6
	10-hydroxy-amitriptyline	0.21, 0.29	<0.20, <0.20
Pesticides	Aminomethylphosphonic Acid [AMPA]	57, 60	47, 48
	Atrazine	30, 31	32, 33
	Glyphosate	24, 25	21, 48
	Metolachlor	18, 18	16, 16
	Desethylatrazine	6.1, 9.5	5.8, 6.8
	Simazine	1.7, 2.9	2.5, 2.6
	Dimethenamid	0.9, 1.0	0.4, 0.4
	Hexachlorobenzene	0.02, 0.03	0.02, 0.03
	Dieldrin	0.02, 0.02	<0.01, <0.01
	Endrin	<0.01, 0.02	<0.01, <0.01
	Endrin Ketone	<0.01, <0.01	<0.01, <0.01
	HCH, alpha (lindane)	<0.01, 0.02	<0.01, 0.03

Targeted analysis of daily grab samples

Analysis of water samples collected in June focussed on the 9 targeted pesticide. Atrazine and metolachlor were consistently detected at all sampling sites, and Imazethapyr was consistently detected in both tributaries but only in 30% and 44% of the samples from the Richelieu River (Chambly and Saint-Ours respectively) sites (**Table 8**). All 9 of the targeted pesticides were detected in the Acadie Tributary and all but imidacloprid were detected in the Huron Tributary

(**Table 8**). In the Richelieu River, 6 pesticides were detected at Chambly and Saint-Ours (AMPA, atrazine, clothianidin, imazethapyr, metolachlor, thiamethoxam) (**Table 8**). Levels of detected pesticides were higher in the water from the tributaries than in water from the Richelieu River. The one exception was AMPA, which was measured at higher concentrations in the Richelieu River than the tributaires in 74% of samples (**Figure 13**).

A general trend of low or non-detectable levels of pesticides which spiked in the days after a large rainfall was observed throughout the month of June. Two major concentration peaks were observed for AMPA, atrazine, clothianidin, glyphosate, imazethapyr, metolachlor and thiamethoxam, following heavy rain events on June 4th (21 mm) and June 18th (32mm) (**Figure 13**). This trend was less consistent for the two remaining chemicals, chlorantraniliprole and imidacloprid, both of which were detected infrequently and only in the tributaries.

Of the nine targeted compounds, only two nicotinoids pesticides (clothianidin and thiamethoxam) were measured at least once above the Canadian freshwater safety threshold for chronic aquatic life (**Table 8**). The other chemicals (atrazine, chlorantraniliprole, glyphosate, imidacloprid, imazethapyr) were at least 1 order of magnitude below the Canadian freshwater safety threshold for chronic aquatic life while metolachlor was close (**Table 8**). There is currently no Canadian freshwater safety threshold for chronic aquatic life for AMPA.

Passive Samplers

Analysis of extracts from passive samplers focussed on hydrophilic organic pesticides and their degradation products. Of the 77 compounds measured in the extracts from the passive samplers, 21 were detected at least once. This included 16 parent compounds and 5 degradates (**Table 10**). A total of 14 compounds (12 parent compounds and 2 degradates) were detected in the Richelieu River (both Chambly and Saint-Ours), of which 6 are herbicides, 5 are insecticides and 3 are fungicides. The remaining compounds, the herbicide linuron and the insecticides diazinon, endrin, chlordane, two of its degradation products chlordane alpha and trans nonachlor and heptachlor degradation product heptachlor epoxy were only detected at Richelieu River Saint-Ours site. For the compounds with available published sampling rates, the calculated time weighted average concentrations of pesticides recovered from the passive samplers were all below the CCME toxicity threshold for chronic aquatic life (**Table 9**). Overall, in the Richelieu River the time

weighted average concentrations of pesticides were all higher at Saint-Ours, which is downstream of Chambly

Table 8. Summary table of toxicity thresholds, detection frequency, ranges and dates of the highest concentrations recorded of pesticide concentrations measured in 2 locations along the Richelieu River (Chambly and Saint-Ours) and two of its tributaries (Acadie and Huron Tributaries). Toxicity thresholds were the Canadian Council of Ministers of the Environment long term water quality guidelines for the protection of aquatic life.

			Acadie				Huron				Chambly				Saint-Ours			
Type	Compound	Toxicity threshold (ng/L)	Detection frequency (%)	range (ng/L)	Average (ng/L)	Date of highest concentration recorded	Detection frequency (%)	range (ng/L)	Average (ng/L)	Date of highest concentration recorded	Detection frequency (%)	range (ng/L)	Average (ng/L)	Date of highest concentration recorded	Detection frequency (%)	range (ng/L)	Average (ng/L)	Date of highest concentration recorded
Herbicides	Aminomethyl-phosphonic acid (AMPA)	NA	32	<150-7231	925	10-Jun	32	<150-3238	584	07-Jun	75	<150-3187	923	08-Jun	84	<150-3940	844	08-Jun
	Atrazine	1800	100	23-811	177	19-Jun*	100	15-569	100	19-Jun*	100	7-38	21	19-Jun*	100	7-257	36	19-Jun*
	Glyphosate	65000	58	<220-969	205	19-Jun*	32	<220-947	131	19-Jun*	0	<220	0	-	0	<220	0	-
	Imazethapyr	8100	100	7-548	140	21-Jun	100	6-658	111	19-Jun*	30	<1-48	5	07-Jun	44	<1-179	14	19-Jun*
	Metolachlor	7800	100	184-6243	1455	05-Jun	100	114-7008	980	19-Jun*	100	10-38	78	07-Jun	100	13-1247	131	19-Jun*
Insecticides	Chlorantranil-iprole	220	5	<9-12	1	07-Jun	21	<9-26	4	06-Jun	0	<9	0	-	0	<9	0	-
	Clothianidin	20	32	<6-199	27	07-Jun	32	<6-198	35	19-Jun*	5	<6-8	0	14-Jun	4	<6-43	2	19-Jun*
	Imidacloprid	230	5	<4-41	2	07-Jun	0	<4	0	-	0	<4	0	-	0	<4	0	-
	Thiamethoxam	20	63	<1-395	57	07-Jun	68	<1-607	69	06-Jun	20	<1-47	6	07-Jun	12	<1-24	3	19-Jun*

*19-Jun was the day after the heaviest rain event.

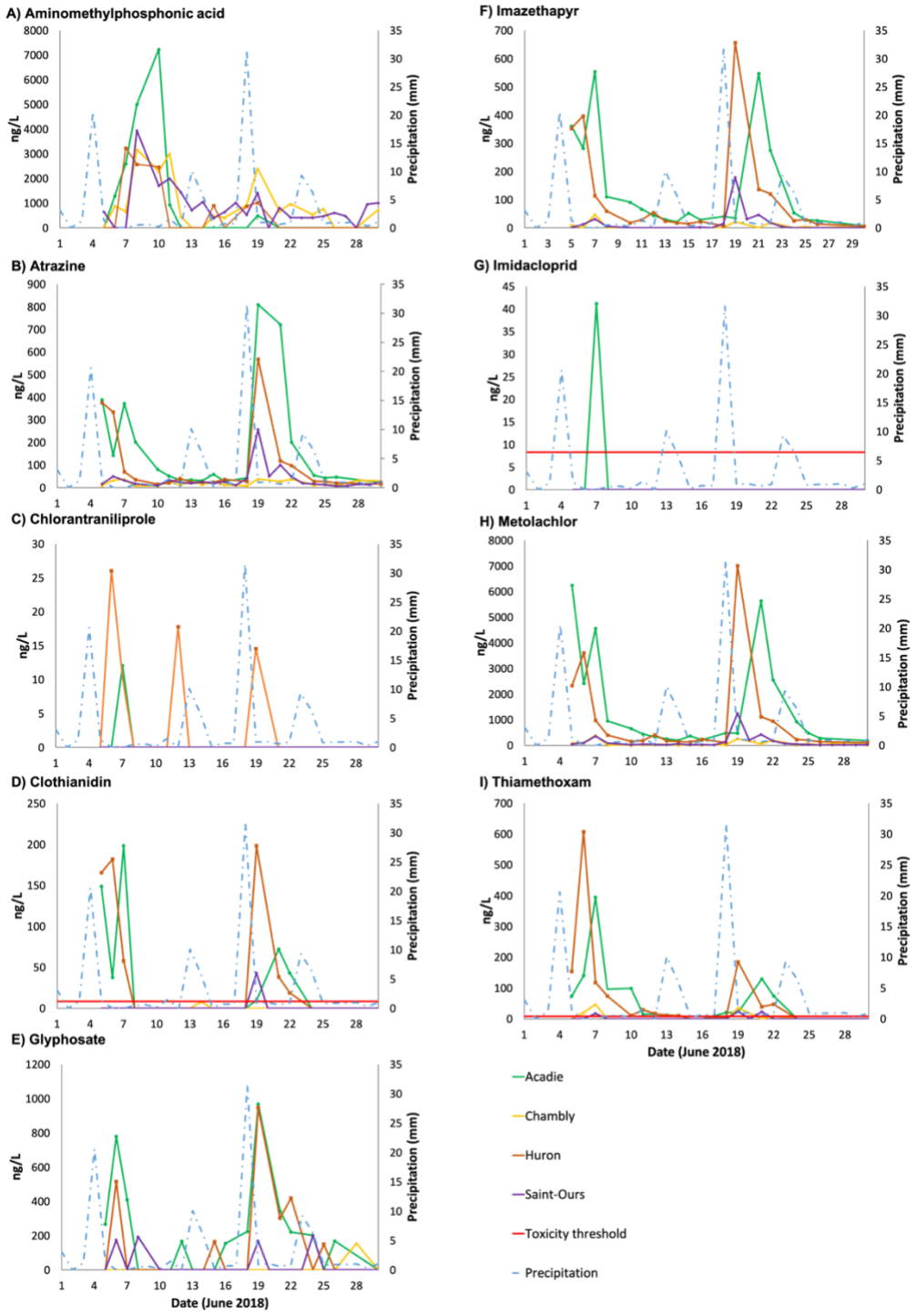

Figure 13: Daily pesticide concentrations (ng/L) measured in 2 locations in the Richelieu River (Chambly and Saint-Ours) and two of its tributaries (Acadie and Huron Rivers) during the river and copper redhorse spawning season. Daily surface water grab samples were hand-collected (4 times 40 mL/per location and per day). Precipitation (mm) during sample collection is also presented. Note that aminomethylphosphonic acid (AMPA) is a degradation product of glyphosate and thiamethoxam is the precursor of clothianidin. Canadian Council of Ministers of the

Environment (CCME) long term water quality guidelines for the protection of aquatic life are shown as solid red lines only for compounds in exceedance of the threshold. Thresholds for other compounds not shown on graph are A) AMPA: not available, B) Atrazine: 1800 ng/L, C) Chlorantraniliprole E) Glyphosate: 65,000 ng/L, F) Imazethapyr: 8100 ng/L, H) Metolachlor: 7800 ng/L.

Table 9. Time weighted daily average pesticide concentrations measured in passive sampler disc deployed in each of copper redhorse spawning grounds. The time weighted average concentrations were calculated using the average concentrations per disc (6 disc per unit) for each of the two field replicates (units) deployed in each location. The long term water quality guidelines for the protection of aquatic life for each compound is from The Canadian Council of Ministers of the Environments (CCME).

Compound (pesticides)	CCME water quality guidelines (ng/L)	Time weighted average concentrations (ng/L)	
		Chambly	Saint-Ours
Ametryn[a]	No data	**0.18**	**0.28**
Atrazine[a]	1800.0	**112**	**201**
Dacthal[a]	No data	**0.10**	**0.13**
Diazinon[b]	4.0	**-**	**0.04**
Dieldrin[b]	4.0	**0.10**	**0.25**
Dimethenamid[a]	5600.0	**4.5**	**13.2**
Endrin[b]	2.3	**-**	**0.02**
Linuron[a]	7000.0	**-**	**1.3**
Metolachlor[a]	7800.0	**56**	**194**
Metribuzin[a]	1000.0	**4.7**	**16.8**
Tebuconazole[c]	No data	**0.45**	**0.49**

- Not detected at this location

[a] Herbicides [b] Insecticides [c] Fungicides

Table 10. Pesticide concentrations per sampler (disc) (ng) measured in passive samplers after a 28- (Chambly) or 29- (Saint-Ours) day deployment in 2 locations along the Richelieu River. These are the absolute concentrations measured in the passive samplers and not daily averages. The letters A and B represent field replicates, two per location, each containing 6 sampling discs.

		Chambly		Saint-Ours	
		A	B	A	B
Herbicides	Atrazine	3120	1560	4260	3860
	Ametryn	7.5	2.9	7.5	8.4
	Dacthal	1.3	0.7	1.2	1.2

	Dimethenamid	229	114	423	540
	Linuron	-	-	28.2	26.5
	Metolachlor	2450	1800	6380	7970
	Metribuzin	92.2	45.6	226	247
Insecticides	Chlordane, alpha (chlordane)*	-	-	0.3	0.2
	Chlordane, gamma	-	-	0.7	0.4
	Diazinon	-	-	2.8	-
	Dieldrin	0.9	0.6	1.8	1.9
	Endosulphan sulfate (endosulphan)*	2	-	2.5	2.5
	Endrin	-	-	0.3	0.1
	Hexachlorocyclohexane, alpha (lindane)*	1.7	0.9	1.5	1.6
	Hexachlorocyclohexane, gamma (lindane)	0.7	-	1	0.9
	Heptachlor (chlordane)	-	0.2	0.1	0.2
	Heptachlor epoxy (heptachlor) (chlordane)*	-	-	0.5	0.5
	Nonachlor, trans (chlordane)*	-	-	0.3	-
Fungicides	Hexachlorobenzene	0.1	0.1	0.2	0.2
	Quintozene	1	0.5	4	-
	Tebuconazole	12.8	6	-	19.7

*These compounds are degradation products of the compound in parenthesis.

4.8 Discussion

June is an important time of year in the Richelieu River as it is a favored breeding time for many fish species, including those at risk. It is also a period of intensive agricultural activity when pesticides can leach from fields and be found at high concentrations in the river water. Here we provide a detailed temporal analysis of pesticides in the Richelieu river and two tributaries throughout the month of June using daily grab samples, passive samplers and off-season sampling. Overall, our results confirm that fish are exposed to a complex mixture of contaminants; a total of 69 compounds were detected in water, including 31 pesticides (12 herbicides, 16 insecticides and 3 fungicides including several that have been banned in Canada), 12 PBDEs and 26 PPCPs. Pesticides concentrations varied considerably (2 orders of magnitude) over time with peaks associated with rain. Two neonicotinoid pesticides, thiamethoxam and clothianidin, were

frequently detected at concentrations above the Canadian freshwater chronic safety threshold for aquatic life.

Targeted compounds

Nine pesticides were selected for targeted analysis due to their previous occurrence in the watershed (Giroux 2015), known toxicity, and our preliminary data. The selected chemicals are good representatives of current use pesticides in modern corn and soy agriculture. All of the targeted pesticides were detected at least once.

Herbicides

Atrazine is one on the most frequently detected herbicides in surface water worldwide (De Souza et al. 2020). This herbicide is an important tool for large-scale agricultural production of corn and soy, the two crops that are most prevalent in the Richelieu watershed. In the Richelieu River and its tributaries, we detected atrazine in all of our samples in all locations during the month of June. Concentrations in tributaries ranged from 15 to 811 ng/L and concentrations in the Richelieu River ranged from 7 to 38 ng/L at Chambly and 7-257 ng/L at Saint-Ours (**Table 8**). The time weighted average calculated from the passive samplers gives concentrations in the same range in the Richelieu River (112 ng/L for Chambly and 201 ng/L for Saint-Ours) (**Table 9**). Similar background concentrations (32 ng/L) of atrazine were also measured in the Richelieu River in December, well outside of the agricultural season (**Table 7**). Previous data demonstrate that atrazine has been ubiquitous in St. Lawrence watershed, which includes the Richelieu River (Giroux 2019, Montiel-León et al. 2019). Levels of atrazine alone measured in the Richelieu River and its tributaries are expected to have minimal, if any, impact on wildlife. Most studies that report deleterious effects from atrazine in aquatic organisms occur at dose one and two order of magnitude higher than what we measured in the environment (Van Der Kraak et al. 2014, Hanson et al. 2019).

The second herbicide that was omnipresent in our samples was metolachlor, which is also used in Canada for corn and soybean crops. Metolachlor is the targeted compound that we measured at highest concentrations, with maximal values of above 7000 ng/L measured in the Huron Tributary (**Table 8**). Similar metolachlor concentrations (10,500 ng/L) were measured in 2016 in the surface

water of an agricultural area in South Georgia, where it was also the most frequently detected herbicide (Glinski et al. 2018). Sub-lethal effects of metolachlor have been reported at doses in 1000 of ng/L. For example, concentration of 10,000 ng/L of metolachlor increased transcription of thyroid-related genes in female juvenile medaka (Oryzias latipes) (Jin et al. 2011). Peaks of metolachlor concentrations have previously been linked to intense rain events (Griffini et al. 1997, Giroux 2019). In the Richelieu River, concentrations of metolachlor ranged from 10-1247 ng/L (**Table 8**). The time weighted average calculated from the passive samplers gives concentrations in the same range for the Richelieu River (56 ng/L for Chambly and 194 ng/L for Saint-Ours) (**Table 9**). Similar background concentrations (17 ng/L) of metolachlor were also measured in the Richelieu River in December, well outside of the agricultural season (**Table 7**). The concentrations of metolachlor measured in the tributaries were very close to the CCME toxicity threshold, however, concentrations in the Richelieu River itself were 7-20 times lower than levels reported to have an effect on fish (Jin et al. 2011).

