CUMULATIVE TOXICITIES OF NEONICOTINOID INSECTICIDES AND THEIR MIXTURES TO SENSITIVE FRESHWATER INSECTS

A Thesis Submitted to the College of

Graduate and Postdoctoral Studies

in Partial Fulfillment of the Requirements

For the Degree of Doctor of Philosophy

In the Toxicology Graduate Program

University of Saskatchewan,

Saskatoon, SK, Canada.

By

Erin Maria Maloney

PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a Postgraduate degree from the University of Saskatchewan, I agree that the Libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, in whole or in part, for scholarly purposes may be granted by the professor or professors who supervised my thesis work or, in their absence, by the Head of the Department or the Dean of the College in which my thesis work was done. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis/dissertation.

Requests for permission to copy or to make other uses of materials in this thesis/dissertation in whole or in part should be addressed to:

Chair of the Toxicology Graduate Program

Toxicology Centre

44 Campus Drive

University of Saskatchewan

Saskatoon, Saskatchewan S7N 5B3 Canada

ABSTRACT

Neonicotinoids are neurotoxic insecticides that are commonly applied to combat agricultural pests. Due to widespread application and select physicochemical characteristics, mixtures of different neonicotinoids are frequently detected in freshwater environments. This is of potential concern because these freshwater habitats are populated with ecologically important benthic macroinvertebrates (e.g. Chironomidae), which are markedly sensitive to neonicotinoid compounds. Despite the likelihood of continuous and/or repeated exposure, previous studies have primarily evaluated the individual toxicities of these neurotoxic compounds. Yet, little is known about how mixtures affect sensitive aquatic insects under real world exposure scenarios. Thus, the objectives of this research were to (1) evaluate acute and chronic toxicities of three commonly used neonicotinoids (imidacloprid (IMI), clothianidin (CLO), and thiamethoxam (TMX)) and their mixtures to Chironomidae using *Chironomus dilutus* as a representative test species, (2) validate single compound and neonicotinoid mixture toxicity predictions to Chironomidae populations under field settings, and (3) identify mechanisms behind species-, life stage-, and compound-specific differences in neonicotinoid toxicity for these sensitive aquatic insects.

To address the first objective of this research, acute (96 h, endpoint = lethality) and chronic (28 d, endpoint = cessation of emergence) laboratory-based toxicity tests were carried out, characterizing the toxicities of IMI, CLO, TMX and their binary and ternary mixtures to larval *C. dilutus*. Using the MIXTOX approach (a statistical technique based on fitting mixture toxicity data to pre-defined mixture models), the nature and magnitude of cumulative toxicity was classified for each neonicotinoid mixture. Several mixtures were found to display cumulative toxicity that significantly deviated from direct, concentration-based additivity. Under acute exposure settings, all IMI-containing mixtures (IMI-CLO, IMI-TMX, and IMI-CLO-TMX) exhibited synergism

when the concentrations of IMI in the solution were dominant (up to 7 %, 28 %, and 6 % decreases in survival, respectively), and some mixtures (IMI-CLO and IMI-TMX) displayed antagonism when the other mixture constituent was dominant (up to 19 % and 30 % increases in survival, respectively). Under chronic exposure settings all binary mixtures demonstrated dose-ratio dependent deviation from direct additivity (concentration addition), displaying synergism at high concentrations of CLO (IMI-CLO: 13 % decrease in emergence) or TMX (CLO-TMX and IMI-TMX: 2 % and 4 % decreases in emergence, respectively) and antagonism at high concentrations of IMI (IMI-CLO and IMI-TMX: 5 %, and 2 % increases in emergence, respectively). Under chronic exposures, the ternary mixture (IMI-CLO-TMX) elicited an overall antagonistic effect (2 % increase in emergence). Thus, laboratory-derived bioassays indicated that under both acute and chronic exposure settings, neonicotinoid mixtures had the potential to display cumulative toxicities that deviated from direct additivity. Furthermore, although toxicities of neonicotinoid mixtures were not exactly parallel across different exposure settings, acute tests generally predicted which mixtures were likely to display significant synergism under chronic exposure settings.

To determine if laboratory-derived predictions could be used to estimate the toxicities of neonicotinoids and their mixtures under more environmentally realistic exposure settings (Objective 2), chronic (56 day), semi-controlled field studies were carried out in a natural wetland in Saskatchewan's Prairie Pothole Region. Using *in situ* limnocorrals fitted with emergence traps, the effects of predicted equitoxic concentrations of IMI, CLO, TMX, and their binary mixtures (concentrations equivalent to 28 d EC_{50} values; mixtures at 1:1 ratio) were characterized for all emerged aquatic insects (endpoint: abundance) and Chironomidae (endpoint: abundance, biomass, and sex ratios) at the community level. In all treated limnocorrals, there were subtle shifts in insect community composition. Furthermore, at concentrations tested, neonicotinoids and their mixtures

significantly impacted Chironomidae abundance and biomass. However, contrary to laboratory predictions, IMI-CLO and IMI-TMX mixtures did not elicit greater-than-additive effects. Furthermore, exposure to IMI, CLO, TMX, and CLO-TMX elicited greater-than-expected declines in Chironomidae abundance and biomass. In addition, CLO significantly shifted sex-ratios of emerged Chironomidae towards female-dominated populations. Thus, although laboratory-derived toxicity estimates could adequately predict relative effects of IMI, CLO, and TMX on Chironomidae populations (e.g. toxicity: IMI ≥ CLO >> TMX), they frequently underestimated the magnitudes of single-compound and neonicotinoid mixture effects under semi-controlled field settings.

To better characterize patterns of observed toxicity (e.g. differences among compounds, species, and life-stages), the binding properties of IMI, CLO, and TMX to their molecular target (nicotinic acetylcholine receptors (nAChRs)) were investigated in Chironomidae (Objective 3). Using radioligand binding studies with tritium-labeled IMI ([^{3}H]-IMI) and unlabeled competitors (IMI, CLO, and TMX), nAChR density and neonicotinoid binding affinity were characterized for and compared across two species (*C. dilutus* and *Chironomus riparius*) at two different life stages (larval and adult). Despite marked differences in neonicotinoid toxicity, there were no significant species-specific differences in neonicotinoid binding or nAChR density. However, there were life stage-specific differences in nAChR density and binding, and compound-specific differences in binding affinity that reflected previously described patterns in neonicotinoid toxicity (e.g. higher larval sensitivity and relative toxicity of IMI ≥ CLO >> TMX). Furthermore, compared to other insects, Chironomidae displayed relatively high densities of nAChRs with high neonicotinoid affinity, which reflected their sensitivity to these insecticides.

Ultimately this work provides a comprehensive characterization of the toxicity of three commonly used neonicotinoid insecticides (IMI, CLO, and TMX) and their mixtures to the sensitive aquatic insect group, Chironomidae. This can help inform regulators and risk assessors focused on assessing risks of neonicotinoids in freshwater environments. Furthermore, by characterizing effects at three levels of biological organization (molecular, individual, and communities), this work provides a basis through which a relative toxicity pathway could be formed, highlighting techniques that could be potentially used to predict large-scale effects for Chironomidae inhabiting neonicotinoid-contaminated aquatic environments. Finally, this work highlights areas worthy of further investigation and provides methodology through which these studies can be carried out, including the characterization of the binding properties and/or expression profiles of nAChRs for other neonicotinoid-sensitive aquatic insects, evaluation of the nAChR binding profiles for other nAChR agonists (e.g. other neonicotinoids, sulfoximines, and butenolides), and further characterization of nAChR binding profiles in Chironomidae (e.g. with α-bungarotoxin or epibatidine) to allow for a more comprehensive, mechanistic understanding of neonicotinoid mixture toxicity.

ACKNOWLEDGEMENTS

There is a common expression that accurately describes the completion of this dissertation: ‘it takes a village to raise a child’. In this case, it would more appropriately be ‘it takes a village to help Erin Maloney finish her Ph.D. thesis’. Truly, this work would not have been possible without the guidance, support, and assistance of many amazing individuals who I am lucky to call my friends, family, colleagues, and mentors. First, I would like to acknowledge my co-supervisors, Drs. Karsten Liber and Christy Morrissey for their boundless support over the last five years. I would like to thank Karsten for plucking me from the mountains of Gangwondo, South Korea, and giving me the opportunity to take on this project. Not only did he patiently assist in my transformation from an English as a Second Language teacher to an ecotoxicologist, he always provided support, scientific knowledge and insight when I needed it the most. I would like to thank Christy for continuously challenging me to be a better researcher. From data analysis to conference presentations, her thought-provoking questions and scientific insight have truly helped me learn and grow, and for that I cannot thank her enough. De même, je voudrais remercier mes collaborateurs à l’Université d’Orléans, Drs. Steeve Thany et Emiliane Taillebois et à la CEA Paris Saclay, Drs. Nicolas Gillet et Denis Servent, pour leur assistance avec le chapitre final de cette thèse. Je suis vraiment reconnaissant pour votre soutien alors que j’ai appris comment parler français, comment faire les manipulations avec radioligands, et comment de naviguer les laboratoires français. A big thank you to my committee members, Drs. Mark Wickstrom, Markus Hecker, Som Niyogi, and Elemir Simko, whose comments, questions and critiques have been essential for the completion of this thesis.

I would like to acknowledge the extensive analytical support provided from Environment Canada throughout the duration of this project. To my co-authors Drs. Kerry Peru and John

Headley, I appreciate your feedback, support, and participation in the completion of this research. Thank you for answering my questions, and always providing me with the analytical support I needed (however last minute or extensive it may have been). A special thanks to Jessica Fehr for her work in the analysis of all my water samples. I appreciate her hard work, excellent attitude, and consistent patience with my erratic analytical requirements and sample deposit schedules.

I have been incredibly lucky to be helped by a number of excellent human beings, without whom this work would not have been impossible. Not too many people would voluntarily spend their days cooped up in a windowless environmental chamber, dragging around giant chains and algae-infested limnocorral equipment, or jumping into freezing cold ponds with me - you are truly a fantastic bunch. Thank you to Iain Phillips at the Saskatchewan Water Security Agency, for his aquatic insect identification assistance. Liber and Morrissey lab members, past and present, for their comments, critiques, friendship and support. Special thanks to Katherine Raes, Blue Markwart, and Stephanie Schiffer, for their help with toxicity testing and chamber MacGyvering, and Egina Malaj and Maira Peixoto-Mendes, for their masterful maneuvering of international Chironomidae importation. Thank you to my field assistants Raiden Brailsford and Ally Gerhart, and my field helpers Stephanie Schiffer, Larry D'Silva, Kristin Bianchini, Michael Cavallaro, Sara Hanson, and Ashley Moate. Through the long days, variable weather, truck issues, canoe mishaps, and constant tick attacks, you managed to remain optimistic and dedicated, which was truly motivating. Merci à mes collèges à l'Université d'Orléans et la Commissariat à l'Énergie Atomique et aux Énergies Alternatives Carine Martin, Anais LeMauff, Alison Catereau, et Jean-Noel Houchatt pour leur aide dans la laboratoire.

I am extremely grateful to the Natural Science and Engineering Research Council of Canada (NSERC), the Toxicology Centre, the University of Saskatchewan, and the Teacher-

Scholar Doctoral Fellowship (University of Saskatchewan) for providing stipend support for my Ph.D. I would also like to thank the Mitacs Globalink Scholarship and Dr. Steeve Thany's group (la laboratoire de Biologie des Ligneux et des Grands Cultures (LBLGC)) for supporting my research exchange with l'Université d'Orléans. Finally, a special thanks to the following agencies for providing the funding required to finish this research: NSERC, Mitacs, the Department of Fisheries and Oceans (DFO), and the Commissariat à l'Énergie Atomique et aux Énergies Alternatives (CEA).

Last but never least, I would like to thank my friends and family. I am truly grateful for my parents, Lisa and Bob who have been constant sources of support and encouragement throughout this long and sometimes arduous journey. Thank you for your love and all the sacrifices you have made to allow me to become the second Dr. Maloney in the family. Thank you to my brothers, Connor and Stephen, who were always there when I needed them. Thank you to Chili-dog for proving that if you are a good boy, dreams can really come true. Finally, thank you to all my friends who have supported me during every step of this process. Thanks to the McKercher kids for always keeping up with the carbides, the Core4 for 'juan' or two excellent adventures, and the Tox Crew for the schemes, dreams, and honestly too many good times to recall. Thank you to my Guelph family for being there throughout the last decade of post-secondary education, to Colleen Parker for answering my many long, rambling calls, to the Yeongwol Crew for always having somewhere for me to stay, and the Habibis for helping me through that final chapter of my thesis. Indeed, I cannot thank you all enough for proving that although there are tall ships and short ships, the best ships are friendships.

DEDICATION

This dissertation is dedicated to all the Chironomidae who lived and died so that this research could be completed. Peace be with you; you brave little macroinvertebrates. I'll always be grateful for your sacrifice.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

°C	Degrees Celsius
α	Alpha
α-BGT	Alpha bungarotoxin
µL	Microlitres
µm	Micrometers
µS/cm	Microsiemens per centimeter
χ^2	Chi-square
^{3}H-IMI	Radiolabeled imidacloprid
^{3}H-TMX	Radiolabeled thiamethoxam
ACh	Acetylcholine
ACE	Acetamiprid
ANOVA	Analysis of variance
AO	Adverse outcome
AOP	Adverse outcome pathway
bw	Body weight
BT	Total binding
BC	Total competitive binding
B_{max}	Maximal binding parameters
c	Chemical concentration
CA	Concentration addition
CLO	Clothianidin
cm	Centimetres

CV	Coefficient of variation
CYP	Cytochrome P450
d	Days
D-L	Dose-level
DIN	Dinotefuran
DMSO	Dimethyl sulfoxide
DO	Dissolved oxygen
DOC	Dissolved organic carbon
D-R	Dose-ratio
DT_{50}	Degradation half-life
EC_{50}	Median effective concentration
ECCC	Environment and Climate Change Canada
EFSA	European Food Safety Authority
EU	European Union
g	Grams
h	Hours
HQ	Hazard quotient
HC5	Hazardous concentration for 5% of species
IA	Independent action
IC_{50}	Median inhibitory concentration
IMI	Imidacloprid
kg	Kilograms
K_D	Dissociation constant

K_i	Inhibition coefficient
K_{oc}	Soil organic carbon-water partitioning coefficient
K_{ow}	Octanol-water partition coefficient
KE	Key event
L	Litres
LC_{50}	Median lethal concentration
LD_{50}	Median lethal dose
LC-MS/MS	Liquid chromatography coupled with tandem mass spectrometry
LOQ	Limit of quantification
min	Minutes
MIE	Molecular initiating event
mol	Moles
mL	Millilitres
mm	Millimetres
mM	Millimolars
mPa	Millipascals
n	Number of replicates
N	Nitrogen
NA	North America
nAChR	Nicotinic acetylcholine receptor
NH_3	Ammonia
NIT	Nitenpyram
NO_3	Nitrate

NS	Non-specific binding
OC	Organic carbon
PMSF	Phenylmethyl sulfonyl fluoride
pM	Picomolar
pmol	Picomole
PO_4	Phosphate
PPR	Prairie Pothole Region
R^2	Correlation of determination
RIVM	Dutch National Institute for Public Health and the Environment
RSS	Residual sum of squares
S/A	Synergism/antagonism
SD	Standard deviation
SE	Standard error
SN	Supernatant
SPE	Solid-phase extraction
SYN	Synergism
THIA	Thiacloprid
TMX	Thiamethoxam
TU	Toxic units
US EPA	United States Environmental Protection Agency
X	Times

NOTE TO READERS

This thesis is organized and formatted to follow the University of Saskatchewan College of Graduate and Postdoctoral Studies guidelines for a manuscript-style thesis. Chapter 1 is a general introduction and literature review, including the project goal and objectives, and Chapter 6 is a synthesis chapter, containing a general discussion and conclusions that tie the chapters together. Chapters 2, 3, 4 and 5 of this thesis are organized as manuscripts for publication in peer-reviewed scientific journals. Chapter 2 has been published in Environmental Toxicology and Chemistry, Chapter 3 has been published in Ecotoxicology and Environmental Safety**,** Chapter 4 has been published in Environmental Pollution, and Chapter 5 will be submitted to Aquatic Toxicology**.** Full citations for the published research manuscripts are provided below. Due to this manuscript-style format, there is some repetition in the introductions and materials and methods sections of the thesis. All tables, figures, supporting information, and references cited in the research chapters of this thesis have been reformatted to the thesis style. References cited in each chapter are combined and listed in the References section of this thesis. Supporting information associated with research chapters are presented in the Appendix section at the end of this thesis.

The content of Chapter 2 was reprinted (adapted) from Environmental Toxicology and Chemistry (http://dx.doi.org/10.1002/etc.3878). E.M. Maloney, C.A. Morrissey, J.V. Headley, K.M. Peru, and K. Liber. "Cumulative toxicity of neonicotinoid insecticide mixtures to *Chironomus dilutus* under acute exposure scenarios" 36: 3091-3101. Copyright (2017), with permission from Wiley. Corrigendum to "Cumulative toxicity of neonicotinoid insecticide mixtures to *Chironomus dilutus* under acute exposure scenarios" [Environ. Toxicol. Chem. (2020) 39: 942-946. Doi: 10.1002/etc.4675], with permission from Wiley.

The content of Chapter 3 was reprinted (adapted) from Ecotoxicology and Environmental Safety (http://dx.doi.org/10.1016/j.ecoenv.2018.03.003). E. M. Maloney, C.A. Morrissey, J.V. Headley, K.M. Peru, and K. Liber. “Can chronic exposure to imidacloprid, clothianidin, and thiamethoxam mixtures exert greater than additive toxicity in *Chironomus dilutus*?” 156: 354-365. Copyright (2018); Corrigendum to "Can chronic exposure to imidacloprid, clothianidin, and thiamethoxam mixtures exert greater than additive toxicity in Chironomus dilutus?" [Ecotoxicol. Environ. Saf. 156C (2019) 354-365], with permission from Elsevier.

The content of Chapter 4 was reprinted (adapted) from Environmental Pollution (https://doi.org/10.1016/j.envpol.2018.09.008). E. M. Maloney, C.A. Morrissey, J.V. Headley, K.M. Peru, and K. Liber. “Neonicotinoid insecticide mixtures: Evaluation of laboratory-based toxicity predictions under semi-controlled field conditions” 243(B): 1727-1739. Copyright (2018), with permission from Elsevier.

The author contributions for each research chapter included:

Erin M. Maloney (University of Saskatchewan) managed and conducted all research and field sampling, performed all statistical and modelling analysis, and drafted each of the manuscripts.

Dr. Christy A. Morrissey (University of Saskatchewan) provided scientific guidance and input, manuscript review, comments and editorial corrections, and funding support and direction for all research.

Dr. Karsten Liber (University of Saskatchewan) provided scientific guidance and input, manuscript review, comments and editorial corrections, and funding support and direction for all research.

Dr. John V. Headley (National Hydrology Research Centre, Environment and Climate Change Canada) provided all analytical support, manuscript review, and comments and editorial corrections for Chapters 2 - 4.

Dr. Kerry M. Peru (National Hydrology Research Centre, Environment and Climate Change Canada) provided all analytical support, manuscript review, and comments and editorial corrections for Chapters 2 - 4.

Dr. Steeve Thany (Le Laboratoire de Biologie des Ligneux et des Grandes Cultures, Université d'Orléans) provided scientific guidance and input, manuscript review, and funding support for Chapter 5.

Dr. Emiliane Taillebois (Le Laboratoire de Biologie des Ligneux et des Grandes Cultures, Université d'Orléans) provided scientific guidance and input, manuscript review, and direction for Chapter 5.

Dr. Nicolas Gillet (Laboratoire de Toxicologie Moléculaire et Biotechnologies, Commissariat à l'Énergie Atomique et aux Énergies Alternatives Paris-Saclay) provided scientific guidance and input, manuscript review, and direction for Chapter 5.

Dr. Denis Servent (Laboratoire de Toxicologie Moléculaire et Biotechnologies, Commissariat à l'Énergie Atomique et aux Énergies Alternatives Paris-Saclay) provided scientific guidance and input, manuscript review, funding and direction for Chapter 5.

CHAPTER 1: GENERAL INTRODUCTION

Preface

This chapter contains a general introduction to neonicotinoid insecticides and a literature review on environmental chemodynamics, aquatic detections, toxicities (single compound and mixture), and modes of action of neonicotinoids along with the environmental risk assessment strategies used for these insecticidal compounds. Chapter 1 also gives an overview of the current knowledge of nicotinic acetylcholine receptors and discusses their diversity in expression and function in insect species. Finally, this chapter includes the overall goals and objectives of this thesis, and the hypotheses tested in subsequent chapters.

1.1 Introduction

Over the past two decades, neonicotinoids have represented the largest-selling and fastest-growing group of insecticides worldwide (Jeschke et al., 2010). Initially developed to counter the growing occurrence of pest resistance to 'classic pesticides' (e.g., organophosphates, carbamates and pyrethroids), neonicotinoids were rapidly adopted due to their high selective toxicity for invertebrate (particularly arthropod) species, versatility in application, and physicochemical characteristics (e.g. low lipid solubility and high persistence in light-limited environments) (Simon-Delso et al., 2015). Between 1991 (when neonicotinoids were introduced) and 2008 (when the most recent survey of global pesticide sales data was published), neonicotinoids had become the dominant insecticide class in the agrochemical market, making up 24 % of all insecticides and 80 % of seed-treatments sold worldwide (Jeschke et al., 2010). Since 2008, global neonicotinoid application has continued to move in an upward trajectory. In the Canadian prairies alone, neonicotinoid application has increased from an estimated 11 million hectares in 2012 (Main et al., 2014), to an estimated 13 million hectares in 2015 (E. Malaj, pers. comm. 2019). Global usage and sales data suggest that similar rising patterns have also been occurring in other regions in North America (NA) (e.g. California), the United Kingdom, the European Union (EU) (e.g. Sweden), and Asia (e.g. Japan) (Simon-Delso et al., 2015). The most recent estimates available indicate that neonicotinoid insecticides are registered for use on over 140 different crops in over 120 different countries (Jeschke et al., 2010). However, as patent protections have recently expired for most neonicotinoid compounds, allowing for the introduction of generic products onto the market, this is likely an underestimation of actual neonicotinoid usage worldwide.

Neonicotinoids are a class of insecticides presently containing seven commercial compounds (active ingredients): imidacloprid (IMI), clothianidin (CLO), thiamethoxam (TMX),

nitenpyram (NIT), acetamiprid (ACE), thiacloprid (THIA), and dinotefuran (DIN), under various product trade names (Jeschke et al., 2010). These compounds are divided into generations based on when they were synthesized. Introduced in 1991, IMI is the only first-generation neonicotinoid (Jeschke et al., 2010). Thus, IMI represents the prototypical neonicotinoid, and remains the most widely researched and the historically most heavily applied compound on the market (Jeschke et al., 2010). All the other compounds listed here (CLO, TMX, ACE, DIN, NIT, THIA) are second- or third-generation compounds, having been introduced after IMI, and formulated to elicit slightly different toxicological effects with slightly different environmental profiles (Simon-Delso et al., 2015). Commonly used to protect seedlings from piercing-sucking pests (e.g. aphids or brown planthoppers), neonicotinoids can be applied as soil-drenches (applied directly to the soil), foliar sprays (sprayed directly on the leaves of crops), or seed treatments (coated on seed prior to planting, conveying systemic protection throughout plant growth) (Elbert et al., 2008; Jeschke et al., 2010). Whereas all neonicotinoids described here can be applied as soil drenches and foliar sprays, only IMI, CLO, and TMX are also commonly applied as seed-treatments (Elbert et al., 2008). Because of this wide versatility in application, IMI, CLO, and TMX also have larger ranges of crop uses (applied on fruit, vegetable, and cereal crops) and broader pest spectra (protecting against pests that target young crops and newly sprouted seedlings) than most of the other compounds (Table 1.1) (Elbert et al., 2008; Jeschke et al., 2010). Thus, these compounds are the most widely applied neonicotinoids in the agricultural sector. Due to their widespread use and heavy application as seed treatments on cereal crops, especially in the Canadian prairies (E. Malaj, pers. comm. 2019), this work will exclusively focus on IMI, CLO, and TMX (Figure 1.1) and the risk these pesticides pose to aquatic environments in arable regions.

Table 1.1 Application profiles of current-use neonicotinoid insecticides. [a]

Neonicotinoid	Number of Crop Uses	Number of Additional Pest Uses [b]	Application Methods
Acetamiprid	60	2	Foliar spray, soil drench.
Clothianidin	40	3	Foliar spray, soil drench, seed treatment.
Dinotefuran	35	3	Foliar spray, soil drench.
Imidacloprid	140	4	Foliar spray, soil drench, seed treatment.
Nitenpyram	12	-	Foliar spray, soil drench.
Thiamethoxam	115	4	Foliar spray, soil drench, seed treatment.
Thiacloprid	50	2	Foliar spray, soil drench, seed treatment. [c]

[a] Adapted from Elbert et al. (2008); Jeschke et al. (2010).

[b] Specific target pest spectrum (outside of the common neonicotinoid spectrum) for each individual compound.

[c] Thiacloprid is only used as a seed treatment in one commercial product (Sonido®, Bayer Crop Science).

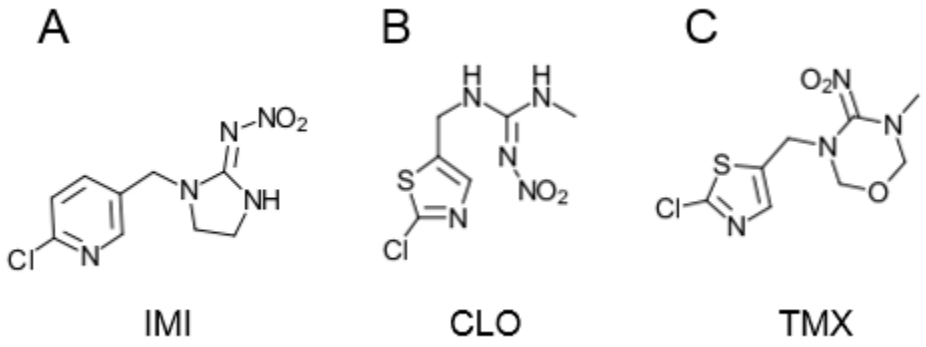

Figure 1.1 Chemical structures of the three most widely applied neonicotinoid insecticides, (A) imidacloprid (IMI), (B) clothianidin (CLO), and (C) thiamethoxam (TMX), demonstrating the structural diversity of different neonicotinoid insecticides.

1.2 Chemical properties and environmental fate of neonicotinoid insecticides

Neonicotinoids are all based on the molecular structure of nicotine but display different degrees of structural diversity. These compounds can exist as five-membered ring systems (e.g. IMI), six-membered ring systems (e.g. TMX), or non-cyclical compounds (e.g. CLO) (Figure 1.1) and can contain one of three pharmacophore moieties (N-nitroguanidine, nitromethylene, or n-cyanominidine), which impart different physicochemical properties (Jeschke et al., 2010). IMI, CLO, and TMX all contain an N-nitroguanidine moiety, which increases the lipophilicity and photostability of these compounds, facilitating their use as efficacious seed treatments, and increasing their stability in soils post-application (Anderson et al., 2015; Jeschke et al., 2010).

The three systemic neonicotinoids of interest (IMI, CLO, and TMX) display physicochemical properties that suggest they are likely to move into nearby aquatic environments following agricultural application. These compounds are all small and non-polar, with low octanol-water and organic carbon partition coefficients ($\log(K_{OW})$ = -0.13 - 0.7 at 25°C; K_{OC} = 84 - 2877 L/kg) (Table 1.2) (Cox et al., 1997; Gupta et al., 2008; Jeschke et al., 2010; Stoughton et al., 2008; United States Environmental Protection Agency, 2003). Furthermore, IMI, CLO and TMX are all highly water soluble (solubilities: 0.33 - 4.10 g/L at 20°C) (Table 1.2) (Bonmatin et al., 2015). Therefore, these compounds are more likely to remain in aquatic fractions than bind to particles and organic carbon, or partition into organic compartments of the soil matrix, allowing them to easily move from areas of application into ground- or surface-waters. Movement into aquatic systems is thought to primarily occur through surface run-off and drainage following major rainfall events (Armbrust and Peeler, 2002; Chiovarou and Siewicki, 2008). However, there is also evidence that suggests that these compounds can be carried into nearby aquatic systems via snowmelt runoff (e.g. soluble and insoluble fractions carried over from soil during the winter)

(Main et al., 2016), leaching into groundwater followed by subsurface discharge (Lamers et al., 2011; Pest Management Regulatory Agency, 2001), decay of treated plants in water bodies (Kreutzweiser et al., 2007), drift of contaminated dust dispersed during seed drilling (Krupke et al., 2012; Nuyttens et al., 2013), and/or the deposition/drift of treated seeds or sprayed insecticides into soil, waterbodies or depressions (Morrissey et al., 2015). Some of these pathways (e.g. runoff, leaching, and snowmelt) are particularly important for neonicotinoid seed treatments which, post-seeding, can move into soils rather than be systemically taken up by target crops. Indeed, studies have found that often > 90% of the active ingredient moves directly into the soil, compared to ~ 5% which is taken up by target crops (Goulson, 2013), rendering a large fraction of applied compound available for movement into nearby aquatic systems.

Movement of neonicotinoids from the soil has been shown to follow a biphasic pattern, with an initial phase of rapid loss (pulse exposure), followed by a secondary phase of slower loss. Within each phase, the rate of loss follows first-order kinetics (Gupta et al., 2008). The initial phase of dissipation from soil can be quite rapid, with aquatic neonicotinoid concentrations peaking within 24 h of application (Armbrust and Peeler, 2002). As sorption of neonicotinoids to soil increases with time, the secondary phase ends when an equilibrium has been reached between adsorbed and free neonicotinoid concentrations in the soil (Gupta et al., 2008). Therefore, a portion of applied neonicotinoid will remain adsorbed to the soil. This can enhance neonicotinoid persistence in terrestrial environments and increase the likelihood of subsequent, nearby aquatic system contamination. Due to their high water solubility, adsorbed neonicotinoid fractions can be transported into aquatic environments through leaching or via events such as rainfall, snowmelt, drainage, and runoff (Wood and Goulson, 2017). Therefore, nearby aquatic environments can be

subjected to a series of pulse neonicotinoid exposures rather than just a single, high concentration pulse exposure following agricultural application.

Neonicotinoids are primarily degraded via photolysis (Canadian Council of Ministers of the Environment, 2007; Peña et al., 2011; Thuyet et al., 2011), with hydrolysis and microbial degradation representing important (yet less prevalent) secondary and tertiary degradation pathways (Gupta et al., 2008; Morrissey et al., 2015). Optimal elimination occurs under acidic or neutral conditions with high light penetration (Sarkar et al., 1999); however, neonicotinoids have been found to persist in light-limited environments (Wood and Goulson, 2017). This is particularly true in terrestrial settings. Neonicotinoids display low volatilities (vapour pressures = 2.0×10^{-4} - 1.3×10^{-7} mPa at 20°C) (Gupta et al., 2008; Stoughton et al., 2008; Uneme, 2011), long terrestrial half-lives (DT_{50}s = 3 - 6931 d) (Goulson, 2013) (Table 1.2), and have been detected in agricultural soils several years after the last application date (Wood and Goulson, 2017). Terrestrial persistence is highly dependent on a number of factors, such as treatment regimen, rate and method of application, temperature, soil characteristics, and concurrent use of fertilizers (Anderson et al., 2015). However, the current weight of evidence indicates that, in most agricultural areas, neonicotinoids are likely to remain in the soil, demonstrating persistence that exceeds annual agricultural cycles (Wood and Goulson, 2017). Similarly, it has been suggested that neonicotinoids can persist in aquatic systems under environmentally relevant conditions. Although reported aquatic half-lives for these insecticides can be quite short (ranging from < 1 - 39.5 days, Table 1.2) (Morrissey et al., 2015), a recent study has found that turbid wetland water can screen out UV light (e.g. via the actions of natural organic matter), severely limiting photolysis (Lu et al., 2015). Indeed, Lu et al. (2015) found that photolytic degradation of TMX was completely restricted in wetland water at depths greater than 8 cm, and suggested that, due to their chemical similarities, the same

effect could occur with other neonicotinoid compounds (e.g. IMI and CLO). Therefore, rather than being rapidly eliminated, it is likely that this reduced photodegradation could occur broadly in wetland environments near areas of agricultural application (e.g. the Prairie Pothole Region (PPR)), allowing neonicotinoids to persist in these aquatic systems.

In aquatic and terrestrial environments, neonicotinoid degradation processes (e.g. photolysis, and microbial activity) can result in the production of metabolites. In aquatic environments, IMI can be degraded into 14 different metabolites, CLO into 5 different metabolites, and TMX into 9 different metabolic products (Simon-Delso et al., 2015). Although a majority of these metabolites are not typically included in monitoring programs or ecotoxicological studies, there has been a focus on certain metabolic products, as they have the potential to contribute to the toxic load of neonicotinoids in aquatic systems. For example, in both aquatic and terrestrial environments, TMX can be metabolized into CLO (Simon-Delso et al., 2015). Therefore, in contaminated environments, rather than TMX existing as a single-compound neonicotinoid contaminant, it is likely to exist as a mixture of CLO and TMX (e.g. (Main, 2016)), which can potentially enhance neonicotinoid toxicity in these systems.

The physicochemical properties and environmental chemodynamics of neonicotinoids indicate that these compounds can pose a risk to aquatic ecosystems. First, aquatic persistence in UV-filtered/light limited environments can result in chronic neonicotinoid exposure to aquatic insects, organisms which have been shown to be markedly sensitive to these insecticides (Raby et al., 2018b). Second, terrestrial persistence indicates that there is a high likelihood for repeated pulse exposure events following initial application. These repeated exposures can result in neonicotinoid accumulation in aquatic insects, increasing internal neonicotinoid concentrations, and therefore eliciting toxicity at lower concentrations than would be expected with single, acute

exposures (Focks et al., 2018). Finally, observed multi-season persistence (Main et al., 2014) and metabolism to other neonicotinoids (e.g. TMX to CLO), combined with agricultural practices (e.g. field rotation), can result in the presence of neonicotinoid mixtures, which may potentially pose a greater risk than single-compound exposures.

1.3 Neonicotinoid detection in aquatic environments

Neonicotinoids insecticides are frequently detected in aquatic environments (Anderson et al., 2015; Hladik and Kolpin, 2015; Morrissey et al., 2015). Due to its widespread historical use, most monitoring programs have focused exclusively on IMI, detecting this neonicotinoid at high frequencies (36 - 100%) in surface waters sampled across North America (Phillips and Bode, 2004; Starner and Goh, 2012; Struger et al., 2017; Xing et al., 2013), South America (Starner and Goh, 2012), Europe (Kreuger et al., 2010; van Dijk, 2010), Asia (Lamers et al., 2011), and Australia (Sánchez-Bayo and Hyne, 2014; Smith et al., 2012). Fewer monitoring programs have focused on quantifying CLO or TMX in aquatic environments. However, recent studies have detected CLO at high frequencies (44 - 93%) in surface waters across North America (Hladik et al., 2018a; Main et al., 2014; Miles et al., 2017; Struger et al., 2017), Australia (Sánchez-Bayo and Hyne, 2014), and Asia (Yamamoto et al., 2012), and TMX at high frequencies (22 - 100%) in surface waters across North America (Anderson et al., 2013; Hladik et al., 2018a; Main et al., 2014; Miles et al., 2017; Struger et al., 2017) and Asia (Yamamoto et al., 2012).

Concentrations of IMI, CLO and/or TMX detected in freshwater aquatic environments tend to vary depending on land use (e.g. agriculture vs. urban) and season. In freshwater environments, surface waters (e.g. wetlands and rivers) that directly drain or receive runoff from agricultural crops are the most susceptible to neonicotinoid contamination (Morrissey et al., 2015).

Table 1.2 Select physicochemical properties of imidacloprid, clothianidin, and thiamethoxam, the three most commonly applied and widely detected neonicotinoid insecticides.

Neonicotinoid	Molecular Weight (g/mol)	Solubility (mg/L; pH 7, 20°C)	Lipophilicity (log (K_{ow}); 25°C)	Soil Affinity (K_{oc}; L/kg)	Vapour Pressure (mPa; 20°C)	Soil Persistence (DT_{50}; d)	Aquatic Half-life (DT_{50}; d)
Imidacloprid	255.7	0.5 - 0.6 [1, 2]	0.6 [1]	248 - 411 [3,4]	2.0×10^{-4} [5]	28 - 1250 [10]	< 1 [11]
Thiamethoxam	291.7	4.1 [1]	-0.1 - 0.7 [1,6]	104 - 2877 [7]	6.6×10^{-6} [6]	7 - 6931 [10,11]	2.7 - 39.5 [11]
Clothianidin	249.7	0.3 [1]	0.7 [1]	84 - 345 [8]	1.3×10^{-7} [9]	3 - 1386 [10,11]	< 1 [11]

[1] Jeschke et al. (2010); [2] Gupta et al. (2002); [3] Cox et al. (1997); [4] Armbrust and Peeler (2002); [5] Stoughton et al. (2008); [6] Gupta et al. (2008); [7] Carbo et al. (2007); [8] United States Environmental Protection Agency (2003); [9] Uneme (2011); [10] Goulson (2013); [11] Morrissey et al. (2015).

Through a comprehensive survey of US streams conducted between 2012 and 2014, Hladik and Kolpin (2015) found that levels of CLO and TMX detected in surface waters were significantly and positively correlated with the amount of surrounding landscape used for agriculture. Similarly, Struger et al. (2017) found that distribution of neonicotinoid insecticides in southern Ontario surface waters were significantly and positively correlated with agricultural activities (IMI detection correlated with greenhouse activity, vegetables, vineyards and orchards; CLO and TMX correlated with row crops). Furthermore, the highest acute concentrations of neonicotinoid contamination (IMI = 10 - 320 µg/L; CLO = 10 - 55.7 µg/L; and TMX = 10 - 225 µg/L) have been detected in surface waters within agricultural watersheds (Wood and Goulson, 2017). Overall, environmental monitoring trends indicate that aquatic neonicotinoid concentrations tend to be highest in the spring, and contamination is associated snowmelt, spring rains, and crop planting (Hladik et al., 2014; Main et al., 2014; Struger et al., 2017). Although neonicotinoids have been frequently detected both prior to the planting seasons in some agricultural areas (e.g. Prairie Pothole Region, Saskatchewan (Main et al., 2016)), and year-round in non-agricultural waterbodies (e.g. Great Lakes tributaries (Hladik et al., 2018a); agricultural sites in the Niagara region in Canada (Struger et al., 2017)), concentrations are generally lower compared to the late spring/early summer seasons. Therefore, in temperate agricultural regions, contamination of aquatic systems with neonicotinoids is most likely to peak in the spring, following spring rainfall and after pesticide application occurs (i.e. in planting/growing seasons).

1.4 Neonicotinoid toxicity

Due to their popularity as agricultural insecticides, potential for persistence, propensity for movement from the area of application, and environmental ubiquity, there is a high probability that non-target organisms inhabiting environments in agricultural regions will be

exposed to neonicotinoid insecticides singly and in combination. To date, a significant amount of the published research on this topic has focused on the toxicity of neonicotinoids to terrestrial pollinators (e.g. bees), as these compounds have been shown to have deleterious individual- and population-level impacts on these sensitive and ecologically important organisms (Wood and Goulson, 2017). However, given the frequent detection of neonicotinoids in aquatic environments, the toxicity of neonicotinoids to non-target aquatic organisms has become an increasingly prevalent issue, especially when trying to understand the broader effects that widespread neonicotinoid contamination could have on biodiversity. Therefore, it is important to understand the mechanisms of neonicotinoid toxicity in non-target organisms, their toxicological effects on aquatic species (direct effects on individuals and populations), and their large-scale effects on aquatic ecosystems (indirect effects on consumers and communities).

1.4.1 Mechanism of neonicotinoid toxicity

Neonicotinoids elicit toxicity by acting on nicotinic acetylcholine receptors (nAChRs) in exposed organisms. As agonist-gated ion channels, localized in post-synaptic membranes of neuronal or neuromuscular junctions (Guyton and Hall, 2006), nAChRs are responsible for rapid-excitatory neurotransmission at cholinergic synapses (Tomizawa and Casida, 2004). In vertebrates nAChRs are located in both the central nervous system (CNS) and the peripheral nervous system (PNS) (Guyton and Hall 2006). In invertebrates, which display varying degrees of nervous system complexities, nAChRs are either similarly dispersed (e.g. across both the CNS or PNS) or exclusively located in diffuse neuropil regions (Matsuda et al. 2001).

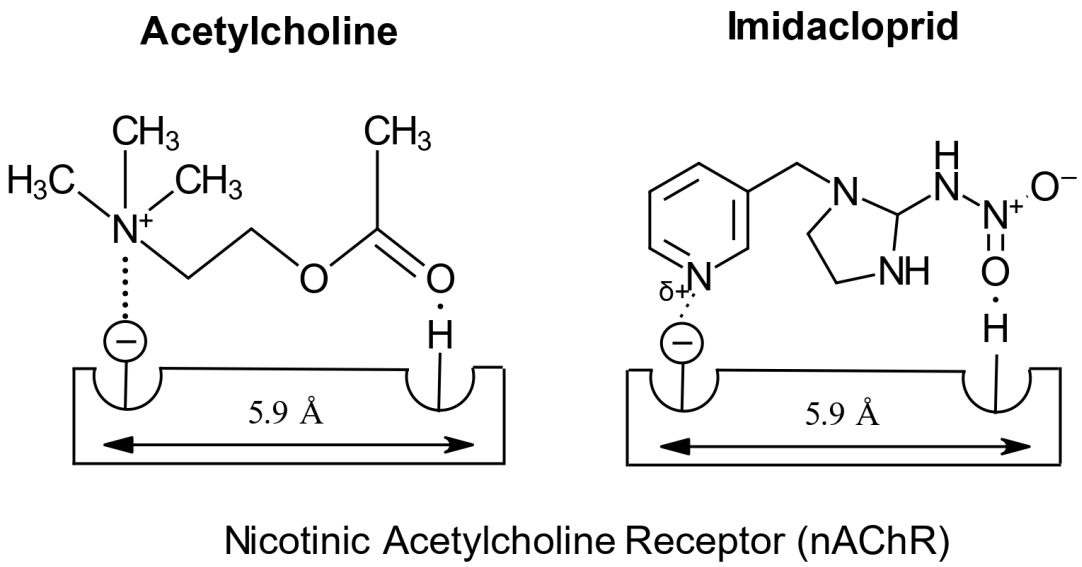

Figure 1.2 Interaction of the hydrogen-donating and anionic sites of insect nicotinic acetylcholine receptors with (A) acetylcholine (ACh) and (B) a representative neonicotinoid, imidacloprid (IMI), demonstrating key structural and binding similarities between the endogenous moiety (ACh) and the insecticidal compound (IMI). Modified from Jeschke and Nauen (2008).

Neonicotinoids elicit toxicity by binding to nAChRs and interfering with neural transmission (Simon-Delso et al., 2015). Normally, nAChRs are activated by the endogenous neurotransmitter acetylcholine (ACh) (Tomizawa and Casida, 2004). Upon ACh binding, nAChRs open ion channels, allowing for an influx of extracellular ions [typically sodium (Na^+) or calcium (Ca^{2+})] and an efflux of intracellular ions [typically potassium (K^+)], which acts to propagate an action potential in post-synaptic neurons or myocytes (Casida and Durkin, 2013; Karlin, 1977). Once the nervous signal is transmitted, acetylcholinesterase (AChE) is released, hydrolyzing ACh at nAChR surfaces, inhibiting ion flow and terminating impulse transmission at cholinergic synapses (Fayuk et al., 2004). Neonicotinoids have key structural similarities to ACh (e.g. quaternary (sp^3) nitrogen atoms, and hydrogen bond acceptors) (Figure 1.2), allowing them to bind to the ACh binding site and activate nAChRs (Jeschke and Nauen, 2008; Tomizawa and Casida, 2004). However, these insecticides are not degraded by AChE (Thany, 2011), thus once bound, neonicotinoids can continuously excite cholinergic neurons, causing a biphasic response (Gupta, 2007; Tomizawa and Casida, 2004). First, neonicotinoid binding excites the cholinergic neuron/myocyte, increasing the frequency of spontaneous discharge, resulting in uncontrollable muscle tremors, cell energy exhaustion, and cell death (Gupta, 2007; Tomizawa and Casida, 2004). This is then followed by neural desensitization to ACh, blocking nerve impulse propagation, resulting in paralysis, loss of normal neuronal or neuromuscular function, and then death (Oliveira et al., 2011; Thany, 2009; Tomizawa and Casida, 2004). Therefore, following exposure neonicotinoid toxicity manifests as seizures, immobility, and then eventual death.

Neonicotinoids demonstrate a high selectivity for target sites (nAChRs) in the insect CNS, and a low affinity for vertebrate nAChRs (Jeschke and Nauen, 2008). This is thought to be due to structural differences between vertebrate and invertebrate receptors. Insect nAChRs have cationic

target sites, whereas vertebrate nAChRs have anionic target sites, tending to preferentially interact with cationic ligands (Tomizawa and Casida, 2004, 2003). Neonicotinoids demonstrate both low protonation at physiological pH and co-planarity, resulting in a conjugated system that facilitates the formation of an electronegative tip at the nitro- or cyano-moiety (Figure 1.2) (Matsuda et al., 2001). Therefore, this negatively charged moiety preferentially interacts with the cationic invertebrate nAChRs and displays limited interaction with its anionic counterpart in vertebrate species (Tomizawa and Casida, 2004, 2003).

Toxicity of neonicotinoids can also be influenced by metabolic processes. Neonicotinoids are metabolized in two phases. Phase I, carried out by cytochrome P450 enzymes (CYP), involves structural alterations (e.g. hydroxylation (ring opening or olefin production), demethylation, nitro-reduction, cyano-hydrolysis, and/or dechlorination) at multiple sites, either detoxifying the compound or producing metabolites that can be more toxic than the parent compounds (e.g. the metabolism of TMX to CLO) (Simon-Delso et al., 2015). Phase II, carried out by a variety of conjugation enzymes (dependent on organism of interest and neonicotinoid of exposure), involves conjugation of the Phase I metabolites, and often leads to the production of less toxic products that are subsequently excreted (Simon-Delso et al., 2015). Therefore, any alteration of the expression or activity of Phase I or II enzymes can influence metabolic pathways, changing the magnitude of toxic effect elicited by neonicotinoid exposure.

1.4.2 Direct toxicity to aquatic organisms and higher-tier consumers

As aquatic macroinvertebrates typically inhabit aquatic systems for a majority or the entirety of their life-cycle, they are both likely to be exposed to neonicotinoid contaminants and susceptible to neonicotinoid toxicity. Indeed, aquatic macroinvertebrates (aquatic insects, macro-crustaceans, and cladocerans) are significantly more sensitive to neonicotinoid toxicity than most

other tested aquatic/terrestrial organisms (Table 1.3). For example, acute geometric mean neonicotinoid LC_{50} values for higher tier aquatic vertebrates (i.e. fish and amphibians) range from 60.8 - 162.8 mg/L, whereas acute geometric mean LC_{50} values for aquatic macroinvertebrates range from 0.006 - 30.4 mg/L (Sanchez-Bayo, 2012). Therefore, aquatic macroinvertebrates can be anywhere from 2 -10^7 times more sensitive to neonicotinoid insecticides than their vertebrate counterparts. Due their neurological similarities to pest species (e.g. nAChR structure and function) (Tomizawa and Casida, 2003), aquatic insects are the most neonicotinoid-sensitive macroinvertebrate group (acute geometric mean neonicotinoid LC_{50} of 6.0 µg/L) (Table 1.3) (Sanchez-Bayo, 2012). Specifically, Ephemeroptera (mayflies), Trichoptera (caddisflies), and Diptera (true flies) are the most sensitive aquatic insect taxa (Table 1.4), with acute geometric mean LC_{50} values of 3.9, 6.9, and 32.9 µg/L, respectively (Morrissey et al., 2015). Interestingly, despite their typical sensitivity to aquatic contaminants and use in toxicity testing (e.g. Wogram and Liess (2001)), cladocerans (e.g. *Daphnia magna*) are relatively insensitive to neonicotinoid toxicity, representing the most resistant aquatic macroinvertebrate order (acute geometric mean neonicotinoid LC_{50} = 43.9 mg/L) (Morrissey et al., 2015).

Some studies have indicated that neonicotinoids can elicit time-dependent toxicities in aquatic invertebrates (i.e. the concentration required to produce lethality was inversely proportional to exposure time). For example, time-dependent lethality has been observed in laboratory-based experiments with mayflies (*Epeorus longimanus*) (Alexander et al., 2007), midges (*Chironomus dilutus*), and amphipods (*Hyallela azteca*) (Stoughton et al., 2008), and Beketov and Liess (2009) found that 24-h, low concentration neonicotinoid pulses (5.4 - 4740 µg/L) could elicit delayed lethality (up to 12 d post exposure) in various aquatic insect species. Time-dependent toxicity is thought to result from a nearly irreversible binding of neonicotinoids

to nAChRs (as described by the Druckrey-Küpfmüller equation), resulting progressive inactivation and eventual death in exposed organisms (Tennekes, 2011, 2010; Tennekes and Sánchez-Bayo, 2013). However, both this proposed mechanism of action and the occurrence of time-dependent toxicity have been contested due to the methods applied to characterize time-weighted effects (e.g. Druckrey-Küpfmüller equations) (Maus and Nauen, 2011) and conflicting results observed in alternative studies (e.g. a lack of neonicotinoid accumulation at target sites and rapid post-exposure recovery following IMI exposure in honeybees and bumblebees) (Cresswell et al., 2014). In fact, a lack of time-dependent toxicity has been observed for some compounds in some aquatic invertebrate species. For example, Bartlett et al. (2019) found that for *H. azteca*, neonicotinoid toxicity did not significantly increase with exposure time. Instead, as seen in *Hexagenia* spp. (Bartlett et al., 2018), toxicological discrepancies tended to stem from differences in endpoint of interest (e.g. survival and growth) (Bartlett et al., 2019). Therefore, although neonicotinoids demonstrate the potential to elicit time-dependent cumulative toxicity, there is no overarching consensus on whether increased exposure time equates to enhanced toxicity for all neonicotinoid compounds and in all exposed organisms.

Under chronic, low-dose exposure scenarios (e.g. via persistence in aquatic systems or repeated low-concentration pulse events) neonicotinoids can also have sub-lethal effects on exposed aquatic invertebrate populations. This can include delayed or reduced emergence (Alexander et al., 2007; Cavallaro et al., 2018; Mohr et al., 2012; J. Pestana et al., 2009; Stoughton et al., 2008), supressed reproduction (Beketov and Liess, 2009; Raby et al., 2018b), decreased growth and development (Alexander et al., 2007; Henrique M V S Azevedo-Pereira et al., 2011; J. Pestana et al., 2009), immobility and decreased locomotion (H.M.V.S. Azevedo-Pereira et al., 2011), impairment of burrowing behaviour (benthic invertebrates) (J. Pestana et al., 2009), and/or

reduced feeding, shredding activity and respiration (Alexander et al., 2007; Henrique M V S Azevedo-Pereira et al., 2011; J. Pestana et al., 2009; J. L. T. Pestana et al., 2009). These sub-lethal effects are of concern as they have the potential to culminate in lethality and/or destabilization of invertebrate populations. Indeed, a number of prior studies have found that low-dose neonicotinoid exposure can influence diversity, richness or abundance of aquatic invertebrates (Colombo and Mohr, 2013; Daisuke Hayasaka et al., 2012; J. L. T. Pestana et al., 2009).

Of the three neonicotinoids of interest here (IMI, CLO, and TMX), IMI is the oldest and (historically) most widely applied neonicotinoid. Therefore, IMI has the best-characterized toxicological profile. However, due to their prevalence in aquatic environments (e.g. Hladik and Kolpin (2015)), a range of recent studies have also evaluated the toxicities of CLO and TMX to non-target aquatic invertebrate species. Whereas IMI and CLO are typically equitoxic (or at least have a relatively comparable toxicity), TMX has a lower toxicity to most aquatic macroinvertebrate taxa (Table 1.4). In a recent review of the published toxicity data for aquatic insects, Morrissey et al. (2015) calculated geometric mean acute LC_{50} values for IMI and CLO that ranged between 25.3 and 26.8 µg/L, and a median toxicity value for TMX of 44.8 µg/L (approximately 2 times higher than that of IMI and CLO) (Table 1.4). Similar results were observed with crustaceans, with TMX displaying a geometric mean acute toxicity (8864.5 µg/L) that was approximately 10 times lower than that of IMI or CLO (587.0 – 842.3 µg/L) (Table 1.4) (Morrissey et al., 2015). This comparative toxicity pattern has been verified for a number of sensitive aquatic organisms. For example, Cavallaro et al. (2017) reported that under chronic exposure settings (40 d) TMX was approximately 10 times less toxic to the aquatic midge *C. dilutus* than CLO or IMI (endpoint = emergence; IMI and CLO EC_{50}s = 0.71 - 1.48 vs. TMX EC_{50} = 23.60). Similarly, under chronic exposure scenarios, Raby et al. (2018a) found that TMX was 2

times less toxic to the mayfly *Neocloeon triangulifer* than IMI or CLO (endpoint = emergence; IMI and CLO EC_{50}s = 0.95 - 1.75 µg/L vs. TMX EC_{50} = 2.18 µg/L). In addition, risk-based rankings of the in-use agricultural pesticides of Canada have indicated that the relative risks of these three seed treatments are as follows: IMI > CLO > TMX. For example, by dividing estimated 96-h environmental concentrations (modeled using application data and physicochemical properties) by the hazard concentration for 5 % of aquatic species (HC_5 values; obtained through species sensitivity distributions), Whiteside et al. (2011) indicated that IMI posed a much higher risk to aquatic invertebrates than CLO or TMX (risk IMI = 4.38 vs. risk CLO and TMX = 0.00 - 0.03). Similarly, by dividing measured environmental concentrations (derived from aquatic monitoring programs) by HC_5 values, Raby et al. (2018a, b) derived hazard quotients (HQs) that indicated that IMI posed the highest risk to invertebrates inhabiting contaminated aquatic environments, followed by CLO and then finally TMX under acute (HQ: IMI > 1.1 ; CLO = 0.1 < HQ < 1.0 ; TMX < 0.1) and chronic (HQ: IMI = 74; CLO = 1 – 1.5; TMX < 0.1) exposure settings.

Neonicotinoids are likely to be metabolized *in vivo* in exposed organisms or biotransformed in the environment (e.g. photolysis, hydrolysis, microbial degradation). Yet, limited studies have focused on characterizing the effects of neonicotinoid metabolites. The most recent evidence indicates that most neonicotinoid metabolites are less acutely toxic than their parent compounds (Malev et al., 2012). However, there are three notable exceptions. TMX can be metabolized into CLO or N-(2-chlorothiazol-5-ylmethyl)-N'-methyl-N-nitroguanidine (CGA-322704) in plant and insect tissues (Morrissey et al., 2015; Simon-Delso et al., 2015), and IMI can be metabolized into 6-chloronicotinic acid (6-NC), which are slightly more toxic then their parent compounds (Malev et al., 2012; Morrissey et al., 2015). Thus, the *in vivo* metabolism or environmental biotransformation of IMI or TMX could potentially enhance their toxic effects.

Table 1.3 Comparative toxicities of neonicotinoid insecticides to non-target terrestrial and aquatic organisms. Adapted from Sanchez-Bayo (2012).

	Taxonomic Group	LC_{50} / LD_{50} *
Aquatic Organisms	Aquatic Insects	0.006 mg/L
	Macrocrustaceans	4.1 mg/L
	Cladocerans	30.4 mg/L
	Fish	60.8 mg/L
	Amphibians	162.8 mg/L
Terrestrial Organisms	Bees	0.00013 mg/organism
	Earthworms	54.0 mg/kg soil
	Birds	659 mg/kg body weight
	Mammals	868 mg/kg body weight

*Toxicities are reported as geometric means of oral LD_{50} (terrestrial organisms) or LC_{50} (aquatic organisms) values.

Table 1.4. Comparative toxicity of neonicotinoid insecticides to common aquatic macro-invertebrates (Crustacea and Insecta) by taxonomic order and neonicotinoid active ingredient. Sensitive taxa from each order are listed in brackets. Adapted from Morrissey et al. (2015) and Sanchez-Bayo (2012).

Taxonomic Group	Order	LC_{50} (µg/L) *	Active ingredient	LC_{50} (µg/L) *
Insecta	Ephemeroptera	3.9	Clothianidin	25.3
	Trichoptera	6.9		
	Diptera	32.9	Imidacloprid	26.8
	(*Chironomus dilutus*)	(9.3)		
	Odonata	55.2		
	Hemiptera	64.9	Thiamethoxam	44.8
	Megaloptera	711.3		
Crustacea	Podocopida	73.6	Imidacloprid	587.0
	Mysida	106.2		
	Amphipoda	235.8	Clothianidin	842.3
	Isopoda	464.8		
	Decapoda	1562.2	Thiamethoxam	8864.5
	Cladocera	23690.0		
	(*Daphnia magna*)	(43926.5)		

*Reported LC_{50} values are geometric means from acute (24 – 96 h) toxicity tests.

1.4.3 Indirect effects on aquatic invertebrate communities and consumers

At concentrations observed in aquatic environments, neonicotinoids are unlikely to directly elicit toxic responses in vertebrate consumers that inhabit or depend on those ecosystems (Gibbons et al., 2015). It is more likely that these higher-tier consumers will be impacted by neonicotinoids indirectly, via impairment of aquatic ecosystem function. As aquatic invertebrates are relatively susceptible to neonicotinoid toxicity (Table 1.3) (Morrissey et al., 2015) and low-concentration neonicotinoid exposure can lead to pronounced effects on diversity and abundance in aquatic invertebrate populations (Colombo and Mohr, 2013; Daisuke Hayasaka et al., 2012; J. L. T. Pestana et al., 2009), widespread contamination of aquatic environments with neonicotinoids could affect the composition and function of aquatic macroinvertebrate communities. In fact, loss of specific aquatic invertebrate populations due to neonicotinoid toxicity has been linked to changes in trophic interactions which could affect aquatic ecosystem function, including decreases in predator abundance (Sánchez-Bayo and Goka, 2009), increases in the relative abundance of competing species (Daisuke Hayasaka et al., 2012), and changes in leaf-litter decomposition rates (Kreutzweiser et al., 2007)**.**

Indirect effects are often hard to measure and suffer from limitations of correlative inferences, therefore only a limited number of studies have focused on characterizing the indirect effects of neonicotinoids on higher-tier organisms that inhabit or rely on aquatic ecosystems. However, as aquatic macroinvertebrates represent important food sources for a range of fish, reptile, amphibian, and avian species, it is possible that effects of neonicotinoids on these species could translate to higher-tier organisms (Robinson et al., 2019). For example, Hayasaka et al. (2012) found that low concentrations of IMI contamination in rice-paddies (~ 1.0 µg/L) could reduce body size in both adult and juvenile medaka fish (*Oryzias latipes*) by influencing the abundance of

aquatic arthropods (which constituted their food source). Several authors have also suggested that this type of indirect effect could occur with avian species, postulating that there is a link between global neonicotinoid use and declining insectivorous bird populations (Gibbons et al., 2015; Goulson, 2014; Hallmann et al., 2014; Mineau and Whiteside, 2013). The observed global decline of insectivorous bird populations is likely multi-factorial. However, food supply (i.e. abundance and availability) has been shown to affect the reproductive success, habitat selection and survival of bird populations (Mineau and Palmer, 2013). Furthermore, previous studies have linked prey-base collapses (resulting from pesticide use) with adverse effects in avian populations (Boatman et al., 2004; Mineau and Whiteside, 2013; Poulin et al., 2010). Therefore, it is possible that neonicotinoids are indirectly affecting avian populations, and neonicotinoid-driven invertebrate loss is contributing to the phenomenon of insectivorous bird decline.

1.5 Invertebrate nicotinic acetylcholine receptors

1.5.1 Molecular structure

Despite the frequent use of the nAChR as a selective target for neurotoxic insecticides like neonicotinoids, the molecular structure of this receptor has not yet been fully characterized in an invertebrate species. Thus, the much better defined vertebrate nAChR is typically used as a basis for understanding the molecular structures of invertebrate nAChRs (Tomizawa and Casida, 2001). Belonging to the cys-loop ligand gated ion channel (cysLGIC) superfamily, nAChRs consist of five homologous subunits arranged around a central ion channel (Figure 1.3A). Each subunit contains four hydrophobic transmembrane domains (TM1 - 4) and a large (~200 amino acid) N-terminal extracellular domain (Millar, 2003) (Figure 1.3a). The N-terminal extracellular domain contains a functionally important Cys-loop motif, made up of two disulfide bond-forming cysteine residues separated by 13 amino acid residues (Jones and Sattelle, 2010), which is thought to play

a significant role in nAChR assembly and continued function (Green and Wanamaker, 1997). The agonist binding site, located at the interface of two subunits (Figure 1.3B), is formed by six of these N-terminal extracellular loops (loops A - F) (Corringer et al., 2000). Loops A - C are composed of an α subunit, whereas loops D - F are composed of either an α or a non-α (e.g. β, γ, δ, or ε) subunit. The α subunits differ from other subunit classes in that they contain two adjacent cysteine residues in their third loop (loop C), which are important for binding acetylcholine or other receptor agonists (Kao and Karlin, 1986). Non-α subunits lack these vicinal cysteines (Jones and Sattelle, 2010). Within each subunit classes there is a range of different subunit subtypes (e.g. within the α class, subtypes α1 - α9 can exist (Jones and Sattelle, 2010)). Thus, a suite of functional nAChRs with different subunit combinations can be formed. In fact, nAChRs can be homomeric, consisting of only one kind of α subunit, or heteromeric, consisting of either multiple types of α subunits or various combinations of α and non-α subunits (Figure 1.3b) (Millar and Gotti, 2009).

1.5.2 nAChR subunit diversity

Diversity of expressed nAChR subunits varies considerably amongst different species. Vertebrates generally have large nAChR gene families, expressing up to 17 distinct subunit types (α1 - 10, β1 - 4, δ, ε, and γ) (Millar, 2003), but invertebrates tend to have very small nAChR gene families, expressing a much smaller range of distinct subunit types (Jones and Sattelle, 2010). Arthropod nAChR gene families are amongst the smallest known, with only 10 - 12 distinct subunit types having been identified to date (Jones et al., 2007; Jones and Sattelle, 2010). In this group, complete nAChR gene families have been characterized for several species: *Drosophila melanogaster* (common fruit fly) (Littleton and Ganetzky, 2000), *Anopheles gambiae* (African malaria mosquito) (Holt et al., 2002; Jones et al., 2005), *Apis mellifera* (honey bee) (Jones et al., 2006; Weinstock et al., 2006), *Tribolium castaneum* (red flour beetle) (Jones and Sattelle, 2007;

Richards et al., 2008), *Bombyx mori* (silk worm) (Shao et al., 2007; Xia et al., 2004), *Chilo supressalis* (rice striped stem borer) (Xu et al., 2017). The number of distinct nAChR subunits depends on taxonomic order, with Diptera (*D. melanogaster* and *A. gambiae*) expressing 10 subunits (Holt et al., 2002; Jones et al., 2005; Littleton and Ganetzky, 2000), Hymenoptera (*A. mellifera*) expressing 11 subunits (Jones et al., 2006; Weinstock et al., 2006), and Coleoptera (*T. castaneum*) and Lepidoptera (*B. mori* and *C. supressalis*) expressing 12 nAChR subunits (Jones and Sattelle, 2007; Richards et al., 2008; Shao et al., 2007; Xia et al., 2004; Xu et al., 2017).

Each of the characterized invertebrate nAChR gene families has been shown to express a group of 7 subunits that is highly conserved across species (> 60 % homology in amino acid sequence) (Jones et al., 2007). Due to their common (and historical) use as a test species, these core subunits are often compared to and classified against the equivalent subunits expressed in *D. melanogaster*: Dα1 - 7 and Dβ1 - 2 (Jones and Sattelle, 2010). Interestingly, along with this core group of conserved subunits, different insect species have been shown to display at least one species-specific divergent subunit that demonstrates low homology to all other known nAChR subunits (< 29 % homology in amino acid sequence) (Jones et al., 2007). Although little is currently known about the impact of these divergent subunits on nAChR function, prior research has indicated that these subunits can influence both ligand binding and ion channel characteristics (Jones and Sattelle, 2010), and thus could potentially influence the pharmacological characteristics of nAChR subtypes and species-specific sensitivity differences to neonicotinoids and their mixtures.

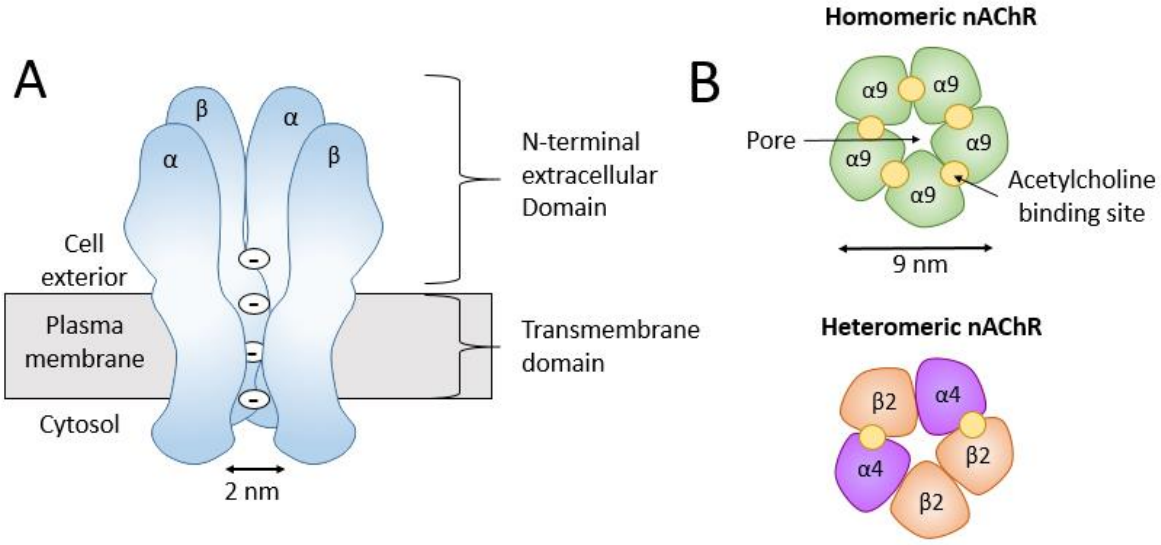

Figure 1.3 Structure of neuronal nicotinic acetylcholine receptors (nAChRs). (A) Schematic model of the pentameric nAChR at the neuronal synapse indicating the ion channel, lined by negative charges, and large N-terminal extracellular and transmembrane domains. (B) Cross section of the receptor, indicating the arrangement of subunits around the pore and the locations of the acetylcholine binding sites. Homomeric nAChRs (top) are composed of only α subunits, whereas heteromeric nAChRs (bottom) are composed of various combinations of α and non-α subunits. Modified from Hendrickson et al. (2013) and Uzman (2001).

1.5.3 Pharmacological subtypes

Three major classes of nAChRs have been identified in vertebrates: muscle, sensory/epithelial, and neuronal. Muscle nAChRs are composed of the following subunits: α1, β1, γ, and either ε or δ (Millar, 2003). Sensory/epithelial nAChRs are homomeric complexes, composed of a combination of either α9 or α10 subunits (Millar, 2003). Neuronal nAChRs are primarily composed of α and β subunits, with exact subunit composition depending on pharmacological subtype (Millar, 2003). Neuronal nAChRs are often subdivided into two different pharmacological subtypes based on the sensitivity of the receptor to the neurotoxin α-bungarotoxin (α-BGT) (Tomizawa and Casida, 2001). α-BGT sensitive nAChRs are heteromeric complexes, composed of a combination of α (α2 - 6) and β (β2 - 6) subunits (Millar, 2003). In contrast, α-BGT insensitive nAChRs can be homomeric or heteromeric complexes; either exclusively composed of α7 or α8 subunits, or of a combination of α and β subunits (α7 - 8, β2 - 4) (Millar, 2003).

As with molecular structure, the genetic and functional composition of pharmacological subtypes have been more sufficiently characterized for vertebrates. Therefore, vertebrate subtypes are often used as a basis for the understanding of nAChR subtype structure and function in invertebrate species. Electrophysiological and molecular structure studies with vertebrate-invertebrate nAChR hybrids (i.e. experimental nAChRs constructed from a combination of known vertebrate and invertebrate subunits) and *in vitro* binding studies with insect proteins and neural tissues have demonstrated that there are different pharmacological subtypes of invertebrate nAChRs, and that subunit composition can influence affinity for and sensitivity to agonists such as neonicotinoid insecticides (Matsuda et al., 2001). Typically, invertebrate nAChRs are first split into two categories based on their response to α-BGT: 1) α-BGT sensitive nAChRs, thought to be relatively insensitive to neonicotinoids, and 2) α-BGT insensitive nAChRs, thought to be relatively

sensitive to neonicotinoids (Salgado and Saar, 2004). Subtype is then further defined based on nAChR responses to particular neonicotinoid agonists. The α-BGT sensitive nAChRs are generally divided into two subtypes based on receptor desensitization post-neonicotinoid exposure: 1) nAChD, which becomes completely desensitized post-neonicotinoid exposure, and 2) nAChN, which do not become desensitized post-neonicotinoid exposure (Salgado and Saar, 2004). The α-BGT insensitive nAChRs are divided into two subtypes based on their response to IMI: 1) nAChR1, which are sensitive to IMI, and 2) nAChR2, which are insensitive to IMI (Simon-Delso et al., 2015). Differences in sensitivity to neonicotinoid exposure between these two nAChR subtypes (nAChR1 and nAChR2) is thought to be due to conformational variation (Bodereau-Dubois et al., 2012). In a resting state nAChR1 remains closed, but upon agonist binding this receptor opens up, allowing an influx of sodium cations (Na^+), which acts to propagate an action potential (Bodereau-Dubois et al., 2012). In contrast, in a resting state nAChR2 remains open, but upon agonist binding this receptor closes, inhibiting the efflux of potassium cations (K^+), which act to propagate an action potential (Bodereau-Dubois et al., 2012). This open/closed resting state variation is thought to influence the binding capabilities of neonicotinoids and thus the response of the receptor to particular neonicotinoid agonists.

Current evidence suggests that neonicotinoids preferentially interact with specific nAChR subtypes. Indeed, prior studies have found that whereas CLO can act on all four nAChR subtypes (nAChD, nAChN, nAChR1, nAChR2) (Calas-List et al., 2012; Thany, 2009), IMI can only act on three subtypes (nAChD, nAChN, nAChR1) (Calas-List et al., 2012; Thany, 2009), and TMX primarily acts on one subtype (nAChN), but can weakly bind to two others as well (nAChR1, nAChR2) (Thany, 2011). Recent evidence also has suggested that there may be another subtype of nAChR preferentially targeted by thiamethoxam (a mixed nicotinic/muscarinic receptor present

in cockroach model species (Thany, 2011), however further studies must be completed to verify the presence of this receptor in other invertebrate organisms.

1.5.4 Diversity in neonicotinoid binding and expression in insects

Expression and activity of nAChRs have been extensively characterized in agricultural pests (e.g. aphids, planthoppers, leafhoppers, locusts, hornworms and budworms), and standard test insects (e.g. cockroaches, fruit flies, and houseflies) through radioligand binding studies (Crossthwaite et al., 2017; Taillebois et al., 2018). Thus, there is strong evidence that different pharmacological nAChR subtypes exist in most insect species (Lapied et al., 1990; Matsuda et al., 2001; Thany and Tricoire-Leignel, 2011). However, these studies have also indicated that there is wide diversity in the pharmacological function and expression of insect nAChRs. For example, neonicotinoid-sensitive nAChR binding appears to significantly differ depending on taxonomic order. Indeed, previous binding studies with radiolabeled IMI ([^{3}H]-IMI) have indicated that dipteran insects (e.g. fruit flies and houseflies) tend to express only one IMI binding site, whereas hemipteran insects (e.g. aphids and hoppers) tend to express two distinct IMI binding sites that differ in their neonicotinoid binding affinity (i.e. high affinity and low affinity binding sites) (Crossthwaite et al., 2017; Taillebois et al., 2018). In addition, nAChR binding and/or expression has been shown to vary depending on the individual species of interest. For example, *Myzus persicae* (green peach aphid) and *Acyrthosiphon pisum* (pea aphid) display distinct differences in IMI-sensitive nAChR affinity, with *A. pisum* generally expressing nAChRs with lower affinities for IMI than *M. persicae* (e.g. dissociation constants (K_D) for *A. pisum* range from 0.2 - 41.7 nM vs. 0.08 - 12.6 nM in *M. persicae*) (Crossthwaite et al., 2017; Nauen et al., 1998a; Shiokawa et al., 1994; Taillebois et al., 2018, 2014). Furthermore, nAChR response has been shown to widely diverge depending on neonicotinoid compound of exposure. Indeed, most studies that have

compared neonicotinoid binding in insects have found that IMI, CLO, and TMX tend to elicit significantly different affinities for nAChRs (e.g. (Taillebois et al., 2014). However, although nAChR expression/pharmacological function is relatively well defined in agricultural pests and experimental insect species, nAChR binding and expression has not been characterized for any aquatic insects to date. Thus, we cannot evaluate whether the previously observed patterns in nAChR expression/binding also apply for non-target insect species. Aquatic insects (e.g. Chironomidae, Ephemeroptera) are both highly sensitive to neonicotinoids (Morrissey et al., 2015) and likely to be repeatedly and /or chronically exposed to them over the course of a growing season (Hladik et al., 2014; Main et al., 2014). Therefore, it is important to understand how and why neonicotinoids elicit their toxic effects in these non-target organisms. In fact, understanding the receptor-level actions of neonicotinoids in aquatic insects could be both commercially and ecologically beneficial. For example, this knowledge could potentially aid in the design of novel neonicotinoids, helping improve the selectivity of these pest control products for target organisms. In addition, it could help further elucidate the mechanisms behind neonicotinoid toxicity in non-target insects and provide further information that can be used in risk assessments of neonicotinoid-contaminated aquatic environments.

1.6 Neonicotinoid mixtures

In aquatic ecotoxicology, the focus is often placed on characterizing the effects of single compounds on sensitive aquatic organisms. This often translates to environmental risk assessment and regulation, where contaminants are often regulated on a single compound basis and risk is typically estimated using techniques that either do not account for the presence of multiple contaminants, or fail to capture the complexity of mixture interactions (e.g. by directly summing chemical concentrations in the environment) (Backhaus and Faust, 2012). However, due to the

frequent, concurrent use of multiple chemicals in most human-dominated landscapes, aquatic environments are more likely to be contaminated with mixtures of chemicals than single compounds (e.g. (Kolpin et al., 2002)). This is certainly the case for neonicotinoid insecticides in aquatic environments. Due to a combination of their physicochemical and environmental properties (e.g. hydrophilicity, propensity for movement from area of application into aquatic systems, extended/multi-season persistence) and current agricultural practices (e.g. field-rotation, co-application of multiple compounds in a formulated product/tank mix, or application of multiple compounds in one watershed), neonicotinoids are likely to exist in mixtures in many aquatic environments. However, the ecotoxicities of neonicotinoid mixtures have rarely been investigated in the published literature. Therefore, there is a need to consider neonicotinoid (IMI, CLO, and TMX) mixtures from the perspective of their prevalence in aquatic environments, their cumulative toxicity to sensitive aquatic species, and the potential risks they pose to aquatic ecosystems.

1.6.1 Prevalence in aquatic environments

Binary and ternary mixtures of neonicotinoid insecticides have been detected at relatively high frequencies in a range of freshwater systems within (or in close proximity to) agricultural watersheds. For example, in a multi-year survey (2012 - 2013) of the Canadian Prairie Pothole Region (PPR), Main (2016) found that 11 - 63 % of wetlands sampled contained mixtures of IMI, CLO, and/or TMX at cumulative concentrations ranging from 0.004 - 1.66 µg/L. In a more recent survey of the Canadian PPR (2017 - 2018) similar trends were found, with Malaj et al. (pers. comm.) detecting binary and ternary mixtures of IMI, CLO, and/or TMX in 40 % of wetlands sampled. Neonicotinoid mixtures have also been detected in other aquatic environments, including other wetlands (0 - 67 % of sampled; cumulative concentrations = 0.008 - 0.7 µg/L) (Smalling et al., 2015), rivers and streams (25 % of sampled; cumulative concentrations = 0.0054 - 0.38 µg/L)

(Hladik and Kolpin, 2015), groundwater (6.1 % of sampled; cumulative concentrations = 0.28 - 1.05 µg/L) (Giroux and Sarrasin, 2011), and other agricultural surface waters (e.g. puddles, ditches, and drains) (100% of sampled; cumulative concentrations = 0.039 - 44.38 µg/L) (Schaafsma et al., 2015). Therefore, there is substantive evidence that, rather than existing as single compounds, IMI, CLO, and TMX are likely to co-exist in aquatic systems. Furthermore, these monitoring data suggest that such neonicotinoid mixtures can sometimes be found at cumulative concentrations that could pose a risk to aquatic insects likely to inhabit contaminated areas, especially when considering their marked sensitivities to individual mixture constituents (Tables 1.3 and 1.4).

1.6.2 Neonicotinoid mixture toxicity

Nearly all prior research on neonicotinoid insecticides and their effects on aquatic organisms has focused on characterizing the toxicity of individual compounds. However, there is both physiological and toxicological evidence that suggests that neonicotinoid mixtures could have cumulative inhibitory actions on insect nAChRs, resulting in enhanced toxicity. First, all neonicotinoid compounds follow similar metabolic pathways (Simon-Delso et al., 2015; Tomizawa and Casida, 2004). Therefore, exposure to multiple compounds could impact levels of enzymes involved in phase I or phase II metabolism (e.g. cytochrome P450 or conjugation system enzymes), which could impede detoxification, enhance persistence at nAChRs, and potentially increase toxicity. Additionally, neonicotinoids have been shown to display differential selectivity for different nAChR subtypes (Calas-List et al., 2012; Oliveira et al., 2011; Thany, 2011, 2009), so exposure to a neonicotinoid mixture could potentially activate a higher frequency of subtypes than exposure to a single compound and enhance toxicity (especially under high concentration exposure scenarios). Thus, when present in mixtures, it is possible that neonicotinoids exert a level of cumulative toxicity that cannot be predicted by single compound exposures.

To date, the cumulative toxicity of neonicotinoid mixtures has only been characterized for a handful of invertebrate species and a limited number of compounds. However, these studies have uncovered some interesting and unexpected cumulative effects. Due to their common mechanism of action, neonicotinoids have been expected to display a cumulative toxicity that can be estimated by direct summation of constituent concentrations (i.e. via the concept of Concentration Addition, Section 1.6.2). Concentration-additive cumulative toxicity has been reported for some sub-lethal endpoints, including body length of the cladoceran, *Daphnia magna,* (imidacloprid-thiacloprid (IMI-THIA) mixture) (Pavlaki et al., 2011) and cocoon production and feeding in the earthworm, *Eisenia fetida*, (IMI-THIA mixture) (Gomez-Eyles et al., 2009). However, other studies have reported that cumulative toxicity can deviate from this concentration-additive model. Loureiro et al. (2010) found that, in *D. magna*, IMI-THIA mixtures could have a greater-than-additive (synergistic) effect on lethality and a less-than-additive (antagonistic) effect on feeding inhibition. Gomez-Eyles et al. (2009) found that, in roundworms (*Caenorhabditis elegans*), IMI-THIA mixtures could elicit synergism at low cumulative doses and antagonism at high cumulative doses (dose-dependent deviation from concentration-additive toxicity) for the endpoint of reproduction. Similarly, Pavlaki et al. (2011) found that, for reproduction in *D. magna*, IMI-THIA mixtures could synergistically deviate from direct additivity until reaching doses above the EC_{50} isobole, where the cumulative toxicity became antagonistic. Using transcriptomic and proteomic endpoints, Dondero et al. (2010) demonstrated that IMI-THIA mixtures could also elicit synergistic cumulative toxicity in marine mussels (*Mytilus galloprovincialis*), with exposure to IMI-THIA mixtures resulting in gene response patterns that outweighed the effects of either IMI or THIA individually. Finally, and in support of these independent findings, Bayer Crop Science has

previously patented the synergistic lethality of binary neonicotinoid mixtures (IMI, CLO and/or THIA) for several target invertebrate species (Andersch, W., Jeschke, P., Thielert, 2010).

Although some studies have evaluated the cumulative effects of neonicotinoid mixtures to aquatic organisms, there are clearly some major knowledge gaps that limit our ability to comprehensively evaluate their risks to aquatic environments: *1) No published studies have evaluated the toxicity of environmentally relevant neonicotinoid mixtures.* Environmental monitoring studies have indicated that IMI, CLO, and TMX are likely to be present in mixtures in aquatic environments (Main, 2016; Schaafsma et al., 2015; Smalling et al., 2015). However, the cumulative toxicities of these three neonicotinoids have yet to be characterized. Indeed, the majority of prior neonicotinoid mixture studies have focused on IMI-THIA mixtures. This is limiting as IMI-THIA mixtures do not represent the most frequently detected neonicotinoid mixtures in surface water environments (Hladik and Kolpin, 2015; Main, 2016; Metcalfe et al., 2019; Schaafsma et al., 2015; Smalling et al., 2015; Struger et al., 2017). Furthermore, it is unclear as to whether THIA is similar enough to CLO and/or TMX (in terms of nAChR subtype specificity and metabolic pathway) to be used as a proxy for these compounds during neonicotinoid mixture assessment. *2. The effects of neonicotinoid mixtures have yet to be characterized for a sensitive, non-target insect species.* Ephemeroptera, Trichoptera, and Diptera have been identified as the most sensitive aquatic insect taxa to neonicotinoid exposure (Table 1.4) (Morrissey et al., 2015). However, the effects of neonicotinoid mixtures have yet to be evaluated for these sensitive organisms. Indeed, a majority of prior neonicotinoid mixture studies have been carried out with standard invertebrate test species (e.g. *D. magna*), which are relatively insensitive to neonicotinoid exposure (Table 1.4) (Morrissey et al., 2015) and physiologically dissimilar to the more sensitive insect species. Thus, previously characterized mixture effects may not adequately describe

cumulative toxicities in organisms likely to be adversely impacted by low concentration neonicotinoid exposures. *3) The molecular mechanisms of neonicotinoid toxicity have yet to be characterized in aquatic insects.* Despite their marked sensitivity, no published studies have attempted to characterize the molecular-level effects of neonicotinoid insecticides in aquatic insects. Prior studies have characterized the molecular mechanisms of neonicotinoid toxicity in other insects (e.g. dipteran test insects like *D. melanogaster* and agricultural pests like *A. pisum* (Taillebois et al., 2018)). However, as nAChR expression, function, and pharmacological properties tend to widely vary amongst different insect species, it is unlikely that these prior studies can be used to validate molecular toxicity pathways in any sensitive aquatic insect species.

1.7 Environmental risk assessment of neonicotinoids and their mixtures

1.7.1 Single compounds

Prospective ecological risk assessment frameworks provided by regulatory agencies (i.e. those primarily used for the risk assessment and registration of plant protection products) also tend to focus on individual compounds. The ecological risk assessment frameworks available from Environment and Climate Change Canada (ECCC) and the US EPA recommend using weight-of-evidence approaches with multiple lines of ecotoxicological evidence (hazard quotients, concentration-response, probabilistic methods, etc.) to evaluate the risk of predicted or measured concentrations of individual pesticides in aquatic environments (Environment and Climate Change Canada, 2012; United States Environmental Protection Agency, 2018). The risk assessment framework recommended by the EFSA is a little more comprehensive, accounting for the cumulative toxicity of pesticide mixtures (European Food Safety Authority, 2013a). However, these risk assessment recommendations are primarily focused on formulated products containing pesticide mixtures, rather than unintentional mixtures found in aquatic systems. Therefore, risk

assessments performed in the EU similarly tend to focus on neonicotinoids as individual compounds. This is widely reflected in published studies and regulatory reports, with ecological risk assessments for neonicotinoid insecticides primarily focusing on individual compounds and the risk that long-term, low concentration exposures pose to sensitive aquatic insect communities (e.g. (Health Canada, 2018a, 2018b; Pest Management Regulatory Agency, 2018a; Raby et al., 2018b).

1.7.2 Neonicotinoid mixtures

In environmental risk assessments, the cumulative toxicities of chemical mixtures are typically predicted based on the toxic mechanisms of action (MOA) of mixture constituents. There are two models that are commonly used to predict the cumulative effects of non-interactive (independent) chemicals: concentration addition (CA) and independent action (IA). Concentration addition is used to predict the cumulative toxicity of chemicals with similar MOA. This model assumes that chemicals in a mixture act as dilutions of each other and predicts joint action by summing concentrations of the mixture constituents, scaled to reflect their relative toxicity (de Zwart and Posthuma, 2005). For example, each mixture constituent can be converted into a toxic unit (TU) (Equation 1.1):

$$\sum TU = \frac{c_i}{EC_{xx}(i)} \qquad \text{(Equation 1.1)}$$

where c represents the concentration of chemical i, and EC_{xx} represents the concentration of chemical c eliciting a particular toxicological effect of interest (e.g. LC_{50}: median lethal concentration). Cumulative toxicity can then be estimated by summing the toxic units of all chemicals in the mixture, with TU = 1 representing an EC_{xx} -level effect. In contrast, IA is used to predict the cumulative toxicity of chemicals with strictly dissimilar MOA. This model assumes

that the effects of each chemical in the mixture are statistically independent and predicts joint action by multiplying the probabilities of responses (de Zwart and Posthuma, 2005). CA models are easier to apply than IA models, and provide a more conservative estimate of toxicological effect, thus CA models are more commonly used in the ecological risk assessment of chemical mixtures (de Zwart and Posthuma, 2005; Deneer, 2000; Verbruggen and Van den Brink, 2010). However, the behaviour of chemicals in a mixture does not always correspond to that predicted by additive mixture models. Toxicological interactions at physiological or biochemical levels, or in toxicokinetic or toxicodynamic phases, can result in a cumulative toxicity that deviates from CA or IA (Altenburger et al., 2013). Sometimes chemical mixtures will exhibit synergism, where the additive model under-predicts mixture toxicity (greater-than-additive toxicity), or antagonism, where the additive model over-predicts mixture toxicity (less-than-additive toxicity) (Rand, 1995). The toxicological deviation of a mixture from direct additivity can also be dependent on cumulative concentration (dose-level dependent deviation), or the ratio of mixture constituents (dose-ratio dependent deviation) (Jonker et al., 2005). In dose-level dependent deviation, deviation from the additive reference model shifts depending on the cumulative concentration of the sample (Jonker et al., 2005). For example, in a mixture displaying dose-level deviation, there could be antagonism at low cumulative dose-levels (low cumulative mixture concentrations) which shifts to synergism at high cumulative dose-levels (high cumulative mixture concentrations). In contrast, in dose-ratio dependent deviation, deviation from the additive reference model can shift depending on the composition of the mixture (Jonker et al., 2005). For example, in a mixture displaying dose-ratio deviation there could be antagonism if chemical A is more prevalent (i.e. at ratios favouring chemical A) and synergism if chemical B is more prevalent (i.e. at ratios favouring chemical B).

Therefore, although chemicals can behave additively when in mixtures, there are many ways in which directly additive mixture models (e.g. IA and CA) could under- or over-predict toxic effect.

Neonicotinoids all act on nAChRs, therefore their cumulative toxicity (when accounted for) is typically characterized using the CA mixture model. However, this assumption has yet to be validated in a sensitive, aquatic invertebrate species. Previous toxicity studies have shown that neonicotinoid mixtures can elicit synergistic or antagonistic toxicities (Gomez-Eyles et al., 2009; Loureiro et al., 2010; Pavlaki et al., 2011), and physiological studies have shown that IMI, CLO, and TMX can have diverse effects on different nAChR subtypes (which may influence the cumulative toxicological response to mixture exposure) (Simon-Delso et al., 2015). Therefore, it is possible that mixtures of these neonicotinoids could elicit cumulative toxicity that deviates from the assumption of directly additivity in sensitive aquatic insects. Further testing is thus necessary to determine if the assumptions being applied in aquatic regulations and environmental risk assessments (e.g. focusing on single compound toxicity and accounting for mixture toxicity using CA) are adequately protective of the ecologically important, sensitive aquatic insect species (e.g. Chironomidae) likely to be chronically and/or repeatedly exposed to mixtures of IMI, CLO, and/or TMX in the natural environment.

1.8 Project summary and rationale

The neonicotinoid insecticides IMI, CLO, and TMX have been detected singly and as mixtures in aquatic ecosystems, where they could potentially pose a risk to non-target larval insects. However, the cumulative toxicities of binary and ternary mixtures of these compounds have yet to be formally characterized for any sensitive or ecologically important aquatic insect species. Furthermore, the molecular mechanisms of neonicotinoid or neonicotinoid mixture toxicity have yet to be evaluated in any sensitive, aquatic insects. Therefore, we cannot definitively determine

whether current environmental regulations and ecological risk assessment practices, which primarily focus on the single compound neonicotinoid toxicity under short-term, laboratory-based exposure scenarios, are adequately protective of aquatic insects inhabiting these neonicotinoid contaminated environments.

1.8.1 Research objectives and hypotheses

The overall goals of the research presented in this thesis were to characterize the cumulative toxicity of neonicotinoid insecticides (IMI, CLO, TMX) and their mixtures under laboratory (Chapters 2 - 3) and field (Chapter 4) based settings, and to investigate how receptor-level binding characteristics may drive toxic effect (Chapter 5). The specific research objectives were:

1. a) Characterize the acute (96 h) cumulative toxicities of binary and ternary mixtures of IMI, CLO, and TMX to larval *Chironomus dilutus* (a model freshwater benthic invertebrate). The goal was to provide a preliminary understanding (proof of principle) of whether neonicotinoid mixture toxicity can be adequately predicted by directly additive mixture models (e.g. concentration addition).

   H_0*:* There is no statistically significant differences between single compounds and their binary and ternary mixtures at theoretically equitoxic concentrations; all cumulative toxicities are adequately predicted by the concentration addition mixture model.

   b) Characterize the chronic (28 d) cumulative toxicities of binary and ternary mixtures of IMI, CLO, and TMX to larval *Chironomus dilutus*, using successful emergence as a toxicological endpoint. The objective was to enhance the understanding of neonicotinoid mixture toxicity under more environmentally relevant exposure scenarios and assess whether chronic neonicotinoid mixture toxicity can be predicted from acute toxicity studies.

H_0: There are no statistically significant differences between single compounds and their binary and ternary mixtures at theoretically equitoxic concentrations; all cumulative toxicities are adequately predicted by concentration addition and acute toxicity studies similarly and adequately predict mixture effects under chronic, sub-lethal exposure scenarios.

2. Evaluate the chronic (28 and 56 d) toxicities of single neonicotinoids (IMI, CLO, and TMX) and their binary mixtures to natural Chironomidae populations in a field-based exposure setting. Endpoints of cumulative emergence and biomass were used to determine if laboratory-derived neonicotinoid toxicity models (single compound and mixture) can successfully predict toxicological effects on aquatic insect communities in an environmentally realistic field setting.

   H_0: Laboratory-derived neonicotinoid toxicity models (for single compounds and mixtures) using *C. dilutus* as a model species successfully predict the toxicities of IMI, CLO, TMX, and their binary mixtures to Chironomidae populations under semi-controlled field exposure conditions.

3. Characterize the binding profiles of neonicotinoid-sensitive nicotinic acetylcholine receptors in Chironomidae using radioligand binding assays. Neonicotinoid binding will be compared in two species (*C. dilutus* and *C. riparius*), at two different life stages (larval and adult), and with three different compounds (IMI, CLO, TMX) to determine if receptor binding characteristics can explain species-, life stage-, and compound-specific patterns in toxicity.

H_0: Neonicotinoid receptor binding affinity will be equivalent among Chironomidae species, adult and larval life stages, and neonicotinoid compounds.

CHAPTER 2: CUMULATIVE TOXICITY OF NEONICOTINOID INSECTICIDE MIXTURES TO *CHIRONOMUS DILUTUS* UNDER ACUTE EXPOSURE SCENARIOS

Preface

This chapter focuses on characterizing the acute cumulative toxicities of binary and ternary mixtures of select neonicotinoid insecticides (imidacloprid, clothianidin, and thiamethoxam) using the larval midge, *Chironomus dilutus*, as a representative aquatic insect species. Neonicotinoid mixture toxicity was investigated through a series of 96-h toxicity tests (endpoint: lethality) with single compounds, binary mixtures, and ternary mixtures. Using the MIXTOX approach, predictive parametric models were fitted using single-compound toxicity data and statistically compared to cumulative toxicity in mixture tests. Results from this chapter demonstrate that, under acute exposure scenarios, neonicotinoid mixture toxicity can deviate from the common assumption of direct additivity (i.e. concentration addition). In fact, it was found that most tested mixtures could display greater- or less-than-additive toxicity to *C. dilutus*. However, cumulative toxicity was highly dependent on mixture composition, with mixtures containing higher concentrations of imidacloprid displaying the greatest propensity for synergism. This work highlights the need to evaluate neonicotinoid mixture toxicity under more environmentally relevant exposure scenarios, and suggests that until further investigations are carried out, the synergistic effects observed here should be considered when setting water quality benchmarks for neonicotinoid compounds.

This chapter was published in Environmental Toxicology and Chemistry, under joint authorship with Christy A. Morrissey (University of Saskatchewan), Karsten Liber (University of Saskatchewan), John V. Headley (National Hydrology Research Centre, Environment and Climate Change Canada), and Kerry M. Peru (National Hydrology Research Centre, Environment and Climate Change Canada). Following publication, a calculation error was found in the mixture

toxicity models. This was corrected, mixture toxicity estimates were re-calculated, and the chapter/manuscript was updated accordingly. The corrected work is presented here, and a corrigendum has been published in Environmental Toxicology and Chemistry.

Maloney, E.M., Morrissey, C.A., Headley, J.V., Peru, K.M., and Liber, K. 2017. Cumulative toxicity of neonicotinoid insecticide mixtures to *Chironomus dilutus* under acute exposure scenarios. Environ Toxicol Chem. 36: 3091-3101. Corrigendum : Environ. Toxicol. Chem. 2020 Apr 39(4): 942 – 946. Doi: 10.1002/etc.4675.

2.1 Introduction

Neonicotinoid insecticides are the fastest-growing and largest-selling group of insecticides worldwide. Used in a multitude of agricultural products, neonicotinoids are commonly applied as seed-treatments, soil drenches, or foliar sprays to protect young crops from biting-sucking pests. Because of their versatility, broad-spectrum insecticidal action, and low toxicity to vertebrates, neonicotinoids have recently come to dominate the agrochemical market, representing more than 24 % of agrochemicals and 80 % of seed-treatments sold worldwide (Jeschke et al., 2010). Of the seven commercially available neonicotinoid compounds, the most commonly applied are the second-generation seed treatments thiamethoxam (TMX) and clothianidin (CLO), and the first-generation seed treatment imidacloprid (IMI) (Simon-Delso et al., 2015). Extensive application has raised concerns about the environmental impacts of these compounds, particularly in aquatic environments surrounding areas of intensive use where multiple neonicotinoids may be found.

The seed treatments IMI, CLO, and TMX display many physicochemical characteristics that facilitate their movement into and persistence in aquatic environments (Morrissey et al., 2015). Following application, a large portion of active ingredient (up to 90 %) moves from the treated seed directly into the soil and soil water (Goulson, 2013). These compounds can then easily move into nearby surface- and ground-water systems via leaching, drainage, run-off, or snowmelt processes (Main et al., 2014). Once in aquatic and terrestrial environments, neonicotinoids can exhibit extended persistence (e.g. TMX has max aquatic and terrestrial half-lives of 43 d and 6931 d, respectively) (Anderson et al., 2015; Goulson, 2013). This has resulted in widespread and frequent detection of IMI, CLO, and TMX residues in diverse waterbodies in Canada (Main et al., 2014; Struger et al., 2017), Australia (Sánchez-Bayo and Hyne, 2014; Smith et al., 2012), United

States (Phillips and Bode, 2004; Starner and Goh, 2012; Xing et al., 2013), Europe (Kreuger et al., 2010; van Dijk, 2010), and Asia (Lamers et al., 2011).

One environmental concern is the impact of these neonicotinoid residues on non-target aquatic organisms. Many aquatic macroinvertebrates are relatively sensitive to these compounds (Morrissey et al., 2015), and thus may be adversely affected by neonicotinoid exposure. Of the aquatic macroinvertebrate taxa, insects that have an aquatic larval stage and emerge as adults (e.g. Ephemeroptera, Trichoptera, and Diptera) are particularly sensitive to neonicotinoids (Morrissey et al., 2015; Van den Brink et al., 2016). Neonicotinoids elicit neurotoxicity in insects by interfering with neural transmission. These compounds bind to and activate post-synaptic nicotinic acetylcholine receptors (nAChRs), continuously exciting cholinergic neurons, resulting in muscle tremors and cell energy exhaustion. This can be followed by neural desensitization to acetylcholine (ACh), blocking neural transmission and resulting in paralysis or lethality (Morrissey et al., 2015). The relative sensitivity of these insect species is of concern, as aquatic insects play important roles in both aquatic and terrestrial ecosystems.

Rather than being present as single compounds, neonicotinoid residues are often found as mixtures in aquatic environments. In a recent survey of the Canadian Prairie Pothole Region, binary or ternary neonicotinoid mixtures were detected in 11 - 63 % of wetlands sampled, with cumulative concentrations ranging from 0.004 to 1.66 µg/L (Main, 2016). Because of the similar mechanism of action among neonicotinoids, mixtures of these insecticides are expected to display directly additive cumulative toxicity (e.g. concentration addition). Indeed, concentration-additive cumulative toxic effects have been reported for binary mixtures of IMI and thiacloprid (THIA) in some invertebrate species (Gomez-Eyles et al., 2009; Pavlaki et al., 2011). However, deviation from this assumption of directly additive cumulative toxicity has also been reported (Gomez-Eyles

et al., 2009; Loureiro et al., 2010; Pavlaki et al., 2011), with the cumulative toxicity of neonicotinoids differing based on test species, toxicological endpoint of interest, and mixture constituents. In Canada, the United States, and the European Union neonicotinoids are currently regulated as single compounds (Canadian Council of Ministers of the Environment, 2007; European Food Safety Authority, 2013a; Morrissey et al., 2015; Smit, 2014; USEPA, 2017), with regulations primarily based on toxicity data for IMI only (Canadian Council of Ministers of the Environment, 2007). This is of concern for two reasons. First, the use of a single-compound toxicity value to protect aquatic organisms does not account for the potential cumulative effects of neonicotinoid mixtures in aquatic environments. Second, TMX and CLO account for most neonicotinoid use across Canada, especially in densely agricultural regions like the Canadian Prairies (Main, 2016). Furthermore, the mixture effects of the neonicotinoid compounds most frequently detected in aquatic environments (IMI, CLO, and TMX) have yet to be formally tested in mixtures with an ecologically relevant test species. Therefore, it is essential to characterize the cumulative toxicities of these neonicotinoid mixtures to understand the impacts of mixture exposures on sensitive aquatic insects, and to determine whether using a single-compound water quality guideline value would be adequately protective of sensitive aquatic life.

In the present study, cumulative toxicities of binary and ternary mixtures of IMI, CLO, and TMX were investigated under acute exposure scenarios to gain a preliminary understanding (proof of principle) of whether neonicotinoid mixture toxicity can be adequately predicted using single-compound toxicity values. A regression-based, dose-response computational method developed by Jonker et al. (2005), MIXTOX, was used to analyze toxicological deviations of the neonicotinoid mixtures from direct additivity (i.e. synergism/antagonism, dose-level dependent deviation, dose-ratio dependent deviation) using a sensitive aquatic insect, *Chironomus dilutus*, as

a representative test species. The objectives of this work were: 1) to assess the relative acute toxicities of the individual neonicotinoid insecticides, IMI, CLO, and TMX; and 2) to characterize the joint acute toxicities of binary and ternary mixtures of IMI, CLO, and TMX, to *C. dilutus* larvae. Because of their common mechanism of action, we hypothesized that these neonicotinoid insecticides would display a cumulative toxicity that may be adequately described by the assumptions of a concentration addition mixture model.

2.2 Materials and methods

2.2.1 Test organisms and culture conditions

Chironomus dilutus were obtained from a laboratory culture maintained at the Toxicology Centre, University of Saskatchewan (Saskatoon, SK, Canada). Organisms were cultured in a controlled environmental chamber with a temperature of 23 ± 1°C, a 16:8-h light: dark photoperiod, and an illumination intensity of 500 - 1000 lux. Cultures were sustained in 20-L aquaria and maintenance was based on the protocol outlined by Environment Canada (Environment Canada, 1997). Culture water consisted of carbon-filtered, bio-filtered, Saskatoon municipal water, aerated in 50-L Nalgene® carboys prior to use. Culture tanks were fed with 15 mL of Nutrafin® (Rolf C. Hagen Inc., Montreal, QC, Canada) fish food slurry (100 g/L) three times a week. Water quality was monitored monthly, with parameters as follows [mean ± standard deviation (SD)]: dissolved oxygen (DO) 7.54 ± 0.55 mg/L; unionized ammonia (NH_3) 0.63 ± 1.34 mg/L; pH 8.13 ± 0.19; conductivity 510 ± 20 µS/cm; total hardness 174 ± 11 mg/L as $CaCO_3$; and alkalinity 127 ± 16 mg/L as $CaCO_3$.

Chironomus dilutus larvae were obtained for experimentation by isolating and breeding adults from the laboratory culture (Stoughton et al., 2008). Adult *C. dilutus* were collected into a 300-mL Erlenmeyer flask via aspiration, and then transferred into a 1-L glass breeding jar

containing 200 mL of culture water, a floating Parafilm® platform, two rectangular plastic pieces of mesh (serving as mating platforms), and a screened lid. Breeding jars were placed in enclosed cardboard containers to deter visual disturbances and left in environmental chambers until egg mass production occurred (up to 48 h). Egg masses were transferred to fresh 20-L glass aquariums containing aerated culture water and a 1-cm layer of washed silica sand (250 - 425 µm). Nutrafin® slurry (5 mL @ 100 g/L) was introduced to tanks at the time of hatch (48 - 96 h post-transfer), and subsequently every two days until time of experimentation. After 6 or 7 d, larvae were transferred to a glass tray and organisms selected for experimental use.

2.2.2 Experimental compounds

Three technical grade neonicotinoids were used as experimental compounds: IMI (98.8% pure; *N*-[1-[(6-chloropyridin-3-yl)methyl]-4,5-dihydroimidazol-2-yl]nitramide), CLO (99.6% pure; 1-[(2-chloro-1,3-thiazol-5-yl)methyl]-2-methyl-3-nitroguanidine), and TMX (98.8% pure; (*NE*)-*N*-[3-[(2-chloro-1,3-thiazol-5-yl)methyl]-5-methyl-1,3,5-oxadiazinan-4-ylidene]nitramide). IMI and CLO were acquired from Bayer Crop Science (Kansas City, MO, USA), and TMX was acquired from Syngenta Crop Protection LLC (Greensboro, NC, USA). Stock solutions were prepared by dissolving the technical product in purified, reverse-osmosis water (Barnstead® Diamond™ NANOpure, 18 megaohm/cm; Barnstead International, Dubuque, IA, USA) and stored in amber glass bottles at 4°C in the dark until experimental use. To avoid degradation and contamination, fresh stock solutions were prepared monthly, and stock solutions were chemically analyzed prior to every experiment.

2.2.3 Experimental procedures

Toxicity tests were performed in a controlled environmental chamber at the Toxicology Centre, University of Saskatchewan. Experimental conditions remained consistent with those used

to culture test animals. Acute (96 h) static toxicity tests were conducted using 300-mL glass beakers containing 50 g of washed, dried 250- to 425-µm silica sand and 200 mL of test solution. Experimental solutions were prepared by spiking 1 L of culture water with concentrated stock solutions to achieve desired test concentrations. Beakers were gently aerated to maintain adequate DO concentrations (> 6 mg/L) and covered with borosilicate glass to prevent neonicotinoid photo-degradation. Ten early-instar (approximately 6 - 7 d old) *C. dilutus* larvae were placed in each beaker and exposed to test solutions for 96 h. To feed test organisms, 60 µL of a 10 g/L Nutrafin® slurry was introduced to each beaker daily. Following the exposure period, live organisms were retrieved and counted to assess survival. Mortality of test organisms in control solutions never exceeded 10 %, thus meeting experimental validity requirements (e.g. > 85 % survival in untreated control (Benoit et al., 1997; Environment Canada, 1997; Organization for Economic Cooperation and Development (OECD), 2011)).

2.2.4 Single-compound toxicity tests

Acute toxicity (median lethal toxicity [LC_{50}]) was assessed for each neonicotinoid compound in single-compound toxicity tests. *Chironomus dilutus* larvae were exposed to anywhere from 6 to 10 concentrations of insecticide (IMI, CLO, or TMX), along with untreated controls. Each treatment was replicated four times (n = 40 organisms/treatment). Nominal concentrations of IMI (0.4 - 20.61 µg/L), CLO (0.4 - 20.61 µg/L), and TMX (0.4 - 482.9 µg/L) were based on range-finding tests and previous studies (Cavallaro et al., 2017; Stoughton et al., 2008).

2.2.5 Binary mixture tests

Mixture tests were designed based on the toxic unit (TU) concept, where a TU was defined as the actual concentration of a chemical (c) divided by its toxicity threshold (in this case the LC_{50}; Equation 2.1).

$$TU = \frac{c}{LC_{50}} \qquad \text{(Eqn. 2.1)}$$

Exposure scenarios were based on a fixed-ray experimental design. Compounds were tested at 5 TU dose-ratios (1:0, 3:1, 1:1, 1:3, 0:1), and 6 dose-levels (ΣTU = 0.25, 0.5, 1.0, 1.5, 2.0, 3.0), yielding 18 different binary mixtures and 12 single-compound exposures (Figure 2.1A-D) along with 6 untreated controls. Nominal exposure concentration ranges were as follows: IMI, 0.29 - 13.89 µg/L; CLO, 0.37 - 17.79 µg/L; and TMX, 3.46 - 166.02 µg/L. The fixed-ray design necessitated reduced replicates (2 per treatment) to allow for an increased number of exposure combinations. As the analysis of the mixture toxicity data was regression based, the statistical strength was maintained via adequate coverage of the toxicological response surface (Jonker et al., 2005).

2.2.6 Ternary mixture tests

The ternary mixture test also followed a fixed-ray experimental design based on the toxic unit concept (Figure 2.1E - F). Mixtures were tested at 10 dose ratios (1:0:0, 0:1:0, 0:0:1, 1:1:1, 2:1:1, 1:2:1, 1:1:2, 2:2:1, 2:1:2, 2:2:1), and 6 dose levels (ΣTU = 0.25, 0.5, 1.0, 1.5, 2.0, 3.0), yielding 42 different ternary mixtures and 18 single-compound exposures along with 8 untreated controls. Each ternary mixture treatment was replicated twice (2 / treatment). Nominal exposure concentration ranges were as follows: IMI 0.23 - 13.89 µg/L; CLO 0.46 - 17.79 µg/L; and TMX 3.46 - 166.02 µg/L.

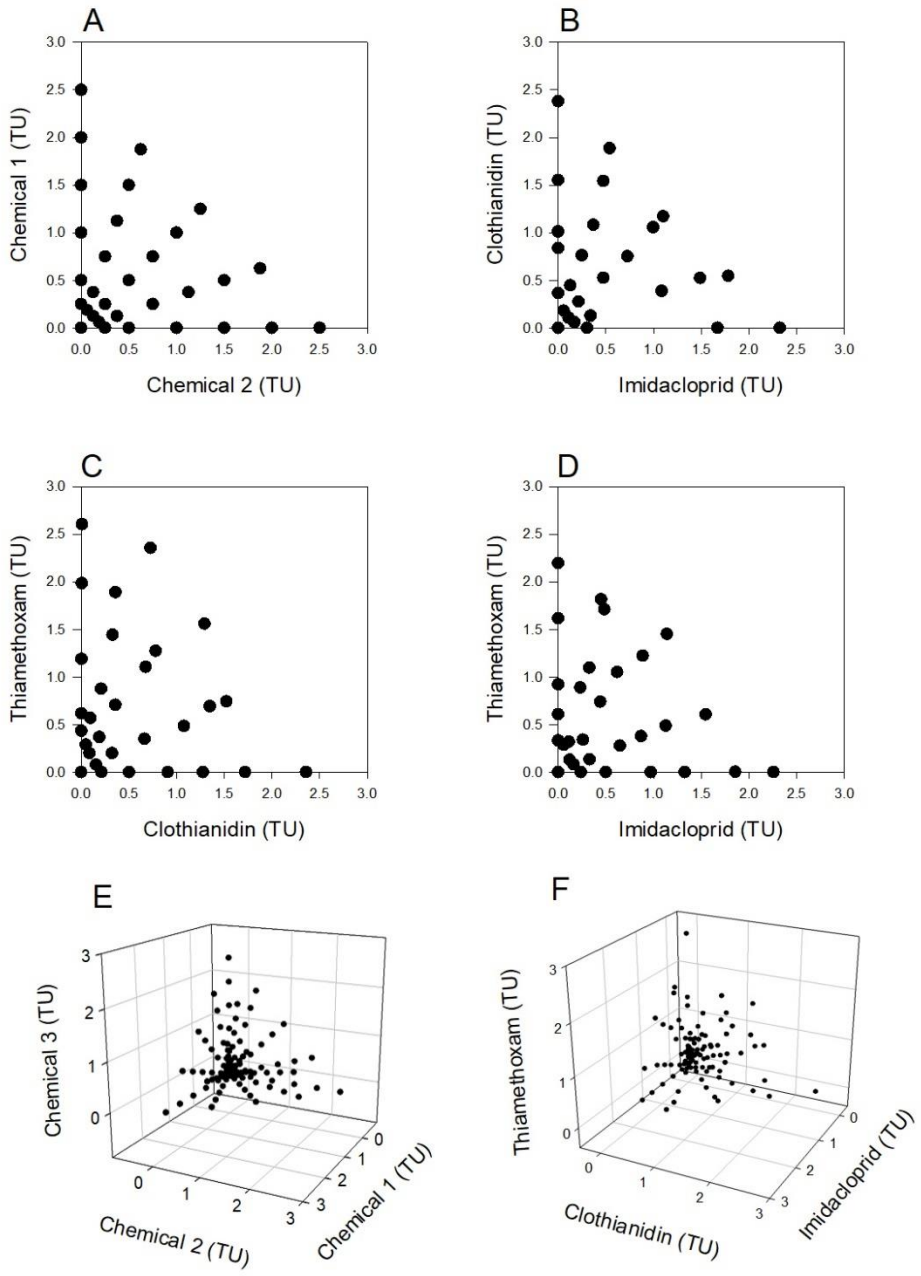

Figure 2.1 Fixed-ray experimental design applied in binary (A) and ternary (E) mixture toxicity tests, compared to actual concentrations of exposure in (B) IMI-CLO, (C) CLO-TMX, (D) IMI-TMX, and (F) IMI-CLO-TMX mixture studies.

*TU = Toxic Units.

2.2.7 Water Quality

Water quality was assessed at the beginning (d 0) and end (d 4) of each test. A 20-mL sample of water was removed from test beakers and analyzed for pH, conductivity, total hardness, and alkalinity. The pH was measured with an ORION® PerpHect LogR meter, model 370 (ORION Research, Beverly, MA, USA), conductivity with an ORION® Conductivity meter, model 170 (ORION Research, Beverly, MA, USA), and hardness and alkalinity using a Hach Digital Titrator, model 16900 (Hach Company, Loveland, CO, USA). Temperature, DO, and ammonia (NH_3) concentrations in test beakers were evaluated every 2 days to ensure adequate experimental conditions were maintained. The DO and temperature were measured with a Thermo ORION® dissolved oxygen meter, model 835 (Thermo Orion, Beverly, MA, USA), and ammonia was measured with a VWR™ SB301 sympHony ISE ammonia meter (VWR International, Ltd. West Chester, PA, USA) paired with a Thermo ORION® 95-12 ammonia electrode (Thermo Orion, Beverly, MA, USA).

2.2.8 Chemical analysis

Test solutions were sampled at the start (d 0) and end (d 4) of each test and analyzed to determine actual concentrations of neonicotinoid exposure. For each treatment, 50 mL of test solution was collected from each replicate beaker, pooled, and stored in a 250-mL amber glass bottle at 4°C until time of analysis. In the single-compound tests, both new (d 0) and old (d 4) water samples were analyzed for each treatment to ensure minimal degradation and constant exposure concentrations. Because of the complexity of the experimental designs for the mixture tests, only a subset of samples (TU = 1.0 at each dose ratio) were analyzed at both d 0 and d 4. The remaining samples were pooled across sample times (d 0 + d 4) prior to analysis.

Samples were analyzed at the National Hydrology Research Centre, Environment and Climate Change Canada, Saskatoon, SK, Canada using methods described in Main et al. (2014). Briefly, analytical standards of IMI, CLO, and TMX were obtained from Chem Service, West Chester, PA, USA. The internal standards (d_4-IMI and d3-TMX) were obtained from CDN Isotopes, Pointe-Claire, QC, Canada. Neonicotinoid concentrations were quantified via solid-phase extraction (SPE) followed by high performance liquid chromatography paired with tandem mass spectrometry (LC-MS/MS). SPE was performed by loading samples onto OASIS® HLB cartridges (Waters, Mississauga, ON, Canada), rinsing with deionized water to remove any salts, and eluting the retained solutes with methanol. The eluted samples were then dried via evaporation, reconstituted in deionized water, and spiked with the internal standards. The LC-MS/MS was performed using a Waters 2695 Alliance HPLC system (Waters Corp., Milford, MA, USA) equipped with a Waters XTerra MS-C8 column (3.5-µm dia. particle size; 2.1- x 100-mm) (Waters Corp., Milford, MA, USA), paired with a Micromass Quattro Premier triple quadrupole mass spectrometer (Waters Corp., Milford, MA, USA) equipped with an electrospray ionization interface (positive ion mode). The mobile phase consisted of an 80/20 mix of solvent A (99.9% water, 0.1% formic acid) and solvent B (90% acetonitrile, 9.9% water, 0.1% formic acid). The injection volume was 20 µL, the flow rate 200 µL/min, and the average run-time was 10 min. Calibration curves were run, allowing for quantification of neonicotinoids to the following mean (± standard deviation [SD]) limits of quantification (LOQ): IMI 0.008 (± 0.002) µg/L, CLO 0.009 (± 0.003) µg/L, TMX 0.015 (± 0.002) µg/L. Mean recoveries from Milli-Q water spiked with neonicotinoid concentrations of 0.125 µg/L were as follows: IMI 88.8 (± 1.5) %, CLO 85.2 (± 1.6) %, TMX 88.7 (± 4.7) %. All measured neonicotinoid concentrations reported were recovery corrected prior to use in statistical analysis and MIXTOX modeling.

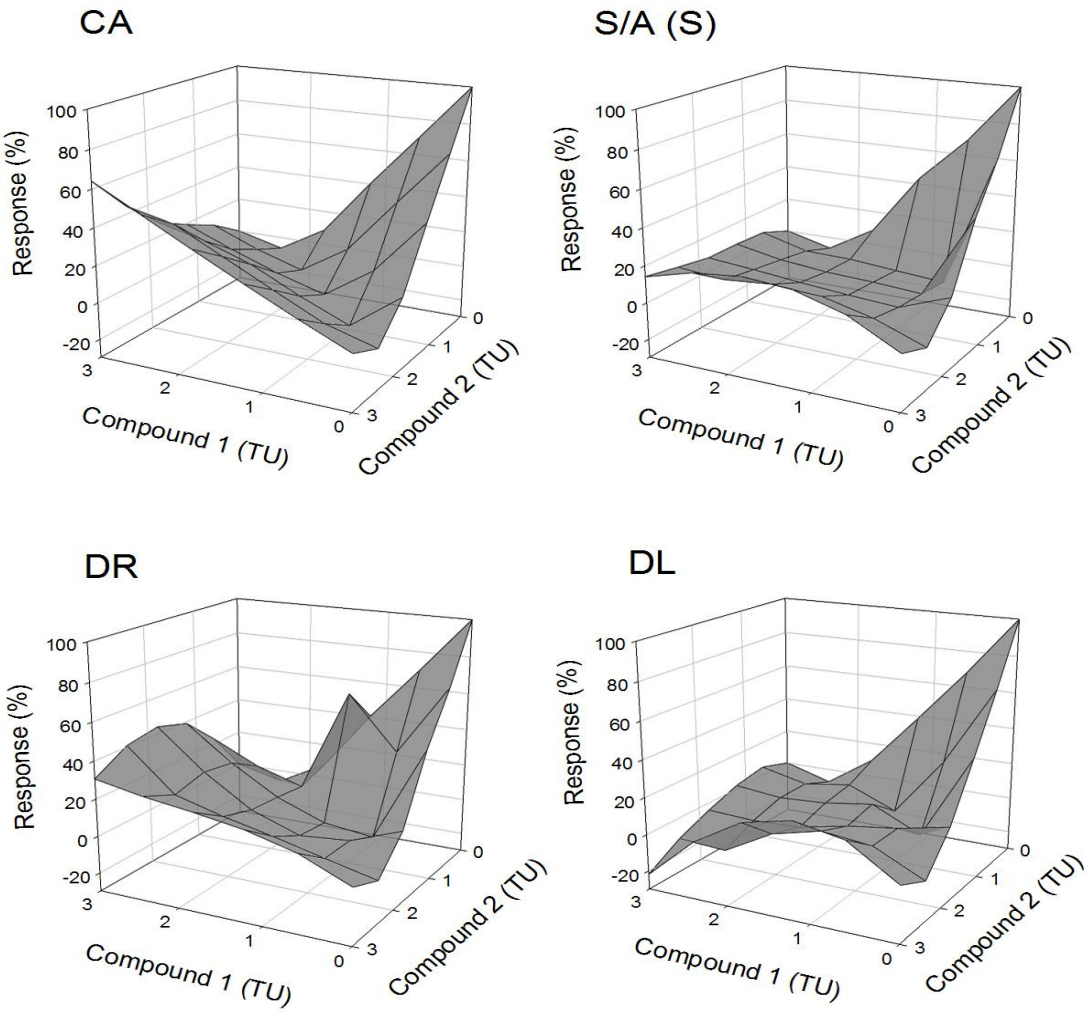

Figure 2.2 3-D Binary mixture dose-response surfaces depicting Concentration Addition (CA) and three deviation patterns from this reference model: no deviation (CA), synergistic deviation (S/A (S)), dose-ratio dependent deviation (DR), and dose-level dependent deviation (DL).