Concentrations of the herbicide imazethapyr, in tributaries ranged from 7 to 658 ng/L and concentrations in the Richelieu River ranged from <MDL to 48 ng/L (**Table 8**). Imazethapyr was not measured in the passive samplers nor in the winter samples. The selective mode of action of imazethapyr, inhibition of acetohydroxyacid synthase, an enzyme which is only found in plants suggests minimal effects on non-vegetal organisms (Solomon et al. 2013), but effects in non-target organisms have been reported (Moraes et al. 2011, Costa et al. 2022). Effects on fish such as reduced acetylcholinesterase enzyme activity, increased oxidative stress and protein catabolism have been reported in *Cyprinus carpio* (Moraes et al. 2011), but at doses approximately 20 times higher than the highest concentration of imazethapyr (657.7 ng/L) that was measured in the tributaries in the current study (**Table 8**). Overall the concentrations of imazethapyr measured in the Richelieu River should have minimal effects on the river fish fauna.

Glyphosate is a non-selective, broad spectrum, post-emergence herbicide, often used in combination with genetically engineered glyphosate resistant crops (Borggaard & Gimsing 2007). In our analysis, glyphosate was never detected in the Richelieu River and was detected in 21% and 42% of the samples from, respectively, the Huron and Acadie Tributaries (**Table 8**). It is most likely that glyphosate was present in the Richelieu but the concentrations were below the detection limits of our instruments (220 ng/L). For example, background levels of glyphosate were detected

in the Richelieu River (24 ng/L at Chambly and 22 ng/L at Saint-Ours) during winter (**Table 7**). Also other analysis of surface water in the Richelieu downstream of Saint-Ours showed that glyphosate was detected in 84% of the samples and at concentrations up to 3000 ng/L (Montiel-Leon 2019). Our relatively high detection limit may explain why our detection frequency in the Huron Tributary is about four times lower than the previous years. For example, from 2015-2017 glyphosate has been detected in 96% of the samples from the Huron Tributary (Giroux 2019). In the tributaries, the concentrations of glyphosate that we measured reached 969 and 947 ng/L in Acadie and Huron respectively (**Figure 13E**). Overall the concentrations of glyphosate measured in the Richelieu River were two order of magnitude below the long term Canadian Council of Ministers of the environment long term water quality guidelines for the protection of aquatic life and would be expected to have minimal effects on the river fish fauna.

Glyphosate and its primary metabolite, AMPA, are most often detected together in the environment (Battaglin et al. 2014). Very little information is available regarding aquatic organism long-term chronic exposure to AMPA. The compound with the highest concentrations in all the locations that we sampled was AMPA, with concentrations peaking at 7231 ng/L in the Acadie Tributary (**Figure 13A**). Available data suggests low chronic toxicity of AMPA to *Daphnia magna* and fathead minnow (*Pimephales promelas*) with no observed adverse effect at concentration of 15mg/L and 12 mg/L, respectively (Levine et al. 2015). This is more than ten thousand times higher than what we measured in the Richelieu river (µg/L). Even though safety thresholds are often set very high for glyphosate since acute toxicity only happens at very high concentrations (mg/L), more information related to chronic long term exposure on aquatic organisms is still needed (Tresnakova et al. 2021).

Insecticides

Chlorantraniliprole is a highly selective insecticide which can have negative effects on untargeted aquatic life (Rodrigues et al. 2015). We detected chlorantraniliprole in the 2 tributaries of the Richelieu River only with the highest concentration being 26 ng/L (**Table 8**). This concentration is about 8 times lower than the level previously reported to affect aquatic arthropods (Rodrigues et al. 2015 & 2016) but 96h exposure of fathead minnow to 25 ng/L of chlorantraniliprole had a negative impact on neuromuscular health through overactivation of the ryanodine receptor

(Stinson et al. 2022). Other sub-lethal effects in fish such as disruption of muscle and gill ATPase (Temiz et al. 2018) and oxidative stress (Rathnamma & Nagaraju al. 2014), were only observed at concentrations in the mg/L range which suggests that concentrations in the Richelieu River are unlikely to have a major impact on fish health.

Neonicotinoids (clothianidin, imidacloprid and thiamethoxam)

Neonicotinoids are broad-spectrum insecticides that are often detected in Canadian waters (Struger et al. 2017). They are designed to irreversibly bind to the insect-specific nicotinic acetylcholine receptors, which means they are highly and indiscriminately toxic to all insects, but in theory have minimal impacts on other taxa (Hladik et al. 2018). Effects on aquatic invertebrate communities are estimated to occur at an average concentration of 35 ng/L (Morrissey et al. 2015). Difficulties establishing guidelines for neonicotinoids in aquatic ecosystems come from large differences in sensitivity among species (Morrissey et al. 2015). For example, toxicity values can vary up to 4 orders of magnitude between different arthropod species (Raby et al. 2018). Current Canadian water quality guidelines set the chronic safety threshold for imidacloprid at 230 ng/L (no data for acute), and the thresholds for thiamethoxam and clothianidin are temporarily set at 430 ng/L (2780ng/L for acute) and 130 ng/L (340 ng/L for acute) respectively.

Overall, thiamethoxam concentration were higher in the tributaries (607 ng/L in Acadie Tributary and 305ng/L in the Huron Tributary) than in the Richelieu River (**Figure 13I**). The concentration in the Acadie Tributary was above the Canadian chronic water quality guideline but below the acute threshold. In both tributaries, concentrations were one order of magnitude higher than the more conservative Morrissey et al. (2015) predicted concentration to have effects on aquatic invertebrate communities. In the Richelieu River, Chambly (46.9 ng/L) and Saint-Ours (24.1 ng/L), highest thiamethoxam concentrations were 9 and 18 times respectively lower than the safety guidelines (**Table 8**). During 2 non-consecutive days, clothianidin values measured in both tributaries (198 ng/L) were above the chronic Canadian safety threshold. Concentrations were also, during multiple days, above the concentration predicted to have effects on aquatic invertebrates communities (Morrissey et al. 2015). In the Richelieu River, clothianidin concentrations only peaked the day following a heavy rain event and highest concentrations were almost 25 times lower at Chambly (8.1 ng/L) and almost 5 times lower at Saint-Ours (43 ng/L) and below all

thresholds (**Figure 13I)**. The occasional exceedance of current Canadian water quality guidelines and often exceedance of the predicted effect concentrations for aquatic invertebrates make the presence of these 2 pesticides concerning. This is mostly relevant in regards to the aquatic invertebrate communities living in the tributaries which are often at the base of the aquatic communities' food web and effects on them could trickle down to other species. Imidacloprid was only detected once, in the Acadie Tributary at 41.2 ng/L, almost one order of magnitude below the chronic safety threshold (**Figure 13G**). Imidacloprid is one of the most widely used neonicotinoid worldwide (Hladik et al. 2018) although it is not often detected in the Richelieu River watershed (Giroux 2019). Imidacloprid was only detected in the Huron Tributary in 2017 but only later in the summer (July and August) at concentrations ranging from 4 to 15 ng/L (Giroux 2019).

Other compounds in the river

In addition to the 9 targeted compounds, the river contained a mixture of at least 22 pesticides (7 herbicides, 12 insecticides and 3 fungicides) that were all detected at levels below the safety thresholds (**Table 10**). The estimated time weighted average concentrations measured in the Richelieu River in ng/L were between 2 and 3 orders of magnitude lower than the Canadian freshwater safety threshold for chronic aquatic life (when available), which are all in µg/L. Concentrations were most likely higher in the tributaries but passive samplers were only deployed in the main river.

Many of the insecticides detected in the passive samples have been banned for agricultural use in Canada and elsewhere. For example, dieldrin, endrin, lindane, heptachlor and nonachlor were detected in the river and are all prohibited by Health Canada (Health Canada 2022). In fact, dieldrin and endrin, 2 organochloride insecticides that have been banned in the U.S. and Canada for almost 30 years. This level of persistence has been previously documented. For example, after a single application in an experimental plot, dieldrin was still measurable in earthworms 45 years later (Beyer & Gale 2013). Concentrations of these persistent compounds were however all very low, in the ng/L range.

Concentrations of pesticides measured in the winter preliminary samples were all below safety thresholds. The pesticides with the highest concentrations measured were AMPA (53 ng/L), atrazine (32 ng/L) and metolachlor (17 ng/L) (Table 1). Concentrations of atrazine and metolachlor

were two order of magnitude below the CCME water quality guidelines for the protection of aquatic life, while no threshold value was available for AMPA.

Compounds other than pesticides were also measured in the winter samples. This included a total of 12 PBDEs and 26 PPCPs (**Table 7**). We did not target these chemicals in the water collected in June, but it is very likely that they are present in the river year round. The 5 compounds detected with the highest concentrations in the Richelieu river water were PPCPs: metformin (517 ng/L), acetaminophen (289 ng/L), caffeine (222 ng/L), iopamidol (217 ng/L), and theophylline (161 ng/L) (**Table 7**). Aquatic environmental safety thresholds for these compounds are not available. We know that the local wastewater treatment plants, which are prone to overflow following heavy rain events, are sources of such pharmaceutical and personal care products to the river (Cliché & Saladzius 2018). For example, a total of 24 pharmaceutical and personal care products and 23 pesticides were previously tentatively identified in the Richelieu River at Saint-Ours in samples collected following a heavy rain event (Marchand et al. 2022). Even though we are here focusing on pesticides, there are a lot of other compounds in the river that may have an effect on wildlife.

Our water analysis focused on the active molecules of 9 current use pesticides, while the river water may contain hundreds if not thousands of chemicals including natural compounds, formulants and various degradation products. These formulants and degradation products can be more toxic than the active or parent compounds. For example, among 14 glyphosate-based herbicides, the most toxic compound was not glyphosate, but the petroleum-based compounds in the formulation. As mentioned previously, degradation products, such as those from metolachlor, can be more toxic than the parent compounds (Coffinet et al. 2012) and remain in the environment for a long time (De Souza et al. 2020).

Concentrations were consistently higher in the summer for most of the pesticides (dimethanamid, dieldrin, endrin, lindane and hexachlorobenzene) detected in both the summer passive samplers and in the winter samples. Simazine was the only pesticide that was measured in the Richelieu River winter samples (2.3 ng/L at Chambly and 2.5 ng/L at Saint-Ours) but not detected in the summer samples (**Table 7 & 10**). This could be explained by the lesser mobility of simazine when compared to similar use herbicides (such as atrazine) or difference in timing of application (Kruger et al. 1996). This means that it will take longer for simazine spring application to reach surface

water, which is in agreement with the previous monitoring which detected simazine only in August (Giroux 2019). Overall, multiple pesticides that were detected in the summer were also present in the winter samples, but at lesser concentrations.

Association of Contamination Pulse with Rain/Source of Contamination

Highest concentrations of pesticides were detected, in all locations, after heavy rain events. Heavy precipitation is known to wash agricultural fields of pesticides leading to spikes in surface water concentrations (USGS 1997, Perez et al. 2017, Struger et al. 2017). We measured concentrations up to 100 times higher than the baseline levels following rain events, with thiamethoxam exhibiting the highest increase in the levels in the Huron Tributary (**Figure 13I & Table 8**). This association between pesticide concentrations and precipitation was observed with neonicotinoid insecticides in Southern Ontario (Struger et al. 2017) and with multiple pesticides in the Maritime region of Canada (Lalonde & Garron 2020). In the latter, a 6-year survey of 13 freshwater stream sites identified acetamiprid, atrazine, chlorantraniliprole, clothianidin, imidacloprid, metolachlor, thiamethoxam as the 7 most often detected pesticides. For all these compounds, the frequency of detection was 2-19% higher during rain events when compared to samples collected during dry weather, and the largest differences in frequency were for clothianidin, imidacloprid and thiamethoxam (Lalonde & Garron 2020). We also detected pesticides more frequently after rain events with neonicotinoids exhibiting the highest pulse (**Figure 13D, G & I**). Most of the pesticides we detected are commonly used in corn and soy cultures (mainly broad spectrum herbicides and neonicotinoid insecticides), which are prevalent in this agricultural-rich region (COVABAR 2015; Giroux 2015 & 2019). Acetamiprid which was detected in the maritime region and absent from our results, targets crops such leafy vegetables and fruit trees, which represents less than 5% of the cultivated crops in the Richelieu (Huron) watershed (Giroux 2022). These major pulses in concentrations showcase the importance of sampling all along spawning season since the timing of the rain (and pesticide spike) could have a big impact on the success of individual fish spawns (including sensitive early developmental stages) and of different species.

Pesticides concentrations, mostly atrazine and metolachlor were also detected in the December winter samples (**Table 7**). In Canada temperate climate, corn and soy are planted in the spring (May and June) (USDA 2022), with herbicide application occurring before, during and after

planting (Krähmer et al. 2021). Rain event runoffs occurring in the two first weeks following application are the main source of pesticides contamination of surface water (CCME 1999). But for the ubiquitous atrazine and metolachlor, the concentrations measured in winter were similar to the baseline concentrations measured in between rain events (**Tables 7 & 8**). The detection of atrazine and metolachlor in winter can most likely be attributed to their long degradation time in water with half-life in water well above 6 months (Senseman 2007; de Souza et al. 2020).

Spatial variations (Tributaries vs. River)

The timing of pesticide peaks was similar in all the sampled locations but peaks and overall concentrations were higher in the tributaries than in the Richelieu River itself (**Figure 13**). Similar trends were previously observed in the Lake Erie basin where smaller tributaries had higher maximum concentrations and greater temporal variability than larger tributaries (Richards & Baker 2013). The higher concentrations detected in the tributaries can most likely be explained by the fact that these smaller and slower watercourses, are directly fed by agricultural drainage. The Richelieu on the other hand, receive most of its water from Lake Champlain and has, overall a much higher dilution factor from higher volume of water and flow. It has also been previously suggested that contaminated ground water source (from agricultural activities) may contribute to the pesticide burden of smaller streams (Lalonde & Garron 2020), but it is unknown if this is applicable to the Richelieu watershed.

In the Richelieu River itself, concentrations of most compounds measured (targeted and passive sampling), were higher at the downstream location of Saint-Ours than in the upstream location of Chambly (**Tables 8 & 9**). Many tributaries, including the Huron and Acadie, feed into the Richelieu between these 2 locations and likely add to the Saint-Ours total pesticide burden (Map if added). A notable exception is thiamethoxam, which had a higher range and maximal concentration at the Richelieu River Chambly site while its degradation product clothianidin (another neonicotinoid insecticide) was higher in the Richelieu River Saint-Ours site (**Tables 8 & 9**), suggesting either slower transport of the compound along the river or potential degradation in between the 2 locations. These major differences in concentrations showcase the importance of sampling all along the watershed and the variability of the pesticide burden within aquatic community habitats.

Difference Between Passive Sampler and Targeted Analysis Results

The daily average results from the passive sampler are only an estimation. The daily average concentrations from the passive samplers were calculated using estimated flow rate and calibration data. Moreover, sampling rates weren't available for some of the compounds that were detected preventing us from calculating daily averages. Instead, available previously published sampling rates and calibration data were used to provide estimated daily averages. It is worth mentioning, that water parameters, such as temperature (Dalton et al. 2014), pH and turbidity (Li et al. 2011) may all effect POCIS individual chemical uptake rate. Although daily average were estimations, they were still under one order of magnitude differences with the data we collected from the daily analysis.

Of the nine targeted compounds, we were able to compare concentrations from two between passive samplers and grab samples. Richelieu River atrazine and metolachlor concentrations calculated from passive samplers (Chambly 112 ng/L and Saint-Ours 201 ng/L) using estimated flow rate data were within one order of magnitude of the concentrations measured directly in the Richelieu River water (Chambly 7-38 ng/L and Saint-Ours 7-257ng/L). The other targeted compounds detected in the Richelieu grab samples but not in the POCIS most likely not captured by the passive samplers. The POCIS we used are able to capture hydrophilic organic contaminants (log kow 4-8) but are not as useful for very hydrophilic compounds (log kow <4). For example, due to their high polarity, neonicotinoids insecticides could not be detected (Xiong et al. 2019). Since our experiment, modifications to the POCIS were developed that permit sampling of neonicotinoids in surface water (Xiong et al. 2019), but these were not used in the current experiment.

Other Source of Contamination

As previously observed in many regions across the globe, pesticide peaks in surface water have been associated with rain events that wash surrounding soils and vegetation (Glinski et al. 2018), but other sources of contamination should not be ignored. For example, the source of some of the contaminants detected may be caused by atmospheric transport rather than local application. The herbicide dacthal that we detected using the passive samplers, is used more in the US than in

Canada and can travel long distances in the atmosphere (Yao et al. 2007). Further investigation is required to better source the contamination of the Richelieu River.

4.9 Conclusion

Our work provided data on the temporal and spatial trends in pesticide concentrations in the Richelieu River and two of its tributaries. Using complementary sampling and analysis methods allowed for the detection of a wider range of compounds. Our data covers the month of June, which includes intensive agricultural activities in the watershed but also the breeding period of fish such as the threatened river redhorse and the endangered copper redhorse.

None of the pesticides measured were found at levels expected to be toxic to fish but two neonicotinoids were above the threshold for the protection of aquatic life. Most of the previous research has been focused on the effects of a single chemical but we detected dozens of pesticides in the Richelieu and mixture risks are frequently ignored in regulatory thresholds. Other ingredients in the pesticide formulation may be more toxic than the pesticides themselves (Lewis 1990; Tsui & Chu 2003; Grisolia et al. 2004). Also many pesticides are known to have additive or even synergistic effects and simultaneous environmental mixture risks are often underestimated (Weisner et al. 2021), making it extremely hard to predict the effects of such complex mixtures on aquatic organisms.