*Adapted from (Jonker et al., 2005). TU = Toxic Units.

2.2.9 Statistical analysis and MIXTOX modeling

Single-compound toxicity was evaluated by fitting survival data to a three-parameter logistic dose-response curve (Equation 2.2) using SigmaPlot statistical software, ver. 11.0 (Systat Software Inc., San Jose, CA, USA). Here the toxicological response (Y_i) is a function of maximum response (Y_{max}), concentration of exposure (c_i), LC_{50}, and the slope of the response curve (β_i):

$$Y_i = \frac{Y_{max}}{1+(C_i/LC_{50})\beta_i} \qquad \text{(Eqn. 2.2)}$$

Single compound LC_{50} values were estimated using the trimmed Spearman-Kärber method (Hamilton et al., 1977) and compared to those derived through fitting the dose-response curve (Equation 2.2) to assess the reliability of parameter estimates. For all mixture tests, a 2-fold difference from Spearman-Kärber LC_{50} estimates was set as the reliability cut-off, with > 2-fold deviation indicating that single-compound data was unreliable for use in further mixture analysis. In this case, mixture tests were repeated to ensure accuracy when evaluating cumulative effect.

Binary mixture toxicity data were analyzed using the MIXTOX approach (Jonker et al., 2005). A descriptive approach to modeling cumulative toxicity of complex mixtures, MIXTOX compares observed data with fitted parametric models of mixture effects, calculated from single-compound toxicity data, thus enabling quantification of the deviation of observed data from reference models of Concentration Addition (CA), which assumes concentration-additive cumulative toxicity, or Independent Action (IA), which assumes response-additive cumulative toxicity, (Figure 2.2; concentration addition shown under CA). Deviation from the reference models could take the form of synergism or antagonism, dose-ratio-dependent deviation, or dose-level-dependent deviation, and was assessed via a step-wise addition of extra parameters, *a* and *b*. The first parameter, *a*, describes a synergistic (greater than expected toxicological effect) or

antagonistic (lower than expected toxicological effect) deviation from CA or IA (Fig. 2.2; synergistic deviation shown under SYN). The models were then further extended with *b*. Two forms of the *b* parameter exist, b_{DR} and b_{DL}, where b_{DR} describes a dose-ratio dependent deviation from the reference model, indicating a shift between synergism and antagonism dependent on the ratio of mixture constituents (Fig. 2.2; dose-ratio dependent deviation shown under DR), and b_{DL} describes a dose-level dependent deviation from the reference model, indicating a shift between synergism and antagonism dependent on the cumulative magnitude of toxic units (Fig. 2.2; dose-level dependent deviation shown under DL). Interpretation of numerical values derived in the MIXTOX analysis can be found in the Appendix (Table A2.1).

Ternary mixture toxicity was analyzed using an extension of the MIXTOX approach, called the Ternary-Plus model (Cedergreen et al., 2012). In this analysis, data from all three binary mixture studies were analyzed alongside the empirical ternary mixture data, to account for the toxicological effects of binary mixtures when predicting ternary-mixture response. A ternary deviation parameter, $a_{1,2,3}$, was introduced, describing the deviation of the measured ternary response surface from the response surface predicted by a combination of binary deviation functions. This parameter describes a synergistic or antagonistic deviation from IA or CA. As the Ternary-Plus model is still in development, this equation could not be further extended to model dose-level and dose-ratio dependent deviations from the synergism/antagonism model. An interpretation of the numerical values derived in the Ternary-Plus model can be found in the Appendix (Table A2.1).

Mean measured (not nominal) neonicotinoid concentrations were used in the MIXTOX analysis to more accurately characterize the cumulative effects of these neonicotinoid mixtures. Adequate coverage of the toxicological response surface was evaluated through scatterplots of

measured concentrations tested in mixture toxicity tests (Figure 2.1). Experimental data were fit to parametric models using maximum likelihood estimation. First, measured data were fit to reference models using the following baseline input values: maximal response (Y_{max}) = 0.98; and slope (β_i) and median effect concentration (LC_{50}) (Equation 2.2) derived for each mixture constituent. Due to their similar mechanisms of action at the nicotinic acetylcholine receptor, mixtures of neonicotinoids were hypothesized to have a cumulative effect that is best described by the CA reference model. However, to comprehensively assess the cumulative toxicity of the neonicotinoid mixtures evaluated in the present study, both reference models (CA and IA) were initially fit to mixture datasets. Models were then further extended with parameters indicating deviation from direct additivity (i.e. synergism/antagonism, dose-level deviation, and dose-ratio deviation). As the sequential addition of parameters resulted in the formation of a series of nested models, the fit of parametric models could be directly assessed through pairwise model comparison and significance testing. Following extension of reference models with additional parameters, improved fit was confirmed by a reduction in the residual deviance (RD) and the statistical significance of this improvement determined via Chi-squared tests (χ^2) with degrees of freedom equal to the difference in number of parameters in the two models. For each mixture, model of best fit was defined as that which most significantly reduced RD compared to the reference model of interest. For each model of best fit, percent deviation (e.g. % increase or decrease from survival) was evaluated by comparing reference model estimates (e.g. estimated survival in CA and/or IA models) to actual survival data at each tested mixture concentration. Further information regarding the derivation and statistical interpretation of parametric MIXTOX models can be found in Jonker et al. (2005).

2.3 Results

2.3.1 Water quality and chemical analysis

Due to their consistency, routine water quality variables were averaged across all single-compound and mixture toxicity tests. Mean values (± SD) were as follows: DO = 7.8 (± 0.4) mg/L; temperature = 23.0 (± 1.3) °C; pH = 8.03 (± 0.13); conductivity = 328 (± 30) µS/cm; total hardness = 108 (± 13) mg/L as $CaCO_3$; and alkalinity = 109 (± 15) mg/L $CaCO_3$. Unionized ammonia concentrations increased over the duration of each test, but remained well below the ammonia 96-h LC_{50} for *C. dilutus* (82 mg N/L) (Schubauer-Berigan, M.K., Monson, P.D., Ankley, 1995), with a mean value (± SD) of 0.66 (± 0.64) mg N/L. Neonicotinoid concentrations did not change significantly throughout the duration of the test (analysis of new and old water) with concentrations (mean ± SD) of IMI, CLO, and TMX on d 4 remaining within 96.7 ± 12.0 %, 100.0 ± 7.8 %, and 102.4 ± 26.1 % of original (d 0) concentrations, respectively. Measured neonicotinoid concentrations were close to nominal concentrations, with measured IMI, CLO, and TMX concentrations (mean ± SD) being within 101.3 ± 14.0 %, 98.6 ± 17.3 %, and 101.7 ± 38.8 % of nominal concentrations, respectively. Neonicotinoid concentrations in control treatments remained lower than the limits of quantification. Measured neonicotinoid concentrations are presented in Tables A2.2 and A2.3.

2.3.2 Single-compound toxicity

Dose-response curves generate from the single compound toxicity tests (Spearman-Kärber method) are shown in Figure 2.3. *Chironomus dilutus* demonstrated the greatest sensitivity to IMI, with a 96-h LC_{50} of 4.63 (3.96 - 5.41) µg/L. CLO displayed similar toxicity, with a 96-h LC_{50} of 5.93 (5.29 - 6.63) µg/L. TMX was the least toxic compound to *C. dilutus*, with a 96-h LC_{50} approximately 10 times lower, at 55.34 (43.98 - 69.64) µg/L.

Single-compound 96-h LC_{50} values were also determined using an alternate method (3-parameter log-logistic curve; Equation 2.2) and for individual mixture constituents in the mixture toxicity tests (MIXTOX fit) (Table 2.1). To evaluate accuracy and replicability, the LC_{50} values for IMI, CLO, and TMX were compared between methods and across single-compound and mixture toxicity tests. For all three compounds, single-compound toxicity remained relatively consistent, with LC_{50} values ranging from 3.55 to 7.19 µg/L for IMI (vs. 4.63 µg/L), 3.70 to 5.91 µg/L for CLO (vs. 5.93 µg/L), and 29.76 to 41.37 µg/L for TMX (vs. 55.34 µg/L). Slight variation in calculated effect levels was likely due to statistical differences between individual methods (e.g. trimmed Spearman-Kärber method (Hamilton et al., 1977) vs. 3-parameter logistic dose response curve fitting (Equation 2.2) vs. MIXTOX fitting (Jonker et al., 2005)) and differences in chosen concentration ranges (in single compound vs. binary mixture tests).

2.3.3 Mixture toxicity

Binary mixture: imidacloprid-clothianidin. Cumulative effects of IMI-CLO mixtures were better described by the CA reference model (RD = 50.0) than the IA reference model (RD = 63.5). Extension of the CA model with the synergism/antagonism (S/A) parameter (a) significantly improved model fit (RD = 45.3, $\chi^2 = 4.74$, $p = 0.03$). Extension of the CA-S/A model with the dose-level (D-L) parameter (b_{DL}) failed to significantly improve model fit (RD = 44.9, $\chi^2 = 0.38$, $p = 0.54$). However, further extension of the CA-S/A model with the dose-ratio (D-R) parameter (b_{DR}) did reduce the RD and significantly improve model fit compared to the CA model (RD = 43.2, $\chi^2 = 6.77$, $p = 0.03$). Therefore, CA-DR was selected as the model of best fit (Figure 2.4A and Table A2.4), with parameters $a = 1.21$ and $b_{DR} = -1.57$ indicating synergism at higher concentrations of IMI (i.e. declines in survival: mean = 1 %; max = 7 %) and antagonism at higher

concentrations of CLO (i.e. increases in survival: mean = 2 %; max = 19 %). The CA-DR model explained 92.0% of the variability in the IMI-CLO mixture data (Figure 2.4B).

Binary mixture: clothianidin-thiamethoxam. Cumulative effects of CLO-TMX mixtures were better described by CA (RD = 43.0) than IA (RD = 77.1). Extension of the CA model with S/A, DL, or DR parameters failed to significantly improve model fit (S/A RD = 43.0, $\chi^2 = 0.01$, $p = 0.92$; DR RD = 43.0, $\chi^2 = 0.07$, $p = 0.96$; DL RD = 43.0, $\chi^2 = 0.01$, $p = 0.99$). Therefore, the model of best fit was CA, with the CLO-TMX mixtures demonstrating concentration additive cumulative toxicity (Figure 2.4C). The CA-S model explained 94.2% of the variability in the CLO-TMX mixture data (Figure 2.4D, Table A2.5).

Binary mixture: imidacloprid-thiamethoxam. Cumulative effects of the IMI-TMX mixtures were better described by IA (RD = 121.1) than CA (RD = 151.5). Extension of the IA model with the S/A parameter significantly improved model fit (RD = 117.0, $\chi^2 = 4.10$, $p < 0.01$). Extension of the IA-S/A model with both DR and DL parameters also further improved model fits. However, IA-DR was the model of best fit for the IMI-TMX mixture data (RD IA-DR = 100.5, $\chi^2 = 16.5$, $p < 0.01$; RD IA-DL = 111.5, $\chi^2 = 5.45$, $p = 0.02$). IA-DR parameters $a = 11.99$ and $b_{DR} = -26.41$ indicated that IMI-TMX mixtures displayed synergism at dose ratios with higher IMI concentrations (i.e. declines in survival: mean = 13 %; max = 28 %), and antagonism at dose ratios with higher TMX concentrations (i.e. declines in survival: mean = 2 %; max = 30 %). Therefore, the model of best fit was IA-DR, with IMI-TMX mixtures demonstrating dose-ratio dependent cumulative toxicity (Figure 2.4E and Table A2.6). This model was found to explain 83.4% of the variability in the IMI-TMX mixture data (Figure 2.4F).

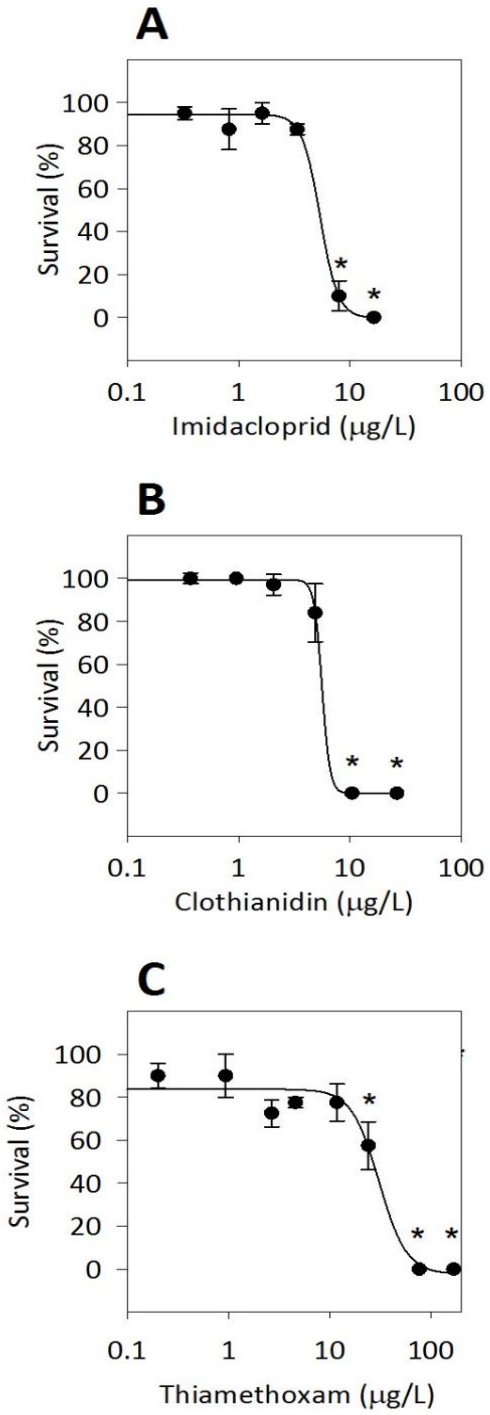

Figure 2.3 Survival (mean ± SD) of *Chironomus dilutus* larvae after exposure to (A) IMI, (B) CLO, and (C) TMX for 96 hours (n = 4 treatments, 10 organisms/treatment).

* Significantly different from the untreated control, calculated via one-way ANOVA with the Tukey post-hoc test ($p < 0.05$).

Table 2.1 Mean lethal concentrations (96-h LC_{50}; µg/L) and slopes (β) for *Chironomus dilutus* larvae after exposure to IMI, CLO, and TMX, calculated using three-parameter log-logistic and MIXTOX fitting methods.

Test Method	Mixture Treatment	Imidacloprid		Clothianidin		Thiamethoxam	
		LC_{50}	β	LC_{50}	β	LC_{50}	β
3-Parameter Log-Logistic [a]	-	5.07	4.67	5.65	14.56	29.76	3.62
MIXTOX [b]	IMI-CLO	7.19	3.62	5.58	21.6	-	-
	CLO-TMX	-	-	5.91	15.78	37.51	30.87
	IMI-TMX	5.88	5.03	-	-	36.20	17.63
	IMI-CLO-TMX	3.55	6.99	3.70	6.79	41.37	7.14

[a] Calculated by fitting a three-parameter dose-response curve, using toxicity data from single compound toxicity tests.

[b] Calculated via maximum-likelihood estimation through MIXTOX analysis, using single compound positive control data from mixture toxicity tests (Jonker et al., 2005).

Ternary mixture: imidacloprid-clothianidin-thiamethoxam. The IMI-CLO-TMX mixtures were better described by IA (RD = 583.4) than CA (RD = 722.5). Extension of this model with S/A parameters significantly improved model fit (RD = 406.3, $\chi^2 = 177.06$, $p < 0.01$), with the parameter $a_{IMI,CLO,TMX} = -50.34$ indicating a synergistic effect across all concentration levels (i.e. decline in survival: mean = 6 %; max = 48 %) and interaction parameters $a_{IMI,CLO} = 4.95$, $a_{CLO,TMX} = -6.33$, and $a_{IMI,TMX} = 0.13$ indicating that the binary mixtures had both antagonistic (IMI-CLO, IMI-TMX) and synergistic (CLO-TMX) contributions to cumulative toxicity. Therefore, the model of best fit was IA-S, with the IMI-CLO-TMX mixtures demonstrating synergistic cumulative toxicity (Table A2.7). This model was found to explain 82.1% of the variation in the IMI-CLO-TMX experimental data (Figure 2.5).

Model fit. In the present study, the strength of model fit is demonstrated in the statistical analysis (Tables A2.4-2.7). Each model presented is the MIXTOX model of best fit, and each fit is statistically significant ($p < 0.05$) compared to the reference model of interest. However, the strength of correlation between modeled and measured data varies between mixture models. Although there is visibly strong correlation between modeled and measured data for IMI-CLO (Figure 2.4B), the correlation between modeled and measured data in CLO-TMX, IMI-TMX, and IMI-CLO-TMX mixtures is visibly less strong (Figures 2.4D, 2.4F, and 2.5). This is potentially because of the complexity of interactions occurring in the insecticide mixtures. The MIXTOX method presents a finite number of mixture models (i.e., CA/IA, S/A, D-R, and D-L for binary mixtures; CA/IA, S/A for ternary mixtures). Thus, it is possible that goodness of fit of the CLO-TMX, IMI-TMX, and IMI-CLO-TMX models could be improved by fitting more complex models of mixture toxicity. However, further research is required to determine how the MIXTOX approach could be extended to increase the complexity of mixture models.

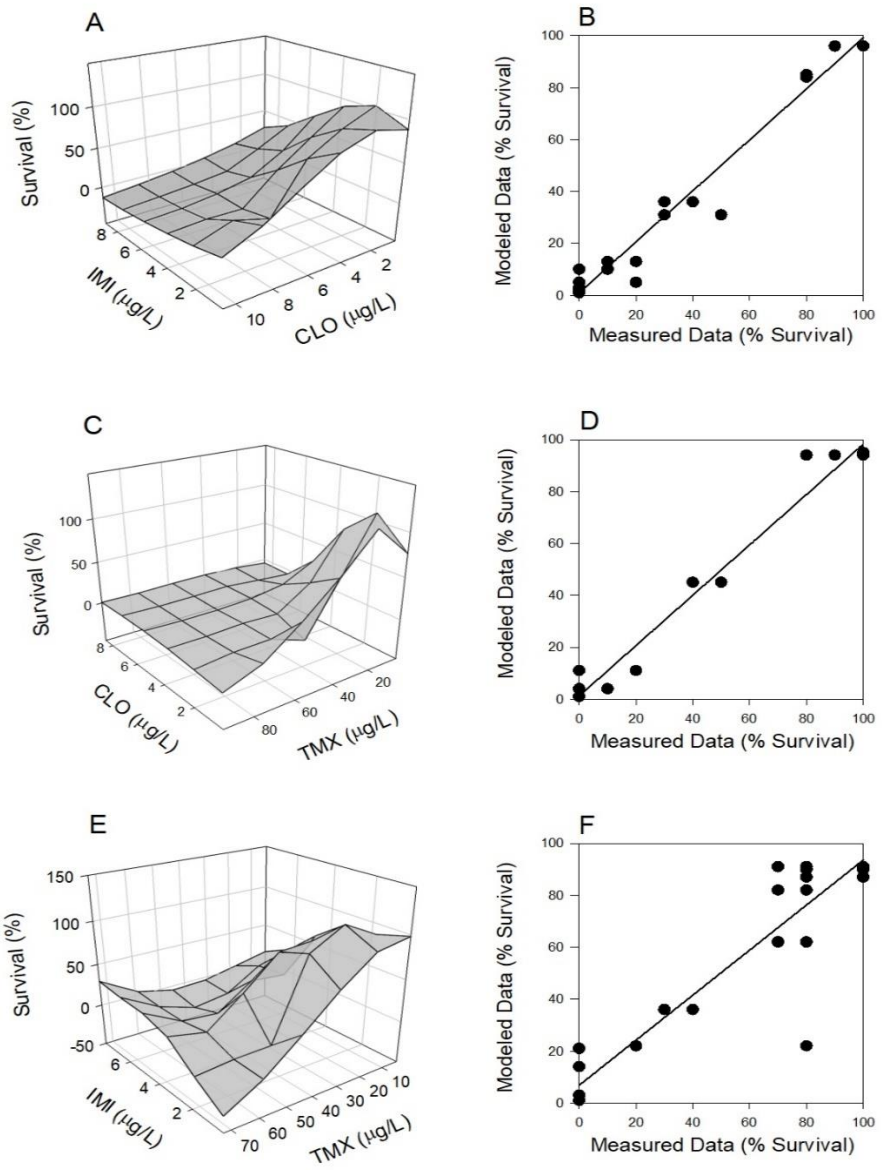

Figure 2.4 Survival (%) of *Chironomus dilutus* larvae following 96-hour exposures to (A) IMI-CLO, (C) clothianidin-thiamethoxam (CLO-TMX), and (E) imidacloprid-thiamethoxam (IMI-TMX) mixtures. Relationship between measured survival data and modeled values for the most statistically significant parsimonious deviation model for (B) IMI-CLO, (D) CLO-TMX, and (F) IMI-TMX mixtures.

*A diagonal line (one-to-one relationship) indicates idyllic model description.

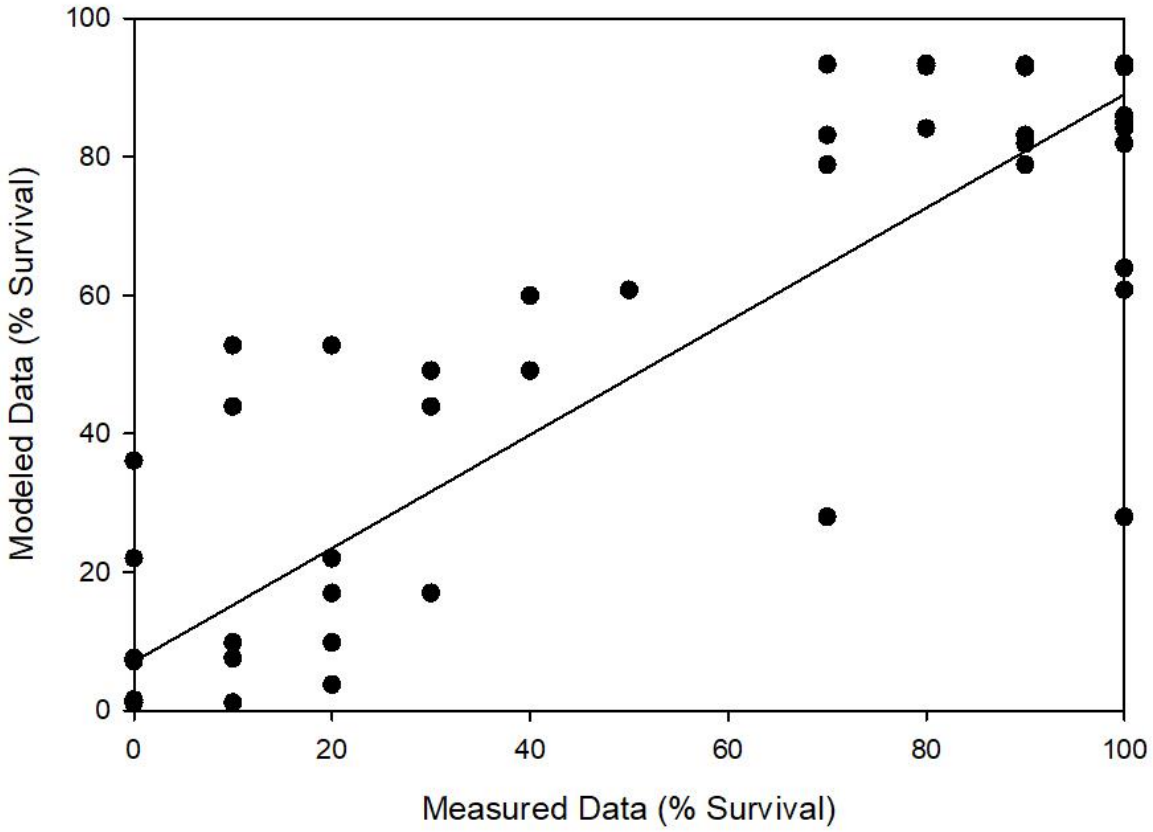

Figure 2.5 Relationship between measured data and modeled values for the most statistically significant parsimonious deviation model for the ternary mixture of IMI, CLO, and TMX.

*A diagonal line (one-to-one relationship) indicates idyllic model description.

2.4 Discussion

2.4.1 Acute toxicity of single neonicotinoid compounds

Although the toxicity of neonicotinoids has been extensively investigated for several non-target invertebrate species, few published studies have compared the toxicities of different neonicotinoid compounds to sensitive insect species under consistent experimental conditions. Furthermore, to our knowledge, this toxicity comparison among multiple neonicotinoids has yet to be reported for *C. dilutus* under acute (96-h) exposure scenarios. In the present study, *C. dilutus* demonstrated the greatest sensitivity to IMI (LC_{50} = 4.63 µg/L), followed by CLO (LC_{50} = 5.93 µg/L), and then TMX (LC_{50} = 55.34 µg/L), which was 10 times less toxic. These relative toxicities are in accordance with what has been previously reported for *C. dilutus* under longer exposure scenarios (Cavallaro et al., 2017). Under sub-chronic (14-d) exposure conditions, *C. dilutus* was found to display the highest sensitivity to IMI (LC_{50} = 1.52 µg/L), followed by CLO (LC_{50} = 2.41 µg/L), and TMX (LC_{50} = 23.6 µg/L) (Cavallaro et al., 2017). After 40 d of exposure, *C. dilutus* displayed slightly higher sensitivity to CLO (median effect concentration [EC_{50}] = 0.28 µg/L) than IMI (EC_{50} = 0.39 µg/L), followed by TMX (EC_{50} = 4.13 µg/L). Other studies with *C. dilutus* have reported similar acute toxicity values to those found in the present study: a 96-h LC_{50} for IMI of 5.75 µg/L (Stoughton et al., 2008) and a slightly lower 96-h LC50 for CLO of 2.32 µg/L (de Perre et al., 2015).

Under acute single-compound exposure scenarios, measured environmental concentrations above these would be expected to elicit lethal toxicity in *C. dilutus* and other sensitive aquatic arthropods (e.g. Ephemeroptera (Van den Brink et al., 2016)). The single-compound toxicity values (96-h LC_{50}) generated from the present study add to the growing body of literature used to evaluate current regulations and will be important for the development of

new water quality guidelines, to ensure protection of sensitive aquatic insects from short-term neonicotinoid exposures.

2.4.2 Cumulative toxicity of neonicotinoid mixtures

Importantly, this study extends beyond the examination of single-compound neonicotinoid toxicity, focusing on characterizing the cumulative toxicity of neonicotinoid mixtures. In the present study, acute exposures of *C. dilutus* to some neonicotinoid mixtures (e.g. IMI-CLO, IMI-TMX, and IMI-CLO-TMX) resulted in cumulative toxicity that deviated from that predicted based on the current mechanistic understanding of the toxicities (concentration addition) of neonicotinoid insecticides to insects. Indeed, whereas CLO-TMX mixtures displayed concentration additive toxicity, IMI-CLO mixtures displayed concentration additive dose-ratio dependent deviation, IMI-TMX mixtures displayed response-additive dose-ratio dependent synergistic deviation, and ternary mixtures (IMI-CLO-TMX) displayed response-additive synergistic deviation.

To our knowledge, no other published studies to date have investigated the cumulative toxicity of neonicotinoid mixtures to any aquatic insect species. However, the deviation from direct additivity observed in the present study is consistent with what has been described for some invertebrate species in other neonicotinoid insecticide mixture studies. In *Caenorhabditis elegans*, a terrestrial roundworm, mixtures of IMI and thiacloprid (THIA), were found to have a dose-level-dependent synergistic effect on reproduction (Gomez-Eyles et al., 2009). In the crustacean, *Daphnia magna*, mixtures of IMI and THIA had a synergistic effect on reproduction (Pavlaki et al., 2011), a synergistic effect on lethality (Loureiro et al., 2010), and an antagonistic effect on feeding inhibition (Loureiro et al., 2010). In addition, Bayer Crop Science has patented synergistic activity of binary mixtures of IMI, THIA, and CLO for control of several invertebrate

pest species (Andersch, W., Jeschke, P., Thielert, 2010). Although the cumulative toxicities of these neonicotinoid insecticide mixtures vary when comparing across compounds, endpoints, and species, the present study, along with other neonicotinoid mixture studies, confirms a general trend of deviation from the hypothesized default CA model under short-term exposure settings.

2.4.3 Mechanistic response to neonicotinoid mixtures

One potential explanation for this observed deviation from the CA model is the action of the mixture constituents at nAChRs. nAChRs are pentameric receptors, with a wide variety of subunits that can be arranged into distinct nAChR subtypes (Simon-Delso et al., 2015). At least two functionally distinct subtypes of nAChRs exist: α-bungarotoxin (α-BGT)-sensitive (neonicotinoid-insensitive) nAChRs, and α-BGT-insensitive (neonicotinoid-sensitive) nAChRs (Simon-Delso et al., 2015). These subtypes have been further categorized based on functional responses to specific neonicotinoid agonists. Within the α-BGT-insensitive nAChR group, subpopulations are defined by their sensitivity to IMI: nAChR1 (IMI-sensitive) and nAChR2 (IMI-insensitive) (Simon-Delso et al., 2015). Within the α-BGT-sensitive nAChR group, subpopulations are categorized based on their ability to bind to and activate these receptor subpopulations. CLO strongly activates both nAChR1 and nAChR2 subtypes and strongly desensitizes cockroach (*Periplaneta americana*) neurons following excitatory action (Salgado and Saar, 2004; Thany, 2009). IMI exclusively activates nAChR1 and desensitizes *P. americana* neurons with the strength of neuronal desensitization dependent on length of exposure (Oliveira et al., 2011; Thany, 2011). TMX weakly interacts with nAChR receptors, with reversible (non-desensitizing) neuronal depolarization effects in *P. americana* (Thany, 2011). Therefore, it is possible that the presence of multiple nAChR subtypes in larval *C. dilutus* could be influencing the observed cumulative toxicological effects of these neonicotinoid mixtures. However, to the

best of our knowledge, the molecular composition and functional characterization of nAChR subtypes in *C. dilutus* have not been investigated. Thus, further research is required to make firmer conclusions regarding the molecular action of neonicotinoid mixtures at the receptor level in aquatic insects such as Chironomidae.

2.4.4 Implications for hazard assessment of neonicotinoid mixtures

The most common approach to hazard assessment of chemical mixtures in the environment involves an assumption of additive joint activity. When mixture constituents have similar sites and modes of action, the chemicals are thought to act as dilutions of each other, and the CA reference model is assumed to most accurately describe cumulative toxicity (Altenburger et al., 2003). All neonicotinoid compounds act on the same general neuronal receptor, with the same general mechanisms of action; therefore, CA was hypothesized to be the model of best fit for binary and ternary mixtures of IMI, CLO, and TMX. However, in the present study, IMI-TMX, and IMI-CLO-TMX mixtures displayed a cumulative toxicity that was better described by the IA reference model. In addition, most neonicotinoid mixtures tested displayed cumulative toxicities that deviated from direct additivity. This deviation from both CA and direct additivity was unexpected. Traditionally, the IA model is assumed to best describe the cumulative toxicity of mixtures of compounds that are strictly dissimilar in their sites and mechanisms of action (Altenburger et al., 2003). In the IA model, cumulative toxicity is determined by summing toxicological responses of test organisms for each mixture constituent. Therefore, mixture effects must be predicted based on toxicological data rather than chemical concentration alone. In the present study, binary and ternary mixtures demonstrated similar deviation patterns when fit with both CA and IA reference models (Tables A2.4-A2.7). However, CA reference models could under-predict the magnitude of deviation from direct additivity. With IMI-CLO mixtures, for

example, the CA model predicted dose-ratio dependent deviation (a = 1.21, b_{DR} = -1.57), whereas the IA model predicted synergism (a = - 6.39; Table A2.4). Similarly, for IMI-CLO-TMX mixtures, the CA model predicted an antagonistic effect (a = 9.26), whereas the IA model predicted a synergistic effect (a = - 49.58) (Table A2.7).

2.4.5 Applications in field settings and risk assessment

As MIXTOX is a descriptive, statistically based data analysis procedure, it cannot be used to directly identify the combination of mechanisms that lead to the cumulative toxicological effects observed in neonicotinoid mixtures (Jonker et al., 2005). Indeed, this analysis is typically carried out with an a priori assumption of mechanism of action of mixture constituents (i.e. the reference model is selected prior to analysis). However, in the present study, we found that cumulative toxicity of most neonicotinoid mixtures investigated deviated from the predicted reference model (based on what is currently assumed about neonicotinoid mode of action). In the literature, there is no consensus on how similar the molecular sites or modes of action of mixture constituents must be to adequately employ either reference model (Altenburger et al., 2003). Consequently, it is difficult to determine what model of mixture toxicity is best to apply in a risk assessment for acute exposures of neonicotinoid insecticide mixtures to aquatic insect species such as *C. dilutus*. We propose the application of a prediction window, incorporating both reference models into a probabilistic prediction of cumulative effects (Altenburger et al., 2003). For risk assessment practices, this information should be further incorporated into a broad dataset of neonicotinoid mixture studies, including a range of neonicotinoid compounds, sensitive aquatic organisms, and exposure scenarios. This will aid in the further development of probabilistic mixture models, allowing for more accurate predictions of the ecotoxicological effects of neonicotinoid mixtures on aquatic insect communities.

Both the prevalence of the neonicotinoid mixtures in aquatic environments (Main et al., 2014) and the antagonistic, synergistic, and additive behaviour of some neonicotinoid mixtures observed in the present study indicate that neonicotinoids should not be regulated as single compounds. The use of neonicotinoid toxic equivalency factors (Cavallaro et al., 2017) represents a reasonable approach for assessing cumulative toxicity; however this may still underestimate risk. Furthermore, although exposure to acutely toxic concentrations of neonicotinoid mixtures could occur, non-target aquatic organisms are more likely to be chronically exposed to low concentrations of neonicotinoids in mixtures (Main, 2016; Morrissey et al., 2015). Therefore, caution should be taken when extrapolating the mixture responses observed in the present study to chronic exposure scenarios. However, until the effects of neonicotinoid mixtures on aquatic insects and arthropod communities under longer term, chronic exposure scenarios are further investigated, the cumulative synergism observed in the present study under acute exposure scenarios should be considered when setting water quality guidelines.

CHAPTER 3: CAN CHRONIC EXPOSURE TO IMIDACLOPRID, CLOTHIANIDIN, AND THIAMETHOXAM MIXTURES EXERT GREATER THAN ADDITIVE TOXICITY IN *CHIRONOMUS DILUTUS?*

Preface

Building on acute studies (Chapter 2), this chapter focuses on characterizing the chronic cumulative toxicities of binary and ternary mixtures of three neonicotinoid insecticides (imidacloprid, clothianidin, and thiamethoxam) using *Chironomus dilutus* as a representative aquatic insect species. Chronic cumulative toxicities were evaluated through a series of 28-d toxicity tests (endpoint: successful emergence) with single compounds, binary mixtures, and ternary mixtures. Using the MIXTOX approach, predictive parametric models were fitted using single-compound toxicity data and statistically compared to observed toxicity in mixture tests. Furthermore, sex ratios of emerged insects were compared between neonicotinoid-exposed (single compound and mixture) and unexposed (controls) organisms to evaluate if exposure to neonicotinoids or their mixtures could cause sex ratio shifts within *C. dilutus* populations. Results from this chapter demonstrate that under chronic exposure scenarios neonicotinoid mixture toxicity can deviate from direct-additivity (concentration addition), eliciting both synergism and antagonism in *C. dilutus* (depending on mixture composition). However, as observed under acute exposure scenarios, in all neonicotinoid mixtures, synergistic and antagonistic potential was relatively limited in magnitude (< 13 % deviation from direct additivity). This research indicates that although cumulative toxicities of neonicotinoid mixtures should be accounted for in current environmental regulations, use of a concentration addition-based approach (accounting for synergistic potential using ~ 10% safety factor) should adequately account for mixture effects. Furthermore, this research highlights the need to investigate the molecular actions of neonicotinoids in non-target insects like *C. dilutus*, to better understand the mechanisms behind

neonicotinoid mixture toxicity and thus improve our ability to assess the risks that these compounds and their mixtures pose to aquatic environments.

This chapter was published in Ecotoxicology and Environmental Safety, under joint authorship with Christy A. Morrissey (University of Saskatchewan), John V. Headley (National Hydrology Research Centre, Environment and Climate Change Canada), Kerry M. Peru (National Hydrology Research Centre, Environment and Climate Change Canada), and Karsten Liber (University of Saskatchewan). Following publication, a calculation error was found in the mixture toxicity models. This was corrected, mixture toxicity estimates were recalculated and the chapter/manuscript was updated accordingly. The corrected work is presented here and a corrigendum has been published in Ecotoxicology and Environmental Safety.

Maloney, E.M., Morrissey, C.A., Headley, J.V., Peru, K.M., and Liber, K. 2018. Can chronic exposure to imidacloprid, clothianidin, and thiamethoxam mixtures exert greater than additive toxicity in *Chironomus dilutus?* Ecotoxicol. Environ. Safe. 156, 354-365.

Corrigendum to "Can chronic exposure to imidacloprid, clothianidin, and thiamethoxam mixtures exert greater than additive toxicity in Chironomus dilutus?" [Ecotoxicol. Environ. Safe. 156C (2018) 354-365].

3.1 Introduction

Environmental monitoring has provided clear evidence that aquatic organisms are often exposed to mixtures of pesticides rather than individual compounds. For example, recent environmental surveys of surface waters in North America (Hladik and Kolpin, 2015; Main et al., 2014; Schreiner et al., 2016), the European Union (Schreiner et al., 2016), Australia (Allinson et al., 2015), and Asia (Zhang et al., 2011) have reported frequent detection of pesticide mixtures, often at cumulative concentrations likely to pose a risk to sensitive aquatic species. Given the numerous potential combinations of pesticides that can be found in aquatic environments, it is not feasible to characterize the toxicity of every potential mixture. Therefore, most ecotoxicological studies, regulatory risk assessments, and water quality guidelines focus on the toxicological effects of single compounds (Barata et al., 2006), and cumulative toxicity is typically estimated directly using additive predictive models based on either concentration or organism response (i.e. Concentration Addition (CA) or Independent Action (IA)) depending on the mechanisms of action of mixture constituents. However, in some scenarios this can underestimate risk, as it does not account for cumulative toxicities that deviate from direct additivity (e.g. synergism). This is particularly relevant for agricultural pesticides in surface waters as they tend to occur in mixtures where a few compounds dominate the overall toxicity and thus are likely to exhibit more severe mixture interactions (i.e. elicit synergistic cumulative toxicity) (Cedergreen, 2014).

Neonicotinoids are a group of insecticides that can exist as simple mixtures in aquatic environments. Commonly applied as seed treatments, foliar sprays and soil drenches, neonicotinoids have been frequently detected as mixtures across Canada and the United States, with cumulative neonicotinoid concentrations ranging from 0.0011 to 1.66 µg/L in wetlands (Main et al., 2014; Smalling et al., 2015), 0.28 to 1.05 µg/L in groundwater (Giroux and Sarrasin, 2011),

0.0054 to 0.38 µg/L in rivers/streams (Hladik and Kolpin, 2015), and 0.039 to 44.38 µg/L in other surface waters (Schaafsma et al., 2015). As neonicotinoids are highly water soluble, large proportions of active ingredient (up to 90%) can move directly into soil and soil water following application (Goulson, 2013). From there, neonicotinoid residues can be transported via leaching, drainage, runoff, or snowmelt into nearby aquatic systems where they can exhibit extended persistence (Main et al., 2014). Multi-season carryover, application of different compounds within the same watershed, and field rotation with different neonicotinoid treated crops has led to the widespread presence of neonicotinoid mixtures in surface waters near areas of intensive agricultural use (Hladik and Kolpin, 2015; Morrissey et al., 2015).

Neonicotinoids are neuroactive insecticides, eliciting toxicity in invertebrates by interfering with neural transmission at post-synaptic nicotinic acetylcholine receptors (nAChR). As all neonicotinoids target nAChRs, the cumulative toxicities of neonicotinoid mixtures are typically estimated through a predictive model based on direct addition of constituent concentrations (i.e. Concentration Addition). However, recent evidence has indicated that neonicotinoid mixtures can exert greater than expected cumulative toxicity (i.e. synergism) in exposed aquatic macro invertebrates (Loureiro et al., 2010; Maloney et al., 2017; Pavlaki et al., 2011). Furthermore, prior studies have demonstrated that neonicotinoids display the potential to chronically or repeatedly contaminate aquatic systems (Hladik et al., 2018a; Main et al., 2014). Therefore, non-target organisms inhabiting these environments have the potential to be chronically exposed to sub-lethal neonicotinoid concentrations throughout their aquatic life stages. This is a concern as, to date, the effects of the neonicotinoid compounds most frequently detected as mixtures in aquatic environments (imidacloprid (IMI), clothianidin (CLO), and thiamethoxam (TMX)) have yet to be formally tested as mixtures under chronic (sub-lethal) exposure conditions.

Therefore, it is currently unknown if the synergism observed under acute exposure conditions holds true under more environmentally realistic conditions, and thus whether current regulatory practices are adequately protective of sensitive aquatic invertebrates.

In this study, we characterized the cumulative toxicities of binary and ternary combinations of three commonly applied neonicotinoids (IMI, CLO, and TMX) under chronic exposure scenarios using a sensitive aquatic insect, *Chironomus dilutus*, as a representative macroinvertebrate test species. Using MIXTOX, a regression-based, dose-response mixture analysis modeling framework (Jonker et al., 2005), we evaluated deviations of neonicotinoid mixture toxicity from direct additivity (i.e., synergism/antagonism, dose-level dependent deviation, dose-ratio dependent deviation). Study objectives included: (1) assessment of the relative single-compound toxicities of IMI, CLO, and TMX under chronic exposure conditions, and (2) characterization of the cumulative chronic toxicities of binary and ternary mixtures of IMI, CLO, and TMX to *C. dilutus*. Current risk assessment and regulatory practices estimate the cumulative toxicity of neonicotinoid mixtures using concentration-additive predictive models. Therefore, we evaluated whether Concentration Addition could adequately characterize neonicotinoid mixture toxicity under chronic exposure scenarios, or whether, as demonstrated under acute exposure scenarios, some of these binary and ternary mixtures could display greater- or less-than-additive cumulative toxicity to *C. dilutus* (Maloney et al., 2017).

3.2 Materials and methods

3.2.1 Experimental organisms and culturing techniques

Chironomus dilutus larvae were obtained from a laboratory culture maintained in a controlled environmental chamber at the Toxicology Centre, University of Saskatchewan, Saskatoon, SK, Canada. Culture maintenance was based on the protocol outlined by Environment

Canada (Environment Canada, 1997) with cultures sustained in 20-L aquaria under a constant temperature (23 ± 1°C), photo-period (16 h light: 8 h dark), and illumination intensity (500 - 1000 lux). Culture water consisted of carbon and bio-filtered Saskatoon municipal water, aerated in a 50-L Nalgene® carboy for > 24 h prior to use. Culture tanks were fed 15 mL of Nutrafin® (Rolf C. Hagen Inc., Montreal, QC, Canada) fish food slurry (100 g/L) three times a week, and culture water was changed weekly. Routine water chemistry was completed, with the following parameters describing the culture water [mean ± standard deviation (SD)]: dissolved oxygen (DO) 7.5 ± 0.6 mg/L; unionized ammonia (NH_3) 0.63 ± 1.34 mg/L; pH 8.1 ± 0.2; conductivity 510 ± 20 µS/cm; total hardness 174 ± 11 mg/L as $CaCO_3$; and alkalinity 127 ± 16 mg/L as $CaCO_3$.

Prior to experimental use, *C. dilutus* larvae were obtained by isolating and breeding adults from the laboratory culture (Stoughton et al., 2008). Briefly, emerged adults were aspirated from culture tanks into a 300-mL Erlenmeyer flask, then transferred into 1-L breeding jars (200 mL of culture water, a small Parafilm® platform, two rectangular pieces of mesh, and a screened lid). Following adult transfer, breeding jars were placed inside cardboard containers, to deter visual disturbances, until egg masses were produced (≤ 2 d). New egg masses (≤ 24 h old) were transferred to new 20-L aquaria containing aerated culture water and 1 cm of washed silica sand (250 - 425 µm). Nutrafin® slurry (5 mL @ 100 g/L) was introduced to the tanks every 2 days, from time of hatch (2 - 3 d post-transfer) until time of experimentation. After 6 or 7 days, early-instar larvae were removed from aquaria and randomly selected for toxicity testing.

3.2.2 Neonicotinoid compounds

Technical grade IMI (98.8% pure; *N*-[1-[(6-chloropyridin-3-yl)methyl]-4,5-dihydroimidazol-2-yl]nitramide), CLO (99.6% pure; 1-[(2-chloro-1,3-thiazol-5-yl)methyl]-2-methyl-3-nitroguanidine), and TMX (98.8% pure; (*NE*)-*N*-[3-[(2-chloro-1,3-thiazol-5-yl)methyl]-

5-methyl-1,3,5-oxadiazinan-4-ylidene]nitramide) were used as experimental compounds in all toxicity tests. IMI and CLO were acquired from Bayer Crop Science (Kansas City, MO, USA) and TMX from Syngenta Crop Protection LLC (Greensboro, NC, USA). Stock solutions were prepared by dissolving the technical product in reverse-osmosis water (Barnstead® Diamond™ NANOpure, 18 megaohm/cm, Barnstead International, Dubuque, IA, USA) and stored in amber glass bottles until experimental use. To avoid degradation and contamination, fresh stock solutions were prepared for each toxicity test. To ensure accuracy in the preparation of test solutions, chemical analysis of stock solutions was performed prior to every experiment (Section 3.2.5).

3.2.3 Toxicity tests

Chronic (28-d) static-renewal toxicity tests were conducted in a controlled environment chamber at the Toxicology Centre, University of Saskatchewan, Saskatoon SK, Canada. Ambient test conditions remained consistent with those used to culture test organisms. Toxicity tests were performed in 300-mL glass beakers containing 50 g of washed, dried silica sand (250 - 425 µm) and 200-mL of test solution. Test solutions were prepared by spiking 1 - 2 L of culture water with concentrated stock solutions to achieve desired test concentrations. Test beakers were gently aerated to maintain adequate concentrations of DO (> 6 mg/L) and covered with borosilicate glass plates to prevent photo-degradation of test compounds.

Ten early-instar *C. dilutus* larvae (approximately 6 - 7 d old) were placed in each beaker and exposed to test solutions for 28 days. Nutrafin® slurry (60 µL/beaker @ 10 g/L) was introduced to each beaker daily to feed the test organisms. The water in all beakers was renewed every 2 - 3 days with freshly prepared test solutions (70 % renewal/beaker) to avoid compound degradation and minimize total ammonia concentrations. Emerged adult *C. dilutus* and moribund pupae were removed and counted daily. Emergence was deemed successful if the adult completely dissociated

from its pupal exuvia and exited the water (Benoit et al., 1997). Surviving adults were evaluated for sex. Male Chironomidae were identified and separated from females via their more slender abdomen, genital appendages, and plumose antennae (Merritt et al., 2008). Pupae were considered moribund if they exhibited paralytic symptoms and failed to respond to manual stimulation. Following the exposure period, all replicates were removed, and the surviving organisms were counted, sexed and assessed for metamorphic stage. Successful emergence was used as the primary toxicological endpoint of interest for both single compound and mixture studies (i.e. MIXTOX analysis). However, sex ratios of successfully emerged adults were also evaluated to determine if neonicotinoid mixtures could alter ecologically relevant endpoints related to reproduction or population dynamics (Cavallaro et al., 2017).

3.2.3.1 Single compound studies

Chronic toxicity (28 d EC_{xx}) was assessed for each neonicotinoid compound in single compound toxicity tests, using lack of successful emergence as a toxicological endpoint. In each test, *C. dilutus* larvae were exposed to six neonicotinoid concentrations (IMI, CLO, or TMX), along with an untreated control. Each treatment was replicated four times (n = 4, 10 organisms/replicate). Nominal concentrations of IMI (0.15 - 5.00 µg/L), CLO (0.4 - 20.16 µg/L), and TMX (1.25 - 40.00 µg/L) were chosen based on previous chronic studies with this species (Cavallaro et al., 2017; Stoughton et al., 2008).

3.2.3.2 Binary mixture studies

Mixture studies used a fixed-ray experimental design. Compounds were tested at five toxic unit (TU) dose-ratios: (1:0, 3:1, 1:1, 1:3, 0:1), and six dose-levels (ΣTU = 0.25, 0.5, 1.0, 1.5, 2.0, 3.0), yielding 18 different binary mixture and 12 single-compound exposures (Figure A3.1A).

Here a TU was defined as concentration (c) divided by its toxicity threshold:

$$TU = \frac{c}{EC_{50}} \qquad \text{(Eqn. 3.1)}$$

In this case, the toxicity threshold chosen was the median effective concentration for lack of successful emergence (28 d EC_{50}) for each chemical.

Nominal concentrations of exposure were as follows; IMI 0.03 - 1.49 µg/L, CLO 0.05 - 2.12 µg/L, and TMX 0.56 - 26.73 µg/L. The fixed ray design necessitates reduced replication to allow for an increased number of exposure concentrations, therefore each treatment was replicated three times (*n* = 3, 10 organisms per replicate). As the mixture toxicity analysis is regression-based, statistical strength was maintained via adequate coverage of the toxicological response surface (Jonker et al., 2005).

3.2.3.3 Ternary mixture study

For the ternary mixture study, the fixed-ray experimental design utilized ten TU dose-ratios (1:0:0, 0:1:0, 0:0:1, 1:1:1, 2:1:1, 1:2:1, 1:1:2, 1:2:2, 2:1:2, and 2:2:1) and six dose-levels (ΣTU = 0.25, 0.5, 1.0, 1.5, 2.0, 3.0), yielding 42 different ternary mixtures and 18-single compound exposures (Figure A3.1B). Nominal concentrations of exposure were as follows: IMI 0.02 - 1.49 µg/L, CLO 0.04 - 2.13 µg/L, and TMX 0.45 - 26.73 µg/L. Each treatment was replicated three times (*n* = 3, 10 organisms per replicate).

3.2.4 Water quality

Water quality was assessed at the beginning (d 0), middle (d 14), and end (d 28) of each test. 20 mL water samples were removed from test beakers and analyzed for pH, conductivity, total hardness, and alkalinity. Water pH was measured with an ORION® PerpHect LogR meter, model 170 (ORION Research, Beverly, MA, USA). Hardness and alkalinity were measured using

a Hach Digital Titrator, model 16900 (Hach Company, Loveland, CO, USA). DO, temperature, and ammonia (NH_3) were measured in old and new water during every solution removal to ensure adequate experimental conditions were maintained. DO and temperature were measured with a Thermo ORION® dissolved oxygen meter, model 835 (Thermo ORION, Beverly, MA, USA), and ammonia was measured with VWR™ SB301 sympHony ISE ammonia meter (VWR International Ltd., West Chester, PA, USA) paired with a Thermo ORION® 95-12 ammonia electrode (Thermo ORION, Beverly, MA, USA).

3.2.5 Neonicotinoid analysis

Over the course of all toxicity tests, test solutions were sampled and analyzed to measure actual concentrations of neonicotinoid exposure. In the single-compound studies, test solutions were routinely sampled over the course of the study (d 0, 3, 6, 12, 15, 22, 28). In the mixture studies, test solutions were sampled at the start (d 0), middle (d 14), and end (d 28) of each exposure period. For each treatment, 80 mL of test solution was collected from each replicate beaker, pooled, and stored in a 250-mL amber glass bottle at 4°C until time of analysis. If measured concentrations fell outside of the expected range (e.g. ≥ 50% difference between measured and nominal) or analysis indicated unexpected neonicotinoid detection, those test beakers were excluded from further mixture analysis. Samples were analyzed at the National Hydrology Research Centre, Environment and Climate Change Canada, Saskatoon, SK. A comprehensive description of analytical methods is available in Main et al. (2014). Briefly, neonicotinoid concentrations were quantified via solid-phase extraction (SPE) followed by high performance liquid chromatography paired with tandem mass spectrometry (LC-MS/MS). The SPE was performed by loading samples onto OASIS® HLB cartridges (Waters, Mississauga, ON, Canada), removing salts with a deionized water rinse, and eluting retained components with methanol. Eluted extracts were then dried via

evaporation, reconstituted in deionized water, and spiked with internal standards (d_4-IMI, and d_3-TMX) obtained from CDN Isotopes, Pointe-Claire, QC, Canada. The LC-MS/MS was comprised of a Waters 2695 Alliance HPLC system (Waters Corp., Milford, MA, USA), equipped with a Waters Xterra MS-C8 column (3.5-µm diameter, particle size; 2.1 x 100-mm) (Waters Corp., Milford, MA, USA), paired with a Micromass Quattro Premier triple quadrupole mass spectrometer (Waters Corp., Milford, MA, USA) equipped with an electrospray ionization interface (positive ion mode). The mobile phase was an 80/20 mix of solvent A (99.9% water, 0.1% formic acid) and solvent B (90% acetonitrile, 9.9% water, 0.1% formic acid). The sample injection volume was 20 µL, the mobile phase flow rate was 200 µL/min, and the average run-time was 10 min. Analytical standards (IMI, CLO, TMX) purchased from Chem Service, West Chester, PA, USA, were used to create calibration curves and determine recoveries. Limits of quantification (LOQ) were as follows: IMI 0.003 - 0.008 µg/L, CLO 0.003 - 0.013 µg/L, and TMX 0.007 - 0.027 µg/L. Recoveries were determined using Milli-Q water spiked with neonicotinoid concentrations of 0.125 µg/L: IMI 85.2 - 104.0 %, CLO 76.1 - 98.8%, and TMX 77.8 - 101.1 %. Measured neonicotinoid concentrations were recovery corrected and averaged across sampling days. Mean measured (not nominal) concentrations were subsequently used in all statistical analysis and MIXTOX modeling.

3.2.6 Data analysis

Single compound toxicity was evaluated by fitting emergence data to a logistic dose-response curve (Equation 3.2) using SigmaPlot statistical software, ver. 11.0 (Systat Software Inc., San Jose, CA, USA). Here the toxicological response (Y_i) is a function of maximum response (Y_{max}), concentration of exposure (c_i), the 28-d EC_{50} estimate, and the slope of the response curve (B_i):

$$Y_i = \frac{Y_{max}}{1+(\frac{C_i}{EC_{50}})B_i} \qquad \text{(Eqn. 3.2)}$$

EC_{50} values were estimated using the trimmed Spearman-Kärber method (Hamilton et al., 1977) and compared to those derived through fitting the dose-response curve (Equation 3.2), and those derived for single compounds using MIXTOX modeling (Jonker et al., 2005) to assess the reliability of parameter estimates. Other effective concentration estimates (EC_{20}, EC_{90}) were obtained through linear interpolation using the US EPA ICp program (Norberg-King, 1993). Effects of neonicotinoid exposures on sex of emerged adults were evaluated by averaging proportions of emerged males and females across treatment replicates. Emergence data were assessed for normality and equality of variance using Shapiro-Wilk and Brown-Forsythe tests ($\alpha = 0.05$), and then differences amongst treatment groups (vs. controls) were assessed using one-way analysis of variance (ANOVA) paired with a Dunnett post-hoc analyses (95% level of confidence, $\alpha = 0.05$). Sex differences of successfully emerged adults were assessed by comparing the mean proportion of emerged males in treatment groups to experimental controls using *z*-tests (95% level of confidence, $\alpha = 0.05$).

Binary mixture toxicity was characterized using the MIXTOX approach (Jonker et al., 2005). The descriptive approach was used to evaluate the cumulative toxicity of neonicotinoid mixtures by comparing measured data, observed in toxicity tests, with fitted parametric models of mixture effects, calculated from single-compound toxicity data. Using the MIXTOX approach, deviation of observed data from the reference model of Concentration Addition (CA; concentration-additive cumulative toxicity) was assessed via a step-wise addition of extra parameters, *a* and *b*. Models were first extended with *a*, describing a synergistic (greater than expected toxicological effect) or antagonistic (lower than expected toxicological effect) deviation

(S/A) from CA. Models were then further extended with b_{DR} and b_{DL}. The b_{DR} parameter describes a dose-ratio dependent deviation from the reference model (DR), indicating a shift between synergism and antagonism dependent on the ratio of mixture constituents. The b_{DL} parameter describes a dose-level dependent deviation from the reference model (DL), indicating a shift between synergism and antagonism dependent on the cumulative magnitude of toxic units. Interpretation of numerical values derived in the MIXTOX analysis and mixture models (CA, S/A, DR, and DL) can be found in the Appendix (Table A2.1).

Ternary mixture toxicity data were analyzed using the Ternary-Plus approach (Cedergreen et al., 2012), an extension of the MIXTOX model optimized for three compound mixtures. In this analysis, data from all three binary mixture studies were analyzed alongside the empirical ternary mixture data, to account for the toxicological effects of binary mixtures when predicting ternary-mixture response. A ternary deviation parameter, $a_{1,2,3}$, was introduced, describing the deviation of the measured ternary response surface from the response surface predicted by a combination of binary deviation functions. This parameter describes a synergistic or antagonistic deviation from CA for the ternary mixture. As the Ternary-Plus model is still in development, this equation could not be further extended to model dose-level and dose-ratio dependent deviations from the synergism/antagonism model. An interpretation of the numerical values derived in the Ternary-Plus model can be found in the Appendix (Table A2.1).

Adequate coverage of the toxicological response surface was evaluated through scatterplots of measured concentrations tested in mixture toxicity tests. Experimental data were fit to parametric models using maximum likelihood estimation. First, measured data were fit to the reference model. Due to their similar mechanism of action at the nicotinic acetylcholine receptor, mixtures of neonicotinoids are typically expected to have a cumulative effect that is best described

by the reference model, Concentration Addition (CA). Therefore, to evaluate whether this assumption is protective of *C. dilutus* and other similarly sensitive aquatic insect species, we used CA as our reference model for all MIXTOX analysis. After fitting to CA, models were further extended with the extra parameters indicating deviation from the reference model (i.e. *a*, b_{DR}, *and* b_{DL}). As the sequential addition of parameters resulted in the formation of a series of nested models, fits of parametric models were directly assessed through pairwise model comparison and significance testing. Following extension of reference models with additional parameters, improved fit was confirmed by a reduction in the residual deviation (RD) and the statistical significance of this improvement determined via Chi-squared tests (χ^2) with degrees of freedom equal to the difference in number of parameters in the two models. For each mixture, model of best fit was defined as that which most significantly reduced RD compared to CA. If a significant deviation from direct additivity was observed, the measured emergence response was directly compared to that predicted by CA to describe the magnitude of deviation (% decrease or increase in emergence). Further information regarding the derivation and statistical interpretation of parametric MIXTOX models can be found in Jonker et al. (2005).