The surges in pesticides may have an impact on the fish species breeding in the Richelieu but even more so in the tributaries where concentrations were higher. Our results suggest that the tributaries contribute large amount of pesticides into the Richelieu River, which in turn pours them into the Saint-Lawrence River before reaching the Atlantic Ocean. Future work should aim to identify the potential effects of environmental mixtures on natural species, and identifying contaminants of concern.

4.10 Supporting information

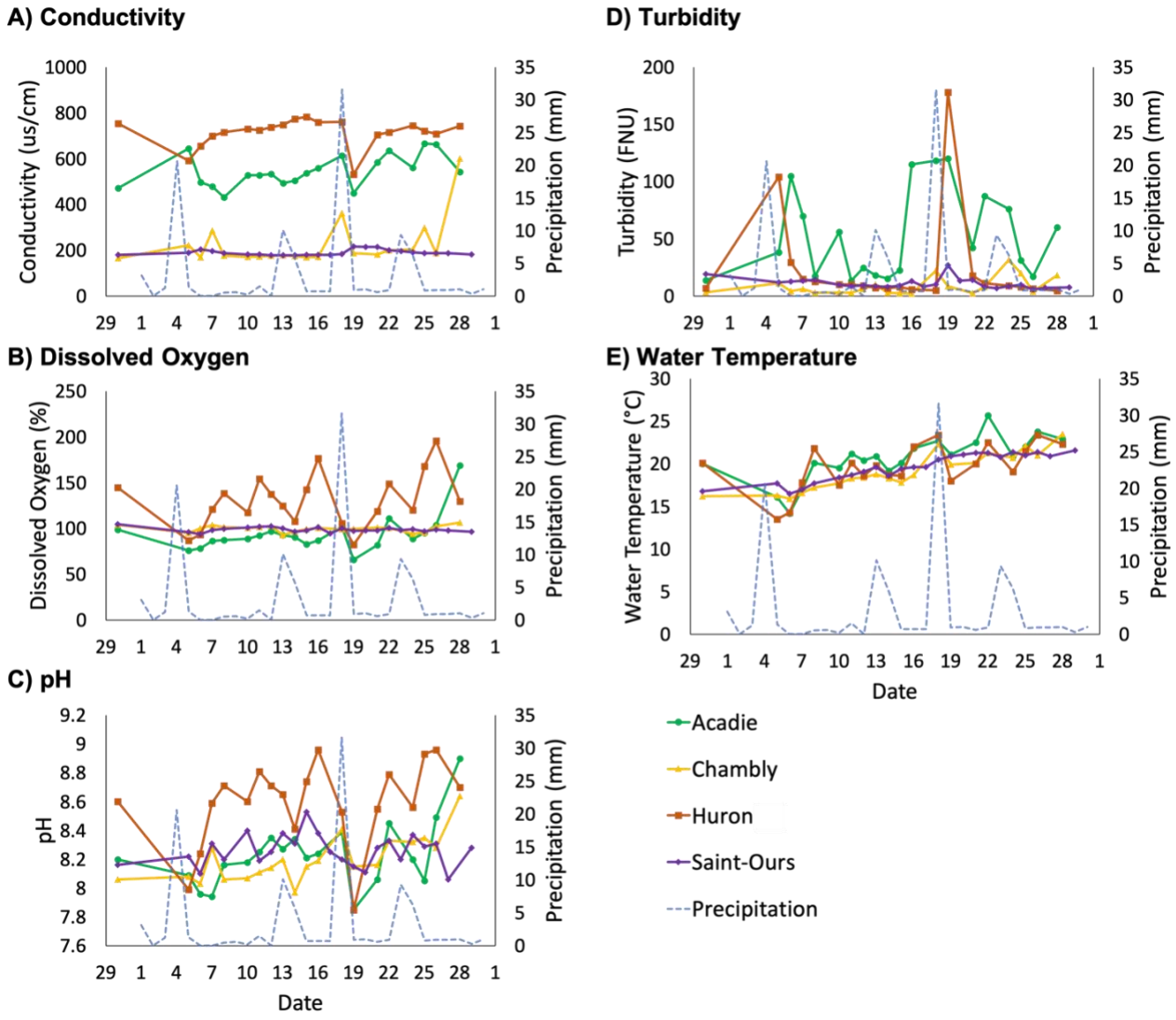

Figure 14: Water parameters, A) conductivity, B) dissolved oxygen, C) pH, D) turbidity and E) temperature, from 2 locations in the Richelieu River (Chambly and Saint-Ours) and two of its tributaries (Acadie and Huron tributaries) during the river and copper redhorse spawning season. Parameters reported are single value taken each day. Daily precipitation data were collected at the l'Assomption station (QC), about 21 km away, by the Meteorological Service of Canada of Environment and Climate Change Canada (https://climate.weather.gc.ca).

Table 11. List of compounds analyzed (pesticides multiresidue analysis) from passive samplers deployed in each of the copper redhorse spawning grounds.

Compounds		
2,4'-DDD	Dacthal	Hexazinone
2,4'-DDE	Desethylatrazine	Linuron
2,4'-DDT	Diazinon	Malathion
4,4'-DDD	Diazinon-Oxon	Methoprene
4,4'-DDE	Dieldrin	Methoxychlor
4,4'-DDT	Dimethenamid	Metribuzin
Alachlor	Dimethoate	Mirex
Aldrin	Disulfoton	Nonachlor, cis-
alpha-Endosulphan	Disulfoton Sulfone	Nonachlor, trans-
Ametryn	Endosulphan Sulphate	Octachlorostyrene
Atrazine	Endrin	Parathion-Ethyl
Azinphos-Methyl	Endrin Ketone	Parathion-Methyl
beta-Endosulphan	Ethalfluralin	Pendimethalin
Butralin	Ethion	Permethrin
Butylate	Fenitrothion	Perthane
Captan	Flufenacet	Phorate
Chlordane, alpha (cis)	Flutriafol	Phosmet
Chlordane, gamma (trans)	Fonofos	Pirimiphos-Methyl
Chlordane, oxy-	HCH, alpha	Quintozene
Chlorothalonil	HCH, beta	Simazine
Chlorpyriphos	HCH, delta	Tebuconazol
Chlorpyriphos-Methyl	HCH, gamma	Tecnazene
Chlorpyriphos-Oxon	Heptachlor	Terbufos
Cyanazine	Heptachlor Epoxide	Triallate
Cypermethrin	Hexachlorobenzene	Trifluralin

Table 12. Sampling rates per compound used for passive sampler analysis.

Compounds	**Sampling rate**	**Published source**
Ametryn	0.339	Alvarez et al. 2007
Atrazine	0.240	Alvarez et al. 2007
Dacthal	0.109	Van Scoy - DaSilva et al. 2015
Diazinon	0.424	Alvarez et al. 2007
Dieldrin	0.086	Alvarez et al. 2007
Dimethenamid	0.435	Bartelt-Hunt et al. 2011
Endrin	0.094	Alvarez et al. 2007
Linuron	0.236	Mazzella et al. 2007
Metolachlor	0.440	Alvarez et al. 2007
Metribuzin	0.168	Alvarez et al. 2007
Tebuconazol	0.240	Alvarez et al. 2007

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Preface to Chapter 5

The previous chapters focused on the ELS effects from exposure to river water in two wild fish species; the river and copper redhorse. We have also looked at the river contamination, its variation across time and space, and which components were present in the environmental mixture. Due to many factors such as legal status, late age of maturity, large size, it was impossible to assess later life effects in either river or copper redhorse.

In this chapter we used a model organism, the zebrafish, to assess the full life cycle effects of ELS exposure to river water. By using zebrafish, we were able to rear the fish to adulthood to assess endpoints throughout their lives. The zebrafish embryos were exposed during ELS only to river water collected during the copper and redhorse spawning season, after a heavy rain event, when pesticide contamination was expected to be the highest. Thereafter, fish were raised in clean laboratory water and we were able to evaluate reproductive outputs and raise a second generation from the exposed fish. Organismal endpoints were also measured in the second generation. This was the first step to understanding the complete life cycle effects of early life exposure to environmental pesticides in laboratory fish, with the further objective of extrapolating the data to wild fish.

Chapter 5

5. Decrease in fertilization rate of zebrafish exposed during early life stage to Richelieu River (QC, Canada) water

5.1 Title Page

Title of the manuscript

Decrease in fertilization rate of zebrafish exposed during early life stage to Richelieu River (QC, Canada) water

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Conflict of interest

The authors declare that there is no conflict of interest.

5.2 List of Figures

5.3 List of Tables

5.4 Abstract

Fish worldwide spawn in waters that are contaminated with complex mixtures of environmental chemicals. This may negatively affect the health of offspring, particularly if exposure occurs at a sensitive early life stage (ELS). Here we study the acute and persistent effects of exposure of zebrafish to pesticide contaminated river water at an early life stage. Zebrafish are used as a surrogate for copper redhorse, an endangered fish that is only known to spawn in the Richelieu River (QC, Canada). During copper redhorse spawning season, surface water samples were collected at two known spawning areas in the Richelieu river (Chambly - downstream and Saint-Ours - upstream) following heavy rain events when pesticide concentrations peak. Based on targeted pesticide analysis, the Saint-Ours sampling site, which is downstream of tributaries fed by agricultural drainage, had higher pesticide concentrations than the upstream site. Zebrafish embryos (3 replicates of 100 embryos each) were exposed to river water (upstream or Saint-Ours) or laboratory water (control group) from < 3hrs post-fertilization until 120 hours post-fertilization. Thereafter, zebrafish were raised to maturity in clean water. Exposure to river water delayed zebrafish hatching compared to controls but had no effect on larval survival or deformities. For fish that were exposed as embryos survival was low in all treatments, most likely due to rearing conditions. In adults, the number of eggs produced was not different among treatment groups. However, fertilization success for the fish exposed to river water was lower than for control fish. This effect was more pronounced in the fish exposed to water from the more contaminated site (65, 72 and 81% for Saint-Ours river water, Chambly river water and control water respectively). The effects were maintained but were more moderate when Saint-Ours river water exposed fish were crossed with control fish, suggesting that both males and females were contributing to this effect. These results suggest that environmentally relevant levels of contaminant present in river water could have lasting impacts on fish fecundity. However, high mortality in the first 60 days across all treatment group suggests that these experiments should be interpreted with cautions.

5.5 Introduction

In agricultural landscape, pesticide concentrations have been shown to peak following heavy rain events with the highest peak often measured in June, a favored time for fish to spawn (Giroux

2019; Marchand et al. 2022). Contamination of spawning grounds and exposure of early life stage (ELS) fish to peaking pesticide concentrations is concerning since early life stages of development are considered to be particularly sensitive to contaminants (Mohammed 2013). Exposure of ELS fish to these contamination peaks could be detrimental to recruitment, but few studies have addressed this concern, particularly in the context of environmentally relevant mixtures .

The Richelieu river is located in south-eastern Québec (Canada), and runs for 124 km through mainly agricultural landscape between Lake Champlain and the Saint-Lawrence River. The Richelieu is home to more than 80 species of fish **(**MFFP 2018) and includes spawning sites of threatened species such as lake sturgeon (*Acipenser fulvescens*) (Thiem et al. 2013), river redhorse (*Moxostoma carinatum*) (COSEWIC 2015) and endangered species such as the copper redhorse (*Moxostoma hubbsi*) (COSEWIC 2014). The only two known spawning grounds of the copper redhorse are located in the Richelieu River (COSEWIC 2014).

In recent years, many contaminants have been detected in the Richelieu river including legacy organic compounds, such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), dioxins, furans (Laliberté 2020), and pharmaceutical and personal care products (Cliché & Saladzius 2018). These are generally detected at concentrations below the provincial criteria for the prevention of contamination of aquatic organism (Laliberté 2020). However, other classes of contaminants such as pesticides have been detected at levels that are concerning. For example, in the Richelieu and its tributaries, neonicotinoids pesticides have been measured at levels that exceed the Canadian Council of Ministers of the Environment (CCME) safety threshold for aquatic life over multiple years in the last decade (Giroux 2022, Marchand et al. 2022). This isn't surprising since 70% of the Richelieu River watershed is dedicated to agriculture (Simoneau & Thibault 2009).

Many listed fish species are spawning in the Richelieu river and we previously found that river water may negatively affects ELS fish. Others have also demonstrated that ELS fish exposure to pesticides has a variety of effects on endpoints such as hatching success and timing (Du Gas et al. 2017; Fiorino et al. 2018), juvenile survival (Vignet 2019), incidence of deformities (Sulukan et al. 2017), immune system deficiencies (Hong et al. 2018), and oxidative stress (Richterova et al. 2015). Exposure to Richelieu river water specifically caused premature hatching in both copper

and river redhorse and decreased survival in copper redhorse larvae but later life effects are unknown (Marchand et al. 2022).

Only few studies have evaluated the later life effects of fish ELS exposure only to pesticides (Wirbisky et al. 2016, Cleary et al. 2019). For example, when zebrafish and Japanese medaka where exposed to atrazine during ELS, reproductive dysfunction and molecular alterations were reported in the adult zebrafish (Wirbisky et al. 2016) and reproductive defects were observed in the medaka grandchildren (Cleary et al. 2019.

These studies focused on a single chemical, while natural waters can contain mixtures of thousands of chemicals. More studies are necessary to better understand the effects of real-world exposure to complex mixtures of pesticides in wild fish (Simonnet-Laprade et al. 2021). This is almost impossible to investigate directly on most threatened wild fish species due to their late age of maturity or large size.

Here, we use the zebrafish (*Danio rerio*), to study persistent effects of ELS exposure to water collected from the Richelieu river. Zebrafish are used as a surrogate for wild species that are spawning in the Richelieu due to their ease of maintenance and short generation time, allowing for a full life cycle assessment. In order to isolate the potential effects of ELS exposure, zebrafish were exposed to river water as embryos and larvae, and then raised in clean water until they reached sexual maturity. Biological endpoints were assessed during the exposure, upon sexual maturation, and in the offspring.

5.6 Materials and Methods

Source of animal and care/maintenance

All work with animals was approved by the McGill University animal care committee (protocol number 2018-7992). On site, a AB wildtype zebrafish colony (from Zebrafish Core Facility, Dalhousie University, Canada) was kept in a recirculating system and fed a commercial diet. Water quality was assessed on a weekly basis. For the first generation, fish were bred in colonies (20-40 individuals) in 5-gallon tanks with a breeding cup (container with a mesh top to prevent the adults from preying on the eggs). Embryos were harvested approximately 2 hours after the initiation of breeding when the light was turned on. Fertilized eggs were removed from the breeding cups and

dipped in a methylene blue solution (2%) for 20 minutes (adapted from Bran et al. 2002) before being rinsed and prepared for treatment. Non-viable embryos were removed at this point.

Source of water and water analysis

Grab water samples (one liter per sample, n = 2 per location) were collected daily in June 2019 during the copper redhorse spawning season at two locations along the Richelieu River. These locations, in the Chambly basin below the Chambly rapids and at Saint-Ours below an artificial dam, are the only two known copper redhorse spawning grounds. Chambly was the upstream sample location on the Richelieu River and will herein be referred to as the upstream site, while Saint-Ours was the downstream site and will be referred to as such. Samples collected from each location on June 16 were selected for the exposure since pesticide concentrations in the Richelieu River are known to peak following heavy rain events and over 12 mm of rain were received on June 14 and 15 (Giroux 2015 & 2019, Marchand et al. 2022). All samples were frozen at -20°C until analysis. The samples were analyzed using targeted liquid chromatography/mass spectrometry (LC-MS) to quantify 9 pesticides (aminomethylphosphonic acid (AMPA), atrazine, clothianidin, chlorantraniliprole, glyphosate, imazethapyr, imidacloprid, metolachlor, thiamethoxam), which are prevalent in the Richelieu surface water (Giroux 2015 & 2019, Marchand et al. 2022). Details of standard solution preparation, sample preparation and instrumental methods are given in Marchand et al. 2022. Briefly, water samples were prepared by spiking a 10 µL internal standard mixture and 100 µL methanol into 890 µL of river water, followed by filtration with a 0.22 µm PTFE syringe filter (Canadian Life Science, Canada) before introduction into the LC-MS. Mass-labeled standards at constant concentrations were used as an internal standard to compensate for any systematic errors due to native compounds (as recommended in USEPA methods (e.g. Method 1694)). Data were collected with an Agilent 1290 Infinity II LC system coupled to the 6545 Quadrupole-Time of Flight–Mass Spectrometry (Q-TOF-MS) (Agilent Technologies, Santa Clara, USA). The LC separation was performed on a CS-C18 column (Agilent Technologies; 2.1 µm × 150 mm). Method performances were assessed in terms of linearity of the calibration response, method detection limits, the method quantification limits, precision, relative recoveries, and matrix effects.

Exposure and Hatching

Zebrafish embryos were exposed from < 3hrs to 120 hours post-fertilization to river water from the upstream site, river water from the downstream site or to control laboratory water that matched river parameters (ultrapure water supplemented with sodium bicarbonate (96 mg/L), magnesium sulfate (60 mg/L), calcium chloride (39 mg/L), and potassium chloride (4 mg/L) (Modified USEPA-821-R-02-12) (**Figure 15A**). For each treatment, 3 replicates of 100 viable embryos were used. For each replicate, embryos were placed in a 250 mL beaker, which was previously solvent-washed and baked (450°C in muffle furnace for 4 hours), and filled with 200 mL of either river (upstream or downstream site) or reconstituted water. The beakers were then placed in a heated water bath (bain-marie) that maintained the temperature of all the beakers at 28°C. Air was gently pumped into each beaker to agitate the surface using a borosilicate glass pipette as dispenser. Hatching, gross deformities (presence/absence) and survival were assessed 3 times daily throughout the exposure period. Dead embryos and larvae were counted and removed. Water parameters were monitored daily.