3.3 Results

3.3.1 Test solutions

Routine water quality of test solutions remained consistent across all toxicity tests with mean (± SD) parameters as follows: DO 7.6 (± 0.3) mg/L, temperature 23.0 (± 1.0) °C, pH 8.1 (± 0.1), conductivity 421 (± 148) µS/cm, total hardness 119 (± 34) mg/L as $CaCO_3$, and alkalinity 117 (± 16) mg/L $CaCO_3$. Due to build-up of excess food and metabolic waste in test beakers, ammonia concentrations increased between water renewals. However, mean concentrations remained well below the unionized ammonia LC_{50} estimate for *C. dilutus* (10 d LC_{50}: 82.4 mg

N/L) at 1.0 (± 0.3) mg N/L (Schubauer-Berigan, M.K., Monson, P.D., Ankley, 1995). Neonicotinoid exposures also remained relatively consistent throughout the duration of each toxicity test. Neonicotinoid concentrations in control treatments were always lower than the limit of quantitation (< LOQ), and the mean measured concentrations of IMI, CLO, and TMX in the insecticide treatments remained within (mean ± SD) 112 ± 20 %, 101 ± 17 %, and 135 ± 34 % of target nominal doses, respectively (Tables A3.1 and A3.2).

3.3.2 Single compound studies

IMI elicited the greatest toxicity in *C. dilutus*, with a 28 d EC_{50} of 0.50 µg/L. CLO demonstrated comparable toxicity with a 28 d EC_{50} of 0.71 µg/L. TMX was markedly less toxic, with a 28 d EC_{50} of 8.91 µg/L. EC_{xx} estimates and confidence intervals are reported in Table 3.1. Toxicity estimates generated using the log-logistic model (Equation 3.2) generated similar median effect levels; 0.43 µg/L (IMI), 1.34 µg/L (CLO), and 8.58 µg/L (TMX) (Table A3.3). Furthermore, there were trends of sex-ratio shifts toward male dominated populations in IMI and CLO treatments (Figure 3A, B). However, the effects of single-compound exposures on sex-ratios were not statistically significant ($p > 0.05$) due to limited sample sizes at higher concentrations.

3.3.3 Mixture studies

To evaluate the reproducibility and accuracy of the laboratory tests, 28 d EC_{50} values for IMI, CLO, and TMX were compared with the single compound test data and presented in Table A3.3. Single compound toxicity remained relatively consistent across all three mixture tests, with 28 d EC_{50} values calculated from the positive controls generally falling within the 95% confidence intervals of single compound EC_{50} estimates. In some cases, EC_{50} estimates were slightly higher than expected. For example, in the IMI-TMX mixture test, the EC_{50} of IMI was slightly higher than that generated in the single compound toxicity test (IMI EC_{50} = 0.81 µg/L vs. 0.50 (0.37 –

0.59) µg/L) and in the IMI-CLO-TMX test the EC_{50} of IMI was slightly higher than expected (IMI EC_{50} = 0.66 µg/L vs. 0.50 (0.37 – 0.59) µg/L). Furthermore, in the CLO-TMX test, the toxicity of TMX fell far outside of the expected range (e.g. cessation of emergence was not achieved at the highest TMX concentration tested (32.5 µg/L vs. 8.91 (5.79 – 12.37) µg/L). Thus, toxicity values generated in the single compound test were used in the CLO-TMX mixture analysis. However, for the rest of the tests, toxicity estimates remained within relatively narrow ranges (e.g. IMI = 3.3-fold; CLO = 2.8-fold; and TMX = 1.4-fold). Thus, single compound and positive control toxicity estimates were considered to be accurate, and appropriate for use in further mixture analyses.

3.3.3.2 Imidacloprid-clothianidin

Measured IMI and CLO concentrations in the binary mixture test show that the toxicological response surface was adequately covered, indicating that reported results are likely to accurately reflect the cumulative effects of the IMI-CLO mixtures (Figure 3.2A).

The residual deviation (RD) for the CA reference model was 177.9. Extension of the model with the synergism/antagonism (S/A) parameter (*a*) significantly improved model fit (RD = 164.6, χ^2 = 13.3, $p < 0.05$; a = 0.54). Extension of CA-S/A with the dose-level (DL) parameter failed to improve model fit (RD = 164.4, χ^2 = 0.21; p = 0.64). However, extension of CA-S/A with the dose-ratio (DR) parameter did significantly improve model fit (RD = 139.3, χ^2 = 25.26, $p < 0.05$), with a = -9.97 indicating greater-than-additive toxicity at higher concentrations of CLO (i.e. decrease in emergence compared to CA: mean = 13 %; max = 49 %) and b_{DR} = 17.82 indicating less-than-additive toxicity at higher concentrations of IMI (i.e. increase in emergence: mean = 5 %; max = 25 %) (Figure 3.2B). This model (CA-DR) explained 68.9% of the variability (R^2 = 0.689) (Figure 3.2C). Additional information on the MIXTOX analysis of the IMI-CLO mixtures can be found in the Appendix (Table A3.4).

Table 3.1 Chronic toxicity endpoints (EC_{20}, EC_{50}, EC_{90}; µg/L) and 95% confidence intervals for successful emergence of *Chironomus dilutus* exposed to technical grade IMI, CLO, and TMX over 28 days, compared to current water quality guidelines in Canada, the US, and the EU.

EC_{xx}	Imidacloprid	Clothianidin	Thiamethoxam
20[a]	0.14 (0.08 – 0.42)	0.34 (0.19 – 0.45)	4.62 (0.85 – 6.70)
50[b]	0.50 (0.37 – 0.59)	0.71 (0.50 – 0.85)	8.91 (5.79 – 12.37)
90[c]	1.02 (0.80 – 1.09)	1.37 (1.22 – 2.08)	17.38 (15.93 – 18.16)
Current Water Quality Guidelines (µg/L)	0.0083 – 0.23 [d-g]	1.10 [d]	35.0 [d]

[a] Concentrations estimated to produce a 20% effect, calculated using the USEPA Inhibition Concentration program (ICp) (USEPA, 1993).
[b] Median effective concentration, calculated using the trimmed Spearman-Kärber method (Hamilton *et al.,* 1977).
[c] Concentrations estimated to produce a 90% effect, calculated using the USEPA ICp method (USEPA, 1993).
[d] USEPA Aquatic Life Benchmarks (USEPA, 2017).
[e] Environment and Climate Change Canada Water Quality Guidelines (Canadian Council of Ministers of the Environment, 2007).
[f] European Food Safety Authority Water Quality Guidelines (European Food Safety Authority, 2006).
[g] Dutch National Institute for Public Health and the Environment Water Quality Guideline (Smit et al., 2015).

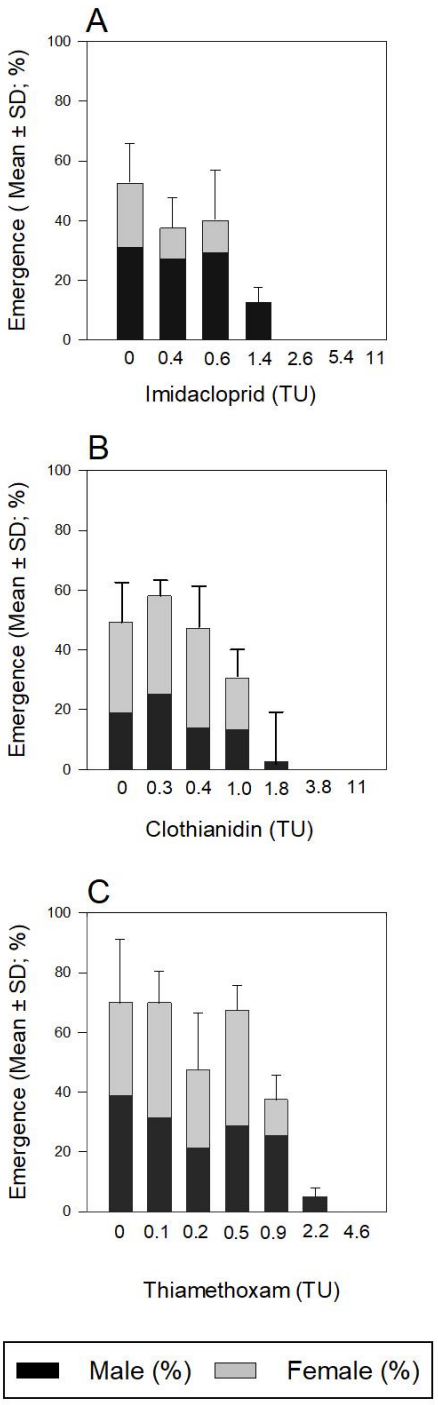

Figure 3.1 Emergence and sex (%) of adult *Chironomus dilutus* after exposure to (A) IMI, (B) CLO, and (C) TMX (C) for 28 days relative to controls (n = 4 treatments, 10 organisms/treatment).

IMI-CLO mixtures did not have any statistically significant effects on sex of emerged *C. dilutus* ($p > 0.05$). However, there were trends of sex ratio shift towards male dominant populations across all dose-ratios and at all concentration-levels (Figure 3.2D).

3.3.3.3 Clothianidin-thiamethoxam

Measured concentrations show that the coverage of the toxicological response surface in the CLO-TMX mixture study was slightly skewed at higher concentrations of TMX, with the highest dose-levels exceeding 3 TU (Figure 3.3A). However, as the cessation of *C. dilutus* emergence occurred at cumulative concentrations less than 3 TU, these higher concentration ranges are not essential for the characterization of cumulative toxicological effects. Therefore, the model presented here should accurately reflect the cumulative effects of the CLO-TMX mixtures.

The RD of the CA reference model was 140.06. Extension of the CA model with the S/A, parameter significantly improved model fit (CA-S/A RD = 93.9, $\chi^2 = 46.2$, $p < 0.01$; $a = 0.84$). Further extension of the CA-S/A model with the DL parameter slightly decreased RD (CA-DR RD = 92.8, $\chi^2 = 1.05$, $p = 0.30$). However, extension of the CA-S/A model with the DR parameter more significantly decreased RD (CA-DR RD = 91.6) compared to CA ($\chi^2 = 47.2$, $p < 0.01$). Therefore, the model of best fit was defined as CA-DR (Figure 3.3B), with $a = 2.15$ indicating negligible less-than-additive toxicity at higher concentrations of CLO (i.e. % increase in overall emergence: mean = 0 %; max = 8 %) and $b_{DR} = -2.15$ indicating greater-than-additive toxicity at high concentrations of TMX (i.e. % decrease in overall emergence: mean = 2 %; max = 13 %). This model was found to explain 84.0 % of the variability in the measured data (Figure 3.3C). A summary of the MIXTOX analysis output for the CLO-TMX mixtures can be found in the Appendix (Table A3.5).

Exposure to CLO-TMX mixtures did not have any statistically significant effects on the sex of emerged *C. dilutus* ($p > 0.05$). However, there were trends of sex ratio shift towards male dominant populations occurring across most dose-ratios with as cumulative concentration increased (Figure 3.3D).

3.3.3.4 Imidacloprid-thiamethoxam

Measured concentrations of IMI and TMX in the mixture study indicated that the toxicological response surface was slightly skewed at higher dose-levels, with the highest measured dose levels in this study exceeding 3 ΣTU (Figure 3.4A). However, cessation of emergence (i.e. EC_{100}) occurred at concentrations lower than 3 ΣTU. Therefore, the measured data at these higher dose-levels (i.e. ΣTU between 3.0 and 4.5) were unnecessary for an accurate characterization of joint toxicity, and the model presented here should accurately reflect the cumulative effects of the IMI-TMX mixtures.

The RD of the CA reference model was 175.5. Extension of the model with the S/A parameter (a) failed to improved model fit (RD = 172.6; $\chi^2 = 2.94$, $p = 0.09$). Similarly, extension of the model with the DL parameter (b_{DL}) failed to improve model fit (RD = 172.6; $\chi^2 = 0.002$; $p = 0.96$). However, extension of the model with the DR parameter did significantly improve the goodness of fit (CA-DR RSS = 168.7, $\chi^2 = 3.86$, $p < 0.05$), with parameters of $a = -0.95$, and $b_{DR} = 3.10$ indicating greater-than-additive toxicity in mixtures with higher concentrations of TMX (i.e. decrease in emergence: mean = 4 %; max = 24 %), and less-than-additive toxicity in mixtures with higher concentrations of IMI (i.e. increase in emergence: mean = 2 %; max = 27 %) (Figure 3.4B). This model (CA-DR) explained 67.1% of variability (Figure 3.4C). A summary of the MIXTOX output for this mixture can be found in the Appendix (Table A3.6).

Exposure to the IMI-TMX mixtures did not have any significant effects on the sex of emerged *C. dilutus* ($p > 0.05$) and did not elicit visible trends of a sex ratio shift (Figure 3.4D).

3.3.3.5 Imidacloprid-clothianidin-thiamethoxam

Measured concentrations of IMI, CLO, and TMX in the ternary mixture study showed that toxicological response surface was adequately covered (Figure 3.5A), indicating that reported results accurately reflect the cumulative effects of IMI-CLO-TMX mixtures.

The RD of the CA reference model was 618.0. Extension of the CA model with the S/A significantly improved fit (CA-S/A RSS = 588.1, $\chi^2 = 29.9$, $p < 0.05$), with $a_{IMI,CLO,TMX} = 0.20$ indicating less-than-additive toxicity (i.e. increase in emergence: mean = 2 %; max = 28 %) in the ternary mixture, and $a_{IMI,CLO} = -1.24$, $a_{CLO,TMX} = 0.18$, and $a_{IMI,TMX} = 0.54$ indicating that the binary mixtures had both synergistic (IMI-CLO, CLO-TMX) and antagonistic (IMI-TMX) contributions to cumulative ternary mixture toxicity. Therefore, the IMI-CLO-TMX mixture displayed slight antagonistic deviation from the CA model (Figure 3.5B). This model was found to explain 65.5% of the variability, with weaker correlation (compared to most binary mixtures) between modeled and measured data (Figure 3.5C). A summary of the MIXTOX output for this mixture can be found in the Appendix (Table A3.7).

Exposure to IMI-CLO-TMX mixtures did not have any statistically significant effects on sex of emerged *C. dilutus* ($p > 0.05$). However, there were trends of sex ratio shift across most tested dose-ratios and dose-levels (Figure 3.2D).

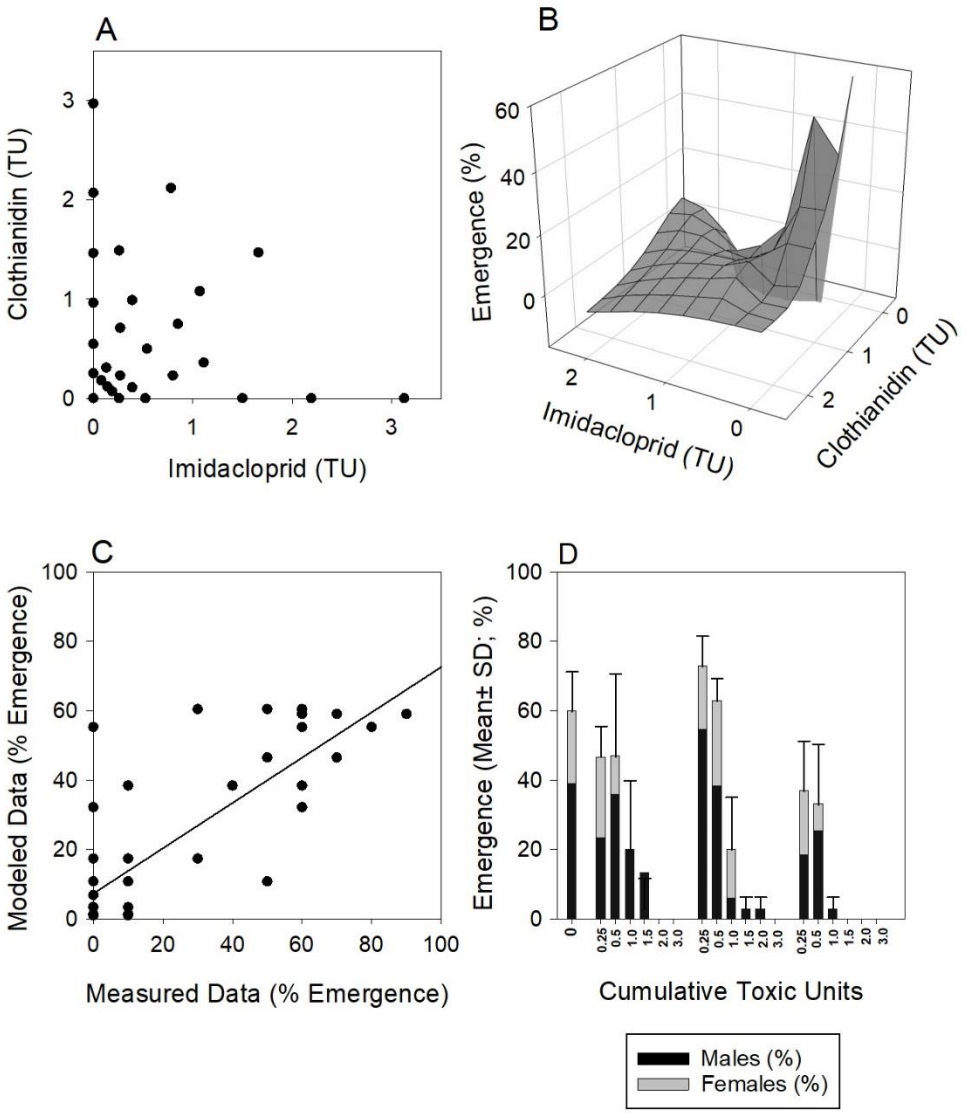

Figure 3.2 Chronic (28-d) exposure to IMI-CLO mixtures demonstrating (A) actual concentrations of exposure, (B) emergence (mean, %) of adult *Chironomus dilutus* relative to control, (C) relationship between measured emergence data and modeled values for the most statistically significant parsimonious deviation from reference model (dose-ratio dependent deviation), and (D) sex of emerged adults (mean ± SD; %) relative to controls.

*A diagonal line (1:1) indicates idyllic model description (C). TU = Toxic Unit.

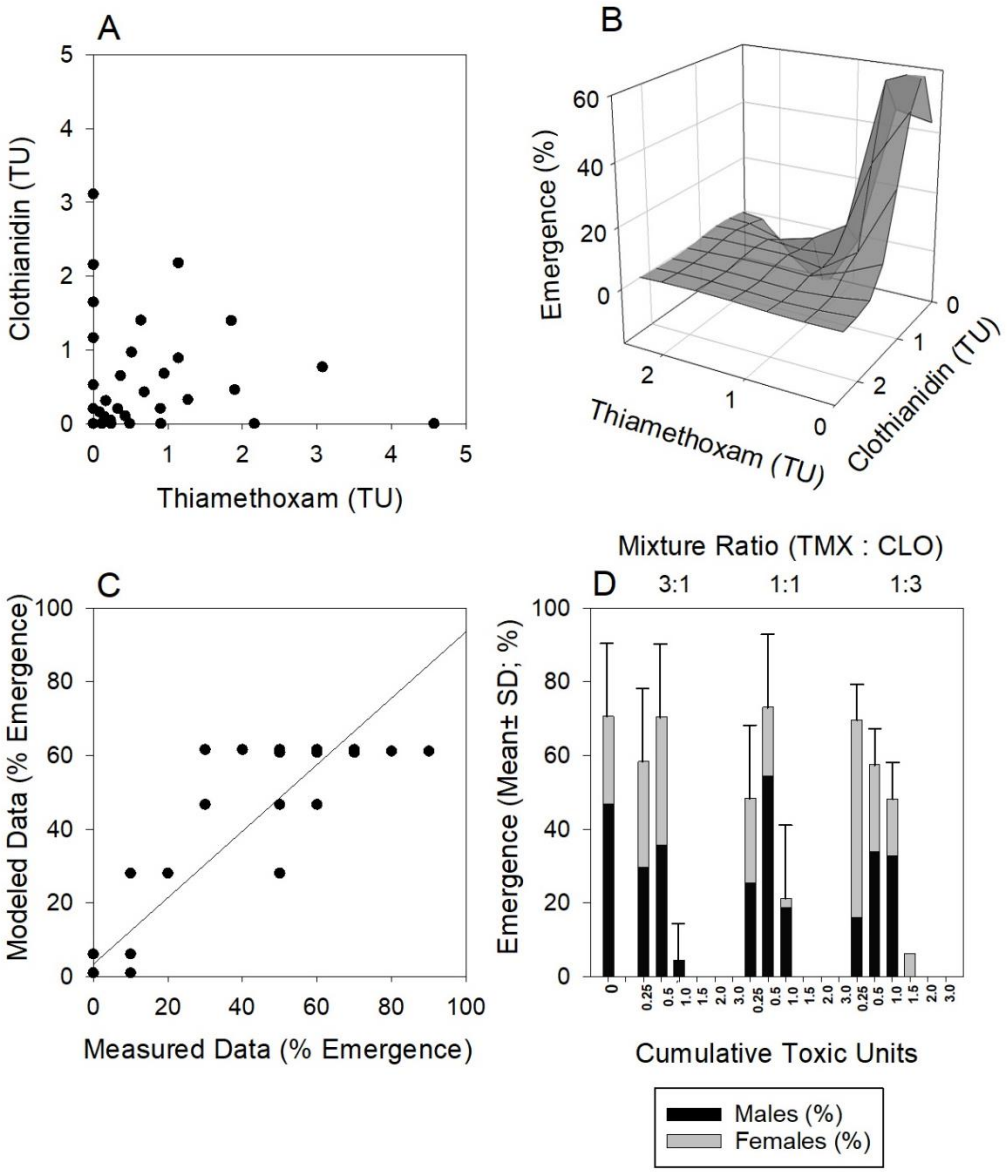

Figure 3.3 Chronic (28-d) exposure to CLO-TMX mixtures demonstrating (A) actual concentrations of exposure, (B) emergence (mean, %) of adult *Chironomus dilutus* relative to controls, (C) relationship between measured survival data and modeled values for the most statistically significant parsimonious deviation from reference model (no deviation, Concentration Addition), and (D) sex of emerged adults relative to controls (mean ± SD; %).

*A diagonal line (1:1) indicates idyllic model description (C). TU = Toxic Unit.

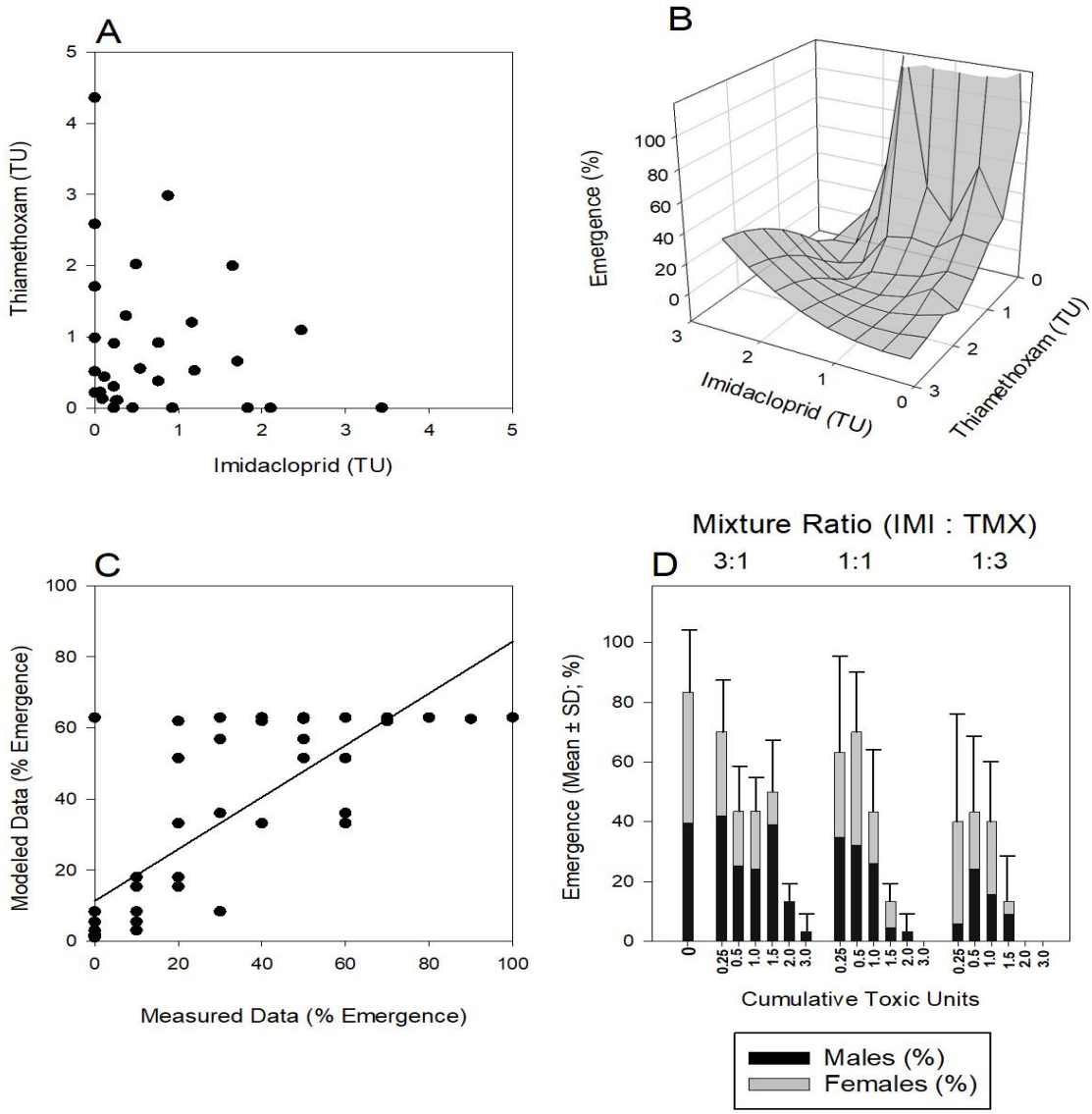

Figure 3.4 Chronic (28-d) exposure to IMI-TMX mixtures demonstrating (A) actual concentrations of exposure, (B) emergence (mean, %) of adult *Chironomus dilutus* relative to controls, (C) relationship between measured survival data and modeled values for the most statistically significant parsimonious deviation from reference model (dose-ratio synergism), and (D) sex of emerged adults (%) relative to controls (mean ± SD; %).

*A diagonal line (1:1) indicates idyllic model description (C). TU = Toxic Unit.

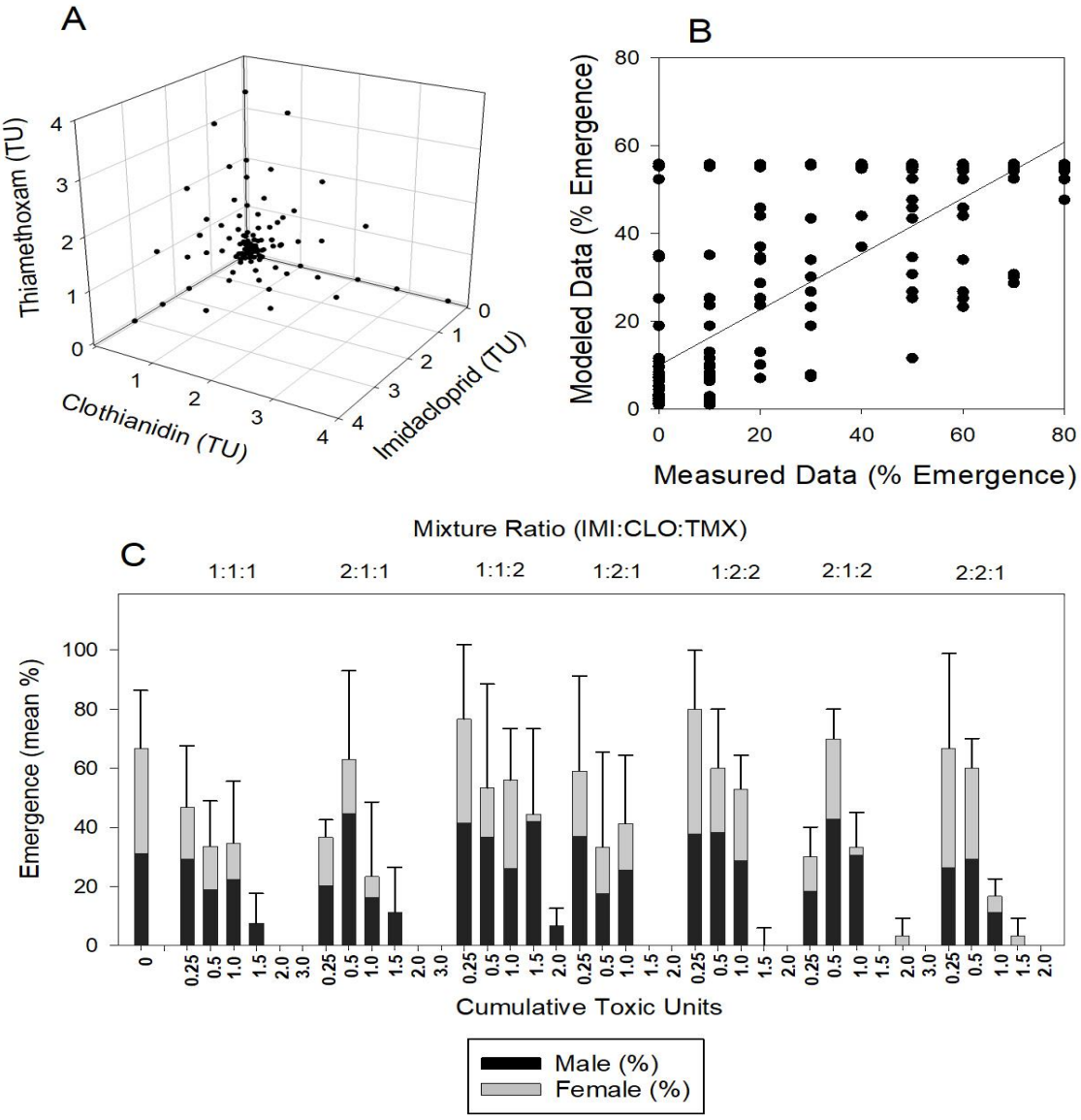

Figure 3.5 Chronic (28-d) exposure to IMI-CLO-TM mixtures demonstrating (A) actual concentrations of exposure, (B) relationship between measured survival data and modeled values for the most statistically significant parsimonious deviation from reference model (no deviation, Concentration Addition), and (C) sex (mean ± SD; %) of emerged adults relative to controls.

* A diagonal line (1:1) indicates idyllic model description (B). TU = Toxic Unit.

3.4 Discussion

3.4.1 Chronic toxicity of single compounds

In aquatic environments, chronic exposure to IMI, CLO, or TMX at concentrations exceeding the toxicity estimates reported here will likely elicit sub-lethal toxicological effects in *C. dilutus* and other sensitive aquatic insect taxa (e.g. Ephemeroptera, Trichoptera, and other Chironomidae species (Morrissey et al., 2015)). In this study, IMI elicited the greatest toxicity in *C. dilutus* (e.g. 28 d EC_{50} = 0.50 µg/L), followed closely by CLO (28 d EC_{50} = 0.71 µg/L). TMX was by far the least toxic neonicotinoid tested, inhibiting emergence only at concentrations 13 - 18 times higher than IMI or CLO (28 d EC_{50} = 8.91 µg/L). The chronic toxicity estimate for IMI is comparable to what has been previously reported for *C. dilutus* in the literature (e.g. a 28-d EC_{50} for IMI in formulation of 0.91 µg/L (Stoughton et al., 2008)). The relative toxicities of IMI, CLO, and TMX also corroborate what has previously been reported for *C. dilutus* under shorter and longer exposure durations. Under acute exposure conditions (96 h), *C. dilutus* demonstrated the greatest sensitivity to IMI (96 h LC_{50} = 4.63 µg/L), followed closely by CLO (96 h LC_{50} = 5.93 µg/L), and then TMX (96 h LC_{50} = 55.34 µg/L) which was 10 times less toxic (Maloney et al., 2017). Under longer chronic exposure conditions (40 d), IMI and CLO displayed similar toxicities (IMI 40 d EC_{50} = 0.39 µg/L; CLO 40 d EC_{50} = 0.28 µg/L), whereas TMX was 15 times less toxic (40 d EC_{50} = 4.13 µg/L) (Cavallaro et al., 2017). Furthermore, the trend towards greater toxicity (lower EC_{50} values) with increased exposure duration with IMI, CLO, and TMX is consistent for a range of other test species, and for other neonicotinoid compounds (Sánchez-Bayo et al., 2016).

3.4.2 Chronic toxicity of imidacloprid, clothianidin, and thiamethoxam mixtures

Importantly, this study focused on characterizing the cumulative toxicity of neonicotinoid insecticide mixtures to determine if single compound toxicity values could adequately predict

neonicotinoid mixture toxicity for *C. dilutus* under chronic exposure scenarios. We found that the toxicities of IMI, CLO, and TMX mixtures could deviate from direct additivity; however cumulative effects varied depending on mixture composition. For the IMI-CLO-TMX mixtures there was only a slightly antagonistic deviation from the CA mixture model (displaying a 2 % increase in emergence). However, all binary neonicotinoid mixtures displayed cumulative toxicities that deviated from direct additivity, depending on the ratio of mixture constituents (dose-ratio dependent deviation). Indeed, IMI-CLO, CLO-TMX, and IMI-TMX mixtures all displayed the potential for greater-than-additivity (displaying 2 - 13 % average decreases in successful emergence compared to that predicted by CA) and less-than-additive toxicity (displaying 2 - 5 % increases in successful emergence compared to that predicted by CA), depending on mixture composition. Therefore, although CA could likely be used to adequately predict mixture toxicity of some mixture compositions, this assumption of direct additivity could underestimate cumulative toxicities of other mixture compositions in aquatic environments (resulting in up to a 13 % greater-than-predicted decrease in successful emergence of *C. dilutus*).

Under acute exposure scenarios, the cumulative toxicities of most binary and ternary mixtures of IMI, CLO, and TMX were found to significantly deviate from directly additive toxicity (Maloney et al., 2017). Here, we found similar patterns of cumulative toxicity for some (but not all) neonicotinoid mixtures under chronic exposure scenarios. For example, as in acute studies IMI-CLO and IMI-TMX mixtures displayed dose-ratio dependent deviation. However, contrary to acute studies (where IMI was the primary driver of dose-ratio dependent synergism), IMI-CLO and IMI-TMX mixtures displayed synergism only at high concentrations of CLO and TMX, respectively. Furthermore, whereas in acute studies the CLO-TMX mixture was found to be concentration-additive, chronic exposures to CLO-TMX resulted in dose-ratio dependent

cumulative toxicity. Finally, under acute settings the IMI-CLO-TMX mixture was found to be mildly synergistic, but under chronic exposure settings this ternary mixture was slightly antagonistic. In addition, the magnitude of synergism tended to differ depending on exposure time. For example, in IMI-CLO and CLO-TMX mixtures the magnitude of synergism increased between acute and chronic exposure settings (e.g. from 1 % to 13 % (IMI-CLO) and from negligible to 2 % (CLO-TMX)) (Maloney et al., 2017). However, the exact opposite was observed in IMI-TMX and IMI-CLO-TMX mixtures, in which the magnitude of synergism decreased between acute and chronic exposure settings (e.g. from 13 % to 5 % (IMI-TMX) and from 2 % synergism to antagonism (IMI-CLO-TMX)) (Maloney et al., 2017). For most mixtures (with the exception of IMI-CLO), these differences in synergistic potential were relatively limited, displaying less than a 10 % difference in magnitude between acute and chronic exposure settings. Therefore, it is likely that for IMI, CLO, and TMX, the chronic toxicities of binary and ternary mixtures can be adequately predicted using data from acute exposure scenarios, as long as the potential for time-weighted enhancement of cumulative toxicity is adequately accounted for.

3.4.3 Consideration of a predictive window for neonicotinoid mixture toxicity

During a risk assessment of chemical mixtures in aquatic environments, CA is typically used as a predictive model when the mixture constituents are mechanistically similar (i.e. have similar sites and modes of action). In contrast, the predictive model Independent Action (IA), assuming response-additive cumulative toxicity, is typically applied when mixture constituents are strictly dissimilar in target sites and mechanism of action (Altenburger et al., 2003). However, in the literature there is no consensus on how similar the target sites or modes of action of mixture constituents must be to properly employ either reference model in an environmental risk assessment (Altenburger et al., 2003). Therefore, when the mechanism of action has not been fully

elucidated, or the molecular target has not been characterized in a species of interest, it can be of benefit to apply both IA and CA in mixture toxicity analysis. Here we used CA as the reference model of interest to evaluate if current CA-based risk assessment techniques are adequately protective of *C. dilutus*. However, we caution that alternative conclusions (i.e. synergism of CLO-TMX or IMI-CLO-TMX mixtures) may be drawn when using IA as a reference model (Tables A3.2 - 5). This may be due to the fact that the molecular target of neonicotinoids has not been characterized in our test species (see Section 3.4.4). Therefore, to be adequately protective of sensitive aquatic insect species, we propose consideration of a prediction window that incorporates both reference models (CA and IA) when interpreting cumulative effects (Altenburger et al. 2003; Maloney et al. 2017), accounting for any potential greater-than-additive effects that may occur resulting from neonicotinoid mixture exposure.

3.4.4 Neonicotinoid action at the nicotinic acetylcholine receptor

Neonicotinoid mixture toxicity can thus deviate from directly additive toxicity. However, the magnitude of deviation varies depending on mixture composition, exposure conditions (i.e. duration and intensity) and reference model applied (CA vs. IA). The reasons for this are not well understood. We hypothesize this may be due to the actions of the neonicotinoid compounds at the nicotinic acetylcholine receptor (nAChR). Neonicotinoids all act on the nAChR, therefore neonicotinoid mixtures should theoretically elicit concentration-additive toxicity in invertebrate species (i.e. mixture effects should be directly predictable from single compound toxicity values). We have determined that, depending on specific composition, mixtures of neonicotinoids can elicit greater-than-additive toxicity in *C. dilutus* under both acute and chronic scenarios. We have previously suggested that the greater-than-additive mixture effects could be a result of neonicotinoid interactions at the receptor level (Maloney et al., 2017). Neonicotinoid research

focusing on target (pest) insect species has found that the nAChR can display a range of functionally distinct subtypes that differ in their response to neonicotinoid exposure. Indeed, IMI, CLO, and TMX have been shown to differ in their abilities to bind to and activate different nAChR subtypes (Calas-List et al., 2012; Oliveira et al., 2011; Salgado and Saar, 2004; Thany, 2011, 2009). Therefore, it is possible that the presence of multiple nAChR subtypes in *C. dilutus* could be influencing the observed cumulative toxicological effects of neonicotinoid mixtures, resulting in the greater-than-additive toxicity observed in most mixtures in our acute and chronic studies. The actions of these compounds on nAChR receptors could also explain the decrease in synergistic magnitude seen in IMI-TMX and IMI-CLO-TMX mixtures between acute and chronic exposure conditions. IMI, CLO, and TMX elicit dose-dependent responses in nAChRs, with higher neonicotinoid concentrations inducing stronger nAChR depolarization (Buckingham et al., 1997; Thany, 2011, 2009). Therefore, the higher concentrations of exposure in the acute study could have elicited stronger responses in the various nAChR subtypes, leading to more dramatic mixture effects than observed in the lower concentration, chronic exposure study. However, as synergism slightly increased between acute and chronic exposures in IMI-CLO and CLO-TMX mixtures, it is likely that other factors are responsible for differences in cumulative toxicity observed between acute and chronic exposure settings. For example, it is possible that differential effects are due to variation in nAChR subtype expression throughout the *C. dilutus* life-cycle, with different proportions of functional subtypes being expressed at different life-stages (i.e. larvae, pupae, adult). Indeed, previous studies with the rice striped stem borer (*Chilo supressalis)* and the pea aphid (*Acyrthosiphon pisum*) have shown that the expression levels of nAChR subunits can vary between developmental stage (Taillebois et al., 2014; Xu et al., 2017). However, to the best of our knowledge, neither the nAChR nor the actions of neonicotinoids on this receptor have yet to be

characterized for any sensitive aquatic insect species at any stage of development. Therefore, further research is required to determine whether the presence and expression of multiple nAChR subtypes in *C. dilutus* can influence their toxicological responses to neonicotinoid mixtures.

3.4.5 Effects of neonicotinoids and their mixtures on sex ratios of emerged adults

Along with effects on successful emergence, this study investigated the impacts of chronic neonicotinoid single and mixture exposures on sex ratios of emerged *C. dilutus*. Chironomidae are sexually reproducing aquatic insects (Armitage, 1995). Therefore sex ratio can be an ecologically relevant toxicity endpoint, as significant sex-ratio skews (e.g. significantly different from the typical 1:1 male: female ratio) can reduce swarming success and egg mass fertility (Armitage, 1995). In turn, this can influence the reproductive success and viability of Chironomidae populations. Therefore, it is important to determine if chronic exposures to neonicotinoids and their mixtures have the potential to influence sex ratios in *C. dilutus*, as was previously reported for *C. dilutus* exposed to single neonicotinoids over longer periods (40 d) (Cavallaro et al., 2017) and to other insecticides such as DDT (Rakotondravelo et al., 2006). Results from this study cannot not lead to the formation of definitive conclusions concerning the effects of neonicotinoid mixtures on sex ratios of emerged adults, as none of the sex ratio trends observed in this study were statistically significant. The lack of statistical significance observed here was likely due to the experimental design of the mixture studies. First, in this study, experimental organisms were exposed to neonicotinoid mixtures for only 28 days. Typically chronic (life-cycle) toxicity tests with *C. dilutus* are conducted over the course of 40 days, and are concluded when emergence is fully completed (Benoit et al., 1997). Therefore, the exposure period used here may have been too short to observe statistically significant effects on the sex of emerged adults (with mean (± SD) control emergence in this study ranging from 46.7 ± 23.9 % to 83.3 ± 20.8 %). Indeed, as

Chironomidae are known to exhibit protandry (with males typically emerging before females), it is likely that this experiment failed to capture the full emergence profile for each tested neonicotinoid and neonicotinoid mixtures. Second, the MIXTOX approach is optimized for a select toxicological endpoint (Jonker et al., 2005), so the concentrations of exposure were tailored for the primary endpoint (inhibition of successful emergence) not sex of emerged adults. Consequently, mortality and emergence inhibition at the higher concentrations may have masked the effects on sex ratios. Therefore, to better characterize the effects of neonicotinoids and their mixtures on the sex ratios of *C. dilutus* and to determine if they have potential to pose multi-generational risks to natural Chironomidae populations, further research should focus on evaluating the effects of neonicotinoids and neonicotinoid mixtures under extended exposure scenarios (to capture full emergence profiles) and using lower neonicotinoid concentrations (to limit lethality in exposed organisms).

3.4.6 Environmental risk assessment and regulatory implications

In Canada, the United States, and the European Union, neonicotinoid water quality guidelines exclusively focus on the toxicities of single compounds (Canadian Council of Ministers of the Environment, 2007; European Food Safety Authority, 2013a; Smit, 2014; USEPA, 2017). For example, Environment and Climate Change Canada (ECCC) currently uses an interim freshwater quality guideline of 0.23 µg active ingredient/L (IMI) (Canadian Council of Ministers of the Environment, 2007). The United States Environmental Protection Agency (US EPA) sets aquatic life benchmarks for chronic neonicotinoid exposures at 0.01 µg/L (IMI), 1.10 µg/L (CLO), and 35 µg/L (TMX) (USEPA, 2017). The European Food Safety Authority (EFSA) has recommended maximum aquatic concentrations of 0.2 µg/L (IMI) (European Food Safety Authority, 2006). Finally, the Dutch National Institute for Public Health and the Environment

(RIVM, Netherlands) has recently derived a chronic water quality guideline of 0.0083 µg/L (IMI) (Smit, 2014). Across all these water quality guidelines, the additive and/or greater-than-additive potential of neonicotinoid mixtures demonstrated here and in previous studies is currently unaccounted for (Gomez-Eyles et al., 2009; Loureiro et al., 2010; Maloney et al., 2017).

MIXTOX is a descriptive, statistically-based computational method. Although this method can elucidate statistically significant differences in the magnitude of cumulative effects, it cannot be used to determine the biological significance of observed mixture toxicity. Biologically significant synergism is commonly defined as more than a two-fold deviation of observed effects from direct additivity (CA) (i.e. the concentration predicted to yield a certain effect is more than two times the concentration actually observed giving the proposed effect) (Cedergreen, 2014). In this study, we observed statistically significant greater-than-additive toxicity in all binary neonicotinoid mixtures (compared to CA). However, as the magnitude of synergism was relatively low (only up to ~ 13%), we cannot make definitive conclusions concerning the likelihood of these mixtures eliciting biologically significant synergistic toxicity in sensitive aquatic insects inhabiting chronically contaminated aquatic environments. Indeed, further studies are required to better characterize the risk neonicotinoid mixtures pose to natural aquatic insect communities. Nonetheless, given the prevalence of mixtures observed in field water quality monitoring (Hladik and Kolpin, 2015; Main, 2016; Schaafsma et al., 2015; Smalling et al., 2015), the weak synergism observed in this study should be considered in the risk assessment and regulation of neonicotinoid insecticides to ensure adequate protection of sensitive, ecologically important aquatic insect species.

The degree of protection of these current water quality guidelines appears to vary, which may or may not be adequate for sensitive aquatic insects (e.g. Chironomidae or Ephemeroptera).

For example, whereas the RIVM recommendation (0.0083 µg/L) is likely to protect *C. dilutus* (and other Chironomidae with similar sensitivities), the US EPA aquatic benchmarks for CLO and TMX exceed the 28-d EC_{50} values we found in this study, and thus are unlikely to be adequately protective. Conversely, the interim guidelines set by ECCC and EFSA focus exclusively on IMI and are set toxic units (0.46 and 0.4, respectively) that have been shown here (for particular mixtures) to elicit significant toxicity. Thus, these guidelines are likely to only offer protection for certain neonicotinoid mixtures, depending on the relative concentrations and ratios of mixture constituents. Chironomidae are important in freshwater ecosystems; typically representing a significant proportion of an aquatic insect community and serving as a major food source for higher-tier organisms (i.e. fish and water-fowl) (Benoit et al., 1997; Environment Canada, 1997), so water quality guidelines should attempt to adequately protect these freshwater insects. We recommend that the toxicity values (i.e. 28-d EC_{50}) and the weak synergism presented in this study be accounted for when setting chronic water quality benchmarks and performing risk assessments for this class of pesticides. Furthermore, in this study, we only considered the direct effects of neonicotinoid mixture exposure on *C. dilutus*. Due to the environmentally controlled, laboratory-based exposure conditions, this study did not account for potential population effects or other external factors that could occur in natural aquatic environments (e.g. temporal flux, wetland drainage, nutrient enrichment, and co-occurrence of other contaminants). Therefore, it is possible that the neonicotinoid mixture toxicity observed here could be different in surface waters containing additional stressors. Although further studies using more environmentally realistic experimental settings are necessary to better characterize the risk of multiple neonicotinoids (and other external factors) in aquatic environments, we believe that accounting for the cumulative toxicity of neonicotinoid mixtures for sensitive aquatic insects like *C. dilutus* in regulatory and

risk assessment practices is a good first step toward protecting the integrity of aquatic ecosystems surrounding areas of intensive neonicotinoid use. Therefore, when assessing the toxicity of neonicotinoid mixtures, the toxic unit concept should be used to estimate effect, and a minimum 10 % safety factor should be included in calculations to account for potential greater-than-additive toxicity.

CHAPTER 4: NEONICOTINOID INSECTICIDE MIXTURES: EVALUATION OF LABORATORY-BASED TOXICITY PREDICTIONS UNDER SEMI-CONTROLLED FIELD CONDITIONS

Preface

This chapter focuses on evaluating the toxicities of single neonicotinoid compounds (imidacloprid, clothianidin, and thiamethoxam) and their binary mixtures to natural aquatic insect communities under semi-controlled field conditions. In previous chapters (Chapters 2 and 3), predictions for neonicotinoid and neonicotinoid mixture toxicity were derived using laboratory-based, single species studies. Here those predictions were evaluated to determine if they adequately account for the toxicity of neonicotinoids and their mixtures to aquatic insect populations under more environmentally realistic exposure conditions. Using *in situ* experimental limnocorral enclosures, placed in an experimental wetland and fitted with emergence traps, natural benthic invertebrate communities were exposed to either single compounds or binary mixtures at theoretically equitoxic concentrations (1 Toxic Unit under the principle of Concentration Addition) for 56 days. Emerged adult insects were collected, identified, sexed and weighed, and using parametric analyses (χ^2 tests and repeat measures and one-way ANOVAs) the impacts of neonicotinoids/mixtures on aquatic insect community composition, and emergence, biomass and sex-ratios of Chironomidae populations were evaluated. Results from this chapter demonstrate that laboratory-derived toxicity predictions cannot adequately predict neonicotinoid and neonicotinoid mixture toxicity to Chironomidae populations, with laboratory studies under-predicting single-compound effect and over-predicting mixture effect. This research emphasizes the need for field-validation of laboratory-derived toxicity predictions, to adequately characterize the effects of aquatic contaminants on benthic invertebrate populations/communities and indicates that the

greater-than-predicted toxicity observed in this study should be accounted for when deriving environmental regulations or conducting risk assessments for neonicotinoid insecticides.

This chapter was published in Environmental Pollution, under joint authorship with Karsten Liber (University of Saskatchewan), John V. Headley (National Hydrology Research Centre, Environment and Climate Change Canada), Kerry M. Peru (National Hydrology Research Centre, Environment and Climate Change Canada), and Christy A. Morrissey (University of Saskatchewan).

Maloney, E.M., Liber, K, Headley, J.V., Peru, K.M., and Morrissey, C.A. 2018. Neonicotinoid insecticide mixtures: evaluation of laboratory-based toxicity predictions under semi-controlled field conditions. Environmental Pollution 243(B):1727-1739.

4.1 Introduction

Neonicotinoid insecticides are frequently detected in global surface waters (Anderson et al., 2015; Morrissey et al., 2015). Widespread use and relatively high application rates have led to the detection of neonicotinoids at concentrations commonly ranging from < 0.001 - 44.38 µg/L in marine (Smith et al., 2012), freshwater lentic (e.g. wetlands and ponds) (Evelsizer and Skopec, 2016; Lamers et al., 2011; Main et al., 2014; Schaafsma et al., 2015), and freshwater lotic (e.g. rivers and streams) (Metcalfe et al., 2019; Struger et al., 2017) ecosystems across the world. Due to a combination of their physicochemical characteristics (e.g. high water solubility, limited degradation in light-limited environments) (Lu et al., 2015) and current agricultural application practices (e.g. field-rotation with different neonicotinoid-treated crops and application of different compounds in the same watershed), neonicotinoids are commonly detected as mixtures. For example, in a recent study of Canada's Prairie Pothole Region (PPR), an ecologically important area containing over a million shallow wetlands, binary or ternary neonicotinoid mixtures were detected in 11 - 63% of wetlands sampled (n = 136 wetlands) at cumulative concentrations ranging from 0.004 to 1.66 µg/L (Main, 2016). Other environmental monitoring studies have reported similar neonicotinoid mixtures in rivers/streams (Hladik and Kolpin, 2015), other surface waters (e.g. ditches) (Schaafsma et al., 2015), and ground waters (Giroux and Sarrasin, 2011) across North America.

Despite the prevalence of neonicotinoid mixtures in aquatic environments, a relatively limited number of studies have focused on characterizing the cumulative toxicity of neonicotinoid mixtures to non-target organisms. As neonicotinoids all have a common mechanism of action, eliciting neurotoxicity by binding to and continuously activating nicotinic acetylcholine receptors (nAChRs), it is typically assumed that neonicotinoid mixtures will elicit directly-additive

cumulative toxicities. Therefore, the risk of neonicotinoid mixtures to non-target organisms is typically estimated using a predictive model based on the direct addition of constituent concentrations (i.e. Concentration Addition). Furthermore, current environmental regulations in Canada, the US, and Europe exclusively focus on single neonicotinoid compounds (Canadian Council of Ministers of the Environment, 2007; Morrissey et al., 2015; Smit, 2014; USEPA, 2017), and exclude potential cumulative effects of neonicotinoid mixtures. However, recent studies have shown that the assumption of directly-additive toxicity may not be adequately protective of non-target organisms. For example, it has been previously reported that binary and ternary mixtures of imidacloprid, clothianidin, and thiamethoxam can have greater- or less-than-additive effects on the freshwater midge *Chironomus dilutus* (*C. dilutus*) under acute (96-h) and chronic (28-d) laboratory conditions (Maloney et al., 2018b, 2017). Similarly, deviation from the assumption of directly additive cumulative toxicity has been reported for mixtures of other neonicotinoids (e.g. imidacloprid-thiacloprid) in laboratory assays using water fleas (*Daphnia magna*) and roundworms *(Caenorhabditis elegans*) (Gomez-Eyles et al., 2009; Loureiro et al., 2010; Pavlaki et al., 2011). However, limited broad-scale conclusions can be drawn from these neonicotinoid mixture toxicity assessments. This is primarily because the cumulative toxicity of neonicotinoid insecticides appears to vary depending on exposure time (e.g. acute vs. chronic), endpoint of interest (e.g. emergence, body length, or mortality), and experimental organism (e.g. *D. magna* vs. *C. dilutus*). Furthermore, most studies have been laboratory-based, focusing on the impacts of neonicotinoid mixtures on single species under environmentally controlled conditions. None of the reported mixture effects have been validated under more field-realistic conditions. Therefore, it is currently unknown how neonicotinoid mixtures may impact the organisms (and populations/communities) that typically inhabit contaminated aquatic environments.

Due to their distinct life-cycle traits (e.g. larvae and pupae are typically aquatic or semi-aquatic and these stages make up a majority of their life cycle), aquatic insects are likely to be exposed to neonicotinoid mixtures in wetland environments receiving agricultural runoff, like the PPR. Furthermore, as they are often physiologically similar to target pests, freshwater insects are likely to be sensitive to neonicotinoid mixture toxicity. Non-biting midges (Chironomidae) have been shown to be particularly sensitive to neonicotinoids (Morrissey et al., 2015). This is of concern because Chironomidae are ecologically important in aquatic environments. As the most ubiquitous and abundant insect family in freshwater ecosystems (Bataille and Baldassarre, 1993), Chironomidae play vital roles as primary consumers and act as food sources for a range of high trophic-level consumers, including predatory insects, fish, water fowl and other insectivorous birds, and bats (Benoit et al., 1997). Despite the high likelihood of their exposure to neonicotinoid mixtures, their sensitivity to neonicotinoids, and their relative importance in freshwater (and proximal terrestrial) ecosystems, the effects of neonicotinoid mixtures on natural Chironomidae populations have yet to be evaluated under field-realistic exposure conditions.

This study aimed to characterize the effects of chronic (28- and 56-d) exposure to three common neonicotinoid insecticides (e.g. imidacloprid (IMI), clothianidin (CLO), and thiamethoxam (TMX)) and their binary mixtures (e.g. IMI-CLO, CLO-TMX, IMI-TMX) to natural insect communities and Chironomidae populations under semi-controlled field conditions using limnocorrals (in-situ shallow wetland enclosures). The effects of single compounds were compared to the effects of binary mixtures at concentrations estimated to elicit equivalent toxicity (1 toxic unit (TU) based on the principle of Concentration Addition, derived from 28-d chronic laboratory studies (Maloney et al., 2018b)). This design allowed for the evaluation current risk assessment and regulatory approaches, investigating whether the use of laboratory-derived, single

compound EC_{50} values are adequately protective of natural Chironomidae populations, or if neonicotinoid mixtures also display greater-than-additive cumulative toxicity under semi-controlled field conditions.

4.2 Materials and Methods

4.2.1 Study site

Experiments were conducted in a single permanent (class V) prairie pothole wetland (Pond 2) at the St. Denis National Wildlife Area (NWA), Saskatchewan, Canada (52°12'N/106°5'W). The St. Denis NWA, currently under the management of Environment and Climate Change Canada, is a protected area primarily used for research and conservation of waterfowl and wetland-dependent fauna. Pond 2 was selected due to its consistent inter-annual central water depth (1.0 – 1.3 m), water quality characteristics (e.g. intermediary pH, conductivity, and dissolved oxygen), high insect secondary production, and water inflow from 'untreated' agricultural land (i.e. unexposed to pesticides). Prior use of this wetland for other semi-controlled field experiments provided historical reference values for insect abundance, composition, peak emergence times, and water quality (Cavallaro et al., 2018). Despite prior limnocorral studies, neonicotinoid contamination was not of concern as the limnocorrals were placed in physically different locations in Pond 2 than used in Cavallaro et al. (2018) (e.g. at the periphery rather than the pond centre) and water quality testing prior to the initiation of this study (~ 16 days of water column monitoring pre-dosing) indicated that there were no traces of neonicotinoid contamination within the isolated water columns of the experimental wetland. Sediment sampling was not carried out to avoid disturbance of the benthic community prior to the exposure period.

4.2.2 Experimental design

Twenty-one custom-built limnocorrals (fixed size: 1.0 x 1.0 m diameter; adjustable depth: < 1.5 m) purchased from Curry Industries Ltd (Winnipeg, MB, Canada) were employed in the experimental wetland. Limnocorral design was adapted from Cavallaro et al. (2018). Polyethylene sleeves were fastened to polyvinyl-encased Styrofoam floats (top), and the open bottom of the sleeves were sealed with a heavy steel chain buried in the sediment (~15 cm depth). Sediment seal was confirmed using an Aqua Scope II™ underwater viewer (Rickly Hydrological Co. Inc., Columbus, OH, USA). Limnocorrals were fitted with custom built aquatic insect emergence traps. These covered the entire open-air limnocorral surface and featured a removable acrylic collection chamber leading to a polypropylene jar containing ~400 mL of 70% ethanol for emerged insect collection. Due to variable weather patterns in the St. Denis NWA region (e.g. strong winds and storms), wooden stakes were secured to the corners via a nylon rope. ABS plumber's pipe rings (10 cm diameter) were used to anchor the limnocorral floats, allowing them to rise and fall with the natural water level. Extra material in the limnocorral walls allowed for small changes in water volume (i.e. expansion without disruption of the sediment seal). Biological heterogeneity within the wetland and seasonal water-depth variation was controlled for by randomizing treatments across three experimental blocks (3 x 7). Limnocorrals were secured at water depths of approximately 0.7 m. Limnocorral volumes, calculated from measured depths (i.e. length x width x height), ranged between 442.3 - 793.8 L (mean: 605.4 ± 92.9 L) and were not significantly different amongst neonicotinoid treatments or experimental blocks (one-way analysis of variance (ANOVA), $p > 0.05$). To avoid disturbing either the limnocorral sleeves or the sediments after the limnocorrals were secured in the experimental wetland, all experimental work (i.e. dosing, water sampling, and insect sampling) was conducted from a canoe.

4.2.3 Neonicotinoids

4.2.3.1 Experimental compounds

Technical grade neonicotinoid insecticides were used as experimental compounds in this study. Imidacloprid (IMI) (98.8% pure: *N*-(1-[(6-chloropyridin-3-yl)methyl-4,5-dihydroimidazol-2-yl]nitramide) and clothianidin (CLO) (99.6% pure: 1-[(2-chloro-1,3-thiazol-5-yl)methyl]-2-methyl-3-nitroguanidine) were acquired from Bayer Crop Science (Kansas City, MO, USA). Thiamethoxam (TMX) (98.8% pure: (*NE*)-*N*-[3-[(2-chloro-1,3-thiazol-5-yl)methyl]-5-methyl-1,3,5-oxadiazinan-4-ylidene]nitramide) was acquired from Syngenta Crop Protection LLC (Greensboro, NC, USA). Stock solutions were prepared by dissolving technical product in reverse-osmosis water (Barnstead® Diamond™ NANOpure, 18 MΩ/cm, Barnstead International, Dubuque, IA, USA) and solutions were stored in amber glass bottles (4°C in the dark) until experimental use.