Rising F0 to maturity

Following the 120-hour exposure, embryos were slowly acclimated to tank water from the recirculating system, then rinsed and raised in clean water in the recirculating system until maturity (**Figure 15**). Survival and deformities were monitored daily throughout the rest of the experiment. Starting at 26 days post-fertilization, embryo length was measured monthly for a period of 18 months. For measurement, fish were placed in a glass beaker with enough water to cover them. The beaker was placed over a graduated scale and a picture was taken from above. ImageJ software (National Institutes of Health) was then used to measure the total length of the fish based on the scale.

Sex ratio and Breeding

Breeding experiments were conducted in three different trials. In trial 1**,** mature fish were sexed through visual assessment. For breeding, a subsample of 3 males and 4 females was randomly selected from each of the three replicates. Fish were bred within their own replicate and treatment groups, i.e., control with control, upstream site with upstream site, downstream site with downstream site (**Figure 15B**). These groups of fish were each placed in a 5-gallon tank with a breeding cup for 18 days. The daily number of eggs produced by each group was recorded. The

fertilization rate and viability of the embryos was assessed under a microscope 4 hours after fertilization and embryos were discarded. Then fish were returned to their respective treatment groups and were provided with a 1-week break in between each breeding experiment (in their respective treatment groups). Embryo production was calculated as number of eggs per female, per day and per cm of length ((total number of eggs collected over the trial)/((number of females)*(days of breeding trial)*(average female size in cm)). Trial 2 was performed following the same procedure as trial 1 except that males from control were matched with females from the downstream site water treatment and vice versa. The same endpoints were measured over 14 days. Trial 3 was identical to trial 1 but lasted only 14 days.

Second generation

To obtain the second generation (F1), F0 fish were bred in their respective treatment groups. Embryos were reared following the same method used for the initial exposure of their parents, but without the exposure to river water; they were all reared in clean laboratory water. The following endpoints were measured in the second generation: hatching time, deformities, survival and length. The second generation was terminated at 30 hpf (**Figure 15B**).

Termination, weight and condition factor

F0 Fish were sacrificed at 552 days post-fertilization (dpf) (18 months post-fertilization) using a tricaine methane-sulfonate (MS-222) solution (250 mg/L buffed with equal weight of sodium bicarbonate). The carcasses were visually inspected, measured and weighed, then Fulton's condition factor ((100*weight (g))/(Length(cm)^3)) was calculated for each individual.

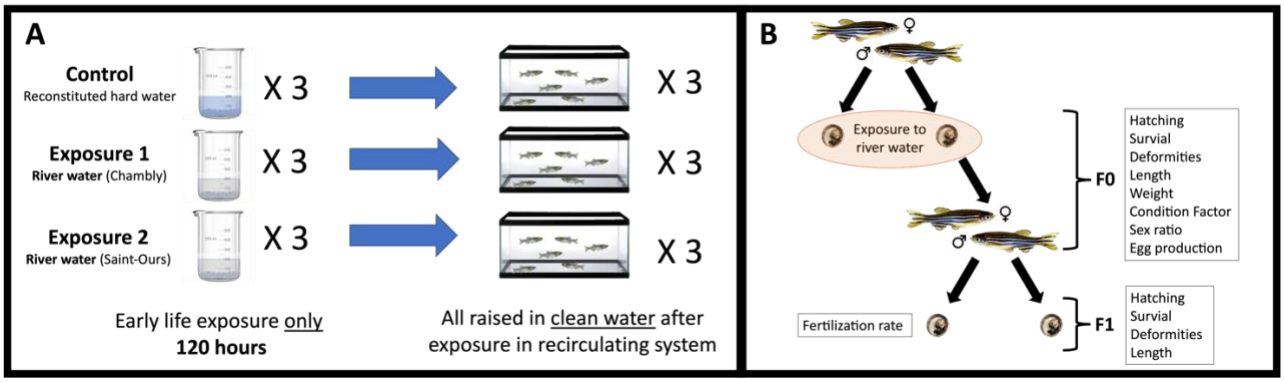

Figure 15. Experimental design used for the early life exposure of zebrafish to river water. **A)** Zebrafish embryos were exposed to river water for 120 hours post-fertilization only, then raised in clean water in a recirculating system. **B)** Following the early life stage exposure, a full life cycle was completed and embryos from the exposed fish (F0) were collected and reared for 30 days. The second generation (F1) was never exposed to river water.

Data analysis

Percentage hatching was calculated as a proportion of the total number of living embryos (hatched larvae/all alive) per time point and per replicate. Mean incidence of deformities and mean survival were calculated as a percentage of the initial number of embryos that were placed in each beaker. All data residuals were tested for normality. Analysis of Variance (ANOVA) and a Bonferroni post-hoc test were performed for daily percentage hatching, incidence of deformities, and survival between replicates using JMP statistical visualization software (SAS Institute). An $\alpha < 0.05$ was considered significant. Percentage hatching data were fitted to a sigmoidal four parameter logistic regression model and the 50% hatch time was calculated using Prism 9 (GraphPad) in hpf. The 50% hatch time values were compared using 95% confidence interval overlap.

5.7 Results

Water analysis

Of the 9 targeted pesticides, 3 were detected in water samples collected at both upstream and downstream sites at roughly equivalent concentrations (Table 1). Imazethapyr (119 ng/L) was only detected in water samples collected at the upstream site and clothianidin (243 ng/L) was only detected at the downstream site. The remaining 4 pesticides (chlorantraniliprole, glyphosate, imidacloprid and thiamethoxam) were not detected at either location (**Table 13**). Only clothianidin was detected at concentrations exceeding the CCME water quality guidelines for the protection of aquatic life.

Hatching

Percentage hatching was high across treatment groups with no significant differences detected. Percentage hatching was 86% (±5) in the control, 82% (±3) in the upstream site water and 77% (±4) in the downstream site water treatments (**Figure 16A**). For the F0 generation, the time to 50% hatch was significantly higher in the upstream site water (64 ± 3.5 hours post-fertilization (hpf)) and in the downstream site water (70 ± 4.7 hpf) treatments than in the control treatment (52 ± 5.3 hpf) (**Figure 16A**). Both the upstream and downstream sites water treatments were significantly different from the control treatment but not from each other. Significant differences in hatching time between river water (both upstream and downstream site) and control were recorded at 3 time points, 48, 52 and 73 dpf.

In the F1 generation, the time for 50% hatch was 45 (± 2.7) hpf for control, 44 (± 4.0) hpf for the upstream site water and 44 (± 3.6) hpf for the downstream site water treatments (**Figure 16B**). There was no statistically significant effect of F0 early life exposure to river water on F1 50% hatch time. However, there was a significant difference in the hatching time in controls between the F0 and the F1 generations ($\alpha = 0.029$).

Survival

Survival to 30 dpf was low for both generations. Following the exposure, zebrafish larvae were still too small for the recirculating system and were kept in static vessels with daily water renewal until large enough. They were raised on dry feed until they were large enough to consume freshly hatched *Artemia*. The static water conditions and early life dry feed diet, probably both contributed to the low survival observed. Another study also reported lesser survival, around 60%, when zebrafish larvae were raised on dry feed exclusively (Farias & Certal 2016). At 60 dpf, fish were placed in a recirculating system and there was a minimal decline in survival after that.

For F0, the percent survival at 30 dpf was 31% (± 12) for the control, 38% (± 6) for the upstream site water and 27% (± 1) for the downstream site water treatments (**Figure 17A**). At the end of the 18 month experiment, the percent survival of the zebrafish was 14% (± 4) for the control, 15% (± 1) for the upstream site water and 12% (± 1) for the downstream site water treatments. There were

no statistically significant differences in survival between treatment groups and control on over the 120h early life exposure to river water nor over the whole 18 months of the experiment.

For F1, which were raised to 30 dpf, the percent survival at the end of that period was 28% (± 3.6) for the control, 27% (± 2.8) for the upstream site water and 29 % (± 1.3) for the downstream site water treatments (**Figure 17B**). The was no significant difference between the treatment nor between the F0 and F1 generations.

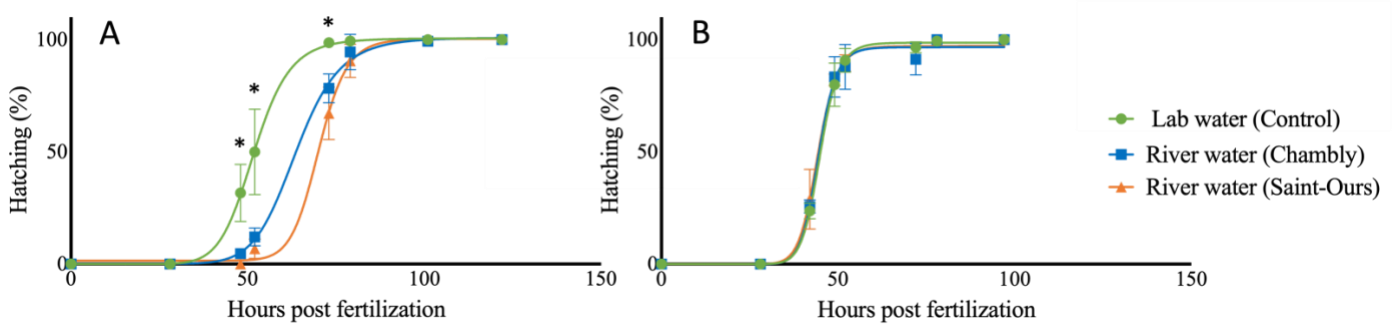

Figure 16. Effects of early life stage exposure to Richelieu River water on timing of hatching of zebrafish embryos. The exposure groups were exposed for 120 hours to river water that was collected at either one of the two locations along the Richelieu River; Chambly, which is upstream and Saint-Ours, which is downstream. Data are expressed as the percentage of live embryos that hatched on a given hour post-fertilization. Each data point represents the average of n=3 replicates, and error bars represent standard deviation. Significant differences between treatments are marked with an asterisk ($\alpha < 0.05$). Included in the data are the **A)** exposed generation (F0), which were exposed to river water during early life stage only and B) their offspring (F1), which were never exposed.

Deformities

Overall incidence of deformities was low for F0 fish and there was no significant differences between treatments (control 3.9% (± 1.4); upstream site water 3.4% (± 1.2); downstream site water 3.7% (± 0.5)). For all the fish that suffered from deformities, none survived beyond the larval stage.

Overall incidence of deformities was higher in the F1 fish and there was significant differences between treatment (control 8.5% (± 3.8); upstream site water 15.7% (± 2.5); downstream site water 16.6% (± 3.4)). Both upstream site water ($\alpha = 0.0369$) and downstream site water ($\alpha = 0.0238$) treatment percentage of deformities was significantly higher than control. Overall, there was also a significant difference in the percentage of deformities between the F0 and the F1 generations ($\alpha < 0.0001$). Edema (pericardial and yolk edema) represented 37% and 45% of the recorded

deformities for the upstream and downstream site water treatments respectively. Craniofacial deformities (shorter jaw, incomplete head formation) represented 32% and 15% and spinal curvature (kyphosis and lordosis) represented 32% and 40%.

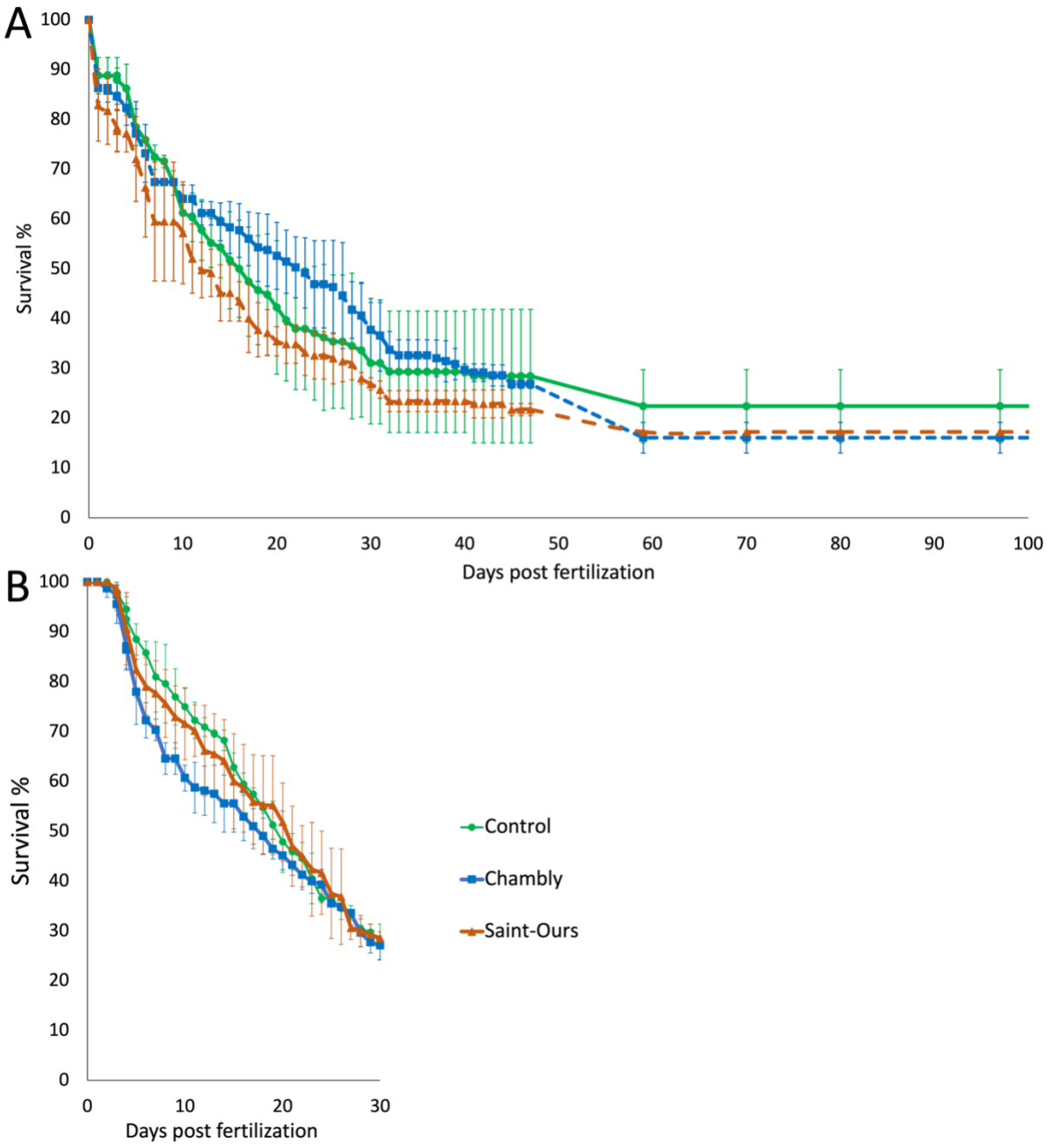

Figure 17. Effects of early life stage exposure to Richelieu River water on survival of zebrafish. The exposure groups were exposed for 120 hours to River water that was collected at either one of the two locations along the Richelieu River; Chambly, which is upstream and Saint-Ours, which is downstream. Data are expressed as the

percentage of live fish on days post-fertilization. Each data point represents the average of n=3 replicates, and error bars represent standard deviation. Significant differences between treatments are marked with an asterisk ($\alpha < 0.05$). Included in the data are the **A)** exposed generation (F0), which were exposed to river water during early life stage only and **B**) their offspring (F1), which were never exposed.

Length

The F0 fish grew steadily throughout the 18 months of the experiment with the growth curves diverging at 80 dpf until about 100 dpf and then remaining parallel. The average total length of the zebrafish after 18 months was 3.40 cm (± 0.62) for the control, 3.03 cm (± 0.57) for the upstream site water and 3.06 cm (± 0.50) for the downstream site water treatment (**Figure 18**). This difference was not statistically significant at any timepoint throughout the experiment.

For F1, the average total length at dpf 30 (at termination) was 0.85 cm (± 1.66) for the control, 0.86 cm (± 1.82) for the upstream site water and 0.87 cm (± 1.82) for the downstream site water treatments. The F0 fish were not measured at 30 dpf but similar lengths were observed at 26 dpf (control 0.87 cm (± 0.40); upstream site water 0.72 cm (± 0.36); downstream site water 0.79 cm (± 0.32)) (**Figure 18**).