4.2.3.2 Neonicotinoid treatments

Limnocorrals were treated with single neonicotinoid compounds (e.g. IMI, CLO, or TMX) or binary mixtures (e.g. IMI-CLO, CLO-TMX, or IMI-TMX) every 4 days for 56 days. Each treatment was replicated three times (n = 3). Three experimental controls (untreated) were also employed, yielding a total of 21 experimental limnocorrals. Neonicotinoid exposures began in spring, following ice-off and complete sediment thaw, and continued into early summer (May-July). Specific exposure lengths (e.g. 28 d) and conditions (e.g. semi-continuous rather than pulse neonicotinoid exposure) were chosen to allow for direct comparison of prior laboratory studies (Maloney et al., 2018b) to this semi-controlled field study. Overall exposure length (56 d) was selected based on a study by Cavallaro et al. (2018) that indicated that peak emergence of indigenous Chironomidae in a Prairie wetland occurred within 50 days of ice-off, thus 56 days was

expected to capture the full life cycle. Therefore, responses of single compound and binary mixture treated limnocorrals were compared at 28 d of exposure (study midpoint) to emulate the exposure lengths of prior laboratory studies, and 56 d of exposure (study cessation) to evaluate effects on the full Chironomidae life cycle.

Neonicotinoid concentrations were selected based on toxicity thresholds (28-d EC_{50} values for successful emergence) determined for a representative aquatic insect species (*Chironomus dilutus)* in previous laboratory studies (Maloney et al., 2018b). To allow for direct comparisons of effect, all treatments with single compounds or binary neonicotinoid mixtures were tested at equivalent toxic unit (TU) dose-ratios (1:1), and dose-levels (ΣTU = 1), yielding three different binary mixtures and three single-compound exposures. Here a TU was defined as the concentration (*c*) of a compound (e.g. IMI, CLO, or TMX) divided by its toxicity threshold (28-d EC_{50}):

$$TU = \frac{c}{EC_{50}} \qquad \text{(Eqn. 4.1)}$$

To determine if binary mixtures elicited greater-than-additive (synergistic) or less-than-additive (antagonistic) effects, toxicological response (e.g. Chironomidae emergence) was compared directly between limnocorrals treated with single compounds and theoretically equitoxic binary mixtures. Under the principle of Concentration Addition, treatments with equivalent ΣTU should yield equivalent toxicological responses (i.e. 50% of control response). Therefore, mixtures eliciting significantly greater toxicological responses than the single compound exposures were deemed 'greater-than-additive' and mixtures eliciting significantly lower toxicological responses than single compound exposures were deemed 'less-than-additive'.

Neonicotinoid concentrations in limnocorrals were measured prior to (pre-dose) and 1 h after (post-dose) dosing events (Section 4.2.3.3). Between dosing periods, treatment-specific

dissipation rates were estimated for each neonicotinoid compound. Dissipation rates were estimated by calculating the mean (± standard deviation) percent of active ingredient remaining in each limnocorral 4 d after dosing. Treatment- and limnocorral-specific 4-d dissipation rates, combined with estimated enclosure volumes, allowed for the concentrations of neonicotinoids in each limnocorral to be estimated and then dosing adjusted accordingly to maintain semi-continuous neonicotinoid exposures. Dosing solutions were prepared fresh for each treatment day by spiking 250 mL of culture water (carbon-filtered, bio-filtered Saskatoon municipal water, aerated in a 50-L Nalgene® carboy for >24 h prior to use) with concentrated neonicotinoid stock solution to achieve nominal test concentrations as follows: IMI (single compound = 0.50 µg/L; in binary mixtures = 0.25 µg/L), CLO (single compound = 0.71 µg/L; in binary mixtures = 0.36 µg/L), and TMX (single compound = 8.91 µg/L; in binary mixtures = 4.46 µg/L). Dosing solutions were then transferred into 250-mL amber bottles and transported in coolers to the study site (to limit thermal and photo-degradation). During each dosing event, test solutions were poured directly into limnocorrals, and each limnocorral was gently stirred with a paddle to ensure adequate mixing and equivalent neonicotinoid distribution throughout. To control for potential disturbance-related effects on aquatic insect communities, untreated controls were subjected to the same treatment as the other limnocorrals (e.g. addition of 250 mL of untreated culture water and stirring on each dosing day). Further mixing occurred through natural turnover within the pond, via wind and temperature-guided density changes.

4.2.3.3 Water sampling and neonicotinoid analysis

Prior to the initiation of the study, water samples were collected from Pond 2 to assess for neonicotinoid contamination (days -12, -8, -4, and -2). Over the course of the experimental period, water samples were collected from each limnocorral and analyzed to measure actual

concentrations of neonicotinoid exposure. Pre- and post-dosing, water samples were collected from each limnocorral (every 4 d over the course of the study). Pre-dose samples were collected immediately before limnocorral dosing. Post-dose samples were collected 1-h after limnocorral dosing (to allow limnocorrals to equilibrate after dosing/mixing events). Subsurface grab water samples were collected in 250-mL amber bottles from the centre of each limnocorral at >30 cm depth below the surface and stored at 4°C in the dark until analysis (within 14 d).

All samples were analyzed at the National Hydrology Research Centre, Environment and Climate Change Canada, Saskatoon, Canada, as described in Main et al. (2014). Briefly, neonicotinoid concentrations were quantified through solid-phase extraction (SPE) followed by high performance liquid chromatography paired with tandem mass spectrometry (LC-MS/MS). Methods adapted by Xie et al. (2011) allowed for simultaneous extraction and determination of IMI, CLO, and TMX concentrations in aqueous samples. SPE was performed using OASIS® HLB cartridges (Waters Corp., Milford, MA, USA). Internal standards (d_4-imidacloprid and d_3-thiamethoxam) were obtained from CDN Isotopes (Pointe-Claire, QC, Canada). LC-MS/MS was performed using a Waters 2695 Alliance HPLC system (Waters Corp., Milford, MA, USA) equipped with a Waters XTerra MS-C8 column, paired with a Micromass Quattro Premier triple quadrupole mass spectrometer (Waters Corp., Milford, MA, USA) equipped with an electrospray ionization interface (positive ion mode). Analytical standards obtained from Chem Service (West Chester, PA, USA) were used to create calibration curves and determine neonicotinoid recoveries. Limits of quantification (LOQ) and recovery correction factors (RC %) were as follows, IMI: LOQ = 0.0018 - 0.0043 µg/L (mean = 0.0028 µg/L), RC = 85.5 - 94.6 % (mean: 90.8 %); CLO: LOQ = 0.0026 - 0.0056 µg/L (mean = 0.0034 µg/L), RC = 65.2 - 74.7 % (mean: 69.8 %); TMX: LOQ = 0.0042 - 0.0077 µg/L (mean = 0.0058 µg/L), RC = 79.2 - 100.9 % (mean: 88.3 %). Measured

neonicotinoid concentrations in limnocorrals were recovery corrected and averaged across sample days. Mean measured concentrations were subsequently used in all statistical analyses.

4.2.4 Water quality

Dissolved oxygen (DO) and temperature were measured in each limnocorral on every dosing day using a hand-held Thermo ORION® dissolved oxygen meter, model 835 (Thermo ORION, Beverly, MA, USA). Due to prior studies indicating their relative stability in the limnocorrals over time (Cavallaro et al., 2018), all other water quality parameters were only assessed at the beginning (d 0), middle (d 28), and end (d 56) of the study. Water samples (50 mL) were removed from limnocorrals and analyzed in the laboratory for nitrate, phosphate, pH, conductivity, total hardness, alkalinity, ammonia (NH_3), and dissolved organic carbon (DOC) (< 12 h after sampling). Phosphate (PO_4) and nitrate (NO_3) were measured with an YSI EcoSense photometer, model 9300 (YSI Inc., Yellow Springs, OH, USA); pH was measured with an ORION® PerpHect LogR meter, model 170; hardness and alkalinity were measured using a Hach Digital Titrator, model 16900 (Hach Company, Loveland, CO, USA); ammonia was measured with a VWR™ SB301 sympHony ISE ammonia meter (VWR International Ltd., West Chester, PA, USA), paired with a Thermo ORION® electrode (Thermo ORION, Beverly, MA, USA); and DOC was measured using a Shimadzu Total Organic Carbon Analyzer (TOC-V), CPN model 5000 (Shimadzu Co., Kyoto, Japan).

4.2.5 Insect sampling, identification, and analysis

Adult insects were collected from the polypropylene sample jars on each emergence trap over the course of the exposure period (every 4 d for 56 d) to obtain cumulative abundance. To determine a baseline level for limnocorral productivity, insects were also collected prior to the dosing period (d -12, -8, -4, -2, and 0). Insects were collected by changing the entire collection jar,

and organisms were stored at 4°C (preserved in 70 % ethanol) until identified and counted. All collected insects were first identified to order. Diptera were further identified to family using dichotomous keys (Merritt et al., 2008). Adult Chironomidae were subsequently sexed. Male chironomids were separated from females via their more slender abdomens, genital appendages, and (typically) plumose antennae (Merritt et al., 2008). To determine the subfamily composition of collected Chironomidae, a subset of samples from control treatments were collected and sent to the Water Security Agency of Saskatchewan, Saskatoon, SK, Canada to be further identified. Briefly, larval head capsules were removed, and soft tissue was dissolved in a 10 % potassium hydroxide (KOH) solution. Head capsules were then mounted on glass slides using Euparal mounting medium and dried for 48 h. Taxa-specific key sand literature were subsequently used to accurately identify unique midge fauna (Diptera: Chironomidae) (Hirvenoja, 1973; Merritt et al., 2008; Oliver, 1976; Oliver and Rousssel, 1983). A voucher series, linking the identified Chironomidae to this research project, was deposited in the Water Security Agency of Saskatchewan Invertebrate Voucher Collection, Saskatoon, SK, Canada. All remaining collected Chironomidae were oven-dried (at 60°C for 24 h) and weighed to evaluate total biomass for each experimental day.

4.2.6 Data analysis

Water quality variables and neonicotinoid concentration data were averaged across time and then compared between treatments using one-way analysis of variance tests (ANOVAs) paired with Tukey's post-hoc analyses for pairwise comparisons between treatments (95 % level of confidence, $\alpha = 0.05$). Initial (baseline) cumulative total insect and Chironomidae abundances in limnocorrals were also compared across treatments using one-way ANOVAs paired with Tukey's post-hoc tests (95 % level of confidence, $\alpha = 0.05$). As baseline insect abundances were low (as it

was relatively early in the spring) and did not significantly differ among treatments (one-way ANOVA, $p > 0.05$) (Table A4.1), baseline insect abundance was not included as a variable in further analyses. Effects of the three experimental wetland blocks on abundance (total insect and Chironomidae) and biomass were tested using two-way ANOVAs, using experimental block and neonicotinoid treatment as factors (95 % level of confidence, $\alpha = 0.05$). Similar to other tested factors, experimental blocks did not significantly affect cumulative total insect or Chironomidae abundance or biomass (two-way ANOVA, $p_{interaction} > 0.05$), therefore block was not included as a variable in further analyses. Prior to comparative analysis, water quality, neonicotinoid concentration and emergence data were evaluated to ensure they met normality (Shapiro-Wilk, $\alpha = 0.05$) and homogeneity of variance (Brown-Forsythe, $\alpha = 0.05$) assumptions.

The mean, cumulative proportion of emerged insects in each order was compared amongst treatments using chi-square tests (χ^2, 95 % level of confidence, $\alpha = 0.05$). Prior to statistical analysis, data (cumulative Chironomidae abundance and biomass) were transformed [log (x + 1)] to meet normality (Shapiro-Wilk, $\alpha = 0.05$) and equality of variance (Brown-Forsythe, $\alpha = 0.05$) assumptions. To determine time-weighted treatment effects, cumulative Chironomidae abundance and biomass were compared across neonicotinoid treated limnocorrals over the course of the study (d 0 - 56) using two-way repeated measures (RM) ANOVA (fixed effect of time + treatment + time x treatment interaction) paired with the Holm-Sidak method for post-hoc pairwise comparisons (95% level of confidence, $\alpha = 0.05$). Cumulative Chironomidae abundance and biomass were analyzed at the two key time points, d 28 (study midpoint, allowing for direct comparisons to prior 28-d laboratory-based tests) and d 56 (study culmination, aimed to capture a more complete Chironomidae life cycle) using univariate statistical tests (i.e. one-way ANOVAs

paired with Tukey's post hoc tests (95 % level of confidence, $\alpha = 0.05$). All statistical analyses were performed using SigmaPlot™ Version 13.0 (Systat Software Inc., San Jose, CA, USA).

4.3 Results

4.3.1 Water quality

General water quality remained relatively consistent across time, and did not significantly deviate amongst individual limnocorrals, across experimental blocks, or amongst neonicotinoid treatments (one-way ANOVA, $p > 0.05$) (Table A4.2). Mean (± SD) water quality parameters during the dosing period were as follows: DO = 5.2 (± 0.7) mg/L, temperature = 20.5 (± 0.5) °C, pH = 8.1 (± 0.0), conductivity = 2934 (± 53) µS/cm^3, total hardness = 1708 (± 59) mg/L as $CaCO_3$, alkalinity = 414 (± 26) mg/L as $CaCO_3$, phosphate = 1.3 (± 0.5) mg/L, nitrate = 3.97 (± 0.89) mg/L, ammonia = 0.7 (± 0.3) mg/L, and DOC = 32.0 (± 2.4) mg/L).

4.3.2 Measured neonicotinoid concentrations

Mean measured IMI, CLO, and TMX concentrations in treated limnocorrals are presented in Table 4.1. Over the course of the study, mean (± SD) measured neonicotinoid concentrations remained within 99.2 ± 28.7 %, 104.4 ± 30.5 %, and 102.8 ± 48.0 % of the target nominal doses, respectively (Tables A4.3 and A4.4). Mean cumulative toxic units (ΣTU) ranged from 0.7 to 1.20 (target ΣTU = 1.0) and were not significantly different between treatments (one-way ANOVA, $p > 0.05$).

Occasional variation in neonicotinoid concentrations occurred between dosing days (e.g. target nominal concentrations were exceeded on some dosing days, with maximum ΣTUs ranging from 1.37 to 2.23 (Table A4.4)). However, these deviations from the target concentrations were relatively consistent across treatments. Despite experimental precautions, there were occasional

detections of low-level neonicotinoid contamination in treated limnocorrals (e.g. TMX detection in IMI treatments in 3/42 analyzed samples) and in experimental controls (Tables A4.3 and A4.4). However, these were typically ΣTU < 0.01 (or < 5 % of the lowest target dose), and were included in the calculated ΣTU, thus it is unlikely that this significantly influenced experimental results. Notably, in all TMX treated limnocorrals, there was slight degradation of TMX into CLO. On average, 0.32 ± 0.43 % of TMX was degraded into CLO (v/v) between 4 d dosing periods.

4.3.3 Insect diversity

Mean (± SD) cumulative proportions of emerged adult insects collected from experimental limnocorrals are presented in Table 4.2. Across treatments, Diptera were the dominant taxa, accounting for 92.5 ± 3.9 % of the total number of emerged insects over the course of the study. Overall, Chironomidae were the most abundant insect family, accounting for 89.3 ± 5.8 % of the total number of emerged insects across treatments. Within the Chironomidae, there was relatively low subfamily diversity: 93.3 ± 3.9 % were Chironominae, 4.3 ± 2.4 % were Orthocladiinae, and 2.4 ± 1.2 % were Tanypodinae. The remaining insect taxa primarily consisted of Odonata (dragonflies and damselflies) (4.4 ± 3.0 %) and Trichoptera (caddisflies) (2.2 ± 1.4 %). A limited number of Hymenoptera (parasitoid wasps) (0.5 ± 0.3 %), Coleoptera (beetles) (0.4 ± 0.4 %) and Ephemeroptera (mayflies) (0.0 ± 0.0 %) were also collected.

Neonicotinoid and neonicotinoid mixture exposure subtly altered insect community composition. For most orders (i.e. Diptera, Hymenoptera, Coleoptera, and Ephemeroptera), the mean proportions of emerged adult insects (d 56, cumulative) were not significantly different between treated and untreated limnocorrals, or amongst single compounds and binary mixtures (Chi-square test, $p > 0.05$). However, statistically significant, treatment-specific differences were found in the cumulative proportions of emerged Trichoptera ($\chi^2 = 25.0$, $p < 0.001$) and Odonata

(χ^2 = 100.6, p < 0.001) over the course of the study (e.g. 56-d cumulative). In all treatments, there were increases in the mean proportions of emerged Trichoptera relative to controls (e.g. 0.03 ± 0.0 % in controls vs. 1.1 ± 0.9 - 4.1 ± 5.5 % in treatments), and in IMI, IMI-CLO, CLO, and CLO-TMX treatments there were significant increases in the mean proportions of emerged Odonata relative to controls (e.g. 1.3 ± 1.1 % in controls vs. 4.8 ± 3.8 % - 9.7 ± 15.3 % in treatments). However, due to the large amount of variation amongst experimental replicates, the cumulative total abundances of Trichoptera and Odonata in treated limnocorrals (Table A4.5) were not significantly different from controls (one-way ANOVA, p > 0.05).

4.3.4 Chironomidae abundance and biomass

4.3.4.1 Time-weighted treatment effects

Cumulative abundance of emerged Chironomidae, the most abundant taxa, increased over time (two-way RM ANOVA, F = 129.8, p < 0.001) (Figure 4.1A). In the time-weighted analysis, treatment was statistically insignificant (two-way RM ANOVA, F = 2.20, p = 0.11). However, there was a significant interaction between treatment and exposure time across all treatments (two-way RM ANOVA, F = 1.39, p = 0.03), indicating that time was an important factor in treatment effects, with effects of neonicotinoid treatments on cumulative abundance becoming more pronounced over time (Figure 4.1A). Similarly, in all treatments, cumulative biomass increased over the course of the study (Figure 4.1B) (two-way RM ANOVA, F = 29.9, p < 0.001) and treatment was statistically insignificant in the time-weighted analysis (two-way RM ANOVA, F = 2.379, p = 0.085), but again there was a significant interaction between treatment and exposure time (two-way RM ANOVA, F = 2.2, p < 0.001). Therefore, for cumulative Chironomidae biomass, exposure time also enhanced treatment effects (Figure 4.1B).

Table 4.1 Mean (± SD) measured neonicotinoid concentrations (µg/L) and toxic units (TU) in single compound and binary neonicotinoid mixture treated limnocorrals over the course of the study period (56 days, 14 measurements). (n = 3 limnocorrals/treatment).

Treatment	**Measured Neonicotinoid Concentrations (µg/L)**			**Toxic Units (TU) [(1)]**			**Cumulative Toxic Units (ΣTU)**
	IMI	CLO	TMX	IMI	CLO	TMX	
CON	<0.01	-	<0.01	<0.01	-	<0.01	<0.01
IMI	0.52 ± 0.16	-	0.01 ± 0.03	1.03 ± 0.32	-	<0.01	1.03 ± 0.32
IMI-CLO	0.25 ± 0.08	0.34 ± 0.12	0.01 ± 0.04	0.49 ± 0.14	0.47 ± 0.17	<0.01	0.97 ± 0.32
CLO	-	0.73 ± 0.21	0.03 ± 0.06	-	1.03 ± 0.30	<0.01	1.03 ± 0.30
CLO-TMX	-	0.39 ± 0.10	5.80 ± 2.40	-	0.55 ± 0.14	0.65 ± 0.28	1.20 ± 0.38
TMX	<0.01	0.02 ± 0.03	9.31 ± 3.7	<0.01	0.03 ± 0.04	1.04 ± 0.42	1.09 ± 0.44
IMI-TMX	0.23 ± 0.07	0.03 ± 0.00	5.27 ± 2.19	0.47 ± 0.14	0.04 ± 0.01	0.58 ± 0.24	1.10 ± 0.37

* All unreported concentrations were lower than the limits of quantification (LOQ): imidacloprid = 0.0028 ± 0.0006 µg/L; clothianidin = 0.0034 ± 0.0008 µg/L; thiamethoxam = 0.0058 ± 0.0009 µg/L.

[(1)] Toxic units were calculated from laboratory-based 28-day EC_{50} values (emergence) previously characterized for *Chironomus dilutus* (Maloney et al., 2018b).

Table 4.2 Mean (± SD) proportions (%) of collected invertebrate taxa in untreated limnocorrals (control) compared to limnocorrals treated with single compounds or binary neonicotinoid mixtures for 56 days (cumulative), (n = 3 limnocorrals/treatment).

Treatment	**Diptera**		**Trichoptera**	**Hymenoptera**	**Odonata**	**Coleoptera**	**Ephemeroptera**
	Total	*Chironomidae*					
Control	98.1 ± 1.2	97.4 ± 2.1	0.3 ± 0.0	0.1 ± 0.0	1.3 ± 1.1	0.2 ± 0.0	0.0 ± 0.0
IMI	87.8 ± 14.6	86.2 ± 13.9	1.9 ± 0.6 [a]	0.4 ± 0.0	9.7 ± 15.3 [a]	0.2 ± 0.3	0.0 ± 0.0
IMI-CLO	93.8 ± 3.4	91.1 ± 5.1	1.1 ± 0.9 [a]	0.1 ± 0.2	4.8 ± 3.8 [a]	0.2 ± 0.3	0.0 ± 0.0
CLO	91.4 ± 2.1	87.5 ± 2.3	2.7 ± 1.6 [a]	0.8 ± 0.2	4.5 ± 1.6 [a]	0.6 ± 0.6	0.1 ± 0.2
CLO-TMX	87.5 ± 9.8	79.5 ± 14.6	4.1 ± 5.5 [a]	1.0 ± 1.6	6.2 ± 9.0 [a]	1.2 ± 0.9	0.0 ± 0.0
TMX	95.4 ± 2.3	93.8 ± 2.6	1.6 ± 1.3 [a]	0.6 ± 0.5	2.2 ± 1.7	0.6 ± 0.2	0.1 ± 0.1
IMI-TMX	93.3 ± 4.5	89.6 ± 7.3	3.6 ± 1.5 [a]	0.5 ± 0.6	1.9 ± 0.8	0.6 ± 0.5	0.0 ± 0.0
Average	92.5 ± 3.9	89.3 ± 5.8	2.2 ± 1.4	0.5 ± 0.3	4.4 ± 3.0	0.4 ± 0.4	0.0 ± 0.0

[a] Significantly different from control (Chi-square test, $p < 0.05$).

4.3.4.2 Single compounds

By d 28 (study midpoint), there were no statistically significant impacts of IMI, CLO, and TMX on cumulative Chironomidae emergence or biomass (relative to the controls) (one-way ANOVA, $p > 0.05$) (Figure 4.1C and D). However, IMI and CLO treatments reduced cumulative emergence by 92.0 ± 2.6 % (IMI) and 67.8 ± 6.5 % (CLO) relative to controls (vs. 50 % expected) (Figure 4.1C). A similar trend occurred for mean cumulative Chironomidae biomass (e.g. 86.2 ± 11.5 % (IMI), and 85.0 ± 6.6 % (CLO) reduction relative to controls) (Figure 4.1D). At the termination of the study (d 56), IMI and CLO treatments elicited statistically significant declines in cumulative Chironomidae emergence (Figure 4.1E) (Tukey post-hoc test, $n = 20$, $F = 5.1$, $p = 0.008$). Indeed, mean cumulative emergences were: 90.0 ± 1.4 % (IMI) and 85.4 ± 5.5 % (CLO) reduced relative to controls. At study termination (d 56), IMI and CLO treatments had also elicited statistically significant declines in the cumulative biomass of emerged Chironomidae (Figure 4.1F) (Tukey post-hoc test, $n = 20$, $F = 3.2$, $p = 0.03$), with mean cumulative biomasses that were 79.6 ± 6.0 % (IMI) and 81.4 ± 8.6 % (CLO) reduced, relative to controls. However, the TMX treatment did not elicit a statistically significant decline in either cumulative Chironomidae emergence or biomass on d 56, demonstrating 71.0 ± 7.9 % (emergence) and 51.7 ± 6.8 % (biomass) reduction relative to controls (Figures 4.1E and F).

4.3.4.3 Binary mixtures

Binary neonicotinoid mixtures elicited effects that deviated from lab-based predictions. On d 28, there were (statistically insignificant) declines in mean cumulative abundance relative to controls (e.g. % mean reduction in emergence (94.1 ± 1.7 % vs. 50 % expected) and biomass (97.5 ± 0.1 %) in the CLO-TMX treated limnocorrals (Figure 4.1C and D). At study termination (d 56), there were statistically significant reductions in Chironomidae emergence (one-way ANOVA, $n =$

20, F = 5.2, $p = 0.008$) and biomass (one-way ANOVA, $n = 20$, F = 3.2, $p = 0.03$) in the CLO-TMX treated limnocorrals, with a reductions of 85.4 ± 12.7 % and 86.5 ± 9.1 % for mean cumulative emergence and mean cumulative biomass relative to controls (Figure 4.1E and F). However, partially due to the high variability in all neonicotinoid (single compound and binary mixture) treatments, declines in emergence and biomass in CLO-TMX treatments were not significantly different from single compound treatments (Tukey post-hoc tests, $p > 0.05$). Therefore, although declines in cumulative abundance and biomass in the CLO-TMX treated limnocorrals were greater-than-predicted, the mixture response could not be categorized as greater-than-additive.

Due to high variation between individual limnocorrals, IMI-CLO and IMI-TMX mixtures did not elicit statistically significant declines in cumulative emergence or biomass on d 28 (Figure 4.1C and D). Indeed, reductions in cumulative emergence were 30.5 ± 67.3 % (IMI-CLO) and 46.0 ± 86.0 % (IMI-TMX) (Figure 4.1E) and reductions in cumulative biomasses were 47.5 ± 20.0 % (IMI-CLO) and 33.4 ± 54.8 % (IMI-TMX) (Figure 4.1F), relative to untreated controls. Toxicological responses in IMI-CLO and IMI-TMX treated limnocorrals were not significantly different from single-compound treatments (Tukey post-hoc tests: $p > 0.05$), thus the mixture effects had to be categorized as directly additive (Concentration Addition).

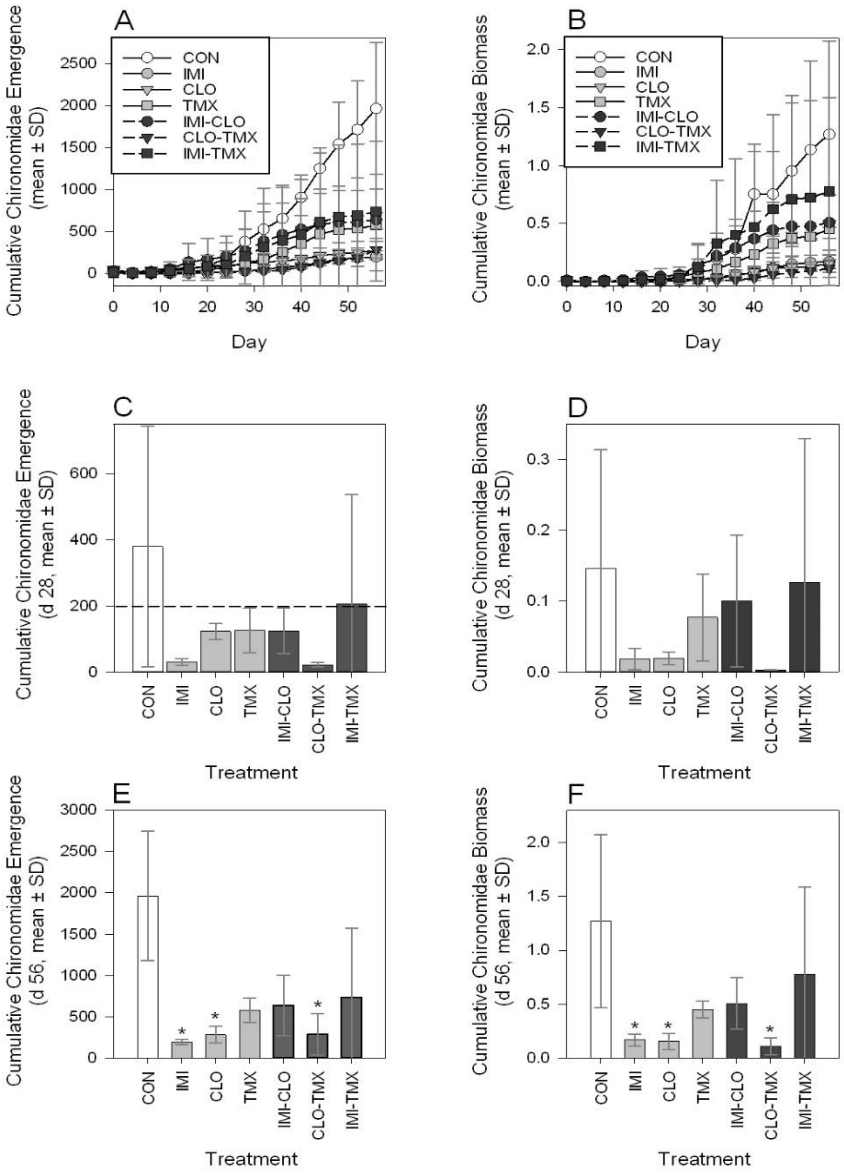

Figure 4.1 Chronic (56-d) exposures of Chironomidae populations to single compounds (imidacloprid (IMI), clothianidin (CLO), and thiamethoxam (TMX)) and binary neonicotinoid mixtures in experimental limnocorrals, compared to untreated controls: (A) mean (± SD) cumulative emergence and (B) biomass over time; (A) mean (± SD) cumulative emergence and (D) biomass at day 28 (study mid-point); and (E) mean (± SD) cumulative emergence and (F) biomass at day 56 (study cessation). (n = 3 limnocorrals/treatment, data.).

***** Asterisk indicates significantly different from untreated control (one-way ANOVA, $p < 0.05$).

**Dashed line (B) indicates predicted effect (Σ Toxic Unit = 1), based on 28-d EC_{50} values (emergence) derived from laboratory studies using the aquatic insect *Chironomus dilutus* as an experimental organism.

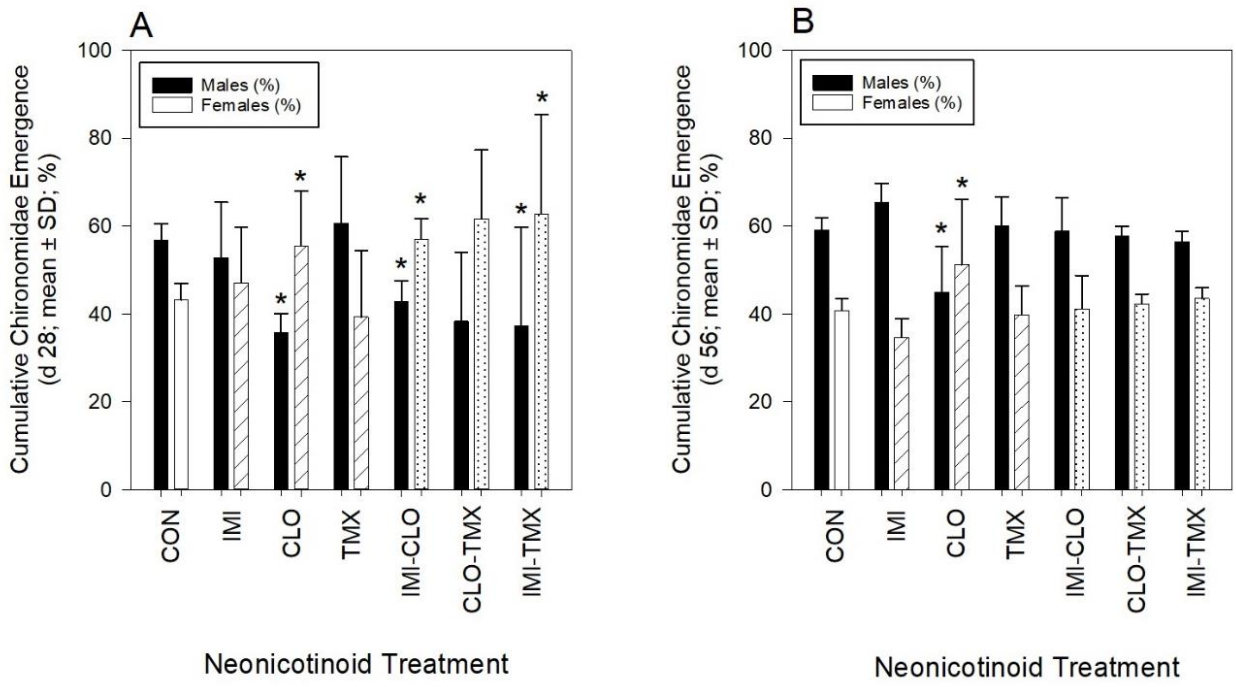

Figure 4.2 Sex of emerged Chironomidae (mean ± SD; %) after exposure to single neonicotinoids and their binary mixtures in experimental limnocorrals for (A) 28 and (B) 56 days. (n = 3 limnocorrals/treatment).

* Asterisk indicates significantly different from control (z-test, $p < 0.05$).

** Sex ratios for specific treatments (i.e. d 28 IMI and CLO, and d 56 CLO) did not equal 100% of total, due to variation of emerged proportions of males/females within treatment.

4.3.5 Chironomidae sex ratios

The impacts of single compound and neonicotinoid mixture exposure on the sex of emerged Chironomidae were also evaluated after 28 d (study midpoint) and 56 d (Figure 4.2A and B). In most neonicotinoid treatments, the cumulative proportions of male and female chironomids were similar to controls. However, there were a few notable exceptions. Statistically significant shifts toward female-dominated Chironomidae populations occurred at d 28 in CLO (55.5 % Female) ($z = 4.29$, $p < 0.001$), IMI-CLO (57.1 % Female) ($z = 3.25$, $p = 0.001$), and IMI-TMX treatments (62.8 % Female) ($z = 2.79$, $p = 0.005$), compared to controls (43.2 % Female) (Figure 4.2A). However, by the end of the study (d 56), with the exception of the CLO treatment (51.2 % Female) compared to controls (43.2 % Female) ($z = 4.32$, $p < 0.001$), sex ratios did not significantly differ between other treatments and controls (Figure 4.2B).

4.4 Discussion

4.4.1 Effects of single and neonicotinoid mixture exposures on aquatic insect communities

In this study, both theoretically equitoxic single-compound and neonicotinoid mixture exposures were found to slightly shift the taxonomic composition of the aquatic insect community. In all neonicotinoid-treated limnocorrals, there were statistically significant increases in the proportions of emerged Trichoptera, and in the IMI, IMI-CLO, CLO, and CLO-TMX treated limnocorrals there were statistically significant increases in the proportions of emerged Odonata. However, largely due to high within-treatment variation, there were no statistically significant increases in the absolute abundances of emerged Trichoptera and Odonata in the treated limnocorrals. Interestingly, although Diptera (e.g. Chironomidae) are known to be sensitive to neonicotinoids (Morrissey et al., 2015), the proportion of emerged dipterans were similar among treatments. This either indicates that effects on dipteran species were masked by variation (due to

the limited number of experimental replicates and variability associated with natural insect community composition), or that (as a taxonomic group) Diptera are not significantly more sensitive than other insect communities. This can be clarified by a thorough examination of the abundance data for all collected insect taxa (Table A4.5). In all neonicotinoid treated limnocorrals, there were marked reductions in emergence of Diptera (and thus Chironomidae) compared to untreated controls. Indeed, in neonicotinoid treated limnocorrals there were 2.6 - 9.8-fold reductions in mean dipteran abundance (and 2.7 - 10-fold reductions in mean chironomid emergence) compared to untreated controls. However, the significance of these differences was likely masked when evaluating proportional differences in emergence amongst insect communities. First, there was a large amount of variation amongst experimental replicates (e.g. 15.4 - 112.0 % variance in mean dipteran emergence and 14.2 - 112.0 % variance in mean chironomid emergence), which likely limited the statistical power of our comparative analysis and/or masked some of the differences between untreated controls and neonicotinoid-treated groups. In addition, in all limnocorrals, Diptera were the most dominant taxa, displaying abundances that far exceeded those of other taxa (e.g. 17.6 - 654-fold higher than that of Trichoptera, Hymenoptera, Odonata, Coleoptera, and Ephemeroptera) (Table A4.5). Thus, although neonicotinoid exposure did markedly reduce emergence of Diptera, effects were likely masked due to the high proportions of this taxon in all limnocorrals.

Although many studies have focused on the effects of anthropogenic disturbance on wetland invertebrate communities, there is currently limited scientific consensus on what shifts in community dynamics are likely to be ecologically significant (Batzer, 2013). In this study, the proportional changes observed in aquatic insect taxa were relatively small, with emergence of Odonata and Trichoptera in the neonicotinoid treated limnocorrals typically being 1 - 8 % higher

than in the controls (and Diptera being ~ 3 - 13 % lower than controls). Furthermore, these Prairie wetland communities were highly dominated by a few dipteran taxa, and the abundances of Odonata and Trichoptera were low in comparison. Similarly, a previous limnocorral study found that neonicotinoid (IMI, CLO, TMX) exposure did not elicit changes in wetland insect community composition (Cavallaro et al., 2018). Therefore, it is difficult to conclude if these shifts in community composition are likely to be ecologically significant and thus these observed community shifts should not be directly interpreted into potential effects on an ecosystem level.

4.4.2 Effects of neonicotinoid exposures on Chironomidae populations

Importantly, this study focused on determining if laboratory-derived toxicity models, developed using a representative aquatic insect species (*Chironomus dilutus*), could adequately predict the toxicity of three individual neonicotinoids and their binary mixtures to natural Chironomidae communities under semi-controlled field conditions. To accomplish this, wetland limnocorrals containing natural Chironomidae populations were exposed to effect-based neonicotinoid concentrations (derived from laboratory-based 28-d EC_{50} values), and cumulative toxicological effects were evaluated over time, focusing on endpoints of emergence and biomass, and at two specific time points [28 d (comparative to laboratory-based studies) and 56 d (aimed at capturing a more complete Chironomidae life-cycle)], focusing on endpoints of emergence, biomass, and sex-ratios (relative to controls). Although there were differences among individual limnocorrals (experimental replicates), effects of chronic single-compound and binary mixture exposures were found to significantly deviate from what was predicted from previous laboratory studies using current environmental risk assessment approaches.

4.4.2.1 Time-weighted treatment effects

This study specifically focused on evaluating neonicotinoid and neonicotinoid mixture toxicity at the two key time points (28- and 56-d). Therefore, this experiment was not specifically designed to focus on time-weighted effects of neonicotinoid insecticides (single compounds and binary mixtures) on Chironomidae populations. However, as a result of the study design (i.e. insect collection every 4 d over the course of the study), an analysis could be performed to evaluate the time-weighted effects of IMI, CLO, TMX and their binary mixtures on cumulative Chironomidae abundance (emergence) and biomass. As expected, we found that Chironomidae emergence and biomass were mainly affected by time (both increasing over the study duration). However, treatment effects were somewhat masked by intra-treatment variation. Indeed, when accounting for individual limnocorral identity, exposure time, and the time-treatment interaction, there were no statistically significant differences in cumulative Chironomidae abundance or biomass amongst the neonicotinoid treatments and experimental controls. Importantly, the interaction between time and treatment was determined to be significant, with treatment effects being highly dependent on exposure duration. This indicates that exposure time is critical when considering the toxicity of neonicotinoid compounds and their mixtures to Chironomidae and other similarly sensitive aquatic insect species. In fact, for neonicotinoids and their mixtures, time-weighted analyses might be more important than concentration- or toxicological response-based analyses in environmental risk assessment. Previous studies with lab-reared Chironomidae have shown that both single compound and neonicotinoid mixture toxicity can significantly vary depending on exposure time (Cavallaro et al., 2017; Maloney et al., 2018b, 2017). Therefore, further studies should be carried out to better evaluate the time-weighted toxicological effects of neonicotinoids and their mixtures to determine

if and how exposure duration and life cycle stage should be incorporated into neonicotinoid risk assessment for sensitive aquatic insect species.

4.4.2.2 Single neonicotinoid compounds

The effects of individual neonicotinoids on natural invertebrate populations are typically characterized using laboratory-based toxicity tests with standard laboratory species (e.g. *Daphnia magna* or *Chironomus dilutus*). However, in this study, laboratory-derived toxicity thresholds for neonicotinoid insecticides (single-compounds) on *C. dilutus* were not adequately predictive of the responses of natural Chironomidae populations. Indeed, most of the single-compound exposures were much more toxic than hypothesized based on the toxic unit approach. Applied neonicotinoid concentrations were chosen to elicit a predicted 50 % decrease in emergence of *C. dilutus* and other similarly sensitive species (e.g. a $\Sigma TU = 1$, based on 28-d EC_{50} values). However, after 28 d of exposure, abundances of emerged Chironomidae in IMI and CLO treated limnocorrals were 42 % and 17.8 % lower than predicted. Although some deviation should be expected (due to sensitivity differences between lab-reared and natural insect species and variation in dosing), the magnitude of deviation in single-compound effects observed in this study from laboratory-predicted toxicity was quite large. Furthermore, similar trends were observed after 56 d of exposure (e.g. 85.4 - 90 % declines in emergence and 86.2 - 85 % declines in the size/weight of Chironomidae, relative to controls), demonstrating the potential extents of IMI and CLO impacts on exposed Chironomidae communities.

4.4.2.3 Binary neonicotinoid mixtures

The effects of neonicotinoid mixtures are commonly estimated using the principle of Concentration Addition (CA). That is, toxicity is predicted by directly summing neonicotinoid concentrations (as toxic equivalents). In prior studies, mixtures of IMI, CLO, and TMX were

shown to elicit greater-than-additive toxicity in *C. dilutus*. Under acute (96 h) exposure scenarios, IMI-CLO and IMI-TMX were found to display cumulative toxicities that deviated from direct additivity, eliciting greater-than-additive (1 - 13 %) or less-than-additive (2 - 3 %) toxicities depending on mixture composition (Maloney et al. 2017)). Similarly, under chronic laboratory-based exposure scenarios, all three binary mixtures displayed cumulative toxicities that deviated from direct additivity, eliciting greater-than-additive (2 - 13 %) or less-than-additive (2 - 6 %) toxicities, depending on mixture composition (Maloney et al., 2018b). Furthermore, for IMI-TMX and IMI-CLO-TMX mixtures, the magnitude of synergism could actually decrease with increased exposure time (Maloney et al., 2018b). However, in this study, the mixture effects (on emergence) did not follow what was predicted by these laboratory-based toxicity tests. Contrary to laboratory predictions, there were no statistically significant differences in toxicological response amongst single compound and theoretically equitoxic neonicotinoid mixture treated limnocorrals, with IMI-CLO, CLO-TMX, and IMI-TMX mixtures displaying directly additive toxicities (i.e. behaving as predicted by CA). In addition, the relative responses to the neonicotinoid mixture treatments varied from what was predicted from laboratory studies. The CLO-TMX mixture, and not IMI-CLO or IMI-TMX mixtures elicited the most significant declines in Chironomidae emergence (e.g. 44 % greater-than-predicted impact on field Chironomidae than that was predicted from 28-d laboratory toxicity tests; 94 % reduction in emergence vs. 50 % predicted).

4.4.2.4 Chironomidae sex ratios

Previous laboratory studies have also indicated that exposure to neonicotinoids and neonicotinoid mixtures could potentially shift sex ratios toward male dominant populations (Cavallaro et al., 2017). Contrary to those laboratory-based predictions, this study found that

chronic neonicotinoid exposure did not shift sex ratios towards a greater proportion of males in natural Chironomidae populations. Exposure to some neonicotinoid treatments did elicit statistically significant shifts in sex-ratios relative to the control. For example, after 28 d of exposure there were shifts toward female-dominant populations in CLO, IMI-CLO, and IMI-TMX treatments. However, for most treatments, any statistically significant sex-ratio differences that were apparent at d 28 became statistically insignificant by d 56. In fact, CLO was the only neonicotinoid treatment that elicited a statistically significant sex-ratio shift over the full course of the study, resulting in an increase in the overall proportion of emerged female Chironomidae (e.g. an average 40.8 % females in the controls vs. 51.2 % females in the CLO treated limnocorrals). Therefore, as with Chironomidae emergence and biomass, the effects of neonicotinoid exposure on Chironomidae sex-ratios significantly deviated from what has been previously observed in laboratory tests.

4.4.3 Deviations from laboratory-predicted toxicity

Under semi-controlled field conditions, single compounds were generally more toxic than predicted by laboratory-based studies. The reasons behind these deviations are unclear, but there are several experimental factors that could have influenced the results. First, neonicotinoid concentrations occasionally fell above or below the target doses (Tables A4.3 and A4.4). One particular example of this was on d 8, where neonicotinoid concentrations were ~ 2 X higher than the target doses (e.g. d 8 measured ΣTUs ranged from 1.37 to 2.23, compared to the target ΣTUs of 1.0). These concentration spikes could have resulted in increased internal concentrations in the exposed organisms, which could have contributed to the greater-than-predicted toxicity observed in some of the neonicotinoid treatments (Focks et al., 2018). However, it is unlikely that these concentration spikes elicited acute toxicity in the exposed organisms, as these peak concentrations

were much lower than concentrations likely to cause lethality in the exposed invertebrates (e.g. IMI peak concentration = 0.94 µg/L vs. IMI 96 h LC_{50} for *C. dilutus* = 4.63 µg/L). In addition, these spikes were relatively consistent across mixture and single-compound treatments, thus any effects should have been equivalent across treatments. Furthermore, although the limnocorrals were occasionally overdosed, they were also occasionally under-dosed (Tables A4.3 and A4.4), with mean limnocorral concentrations remaining within the target ΣTU over the course of the study (Table 4.1). Thus, internal concentrations in the exposed organisms were likely stabilized over the course of the study. In short, although these concentration spikes could have contributed to the observed deviations from predicted toxicity, there were likely other contributing factors that influenced the experimental results.

Another reason for the observed greater-than-predicted toxicity in the single compound treatments could be that there are species-specific differences in neonicotinoid sensitivity amongst exposed Chironomidae. Although this has yet to be confirmed, previous studies have shown that sensitivity to water quality (e.g. both natural and anthropogenic stressors) greatly varies among different chironomid species (Odume and Muller, 2011; Orendt, 1999). Furthermore, species-specific differences in physiology, reproduction (e.g. multivoltine vs. univoltine), life-cycle length, and behaviour, along with ecological preferences, could result in different sensitivities to neonicotinoid insecticides. Unfortunately, since the biomass of the emerged Chironomidae was quantified using a destructive technique, there was no way to verify whether differences in species composition in the treated limnocorrals significantly contributed to these differential effects. In addition, the doses applied in this study were based on laboratory studies that used only one Chironomidae species (*C. dilutus*) in an environmentally controlled setting (Maloney et al., 2018b). In this semi-controlled field study, natural biotic and abiotic factors (i.e. predatory stress,

community dynamics, and species-specific differences in response) were present that could have influenced neonicotinoid toxicity. Thus, while this experiment offers an initial bridge between laboratory- and field-based toxicity studies, further studies are needed to determine how these biotic and abiotic factors could influence the species-specific toxicity of neonicotinoids to wetland insects.

Importantly, the response of natural Chironomidae populations to neonicotinoid mixtures also deviated from that predicted from laboratory mixture studies. This was primarily due to the high amount of variability among replicate limnocorrals, particularly in the mixture treatments, which potentially limited the interpretation of mixture effects. For example, for Chironomidae emergence (56 d exposure), the coefficient of variation (CV) for experimental controls was 39.9 % and for the single compound treatments CVs ranged from 14.2 to 27.4 % (Table A4.6). However, for the mixtures, variance was 2 - 4 times higher, with CVs ranging from 57.1 to 111.9 % (Table A4.6). Therefore, it is possible that mixture effects did occur, but they were masked by this large variation. Although variance was not as significant for Chironomidae biomass (Table A4.6), there was still a trend of higher variability in the mixtures (38.1 - 82.4 %) when compared to the single compound treatments (14.0 - 46.3 %). Increased variability can be an indicator of an ecological system experiencing stress (Forbes et al., 1995; Orlando and Guillette, 2001), so it is possible that these neonicotinoid mixtures were negatively impacting Chironomidae populations. Finally, the lack of greater-than-additive mixture effects observed in this study could also have been a factor of experimental design. Due to physical, practical, and financial constraints, the experimental design used in this study was kept relatively simple (i.e. mixtures were only tested at one dose-level ($\Sigma TU = 1.0$) and one dose-ratio (1:1)). However, as prior studies have demonstrated, mixtures tend to behave variably depending on composition and cumulative

concentration (Jonker et al., 2005; Maloney et al., 2017), as well as exposure time (Maloney et al., 2018b). Effects in this study were found to be time dependent, therefore the simplified exposure regime may have failed to capture deviations from direct additivity, as seen in previous laboratory-based studies. Further studies should thus focus on characterizing the toxicity of neonicotinoid mixtures under field-realistic settings with more extensive exposure ranges and ratios (i.e. aim to better characterize the toxicological response surface) and more replication, to determine if synergistic or antagonistic mixture effects do occur and if they pose a risk to natural Chironomidae populations.

4.4.4 Relevance to global water quality regulations

The neonicotinoid concentrations employed in this study were specifically chosen to evaluate if laboratory-based predictions of mixture toxicity would adequately translate to semi-controlled field conditions. Therefore, the doses applied were effect-based (designed to be equitoxic if the principle of Concentration Addition held (e.g. $\Sigma TU = 1$)), and in some cases higher than neonicotinoid concentrations typically observed in the field. In aquatic environments, IMI, CLO, TMX and their mixtures have been detected at maximum cumulative toxic units of 0.09 (agricultural surface waters) (Schaafsma et al., 2015; Smalling et al., 2015), 0.36 (rivers and streams) (Hladik and Kolpin, 2015), 4.38 (wetlands) (Main, 2016), and 32.0 (groundwater) (Giroux and Sarrasin, 2011). Some of the concentrations employed here were either equivalent to or lower than current neonicotinoid water quality guidelines. For example, in this study there were significant reductions in Chironomidae emergence and biomass in limnocorrals treated with CLO at concentrations (0.34 µg/L, mixtures; 0.73 µg/L, single compounds) substantially lower than the current United States Environmental Protection Agency (US EPA) aquatic life benchmark (1.10 µg/L) (USEPA, 2017). Similarly, in the TMX-treated limnocorrals, concentrations applied ranged

from 5.27 (mixtures) to 9.31 µg/L (single-compound), which are much lower than the current US EPA aquatic life benchmark of 17.5 µg/L (USEPA, 2017). Regulations for IMI are more stringent, thus the concentrations applied here were higher than most chronic water quality benchmarks (Smit, 2014; USEPA, 2017). However, IMI concentrations used in the IMI-CLO and IMI-TMX mixtures (0.23 - 0.25 µg/L) were approximately equivalent to current Canadian and European water quality guidelines (0.23 and 0.20 µg/L, respectively) (Canadian Council of Ministers of the Environment, 2007; European Food Safety Authority, 2006). Therefore, at concentrations equivalent to current water quality guidelines, Chironomidae emergence and biomass could potentially be affected in aquatic environments chronically contaminated with some single compounds (CLO and TMX), and all binary neonicotinoid mixtures. For example, an environmental monitoring survey of the Canadian PPR carried out by Main et al. (2014) showed that 5.5 - 8.2 % of wetland samples collected in early summer (June 2012 and 2013) contained neonicotinoid concentrations that were lower than current water quality guidelines, but cumulatively exceeded concentrations that were shown to significantly reduce Chironomidae emergence (under chronic exposure scenarios) in this study.

To be adequately protective of natural Chironomidae and similarly sensitive taxa (e.g. Ephemeroptera), the current aquatic regulatory values should be modified to account for enhanced neonicotinoid toxicity observed under field conditions. The US EPA and the National Institute for Public Health and the Environment (RIVM, Netherlands), have set chronic aquatic life benchmarks for IMI of 0.01 and 0.0083 µg/L, respectively (Smit, 2014; USEPA, 2017). According to this study (and previous laboratory toxicity tests), these benchmarks would be protective for natural Chironomidae populations and similarly sensitive aquatic insects. Therefore, other regulatory bodies (e.g. Canadian Council of Ministers of the Environment and the European Food

Safety Authority) should consider employing similar regulatory guidelines for IMI concentrations in aquatic systems. In previous studies, CLO and IMI have been shown to be approximately equitoxic to natural Chironomidae populations. Thus, to be adequately protective of Chironomidae species, water quality guidelines for IMI and CLO should be set at similar levels. Therefore, CLO chronic toxicity benchmark in the range of 0.0083 - 0.01 µg/L is recommended (Smit, 2014; USEPA, 2017). Under both laboratory and field conditions, TMX appears to be ~ 10 times less toxic to Chironomidae than IMI or CLO. Therefore, a freshwater, chronic toxicity benchmark for TMX that does not exceed 0.1 µg/L is recommended. Although this is likely protective for Chironomidae, it does not consider other more sensitive species (e.g. Ephemeroptera (Van den Brink et al., 2016)). Therefore, further studies with other sensitive aquatic invertebrates should be considered when deriving and modifying current water quality benchmarks. Importantly, along with deriving regulatory recommendations for neonicotinoids and their mixtures for the protection of some important non-target aquatic insect species, this work stresses the importance of complementary field-based studies to better characterize toxic responses elicited by neonicotinoids and their mixtures when setting regulatory guidelines.

CHAPTER 5: LINKING NEONICOTINOID TOXICITY TO NICOTINIC ACETYLCHOLINE RECEPTOR BINDING AND EXPRESSION IN CHIRONOMIDAE

Preface

This chapter builds upon previous research demonstrating species-, compound- and life-stage specific differences in neonicotinoid toxicity (Chapters 2 - 4) by evaluating binding profiles of imidacloprid, clothianidin, and thiamethoxam to chironomid nicotinic acetylcholine receptors (nAChR). Using radioligand binding assays with [^{3}H]-imidacloprid, nAChR density and binding affinity were compared between two different midge species (*Chironomus riparius* vs. *Chironomus dilutus*), at two different life stages (larval vs. adult), with the three different neonicotinoid insecticides of interest (imidacloprid vs. clothianidin vs. thiamethoxam). Results indicated that some (but not all) previously observed toxicological patterns likely result from differential nAChR expression and/or neonicotinoid binding. For example, larval organisms displayed significantly higher nAChR densities and binding affinities than adults and relative binding affinities of the three neonicotinoid compounds were similar to their relative toxicities in *C. riparius* and *C. dilutus*, indicating that life stage- and compound-specific toxicity is likely influenced by receptor binding characteristics. However, there were no significant differences in receptor binding or density between the two chironomid species, indicating that species-level differences in sensitivity are unlikely to be driven by differential nAChR binding profiles. Furthermore, comparison of Chironomidae binding data (generated in this study) to those previously derived for other insects (agricultural pests and non-target dipteran species) indicated that Chironomidae tend to display relatively high densities of nAChR that bind neonicotinoids with relatively high affinity. This provides one potential explanation for the marked sensitivity of Chironomidae to neonicotinoid insecticides. This chapter presents novel information, yielding

mechanistic explanations for previously observed patterns of neonicotinoid toxicity in Chironomidae and presents new techniques that can help guide future research focusing on evaluating the mechanistic effects of neurotoxic compounds in aquatic insect species.

This chapter will be submitted to Aquatic Toxicology, under joint authorship with Karsten Liber (University of Saskatchewan), Christy A. Morrissey (University of Saskatchewan), Emiliane Taillebois (Université d'Orléans), Dennis Servent (Commissariat à l'Énergie Atomique et aux Énergies Alternatives), Nicholas Gilles (Commissariat à l'Énergie Atomique et aux Énergies Alternatives), and Steeve H. Thany (Université d'Orléans).

5.1 Introduction

Neonicotinoids are a group of neurotoxic insecticides, commonly applied to protect young crops against biting-piercing agricultural pests. These compounds display broad pest control spectra, low mammalian toxicity, and are highly versatile in application (Jeschke and Nauen, 2008). Thus, over the past two decades, neonicotinoids have come to dominate global agrochemical markets (Jeschke et al., 2010). Originally based on the molecular structure of nicotine, neonicotinoids elicit toxicity in exposed insects by acting on nicotinic acetylcholine receptors (nAChR) (Kagabu, 2011). These compounds are structurally similar to the endogenous nAChR agonist acetylcholine (ACh) (Jeschke and Nauen, 2008), so they can bind directly to agonist binding sites, activating the receptor and propagating action potentials. However, unlike ACh, neonicotinoids are not degraded by acetylcholinesterase (Thany, 2011). Thus, once bound, these insecticides can continuously stimulate action potentials, progressively causing uncontrollable muscle tremors, cell energy exhaustion, paralysis, and eventually death in exposed insects (Tomizawa and Casida, 2004).

Insect central nervous systems typically contain high densities of nAChRs (Jones and Sattelle, 2010; Millar and Denholm, 2007). Thus, along with being the primary molecular targets of neonicotinoids, nAChRs are likely to be highly important for normal physiological function. Responsible for fast transmission at neuronal and neuromuscular junctions (Jones and Sattelle, 2010; Millar and Denholm, 2007), nAChRs are composed of five related subunits arranged around a central cation selective pore (Hendrickson et al., 2013; Uzman, 2001). Each subunit contains four hydrophobic transmembrane domains (TM1 - 4), and a large (~ 200 amino acid) N-terminal extracellular domain (Millar, 2003). Agonist binding sites are located at the interface of two adjacent subunits (Corringer et al., 2000). Insect nAChRs are difficult to stably express using *in*

vivo and *in vitro* techniques, thus they remain poorly characterized (Tomizawa and Casida, 2001). However, genetic analyses have indicated that insects tend to express a core group of nAChR subunits that is highly conserved across species (> 60% homology in amino acid sequence), and at least one species-specific subunit (< 20 % homology in amino acid sequence), which is thought to contribute to species-specific differences in nAChR function (Jones et al., 2007).

Binding properties and densities of insect nAChRs are typically investigated by evaluating receptor response to standard agonists (e.g. α-bungarotoxin (α-BGT), and epibatidine) and/or well-characterized neonicotinoid insecticides (e.g. imidacloprid (IMI), clothianidin (CLO), thiamethoxam (TMX)) (Taillebois et al., 2018). These neuro-pharmacological studies have indicated that there are different functional subtypes of insect nAChRs, which can influence affinity for and sensitivity to agonists like neonicotinoid insecticides (Matsuda et al., 2001), and that binding and receptor density tend to vary between taxonomic orders, individual species, and different life-stages (Crossthwaite et al., 2017; Eastham et al., 1998; Schloss et al., 1988; Taillebois et al., 2018). However, the majority of neonicotinoid research has focused on insect targets (i.e. agricultural pests, standard insect test species, and disease vectors) (Crossthwaite et al., 2017; Taillebois et al., 2018). In fact, there are no published studies in the open literature which have characterized the functional expression of nAChRs in non-target insect species that can be unintentionally exposed to neonicotinoids. Thus, it is currently unknown if these functional subtypes also exist in insect species and if functional expression of the nAChRs can influence affinity for and sensitivity to neonicotinoids.

Of recent concern are the ecotoxicological effects of neonicotinoids on aquatic insects (Sánchez-Bayo et al., 2016). Due to their unique physicochemical properties (e.g. high water solubilities and poor degradation in light-limited environments) (Jeschke et al., 2010; Lu et al.,

2015), neonicotinoids can easily move into nearby aquatic systems following agricultural application (Goulson, 2013). Thus, aquatic insects (who spend most their life cycles in aquatic systems) can be unintentionally subjected to repeated and/or prolonged exposures to these neurotoxic insecticides. One group of aquatic insects that has been shown to be markedly sensitive to neonicotinoids are Chironomidae (Raby et al., 2018a). Chironomidae are highly abundant, diverse, and ecologically important, representing food sources for birds and fish (Benoit et al., 1997; Oliver, 1971). Therefore, it is important to comprehensively characterize the risk that neonicotinoids may pose to these sensitive and ecologically important insects. Recently, a large number of studies have focused on evaluating the impacts of neonicotinoids on Chironomidae (e.g. Cavallaro et al. 2017; Maloney et al. 2018a; Raby et al. 2018a). However, toxicological effects tend to vary depending on individual species, length of exposure, and neonicotinoid compound (Raby et al., 2018a, 2018b), which can make it difficult to predict population-level effects using laboratory-derived toxicity data (e.g. Maloney et al. 2018a).

In this study, we aimed to move beyond standard ecotoxicity testing and characterize potential neuropharmacological drivers of these ecotoxicological patterns in Chironomidae. Our objectives were to: 1) use radioligand binding assays to characterize nAChR densities and binding profiles in two chironomid species (*Chironomus riparius* and *Chironomus dilutus*), at two distinct life-stages (larval and adult), using three common neonicotinoids (IMI, CLO, and TMX); and 2) compare our collected data to those previously reported for agricultural pests and standard test insects with differing neonicotinoid sensitivity. By investigating some of the underlying mechanisms driving the expression of neonicotinoid toxicity in non-target aquatic insects, this study focused on providing information that could both help inform future nAChR insecticide development and guide predictive risk assessments for neonicotinoids in aquatic environments.

5.2 Materials and Methods

5.2.1 Experimental organisms

Chironomus dilutus were obtained as egg cases from Aquatic Biosystems Inc. (Fort Collins, CO, USA) and reared in an environmentally controlled chamber at the Toxicology Centre, University of Saskatchewan, Canada. *Chironomus riparius* were obtained as egg cases from the Centre Ecotox (EPFL ENAC IIE-GE, Lausanne, Switzerland) and grown in an environmentally controlled chamber at Laboratoire de Biologie des Ligneux et des Grandes Cultures (LBLGC, Université d'Orléans, Orléans, France). Consistent with published protocols (Environment Canada, 1997), Chironomidae were reared in 50-L aquaria (3 - 6 egg cases/tank) under constant temperature (23 ± 1°C), photo-period (16 h light: 8 h dark), and illumination (500 - 1000 lux). *Chironomus dilutus* cultures were reared in aquaria containing 30 L of culture water, which consisted of carbon and bio-filtered Saskatoon municipal water, aerated in a 50-L Nalgene® carboy for >24 h prior to use. *Chironomus riparius* cultures were reared in aquaria containing 5 or 10 L of culture water, which consisted of reconstituted deioinized water (0.37 mM $CaSO_4$, 0.45 mM $CaCl_2$, 0.25 mM $MgSO_4$, 1.14 mM $NaHCO_3$, 0.05 mM KCl) (United States Environmental Protection Agency, 1994), aerated in a 20-L container for >24 h prior to use. For both Chironomidae cultures, water was renewed every 2 - 3 days and organisms were fed ~ 300 mg of Nutrafin® (Rolf C. Hagen Inc., Montreal, QC, Canada) fish food every 1 - 2 days. Water chemistry analyses were routinely completed, yielding the following results [mean ± standard deviation (SD)]: dissolved oxygen (DO) 6.91 ± 1.4 mg/L; temperature 21.05 ± 1.1 °C; unionized ammonia (NH_3) 0.93 ± 1.88 mg/L; pH 7.93 ± 0.43; conductivity 418 ± 5 µS/cm; total hardness 115 ± 42 mg/L as $CaCO_3$; alkalinity 127 ± 39 mg/L as $CaCO_3$; and total chlorine 0.01 ± 0.02 mg/L. Experimental organisms were isolated at two different life stages: larval and adult. Larvae were

siphoned directly from culture tanks when they were in approximately third-instar (17 days post-hatch for *C. dilutus* and 12 days post-hatch for *C. riparius*) and removed from their cases prior to further analysis. Adult Chironomidae were removed from culture tanks following successful emergence (daily) via manual aspiration. Following isolation, experimental organisms at both life stages were flash-frozen in liquid nitrogen and stored at -80 °C until further use.

5.2.2 Membrane protein preparation

Membrane proteins were isolated from whole, frozen insects at the LBLGC, Université d'Orléans, France. To identify the optimal methods to use in membrane protein extraction, preliminary studies were completed using whole, frozen Chironomidae larvae (mixed culture) under a range of extraction conditions (Table A5.1) (Liu and Casida, 1993; Taillebois et al., 2018; Wiesner and Kayser, 2000). Membrane protein extraction was ultimately carried out using methods adapted from Wiesner and Kayser (2000) and Taillebois et al. (2014). The dissociation medium (pH = 7.0) was composed of 20 mM of sodium phosphate, 150 mM of sodium chloride, 1 mM of ethylenediaminetetraacetic acid (EDTA), 0.1 mM of phenylmethyl sulfonyl fluoride (PMSF), 2 µg of pepstatin (dissolved in methanol), 2 µg chymostatin (dissolved in dimethyl sulfoxide (DMSO)), and 2 µg leupeptin (dissolved in deionized water). Experimental organisms were homogenized in dissociation medium with a pellet pestle motor (4°C, 6 mL/g of insects). Samples were then centrifuged for 10 minutes at 1000 *g* (4°C), and the supernatant collected (SN1). The pellet was then resuspended in dissociation medium (4°C, 3 mL/sample) and the solution homogenized with a pellet pestle motor (on ice). The homogenized sample was then centrifuged again (10 min, 1000 *g*, 4°C) and the supernatant collected (SN2). Supernatants (SN1 and SN2) were combined and then ultra-centrifuged for 30 min at 43000 *g* (4°C). The precipitated pellet was then washed with cold dissociation medium (1 mL) and ultra-centrifuged again (30 min,

43000 *g*, 4°C). The final protein pellet was resuspended in cold dissociation medium (2-5 mL/sample, 4°C). Total protein was quantified via Pierce™ Coomassie (Bradford) Protein Assays (Thermo Scientific, Pierce Biotechnology, Rockford, IL, USA) using bovine serum albumin as the protein standard. Following quantification, membrane preparations were stored at -80°C until further use.

5.2.3 Binding assays

Binding assays were completed at the Service d'Ingénierie Moléculaire des Protéines (SIMOPRO), Commissariat à l'Énergie Atomique et aux Énergies Alternatives (CEA) Paris Saclay, Gif-Sur-Yvette, France. Due to practical and financial constraints associated with radioligand binding assays, this study exclusively focused on characterizing the binding profiles of IMI-sensitive nAChRs (which are typically classified as the 'neonicotinoid-sensitive' receptor subtypes) (Crossthwaite et al., 2017; Taillebois et al., 2018). Thus, [^{3}H]-imidacloprid ([^{3}H]-IMI) (40 Ci/mmol, American Radiolabeled Chemicals, St. Louis, MO, USA) was exclusively used as the radiotracer for all binding experiments. Saturation and competition binding experiments were carried out under consistent experimental conditions. Binding reactions had a final volume of 200 µL, and Tris-HCl (10 mM, pH = 7.4) was used as the reaction buffer. Reactions were incubated in 96-well microplates for 3 h at room temperature (23°C) and terminated via rapid vacuum filtration using GF/C glass microfiber filter plates presoaked in 0.5% polyethyleneimine. Following reaction termination, filter plates were rapidly rinsed (< 20 s) with cold Tris-HCl buffer (10 mM, pH = 7.4) and dried. Scintillation liquid was added to each well (20 µL/well) (Perkin-Elmer, Waltham, MS, USA) and plates immediately counted on a β-counter (TopCount NXT, Hewlett Packard, San Diego CA, USA).

Saturation binding studies were carried out to characterize and compare receptor density and ligand ([^{3}H]-IMI) affinity in experimental organisms. Larval saturation binding experiments were carried out by incubating 2.5 - 5 µg of total membrane protein (varying between independent experiments) with [^{3}H]-IMI concentrations ranging from 6.8 pM to 20 nM, to obtain complete binding curves. Adult saturation binding experiments were carried out by incubating 10 µg of total membrane protein with [^{3}H]-IMI concentrations ranging from 22.9 pM to 50 nM, to obtain complete binding curves. At each concentration of [^{3}H]-IMI, non-specific binding (NS) was measured by adding 10 µM of unlabeled IMI to a subset of samples prior to membrane incubation. To account for potential quenching effects, blank filter (BF) binding was measured by adding only [^{3}H-IMI] to a subset of wells. Each saturation test was replicated twice, with two total binding (BT), two NS replicates, and two BF replicates per [^{3}H]-IMI concentration (n = 4 BT and 4 NS / [^{3}H]-IMI concentration).

Competition binding assays were carried out to characterize and compare receptor binding to three different neonicotinoid insecticides of ecotoxicological interest (IMI, CLO, TMX) in *C. dilutus* and *C. riparius*. Due to experimental constraints (e.g. assay cost and availability of protein) and ecotoxicological considerations (e.g. limited neonicotinoid exposure at adult life stages), competition binding assays were only carried out using larval organisms. Competitive binding experiments were carried out by incubating 2.5 - 10 µg of total membrane protein (varying between independent experiments) with a fixed concentration of radiolabeled [^{3}H]-IMI (0.5 nM) and unlabelled competitors (IMI, CLO, TMX) at a range of concentrations to obtain complete inhibition curves. Concentrations of unlabeled competitors (*C. riparius:* IMI/CLO = 0.10 nM - 217 nM, TMX = 0.64 nM - 38.23 µM; *C. dilutus*: IMI/CLO = 3.4 pM - 24 nM, TMX = 0.53 nM - 3.51 µM) were chosen based on prior binding studies (Crossthwaite et al., 2017; Taillebois et al.,

2018) and preliminary range-finding tests. To quantify non-specific binding (NS; addition of 10 µM of unlabeled IMI), quenching effects (BF), and total binding (BT_{comp}; experimental controls, no added competitor), an additional set of reactions (*n* = 3 BT, 3 NS, and 2 BF / competition experiment) were run for each competition binding experiment. Each competition test was replicated twice, with two or three competitive binding (BC) replicates per concentration of unlabeled competitor (*n* = 5 - 6 BC / competitor concentration).

5.2.5 Data analysis

Analysis of data from binding experiments was performed using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA). To account for differing quantities of protein used between independent experiments, binding data were normalized prior to analysis. In saturation binding assays, binding data were normalized to protein concentration (i.e. analyzed as pmol of bound ^{3}H-IMI/mg protein). In competition binding assays, binding data were normalized to total binding in experimental controls (i.e. analyzed as % bound ^{3}H-IMI relative to BC). Prior to analysis, binding data were corrected to account for biological activity of the radioligand (Figure A5.1) and evaluated for ligand depletion (if ligand depletion was > 30%, data point was considered unreliable thus was repeated and/or excluded from analysis).

Normalized binding data were analyzed via non-linear regression. For saturation binding, maximal binding parameters (B_{max}; indicative of receptor density) and dissociation constants (K_D; indicative of ligand affinity) were derived by fitting specific binding data to Equation 5.1:

$$Y = \frac{B_{max} \times [L^*]}{K_D + [L^*]} \qquad \text{(Eqn. 5.1)}$$

where Y represents the concentration of [^{3}H]-IMI bound (pmol/mg protein) and [L*] represents the concentration of ^{3}H-IMI (nM). Scatchard plots were used to further visualize model fits.

Briefly, the data were transformed and total amount of bound ligand ([Bound]) was plotted against the amount of bound ligand divided by its free concentration ([Bound]/[Free]). Axis intercepts were derived using previously computed saturation binding parameters (Y axis: X = 0, Y = B_{max}/K_D; X intercept: X = B_{max}, Y = 0).

For competition binding assays, the median inhibitory concentrations of unlabeled IMI, CLO, and TMX (IC_{50}; indicative of functional strength of competitor) were calculated by fitting specific binding data to Equation 5.2, and inhibition coefficients (K_i; indicative of competitor affinity for receptor) were derived using the Cheng and Prusoff equation (Equation 5.3):

$$Y = Y_{min} + \frac{(Y_{max} - Y_{min})}{(1 + 10^{(x - \log(IC_{50}))})} \qquad \text{(Eqn. 5.2)}$$

$$K_i = \frac{IC_{50}}{1 + (\frac{[L^*]}{K_D})} \qquad \text{(Eqn. 5.3)}$$

where Y represents the percentage of bound ^{3}H-IMI relative to the untreated control (BT in competition binding assay), *x* represents the logarithmic concentration of the competitor (log[ ], nM), IC_{50} represents the median inhibitory concentration of the competitor, [L*] represents the concentration of ^{3}H-IMI (nM), and K_D is the dissociation constant for ^{3}H-IMI (Equation 5.1).

Statistical analysis was performed using SigmaPlot 14 (Systat Software Inc., San Jose, CA, USA). One-way analyses of variance (ANOVA) analyses paired with Tukey's post-hoc tests (α = 0.05) were used to characterize statistical differences between binding parameters. For saturation binding assays, binding parameters (K_D and B_{max}) were compared between life stages (larvae vs. adult) and across Chironomidae species (*C. dilutus* vs. *C. riparius*). In competition binding assays, K_i and IC_{50} values were compared among neonicotinoid compounds (IMI vs. CLO vs. TMX) and between species (*C. dilutus* vs. *C. riparius*).

5.2.6 Comparison of Chironomidae nAChR binding profiles to other Insecta

To investigate whether the marked neonicotinoid sensitivity in Chironomidae is likely to be receptor mediated (i.e. arise due to differences in nAChR density or neonicotinoid binding), results obtained in saturation binding assays were compared to those previously derived for other insect species. Specifically, binding and toxicity data were collated from the published literature, and receptor densities, binding affinities, and IMI toxicity were compared between Chironomidae, agricultural pests, and other dipteran insects commonly used in efficacy/mode of action testing. Binding and nAChR density data were primarily derived from two recent literature reviews, Crossthwaite et al. (2017) and Taillebois et al. (2018), and included five hemipteran pests (*Myzus persicae* (green peach aphid), *Acyrthosiphon pisum* (pea aphid), *Aphis craccivora* (cotton aphid), *Nephotettix cincticeps* (green rice leafhopper), and *Nilaparvata lugens* (brown planthopper)) one orthoperan pest (*Locusta migratoria* (migratory locust)), two lepidopteran pests (*Manduca sexta* (tobacco hornworm) and *Heliothis virescens* (tobacco budworm)), and two dipteran test insects (*Drosophila melanogaster* (common fruit fly) and *Musca domestica* (housefly)). Hemipteran and orthopteran species tend to display two binding sites, one with high neonicotinoid affinity and one with low neonicotinoid affinity (Crossthwaite et al., 2017; Taillebois et al., 2018). For each species, binding data were averaged and presented as mean ± standard error (SE). Acute IMI toxicity data (24 - 96 h L/EC_{50}; endpoint = lethality or immobility) were also collated from a range of studies and compared across insect species (Abbas et al., 2015; Abd-Ella, 2014; Eure et al., 2018; European Food Safety Authority (EFSA), 2014; Frantzois et al., 2008; Jairin et al., 2005; Kaufman et al., 2006; Lagadic et al., 1993; Maloney et al., 2017; Matsuda et al., 2009; Nauen et al., 1998b, 1998a; Ohkawara et al., 2002; Parkinson et al., 2017; Posthuma-Doodeman, 2008; Raby et al., 2018a; Taillebois et al., 2014; Tang et al., 2013; Wang et al., 2009; White et al., 2007). To

ensure comparability to Chironomidae toxicity estimates, toxicity data were preferentially selected from studies that presented results in liquid concentration units (e.g. ng/L - mg/L). Due to the wide range of methods applied across test species (e.g. aquatic toxicity tests, leaf dip bioassays, and artificial feed assays), there was occasionally significant variability in reported toxicity values. Therefore, to be conservative, the lowest reported toxicity estimate was used to compare toxicological effects between Chironomidae and the other insect species.

5.3 Results

5.3.1 Saturation binding

Complete saturation binding curves (mean ± standard error (SE); Figure 5.1) were obtained for *C. riparius* and *C. dilutus* at both larval and adult life stages, allowing for the derivation of binding affinity (K_D) and receptor densities (B_{max}) (presented as mean, (95 % confidence intervals); Table 5.1) for all tested organisms. Overall, experimental data provided good fits for saturation binding curves (R^2 = 0.84 - 0.92) for both larval and adult binding assays (Figure 5.1, Table 5.1). Saturation binding assays revealed that both Chironomidae species displayed a single ^{3}H-IMI binding site (Figure 5.1), which was supported by a lack of slope change observed in Scatchard representations (Figure 5.1, insets).

In both Chironomidae, larval organisms expressed significantly higher densities of nAChRs than adult organisms (*C. riparius*, q = 15.1, $p < 0.001$; *C. dilutus*, q = 24.0, $p < 0.001$; Table 5.1). For *C. riparius* there was approximately a 3-fold difference in nAChR density between life stages (Figures 5.1A, C), with larvae displaying a B_{max} of 5.10 (4.43 - 5.77) pmol/mg membrane protein and adults displaying a B_{max} of 1.57 (1.37 - 1.78) pmol/mg membrane protein. For *C. dilutus* there was approximately a 7-fold difference in nAChR density (Figures 5.1B, D), with larvae displaying a B_{max} of 6.52 (5.88 - 7.17) pmol/mg of membrane

protein and adults displaying a B_{max} of 0.93 (0.80 - 1.05) pmol/mg membrane protein. Similarly, in both species, larval nAChR were found to have higher affinities for IMI than adults (Table 5.1). For *C. riparius*, differences in receptor affinity were not statistically significant ($q = 2.3$, $p > 0.05$), with larvae displaying a K_D of 0.20 (0.08 - 0.31) and adults displaying a K_D of 0.49 (0.23 - 0.74) (Table 5.1). However, for *C. dilutus*, differences in receptor affinity were statistically significant (one-way ANOVA, $q = 4.9$, $p = 0.02$), with larvae displaying a K_D of 0.24 (0.15 - 0.34) nM, and adults displaying a mean K_D of 0.87 (0.42 - 1.32) (Table 5.1).

Comparison of saturation binding parameters between *C. dilutus* and *C. riparius* indicated that there were only slight differences in nAChR density or IMI binding affinity between the two species (Table 5.1). At the larval stage there were minor, but statistically significant, species-level differences in receptor density, with *C. dilutus* displaying an approximately 1.2-fold higher density of nAChR than *C. riparius* ($q = 6.1$, $p = 0.005$; Figures 5.1A, B) There were also species-level differences in receptor affinity at the adult life stage, with *C. dilutus* displaying an approximately 2-fold lower affinity for IMI than *C. riparius* ($q = 4.9$, $p = 0.02$; Figures 5.1C, D). No other binding parameters (e.g. larval affinity, adult density) were significantly different between the two chironomid species ($q = 0.311$ - 2.95, $p > 0.05$).

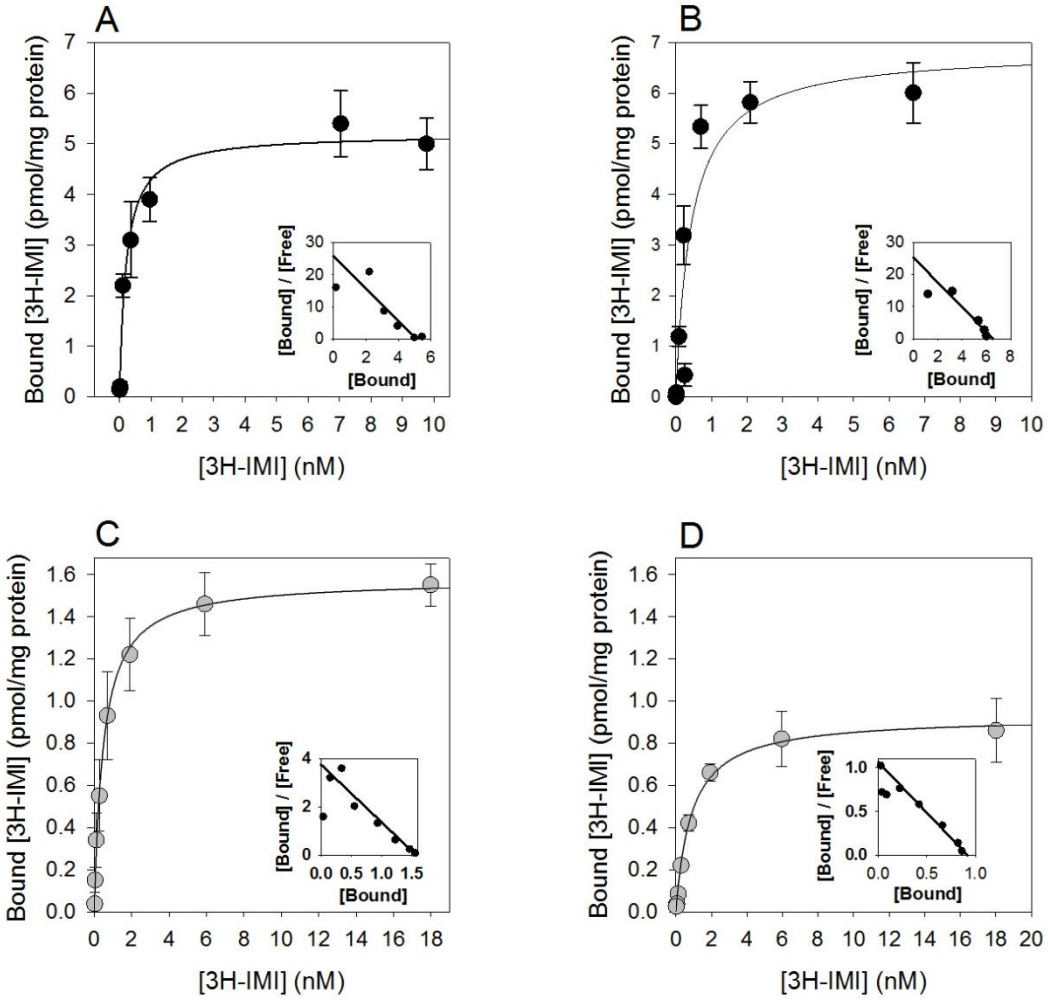

Figure 5.1 Saturation curves demonstrating specific binding of [^{3}H]-imidacloprid (3H-IMI) to membrane protein extracted from two different Chironomidae species, at two different life stages; *Chironomus riparius* larvae (A) and adults (C), and *Chironomus dilutus* larvae (B) and adults (D). Binding (total vs. bound [3H]-IMI) are presented as mean ± standard error (SE) of four experimental replicates. Scatchard plots (insets) are presented to visualize model fits.

Table 5.1 Saturation binding of [^{3}H]-imidacloprid to membrane protein of two Chironomidae (*Chironomus riparius* and *Chironomus dilutus)* at two different life-stages (larval and adult). [*]

Species	***C. riparius***		***C. dilutus***	
Life-stage	Larvae	Adults	Larvae	Adults
K_D (nM)[**]	0.20 [a] (0.08 - 0.31)	0.49 [a] (0.23 - 0.74)	0.24 [a] (0.15 - 0.34)	0.87 [b] (0.42 - 1.32)
B_{max} (pmol/mg) [**]	5.10 [a] (4.43 - 5.77)	1.57 [b] (1.37 - 1.78)	6.52 [c] (5.88 - 7.17)	0.93 [b] (0.80 - 1.05)
R^2	0.85	0.84	0.92	0.88

[*] Saturation binding parameters were estimated from four experimental replicates. Data presented as mean estimates, and 95% confidence intervals are presented in parentheses.

[**] Binding parameters (K_D and B_{max}) compared across species and life-stages using one-way ANOVAs paired with Tukey's post-hoc analyses. Significant differences ($p < 0.05$) are indicated with different letters.

5.3.4 Competition binding

Complete competition binding curves (mean ± SE, Figure 5.2) were obtained for each tested neonicotinoid, allowing nAChR binding affinity (Ki) and functional strength (IC_{50}) (presented as mean (95 % CI)) to be derived for IMI, CLO, and TMX in both *C. riparius* and *C. dilutus* (Table 5.2). Overall, good fits were obtained for competition binding curves (R^2 = 0.94 - 0.97), indicating that derived parameters accurately reflected experimental data (Table 5.2).

Competition binding studies indicated that in both larval Chironomidae there were compound-specific differences in nAChR binding affinity and functional strength. In *C. riparius*, IMI and CLO exhibited analogous binding profiles, displaying high affinities for nAChR (IMI K_i = 0.50 (0.39 - 0.65) nM vs. CLO K_i = 0.43 (0.30 - 0.62) nM; q = 1.1, $p > 0.05$) and relatively high functional strengths (IMI IC_{50} = 1.78 (1.38 - 2.31) nM vs. CLO IC_{50} = 1.53 (1.06 - 2.19) nM); q = 1.1, $p > 0.05$). TMX, on the other hand, exhibited a much weaker binding profile than IMI and CLO, with a significantly lower nAChR affinity (TMX K_i = 43.36 (35.09 - 53.50) nM; q = 30.6 – 31.7, $p < 0.001$) and a significantly lower functional strength than the other competitors (TMX IC_{50} = 153.90 nM (124.60 - 190.20); q = 30.7 – 31.8, $p < 0.001$) (Table 5.2, Figure 5.2A). In *C. dilutus*, competitive binding patterns were slightly different. CLO exhibited a significantly stronger binding affinity to nAChRs than IMI (CLO K_i = 0.21 (0.14 - 0.29) nM vs. IMI K_i = 0.42 (0.33 - 0.54) nM; q = 4.6, p = 0.04) and displaying a significantly higher functional strength (CLO IC_{50} = 0.63 (0.43 - 0.90) nM vs. IMI IC_{50} = 1.29 (1.01 - 1.65) nM; q = 4.6, p = 0.03). Similar to that observed for *C. riparius*, TMX had a much weaker binding profile than either IMI or CLO in *C. dilutus*, exhibiting a significantly lower affinity (TMX K_i = 45.54 (31.44 – 65.97) nM; q = 29.4 - 34.0, $p < 0.001$) and functional strength than the other competitors (TMX IC_{50} = 140.00 (96.90 – 203.40) nM); q = 29.5 - 34.0, $p < 0.001$) (Table 5.2, Figure 5.2B).

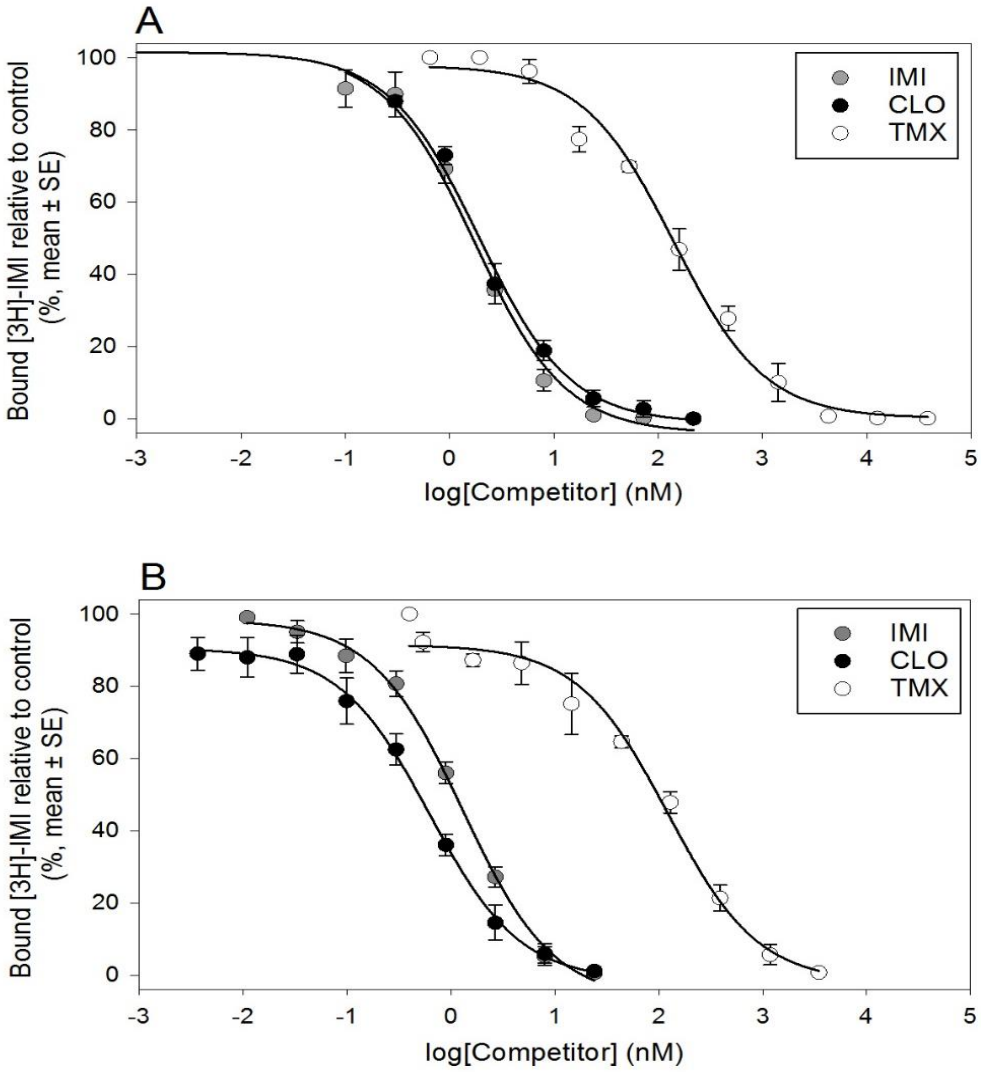

Figure 5.2 Competitive inhibition of [^{3}H]-imidacloprid (3H-IMI) in response to differential exposure to imidacloprid (IMI), clothianidin (CLO), and thiamethoxam (TMX). Competitive inhibition was measured in membrane protein extracted from larval Chironomidae of two species: (A) *Chironomus riparius*, and (B) *Chironomus dilutus*. Data are presented as mean ± standard error (SE) of five or six experimental replicates (n = 5 - 6).

Table 5.2 Competitive binding of [^{3}H]-imidacloprid and unlabelled neonicotinoids, imidacloprid (IMI), clothianidin (CLO), and thiamethoxam (TMX), to membrane protein isolated from larvae of two Chironomidae species (*Chironomus riparius* and *Chironomus dilutus*) compared to their acute toxicities (48-96 h LC/EC_{50} values; endpoint = lethality or immobility).

	C. riparius			***C. dilutus***		
Competitor	IMI	CLO	TMX	IMI	CLO	TMX
IC_{50} (nM)*	1.78 [a] (1.38 - 2.31)	1.53 [a] (1.06 - 2.19)	153.90 [b] (124.60 - 190.20)	1.29 [a] (1.01 - 1.65)	0.63 [c] (0.43 - 0.90)	140.00 [b] (96.90 - 203.40)
K_i (nM)*	0.50 [a] (0.39 - 0.65)	0.43 [a] (0.30 - 0.62)	43.36 [b] (35.09 - 53.50)	0.42 [a] (0.33 - 0.54)	0.21 [c] (0.14 - 0.29)	45.54 [b] (31.44 - 65.97)
R^2	0.97	0.95	0.97	0.97	0.94	0.94
Acute L/EC_{50} (µg/L) **	12.94	21.80	55.50	4.63	3.30	45.00

* Competitive binding parameters (IC_{50} and K_i) were estimated from six experimental replicates, and 95 % confidence intervals are presented in parentheses. K_i calculations considered K_D parameters derived in saturation binding experiments (K_D *C. riparius* = 0.20; K_D *C. dilutus* = 0.24) and concentration of ^{3}H-IMI (0.51 nM). Significant differences in IC_{50} and K_i were evaluated using one-way ANOVA ($p < 0.05$) and are indicated by different letters.

** Acute toxicity values presented are derived from regulatory risk assessments (used in species sensitivity distributions, SSDs) carried out by the European Food Safety Authority and the Pest Management Regulatory Agency (PMRA, Health Canada).) (European Food Safety Authority (EFSA), 2014; Pest Management Regulatory Agency, 2018b, 2018c).

There were limited differences in neonicotinoid binding between the two Chironomidae (Table 5.2; Figure 5.2). For IMI and TMX, competition binding assays indicated that there were no significant differences in affinity (q = 0.3 - 1.2, $p > 0.05$) or functional strength (q = 0.6 - 2.1, $p > 0.05$) between *C. riparius* and *C. dilutus*. However, minor species-specific differences in functional strength and binding affinity were observed for CLO, which had a significantly higher affinity for nAChRs (K_i *C. dilutus* = 0.21 (0.14 - 0.29) nM vs. K_i *C. riparius* = 0.43 (0.30 - 0.62) nM; q = 4.9, p = 0.02) and functional strength (IC_{50} *C. dilutus* = 0.63 (0.43 - 0.90) vs. IC_{50} *C. riparius* = 1.53 (1.06 - 2.19); q = 5.9, p = 0.004) in larval *C. dilutus* than in larval *C. riparius*.

5.3.5 Receptor binding, affinity, and toxicity of imidacloprid across different Insecta

Differences in IMI affinity (K_D; mean ± SE), nAChR density (B_{max}; mean ± SE), and IMI acute toxicity (L/EC_{50}) among Chironomidae, agricultural pests, and other dipteran test insects are presented in Figure 5.3 and Table A5.2. Chironomidae nAChRs appeared to have relatively high affinities for IMI (K_D = 0.2 - 0.24 nM), exhibiting K_D values that were markedly lower than any other previously tested Diptera (K_D = 2.96 ± 0.94 - 4.04 ± 0.98 nM) or Lepidoptera (K_D = 1.30 - 1.51 nM), and that fell within the range of high affinity binding sites observed in Hemiptera (K_D = 0.0035 - 0.9 ± 0.3 nM) and Orthoptera (K_D = 0.18 ± 0.02 nM) (Figure 5.3A). Furthermore, Chironomidae displayed nAChR at much higher densities (B_{max} = 5098 - 6522 fmol/mg) than other Diptera (B_{max} = 523 ± 74 - 1018 ± 325 fmol/mg), Lepidoptera (B_{max} = 134 - 150 fmol/mg), Orthoptera [B_{max} = 290 ± 46 fmol/mg (low affinity sites); 131 ± 22 fmol/mg (high affinity sites)] or Hemiptera [B_{max} = 0.43 - 1151 ± 125 fmol/mg (low affinity sites); 0.0035 - 0.9 ± 0.3 fmol/mg (high affinity sites)) (Figure 5.3B). Finally, prior studies indicated that Chironomidae were much more sensitive to IMI than any of the other insect species, displaying acute toxicity values (L/EC_{50} = 4.6 - 12.9 µg/L) much lower than observed in Hemiptera (L/EC_{50} = 30 - 1160 µg/L) and

Lepidoptera (L/EC_{50} = 976 µg/L), as well as other dipteran insects (L/EC_{50} = 194.7 - 6700 µg/L) (Figure 5.3C). Unfortunately, the toxicological information on *L. migratoria* and *H. virescens* is currently unavailable. Thus, a direct comparison of neonicotinoid toxicity could not be carried out between these two pest species and the tested Chironomidae.

5.4 Discussion

5.4.1 Species- and Life-Stage Specific Differences in Receptor Density and Binding Affinity

Chironomidae are a highly diverse taxonomic family, composed of an estimated 1231 different species (Interagency Taxonomic Information System (ITIS), 2019), which display different sensitivities to neonicotinoid compounds. For example, *C. dilutus* and *C. riparius* have been shown to demonstrate 2- to 21-fold differences in IMI toxicity (e.g. acute L/EC_{50} ranges: *C. riparius* = 12.94 - 55.20 µg/L vs. *C. dilutus* = 2.65 - 5.75 µg/L) (European Food Safety Authority (EFSA), 2014; Maloney et al., 2017; Morrissey et al., 2015). As IMI is thought to primarily elicit toxicity by binding to and activating nAChRs, one logical hypothesis is that these species-specific differences are driven by differential IMI binding to nAChR or expression of nAChR at different densities. Through saturation binding analysis, this study investigated whether nAChR density or binding characteristics were likely to significantly influence species-level differences in IMI toxicity. *C. riparius* and *C. dilutus* were found to display similar densities of nAChR that bound to IMI with similar affinities. Although there were species-specific differences at some life stages (e.g. nAChR density was higher in larval *C. dilutus* than in larval *C. riparius*, and receptor affinity was lower in adult *C. dilutus* than in adult *C. riparius*), they were relatively limited in magnitude (e.g. 1.2- to 2-fold differences in density and expression, respectively).

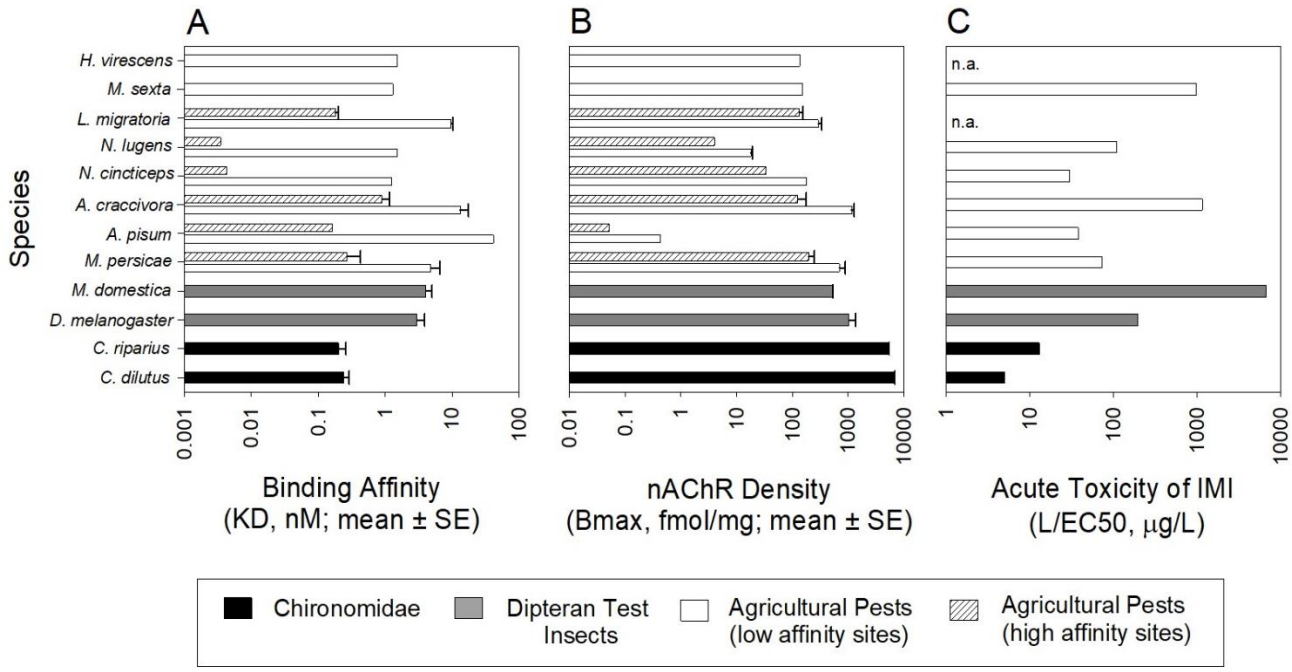

Figure 5.3 Species-level differences in (A) nicotinic acetylcholine receptor (nAChR) affinity for imidacloprid (IMI) (mean ± standard error (SE) [a]; log-scale), (B) nAChR density (mean ± SE [a]; log-scale); and (C) acute IMI toxicity (24 - 96 h L/EC_{50}, endpoint = immobility or lethality; log-scale) amongst Chironomidae, other dipteran test insects, and agricultural pest species (Hemiptera, Orthoptera, and Lepidoptera) [b].

* n.a. = Data were unavailable or unsuitable for direct comparison to other insect species.

[a] Sample sizes (*n*) used to derive SE are presented in Table A5.2.

[b] Low and high affinity sites are presented separately for hemipteran and orthopteran pests.

The results of this study suggest that for *C. riparius* and *C. dilutus*, differences in IMI toxicity are unlikely to be driven by differences in the densities or binding properties of expressed nAChRs. Thus, it is likely that differences in IMI toxicity are the result of other species-level differences. For example, it is possible that there are species-specific differences in neonicotinoid metabolism or elimination. In insects, neonicotinoids are thought to be first metabolized by cytochrome P450 enzymes (CYP; Phase I) and then conjugated by various enzymes, dependent on the species (Phase II) (Simon-Delso et al., 2015). Thus, variation in CYP or Phase II enzyme expression between *C. dilutus* and *C. riparius* could result in differential IMI activity. In fact, some studies have indicated that there are metabolic differences between *C. riparius* and *C. dilutus*. For example, glutathione-s-transferase (GST) activity and expression have been shown to differ between these two Chironomidae, with *C. riparius* displaying lower GST activity (in response to 1-chloro-2,4-dinitrobenzene exposure) and more GST transcripts than *C. dilutus* (Katagi and Tanaka, 2016). Furthermore, *in vivo* metabolism studies have demonstrated that *C. riparius* and *C. dilutus* differentially metabolize the insecticide chlorpyrifos, demonstrating different CYP-mediated metabolic reactions (Katagi and Tanaka, 2016). Therefore, it is possible that differences in IMI toxicity are the result of species-specific differences in neonicotinoid metabolism and/or elimination. However, as neonicotinoid metabolism has yet to be comprehensively characterized for and/or compared across different chironomid species, further studies are required to determine whether these factors are likely to drive species-level differences in IMI toxicity.

This study also investigated life-stage level differences in IMI binding characteristics and nAChR density. Both nAChR density and IMI binding affinity were found to be significantly influenced by life stage, with adult organisms expressing nAChRs at significantly lower densities than larvae (e.g. nAChR density was 3 - 7-fold lower in adults than larvae), and adult nAChRs

displaying significantly lower affinities for IMI than larval nAChR (e.g. affinity was 2.5 - 3.6-fold lower in adults than larvae). This indicates that throughout their development Chironomidae likely undergo significant neurophysiological changes, ultimately influencing nAChR expression and function. In terms of direct neonicotinoid toxicity, this information has limited significance. Adult Chironomidae tend to have short life spans (relative to other developmental stages), limited contact with neonicotinoid-contaminated aquatic environments, and reduced feeding capabilities (Oliver, 1976). Thus, adults are unlikely to be directly exposed to neonicotinoids. In fact, as there is currently no published data examining the toxicity of neonicotinoids to adult Chironomidae, the life-stage level differences in nAChR density and IMI-affinity cannot be directly linked to differences in neonicotinoid toxicity. However, this information can potentially help explain some of the toxicological patterns observed for neonicotinoids in the aquatic portions of Chironomidae life cycles. Previous chronic toxicity studies with larval *C. dilutus* have noted that reduction in emergence was often linked to death at the pupal life stage (Cavallaro et al. pers. comm.) indicating that Chironomidae undergoing metamorphosis may be particularly sensitive to neonicotinoid exposure. Results presented here suggest that throughout their life cycles, *C. riparius* and *C. dilutus* can undergo developmental changes that influence binding properties and nAChR expression. Thus, it is possible that pupal Chironomidae express higher concentrations of IMI-sensitive nAChR with higher affinities for IMI and other neonicotinoids, enhancing their sensitivity to these insecticidal compounds. Unfortunately, due to difficulties associated with the pupae isolation and membrane protein extraction, we were unable validate this hypothesis. Thus, further studies are necessary to characterize the role that nAChR expression and binding activity play in neonicotinoid toxicity at different stages of chironomid development.

5.4.2 Relative Binding Affinities of Imidacloprid, Clothianidin, and Thiamethoxam

Neonicotinoids are a group of seven neurotoxic insecticides, encompassing a range of systemic compounds that can be toxic to sensitive aquatic insects like Chironomidae. Within this class of insecticides, IMI is considered the 'classic' (first generation) neonicotinoid compound, as it was the first compound to be commercially produced and, historically, has been the most widely used (Jeschke and Nauen, 2008). However, in recent years other systemic (second generation) neonicotinoids (i.e. clothianidin (CLO) and thiamethoxam (TMX)) have also become dominant global agrochemicals. In fact, recent environmental monitoring studies have indicated that CLO and TMX are more likely to be detected in freshwater systems (Hladik and Kolpin, 2015; Main et al., 2014), with TMX currently representing the most commonly detected neonicotinoid in western Canadian agricultural regions (Malaj et al. pers comm.). Prior studies have demonstrated that there are significant compound-specific differences in neonicotinoid toxicity to *C. dilutus* and *C. riparius* (European Food Safety Authority, 2013b; Maloney et al., 2017; Pest Management Regulatory Agency, 2018b, 2018a; Raby et al., 2018a). However, limited studies have focused on why these compound-specific toxicological differences occur in these non-target aquatic species. Thus, along with IMI, this study characterized and compared the binding profiles of CLO and TMX in larval Chironomidae and found that compound-specific toxicity differences correlated with differences in nAChR binding (Table A5.3). For example, TMX, which elicits similar toxicities in *C. riparius* and *C. dilutus* (i.e. 48 - 96 h L/EC_{50}s: *C. dilutus* = 45.0 µg/L vs. *C. riparius* = 55.50 µg/L) (Pest Management Regulatory Agency, 2018b), was found to have similar affinities for IMI-sensitive nAChRs in these two insect species. Similarly, CLO, which has been found to be more toxic to *C. dilutus* than *C. riparius* (i.e. 48 - 96 h L/EC_{50}s; *C. dilutus* = 3.30 µg/L vs. *C. riparius* = 21.80 µg/L) (Pest Management Regulatory Agency, 2018c), was found to have a higher

affinity for *C. dilutus* nAChRs. In addition, compound-specific differences in toxicity were in accordance with their relative affinities for chironomid nAChR. In previous toxicity studies, IMI has been shown to display the highest toxicity to *C. riparius* and *C. dilutus* (i.e. 48 - 96 h L/EC50s: *C. dilutus* = 4.63 µg/L; *C. riparius* = 12.94 µg/L), followed closely by CLO (i.e. 48 - 96 h L/EC50s: *C. dilutus* = 3.30 µg/L; *C. riparius* = 21.80 µg/L), and then finally TMX which is much less toxic than the other two compounds (i.e. 48 - 96 h L/EC50s: *C. dilutus* = 45.0 µg/L; *C. riparius* = 55.50 µg/L) (Canadian Council of Ministers of the Environment 2007; Maloney et al. 2018; Pest Management Regulatory Agency 2018a, c). Correspondingly, CLO tended to bind to chironomid nAChR with the highest affinity, followed closely by IMI, and then finally TMX, which demonstrated a much lower binding affinity than the other two compounds (i.e. relative binding affinity: CLO ≥ IMI >> TMX). These findings confirm that binding affinity likely plays a significant role in compound-specific differences in neonicotinoid toxicity. Furthermore, these findings indicate that binding affinity could potentially be used to predict the relative toxicities of IMI, CLO, and TMX to Chironomidae. However, there were slight differences in relative neonicotinoid toxicity and relative binding affinity patterns. For example, prior studies have demonstrated that TMX is between 2.5 and 13-fold less toxic than IMI or CLO in *C. riparius* and *C. dilutus*, but in this study TMX binding affinity was found to be 87 - 217-fold lower than that of IMI or CLO (Table A5.3). This could be because this study only used one radiolabeled nAChR agonist ([^{3}H]-IMI). Previous binding studies with radiolabeled α-bungarotoxin ([^{125}I-α-BGT), have identified distinct nAChR binding sites in *D. melanogaster*, *Myzus persicae*, *Aphis craccivora*, and *Acyrthosiphon pisum* with different neonicotinoid binding affinities (Taillebois et al., 2014; Tomizawa et al., 2005; Wiesner and Kayser, 2000; Zhang et al., 2004). These insects display both α-BGT sensitive nAChR, which can competitively bind CLO and TMX (but not IMI),

and α-BGT insensitive nAChR, which can competitively bind all three compounds (e.g. Taillebois et al., 2014). Similarly, binding studies with radiolabeled thiamethoxam (^{3}H-TMX) have demonstrated that (at least in aphids) there are distinct nAChR binding sites for IMI and TMX, which are highly specific to each compound (Kayser et al., 2016). Thus, it is possible that the binding affinities obtained in this study did not perfectly correlate with toxicity because the use of a single radiolabeled ligand could not fully capture the binding profiles of these three neonicotinoid compounds. Indeed, future studies should focus on expanding the nAChR binding profile presented here by carrying out binding assays with other relevant agonists (e.g. α-BGT or epibatidine). Not only could this enhance our understanding of how neonicotinoids like IMI, CLO, and TMX exert their toxic effects in Chironomidae, but it could also help explain previously observed mixture effects (Maloney et al. 2017, 2018) and be potentially used to construct predictive models describing the ecotoxicity of different neonicotinoids to sensitive aquatic insects.

5.4.3 Receptor Densities and Neonicotinoid Binding in Chironomidae Relative to Other Insects

The expression and neonicotinoid binding characteristics of insect nAChRs have been widely investigated in agricultural pest species (e.g. aphids, planthoppers, locusts, budworms, and hornworms) and standard test insects (e.g. fruit flies and houseflies) (Taillebois et al., 2018). Thus, most available data concerning the expression and activity of insect nAChRs have been derived from terrestrial insects, which may not be comparable to Chironomidae in terms of physiology and life history traits. Regardless, this study found that some of the binding characteristics of chironomid nAChR were similar to what has been previously observed for other taxonomically related species. For example, as observed with other dipteran species like *D. melanogaster* (fruit fly), *M. domestica* (house fly), and *Lucilia sericata* (blow fly) (Lind et al., 1998; Liu and Casida,

1993; Ohkawara et al., 2002; Taillebois et al., 2018), *C. riparius* and *C. dilutus* expressed IMI-sensitive nAChR with only one binding site. This finding supports a current hypothesis in invertebrate neurobiology: that there are distinct and predictable differences in the expression and activity of insect nAChR between taxonomic orders, with Hemiptera and Orthoptera expressing two distinct IMI binding sites (high and low affinity) and other orders (e.g. Diptera and Lepidoptera) expressing a single IMI binding site (Crossthwaite et al., 2017; Taillebois et al., 2018). However, due to key differences in membrane protein extraction methodology (e.g. the typical use of Triton-X in dipteran studies, but not with other insects), there is still some doubt surrounding the certainty of this hypothesis (Crossthwaite et al., 2017). In this study, the membrane protein extraction methods were primarily derived from aphid radioligand binding studies (Taillebois et al., 2014; Wiesner and Kayser, 2000). Thus, it is unlikely that the membrane protein techniques applied significantly influenced our characterization of nAChR binding profiles. Furthermore, in both *C. riparius* and *C. dilutus* and at both larval and adult life stages only a single IMI binding site was characterized. Thus, it is highly likely that (as hypothesized) these dipteran species express nAChR with a single binding site. However, it must be acknowledged that Chironomidae comprise a large taxonomic group, and only two species were used in this study. Therefore, further data are required before a conclusion can be made about whether (as observed with other dipteran species) taxonomic-mediated differences in nAChR binding properties apply for this insect family.

Due to the breadth of data that has been published concerning the pharmacological effects of neonicotinoids in different invertebrate species, the IMI binding properties and nAChR densities derived for Chironomidae could be compared to those previously characterized for agricultural pests and other commonly used test insects. Furthermore, through a comprehensive literature

search IMI toxicity data could be collated across these different insect species. This comparison has yielded some potentially important insights into the relative sensitivity of Chironomidae to neonicotinoids. Both *C. riparius* and *C. dilutus* were found to display IMI binding affinities that fell within the range of high-affinity binding sites previously characterized in hemipteran and orthopteran species (e.g. aphids, planthoppers, and locusts). However, whereas these agricultural pests tend to display low densities of high affinity receptors, the Chironomidae studied here displayed significant densities of high affinity, IMI-sensitive nAChRs. The presence of relatively high densities of high affinity IMI-sensitive receptors could (at least partially) explain why IMI tends to be highly toxic to these aquatic organisms. As neonicotinoids are thought to primarily act on nAChRs (Tomizawa and Casida, 2003), it is possible that they are particularly efficacious in Chironomidae because there is a plethora of neuronal receptors that they can efficiently bind to, enhancing the likelihood of a toxic effect. This hypothesis is supported by recent studies focused on insecticide resistance in agricultural pests. Loss of high affinity IMI binding sites has been shown to result in IMI resistance, and entire populations of neonicotinoid resistant Hemiptera (e.g. *M. persicae* and *Aphis gossypii*) have been shown to have limited (or zero) expression of these high affinity nAChRs (Bass et al., 2011; Crossthwaite et al., 2017). However, Chironomidae display other, specific traits that likely enhance their sensitivity to neonicotinoid exposure. For example, Chironomidae spend the majority of their life cycle at the sediment-water interface and tend to be spatially static (Benoit et al., 1997), thus are at a high risk for continuous and/or repeated neonicotinoid exposure during sensitive developmental stages. In addition, larval Chironomidae are thought to have large nervous systems, that make up a considerable proportion of their bodies (Ospina-Pérez et al., 2019; Richardi et al., 2015). Thus, it is likely that at larval and pupal life-stages (when these insects are highly susceptible to neonicotinoid exposure), neonicotinoids have

more significant neurotoxic effects. Finally, Chironomidae could display marked differences in neonicotinoid metabolism (e.g. enzyme expression and/or cytochrome P450 activity) compared to other insect species. Although no published studies have directly compared metabolic enzyme activity in Chironomidae to that of other insects, various studies have described insecticide metabolism in aquatic insects and amphipods (e.g. *Hyallela spp*. and *Daphnia magna*) (Katagi and Tanaka, 2016). These studies have demonstrated that there can be differences in the concentrations and activities of different Phase I and Phase II metabolic enzymes (e.g. CYP, aldrin epoxidase, phosphotriesterase, and glutathione-S-transferase) amongst aquatic insects and between insects and amphipods (Katagi and Tanaka, 2016). Therefore, it is likely that these differences also exist between Chironomidae and terrestrial species (e.g. agricultural pests and other dipteran insects), contributing to the toxicological differences between these insects. Since the present study focused on nAChR density and binding characteristics, it was not possible to explore other factors that may contribute to chironomid sensitivity to neonicotinoids like IMI. However, as these potential physiological, behavioural, and metabolic differences likely play significant roles in neonicotinoid effects, future studies should focus on characterizing and comparing these differences amongst insect species, so that we can gain a better understanding of the species-level differences in neonicotinoid toxicity.

5.4.4 Potential Ecotoxicological Implications for nAChR-Selective Insecticides

Due to their widespread use and physicochemical characteristics (e.g. high water solubility, high leaching potential, minimal photodegradation in light-limited settings) neonicotinoids are highly prevalent in aquatic environments near agricultural areas (Morrissey et al., 2015). Therefore, aquatic organisms in such areas are likely to be continuously or repeatedly exposed to these insecticidal compounds (Main et al., 2014). However, this was the first study (in the

published literature) to investigate receptor binding characteristics of neonicotinoid insecticides in an aquatic insect species. Understanding the actions of neonicotinoids at the receptor level is important, as it allows us to gain a better understanding of the ecotoxicity of these insecticidal compounds in non-target organisms. Through this study it was possible to derive some conclusions concerning how neonicotinoid binding at the nAChR can influence neonicotinoid toxicity in Chironomidae. In addition, this study presents novel information that likely has broader ecotoxicological implications for nAChR-selective insecticides. One of the major findings was that both Chironomidae species (*C. riparius* and *C. dilutus*) expressed high densities of nAChR with high neonicotinoid binding affinities. Thus, it is possible that Chironomidae will be highly sensitive to other (non-neonicotinoid) nAChR-selective insecticide products. Indeed, any products that also act on the neonicotinoid-sensitive nAChR subtypes characterized here will likely demonstrate a high potential to elicit adverse ecotoxicological effects in these non-target aquatic insects. This requires further consideration, as insect nAChRs appear to be a current mechanistic focus in agrochemical development, with several novel nAChR-selective insecticide classes (e.g. butenolides, sulfoximines, and mesionic compounds) having recently been introduced into the global market (Ihara et al., 2017). Although it is not possible to directly use the present data to predict the toxicity of these newer nAChR-selective insecticides, the present findings provide some support for conducting further testing to determine how these newer insecticides may influence Chironomidae inhabiting agricultural watersheds. This is especially relevant since neonicotinoids are currently undergoing regulatory review or removal due to their adverse impacts on aquatic insects and the ecosystems that they inhabit (Health Canada 2018b; Pest Management Regulatory Agency 2018b, c). Therefore, to ensure adequate and continuous protection of sensitive and ecologically important aquatic insects like Chironomidae, the information presented here should

be taken under consideration when both evaluating the likely environmental impacts of nAChR agonists such as neonicotinoids and their potential replacement products during initial product registration, regulation, and risk assessment.

CHAPTER 6: NEONICOTINOID INSECTICIDES: WHAT WE HAVE LEARNED AND WHERE WE CAN GO FROM HERE

6.1 Introduction

Benthic macroinvertebrates are an extremely diverse group of organisms that play important roles in aquatic ecosystems. Present in a wide range of environments (e.g. freshwater and marine systems, lotic and lentic environments, and geographic regions ranging from the near-Arctic to the near-Antarctica), these aquatic organisms represent important food sources for higher-tier predators like fish and birds, and help maintain microbial communities and nutrient cycling by mixing surface sediments and breaking down organic detritus (Wallace and Webster, 1996). Due to specific life-history characteristics and habitat preferences, benthic macroinvertebrates are often at risk of continuous and/or repeated exposure to aquatic contaminants. Over the last ten years, one group of aquatic contaminants that has become a concern is neonicotinoid insecticides. Recent evidence has indicated that due to high rates of application and widespread use, neonicotinoids are present in aquatic systems, both as single compounds and in mixtures (Hladik et al., 2018b; Main, 2016; Main et al., 2014). Furthermore, neonicotinoids have been shown to demonstrate minimal degradation in light-limited environments (e.g. > 8 cm of pond water) (Lu et al., 2015), and thus can persist in aquatic zones that benthic macroinvertebrates are likely to inhabit (e.g. the sediment-water interface). Indeed, previous studies have shown that neonicotinoids can display long terrestrial half-lives (up to 6931 d (Goulson, 2013)) and remain in aquatic systems for multiple agricultural seasons (Main et al., 2014), indicating their propensity for repeated/chronic contamination of aquatic systems. Of the wide range of benthic macroinvertebrates, aquatic insects appear to display the highest sensitivity to neonicotinoids (Morrissey et al., 2015). In particular, Chironomidae (aquatic midges) are markedly susceptible to neonicotinoid toxicity (Raby et al., 2018a). However, despite the

likelihood of neonicotinoid mixtures existing in contaminated environments, neonicotinoid mixture toxicity has not been previously characterized in Chironomidae. Furthermore, despite their marked sensitivity to these neurotoxic compounds, the pharmacological drivers of neonicotinoid toxicity have not been investigated in these aquatic insects. Thus, the specific goals of this thesis were to evaluate the toxicity of neonicotinoid mixtures to Chironomidae, at both an individual species (acute and chronic) and a population level, and to explore some of the mechanisms behind neonicotinoid toxicity by evaluating species-, life stage-, and compound-specific differences in neonicotinoid binding to nicotinic acetylcholine receptors (nAChR) in these sensitive aquatic organisms.