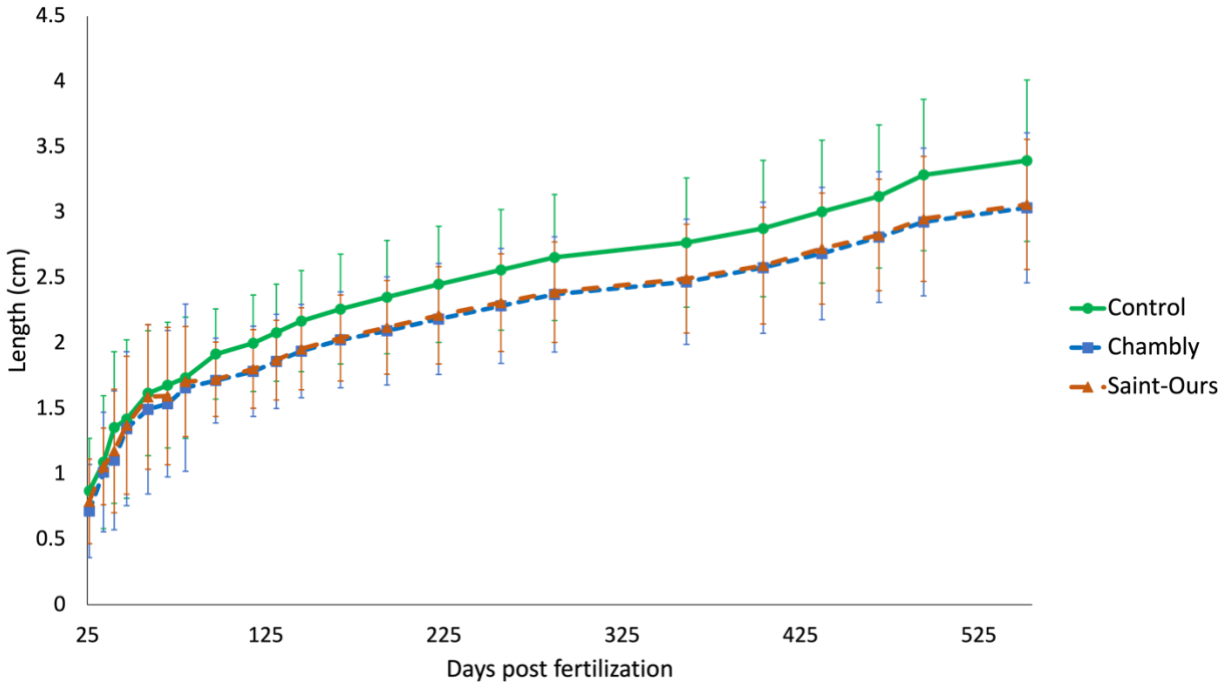

Figure 18. Effects of early life stage exposure to Richelieu River water on length of zebrafish. The exposure groups were exposed for 120 hours to river water that was collected at either one of the two locations along the Richelieu River; Chambly, which is upstream and Saint-Ours, which is downstream. Data are expressed as the

total length of fish on days post-fertilization. Each data point represents the average of n=3 replicates, and error bars represent standard deviation. Included in the data are the exposed generation (F0), which were exposed to river water during early life stage only.

Weight and condition factor

The average total weight of the zebrafish after 18 months was 0.40 g (± 0.24) for the control, 0.32 g (± 0.22) for the upstream site water and 0.32 g (± 0.15) for the downstream site water treatments (**Figure 19B**). Overall, the zebrafish from the control group were heavier than the fish of both river water treatment groups but there was no statistically significant difference between the groups. The average Fulton's condition factor of the zebrafish after 18 months was 1.08 (± 0.31) for the control, 1.17 (± 0.53) for the upstream site water and 1.19 (± 0.43) for the downstream site water treatments (**Figure 19C**). There was no statistically significant difference in condition factor between the groups.

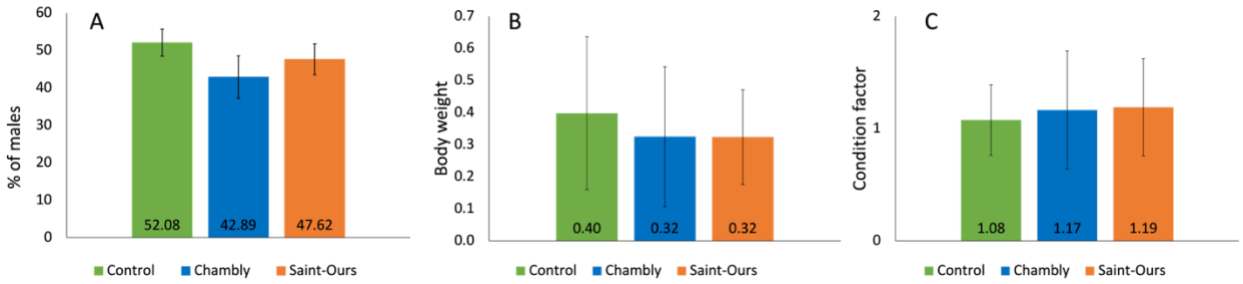

Figure 19. Effects of early life stage exposure to Richelieu River water on sex ratio (percentage of males), body weight and Fulton's condition factor of zebrafish. The exposure groups were exposed for 120 hours to river water that was collected at either one of the two locations along the Richelieu River; Chambly, which is upstream and Saint-Ours, which is downstream. Data are expressed as the total length of fish on days post-fertilization. Each data point represents the average of n=3 replicates, and error bars represent standard deviation. Significant differences between treatments are marked with an asterisk ($\alpha < 0.05$). Included in the data are the exposed generation (F0), which were exposed to river water during early life stage only.

Sex ratio

Overall the proportion of male to female was close to 50% and there was no significant effect of early life exposure to river water on the fish sex ratio (control 52.1% (± 3.6); upstream site water 42.9% (± 5.7); downstream site water 47.6% (± 4.1)) (**Figure 19A**).

Breeding and fertilization rate

For trial 1, the average number of eggs produced per day over 18 days, per centimeter of female zebrafish was 3.7 (± 0.8) for the control, 4.0 (± 0.4) for the upstream site water and 4.1 (± 0.9) for the downstream site water treatments (**Figure 20B** and **Table 14**). There was no statistically significant effect on the average number of eggs produced per day, per centimeter of female zebrafish resulting from early life exposure to river water. The percentage of fertilized and viable embryos 4h post-fertilization was 81% (± 3) for the control, 72% (± 3) for the upstream site water and 65% (± 4) for the downstream site water treatments (**Figure 20A**). There was a statistically significant difference between the control and the 2 river water treated groups (upstream site water $\alpha = 0.0175$; downstream site water $\alpha = 0.0012$) which exhibited a lower fertilization rate. There was also a significant difference between upstream site water and downstream site water treatments ($\alpha = 0.0462$).

For trial 2, which was a cross between control fish and downstream site water treated fish, the average number of eggs produced per day, per centimeter of female zebrafish was 3.0 (± 0.7) for downstream site water treated females with control males, and 3.1 (± 1.1) for control females with downstream site water treated males for Saint-Ours (**Figure 20F**). There was no statistically significant effect on the average number of eggs produced per day, per centimeter of female zebrafish resulting from each cross nor when compared with the control average from the other 2 breeding trials. The percentage of fertilized and viable embryos 4 hpf was 74% (± 0.7) for downstream site water treated females with control males, and the percentage of fertilized and viable embryos 4h post-fertilization was 73% (± 1.1) for control females with downstream site water treated males (**Figure 20E**). There was no statistically significant effect on the percentage of fertilized and viable embryos 4h post-fertilization between the two crosses but downstream site water treated females with control males ($\alpha = 0.0062$) and control females with downstream site water treated males ($\alpha = 0.0032$) were both significantly lower than the controls from the other two breeding trials.

For trial 3, the average number of eggs produced per day, per centimeter of female zebrafish was 3.3 (± 0.9) for the control, 3.9 (± 0.5) for the upstream site water and 3.6 (± 0.4) for the downstream site water treatments (**Figure 20D**). There was no statistically significant effect on the average number of eggs produced per day, per centimeter of female zebrafish resulting from early life exposure to river water. The percentage of fertilized and viable embryos 4h post-fertilization was

83% (± 5) for the control, 73% (± 3) for the upstream site water and 64% (± 5) for the downstream site water treatments (**Figure 20C).** There was, also, a statistically significant difference between the control and the 2 river water treated groups (upstream site water $\alpha = 0.0309$; downstream site water $\alpha = 0.0016$) which exhibited a lower fertilization rate. There was also a significant difference between upstream and downstream stream sites water treatments ($\alpha = 0.0381$).

Females that failed to release embryos were observed in all the replicate of both exposure groups (upstream site water (11.2 % ± 3.6) and downstream site water (16.7 % ± 5.8)), but none in control (**Figure 21**). Embryos could be manually released from the female by gently massaging the female's underside, but they were incapable of releasing embryos naturally (during spawning). Due to their inability to releasee embryos, these females were not included in the breeding trials and were euthanized to prevent rupture of the abdominal wall from embryo reclusion.

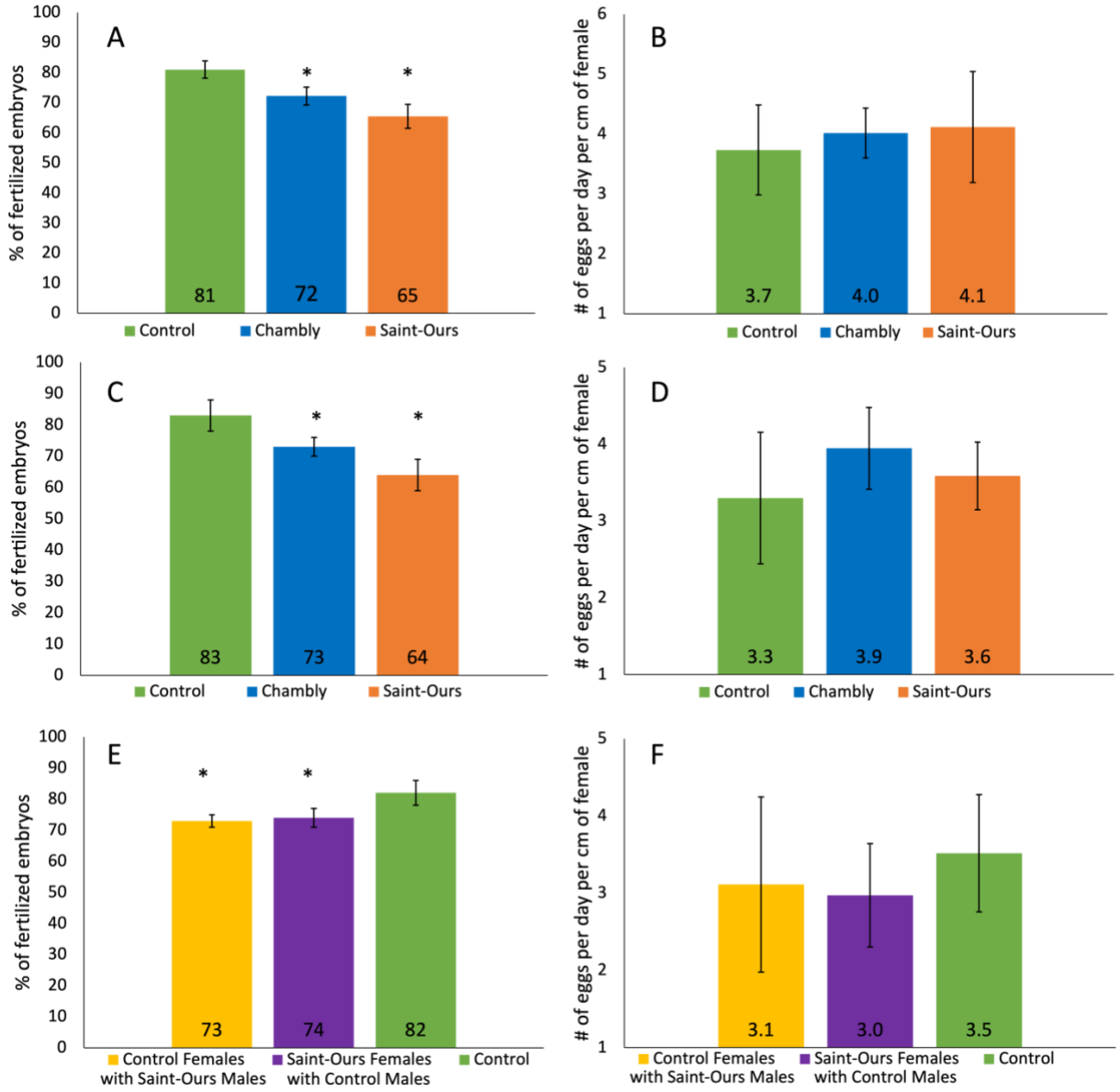

Figure 20. Effects of early life stage exposure to Richelieu River water on the percentage of fertilized embryos and the number of eggs per day produced per centimeter of female zebrafish. The exposure groups were exposed for 120 hours to River water that was collected at either one of the two locations along the Richelieu River; Chambly, which is upstream and Saint-Ours, which is downstream. Data are expressed as the total length of fish on days post-fertilization. The viability of the embryos was assessed under the microscope four hours after fertilization, the number of fertilized viable embryos was recorded. Each data point represents the average of n=3 replicates, and error bars represent standard deviation. Significant differences between treatments are marked with an asterisk ($\alpha < 0.05$). Included in the data are the exposed generation (F0), which were exposed to river water during early life stage only. Three breeding trials were performed, the first one **A)** & **B)** lasted 18 days and the second **C)** & **D)** lasted 14 days. Between the two breeding trials, males from control were matched with females from Saint-Ours and vice versa **E)** & **F**), and the same endpoints were measured over 14 days.

Table 13. Pesticide concentrations in ng/L measured in the Richelieu River samples used for zebrafish early life stage exposure. Chlorantraniliprole, glyphosate, imidacloprid and thiamethoxam were also targeted but not detected. Clothianidin was not detected in Chambly and Imazethapyr was not detected in Saint-Ours.

Compounds	CCME Toxicity threshold	Richelieu River (Chambly)	Richelieu River (Saint-Ours)
AMPA	**NA***	**1006**	**1361**
Atrazine	**1800**	**18**	**19**
Clothianidin	**120**	**ND****	**243**
Imazethapyr	**NA**	**119**	**ND****
Metolachlor	**7800**	**63**	**50**

***NA: Not available**

****ND: Not detected**

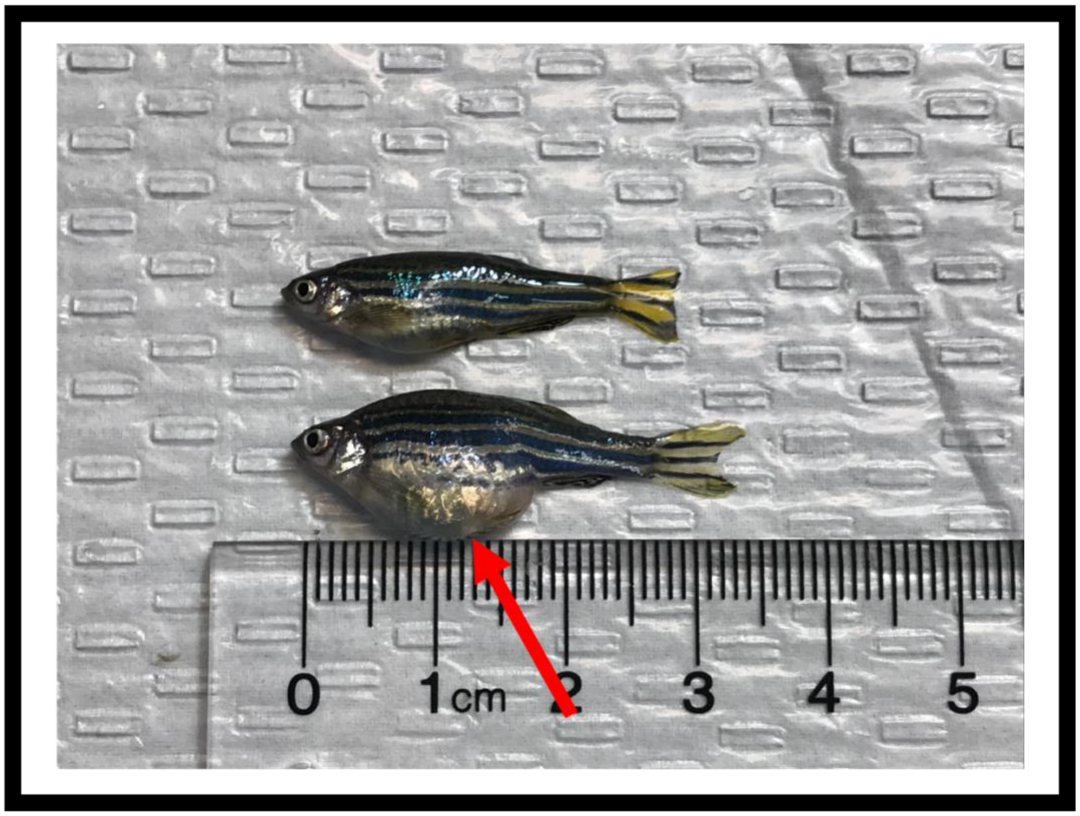

Figure 21. Picture of a female zebrafish with embryos accumulating in the abdominal cavity (red arrow) compared to a regular fish. Females that failed to release embryos were observed in both river water exposure groups but not in control. Embryos could be manually released from the female by gently massaging the female underside, but they were incapable of releasing embryos naturally (during spawning). Due to their inability to release embryos, these females were not included in the breeding trials and were euthanized to prevent rupture of the abdominal wall from embryos reclusion.

Table 14. Summary of reproductive endpoints assessed in zebrafish exposed during early life stages to Richelieu River water.

	Treatment	Average female size	numbers of eggs/female/day	% viable fertilized eggs	eggs/day/cm of female
1st breeding trial	Control	4.8 ± 0.3	13.0 ± 2.5	81 ± 3	2.7 ± 0.5
	Chambly	4.4 ± 0.4	12.0 ± 2.0	72 ± 3	2.7 ± 0.5
	Saint-Ours	4.8 ± 0.2	12.3 ± 1.8	65 ± 4	2.5 ± 0.3
2nd breeding trial	Control	4.8 ± 0.3	11.5 ± 2.8	83 ± 5	2.4 ± 0.6
	Chambly	4.4 ± 0.4	11.8 ± 2.3	73 ± 3	2.7 ± 0.6
	Saint-Ours	4.8 ± 0.2	10.7 ± 0.7	64 ± 5	2.2 ± 0.1
Cross breeding trial	Control males x Saint-Ours females	4.8 ± 0.2	10.0 ± 2.4	74 ± 3	2.1 ± 0.5
	Saint-Ours males x control females	4.8 ± 0.3	9.5 ± 2.5	73 ± 2	2.0 ± 0.5

5.8 Discussion

Summary of basic finding

Our experiment exposed zebrafish during ELS to an environmental mixture (river water) of contaminants from local surface water. We confirmed the presence of pesticides in the river water and observed minimal effects on ELS fish but persistent effects on their reproduction as adults. Our results demonstrated that ELS exposure to river water can have later life effects in fish. This is important since the sampling locations are important fish spawning grounds, and also the only spawning grounds of the endangered copper redhorse.