6.2 Synthesis, major findings and study limitations

6.2.1 Acute, cumulative toxicity of neonicotinoid mixtures

Previous studies have indicated that neonicotinoids often exist in mixtures in aquatic environments (Hladik and Kolpin, 2015; Main et al., 2014). However, limited studies have focused on evaluating the toxicity of these mixtures to non-target aquatic insects. Thus, Chapter 2 focused on characterizing the acute toxicities of binary and ternary mixtures of three neonicotinoids commonly detected in aquatic systems (imidacloprid (IMI), clothianidin (CLO), and thiamethoxam (TMX)) to larval *Chironomus dilutus*, focusing on lethality as a toxicological endpoint. Under acute exposure scenarios, the cumulative toxicities of some mixtures (e.g. IMI-CLO, IMI-TMX, and IMI-CLO-TMX) were found to deviate from direct additivity, with IMI-CLO-TMX mixtures demonstrating synergism and IMI-CLO and IMI-TMX displaying dose-ratio dependent toxicity (displaying synergism at high concentrations of IMI, and antagonism at higher concentrations of the other mixture constituent). However, in general, these deviations from direct additivity were relatively small (e.g. synergistic effects = 5 - 30 % increases in survival;

antagonistic effects = 2 - 19 % decreases in survival), falling well below synergism cutoffs previously defined by Cedergreen (2014) (e.g. synergism considered substantial and reproducible if there is an overall ≥ 2-fold difference between effect and CA model) and the European Food Safety Authority (2013a) (e.g. synergism considered substantial and reproducible if there is an overall ≥ 5-fold difference between effect and CA model). Therefore, although these neonicotinoid mixtures could elicit synergism under some exposure scenarios, there was no overall trend indicating that neonicotinoid mixtures are likely to be substantially and/or reproducibly synergistic in *C. dilutus*. However, there were a few limitations in this acute mixture toxicity study. The study design used (MIXTOX) favours the examination of a wide range of mixture concentrations over experimental replication. Therefore, there was a large amount experimental variation, especially in the partial response ranges (i.e. lethality between 1 and 99 %). Although this is unlikely to dramatically influence overall conclusions about the magnitude of deviation from additivity, this high variation could have masked some more subtle mixture toxicity interactions. Furthermore, due to the number of tests required and the complexity of running fixed-ray design mixture tests, these tests were not repeated. Therefore, it is difficult to determine whether results are exactly reproducible, or whether further testing with neonicotinoid mixtures of slightly different compositions (e.g. different cumulative concentrations or mixture constituent ratios) would yield slightly different results. Indeed, to ensure that all potential mixture effects are adequately captured and to increase the certainty associated with reported mixture effects, future neonicotinoid (or other) mixture studies should use a higher number of replicates (i.e. > 2) in each mixture toxicity test, and aim to carry out multiple full test replicates for each mixture assessed.

6.2.3 Chronic toxicity of neonicotinoid mixtures and acute-to-chronic mixture extrapolation

Due to their distinct physicochemical characteristics, neonicotinoids can persist in light-limited areas of aquatic ecosystems (Lu et al., 2015). Thus, aquatic insects like Chironomidae can be chronically exposed to neonicotinoids and their mixtures. However, limited studies have focused on evaluating the cumulative chronic toxicity of neonicotinoid mixtures to these sensitive aquatic insects. Furthermore, although acute toxicity data are often used to predict chronic toxicity trends, no published studies have focused on evaluating whether mixture toxicity trends could be extrapolated from acute to chronic exposure scenarios in Chironomidae. Thus, Chapter 3 was focused on characterizing the chronic toxicities of binary and ternary mixtures of IMI, CLO, and TMX to larval *C. dilutus*, focusing on successful emergence as the toxicological endpoint. Under chronic exposure settings, all neonicotinoid mixtures were found to display statistically-significant deviations from directly additive toxicity (CA), with binary mixtures demonstrating dose-ratio dependent toxicities and ternary mixtures displaying antagonism. However, although these effects were statistically significant, they were relatively limited in magnitude, (e.g. 2 - 13 % synergism and 2 - 5 % antagonism) and only occurred under certain exposure conditions (e.g. synergism only occurred at relatively high concentrations of CLO (IMI-CLO) or TMX (CLO-TMX, IMI-TMX) and antagonism only occurred at relatively high concentrations of IMI (IMI-CLO, IMI-TMX)). Comparison of these chronic mixture toxicity estimates to those derived from acute studies (Chapter 2), indicated that chronic mixture toxicity for binary neonicotinoid mixtures could likely be extrapolated from acute exposure data with reasonable accuracy. Furthermore, neither single compounds nor neonicotinoid mixtures elicited statistically significant shifts in the sex-ratio of emerged adults.

There were some limitations in the study design that could have influenced derived results. Previous studies have indicated that chronic tests with *C. dilutus* can take up to 50 days (total) under control settings, with contaminant-treated populations occasionally taking longer (Benoit et al., 1997). Due to practical constraints, chronic exposure studies were limited to 28 days (ending 35 days into the *C. dilutus* life cycle). This resulted in reduced overall emergence (i.e. the mean (± standard deviation) emergence in control treatments was 64.6 ± 9.5 % across all 28 d chronic studies vs. > 90 % emergence in controls in previous 40 d studies (Cavallaro et al., 2017)) and could have limited the capacity of the study to capture the full life-cycle effects of neonicotinoids and their mixtures in exposed Chironomidae. This could have influenced the study in two major ways. First, in all the chronic MIXTOX models, the fit was worse than for the acute models (typically $R^2 \leq 0.80$). Indeed, despite an increased number of replicates (n = 3/concentration vs. n = 2/concentration in acute exposures), the variability was much higher in the chronic mixture studies than equivalent acute tests. It is likely that this enhanced study variability was (at least partially) due to the truncated study lengths, which could have masked some potential delayed emergence and skewed study results. Second, in this study there were no significant effects on the sex-ratio of emerged adults. Chironomidae generally exhibit protandry, with males typically emerging around 5 days before females (Benoit et al., 1997). Thus, it is possible that sex-ratio effects were not observed here, as shown previously (Cavallaro et al., 2017), as the chosen length of exposure failed to capture the full profile of emergence for both sexes. Furthermore, due to their complexity and length, none of the chronic mixture toxicity tests were repeated. Thus, it is difficult to determine whether the minor deviations from directly additive toxicity are reproducible, or whether further testing with neonicotinoid mixtures of slightly different compositions (e.g. different cumulative concentrations or mixture constituent ratios) would yield slightly different

results. Future studies should therefore focus on improving model fits, more comprehensively evaluating sex-ratio effects, and enhancing certainty associated with observed effects, by extending chronic mixture experiments to allow for complete emergence of exposed Chironomidae (i.e. 40 – 50 d exposures vs. the 28 d exposure period used here) and by carrying out multiple full test replicates for each neonicotinoid combination.

6.2.4 Extrapolating laboratory-derived mixture predictions to field-based settings

To maintain data quality standards and allow for adequate reproducibility, laboratory toxicity tests with aquatic insects are typically completed under consistent temperatures, photoperiods, illumination intensities, and water quality standards. These tests typically focus on characterizing toxicity in a single, sensitive species, which is often supposed to be representative of an entire aquatic insect population. As physicochemical factors are inherently variable and population/community dynamics can significantly influence ecotoxicological effect (Schmitt-Jansen et al., 2008), laboratory-based studies can over- or under-estimate actual ecotoxicity in contaminated aquatic environments. Therefore, it is important to validate laboratory observations under field settings to determine if laboratory-derived toxicity estimates are likely to adequately reflect real-world ecotoxicological effects. Thus Chapter 4, focused on evaluating laboratory-based predictions of chronic neonicotinoid toxicity (single compound and binary mixtures) to Chironomidae under field-based settings using *in situ* limnocorrals. Contrary to laboratory-based predictions (Chapters 2 and 3), there was no evidence of greater-than-additive toxicity in IMI-CLO and IMI-TMX mixtures for natural Chironomidae populations. In addition, some neonicotinoid treatments (IMI, CLO, and CLO-TMX) elicited greater-than-predicted effects on Chironomidae populations. Therefore, the laboratory-derived toxicity estimates did not adequately predict field-based effects. However, an analysis of the variation in observed results yielded some potential

insight concerning this lack of field-predictability in mixture effects. All mixture-treated limnocorrals displayed much higher variabilities than single compound treatments. This could indicate that neonicotinoid mixtures had species-level effects on Chironomidae that were not captured in the community-level analysis. In fact, one significant limitation in this study was that the dipteran insects were only characterized to family level before the samples were oven-dried and weighed to evaluate biomass. Further characterization of the collected Chironomidae (i.e. to subfamily or species level) could have enhanced the understanding of how IMI, CLO, TMX and their mixtures affect these sensitive aquatic insects, and improved the predictability of laboratory-derived toxicity estimates. Indeed, future studies should consider digging deeper into the species-level effects of neonicotinoids and their mixtures under field conditions. This could lead to both a better evaluation of why there was a disparity between our laboratory-derived predictions and field-observed effects and improve predictive capabilities, allowing for a more accurate estimation of the toxicological effects of neonicotinoid insecticides on sensitive insects inhabiting contaminated aquatic environments.

6.2.5 Influence of receptor-level binding characteristics on neonicotinoid toxicity

Chironomidae display marked differences in neonicotinoid sensitivity based on individual species, life stage, and neonicotinoid compound (Cavallaro et al., 2017; Maloney et al., 2018a, 2018b; Morrissey et al., 2015). However, despite their ecological importance and marked sensitivity to these aquatic contaminants, no published studies have investigated how or why neonicotinoids elicit these different toxicological effects in Chironomidae. Thus Chapter 5 aimed to bridge this data gap by evaluating the species-, life stage-, and compound-specific differences in neonicotinoid binding to chironomid nicotinic acetylcholine receptors (nAChRs). Specifically, binding profiles of IMI-sensitive nAChRs were compared between two standard test species

(*Chironomus riparius* and *Chironomus dilutus*) at two different life stages (larval and adult) with three different neonicotinoid compounds (IMI, CLO, TMX). Interestingly, neonicotinoid binding profiles could potentially explain some, but not all, of the toxicological differences previously observed in Chironomidae. For example, there were no significant differences in neonicotinoid binding between *C. dilutus* and *C. riparius*, indicating that neonicotinoid binding to the nAChR is unlikely to be the reason for species-specific differences in toxicity. However, life-stage specific differences in nAChR binding profiles were observed. Furthermore, larval organisms displayed significantly higher densities of nAChRs with significantly higher neonicotinoid affinities than adults. This indicated that nAChR expression/function likely varies throughout the Chironomidae life-cycle, which could result in life-cycle level differences in neonicotinoid sensitivity. Finally, there were compound-specific differences in nAChR binding, with binding profiles of IMI, CLO, and TMX positively correlating with neonicotinoid toxicity, indicating that in Chironomidae the affinity with which neonicotinoids bind to IMI-sensitive nAChRs is (at least partially) responsible for their relative toxicities. Interestingly, Chironomidae were also found to display relatively high densities of relatively high affinity nAChRs compared to other insects (e.g. agricultural pests and standard test insects), which likely plays a significant role in their marked sensitivities to neonicotinoid insecticides.

This study had one major limitation that could have influenced the interpretations of life stage-, species-, and compound-level relative toxicity patterns. Prior research has indicated that insects display different nAChR subtypes that respond to specific agonists (i.e. [^{125}I]-α-bungarotoxin, [^{3}H]-imidacloprid, and [^{3}H]-thiamethoxam) (Crossthwaite et al., 2017; Kayser et al., 2016; Taillebois et al., 2018). However, due to practical constraints, nAChR binding was only investigated using one radioligand ([^{3}H]-IMI). Thus, the full binding profiles of Chironomidae

nAChR were not adequately captured. This likely limited potential interpretations of the toxicological effects of neonicotinoids and their mixtures. Without a detailed profile of nAChR subtype expression, patterns of cumulative toxicity observed in laboratory-based mixture studies could not be adequately interpreted. Therefore, in future studies, multiple, standard nAChR agonists (i.e. [^{3}H]-imidacloprid, [^{125}I]-α-bungarotoxin, and [^{3}H]-thiamethoxam) should be used to characterize nAChR in non-target insects of interest, so that the relationships between neonicotinoid binding and toxicity can be fully investigated.

6.3 Importance of research and research contributions

6.3.1 Comprehensive characterization of neonicotinoid mixture toxicity in Chironomidae

Despite the fact that neonicotinoid mixtures are commonly detected in aquatic environments (Hladik and Kolpin, 2015; Main, 2016) and that aquatic insects are markedly sensitive to these compounds (Morrissey et al., 2015), no other published studies have attempted to evaluate the cumulative toxicities of neonicotinoid mixtures in Chironomidae. This work is the first to investigate neonicotinoid mixture toxicity in these sensitive and ecologically important aquatic insects. By comprehensively characterizing the toxicological profiles of these insecticide mixtures under acute and chronic exposure settings, this work has enhanced our understanding of how mixtures of IMI, CLO, and TMX can affect Chironomidae inhabiting contaminated environments. Through a series of laboratory- and field-based studies, this thesis has shown that although neonicotinoid mixtures could elicit greater-than-additive toxicity under some exposure scenarios, the toxicities of IMI, CLO, and/or TMX mixtures to chironomid populations/communities are likely to be adequately predicted via Concentration Addition (CA) (Equation 6.1):

$$\sum TU = \frac{C_i}{EC_{50,i}} \qquad 6.1$$

where mixture constituents are assumed to be directly additive and cumulative effect (ΣTU) is evaluated by summing neonicotinoid concentrations (c_i) scaled to their relative toxicities ($EC_{50,i}$) (Altenburger et al., 2013). Based on these findings, a model has been derived that could be used to estimate the cumulative toxicities of IMI, CLO, and/or TMX mixtures to Chironomidae in environmentally realistic settings (Equation 6.2):

$$\sum TU = \left(\frac{C_{imi}}{0.51} + \frac{C_{clo}}{0.71} + \frac{C_{tmx}}{8.91}\right) x\ 5 \qquad 6.2$$

This model is primarily based on the concept of CA, but also aims to be conservative, accounting for potential greater-than-additive toxicity. In this neonicotinoid mixture model (Equation 6.2), ***c*** represents neonicotinoid (IMI, CLO, or TMX) concentration (µg/L), the denominator values (**0.51, 0.71,** and **8.91**) are the laboratory-derived, 28-d EC_{50} values for successful emergence (in µg/L) (Chapter 3), and '**5**' is the recommended safety/uncertainty factor. This recommended safety factor is intended to account for both the subtle mixture effects observed in laboratory-based toxicity tests (up to 28 % greater-than-additive toxicity in some IMI-TMX mixtures) and the greater-than-predicted effects observed for both single compounds (IMI and CLO) and the CLO-TMX mixture under field settings (approximately a 5X greater-than-predicted cessation in emergence). This proposed mixture model would provide toxicity estimates in terms of Toxic Units (TU), which directly translate back to laboratory-derived EC values. For example, a TU of 0.1 would translate to an EC_5 (i.e. an estimated 5% cessation of emergence). By comparing derived TUs to predefined toxicity/hazard quotients, this neonicotinoid mixture model could be directly used to evaluate the risks that IMI, CLO, and/or TMX mixtures pose to Chironomidae in contaminated aquatic environments. This research has the potential to be particularly impactful for risk assessors and regulators in Canada and the United States, where these compounds are still registered for use (Pest Management Regulatory Agency, 2019a, 2019b, 2016; United States Environmental

Protection Agency, 2019), and heavily applied to agricultural crops (Hladik et al., 2018a). In fact, the continued use of IMI, CLO, and TMX in North America makes this characterization of neonicotinoid mixture toxicity and associated proposed mixture toxicity model particularly pertinent. However, there is room for improvement in both the predictive power of this neonicotinoid mixture toxicity model and the comprehensiveness of my neonicotinoid mixture toxicity characterization. Therefore, this research can also help inform future studies focusing on characterizing neonicotinoid mixture toxicity for non-target insects, benefiting researchers by providing a template through which future mixture toxicity assessments can be carried out.

6.3.2 Development of a relative toxicity pathway for imidacloprid, clothianidin, and thiamethoxam

Over the past decade, the toxicities of neonicotinoids to aquatic insects have been extensively investigated. The acute and chronic effects of these insecticides have been relatively well characterized in laboratory-based toxicity studies, e.g. (Finnegan et al., 2017; Raby et al., 2018a, 2018b), and their population- and community-level effects have been extensively assessed through field-based *in situ* or environmental monitoring analyses (Basley and Goulson, 2018; Cavallaro et al., 2018; Kreuger et al., 2010; Maloney et al., 2018a; Sánchez-Bayo et al., 2016; Sánchez-Bayo and Hyne, 2014; Starner and Goh, 2012). However, although the nAChR represents the primary molecular target of neonicotinoids (Tomizawa and Casida, 2004), the study carried out in Chapter 5 was the first to evaluate nAChR binding characteristics in an aquatic insect species. This has contributed to the current understanding of Chironomidae neurophysiology, enhanced the understanding of neonicotinoid interactions at different life-stages and in different chironomid species, and presented a partial explanation for the enhanced neonicotinoid-sensitivity that these aquatic insects display. Thus, this work characterizing nAChR binding profiles in *C. riparius* and

C. dilutus represents an important contribution to the fields of pesticide ecotoxicology and invertebrate neurobiology. Furthermore, this information can be combined with individual toxicity and population-level effects observed in previous studies (Chapters 2 - 4) and used to develop relative toxicological pathways for these insecticides.

In this thesis, the effects of IMI, CLO, and TMX were characterized at three levels of biological organization: molecular (nAChR binding), individual (lethality/cessation of emergence), and population (decline of Chironomidae abundance). By linking these effects and using previously established molecular pathways of neonicotinoid toxicity (Tomizawa and Casida, 2004), a putative toxicological pathway for neonicotinoids was constructed based on the adverse outcome pathway (AOP) framework (Ankley et al., 2010). Sequentially linking molecular and cellular events (known as molecular initiating events (MIE)) to key physiological effects (known as Key Events (KE)) and adverse toxicological outcomes (AO), AOP frameworks provide structured representations of biological events leading to toxicity in organisms exposed to xenobiotic agents (Ankley et al., 2010). As such, this pathway (Figure 6.1 A) links nAChR agonism (neonicotinoid binding) to population-level effects (decline in Chironomidae abundance) (Figure 6.1 B). Here nAChR agonism (neonicotinoid binding; defined as the MEI), leads to continuous nAChR activation (KE 1) and neuronal desensitization and/or cell energy exhaustion (KE 2), eventually causing nervous system failure (AO 1), paralysis and lethality (AO 2), and population decline (AO 3). As the effects of IMI, CLO, and TMX were characterized at each biological level tested (molecular, individual and population/community), this putative toxicological pathway could be expanded (Figure 6.1 C) focusing specifically on relative effect. Here, neonicotinoid effects are linked from receptor- (i.e. nAChR binding affinity) to population-levels (i.e. declines in abundance), and magnitude is scaled to that of IMI. For all three compounds,

relative nAChR binding affinity translates to population-level effects reasonably well. For example, TMX binds to IMI-sensitive nAChR with a 10 times lower affinity than IMI, which translates to 14 times lower toxicity in exposed individuals, and 50 times lower effects on Chironomidae populations than those observed with IMI (Figure 6.1). For CLO, there was a similar linear relationship between relative receptor affinity and population-level effects. CLO binds to nAChR with similar affinity to IMI (< 2-fold difference in IMI-sensitive nAChR affinity), is approximately equitoxic to IMI in exposed individuals (< 2-fold difference in acute/chronic toxicity), and elicits comparable effects on Chironomidae populations in field-based exposure settings (2-fold difference in population-level effects) (Figure 6.1).

This relative toxicity pathway demonstrates that there is a quantifiable link between affinity at the nAChR and magnitude of adverse effect in Chironomidae populations, furthering our specific understanding of how IMI, CLO, and TMX elicit effects in Chironomidae. Furthermore, this framework shows that there are significant differences in the toxicities of IMI, CLO, and TMX across multiple levels of biological organization, demonstrating how relative predictive pathways (like those presented here) could be used to evaluate the ecotoxicological effects of neonicotinoids in aquatic environments. In addition, this work highlights how nAChR characterization could be used in the evaluation of neonicotinoids or other nAChR agonists (e.g. sulfoximine, or butenolides) to Chironomidae or other sensitive aquatic insect species (e.g. Trichoptera or Ephemeroptera). Finally, by emphasizing novel avenues of study and presenting a ‘template’ for future pesticide research (beyond neonicotinoids), this work offers an alternative, multidisciplinary approach to pesticide ecotoxicology, which could help drive further progress in this field.

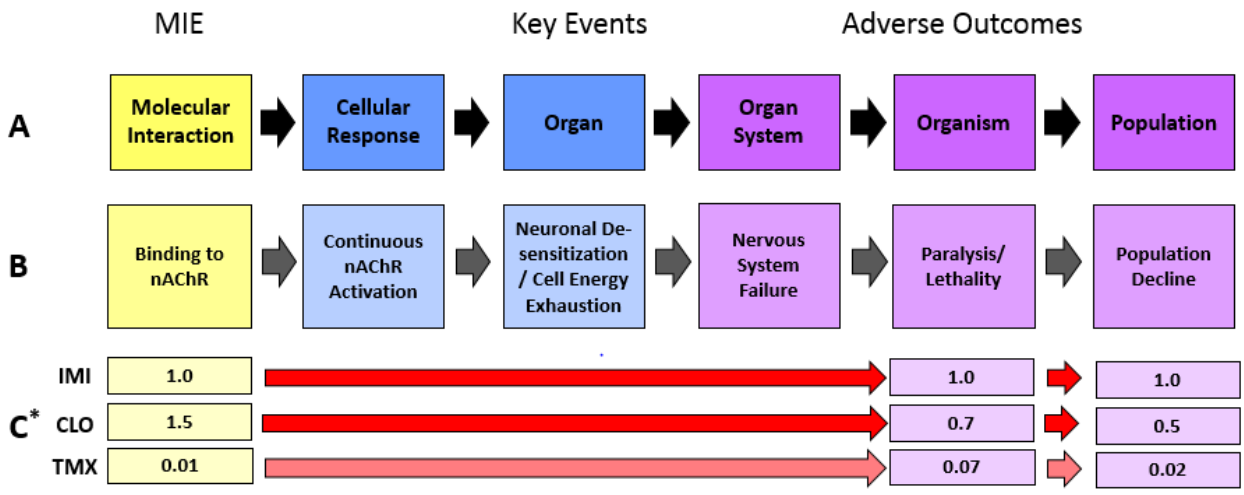

Figure 6.1 Putative toxicological pathways comparing the effects of commonly used systemic neonicotinoids in Chironomidae. Based on the (A) adverse outcome pathway (AOP) concept, this toxicological pathway links (B) nicotinic acetylcholine receptor (nAChR) binding to population declines in these aquatic insects, and estimates how (C) differences in receptor binding among imidacloprid (IMI), clothianidin (CLO) and thiamethoxam (TMX) translate into effects at the individual and population levels.

* Effects expressed relative to that of imidacloprid.

** MIE – molecular initiating event.

Unfortunately, there are some distinct limitations in the relative toxicity pathway generated here. First, although it is fairly logical, this pathway of relative toxicity is not directly predictable (i.e. there is not a 1:1 relationship between relative effect at the individual level and relative effect at the population/community level). It is likely that this is due to chemical, molecular, biological, or ecological factors that were not considered in this thesis. For example, although neonicotinoid metabolism is likely to play a significant role in relative effect, this work did not specifically investigate the metabolic differences that likely exist between different neonicotinoid compounds, different Chironomidae species, and different life-stages. Thus, more research is required to characterize how factors like metabolism contribute to the toxicity of neonicotinoids before this relative toxicity can be applied to predict the large-scale effects of neonicotinoids on aquatic insects like Chironomidae. In addition, the toxicity data generated in this thesis cannot be used to mechanistically explain why there was greater-than-additive neonicotinoid mixture toxicity for some neonicotinoid compounds. The radioligand binding studies used to characterize nAChR density and binding characteristics only used one agonist ([^{3}H]-IMI), thus these data could not be used to fully characterize the expression of different nAChR subtypes (e.g. IMI-sensitive and IMI-insensitive). It is possible that the relative expression of different nAChR subtypes influences the toxicological effects of neonicotinoid mixtures in Chironomidae. In fact, previous studies have suggested that IMI, CLO, and TMX can demonstrate different binding profiles depending on nAChR subtype (Taillebois et al., 2014), which could amplify single-compound toxicity effects, resulting in neonicotinoid mixture toxicity that deviates from direct, concentration based additivity. Although the analyses presented here cannot be used to determine whether mixture toxicity differences result from receptor binding characteristics, they do lay a foundation for future studies focused on further investigating the pharmacological effects of neonicotinoid mixtures. For

example, the methods and results presented here could be used as a basis for further study of the effects of neonicotinoid mixtures at multiple levels of biological organization. Thus, along with presenting a putative relative toxicity pathway, this thesis presents a template and a platform that can be used to inform future research on binary and ternary mixtures of IMI, CLO, and/or TMX.

6.4 Future research recommendations

This research addressed several important issues in the field of pesticide ecotoxicology, providing answers to some of the questions risk assessors and regulators have posed concerning the effects of neonicotinoid insecticides on aquatic organisms. However, it also illuminated many potential avenues of future investigation. These potential research topics span a range of fields, from ecology to neurobiology, but all focus on improving our understanding of how current-use neuroactive pesticides could influence ecologically important aquatic insects inhabiting agriculturally intensive areas. First, the mixture toxicity model presented here is relatively limited in scope, focusing on only one aquatic insect community and only three neonicotinoid compounds. Due to their hydrophilicity and potential for environmental persistence (Morrissey et al., 2015), neonicotinoids are likely to exist in the aquatic compartment and affect a range of aquatic insect species. Indeed, previous studies have demonstrated that other aquatic insects (e.g. Trichoptera and Ephemeroptera) are likely to demonstrate equivalent or higher sensitivity to these neonicotinoid compounds than Chironomidae (Raby et al., 2018a, 2018b; Van den Brink et al., 2016). Therefore, the mixture toxicity characterization presented here should be expanded to include effects of neonicotinoid mixtures on these sensitive aquatic species. Furthermore, IMI, CLO, and TMX are only three of six neonicotinoid compounds currently registered for use as pest control products. Other neonicotinoid compounds like acetamiprid, thiacloprid, and dinotefuran, which are more commonly used in fruit-producing regions, display similar physicochemical and

toxicological characteristics (Jeschke et al., 2010)**.** Thus, future studies should focus on comprehensively characterizing the cumulative effects of mixtures of other neonicotinoid or neonicotinoid-derived compounds (e.g. mixtures containing thiacloprid, acetamiprid, nitenpyram, dinotefuran, sulfoxaflor, or flupyradifurone). Second, over the course of this study, two predictive pathways were established: one focusing on neonicotinoid mixture toxicity (single-species to population) and the other focusing on the relative effects of IMI, CLO, and TMX (molecular to population). These predictive pathways would greatly benefit from further understanding of the ecology of aquatic insects. For example, our understanding of population dynamics in aquatic insect communities is relatively limited (Lancaster and Downes, 2018), so it is difficult to expand these predictive models beyond the population level. Furthermore, although recent research has improved our understanding of Chironomidae population dynamics in the Prairie Pothole Region (Williams and Sweetman, 2019), data concerning how the loss of individual species influences Chironomidae communities are still limited. Thus, it is difficult to determine why there were some deviations between our single-species mixture toxicity predictions and effects on populations observed in *in situ* field studies. Future studies should focus on better understanding aquatic insect ecology so that the ecotoxicological effects of insecticidal contaminants like neonicotinoids can be better characterized. In addition, the predictive pathways presented in this thesis would benefit from further understanding of the specific molecular effects of neonicotinoid insecticides in non-target insect species. Our current understanding of aquatic insect nAChRs and how neonicotinoids interact with these neuronal receptors is still limited. For example, IMI, CLO, and TMX interactions were only characterized with IMI-sensitive nAChRs in this thesis (Chapters 5). As shown with agricultural pests and other standard test insects there are likely other nAChR subtypes (e.g. α-BGT sensitive) that were not investigated here (Crossthwaite et al., 2017). In this thesis,

nAChR responses were only characterized in two related aquatic insect species. However, a broad range of aquatic insects are likely to be exposed to neonicotinoids in contaminated aquatic environments, some of which are also markedly sensitive to neonicotinoid insecticides (e.g. Trichoptera and Ephemeroptera) (Morrissey et al., 2015; Raby et al., 2018a; Van den Brink et al., 2016). Thus, future studies should focus on characterizing nAChR subtypes in other aquatic insect species that may be at risk of neonicotinoid exposure. This is especially important as a large proportion of current-use insecticides exert toxicity by directly acting on insect nAChRs, so understanding the function of these neuronal receptors will improve our ability to understand and characterize toxicological effects of new and existing pest control products.

6.5 Conclusions and Final Perspectives

Chironomidae are comprised of an estimated 1231 species worldwide (Pape et al., 2011), making them one of the most taxonomically rich aquatic insect families in existence. These insects are functionally important in aquatic ecosystems and widely distributed across a range of aquatic environments (Armitage, 1995). Therefore, midges are important sentinels when considering how aquatic contaminants can influence exposed ecosystems. This is especially true for neonicotinoid insecticides, which are both demonstrably toxic to Chironomidae and widely distributed in aquatic environments. This thesis has comprehensively evaluated the cumulative mixture toxicity and the relative effects of imidacloprid, clothianidin and thiamethoxam to Chironomidae, providing relevant information that can be used in future risk assessment and regulation of these products. However, this research also highlighted gaps in our knowledge of neonicotinoids and their effects on non-target aquatic insects. Indeed, in the context of changing environmental regulations, novel insecticide introduction, and fluctuating global environmental conditions, Chironomidae represent an ideal taxon to use in the evaluation of the environmental impacts of pest control products. As

these aquatic insects are both ecologically important and frequently exposed to pesticides, future research focused on Chironomidae may benefit our understanding of how widespread use of agricultural insecticides is affecting aquatic communities.

REFERENCES

Abbas, N., Ijaz, M., Shad, S.A., Khan, H., 2015. Stability of Field-Selected Resistance to Conventional and Newer Chemistry Insecticides in the House Fly, Musca domestica L. (Diptera: Muscidae). Neotrop. Entomol. 44, 402–409. https://doi.org/10.1007/s13744-015-0290-9

Abd-Ella, A.A., 2014. Toxicity and persistence of selected neonicotinoid insecticides on cowpea aphid, Aphis craccivora Koch (Homoptera: Aphididae). Arch. Phytopathol. Plant Prot. 47, 366–376. https://doi.org/10.1080/03235408.2013.809919

Alexander, A.C., Heard, K.S., Culp, J.M., 2007. Emergent body size of mayfly survivors. Freshw. Biol. 53, 171–180. https://doi.org/10.1111/j.1365-2427.2007.01880.x

Allinson, G., Zhang, P., Bui, A.D., Allinson, M., Rose, G., Marshall, S., Pettigrove, V., 2015. Pesticide and trace metal occurrence and aquatic benchmark exceedances in surface waters and sediments of urban wetlands and retention ponds in Melbourne, Australia. Environ. Sci. Pollut. Res. 22, 10214–10226. https://doi.org/10.1007/s11356-015-4206-3

Altenburger, R., Backhaus, T., Boedeker, W., Faust, M., Scholze, M., 2013. Simplifying complexity: Mixture toxicity assessment in the last 20 years. Environ. Toxicol. Chem. 32, 1685–1687. https://doi.org/10.1002/etc.2294

Altenburger, R., Nendza, M., Schüürmann, G., 2003. Mixture toxicity and its modeling by quantitative structure-activity relationships. Environ. Toxicol. Chem. 22, 1900. https://doi.org/10.1897/01-386

Andersch, W., Jeschke, P., Thielert, W., 2010. Combination of methiocarb and one or more compounds selected from thiacloprid, thiamethoxam, acetamiprid, nitenpyram, and dinotefuran: effective animal pests control and for plant seed dressing. US 8916613 B2.

Anderson, J.C., Dubetz, C., Palace, V.P., 2015. Neonicotinoids in the Canadian aquatic environment: A literature review on current use products with a focus on fate, exposure, and biological effects. Sci. Total Environ. 505, 409–422. https://doi.org/10.1016/j.scitotenv.2014.09.090

Anderson, T.A., Salice, C.J., Erickson, R.A., McMurry, S.T., Cox, S.B., Smith, L.M., 2013. Effects of landuse and precipitation on pesticides and water quality in playa lakes of the southern high plains. Chemosphere 92, 84–90. https://doi.org/https://doi.org/10.1016/j.chemosphere.2013.02.054

Ankley, G.T., Bennett, R.S., Erickson, R.J., Hoff, D.J., Hornung, M.W., Johnson, R.D., Mount, D.R., Nichols, J.W., Russom, C.L., Schmieder, P.K., Serrrano, J.A., Tietge, J.E., Villeneuve, D.L., 2010. Adverse outcome pathways: A conceptual framework to support ecotoxicology research and risk assessment. Environ. Toxicol. Chem. 29, 730–741. https://doi.org/10.1002/etc.34

Arain, M.S., Hu, X.-X., Li, G.-Q., 2014. Assessment of Toxicity and Potential Risk of Butene-fipronil Using Drosophila melanogaster, in Comparison to Nine Conventional Insecticides. Bull. Environ. Contam. Toxicol. 92, 190–195. https://doi.org/10.1007/s00128-013-1155-8

Armbrust, K.L., Peeler, H.B., 2002. Effects of formulation on the run-off of imidacloprid from turf. Pest Manag. Sci. 58, 702–706. https://doi.org/10.1002/ps.518

Armitage, P.D., 1995. The Chironomidae: Biology and Ecology of Non-biting Midges. Chapman & Hall, London, UK.

Azevedo-Pereira, Henrique M V S, Lemos, M.F.L., Soares, A.M.V.., 2011. Behaviour and Growth of Chironomus riparius Meigen (Diptera: Chironomidae) under Imidacloprid Pulse and Constant Exposure Scenarios. Water. Air. Soil Pollut. 219.

Azevedo-Pereira, H.M.V.S., Lemos, M.F.L., Soares, A.M.V.M., 2011. Effects of imidacloprid exposure on Chironomus riparius Meigen larvae: Linking acetylcholinesterase activity to behaviour. Ecotoxicol. Environ. Saf. 74, 1210–1215. https://doi.org/https://doi.org/10.1016/j.ecoenv.2011.03.018

Backhaus, T., Faust, M., 2012. Predictive Environmental Risk Assessment of Chemical Mixtures: A Conceptual Framework. Environ. Sci. Technol. 46, 2564–2573. https://doi.org/10.1021/es2034125

Barata, C., Baird, D.J., Nogueira, A.J.A., Soares, A.M.V.M., Riva, M.C., 2006. Toxicity of binary mixtures of metals and pyrethroid insecticides to Daphnia magna Straus. Implications for multi-substance risks assessment. Aquat. Toxicol. 78, 1–14. https://doi.org/10.1016/j.aquatox.2006.01.013

Bartlett, A.J., Hedges, A.M., Intini, K.D., Brown, L.R., Maisonneuve, F.J., Robinson, S.A., Gillis, P.L., de Solla, S.R., 2019. Acute and chronic toxicity of neonicotinoid and butenolide insecticides to the freshwater amphipod, Hyalella azteca. Ecotoxicol. Environ. Saf. 175, 215–223. https://doi.org/https://doi.org/10.1016/j.ecoenv.2019.03.038

Bartlett, A.J., Hedges, A.M., Intini, K.D., Brown, L.R., Maisonneuve, F.J., Robinson, S.A., Gillis, P.L., de Solla, S.R., 2018. Lethal and sublethal toxicity of neonicotinoid and butenolide insecticides to the mayfly, Hexagenia spp. Environ. Pollut. 238, 63–75. https://doi.org/https://doi.org/10.1016/j.envpol.2018.03.004

Basley, K., Goulson, D., 2018. Neonicotinoids thiamethoxam and clothianidin adversely affect the colonisation of invertebrate populations in aquatic microcosms. Environ. Sci. Pollut. Res. https://doi.org/10.1007/s11356-017-1125-5

Bass, C., Puinean, A.M., Andrews, M., Cutler, P., Daniels, M., Elias, J., Paul, V.L.,

Crossthwaite, A.J., Denholm, I., Field, L.M., Foster, S.P., Lind, R., Williamson, M.S., Slater, R., 2011. Mutation of a nicotinic acetylcholine receptor β subunit is associated with resistance to neonicotinoid insecticides in the aphid Myzus persicae. BMC Neurosci. 12, 51. https://doi.org/10.1186/1471-2202-12-51

Bataille, K.J., Baldassarre, G.A., 1993. Distribution and abundance of aquatic macroinvertebrates following drought in three prairie pothole wetlands. Wetlands 13, 260–269.

Batzer, D.P., 2013. The seemingly intractable ecological responses of invertebrates in North American Wetlands: A review. Wetlands 33, 1–15. https://doi.org/10.1007/s13157-012-0360-2

Beketov, M.A., Liess, M., 2009. Acute and delayed effects of the neonicotinoid insecticide thiacloprid on seven freshwater arthropods. Environ. Toxicol. Chem. 27, 461–470. https://doi.org/10.1897/07-322R.1

Benoit, D.A., Sibley, P.K., Juenemann, J.L., Ankley, G.T., 1997. *Chironomus tentans* life-cycle test: Design and evaluation for use in assessing toxicity of contaminated sediments. Environ. Toxicol. Chem. 16, 1165–1176. https://doi.org/10.1002/etc.5620160611

Boatman, N.D., Brickle, N.W., Hart, J.D., Milsom, T.I.M.P., Morris, A.J., Murray, A.W.A., Murray, K.A., Robertson, P.A., 2004. Evidence for the indirect effects of pesticides on farmland birds. Ibis (Lond. 1859). 146, 131–143. https://doi.org/10.1111/j.1474-919X.2004.00347.x

Bodereau-Dubois, B., List, O., Calas-List, D., Marques, O., Communal, P.-Y., Thany, S.H., Lapied, B., 2012. Transmembrane potential polarization, calcium influx, and receptor conformational state modulate the sensitivity of the imidacloprid-insensitive neuronal insect

nicotinic acetylcholine receptor to neonicotinoid insecticides. J. Pharmacol. Exp. Ther. 341, 326–39. https://doi.org/10.1124/jpet.111.188060

Bonmatin, J.-M., Giorio, C., Girolami, V., Goulson, D., Kreutzweiser, D.P., Krupke, C., Liess, M., Long, E., Marzaro, M., Mitchell, E.A.D., Noome, D.A., Simon-Delso, N., Tapparo, A., 2015. Environmental fate and exposure; neonicotinoids and fipronil. Environ. Sci. Pollut. Res. Int. 22, 35–67. https://doi.org/10.1007/s11356-014-3332-7

Buckingham, S., Lapied, B., Corronc, H., Sattelle, F., 1997. Imidacloprid actions on insect neuronal acetylcholine receptors. J. Exp. Biol. 200, 2685–92.

Burgess, E.R., King, B.H., 2015. Compatibility of the Parasitoid Wasp Spalangia endius (Hymenoptera: Pteromalidae) and Insecticides against Musca domestica (Diptera: Muscidae) as Evaluated by a New Index. J. Econ. Entomol. 108, 986–992.

Calas-List, D., List, O., Thany, S.H., 2012. Nornicotine application on cockroach dorsal unpaired median neurons induces two distinct ionic currents: Implications of different nicotinic acetylcholine receptors. Neurosci. Lett. 518, 64–68. https://doi.org/10.1016/j.neulet.2012.04.058

Canadian Council of Ministers of the Environment, 2007. Canadian Water Quality Guidelines for the Protection of Aquatic Life - Imidacloprid. Can. Environ. Qual. Guidel. 1–8.

Carbo, L., Martins, E.L., Dores, E.F.G.C., Spadotto, C.A., Weber, O.L.S., De-Lamonica-Freire, E.M., 2007. Acetamiprid, carbendazim, diuron and thiamethoxam sorption in two Brazilian tropical soils. J. Environ. Sci. Heal. Part B 42, 499–507. https://doi.org/10.1080/03601230701389439

Casida, J.E., Durkin, K.A., 2013. Neuroactive Insecticides: Targets, Selectivity, Resistance, and Secondary Effects. Annu. Rev. Entomol. 58, 99–117. https://doi.org/10.1146/annurev-ento-

120811-153645

Cavallaro, M.C., Liber, K., Headley, J. V., Peru, K.M., Morrissey, C.A., 2018. Community-level and phenological responses of emerging aquatic insects exposed to three neonicotinoid insecticides: an in situ wetland limnocorral approach. Environ. Toxicol. Chem. in press.

Cavallaro, M.C., Morrissey, C.A., Headley, J. V., Peru, K.M., Liber, K., 2017. Comparative chronic toxicity of imidacloprid, clothianidin, and thiamethoxam to Chironomus dilutus and estimation of toxic equivalency factors. Environ. Toxicol. Chem. 36, 372–382. https://doi.org/10.1002/etc.3536

Cedergreen, N., 2014. Quantifying synergy: A systematic review of mixture toxicity studies within environmental toxicology. PLoS One 9. https://doi.org/10.1371/journal.pone.0096580

Cedergreen, N., Sørensen, H., Svendsen, C., 2012. Can the joint effect of ternary mixtures be predicted from binary mixture toxicity results? Sci. Total Environ. 427–428, 229–237. https://doi.org/10.1016/j.scitotenv.2012.03.086

Chiovarou, E.D., Siewicki, T.C., 2008. Comparison of storm intensity and application timing on modeled transport and fate of six contaminants. Sci. Total Environ. 389, 87–100. https://doi.org/https://doi.org/10.1016/j.scitotenv.2007.08.029

Cho, S.-R., Koo, H.-N., Yoon, C., Kim, G.-H., 2011. Sublethal effects of flonicamid and thiamethoxam on green peach aphid, Myzus persicae and feeding behavior analysis. J. Korean Soc. Appl. Biol. Chem. 54, 889–898. https://doi.org/10.1007/BF03253177

Colombo, V., Mohr, S., 2013. Structural Changes in a Macrozoobenthos Assemblage After Imidacloprid Pulses in Aquatic Field-Based Microcosms Structural Changes in a Macrozoobenthos Assemblage After Imidacloprid Pulses in Aquatic Field-Based

Microcosms. https://doi.org/10.1007/s00244-013-9940-2

Corringer, P.-J., Novère, N. Le, Changeux, J.-P., 2000. Nicotinic Receptors at the Amino Acid Level. Annu. Rev. Pharmacol. Toxicol. 40, 431–458. https://doi.org/10.1146/annurev.pharmtox.40.1.431

Cox, L., Koskinen, W.C., Yong Yen, P., 1997. Sorption-desorption of Imidacloprid and its Metabolites in Soil. Food Chem 45, 1468–1472.

Cresswell, J.E., Robert, F.-X.L., Florance, H., Smirnoff, N., 2014. Clearance of ingested neonicotinoid pesticide (imidacloprid) in honey bees (Apis mellifera) and bumblebees (Bombus terrestris). Pest Manag. Sci. 70, 332–337. https://doi.org/10.1002/ps.3569

Crossthwaite, A.J., Bigot, A., Camblin, P., Goodchild, J., Lind, R.J., Slater, R., Maienfisch, P., 2017. The invertebrate pharmacology of insecticides acting at nicotinic acetylcholine receptors 42, 67–83. https://doi.org/10.1584/jpestics.D17-019

de Perre, C., Murphy, T.M., Lydy, M.J., 2015. Fate and effects of clothianidin in fields using conservation practices. Environ. Toxicol. Chem. 34, 258–265. https://doi.org/10.1002/etc.2800

de Zwart, D., Posthuma, L., 2005. Complex mixture toxicity for single and multiple species: Proposed methodologies. Environ. Toxicol. Chem. 24, 2665–2676. https://doi.org/10.1897/04-639R.1

Deneer, J.W., 2000. Toxicity of mixtures of pesticides in aquatic systems. Pest Manag. Sci. 56, 516–520. https://doi.org/10.1002/(SICI)1526-4998(200006)56:6<516::AID-PS163>3.0.CO;2-0

Dondero, F., Negri, A., Boatti, L., Marsano, F., Mignone, F., Viarengo, A., 2010. Transcriptomic and proteomic effects of a neonicotinoid insecticide mixture in the marine mussel (Mytilus

galloprovincialis, Lam.). Sci. Total Environ. 408, 3775–3786. https://doi.org/10.1016/j.scitotenv.2010.03.040

Eastham, H.M., Lind, R.J., Eastlake, J.L., Clarke, B.S., Towner, P., Reynolds, S.E., Wolstenholme, A.J., Wonnacott, S., 1998. Characterization of a nicotinic acetylcholine receptor from the insect Manduca sexta. Eur. J. Neurosci. 10, 879–889. https://doi.org/10.1046/j.1460-9568.1998.00095.x

Elbert, A., Haas, M., Springer, B., Thielert, W., Nauen, R., 2008. Applied aspects of neonicotinoid uses in crop protection. Pest Manag. Sci. 64, 1099–1105. https://doi.org/10.1002/ps.1616

Environment and Climate Change Canada, 2012. Ecological Risk Assessment Guidance.

Environment Canada, 1997. Biological Test Method: Test for Survival and Growth in Sediment Using the Larvae of Freshwater Midges (Chironomus tentans or Chironomus riparius). Ottawa, Ontario.

Eure, E.R., Nelson, P.N., Sorenson, C.E., 2018. Ovicidal Activity of Acetamiprid and Imidacloprid Against Manduca Sexta (Lepidoptera: Sphingidae). J. Entomol. Sci. 53, 400–401.

European Food Safety Authority, 2013a. Guidance on tiered risk assessment for plant protection products for aquatic organisms in edge-of-field surface waters. EFSA J. 11, 3290. https://doi.org/10.2903/j.efsa.2013.3290.Available

European Food Safety Authority, 2013b. Conclusion on the peer review of the pesticide risk assessment of the active substance flubendiamide. Parma, Italy.

European Food Safety Authority, 2006. Conclusion regarding the peer review of the pesticide risk assessment of the active substance 2007, 1–67.

European Food Safety Authority (EFSA), 2014. Conclusion on the peer review of the pesticide risk assessment for aquatic organisms for the active substance imidacloprid. EFSA J. 12, 3835. https://doi.org/10.2903/j.efsa.2014.3835

Evelsizer, V., Skopec, M., 2016. Pesticides, Including Neonicotinoids, in Drained Wetlands of Iowa's Prairie Pothole Region. Wetlands 1–12. https://doi.org/10.1007/s13157-016-0796-x

Fayuk, D., Yakel, Jerrel, L., 2004. Regulation of Nicotinic Acetylcholine Receptor Channel Function by Acetylcholinesterase Inhibitors in Rat Hippocampal CA1 Interneurons. Mol. Pharmacol. 66, 658 LP – 666.

Finnegan, M.S., Baxter, L.R., Maul, J., Hanson, M.L., Hoekstra, P.F., 2017. Comprehensive Characterization of the Acute and Chronic Toxicity of the Neonicotinoid Insecticide Thiamethoxam To a Suite of Aquatic Primary Producers, Invertebrates, and Fish. Environ. Toxicol. Chem. https://doi.org/10.1002/etc.3846

Focks, A., Belgers, D., Boerwinkel, M.-C., Buijse, L., Roessink, I., den Brink, P.J., 2018. Calibration and validation of toxicokinetic-toxicodynamic models for three neonicotinoids and some aquatic macroinvertebrates. Ecotoxicology. https://doi.org/10.1007/s10646-018-1940-6

Forbes, V.E., Moller, V., Depledge, M.H., 1995. Intrapopulation Variability in Sublethal Response to Heavy Metal Stress in Sexual and Asexual Gastropod Populations. Funct. Ecol. 9, 477–484. https://doi.org/10.2307/2390012

Frantzois, G., Paptsiki, K., Sidiropoulou, B., Lazaridis, I., Theophilidis, G., Mavargani-Tsipidou, P., 2008. Evaluation of insecticidal and genotoxic effects of imidacloprid and acetochlor in Drosophila melanogaster. J. Appl. Entomol. 132, 583–590.

Gibbons, D., Morrissey, C., Mineau, P., 2015. A review of the direct and indirect effects of

neonicotinoids and fipronil on vertebrate wildlife 103–118. https://doi.org/10.1007/s11356-014-3180-5

Giroux, I., Sarrasin, B., 2011. Pesticides et nitrates dans l'eau souterraine près de cultures de pommes de terre - Échantillonnage dans quelques régions du Québec en 2008 et 2009. https://doi.org/ISBN 978-2-550-61396-1

Gomez-Eyles, J.L., Svendsen, C., Lister, L., Martin, H., Hodson, M.E., Spurgeon, D.J., 2009. Measuring and modelling mixture toxicity of imidacloprid and thiacloprid on Caenorhabditis elegans and Eisenia fetida. Ecotoxicol. Environ. Saf. 72, 71–79. https://doi.org/10.1016/j.ecoenv.2008.07.006

Goulson, D., 2014. Pesticides linked to bird declines. Nature 511, 295.

Goulson, D., 2013. An overview of the environmental risks posed by neonicotinoid insecticides. J. Appl. Ecol. 50, 977–987. https://doi.org/10.1111/1365-2664.12111

Green, W.N., Wanamaker, C.P., 1997. The Role of the Cystine Loop in Acetylcholine Receptor Assembly. J. Biol. Chem. 272, 20945–20953. https://doi.org/10.1074/JBC.272.33.20945

Gupta, R.C., 2007. Veterinary Toxicology: Basic and Clinical Principles, 1st ed. Elsevier Inc., Oxford, UK.

Gupta, S., Gajbhiye, V.T., Gupta, R.K., 2008. Soil Dissipation and Leaching Behavior of a Neonicotinoid Insecticide Thiamethoxam. Bull. Environ. Contam. Toxicol. 80, 431.

Gupta, S., Gajbhiye, V.T., Kalpana, Agnihotri, N.P., 2002. Leaching behaviour of imidacloprid formulations in soil. Bull. Environ. Contam. Toxicol. 68, 502–8.

Guyton, A.C., Hall, J.E., 2006. Textbook of Medical Physiology, 11th ed. Elsevier Saunders, Amsterdam, NL.

Hallmann, C.A., Foppen, R.P.B., van Turnhout, C.A.M., de Kroon, H., Jongejans, E., 2014.

Declines in insectivorous birds are associated with high neonicotinoid concentrations. Nature 511, 341.

Hamilton, M.A., Russo, R.C., Thurston, R. V, 1977. Trimmed Spearman-Karber method for estimating median lethal concentration in toxicity bioassays . Environ. Sci. Technol. 11, 714–719. https://doi.org/10.1021/es60140a017

Hayasaka, Daisuke, Korenaga, T., Sanchez-Bayo, F., Goka, K., 2012. Differences in ecological impacts of systemic insecticides with different physicochemical properties on biocenosis of experimental paddy fields. Ecotoxicology 21, 191–201.

Hayasaka, D, Korenaga, T., Suzuki, K., Saito, F., Sánchez-Bayo, F., Goka, K., 2012. Cumulative ecological impacts of two successive annual treatments of imidacloprid and fipronil on aquatic communities of paddy mesocosms. Ecotoxicol. Environ. Saf. 80, 355–362. https://doi.org/https://doi.org/10.1016/j.ecoenv.2012.04.004

Health Canada, 2018a. Special Review of Clothianidin Risk to Aquatic Invertebrates: Proposed Decision for Consultation.

Health Canada, 2018b. Proposed Re-evaluation Decision PRVD2018-12, Imidacloprid and its Associated End-use Products: Pollinator Re-evaluation. https://doi.org/H113-27/2018-12E-PDF

Hendrickson, L., Guildford, M., Tapper, A., 2013. Neuronal Nicotinic Acetylcholine Receptors: Common Molecular Substrates of Nicotine and Alcohol Dependence. Front. Psychiatry.

Hirvenoja, M., 1973. Revision der Gattung Cricotopus van der Wulp und ihrer Verwandten (Diptera: Chironomidae). Ann. Zool. Fennici 10, 1–363.

Hladik, M., Kolpin, D., 2015. First national-scale occurrence of neonicotinoid insecticides in streams across the U.S.A. Environ. Chem. 12–20. https://doi.org/10.1071/EN15061

Hladik, M.L., Corsi, S.R., Kolpin, D.W., Baldwin, A.K., Blackwell, B.R., Cavallin, J.E., 2018a. Year-round presence of neonicotinoid insecticides in tributaries to the Great Lakes, USA. Environ. Pollut. 235, 1022–1029. https://doi.org/https://doi.org/10.1016/j.envpol.2018.01.013

Hladik, M.L., Kolpin, D.W., Kuivila, K.M., 2014. Widespread occurrence of neonicotinoid insecticides in streams in a high corn and soybean producing region, USA. Environ. Pollut. 193, 189–196. https://doi.org/10.1016/j.envpol.2014.06.033

Hladik, M.L., Main, A.R., Goulson, D., 2018b. Environmental Risks and Challenges Associated with Neonicotinoid Insecticides. Environ. Sci. Technol. 52, 3329–3335. https://doi.org/10.1021/acs.est.7b06388

Holt, R.A., Subramanian, G.M., Halpern, A., Sutton, G.G., Charlab, R., Nusskern, D.R., Wincker, P., Clark, A.G., Ribeiro, J.C., Wides, R., Salzberg, S.L., Loftus, B., Yandell, M., Majoros, W.H., Rusch, D.B., Lai, Z., Kraft, C.L., Abril, J.F., Anthouard, V., Arensburger, P., Atkinson, P.W., Baden, H., de Berardinis, V., Baldwin, D., Benes, V., Biedler, J., Blass, C., Bolanos, R., Boscus, D., Barnstead, M., Cai, S., Center, A., Chatuverdi, K., Christophides, G.K., Chrystal, M.A., Clamp, M., Cravchik, A., Curwen, V., Dana, A., Delcher, A., Dew, I., Evans, C.A., Flanigan, M., Grundschober-Freimoser, A., Friedli, L., Gu, Z., Guan, P., Guigo, R., Hillenmeyer, M.E., Hladun, S.L., Hogan, J.R., Hong, Y.S., Hoover, J., Jaillon, O., Ke, Z., Kodira, C., Kokoza, E., Koutsos, A., Letunic, I., Levitsky, A., Liang, Y., Lin, J.-J., Lobo, N.F., Lopez, J.R., Malek, J.A., McIntosh, T.C., Meister, S., Miller, J., Mobarry, C., Mongin, E., Murphy, S.D., O'Brochta, D.A., Pfannkoch, C., Qi, R., Regier, M.A., Remington, K., Shao, H., Sharakhova, M. V, Sitter, C.D., Shetty, J., Smith, T.J., Strong, R., Sun, J., Thomasova, D., Ton, L.Q., Topalis, P., Tu, Z., Unger, M.F.,

Walenz, B., Wang, A., Wang, J., Wang, M., Wang, X., Woodford, K.J., Wortman, J.R., Wu, M., Yao, A., Zdobnov, E.M., Zhang, H., Zhao, Q., Zhao, S., Zhu, S.C., Zhimulev, I., Coluzzi, M., della Torre, A., Roth, C.W., Louis, C., Kalush, F., Mural, R.J., Myers, E.W., Adams, M.D., Smith, H.O., Broder, S., Gardner, M.J., Fraser, C.M., Birney, E., Bork, P., Brey, P.T., Venter, J.C., Weissenbach, J., Kafatos, F.C., Collins, F.H., Hoffman, S.L., 2002. The Genome Sequence of the Malaria Mosquito Anopheles gambiae. Science (80-.). 298, 129 LP – 149. https://doi.org/10.1126/science.1076181

Ihara, M., Buckingham, S.D., Matsuda, K., Sattelle, D.B., 2017. Modes of Action, Resistance and Toxicity of Insecticides Targeting Nicotinic Acetylcholine Receptors. Curr. Med. Chem. 24, 2925–2934. https://doi.org/10.2174/0929867324666170206142019

Interagency Taxonomic Information System (ITIS), 2019. Chironomidae [WWW Document]. ITIS Rep. URL https://www.itis.gov (accessed 11.12.19).

Jairin, J., Kojima, N., Nagata, T., 2005. Insecticide Resistance of the Green Rice Leafhopper, Nephotettix cincticeps, to the Systemic Insecticides Used for Seeding-Box Application. ScienceAsia 31, 151. https://doi.org/10.2306/scienceasia1513-1874.2005.31.151

Jeschke, P., Nauen, R., 2008. Neonicotinoids—from zero to hero in insecticide chemistry. Pest Manag. Sci. 64, 1084–1098. https://doi.org/10.1002/ps.1631

Jeschke, P., Nauen, R., Schindler, M., Elbert, A., 2010. Overview of the Status and Global Strategy for Neonicotinoids (dagger). J. Agric. Food Chem. 1–7. https://doi.org/10.1021/jf101303g

Jones, A.K., Brown, L.A., Sattelle, D.B., 2007. Insect nicotinic acetylcholine receptor gene families: from genetic model organism to vector, pest and beneficial species. Invertebr. Neurosci. 7, 67–73. https://doi.org/10.1007/s10158-006-0039-6

Jones, A.K., Grauso, M., Sattelle, D.B., 2005. The nicotinic acetylcholine receptor gene family of the malaria mosquito, Anopheles gambiae. Genomics 85, 176–187. https://doi.org/https://doi.org/10.1016/j.ygeno.2004.09.001

Jones, A.K., Raymond-delpech, V., Thany, S.H., Gauthier, M., Sattelle, D.B., 2006. The nicotinic acetylcholine receptor gene family of the honey bee, Apis mellifera 1422–1430. https://doi.org/10.1101/gr.4549206.

Jones, A.K., Sattelle, D.B., 2010. Diversity of insect nicotinic acetylcholine receptor subunits, in: Insect Nicotinic Acetylcholine Receptors. Springer US, New York, NY, pp. 25–43.

Jones, A.K., Sattelle, D.B., 2007. The cys-loop ligand-gated ion channel gene superfamily of the red flour beetle, Tribolium castaneum. BMC Genomics 8, 327. https://doi.org/10.1186/1471-2164-8-327

Jonker, M.J., Svendsen, C., Bedaux, J.J.M., Bongers, M., Kammenga, J.E., 2005. Significance testing of synergistic/antagonistic, dose level-dependent, or dose ratio-dependent effects in mixture dose-response analysis. Environ. Toxicol. Chem. 24, 2701–2713. https://doi.org/10.1897/04-431r.1

Kagabu, S., 2011. Discovery of Imidacloprid and Further Developments from Strategic Molecular Designs. Agric. Food Chem. 59, 2887–2896. https://doi.org/10.1021/jf101824y

Kao, P.N., Karlin, A., 1986. Acetylcholine receptor binding site contains a disulfide cross-link between adjacent half-cystinyl residues. J. Biol. Chem. 261, 8085–8088.

Karlin, A., 1977. Nicotinic acetylcholine receptors, in: Methods in Enzymology. Academic Press, pp. 582–590. https://doi.org/https://doi.org/10.1016/S0076-6879(77)46072-5

Katagi, T., Tanaka, H., 2016. Metabolism, bioaccumulation, and toxicity of pesticides in aquatic insect larvae. J. Pestic. Sci. 41, 25–37. https://doi.org/10.1584/jpestics.D15-064

Kaufman, P.E., Gerry, A.C., Rutz, D.A., Scott, J.G., 2006. Monitoring susceptibility of house flies (Musca domestica L.) in the United States to imidacloprid. J. Agric. Urban Entomol. 23, 195–200.

Kayser, H., Lehmann, K., Gomes, M., Schleicher, W., Dotzauer, K., Moron, M., Maienfisch, P., 2016. Binding of imidacloprid, thiamethoxam and N-desmethylthiamethoxam to nicotinic receptors of Myzus persicae: pharmacological profiling using neonicotinoids, natural agonists and antagonists. Pest Manag. Sci. 72, 2166–2175. https://doi.org/10.1002/ps.4249

Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., Buxton, H.T., 2002. Pharmaceuticals, Hormones, and Other Organic Wastewater Contaminants in U.S. Streams, 1999−2000: A National Reconnaissance. Environ. Sci. Technol. 36, 1202–1211. https://doi.org/10.1021/es011055j

Kreuger, J., Graaf, S., Patring, J., Adielsson, S., 2010. Pesticides in surface water in areas with open ground and greenhouse horticultural crops in Sweden 2008. Swedish Univ. Agric. Sci. 49.