Water analysis

Measured water concentrations of AMPA, atrazine, and metolachlor were similar between upstream and downstream sites. Chlorantraniliprole, glyphosate, imidacloprid and thiamethoxam were not detected in either location. Since many tributaries that bring pesticides meet the river between the two sampling locations, we expected contamination to be higher at the downstream site. Only clothianidin was higher in downstream site (243 ng/L) than in the upstream site (<6 ng/L). Of all the targeted pesticides, the clothianidin concentration was the only one above the Canadian chronic toxicity threshold for aquatic life; it was approximately two times higher than the threshold (120 ng/L) (Health Canada 2021) (**Table 13**). Imazethapyr on the other hand was only detected at the upstream site but there is currently no Canadian chronic toxicity threshold available for imazethapyr for aquatic life.

Hatching

When exposed to Richelieu River water, zebrafish hatching was delayed when compared to control. Changes in hatching time is a sub-lethal indicator of environmental stress in ELS fish (Barton et al. 2002). In a previous experiment we exposed the endangered copper redhorse (*Moxostoma hubsii*) and the threatened river redhorse (*Moxostoma carinatum*) ELS to river water from the Richelieu in 2018 (Marchand et al. 2022). Both species hatched almost 24h prematurely when compared to control (Marchand et al. 2022). Here, we observed the opposite effect in zebrafish; exposed groups showed delayed hatching when compared to control (**Figure 16**). Species differences in hatching time during pesticide exposure have previously been documented.

For example, when both zebrafish and common carp (Cyprinus carpio) ELS were exposed to glyphosate (50 mg/L), the zebrafish hatched prematurely while the carp exhibited delays in hatching (Fiorino et al. 2018). Clothianidin was the only pesticide that we measured above the CCME toxicity threshold for protection of the aquatic life but early life exposure of sockeye salmon (Oncorhynchus nerka) to up to 150 µg/L clothianidin had no effect on hatching (Marlatt et al. 2019). Hatching delay may have been caused by exposure to a mixture rather than to a single compound. A similarly delayed hatching at 48 and 57 hours post-fertilization was previously reported in zebrafish exposed to 5, 10 and 50% municipal wastewater effluent (Gauthier & Vijayan 2020). Even though we targeted only pesticides and did not measure the full spectrum of contaminants, we know that other anthropogenic contaminants, such as polybrominated diphenyl ethers, pharmaceuticals and personal care procucts and unidentified compounds, are in the river water (Marchand et al, in preparation see Chapter 4). Many contaminants probably reach the river through the wastewater treatment plants which are known to overflow following heavy rain events (Cliché & Saladzius 2018). The absence of differences in hatching time in the unexposed offspring confirms that the river water was the cause of the observed effects (**Figure 16**). Further research is needed to be able to link specific contaminants of the mixture to the effects that we observed. The potential consequences of delayed hatching are unclear but longer time in the egg could potentially lead to increased predation (Barton et al. 2002).

ELS effects (survival, deformities, morphometrics and sex ratio)

Survival (Wang et al. 2017), deformities (Lin Sun et al. 2009; Mhadhbbi & Beiras 2012), and alterations to growth (Wirbisky et al. 2016) or sex ratio (Teather et al. 2005) have all been reported to be affected by ELS exposure to pesticides in fish, albeit at higher doses than the concentrations that zebrafish were exposed to here. In our study, no direct effects of exposure to the environmental mixture present in river water were observed on these endpoints (**Table 14**).

Persistent effects (reproduction and F1 deformity rate)

Zebrafish raised to maturity in clean water following ELS exposure to river water exhibited reduced fertility compared to controls. No effect on egg production was observed, but a significantly lower proportion of fertilized embryos was observed (**Figure 20**). Both males and females from the exposed group seem to have been contributing to the decrease in fertilization

rates. The cross breeding of male from control with female from downstream site water treatment and vice versa resulted in a fertilization rate halfway between downstream site water treatment and control for both (**Figure 20** & **Table 14**). This suggests that both males and females that were exposed during ELS had decreased fertility. The mechanisms behind this are still unknown, as is which components of the mixture are driving the effects. Many of the pesticides that we measured have the potential to influence reproduction and fertility since they are known endocrine disruptors. For example, exposure of adult zebrafish to 300 µg/L metolachlor had endocrine disruption effects and reduced production of sex hormones (Ou-Yang et al. 2022). Also, atrazine is an endocrine disruptor that has a consistent effect of demasculinization and feminization of males among vertebrates (Hayes et al. 2011). Gonadal abnormalities were observed in fathead minnow exposed to concentrations of atrazine as low as 500 ng/L (Tillitt et al. 2010).Previously observed changes in sex hormones, gonadal abnormalities and chromosomal abnormalities in spermatogonia (Tilllitt et al 2010; Papoulias et al. 2014) could lead to lower fertilization success. Breeding behavior was not assessed in this experiment, but previous studies have reported alteration of breeding behavior in fish following pesticide exposure (Moore & Lower 2001; Shenoy 2014). Further studies could investigate the cause of this reduced fertility using histology or gene expression.

Females with abdominal swelling, that failed to release their embryos, were observed in both treatment groups (**Figure 21**). The same condition was observed in female zebrafish that had been exposed to 30 µg/L of atrazine during embryonic development (Wirbisky et al. 2016). This dose is 3-fold higher than the concentration of atrazine that we measured in the river but changes in sex hormones levels or to gonadal tissues were also reported in other measured compound as previously mentioned. The sum of effects of the many chemicals present in the river may have a similar effect on female abdominal swelling. The consequence of this could be dramatic since the females were unable to spawn volitionally and their abdominal wall could rupture from the increase in ovary size.

Larval deformities, were observed in F1, but not in F0 even though these were the fish that were directly exposed. No other multigeneration study reported a similar increase in the rate of deformities following exposure to pesticides, but ELS exposure to atrazine. 0.3, 3 and 30 ug/L caused alterations of head length to body ratio in zebrafish unexposed offspring (Wirbisky et al.

2016). There is increasing awareness of the importance of both trans- and multigenerational effects of endocrine-disrupting chemicals including pesticides at environmentally relevant levels (Robaire et al. 2022, De Courten et al. 2020). To better determine the long term effects of river water on wild fish populations, future studies could span additional generations to also assess the unexposed germline.

Gradation of effects (upstream to downstream)

The magnitude of the effects that we observed followed the expected gradient of contamination. For example, the delayed hatching (**Figure 16**) and the decrease in fertilization rates were observed in both exposure groups (**Figure 20**), but the effect size was larger in fish exposed to water from the downstream site than the upstream site. Previous water analysis did show that the downstream site, was the most contaminated location of the two (Marchand et al, in preparation Chapter 4). Our results were not as clear since only 5 of the 9 pesticides targeted were detected and only one was higher in the downstream site water (**Table 13**). However, it is still reasonable to assume, even if we did not measure it, that the downstream site water contaminant mixture is more complex than the one from the upstream site, and all the contaminants present in the water at the upstream site will most likely end up passing through the downstream site. And more contaminants will be added up along the way.

Limitations

Here we focused on ELS exposure, but fish in the wild would be exposed to fluctuating levels of contamination throughout their lives. Here, the exposure was environmentally realistic in terms of using real water samples but river contamination varies spatially but also across time (Marchand et al. 2022). Even daily concentrations can vary up to 100-fold (Marchand et al. in preparation, see Chapter 4). This is also true for seasonal and yearly fluctuations which may be caused by changes in the activities in the watershed but also changes in agricultural practices.

The low survival rate to 60 days in all treatment groups including control is a major limitation. At 60 dps, survival was 14 ± 4% for the control, 15 ± 1% for the upstream site water and 12 ± 1% for the downstream site water. There are no specific guidelines for survival in a long term experiment as the one presented here, but for the 28 days juvenile fish growth test (OECD 215), survival above

90% in control is required for validation. The mortality was high due to sub-optimal diet and rearing conditions, that were equivalent for all the treatments. Nevertheless, because the fish were raised in sub-optimal conditions for the first 30 days of life, later life effects reported here should be interpreted with caution. Moreover, low survival had effect on the reproductive assay since the number of fish used to assess the effects on breeding was small. In each replicate, the sub sample of fish used was of 3 males and 4 females. Given these considerations, the experiment should be replicated to confirm the results reported here.

5.9 Conclusion

Our work sheds light on the effects of ELS exposure to real-world mixtures in zebrafish. We provided evidence that the ELS exposure to river water, can directly impact larvae, but also lead to a reduction of fertilization rate in adults that were raised to maturity in clean water. A reduction in egg fertilization, such as the 15% observed in the ELS fish exposed to water from the downstream site could be detrimental to wild populations of fish spawning in the Richelieu. There also was a gradation of effect from downstream to upstream which means that ELS of fish from spawning at the former are more likely to have more severe effects than those from the latter. Due to the high variability of contaminant mixtures, more research is required to determine if the effects are always present and if similar effects can be observed in native fish species. Similar studies should be repeated in the future, with water from the Richelieu but also from other water bodies to evaluate the real impact of environmental mixtures. Future work will be aimed at clarifying the individual effects of contaminants present in the Richelieu River on wild fish ELS. Identifying how the fertilization rate was affected is also an essential objective for future research.

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Preface to Chapter 6

In the previous chapters we determined that exposure to river water during ELS causes effects in wild (river and copper redhorse) and laboratory fish (zebrafish). We have also determined that pesticide concentrations are of concern in the Richelieu River, where the only known copper redhorse spawning grounds are located. From the literature review, we also know that many pesticides may be causing the effects we observed, even though effects were usually reported at concentrations higher than those we measured in the river.

In this chapter we tried to link the previously reported effects to specific contaminant exposure. For this, we performed a laboratory experiment during which we exposed river and copper redhorse to one of four contaminants of concern in a laboratory setting. The goal was to determine if the pesticides that were the most detected or at the highest concentrations were driving the previously reported effects by removing the environmental mixture variables. This was the last step in determining the effects of ELS exposure to environmental pesticides in wild and laboratory fishes.

Chapter 6

6. Assessment of 4 current use pesticides in early life stage of two listed *Moxostoma* species: the river and copper redhorse

6.1 Title Page

Title of the manuscript:

Assessment of 4 current use pesticides in early life stage of two listed *Moxostoma* species: the river and copper redhorse

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Conflict of Interest

The authors declare that there is no conflict of interest.

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6.2 List of Tables

6.3 Abstract

Fish living in agricultural landscapes are at high risk of exposure to pesticides. In waterways such as the Richelieu River (Quebec, Canada), pesticide application and spring run-off coincide with the spawning season for many fishes, including 2 listed fish, the river redhorse (*Moxostoma carinatum*) and the copper redhorse (*Moxostoma hubbsi*). Previous research has demonstrated that early life stage exposure to river water caused early hatching in both redhorse species and decrease of survival of copper redhorse. Here we tried to link these effects to four pesticides of concern that were detected in the river water. Three replicates of river (n = 32) and copper redhorse (n =30) embryos were exposed for 14 days to environmentally relevant concentrations (0.3, 3 and 30 µg/L) of the three individual herbicides (atrazine, glyphosate and metolachlor) or to 0.03, 0.3 and 3 µg/L of the neonicotinoid insecticide clothianidin. During the exposure, deformities, hatching and survival were monitored daily and no effect from treatment was observed. Our work demonstrated that river and copper redhorse did not exhibited organismal level signs of response at environmental levels of the active molecule of four pesticides extensively present in their spawning habitats. We provided evidence that, individually, these compounds do not explain the dysregulation of hatching time and decrease of survival previously observed following exposure to river water.

6.4 Introduction

Fish living in agricultural landscapes are at high risk of exposure to pesticides. In waterways such as the Richelieu River (Quebec, Canada), pesticide application and spring run-off correspond with peak spawning season for many fishes, including some threatened or endangered species. The only 2 known spawning grounds of the endangered copper redhorse (*Moxostoma hubbsi*) are located in the Richelieu, and this river includes habitat for other listed species such as the lake sturgeon (*Acipenser fulvescens*) (Thiem et al. 2013) and the river redhorse (*Moxostoma carinatum*) (COSEWIC 2015). Approximately 70% of the Richelieu River watershed is dedicated to agriculture (Simoneau & Thibault 2009) and pesticide concentrations have previously been measured above toxicity threshold for aquatic organisms (Giroux 2015 & Giroux 2022). This is of concern since listed species tend to be more sensitive to environmental contaminants (Sappington

et al. 2001, Besser et al. 2005, Dwyer et al. 2005), and early life stages are often the most vulnerable (Mohammed 2013).

Pesticide exposure to fish early life stage may cause a number of effects such as decreased survival (Wang et al. 2017), increased incidence of deformities (Lazhar et al. 2012; Mhadhbi & Beiras 2012) and alteration of hatching (Schweiser et al. 2019; Liu et al. 2022), behavior (Liu et al. 2016) and biochemical pathways (Richterova et al. 2015; Jin et al. 2015). Most effects were often reported at pesticide concentrations above what is measured in the environment but our previous research suggests that river water could cause measurable effects to ELS fish. We showed that early life stage (ELS) exposure of copper redhorse and river redhorse (Moxostoma carinatum), using constantly renewed river water from one of the copper redhorse spawning grounds along the Richelieu River, showed premature hatching in both species and increased mortality in copper redhorse larvae (Marchand et a. 2022). A follow up study, using zebrafish, exposed to Richelieu water exhibited delayed hatching and later life effects resulting from ELS exposure. A decrease in the adult fertilization rate of up to 15% was detected and a higher incidence of deformities in the offspring of the parents that were exposed only during ELS when compared to control (Marchand et al. Zebrafish). Here, we investigate whether the pesticides detected most frequently in the mixture are acutely toxic to ELS fish.

This means that pesticide contamination could have an effect on the species' natural recruitment and a previous experiment has demonstrated that river water may have negative effects in copper and river redhorse (Marchand et al. 2022). The copper redhorse (*Moxostoma hubbsi*) and river redhorse (*Moxostoma carinatum*) are Catostomidae endemic to eastern north America that feed extensively on molluscs (COSEWIC 2014, COSEWIC 2015). A monitoring and conservation program for the copper redhorse, which includes artificial breeding, was set up in 2004 by Quebec government's Ministère des Forêts, de la Faune et des Parcs (MFFP). But the path to recovery has been slow and the importance of the agricultural industry in its essential breeding habitats may play a role but the absence of toxicity data for copper and river redhorse make it hard to evaluate.

We found that exposure to river water may have a negative impact on reproduction and recruitment. Many contaminants have been detected in the Richelieu River, including legacy organics such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs),

dioxins, and furans (Laliberté 2020), pharmaceutical and personal care products (PPCPs) (Marchand et al. 2022, Chapter 4) and pesticides (Giroux 2019, Marchand et al. 2022, Chapter 4). Most of these contaminants were detected at levels below aquatic life toxicity thresholds but pesticides such as atrazine, clothianidin, metolachlor, thiamethoxam were often detected at levels exceeding these thresholds (Giroux 2015, Giroux 2019, Chapter 4). Here we focus on four pesticides, atrazine, clothianidin, glyphosate and metolachlor, which had the highest detection frequency or that were the most often exceeding toxicity threshold (Giroux 2015, Giroux 2019, Chapter 4). Although effects that could be detrimental to the recovery of the species were detected, we were not able to determine which components of the river were driving the observed effects. It is essential to determine which contaminants are driving the previously reported effects to better protect listed aquatic species.

The contaminants driving the effects are unknown but due to agriculture being the main vocation in the watershed, pesticides are likely good candidates. To investigate this possibility, we conducted a laboratory pesticide exposure to assess the effects of 3 herbicides (atrazine, glyphosate and metolachlor) and one neonicotinoid insecticide (clothianidin) on ELS of 2 listed species: the threatened river redhorse and the endangered copper redhorse. The data will improve our understanding of both river and copper redhorse sensitivity to environmental contaminants, provide the first toxicity data for these two listed species and help determine priority contaminants that may be impacting their recovery.

6.5 Materials and Methods

Chemical Stocks

Pesticide dosing solutions were prepared by dissolving atrazine (CAS number and purity), clothianidin (CAS number and purity) or metolachlor (CAS number and purity) in methanol and glyphosate (CAS number and purity) in ultra-pure water. Nominal concentrations of 2.55 g/L for atrazine, glyphosate and metolachlor and of 0.255 g/L for clothianidin were achieved. Two lower dose solutions were prepared by 10-fold serial dilution from the high dose solution for each of the chemicals.

Source of Animals

All work with animals was approved under animal use protocols from MFFP and McGill University (2018-7992). Research and collection permits were obtained from Parks Canada, Fisheries and Oceans Canada (SARA; MPO-LEP-QC-19-006 & DFO-SARA-QC-22-001) and MFFP (SEG; 2019-04-23-2564-16-S-P & 2022-05-16-3278-16-S-FP). The MFFP artificial breeding program provided newly fertilized embryos for both river and copper redhorse. Wild parent fish were caught during their respective spawning season at the Vianney-Legendre fishway, a fish ladder located beside the St-Ours dam. Details of artificial reproduction were provided in Marchand et al. 2022. Embryos used for this experiment were siblings i.e. offspring of a single pair. Once the embryo's shell was hardened, (approximately 2 hours post-fertilization), they were transported in reconstituted hard water (ultrapure water supplemented with sodium bicarbonate (96 mg/L), magnesium sulfate (60 mg/L), calcium chloride (39 mg/L), and potassium chloride (4 mg/L) (Modified USEPA-821-R-02-12) that matched river physicochemical parameters to McGill University Macdonald Campus in an insulated container. River redhorse embryos were provided in June 2019 and copper redhorse embryos were from 2022.

Experimental Design and Fish Maintenance

Upon reception, river and copper redhorse embryos were divided equally into a control group, a solvent control (methanol or water) and low, medium and high concentration groups for each of the 4 chemicals. For each treatment group, embryos of each species were housed separately in 3 replicate tumblers (Ziss Aqua), each containing 30 embryos of copper or 32 embryos of river redhorse. Each fish egg tumbler was placed in a 1 L borosilicate glass beaker containing 950 mL of pre-cooled reconstituted hard water. The beakers were kept, in a random pattern, inside a walk-in fridge that was adjusted daily to match river temperatures. A central air system was used to power the fish egg tumblers and maintain good oxygenation and a gentle rocking motion of the embryos. As soon as the larvae were free swimming, the fish egg tumblers were removed, but oxygenation was maintained. Water quality parameters were recorded daily.

As soon as possible (approximately 6-8 hours post-fertilization), water in beakers was dosed with 10 µL of the appropriate solution to achieve 0 (solvent control (10 µL of methanol)), 0.3, 3 and 30 µg/L of either atrazine, glyphosate and metolachlor or 0 (solvent control), 0.03, 0.3 and 3 µg/L of clothianidin. These pesticide concentrations were selected to mimic baseline levels detected in the

Richelieu River (low), river levels following a rain event (medium), and tributary levels following a rain event (high) (Giroux 2018, Chapter 4).

Endpoints Measured

Fish embryos and larvae of each species were maintained until 14 days post-fertilization (dpf), just before the complete resorption of the yolk sac, with no renewal of the water. Timing of embryos hatching, incidence of deformities in embryos or larvae, and survival were assessed daily at the same time each day. Embryos were considered hatched when the larval body was fully extended and completely free of the egg membrane.

Embryo and larval size, shape and development stage were observed. In larvae, deformities such as edema, craniofacial malformation, spinal curvature (lordosis and kyphosis) and yolk sac malformation were recorded. Photographs of deformed embryos were taken daily.

Dead embryos and larvae were removed from tumblers or beakers as soon as they were noticed. Embryos and larvae that were discolored, coagulated, showed shell damage, or were not showing movement and/or heartbeat, were considered dead. Percent survival was calculated as the daily total number of live embryos and larvae over the initial number of embryos.

Data Analysis

Daily percentage hatching was calculated as a proportion of the total number of living embryos (hatched larvae/all alive) per day and per replicate. Daily percentage hatching data were fitted to a sigmoidal four parameter logistic regression and the 50% hatch time was calculated using AAT bioquest LC50 calculator (AAT Bioquest 2022). Mean incidence of deformities and mean survival were calculated as a percentage of the initial number of embryos that were placed in each tumbler. All data residuals were tested for normality. Analysis of Variance (ANOVA) and a Tukey's post-hoc test were performed for daily percentage hatching, 50% hatch time, incidence of deformities, and survival between treatments using JMP statistical visualization software (SAS Institute). An $\alpha < 0.05$ was considered significant.

6.6 Results

River Redhorse

Hatching Time

River redhorse embryos started to hatch, on average, on the sixth dpf except for those exposed to the low concentration of atrazine, the low and medium concentrations of clothianidin, the medium and high concentrations of glyphosate, and the medium concentration of metolachlor. Those started to hatch on the seventh dpf. River redhorse embryos were all hatched on the seventh dpf in control and in those exposed to the high concentration of metolachlor. The remaining treatments of river redhorse embryos were all hatched on the eight dpf except for the low concentration of atrazine and the low, medium and high concentrations of metolachlor which were all hatched on the nineth dpf; the high concentration of clothianidin and medium concentration of glyphosate were all hatched on the eleventh dpf; and the high dose of glyphosate which were all hatched on the thirteenth dpf. Overall, there was no significative statistical difference in river redhorse hatching time between control and treatments.

For all treatments, the average 50% hatch time of river redhorse ranged from 6.2 to 6.7 dpf (**Table 15**). For the river redhorse average 50% hatch time, there was no significative statistical difference between control and treatments. There was a significative statistical difference in the average 50% hatch time between the atrazine low and high concentrations ($\alpha = 0.028$) (**Table 15**).

Deformities

For all treatments, the average deformity rate of river redhorse ranged from 1.0 to 4.2% (**Table 15**). For the river redhorse average deformity rate, there was no significative statistical difference between control and treatments.

Survival

For all treatments, the average survival of river redhorse at the end of the exposure (14 days post-fertilization) ranged from 81.3 to 93.8% (**Table 15**). For the river redhorse average survival, there was no significative statistical difference between control and treatments. There was a significative statistical difference in the average survival between the atrazine and clothianidin medium concentrations ($\alpha = 0.008$).

Copper Redhorse

Hatching Time

Copper redhorse embryos started to hatch, on average, on the seventh dpf, except for those exposed to the medium and high concentrations of clothianidin and the low and medium concentrations of metolachlor which started to hatch on the third dpf; and the high atrazine concentration which started to hatch on the fourth dpf. The three treatments that started to hatch early were driven by one replicate each in which hatching started earlier. The other two hatched at a similar time as the control group. Copper redhorse embryos were all hatched on the eighth dpf in all treatment groups except atrazine and clothianidin high concentration and metolachlor low concentration. Those were all hatched on the seventh dpf. Overall, there was no significative statistical difference in copper redhorse hatching time between control and treatments.

For all treatments, the average 50% hatch time of copper redhorse ranged from 5.1 to 7.0 dpf (**Table 15**). For the copper redhorse average 50% hatch time, there was no significative statistical difference between control and treatments.

Deformities

For all treatments, the average deformity rate of copper redhorse ranged from 0.0 to 4.4 % (**Table 15**). For the copper redhorse average deformity rate, there was no significative statistical difference between control and treatments.

Survival

For all treatments, the average survival of copper redhorse at the end of the exposure (14 days post-fertilization) ranged from 93.3 to 100% (**Table 15**). For the copper redhorse average survival, there was no significative statistical difference between control and treatments.

Difference Between Species

For all the endpoints measured, hatching, deformities and survival, there was no significative statistical difference between the river and copper redhorse.

6.7 Discussion

Our study used an ELS laboratory exposure to pesticides to determine the toxicity of four current use pesticide on the threatened river redhorse and the endangered copper redhorse. At environmental concentrations, the four pesticides were not acutely toxic to either of the two fish species. The experimental design allowed us to control the contaminant exposure while maintaining river conditions such as water physico-chemical parameters and temperature. Both river and copper redhorse adapted well to the laboratory system, which successfully maintained favorable conditions for embryo development, hatching, and early larval development. For both species, survival in control and solvent control was above 90% (**Table 15**).

Previous Environmental Exposure

The pesticide concentrations that the copper and river redhorse ELS were exposed to here were environmentally relevant. For example, concentrations of atrazine (Gammon et al. 2005, Liu et al. 2016), glyphosate (Tresnakova et al. 2021) and metolachlor (Vryzas et al. 2011, Quintaneiro et al, 2017) around the globe are usually reported in µg/L. For neonicotinoids such as clothianidin, concentrations range from ng/L to µg/L (Moschet et al. 2014; Anderson et al. 2015; Morrissey et al. 2015). Concentrations concentrations in the Richelieu River and its tributaries are slightly lower and ranged from 0.007 to 0.811 µg/L, 0.06 to 0.198 µg/L, 0.22 to 0.969 µg/L and 0.01 to 7.008 µg/L for atrazine, clothianidin, glyphosate and metolachlor respectively (Marchand et al. chemistry paper). The concentrations used in this experiment 0.003 to 3 µg/L, were therefore environmentally relevant, while the high concentration (30 µg/L) could still represent a worst-case scenario for the Richelieu river. Overall, the exposure did mimic a single compound exposure representative of what wild fish ELS would be exposed to near their spawning grounds.

Table 15. Effects of river redhorse and copper redhorse early life exposure to atrazine, clothianidin, glyphosate or metolachlor on hatching time, deformity rate and survival. Solvent control used was methanol and low, medium and high concentrations of atrazine, glyphosate and metolachlor were 0.3 µg/L, 3 µg/L and 30 µg/L respectively and 0.03 µg/L, 0.3 µg/L and 3 µg/L for clothianidin. Exposure started six to eight hours after fertilization and lasted 14 days. Each column represents the average of n=3 replicates and error bars represent standard deviation. Data shown are **A & B)** 50% hatch time (time required for 50% of the embryos to hatch), **C & D)** deformities percentage and **E & F)** survival expressed as the percentage of live zebrafish at the end of the exposure. No significant effects from any treatments were observed.

Treatment	Concentration	River Redhorse			Copper Redhorse		
		EC50 Hatch (dpf)	% Deformities	% survival	Ec50 Hatch	% Deformities	% survival
Controls	Control	6.4 ± 0.1	2.1 ± 1.8	93.8 ± 6.3	6.7 ± 0.4	1.1 ±1.9	96.7 ± 3.3
	Solvent control	6.5 ± 0.2	1.0 ± 1.8	90.6 ± 5.4	6.6 ± 0.1	0.0 ± 0.0	100.0 ± 0.0
Atrazine	0.3 µg/L	6.7 ± 0.0	2.1 ± 1.8	89.6 ± 3.6	6.7 ± 0.2	1.1 ± 1.9	98.9 ± 1.9
	3 µg/L	6.5 ± 0.0	2.1 ± 1.8	97.9 ± 3.6	6.6 ± 0.1	1.1 ± 1.9	95.6 ± 5.1
	30 µg/L	6.2 ± 0.4	2.8 ± 5.4	87.5 ± 3.1	6.4 ± 0.2	1.3 ± 0.0	95.6 ± 3.8
Clothianidin	0.03 µg/L	6.5 ± 0.1	1.0 ± 3.1	91.7 ± 6.5	6.7 ± 0.3	2.2 ± 1.9	96.7 ± 3.3
	0.3 µg/L	6.6 ± 0.1	3.1 ± 3.1	81.3 ± 3.1	6.6 ± 0.2	1.1 ± 1.9	97.8 ± 3.8
	3 µg/L	6.7 ± 0.3	3.1 ± 5.4	91.7 ± 4.8	5.1 ± 2.3	0.0 ± 0.0	93.3 ± 11.5
Glyphosate	0.3 µg/L	6.4 ± 0.1	4.2 ± 1.8	87.5 ± 3.1	6.5 ± 0.1	0.0 ± 0.0	100.0 ± 0.0
	3 µg/L	6.5 ± 0.1	3.1 ± 3.1	88.5 ± 7.9	6.7 ± 0.4	2.2 ± 1.9	95.6 ± 5.1
	30 µg/L	6.7 ± 0.0	2.1 ± 1.8	91.7 ± 1.8	6.7 ± 0.0	1.1 ± 1.9	96.7 ± 3.3
Metolachlor	0.3 µg/L	6.5 ± 0.1	3.1 ± 0.0	88.5 ± 6.5	6.4 ± 0.1	0.0 ± 0.0	97.8 ± 1.9
	3 µg/L	6.6 ± 0.1	4.2 ± 3.6	92.7 ± 1.8	5.3 ± 2.5	4.4 ± 7.7	93.3 ± 11.5
	30 µg/L	6.5 ± 0.1	2.1 ± 1.8	90.6 ± 0.0	7.0 ± 0.3	1.1 ± 1.9	97.8 ± 1.9

Four chemicals were assessed here and overall, no effects on all measured endpoints on either river or copper redhorse ELS were observed (Table 15). The compounds tested were good candidates because of the overtly high detection frequency in the watershed, but also because of their known effects on fish. For example, there is a consensus that atrazine (Wiegand et al. 2000, Wiegand et al. 2001, Adeyemi et al. 2015, Blahova et al. 2020), glyphosate (Bortagaray et al. 2009, Dornelles Zebral et al. 2017, Fiorino et al. 2018) and metolachor (Quintaneiro et al. 2017) are embryotoxic, dysregulate hatch and increase the incidence of deformities in fish ELS. Little information is available for clothianidin itself, but its parent compound thiamethoxam also has the same effects (Yang et al. 2020, Hasan et al. 2022, Victoria et al. 2022). However, these effects are reported at concentrations usually in the mg/L range (Giddings et al. 2005, Bortagaray et al. 2009, Quintaneiro et al. 2017, Yang et al. 2020), which is two order of magnitude higher than what was measured in the environment and used here.

We previously reported that exposure of early life stage fish to water from the Richelieu river was associated with decreased survival, dysregulation of hatching (Marchand et al. 2022) and reproduction (Marchand et al. ZB). Here both copper and river redhorse embryos were exposed to relevant concentrations of single pesticide selected from the environmental mixture and no effects were reported on organismal endpoints. From previous exposure, organismal effects were standing out while only a small subset of genes were found to be differentially expressed (Marchand et al. 2022). The focus here was set on these organismal changes and not on molecular or behavioral endpoints, which however may be more sensitive to perturbation. Reproduction was not assessed due to the late age of maturity of the tested fish species, but assessment of reproductive marker may be an interesting endpoint for future work. Although multiple lines of evidence point to developmental consequences of exposure to river water, we were not able to determine which component of the mixture is driving these effects.

The four pesticides tested could not be linked to previously reported effects following river water exposure (**Table 15**), but river water is a mixture of numerous chemicals. Here, only the active ingredients of 4 pesticides were tested. Pesticides are often applied as a formulations, containing either single or multiple active ingredients and formulants (to improve its physical characteristics). Previous studies have demonstrated that these formulations and formulants can be as, or even more toxic to fish than the active ingredients (Beggel et al. 2010, Guilherme et al. 2012, Defarge et al.

2018). Also, additive and synergistic effects have previously been reported with pesticides (Perez et al. 2013, Zhang et al. 2017, Wang et al. 2017), but also between pesticides and other contaminants (Nunes et al. 2014). Richelieu River water contains numerous other chemicals, either from natural or anthropogenic sources, such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and dioxins and furans (Laliberté 2020). Previous untargeted screening of the Richelieu River revealed hundreds of compounds, but only a few could be identified (Chapter 4). It is possible that something other than pesticides is driving the effects previously described, future research should focus on the mixture effects from the many chemicals present in the Richelieu River.

The conclusion from a previous study was that copper redhorse are more sensitive than river redhorse (Marchand et al. 2022). Here, we weren't able to confirm or deny this hypothesis. So far, both river and copper redhorse were equally insensitive to the tested doses of the four pesticides assessed (**Table 15**). Listed fish species are often more sensitive than the species commonly used in toxicity testing (Sappington et al. 2001, Besser et al. 2005, Dwyer et al. 2005). Our investigation stands in accordance with previous studies demonstrating that biological effects were only observed in concentrations beyond environmental relevance. However, due to the inherent increased sensitivity of listed species, direct testing of chemicals is the best alternative to assess species specific risks (Dwyer et al. 2005).

Limitation

The experiment was performed on individuals with limited genetic diversity. Animals used for the laboratory exposure were all siblings from a single cross and did not represent the genetic diversity of both species. The experiment may have overlooked the fact that some individuals from the same population can be more or less sensitive to contaminants (Nacci et al. 1999). However, the use of siblings allowed for all embryos to be exposed at the same time and prevented genetic background from being another variable.

The pesticides were only dosed once at the beginning of the experiment and the concentrations weren't renewed over the span of the exposure. This may have led to decreased concentrations over time from deterioration of the parent molecules. However, river water often receives pesticides as pulses following rain events, which was mimicked here. More studies are needed to

determine how the timing of these pulses in relation to the ELS developmental stage impacts effects observed.

6.8 Conclusion

Our work demonstrated that river and copper redhorse did not exhibited organismal level signs of response at environmental levels of the active molecule of four pesticides extensively present in their spawning habitats. We provided evidence that, individually, these compounds do not explain the dysregulation of hatching time and decrease of survival previously observed following exposure to river water. Previously reported effects in zebrafish exposed during ELS were related to reproduction (Chapter 5), further investigation on the effects of these compounds on reproductive markers are warranted. Future work should still be aimed at clarifying the individual effects of other contaminants present in the Richelieu River on fish ELS. Future exposure studies focusing on the effects of mixture and transcriptomics are already underway. The identification of the environmental contaminants from the copper redhorse spawning grounds that are driving the previously described effects would allow for better protection measures to be put into place to help the copper redhorse recovery.

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Chapter 7

7. Discussion

7.1 Restatement of research problem and research questions

Contamination of surface water by pesticides is of increasing concern and a threat to biodiversity (Relyea 2005). The copper redhorse (*Moxostoma hubbsi*), an endangered species, is especially vulnerable since its only known spawning grounds are in the Richelieu River, at the heart of a heavily agriculture area. Even with a protective status and a recovery program using artificial breeding, the road to recovery for the species has been slow. A potential culprit for this slow recovery is pesticide contamination of the spawning grounds, with its peak concentrations coinciding precisely with copper redhorse spawning season and early development. This could have an effect on the whole species, since all the population's offspring are exposed, during ELS, a most sensitive stage, to peaking pesticide concentrations. Here we assess if pesticide contamination of surface water is a concern for ELS fish using the copper redhorse, river redhorse and zebrafish. Using a complementary suite of *in-situ* and laboratory exposure experiments, we investigated the role of pesticide contamination in impeding recovery of the endangered copper redhorse by testing the hypothesis that river water has negative effects on fish ELS.

7.2 Summary of key findings

The overall finding of this thesis is that fish ELS exposure to river water resulted in immediate ELS effects and persistent later life effects. An *in-situ* riverside exposure on the threatened river redhorse and the endangered copper redhorse was performed to assess the effects of exposure to constantly renewed river water from their spawning grounds. This study found that ELS exposure to river water caused early hatching in both species and a decrease in survival in copper redhorse larvae (Chapter 3). Since river redhorse and copper redhorse reach such a large size and have a late age of maturity (> 10 years), zebrafish were used to evaluate the full life cycle effects of ELS exposure to river water (Chapter 5). Exposure to river water caused delayed hatching of zebrafish embryos following the river contamination gradient (Chapter 5). A decrease in fertilization rate, also following the contamination gradient of the Richelieu River, was observed in the adult zebrafish that were exposed as ELS when compared to control (Chapter 5). An increased incidence of deformities was also observed in the offspring of the exposed zebrafish (Chapter 5). Following

a 14-day exposure to constantly renewed river water, dysregulation of copper redhorse genes involved in injury and immune response and upregulation of CYP1A1, a gene involved in the detoxification mechanism, were measured (Chapter 3). In an attempt to link the observed changes with specific contaminants, a laboratory ELS exposure of river redhorse and copper redhorse to the four most prevalent pesticides detected in the Richelieu water was performed (Chapter 6). The results of the laboratory exposure study did not show any effect from exposure to environmentally relevant and higher concentrations of atrazine, clothianidin, glyphosate or metolachlor (Chapter 6).

Richelieu River water contained pesticide concentrations above toxicity thresholds for aquatic life and concentrations peaked after heavy rain events. To assess pesticide contamination, the river water was sampled during the river and copper redhorse spawning season using daily grab samples and passive samplers (Chapter 4). These samples were analyzed by targeting pesticides of concern, by targeting pesticides in general and by un-targeted screening (Chapter 4). The neonicotinoids clothianidin and thiamethoxam were detected at concentrations above the toxicity threshold for aquatic life in the 2 years of sampling (Chapters 4 & 5). Concentrations of pesticides were higher in the tributaries than in the main river. Following heavy rain events, concentrations of most of the pesticides in the water peaked from 10-100 folds higher than concentrations measured over dry weather (Chapter 4). Overall, Richelieu River water, including the only known copper redhorse spawning grounds, contained a rich mixture of contaminants, which can have effects on fish ELS.

7.3 Answer to thesis question

With this in mind, we can confirm that fish exposure to river water during ELS causes a suite of negative outcomes, which could potentially have species level effects. However, these effects could not yet be specifically linked to pesticides or a single compound.

7.4 Interpretation of findings

The results, observed in river redhorse, copper redhorse and zebrafish ELS exposed to Richelieu River water, are consistent with pesticide exposure. For example, exposure to individual pesticide during ELS has been associated with effects on deformity rate (Sulukan et al. 2017), hatching time (Du Gas et al. 2017; Fiorino et al. 2018), survival (Vignet 2019) and immune response (Hong et al. 2018). Later life effects observed in zebrafish confirm that ELS exposure can have persistent

effects. However, the findings don't match those of previous studies. For example, exposure to 500 ng/L of atrazine caused a decrease in egg production but no effect on fertilization rate in fathead minnows (Tilllitt et al 2010) and Japanese medaka (Papoulias et al. 2014), while we observed the opposite, no effect on egg production but a decrease in fertilization rate. Many studies have found that pesticide exposure can cause alterations to fish reproductive systems and hormones (Tilllitt et al 2010; Hayes et al. 2011; Papoulias et al. 2014; Ou-Yang et al. 2022), which may have led to the reduced fertilization rate that we observed. However, these studies focused on single pesticide exposure while here we used an environmental mixture containing numerous contaminants with many unknown properties.

Studies using environmental mixtures and ELS environmental exposure have reported effects ranging from sublethal (Bony et al. 2008, Marlatt et al. 2016, Gauthier & Vijayan 2020) to complete mortalities (Tian et al. 2022), but these are not consistently linked to pesticide exposure. In fact, only in one case were the effects driven by the pesticides present of the mixture (Bony et al. 2008). Other contaminants present in the Richelieu River water may be causing or contributing to the effects observed. contaminants such as pharmaceutical and personal care products (Chapter 4), persistent organic pollutants and polycyclic aromatic carbons (Laliberté 2020), can also have effects on fish hatching (Le Bihanic et a. 2014B, Gauthier & Vijayan 2020), deformity rate (Le Bihanic et a. 2014B), survival rate (Johnson et al. 2013, Le Bihanic et al. 2014A), reproduction (Johnson et al. 2013) and immune response (Johnson et al. 2013). Some of these contaminants, for example pharmaceutical and personal care products, also peak in the river following heavy rain events due to the overflowing of wastewater treatment plants along the Richelieu River (Cliché & Saladzius 2018). This means that many contaminants, which can have similar deleterious effects on fish ELS can be pulsing at the same time as pesticides. The fact that exposure to river water caused effects in multiple fish species combined with the fact that many contaminants could be causing the effects warranted the chemistry investigation of the river water and a laboratory exposure to compounds of interest.

7.5 River chemistry

The pesticide concentrations detected in the Richelieu River matched concentrations expected in a watershed dominated by agriculture. For example, pesticides detected, concentrations and

detection frequency were comparable to those measured across many agricultural sites in the United States (Hladik et al. 2014, Ryberg & Gilliom 2015, Medali et al. 2020, Stackpoole et al. 2021), Ontario (Struger et al. 2017) and Quebec (Giroux et al. 2022). Overall concentrations were higher in the tributaries, which seems sensible since they are smaller water bodies and are directly fed by agricultural drainage. Concentrations of most pesticides were below water toxicity thresholds to aquatic life. The notable exception was neonicotinoid pesticide concentrations, which were of concern. For example, concentrations of neonicotinoids above 35 ng/L have been estimated to be harmful to aquatic invertebrates (Morrissey et al. 2015). Concentrations in the river and the tributaries have been measured at up to 600 ng/L, more than 1-order of magnitude above this threshold. Aquatic arthropods are important from a biodiversity standpoint, but also because they represent an important prey item for many fish (Colllier et al. 2016). For example, in Lake Shinji in Japan, the collapse of two commercially important fish species feeding on arthropods was linked to neonicotinoid contaminations causing a decline of the lake arthropod population (Yamamuro et al. 2019). Adult copper redhorse feed almost exclusively on molluscs but juveniles feed extensively on aquatic arthropods (Branchaud 1995, COSEWIC 2004). The peak of pesticide concentrations in the Richelieu River during the spawning season and early development may deprive the young fish of that first food source, possibly starving them and affecting the whole species yearly recruitment.

In June, pesticide concentrations in the river and the tributaries were found to peak following heavy rain events. This is a well-known phenomenon described in many agricultural zones around the world (Zhang et al. 1997, Konstantinou et al. 2006, Pätzold et al. 2007, Giroux 2022). At this time of year, the bare soil around the newly planted seedlings has limited water retention capacity, leading to large run-off to nearby rivers. Peaks tend to decrease later in the season as crops grow. The seed's coating, which mainly consists of neonicotinoids also contributes to early summer pulses (Radolinski et al. 2019). The peaks in concentrations were found to coincide precisely with many listed species' spawning and early development.

7.6 Laboratory Exposure

When looking at individual pesticide exposure, our findings are consistent with the literature in the sense that doses of single pesticides must be higher than those measured in the environment to

elicit noticeable effects (sterauer & Köhler 2008; Mhadhbbi & Beiras 2012; Sulukan et al. 2017). However, subtle effects resulting from environmentally relevant concentrations have previously been reported. For example, increases in whole body 17B-estradiol was measured in sockeye salmon (*Oncorhynchus nerka*) at 150 ng/L of clothianidin (Marlatt et al. 2019). Here we focus on biological rather than molecular endpoints. However the endpoints assessed were not affected by the tested pesticide concentrations. Effects on hatching and mortality that were observed during river water exposure were not replicated by the laboratory exposure to single pesticides. The pesticide concentrations used in the laboratory exposure were similar to concentrations measured in the Richelieu River, the tributaries and one magnitude greater. Even with the higher concentrations, no effects were observed. But when exposed to river water, with lesser concentrations of these same individual pesticides, biological effects were reported. This leads us to conclude that the tested pesticides alone, could not explain the previously reported effects in river redhorse, copper redhorse and zebrafish.

Overall, our results, when put together, seem to indicate that no single pesticide was driving the observed effects, but a mixture of pesticides or other contaminants and stressors likely were. Previous studies have demonstrated cumulative and synergistic effects of contaminants in fish. One such example is atrazine and chlorpyrifos, when present together in water, were more toxic than by the simple principle of addition (Perez et al. 2013). Here we are working with river water, which is a mixture of hundreds, if not thousands of chemicals. The interactions within this mixture of compounds, including banned pesticides, legacy contaminants and more, and between all the different classes of chemicals are still unknown. Due to its ever-changing nature, river water contamination is hard to quantify and generalize. Yet we do know that this water can have immediate and persistent effects on fish ELS.

7.7 Implications

Our data provide evidence that ELS exposure to real world mixtures has deleterious effects on fish. This is especially worrying for listed species, such as the copper redhorse, which are often already facing other challenges. The copper redhorse for example faces habitat fragmentation and destruction, but is also vulnerable because of its late age of maturity, late spawning period and specific diet (COSEWIC 2004). Also because of a late spawning period, its spawning and early

development coincide with the highest pulse in pesticide contamination. We have therefore demonstrated that actual contamination of surface water in copper redhorse spawning grounds can be added to the list of threats.

Although we detected effects across the three assessed species, we noted different levels and types of effects in each species. Listed species have often been reported as more sensitive (Sappington et al. 2001) which is consistent with our results. For example, following ELS exposure to river water, increase in mortality was only reported in copper redhorse and not in river redhorse. However, it is worth mentioning that copper and river redhorse exposure, during the *in-situ* experiment (Chapter 3), did not completely overlap, which means that river redhorse and copper redhorse were not exposed to the exact same levels of contamination nor at the same developmental time. Also, a notable difference in hatching times in the exposed group was observed between the two redhorse species, which hatched prematurely and the zebrafish, which hatched later than the controls. Such species differences have previously been reported. For example, when exposed to the same dose of glyphosate, zebrafish hatched prematurely while common carp hatched belatedly (Fiorino et al. 2018). So far, our results seem to suggest that copper redhorse are more sensitive than river redhorse and zebrafish.

It is difficult to link more subtle effects such as changes in gene expression or timing of hatching to population level effects. We observed reduction in fertilization rate of the zebrafish exposed to the river water. Reduced reproductive output could easily have negative effects on the already relatively small copper redhorse population because the yearly recruitment all comes from the Richelieu spawning sites. But previous studies on fish inhabiting contaminated areas have demonstrated that fish can show plasticity and adaptation to contamination in their environment (Hamilton et al. 2015). This means that copper redhorse may have adapted, to a certain degree, to the contamination of their environment and that the later life effects observed in the laboratory fish may not be exactly the same in wild fish. Further studies could investigate the effects of ELS exposure on reproductive markers in wild fish such as copper redhorse.

Our screening of the river water revealed many contaminants including legacy contaminants that were banned decades ago. The implication of this is that, even though contaminants were banned, they are still part of the mixture and may still have effects on wildlife. Although the concentrations

were in the low ng/L. There is still a gap in toxicological studies between the two, meaning that studies tend to focus on one (legacy or emerging) rather than the whole. Legacy contaminants are persistent, which is often the reason why they fell out of favor, but it is also why we can still detect them. Few studies have evaluated the levels or potential effects of legacy contaminants and pesticides together in fish (Ren et al. 2013, Vigano et al. 2015, Sun et al. 2018), and information is even more scarce for fish ELS and listed species. This means that there are still numerous unknowns in terms of exposure to environmental mixtures in fish.

7.8 Limitations

Due to copper redhorse unique biology, we should be careful when generalizing effects to other fish species. The Richelieu River houses more than 80 species of fish (MFFP 2018), with variable natural life histories, different breeding seasons, different sizes, etc. Copper redhorse have a very late spawning season, which means that other fish spawning seasons do not necessarily coincide with the pulse in pesticides. Two other fish species spawning in the Richelieu, the walleye (*Sander vitreus*) (Bowles et al. 2020) and the yellow perch (*Perca flavescens*) (Leclerc et al. 2008), spawn in spring (late April to early May), more than a month before the copper redhorse. Water used for our exposure was collected in June and may not represent the exposome of other fish species ELS. Even though the Richelieu River represents a typical river in an agricultural landscape, every watershed is different.

Another important consideration is that the control water used in the river water exposure study was not a perfect match to river water characteristics. Even though most of the water physiochemical properties were similar, the salts used to reconstitute the water hardness are unlikely to be in the same ratio or concentrations as those present naturally in the river. But since ELS survival was generally high for all species in both control and river water, it is unlikely that these differences in chemistry could explain the results, but they may have had an effect. Further studies could use filtered river water or river water collected when pesticide concentrations are expected to be the lowest.

We used zebrafish as a surrogate species to evaluate full life cycle effects. Zebrafish are significantly biologically and phylogenetically different from *Moxostoma*. For example, zebrafish are a tropical fish with a short generation time while *Moxostoma* are from temperate climate and

usually have a late age of maturity (COSEWIC 2004). However, it would have been unrealistic to transpose these experiments onto river or copper redhorse for many logistical reasons such as their large size, limited number in the wild and their late age of maturity. But the MFFP artificial breeding program is currently building a livestock of copper and river redhorse, which may provide an opportunity in the future for further testing, and testing amongst different life stages.

The genetic diversity of the redhorse tested represented only a small subsample of the population. For example, offspring of a single pair from each species were used in the *in-situ* and the laboratory exposure. It is not uncommon for individuals of the same population to exhibit different levels of sensitivity (Nacci et al. 1999). However the use sibling was intentional and advantageous since it allowed for individuals with similar prenatal and genetic backgrounds to be assessed in different conditions. But further research on these species will allow us to draw better conclusions.

Here, we used a realistic environmental mixture for our exposure, but the exposure period was limited to ELS, which does not represent the complete exposome. Wild fish are exposed to contaminants throughout their life cycle. Also, water from the spawning grounds does not necessarily represent the contamination that later life stages are exposed to since fish can migrate over long distances (COSEWIC 2004). We observed effects resulting from the ELS exposure but one could argue that constant exposure during the whole life of the organism could have more effects. The fact that effects resulting from ELS exposure suggests that more protective measures should be put into place during that time to improve the chances of recovery in the copper redhorse.

7.9 Practical applications

Lasting effects from exposure to environmental mixtures should be taken into consideration when issuing protective areas or changes in legislation regarding the protection of spawning grounds of listed species. Ours results demonstrated that peaks in pesticide contamination coincide precisely with copper redhorse and other listed fish species spawning periods, hence risking exposure of their early life stage to the highest concentrations of chemicals. Protection of early life stages could go a long way in terms of conservation since actual levels of contamination already have effects on wildlife.

Our results demonstrate that single pesticide environmental toxicity thresholds may not be adequate to truly protect wildlife or listed species. Most of the contaminants present in the

Richelieu River were below such criteria. Our results show that prevalent single compounds, in this case pesticides, failed to replicate effects from environmental toxicity. Establishing toxicity thresholds per compound, as currently done is a good start to identify contaminants of concern. But the richness of the environmental mixture which has often been shown to have additive or synergistic effects, should not be overlooked.

7.10 Recommendations

Despite these promising results, questions remain on the identity of the contaminant(s) driving the effects. Further investigation should continue with the identification of priority contaminants and individual toxicity testing. A useful next step would be to investigate the effects on contaminant mixtures in fish ELS since it is unlikely that a single contaminant went undetected and was driving the effects.

Future experiments focusing on the Richelieu River mixtures should use fractionation of the water in an effort to find which contaminant fraction can be linked to the observed effects. This method, such as used by Tian et al. 2021, to link 6PPD quinone to coho salmon mortality, could be useful in this case. However, working with sublethal versus lethal effects may require some adjustments such as artificially increasing the potency of the environmental mixture by reducing the volume of water while maintaining the contamination. This would hopefully elicit effects that would be easier to detect, which would allow facilitate the assessment of the mixture fractions.

There is also abundant room for further progress in determining later life effects from ELS exposure in listed fish, or even in fish that are not usually used as model organisms. These fish, which are often of small size and with rapid generation time, are not necessarily good representatives of other species of larger size or with very different natural histories. However, I acknowledge their usefulness in wide toxicity screenings, but the use of other species should also be considered once contaminants of interest have been decided.

Environmental mixtures detected in surface water are ever changing and continuous sampling and analysis should still be carried out to help bridge the gap between contamination and effects on fish recruitment. This is especially important for listed species, for which important budgets are often allocated: it is essential to know if the environment can actually sustain their population. If not, more stringent contamination thresholds should be implemented and enforced.

Our results demonstrate that metabolomics such as RNA sequencing could be used to assess the effects on listed fish species, but the limited amount of information available to non-model species is still scarce. Due to these limitations, results must be expressed with caution since genes used or referenced are often from species that can be phylogenetically distant. However, these methods should be used, as the more they are used the more data will be available to build libraries. A follow up on the dysregulation of immune genes is also warranted.

Chapter 8

8. Final Conclusion and Summary

The work presented in this thesis provided evidence that exposure of fish ELS to river water can have both immediate and persistent effects. The effects observed in zebrafish, river redhorse and copper redhorse ranged from alteration of hatching and gene expression, decreased survival and fertilization rate to increased incidence of deformities in offspring. This is important since this is the actual environmental mixture to which fish are exposed in the wild. Our results indicate that ELS exposure to river water could contribute to the lack of recovery of the endangered copper redhorse, but also on other fish spawning in the Richelieu river. There are many challenges when working with endangered species, but this work is essential since endangered species may be the most impacted by contamination and yet the least studied. Valuable insights for the conservation of the river and copper redhorse were gained through this series of experiments, but more research is needed to link the observed effects to specific components of the environmental mixture.

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