Kreutzweiser, D., Good, K., Chartrand, D., Scarr, T., Thompson, D., 2007. Non-target effects on aquatic decomposer organisms of imidacloprid as a systemic insecticide to control emerald ash borer in riparian trees. Ecotoxicol. Environ. Saf. 68, 315–325. https://doi.org/https://doi.org/10.1016/j.ecoenv.2007.04.011

Krupke, C.H., Hunt, G.J., Eitzer, B.D., Andino, G., Given, K., 2012. Multiple Routes of Pesticide Exposure for Honey Bees Living Near Agricultural Fields. PLoS One 7, e29268.

Lagadic, L., Bernard, L., Leicht, W., 1993. Topical and oral activities of imidacloprid and cyfluthrin against susceptible laboratory strains of Heliothis virescens and Spodoptera littoralis. Pest Manag. Sci. 38, 323–328.

Lamers, M., Anyusheva, M., La, N., Nguyen, V.V., Streck, T., 2011. Pesticide Pollution in Surface- and Groundwater by Paddy Rice Cultivation: A Case Study from Northern Vietnam. CLEAN - Soil, Air, Water 39, 356–361. https://doi.org/10.1002/clen.201000268

Lancaster, J., Downes, B.J., 2018. Aquatic versus Terrestrial Insects: Real or Presumed Differences in Population Dynamics? Insects 9, 157. https://doi.org/10.3390/insects9040157

Lapied, B., Le Corronc, H., Hue, B., 1990. Sensitive nicotinic and mixed nicotinic-muscarinic receptors in insect neurosecretory cells. Brain Res. 533, 132–136. https://doi.org/https://doi.org/10.1016/0006-8993(90)91805-Q

Lind, R.J., Clough, M.S., Reynolds, S.E., Earley, F.G.P., 1998. [3 H] Imidacloprid Labels High- and Low-Affinity Nicotinic Acetylcholine Receptor-like Binding Sites in the Aphid Myzus persicae (Hemiptera : Aphididae). Pestic. Biochem. Physiol. 62, 3–14.

Littleton, J.T., Ganetzky, B., 2000. Ion Channels and Synaptic Organization: Analysis of the Drosophila Genome. Neuron 26, 35–43. https://doi.org/10.1016/S0896-6273(00)81135-6

Liu, M.Y., Casida, J.E., 1993. High Affinity Binding of [3H]Imidacloprid in the Insect Acetylcholine Receptor. Pestic. Biochem. Physiol. 46, 40–46. https://doi.org/https://doi.org/10.1006/pest.1993.1034

Loureiro, S., Svendsen, C., Ferreira, A.L.G., Pinheiro, C., Ribeiro, F., Soares, A.M.V.M., 2010. Toxicity of three binary mixtures to daphnia magna: Comparing chemical modes of action and deviations from conceptual models. Environ. Toxicol. Chem. 29, 1716–1726. https://doi.org/10.1002/etc.198

Lu, Z., Challis, J.K., Wong, C.S., 2015. Quantum Yields for Direct Photolysis of Neonicotinoid Insecticides in Water: Implications for Exposure to Nontarget Aquatic Organisms. Environ. Sci. Technol. Lett. 2, 188–192. https://doi.org/10.1021/acs.estlett.5b00136

Main, A.R., 2016. Spatiotemporal Patterns of Distribution and Drivers of Neonicotinoid fate in Canadian Prairie Pothole Wetlands. University of Saskatchewan.

Main, A.R., Headley, J. V., Peru, K.M., Michel, N.L., Cessna, A.J., Morrissey, C.A., 2014. Widespread use and frequent detection of neonicotinoid insecticides in wetlands of Canada's prairie pothole region. PLoS One 9. https://doi.org/10.1371/journal.pone.0092821

Main, A.R., Michel, N.L., Cavallaro, M.C., Headley, J. V., Peru, K.M., Morrissey, C.A., 2016. Snowmelt transport of neonicotinoid insecticides to Canadian Prairie wetlands. Agric. Ecosyst. Environ. 215, 76–84. https://doi.org/10.1016/j.agee.2015.09.011

Malaj, E., Liber, K., Morrissey, C.A., n.d. Spatial distribution of agricultural pesticide use and predicted wetland exposure in the Canadian Prairie Pothole Region. Sci. Total Environ.

Malev, O., Klobučar, R.S., Fabbretti, E., Trebše, P., 2012. Comparative toxicity of imidacloprid and its transformation product 6-chloronicotinic acid to non-target aquatic organisms: Microalgae Desmodesmus subspicatus and amphipod Gammarus fossarum. Pestic. Biochem. Physiol. 104, 178–186. https://doi.org/10.1016/j.pestbp.2012.07.008

Maloney, E.M., Liber, K., Headley, J.V., Peru, K.M., Morrissey, C.A., 2018a. Neonicotinoid insecticide mixtures: evaluation of laboratory-based toxicity predictions under semi-controlled field conditions. Environ. Pollut. Accepted.

Maloney, E.M., Morrissey, C.A., Headley, J.V., Peru, K.M., Liber, K., 2018b. Can chronic exposure to imidacloprid, clothianidin, and thiamethoxam mixtures exert greater than additive toxicity in Chironomus dilutus? Ecotoxicol. Environ. Saf. 156, 354–365. https://doi.org/10.1016/j.ecoenv.2018.03.003

Maloney, E.M., Morrissey, C.A., Headley, J.V., Peru, K.M., Liber, K., 2017. Cumulative toxicity of neonicotinoid insecticide mixtures to Chironomus dilutus under acute exposure

scenarios. Environ. Toxicol. Chem. 36. https://doi.org/10.1002/etc.3878

Matsuda, K., Buckingham, S.D., Kleier, D., Rauh, J.J., Grauso, M., Sattelle, D.B., 2001. Neonicotinoids: insecticides acting on insect nicotinic acetylcholine receptors. Trends Pharmacol. Sci. 22, 573–580. https://doi.org/10.1016/S0165-6147(00)01820-4

Matsuda, K., Kanaoka, S., Akamatsu, M., Sattelle, D.B., 2009. Diverse Actions and Target-Site Selectivity of Neonicotinoids: Structural Insights. 2Molecular Pharmacol. 76, 1–10.

Maus, C., Nauen, R., 2011. Response to the publication: Tennekes HA (2010): The significance of the Druckrey-Kupfmuller equation for risk assessment - The toxicity of neonicotinoid insecticides to arthropods is reinforced by exposure time. Toxicology 280, 176–7.

Merritt, R.W., Cummins, K.W., Berg, M.B., 2008. An Introduction to the Aquatic Insects of North America, 4th ed. Kendall Hunt Publishing Company, Dubuque, IA.

Metcalfe, C.D., Helm, P., Paterson, G., Kaltenecker, G., Murray, C., Nowierski, M., Sultana, T., 2019. Pesticides related to land use in watersheds of the Great Lakes basin. Sci. Total Environ. 648, 681–692. https://doi.org/https://doi.org/10.1016/j.scitotenv.2018.08.169

Miles, J.C., Hua, J., Sepulveda, M.S., Krupke, C.H., Hoverman, J.T., 2017. Effects of clothianidin on aquatic communities: Evaluating the impacts of lethal and sublethal exposure to neonicotinoids. PLoS One 12, 1–24. https://doi.org/10.1371/journal.pone.0174171

Millar, N.S., 2003. Assembly and subunit diversity of nicotinic acetylcholine receptors. Biochem. Soc. Trans. 31, 869 LP – 874. https://doi.org/10.1042/bst0310869

Millar, N.S., Denholm, I., 2007. Nicotinic acetylcholine receptors: targets for commercially important insecticides. Invertebr. Neurosci. 7, 53–66.

Millar, N.S., Gotti, C., 2009. Diversity of vertebrate nicotinic acetylcholine receptors.

Neuropharmacology 56, 237–246. https://doi.org/https://doi.org/10.1016/j.neuropharm.2008.07.041

Mineau, P., Palmer, C., 2013. The Impact of the Nation's Most Widely Used Insecticides on Birds, in: American Bird Conservancy. pp. 1–98.

Mineau, P., Whiteside, M., 2013. Pesticide Acute Toxicity Is a Better Correlate of U.S. Grassland Bird Declines than Agricultural Intensification. PLoS One 8, e57457.

Mohr, S., Berghahn, R., Schmiediche, R., Hübner, V., Loth, S., Feibicke, M., Mailahn, W., Wogram, J., 2012. Macroinvertebrate community response to repeated short-term pulses of the insecticide imidacloprid. Aquat. Toxicol. 110–111, 25–36. https://doi.org/https://doi.org/10.1016/j.aquatox.2011.11.016

Morrissey, C.A., Mineau, P., Devries, J.H., Sanchez-Bayo, F., Liess, M., Cavallaro, M.C., Liber, K., 2015. Neonicotinoid contamination of global surface waters and associated risk to aquatic invertebrates: A review. Environ. Int. 74, 291–303. https://doi.org/10.1016/j.envint.2014.10.024

Nauen, R., Hungenberg, H., Tollo, B., Tietjen, K., Elbert, A., 1998a. Antifeedant Effect, Biological Efficacy and High Affinity Binding of Imidacloprid to Acetylcholine Receptors in Myzus persicae and Myzus nicotianae. Pestic. Sci. 53, 133–140.

Nauen, R., Tietjen, K., Wagner, K., Elbert, A., 1998b. Efficacy of plant metabolites of imidacloprid against Myzus persicae and Aphis gossypii (Homoptera: Aphididae). Pestic. Sci. 52, 53–57. https://doi.org/10.1002/(SICI)1096-9063(199801)52:1<53::AID-PS621>3.0.CO;2-6

Norberg-King, T.J., 1993. An Interpolation Estimate for Chronic Toxicity: The ICp Approach. Duluth, MN, USA.

Nuyttens, D., Devarrewaere, W., Verboven, P., Foqué, D., 2013. Pesticide-laden dust emission and drift from treated seeds during seed drilling: a review. Pest Manag. Sci. 69, 564–575. https://doi.org/10.1002/ps.3485

Odume, O.N., Muller, W.J., 2011. Diversity and structure of Chironomidae communities in relation to water quality differences in the Swartkops River. Phys. Chem. Earth 36, 929–938. https://doi.org/10.1016/j.pce.2011.07.063

Ohkawara, Y., Akayama, A., Matsuda, K., Andersch, W., 2002. Clothianidin: a novel broad-spectrum neonicotinoid insecticide, in: Brighton Crop Protection Conference: Pests and Diseases. British Crop Protection Council, Brighton, UK, pp. 51–58.

Oliveira, E.E., Schleicher, S., Büschges, A., Schmidt, J., Kloppenburg, P., Salgado, V.L., 2011. Desensitization of nicotinic acetylcholine receptors in central nervous system neurons of the stick insect (Carausius morosus) by imidacloprid and sulfoximine insecticides. Insect Biochem. Mol. Biol. 41, 872–880. https://doi.org/10.1016/j.ibmb.2011.08.001

Oliver, D.R., 1976. Chironomidae (Diptera) of Char Lake, Cornwallis Island, NWT with descriptions of two new species. Can. Entomol. 108, 1053–1064.

Oliver, D.R., 1971. Life History of the Chironomidae. Annu. Rev. Entomol. 16, 211–230. https://doi.org/10.1146/annurev.en.16.010171.001235

Oliver, D.R., Rousssel, M.E., 1983. The Genera of Larval Midges of Canada. Diptera: Chironomidae. Ottawa, Ontario, Canada.

Orendt, C., 1999. Chironomids as bioindicators in acidified streams: a contribution to the acidity tolerance of chironomid species with a classification in sensitivity classes. Int. Rev. Hydrobiol. 84, 439–449.

Organization for Economic Cooperation and Development (OECD), 2011. Chironomus sp.,

Acute Immobilisation Test. OECD Publishing, p. 17.

Orlando, E.F., Guillette, L.J., 2001. A re-examination of variation associated with envrionmentally stressed organisms. Hum. Reprod. Update 7, 265–272.

Ospina-Pérez, E.M., Campeón-Morales, O.I., Richardi, V.S., Rivera-Páez, F.A., 2019. Histological description of immature Chironomus columbiensis (Diptera: Chironomidae): A potential contribution to environmental monitoring. Microsc. Res. Tech. 82, 1277–1289. https://doi.org/10.1002/jemt.23278

Pape, T., Blagoderov, V., Mostovski, M.B., 2011. Order Diptera Linnaeus, 1758, in: Zang, Z.-Q. (Ed.), Animal Biodiversity: An Outline of Higher-Level Classification and Survey of Taxonomic Richness. Magnolia Press, pp. 222–229.

Parkinson, R.H., Little, J.M., Gray, J.R., 2017. A sublethal dose of a neonicotinoid insecticide disrupts visual processing and collision avoidance behaviour in Locusta migratoria. Sci. Rep. 7, 936.

Pavlaki, M.D., Pereira, R., Loureiro, S., Soares, A.M.V.M., 2011. Ecotoxicology and Environmental Safety Effects of binary mixtures on the life traits of Daphnia magna. Ecotoxicol. Environ. Saf. 74, 99–110. https://doi.org/10.1016/j.ecoenv.2010.07.010

Peña, A., Rodríguez-Liébana, J.A., Mingorance, M.D., 2011. Persistence of two neonicotinoid insecticides in wastewater, and in aqueous solutions of surfactants and dissolved organic matter. Chemosphere 84, 464–470. https://doi.org/https://doi.org/10.1016/j.chemosphere.2011.03.039

Pest Management Regulatory Agency, 2019a. Registration Decision (RD2019-06): Thiamethoxam and associated seed treatment end-use products. Ottawa, Ontario.

Pest Management Regulatory Agency, 2019b. Registration Decision (RD2019-05): Clothianidin

and associated end-use products. Ottawa, Ontario.

Pest Management Regulatory Agency, 2018a. Special Review of Thiamethoxam Risk to Aquatic Invertebrates: Proposed Decision for Consultation. Ottawa, Ontario.

Pest Management Regulatory Agency, 2018b. Proposed Special Review Decision. PSRD2018-02. Special Review of Thiamethoxam Risk to Aquatic Invertebrates : Proposed Decision for Consultation. Ottawa, Ontario.

Pest Management Regulatory Agency, 2018c. Proposed Special Review Decision. PSRD2018-01. Special Review of Clothianidin Risk to Aquatic Invertebrates : Proposed Decision for Consultation. Ottawa, Ontario.

Pest Management Regulatory Agency, 2016. PRVD2016-20 Proposed Re-evaluation Decision: Imidacloprid. Ottawa, Ontario.

Pest Management Regulatory Agency, 2001. REG2001-11: Imidacloprid [WWW Document]. URL http://publications.gc.ca/collections/Collection/H113-7-2001-11E.pdf. (accessed 11.12.18).

Pestana, J., Loureiro, S., Baird, D.J., Soares, A.M.V.M., 2009. Fear and loathing in the benthos: Responses of aquatic insect larvae to the pesticide imidacloprid in the presence of chemical signals of predation risk. Aquat. Toxicol. 93, 138–149. https://doi.org/https://doi.org/10.1016/j.aquatox.2009.04.008

Pestana, J.L.T., Alexander, A.C., Culp, J.M., Baird, D.J., Cessna, A.J., Soares, A.M.V.M., 2009. Structural and functional responses of benthic invertebrates to imidacloprid in outdoor stream mesocosms. Environ. Pollut. 157, 2328–2334. https://doi.org/https://doi.org/10.1016/j.envpol.2009.03.027

Phillips, P.J., Bode, R.W., 2004. Pesticides in surface water runoff in south-eastern New York

State, USA: seasonal and stormflow effects on concentrations. Pest Manag. Sci. 60, 531–543. https://doi.org/doi:10.1002/ps.879

Posthuma-Doodeman, C.J.A.M., 2008. Environmental risk limits for imidacloprid (RIVM Letter Report). Bilthoven, the Netherlands. https://doi.org/601716018/2008

Poulin, B., Lefebvre, G., Paz, L., 2010. Red flag for green spray: adverse trophic effects of Bti on breeding birds. J. Appl. Ecol. 47, 884–889. https://doi.org/10.1111/j.1365-2664.2010.01821.x

Raby, M., Nowierski, M., Perlov, D., Zhao, X., Hao, C., Poirier, D.G., Sibley, P.K., 2018a. Acute toxicity of 6 neonicotinoid insecticides to freshwater invertebrates. Environ. Toxicol. Chem. 37, 1430–1445. https://doi.org/10.1002/etc.4088

Raby, M., Zhao, X., Hao, C., Poirier, D.G., Sibley, P.K., 2018b. Chronic toxicity of 6 neonicotinoid insecticides to Chironomus dilutus and Neocleon triangulifer. Environ. Toxicol. Chem. 37, 2727–2739. https://doi.org/10.1002/etc.4234

Rakotondravelo, M.L., Anderson, T.D., Charlton, R.E., Zhu, K.Y., 2006. Sublethal effects of three pesticides on activities of selected target and detoxification enzymes in the aquatic midge, Chironomus tentans (Diptera: Chironomidae). Arch. Environ. Contam. Toxicol. 51, 360–366. https://doi.org/10.1007/s00244-005-0227-0

Rand, G.M., 1995. Fundamentals of Aquatic Toxicology: Effects, Environmental Fate and Risk Assessment, 6th ed. Taylor & Francis, Washington, DC.

Richardi, V.S., Vicentini, M., Rebechi, D., Fávaro, L.F., Navarro-Silva, M.A., 2015. Morpho-histological characterization of immature of the bioindicator midge Chironomus sancticaroli Strixino and Strixino (Diptera, Chironomidae) . Rev. Bras. Entomol. .

Richards, S., Gibbs, R.A., Weinstock, G.M., Brown, S.J., Denell, R., Beeman, R.W., Gibbs, R.,

Beeman, R.W., Brown, S.J., Bucher, G., Friedrich, M., Grimmelikhuijzen, C.J.P., Klingler, M., Lorenzen, M., Richards, S., Roth, S., Schröder, R., Tautz, D., Zdobnov, E.M., Muzny, D., Gibbs, R.A., Weinstock, G.M., Attaway, T., Bell, S., Buhay, C.J., Chandrabose, M.N., Chavez, D., Clerk-Blankenburg, K.P., Cree, A., Dao, M., Davis, C., Chacko, J., Dinh, H., Dugan-Rocha, S., Fowler, G., Garner, T.T., Garnes, J., Gnirke, A., Hawes, A., Hernandez, J., Hines, S., Holder, M., Hume, J., Jhangiani, S.N., Joshi, V., Khan, Z.M., Jackson, L., Kovar, C., Kowis, A., Lee, S., Lewis, L.R., Margolis, J., Morgan, M., Nazareth, L. V, Nguyen, N., Okwuonu, G., Parker, D., Richards, S., Ruiz, S.-J., Santibanez, J., Savard, J., Scherer, S.E., Schneider, B., Sodergren, E., Tautz, D., Vattahil, S., Villasana, D., White, C.S., Wright, R., Park, Y., Beeman, R.W., Lord, J., Oppert, B., Lorenzen, M., Brown, S., Wang, L., Savard, J., Tautz, D., Richards, S., Weinstock, G., Gibbs, R.A., Liu, Y., Worley, K., Weinstock, G., Elsik, C.G., Reese, J.T., Elhaik, E., Landan, G., Graur, D., Arensburger, P., Atkinson, P., Beeman, R.W., Beidler, J., Brown, S.J., Demuth, J.P., Drury, D.W., Du, Y.-Z., Fujiwara, H., Lorenzen, M., Maselli, V., Osanai, M., Park, Y., Robertson, H.M., Tu, Z., Wang, J., Wang, S., Richards, S., Song, H., Zhang, L., Sodergren, E., Werner, D., Stanke, M., Morgenstern, B., Solovyev, V., Kosarev, P., Brown, G., Chen, H.-C., Ermolaeva, O., Hlavina, W., Kapustin, Y., Kiryutin, B., Kitts, P., Maglott, D., Pruitt, K., Sapojnikov, V., Souvorov, A., Mackey, A.J., Waterhouse, R.M., Wyder, S., Zdobnov, E.M., Zdobnov, E.M., Wyder, S., Kriventseva, E. V, Kadowaki, T., Bork, P., Aranda, M., Bao, R., Beermann, A., Berns, N., Bolognesi, R., Bonneton, F., Bopp, D., Brown, S.J., Bucher, G., Butts, T., Chaumot, A., Denell, R.E., Ferrier, D.E.K., Friedrich, M., Gordon, C.M., Jindra, M., Klingler, M., Lan, Q., Lattorff, H.M.G., Laudet, V., von Levetsow, C., Liu, Z., Lutz, R., Lynch, J.A., da Fonseca, R.N., Posnien, N., Reuter, R., Roth, S., Savard, J., Schinko, J.B.,

Schmitt, C., Schoppmeier, M., Schröder, R., Shippy, T.D., Simonnet, F., Marques-Souza, H., Tautz, D., Tomoyasu, Y., Trauner, J., Van der Zee, M., Vervoort, M., Wittkopp, N., Wimmer, E.A., Yang, X., Jones, A.K., Sattelle, D.B., Ebert, P.R., Nelson, D., Scott, J.G., Beeman, R.W., Muthukrishnan, S., Kramer, K.J., Arakane, Y., Beeman, R.W., Zhu, Q., Hogenkamp, D., Dixit, R., Oppert, B., Jiang, H., Zou, Z., Marshall, J., Elpidina, E., Vinokurov, K., Oppert, C., Zou, Z., Evans, J., Lu, Z., Zhao, P., Sumathipala, N., Altincicek, B., Vilcinskas, A., Williams, M., Hultmark, D., Hetru, C., Jiang, H., Grimmelikhuijzen, C.J.P., Hauser, F., Cazzamali, G., Williamson, M., Park, Y., Li, B., Tanaka, Y., Predel, R., Neupert, S., Schachtner, J., Verleyen, P., Raible, F., Bork, P., Friedrich, M., Walden, K.K.O., Robertson, H.M., Angeli, S., Forêt, S., Bucher, G., Schuetz, S., Maleszka, R., Wimmer, E.A., Beeman, R.W., Lorenzen, M., Tomoyasu, Y., Miller, S.C., Grossmann, D., Bucher, G., 2008. The genome of the model beetle and pest Tribolium castaneum. Nature 452, 949–955. https://doi.org/10.1038/nature06784

Robinson, S.A., Richardson, S.D., Dalton, R.L., Maisonneuve, F., Bartlett, A.J., de Solla, S.R., Trudeau, V.L., Waltho, N., 2019. Assessment of Sublethal Effects of Neonicotinoid Insecticides on the Life-History Traits of 2 Frog Species. Environ. Toxicol. Chem. 38, 1967–1977. https://doi.org/10.1002/etc.4511

Salgado, V.L., Saar, R., 2004. Desensitizing and non-desensitizing subtypes of alpha-bungarotoxin- sensitive nicotinic acetylcholine receptors in cockroach neurons. J. Insect Physiol. 50, 867–879. https://doi.org/10.1016/j.jinsphys.2004.07.007

Sanchez-Bayo, F., 2012. Insecticides mode of action in relation to their toxicity to non-target organisms. J. Environ. Anal. Toxicol. S4, 1–11.

Sánchez-Bayo, F., Goka, K., 2009. Ecological effects of the insecticide imidacloprid and a

pollutant from antidandruff shampoo in experimental rice fields. Environ. Toxicol. Chem. 25, 1677–1687. https://doi.org/10.1897/05-404R.1

Sánchez-Bayo, F., Goka, K., Hayasaka, D., 2016. Contamination of the Aquatic Environment with Neonicotinoids and its Implication for Ecosystems. Front. Environ. Sci. 4, 1–14. https://doi.org/10.3389/fenvs.2016.00071

Sánchez-Bayo, F., Hyne, R. V., 2014. Detection and analysis of neonicotinoids in river waters – Development of a passive sampler for three commonly used insecticides. Chemosphere 99, 143–151. https://doi.org/10.1016/J.CHEMOSPHERE.2013.10.051

Sarkar, M.A., Biswas, P.K., Roy, S., Kole, R.K., Chowdhury, A., 1999. Effect of pH and type of formulation on the persistence of imidacloprid in water. Bull. Environ. Contam. Toxicol. 63, 604–609.

Schaafsma, A., Limay-Rios, V., Baute, T., Smith, J., Xue, Y., 2015. Neonicotinoid insecticide residues in surface water and soil associated with commercial maize (corn) fields in Southwestern Ontario. PLoS One 10, 1–21. https://doi.org/10.1371/journal.pone.0118139

Schloss, P., Hermans-Borgmeyer, I., Betz, H., Gundelfinger, E.D., 1988. Neuronal acetylcholine receptors in Drosophila: the ARD protein is a component of a high-affinity alpha-bungarotoxin binding complex. EMBO J. 7, 2889–2894. https://doi.org/10.1002/j.1460-2075.1988.tb03146.x

Schmitt-Jansen, M., Veit, U., Dudel, G., Altenburger, R., 2008. An ecological perspective in aquatic ecotoxicology: Approaches and challenges. Basic Appl. Ecol. 9, 337–345. https://doi.org/https://doi.org/10.1016/j.baae.2007.08.008

Schreiner, V.C., Szocs, E., Bhowmik, A.K., Vijver, M.G., Schafer, R.B., 2016. Pesticide mixtures in streams of several European countries and the USA. Sci. Total Environ. 573,

680–689. https://doi.org/10.1016/j.scitotenv.2016.08.163

Schubauer-Berigan, M.K., Monson, P.D., Ankley, G.T., 1995. Influence of pH on the toxicity of ammonia to Chironomus tentans and Lumbriculus variegatus. Environ. Toxicol. Chem. 14, 713–717.

Shao, Y.-M., Dong, K., Zhang, C.-X., 2007. The nicotinic acetylcholine receptor gene family of the silkworm, Bombyx mori. BMC Genomics 8, 324. https://doi.org/10.1186/1471-2164-8-324

Shiokawa, K., Tsuboi, S.-I., Iwaya, K., Moriya, K., 1994. Development of a Chloronicotinyl Insecticide, Imidacloprid. J. Pestic. Sci. 19, S209.

Simon-Delso, N., Pisa, L., Van der Sluijs, J.P., Amaral-Rogers Buglife, V., Belzunces, L.P., Bonmatin, J.M., Downs, C., Furlan, L., Gibbons, D.W., Giorio, C., Girolami, V., Goulson, D., Kreutzweiser, D.P., Krupke, C.H., Long, E., Liess, M., McField, M., 2015. Worldwide Integrated Assessment of the Impact of Systemic Pesticides on Biodiversity and Ecosystems. Env. Sci Pollut Res. https://doi.org/10.1007/s11356-014-3470-y

Smalling, K.L., Reeves, R., Muths, E., Vandever, M., Battaglin, W.A., Hladik, M.L., Pierce, C.L., 2015. Pesticide concentrations in frog tissue and wetland habitats in a landscape dominated by agriculture. Sci. Total Environ. 502, 80–90. https://doi.org/10.1016/j.scitotenv.2014.08.114

Smit, C.E., 2014. Water quality standards for imidacloprid. Proposal for an update according to the Water Water quality standards for imidacloprid 94 pp.

Smit, C.E., Posthuma-Doodeman, C.J.A.M., van Vlaardingen, P.L.A., de Jong, F.M.W., 2015. Ecotoxicity of Imidacloprid to Aquatic Organisms: Derivation of Water Quality Standards for Peak and Long-Term Exposure. Hum. Ecol. Risk Assess. An Int. J. 21, 1608–1630.

https://doi.org/10.1080/10807039.2014.964071

Smith, R., Middlebrook, R., Turner, R., Huggins, R., Vardy, S., Warne, M., 2012. Large-scale pesticide monitoring across Great Barrier Reef catchments – Paddock to Reef Integrated Monitoring, Modelling and Reporting Program. Mar. Pollut. Bull. 65, 117–127. https://doi.org/10.1016/J.MARPOLBUL.2011.08.010

Starner, K., Goh, K.S., 2012. Detections of the neonicotinoid insecticide imidacloprid in surface waters of three agricultural regions of California, USA, 2010-2011. Bull. Environ. Contam. Toxicol. 88, 316–321. https://doi.org/10.1007/s00128-011-0515-5

Stoughton, S.J., Liber, K., Culp, J., Cessna, A., 2008. Acute and chronic toxicity of imidacloprid to the aquatic invertebrates Chironomus tentans and Hyalella azteca under constant- and pulse-exposure conditions. Arch. Environ. Contam. Toxicol. 54, 662–673. https://doi.org/10.1007/s00244-007-9073-6

Struger, J., Grabuski, J., Cagampan, S., Sverko, E., McGoldrick, D., Marvin, C.H., 2017. Factors influencing the occurrence and distribution of neonicotinoid insecticides in surface waters of southern Ontario, Canada. Chemosphere 169, 516–523. https://doi.org/https://doi.org/10.1016/j.chemosphere.2016.11.036

Taillebois, E., Beloula, A., Quinchard, S., Jaubert-Possamai, S., Daguin, A., Servent, D., Tagu, D., Thany, S.H., Tricoire-Leignel, H., 2014. Neonicotinoid binding, toxicity and expression of nicotinic acetylcholine receptor subunits in the aphid Acyrthosiphon pisum. PLoS One 9. https://doi.org/10.1371/journal.pone.0096669

Taillebois, E., Cartereau, A., Jones, A.K., Thany, S.H., 2018. Neonicotinoid insecticides mode of action on insect nicotinic acetylcholine receptors using binding studies. Pestic. Biochem. Physiol. 151, 59–66. https://doi.org/10.1016/j.pestbp.2018.04.007

Tang, L.-D., Wu, J.-H., Ali, S., Ren, S.-X., 2013. Establishment of Baseline Toxicity Data to Different Insecticides for Aphis craccivora Koch and Rhopalosiphum maidis (Fitch) (Homoptera: Aphididae) by Glass Tube Residual Film Technique. Pak. J. Zool. 45, 411–415.

Tennekes, H.A., 2011. The significance of the Druckrey-Kupfmuller equation for risk assessment - The toxicity of neonicotinoid insecticides to arthropods is reinforced by exposure time: Responding to a Letter to the Editor by Drs. C. Maus and R. Nauen of Bayer CropScience AG. Toxicology 280, 173–5.

Tennekes, H.A., 2010. The Significance of the Druckrey-Küpfmüller Equation for Risk Assessment - The Toxicity of Neonicotinoid Insecticides to Arthropods is Reinforced by Exposure Time. Toxicology 276, 1–4. https://doi.org/https://doi.org/10.1016/j.tox.2010.07.005

Tennekes, H.A., Sánchez-Bayo, F., 2013. The molecular basis of simple relationships between exposure concentration and toxic effects with time. Toxicology 309, 39–51. https://doi.org/https://doi.org/10.1016/j.tox.2013.04.007

Thany, S.H., 2011. Thiamethoxam, a poor agonist of nicotinic acetylcholine receptors expressed on isolated cell bodies, acts as a full agonist at cockroach cercal afferent/giant interneuron synapses. Neuropharmacology 60, 587–592. https://doi.org/10.1016/j.neuropharm.2010.12.008

Thany, S.H., 2009. Agonist actions of clothianidin on synaptic and extrasynaptic nicotinic acetylcholine receptors expressed on cockroach sixth abdominal ganglion. Neurotoxicology 30, 1045–1052. https://doi.org/10.1016/j.neuro.2009.06.013

Thany, S.H., Tricoire-Leignel, H., 2011. Emerging Pharmacological Properties of Cholinergic

Synaptic Transmission: Comparison between Mammalian and Insect Synaptic and Extrasynaptic Nicotinic Receptors. Curr. Neuropharmacol. 9, 706–714. https://doi.org/10.2174/157015911798376343

Thuyet, D.Q., Watanabe, H., Yamazaki, K., Takagi, K., 2011. Photodegradation of Imidacloprid and Fipronil in Rice–Paddy Water. Bull. Environ. Contam. Toxicol. 86, 548–553. https://doi.org/10.1007/s00128-011-0243-x

Tomizawa, M., Casida, J.E., 2004. Neonicotinoid Insecticide Toxicology: Mechanisms of Selective Action. Annu. Rev. Pharmacol. Toxicol. 45, 247–268. https://doi.org/10.1146/annurev.pharmtox.45.120403.095930

Tomizawa, M., Casida, J.E., 2003. Selective Toxicity of Neonicotinoids Attributable to Specificity of Insect and Mammalian Nicotinic Acetylcholine Receptors. Annu. Rev. Entomol. 48, 339–364. https://doi.org/10.1146/annurev.ento.48.091801.112731

Tomizawa, M., Casida, J.E., 2001. Structure and diversity of insect nicotinic acetylcholine receptors. Pest Manag. Sci. 57, 914–922. https://doi.org/10.1002/ps.349

Tomizawa, M., Millar, N.S., Casida, J.E., 2005. Pharmacological profiles of recombinant and native insect nicotinic acetylcholine receptors. https://doi.org/10.1016/j.ibmb.2005.08.006

Uneme, H., 2011. Chemistry of Clothianidin and Related Compounds. J. Agric. Food Chem. 59, 2932–2937. https://doi.org/10.1021/jf1024938

United States Environmental Protection Agency, 2019. Pesticide Product and Label System [WWW Document]. URL https://iaspub.epa.gov/apex/pesticides/f?p=PPLS:1 (accessed 10.5.19).

United States Environmental Protection Agency, 2018. Ecological Risk Assessment for Pesticides: Technical Overview [WWW Document]. URL https://www.epa.gov/pesticide-

science-and-assessing-pesticide-risks/ecological-risk-assessment-pesticides-technical (accessed 8.20.18).

United States Environmental Protection Agency, 2003. Pesticide Fact Sheet: Clothianidin [WWW Document]. URL https://www3.epa.gov/pesticides/chem_search/reg_actions/registration/fs_PC-044309_30-May-03.pdf (accessed 11.20.18).

United States Environmental Protection Agency, 1994. Methods for Measuring the Toxicity and Bioaccumulation of Sediment-Associated Contaminants with Freshwater Invertebrates. Duluth, MN, USA.

USEPA, 2017. Aquatic Life Benchmarks for Pesticide Registration [WWW Document]. URL www.epa.gov/pesticide-science-and-assessing-pesticide-risks/aquatic-life-benchmarks-pesticide-registration (accessed 7.25.17).

Uzman, A., 2001. Molecular Cell Biology (4th edition). Biochem. Mol. Biol. Educ. 29, 126–128. https://doi.org/doi: 10.1016/s1470-8175(01)00023-6

Van den Brink, P.J., Van Smeden, J.M., Bekele, R.S., Dierick, W., De Gelder, D.M., Noteboom, M., Roessink, I., 2016. Acute and chronic toxicity of neonicotinoids to nymphs of a mayfly species and some notes on seasonal differences. Environ. Toxicol. Chem. 35, 128–133. https://doi.org/10.1002/etc.3152

van Dijk, T.C., 2010. Effects of neonicotinoid pesticide pollution of Dutch surface water on non - target species abundance 1–77.

Verbruggen, E.M.J., Van den Brink, P.J., 2010. Review of recent literature concerning mixture toxicity of pesticides to aquatic organisms of pesticides to aquatic organisms. Natl. Inst. Public Heal. Environ. 1–33.

Wallace, J.B., Webster, J.R., 1996. The role of macroinvertebrates in stream ecosystem function. Annu. Rev. Entomol. 41, 115.

Wang, Y.H., Liu, X.G., Zhu, Y.C., Wu, S.G., Li, S.Y., Chen, W.M., Shen, J.L., 2009. Inheritance mode and realized heritability of resistance to imidacloprid in the brown planthopper, Nilaparvata lugens (Stål) (Homoptera: Delphacidae). Pest Manag. Sci. 65, 629–634. https://doi.org/10.1002/ps.1720

Weinstock, G.M., Robinson, G.E., Gibbs, R.A., Weinstock, G.M., Weinstock, G.M., Robinson, G.E., Worley, K.C., Evans, J.D., Maleszka, R., Robertson, H.M., Weaver, D.B., Beye, M., Bork, P., Elsik, C.G., Evans, J.D., Hartfelder, K., Hunt, G.J., Robertson, H.M., Robinson, G.E., Maleszka, R., Weinstock, G.M., Worley, K.C., Zdobnov, E.M., Hartfelder, K., Amdam, G. V, Bitondi, M.M.G., Collins, A.M., Cristino, A.S., Evans, J.D., Michael, H., Lattorff, G., Lobo, C.H., Moritz, R.F.A., Nunes, F.M.F., Page, R.E., Simões, Z.L.P., Wheeler, Diana, Carninci, P., Fukuda, S., Hayashizaki, Y., Kai, C., Kawai, J., Sakazume, N., Sasaki, D., Tagami, M., Maleszka, R., Amdam, G. V, Albert, S., Baggerman, G., Beggs, K.T., Bloch, G., Cazzamali, G., Cohen, M., Drapeau, M.D., Eisenhardt, D., Emore, C., Ewing, M.A., Fahrbach, S.E., Forêt, S., Grimmelikhuijzen, C.J.P., Hauser, F., Hummon, A.B., Hunt, G.J., Huybrechts, J., Jones, A.K., Kadowaki, T., Kaplan, N., Kucharski, R., Leboulle, G., Linial, M., Littleton, J.T., Mercer, A.R., Page, R.E., Robertson, H.M., Robinson, G.E., Richmond, T.A., RodriguezZas, S.L., Rubin, E.B., Sattelle, D.B., Schlipalius, D., Schoofs, L., Shemesh, Y., Sweedler, J. V, Velarde, R., Verleyen, P., Vierstraete, E., Williamson, M.R., Beye, M., Ament, S.A., Brown, S.J., Corona, M., Dearden, P.K., Dunn, W.A., Elekonich, M.M., Elsik, C.G., Forêt, S., Fujiyuki, T., Gattermeier, I., Gempe, T., Hasselmann, M., Kadowaki, T., Kage, E., Kamikouchi, A.,

Kubo, T., Kucharski, R., Kunieda, T., Lorenzen, M., Maleszka, R., Milshina, N. V, Morioka, M., Ohashi, K., Overbeek, R., Page, R.E., Robertson, H.M., Robinson, G.E., Ross, C.A., Schioett, M., Shippy, T., Takeuchi, H., Toth, A.L., Willis, J.H., Wilson, M.J., Robertson, H.M., Zdobnov, E.M., Bork, P., Elsik, C.G., Gordon, K.H.J., Letunic, I., Hackett, K., Peterson, J., Felsenfeld, A., Guyer, M., Solignac, M., Agarwala, R., Cornuet, J.M., Elsik, C.G., Emore, C., Hunt, G.J., Monnerot, M., Mougel, F., Reese, J.T., Schlipalius, D., Vautrin, D., Weaver, D.B., Gillespie, J.J., Cannone, J.J., Gutell, R.R., Johnston, J.S., Elsik, C.G., Cazzamali, G., Eisen, M.B., Grimmelikhuijzen, C.J.P., Hauser, F., Hummon, A.B., Iyer, V.N., Iyer, V., Kosarev, P., Mackey, A.J., Maleszka, R., Reese, J.T., Richmond, T.A., Robertson, H.M., Solovyev, V., Souvorov, A., Sweedler, J. V, Weinstock, G.M., Williamson, M.R., Zdobnov, E.M., Evans, J.D., Aronstein, K.A., Bilikova, K., Chen, Y.P., Clark, A.G., Decanini, L.I., Gelbart, W.M., Hetru, C., Hultmark, D., Imler, J.-L., Jiang, Haobo, Kanost, M., Kimura, K., Lazzaro, B.P., Lopez, D.L., Simuth, J., Thompson, G.J., Zou, Z., De Jong, P., Sodergren, E., Csűrös, M., Milosavljevic, A., Johnston, J.S., Osoegawa, K., Richards, S., Shu, C.-L., Weinstock, G.M., Elsik, C.G., Duret, L., Elhaik, E., Graur, D., Reese, J.T., Robertson, H.M., Robertson, H.M., Elsik, C.G., Maleszka, R., Weaver, D.B., Amdam, G. V, Anzola, J.M., Campbell, K.S., Childs, K.L., Collinge, D., Crosby, M.A., Dickens, C.M., Elsik, C.G., Gordon, K.H.J., Gramates, L.S., Grozinger, C.M., Jones, P.L., Jorda, M., Ling, X., Matthews, B.B., Miller, J., Milshina, N. V, Mizzen, C., Peinado, M.A., Reese, J.T., Reid, J.G., Robertson, H.M., Robinson, G.E., Russo, S.M., Schroeder, A.J., St Pierre, S.E., Wang, Y., Zhou, P., Robertson, H.M., Agarwala, R., Elsik, C.G., Milshina, N. V, Reese, J.T., Weaver, D.B., Worley, K.C., Childs, K.L., Dickens, C.M., Elsik, C.G., Gelbart, W.M., Jiang, Huaiyang, Kitts, P., Milshina, N. V, Reese, J.T.,

Ruef, B., Russo, S.M., Venkatraman, A., Weinstock, G.M., Zhang, L., Zhou, P., Johnston, J.S., Aquino-Perez, G., Cornuet, J.M., Monnerot, M., Solignac, M., Vautrin, D., Whitfield, C.W., Behura, S.K., Berlocher, S.H., Clark, A.G., Gibbs, R.A., Johnston, J.S., Sheppard, W.S., Smith, D.R., Suarez, A. V, Tsutsui, N.D., Weaver, D.B., Wei, X., Wheeler, David, Weinstock, G.M., Worley, K.C., Havlak, P., 2006. Insights into social insects from the genome of the honeybee Apis mellifera. Nature 443, 931–949. https://doi.org/10.1038/nature05260

White, W.H., McCoy, C.M., Meyer, J.A., Winkle, J.R., Plummer, P.R., Kemper, C.J., Starkey, R., Snyder, D.E., 2007. Knockdown and Mortality Comparisons Among Spinosad-, Imidacloprid-, and Methomyl-Containing Baits Against Susceptible Musca domestica (Diptera: Muscidae) Under Laboratory Conditions. J. Econ. Entomol. 100, 155–163. https://doi.org/10.1603/0022-0493(2007)100[155:kamcas]2.0.co;2

Whiteside, M., Mineau, P., Morrison, C., Knopper, L.D., 2011. Comparison of a score-based approach with risk-based ranking of in-use agricultural pesticides in canada to aquatic receptors. Integr. Environ. Assess. Manag. 4, 215–236. https://doi.org/10.1897/IEAM_2007-056.1

Wiesner, P., Kayser, H., 2000. Characterization of nicotinic acetylcholine receptors from the insects Aphis craccivora, Myzus persicae, and Locusta migratoria by radioligand binding assays: Relation to thiamethoxam action. J. Biochem. Mol. Toxicol. 14, 221–230. https://doi.org/10.1002/(SICI)1099-0461(2000)14:4<221::AID-JBT7>3.0.CO;2-6

Williams, N., Sweetman, J., 2019. Effects of neonicotinoids on the emergence and composition of chironomids in the Prairie Pothole Region. Environ. Sci. Pollut. Res. 26, 3862–3868. https://doi.org/10.1007/s11356-018-3683-6

Wogram, J., Liess, M., 2001. Rank Ordering of Macroinvertebrate Species Sensitivity to Toxic Compounds by Comparison with That of Daphnia magna 360–367. https://doi.org/10.1007/s00128-001-0133-8

Wood, T.J., Goulson, D., 2017. The environmental risks of neonicotinoid pesticides: a review of the evidence post 2013. Environ. Sci. Pollut. Res. Int. 24, 17285–17325. https://doi.org/10.1007/s11356-017-9240-x

Xia, Q., Zhou, Z., Lu, C., Cheng, D., Dai, F., Li, B., Zhao, P., Zha, X., Cheng, T., Chai, C., Pan, G., Xu, Jinshan, Liu, C., Lin, Y., Qian, J., Hou, Y., Wu, Z., Li, Guanrong, Pan, M., Li, Chunfeng, Shen, Y., Lan, X., Yuan, L., Li, T., Xu, H., Yang, G., Wan, Y., Zhu, Y., Yu, M., Shen, W., Wu, D., Xiang, Z., Yu, J., Wang, Jun, Li, R., Shi, J., Li, H., Li, Guangyuan, Su, J., Wang, X., Li, Guoqing, Zhang, Zengjin, Wu, Q., Li, Jun, Zhang, Q., Wei, N., Xu, Jianzhe, Sun, H., Dong, L., Liu, D., Zhao, S., Zhao, X., Meng, Q., Lan, F., Huang, X., Li, Y., Fang, L., Li, Changfeng, Li, D., Sun, Y., Zhang, Zhenpeng, Yang, Z., Huang, Y., Xi, Y., Qi, Q., He, D., Huang, H., Zhang, X., Wang, Z., Li, W., Cao, Y., Yu, Y., Yu, H., Li, Jinhong, Ye, Jiehua, Chen, H., Zhou, Y., Liu, B., Wang, Jing, Ye, Jia, Ji, H., Li, Shengting, Ni, P., Zhang, J., Zhang, Y., Zheng, H., Mao, B., Wang, W., Ye, C., Li, Songgang, Wang, Jian, Wong, G.K.-S., Yang, H., 2004. A Draft Sequence for the Genome of the Domesticated Silkworm (Bombyx mori). Science (80-.). 306, 1937–1940. https://doi.org/10.1126/science.1102210

Xie, W., Han, C., Qian, Y., Ding, H., Chen, X., Xi, J., 2011. Determination of neonicotinoid pesticides residues in agricultural samples by solid-phase extraction combined with liquid chromatography–tandem mass spectrometry. J. Chromatogr. A 1218, 4426–4433. https://doi.org/10.1016/J.CHROMA.2011.05.026

Xing, Z., Chow, L., Rees, H., Meng, F., Li, S., Ernst, B., Benoy, G., Zha, T., Hewitt, L.M., 2013. Influences of Sampling Methodologies on Pesticide-Residue Detection in Stream Water. Arch. Environ. Contam. Toxicol. 64, 208–218. https://doi.org/10.1007/s00244-012-9833-9

Xu, G., Wu, S.F., Teng, Z.W., Yao, H.W., Fang, Q., Huang, J., Ye, G.Y., 2017. Molecular characterization and expression profiles of nicotinic acetylcholine receptors in the rice striped stem borer, Chilo suppressalis (Lepidoptera: Crambidae). Insect Sci. 24, 371–384. https://doi.org/10.1111/1744-7917.12324

XueXiang, R., Gang, W., YiMing, Z., KaiYun, W., Jie, W., 2011. The toxicity and effects of sublethal doses on detoxifying enzymes of clothianidin to Myzus persicae (Sulzer) (Hemiptera: Aphididae). Acta Entomol. Sin. 54, 299–305.

Yamamoto, A., Terao, T., Hisatomi, H., Kawasaki, H., Arakawa, R., 2012. Evaluation of river pollution of neonicotinoids in Osaka City (Japan) by LC/MS with dopant assisted photoionisation. J. Environ. Monit. 14, 2194.

Zhang, N., Tomizawa, M., Casida, J.E., 2004. Drosophila nicotinic receptors: Evidence for imidacloprid insecticide and α-bungarotoxin binding to distinct sites. Neurosci. Lett. 371, 56–59. https://doi.org/10.1016/j.neulet.2004.08.040

Zhang, W., Jiang, F., Ou, J., 2011. Global pesticide consumption and pollution : with China as a focus. Proc. Int. Acad. Ecol. Environ. Sci. 1, 125–144. https://doi.org/http://dx.doi.org/10.0000/issn-2220-8860-piaees-2011-v1-0012

APPENDIX

*Supplemental material and information for this thesis are provided in this Appendix (23 tables and 1 figure total). Data include definitions of MIXTOX parameters, comparisons of mean measured and nominal concentrations in single compound and mixture experiments, and parameters for single-compound and mixture toxicity models for laboratory-based experiments (Chapters 2 and 3). Abundance of emerged insects (total and specific taxa) in treated limnocorrals, water quality parameters, comparisons of mean measured and nominal neonicotinoid concentrations, and coefficients of variation for Chironomidae abundance for field-based experiments (Chapter 4) are also presented in this section. For Chapter 5, data presented include a comparison of membrane protein extraction protocols, derived biological activity of Chironomidae membrane protein, and a comparison of previously derived competition binding parameters for agricultural pest species and commonly used test insects to that derived for Chironomidae in this study.

Table A2.1 Interpretation of parameters substituted into concentration addition (CA) or independent action (IA) reference models that act as functional definitions of deviation patterns.

Parameter	CA	IA	Interpretation
		Synergism/Antagonism	
a	< 0	< 0	Synergism
	> 0	> 0	Antagonism
		Dose-Ratio Dependent Deviation	
a	< 0	< 0	Synergism, except for mixture ratios where positive b_{DR} indicates antagonism
	> 0	> 0	Antagonism, except for mixture ratios where negative b_{DR} indicates synergism
b_{DR}	< 0	< 0	Antagonism, where the toxicity of the mixture is caused mainly by chemical 1
	> 0	> 0	Synergism, where the toxicity of the mixture is caused mainly by chemical 1
		Dose-Level Dependent Deviation	
a	< 0	< 0	Synergism at low dose level, antagonism at high dose level
	> 0	> 0	Antagonism at low dose level, synergism at high dose level
b_{DL}	>1	> 2	Change at lower dose level than LC_{50}
	= 1	= 2	Change at LC_{50}
	$0 < b_{DL} < 1$	$1 < b_{DL} < 2$	Change at higher dose level than LC_{50}
	< 0	< 2	No change, but magnitude of synergism/antagonism is dose-level (CA) or effect-level (IA) dependent
		Ternary-Plus Model	
$a_{1,2}$	< 0	< 0	Synergism when mixture solely consists of compounds 1 and 2
	> 0	> 0	Antagonism when mixture solely consists of compounds 1 and 2
$a_{1,3}$	< 0	< 0	Synergism when mixture solely consists of compounds 1 and 3
	> 0	> 0	Antagonism when mixture solely consists of compounds 1 and 3
$a_{2,3}$	< 0	< 0	Synergism when mixture solely consists of compounds 2 and 3

	> 0	> 0	Antagonism when mixture solely consists of compounds 2 and 3
	< 0	< 0	Synergism of ternary mixture - compared to isobole plane predicted by binary deviations
$a_{1,2,3}$	> 0	> 0	Antagonism of ternary mixture - compared to isobole plane predicted by binary deviations

*Adapted from Jonker et al. (2005), and Cedergreen et al. (2012).

SI Table A2.2 Mean (± SD) measured neonicotinoid concentrations (µg/L) for single compound 96 h *Chironomus dilutus* toxicity tests.

Imidacloprid		**Clothianidin**		**Thiamethoxam**	
Nominal	*Measured*	*Nominal*	*Measured*	*Nominal*	*Measured*
Control	< LOQ[a]	Control	< LOQ[a]	Control	< LOQ[a]
0.4	0.33 ± 0.03	0.4	0.37 ± 0.00	0.88	0.24 ± 0.09
0.88	0.82 ± 0.12	0.88	0.95 ± 0.00	1.94	0.96 ± 0.15
1.94	1.63 ± 0.03	1.94	2.06 ± 0.11	4.26	2.72 ± 0.35
4.26	3.37 ± 0.26	4.26	4.89 ± 0.18	9.37	4.69 ± 0.61
9.37	7.98 ± 0.18	9.37	10.49 ± 0.17	20.61	11.90 ± 0.88
20.61	16.45 ± 0.69	20.61	26.49 ± 2.04	45.34	24.25 ± 0.76
-	-	-	-	99.77	77.50 ± 5.65
-	-	-	-	219.5	168.5 ± 0.51

[a] Limits of quantification (LOQ): imidacloprid = 0.005 µg/L, clothianidin = 0.006 µg/L, thiamethoxam = 0.02 µg/L.

Table A2.3 Mean measured neonicotinoid concentrations (µg/L) for binary and ternary mixture 96 h *Chironomus dilutus* toxicity tests.

Imidacloprid		**Clothianidin**		**Thiamethoxam**	
Nominal	*Measured*	*Nominal*	*Measured*	*Nominal*	*Measured*
		Imidacloprid-Clothianidin			
Control	< LOQ[a]	Control	< LOQ[a]	-	-
1.16	1.54	-	-	-	-
6.95	8.48	-	-	-	-
13.89	11.78	-	-	-	-
-	-	1.48	2.06	-	-
-	-	2.97	4.74	-	-
-	-	5.93	5.72	-	-
-	-	8.90	8.78	-	-
-	-	11.86	13.45	-	-
0.87	0.87	0.37	0.33	-	-
1.74	1.73	0.74	0.72	-	-
3.47	5.51	1.48	2.19	-	-
5.21	7.55	2.22	2.97	-	-
6.95	9.04	2.97	3.08	-	-
0.58	0.55	0.74	0.60	-	-
1.16	1.09	1.48	1.56	-	-
2.32	2.41	2.97	2.97	-	-
3.47	3.69	4.45	4.25	-	-
4.36	5.05	5.93	5.98	-	-
6.95	5.58	8.90	6.63	-	-
0.29	0.30	1.11	1.02	-	-
0.58	0.64	2.22	2.53	-	-

1.16	1.26	4.45	4.31	-	-
1.74	1.88	6.67	6.12	-	-
2.32	2.40	8.90	8.72	-	-
3.47	2.74	13.30	10.67	-	-

Clothianidin-Thiamethoxam

-	-	Control[a]	< LOQ	Control[a]	< LOQ
-	-	1.48	1.19	-	0.028
-	-	2.97	2.84	-	-
-	-	5.93	5.13	-	-
-	-	8.90	7.21	-	-
-	-	11.86	9.71	-	-
-	-	14.83	13.32	-	-
-	-	-	0.01	13.84	17.30
-	-	-	0.01	27.67	24.60
-	-	-	0.02	55.34	47.57
-	-	-	0.04	83.01	79.4
-	-	-	0.05	110.68	104.2
-	-	-	0.29	138.35	126.8
-	-	1.11	0.87	3.46	3.05
-	-	2.22	1.83	6.92	7.90
-	-	4.45	3.75	13.84	13.95
-	-	6.67	6.08	20.75	19.3
-	-	8.90	8.6	27.70	29.7
-	-	0.74	0.49	6.92	7.92
-	-	1.48	1.07	13.80	14.7
-	-	2.97	2.02	27.70	28.19
-	-	4.45	3.82	41.50	44.20

-	-	5.93	4.41	55.30	50.9
-	-	7.41	7.30	69.20	62.3
-	-	0.37	0.28	10.40	11.5
-	-	0.74	0.55	20.80	22.70
-	-	1.48	1.18	41.50	35.00
-	-	2.22	1.86	62.30	57.7
-	-	2.97	2.03	83.00	75.6
-	-	3.71	4.10	103.80	94.2

Imidacloprid-Thiamethoxam

Control	< LOQ [a]	-	-	Control	< LOQ [a]
1.16	1.22	-	-	-	-
2.32	2.53	-	-	-	-
4.63	4.92	-	-	-	-
6.95	6.73	-	-	-	-
9.26	9.42	-	-	-	-
11.58	11.45	-	-	-	-
-	-	-	-	13.84	13.26
-	-	-	-	27.67	24.30
-	-	-	-	55.34	36.85
-	-	-	-	83.0	64.6
-	-	-	-	110.68	87.85
-	-	-	-	138.35	126.73
0.87	0.82	-	-	3.46	3.23
1.74	1.67	-	-	6.92	5.31
3.47	3.29	-	-	13.84	11.09
5.21	4.41	-	-	20.75	15.09
6.95	5.73	-	-	27.67	19.44

8.68	6.80	-	-	34.59	24.17
0.58	0.61	-	-	6.92	5.16
1.16	1.32	-	-	13.84	13.53
2.32	2.24	-	-	27.67	29.53
3.47	3.14	-	-	41.51	42
4.63	4.50	-	-	55.34	48.9
5.79	5.78	-	-	69.18	58
0.29	0.30	-	-	10.38	11.45
0.58	0.57	-	-	20.75	12.7
1.16	1.18	-	-	41.51	35.5
1.74	1.66	-	-	62.26	43.8
2.32	2.46	-	-	83.01	68.4
2.89	2.29	-	-	103.76	72.6

Imidacloprid-Clothianidin-Thiamethoxam

Control	< LOQ [a]	Control	< LOQ [a]	Control	< LOQ [a]
1.16	1.25	-	-	-	-
2.32	2.95	-	-	-	-
4.63	4.86	-	-	-	-
6.95	6.76	-	-	-	-
9.26	8.41	-	-	-	-
12.89	17.70	-	-	-	-
-	-	1.48	1.67	-	-
-	-	5.93	3.68	-	-
-	-	8.90	8.78	-	-
-	-	11.86	14.30	-	-
-	-	17.79	27.54	-	-
-	-	-	-	13.84	13.58

-	-	-	-	55.34	50.95
-	-	-	-	83.01	58.1
-	-	-	-	110.68	109.4
-	-	-	-	166.02	157
0.39	0.42	0.49	0.46	4.61	3.89
1.54	1.51	1.98	2.09	18.45	17.60
2.32	2.29	2.97	3.25	27.67	28.3
3.09	2.74	3.95	4.19	36.89	32.6
4.63	4.96	5.93	7.18	55.34	53.1
0.58	0.56	0.37	0.32	3.46	3.99
2.32	1.94	1.48	1.34	13.84	15.65
3.47	2.89	2.22	2.03	20.75	20.9
4.63	4.54	2.97	2.82	27.67	35.2
6.95	6.19	4.45	4.95	41.51	49.6
1.16	1.01	2.97	2.84	13.84	13.97
1.74	1.45	4.45	3.77	20.75	18.60
2.32	2.49	5.93	5.88	27.67	26.96
3.47	3.37	8.89	8.87	41.5	49.73
0.3	0.31	0.37	0.37	6.92	8.09
1.16	1.09	1.48	1.48	27.67	28.71
1.74	1.76	2.22	2.67	41.51	35.8
2.32	2.54	2.97	3.27	55.34	56.6
3.47	3.23	4.45	5.69	83.01	75.8
0.23	0.25	0.59	0.59	5.53	11.1
0.46	0.55	1.19	1.06	11.07	21.98
0.93	1.07	2.37	2.32	22.14	20.45
1.39	1.51	3.56	3.57	33.20	26
1.85	2.05	4.74	4.60	44.27	30.5

2.78	2.74	7.12	6.58	66.41	51.5
0.46	0.49	0.30	0.30	5.53	9.20
0.93	0.95	0.59	0.57	11.07	20.4
1.85	1.90	1.19	1.16	22.14	20.3
2.78	2.65	1.78	1.81	33.2	25.9
3.7	3.93	2.37	2.13	44.27	39.0
5.56	5.88	3.56	3.83	66.41	47.2
0.46	0.48	0.59	0.66	2.77	6.6
0.93	0.85	1.19	1.24	5.53	12.7
1.85	2.03	2.37	2.62	11.07	19.20
2.78	2.79	3.56	4.10	16.6	13.7
3.7	3.45	4.7	4.82	22.14	17.4
5.56	5.58	7.12	7.38	33.20	32.9
0.77	0.80	0.99	0.98	9.22	16.9
1.16	1.34	0.74	0.84	6.92	12.1
0.58	0.64	1.48	1.32	6.92	14.0
0.29	0.29	0.74	0.65	3.46	5.9
0.58	0.59	0.74	0.61	13.84	30.2

[a] Limits of quantification (LOQ): imidacloprid = 0.003 – 0.008 µg/L, clothianidin = 0.003 – 0.017 µg/L, thiamethoxam = 0.005 – 0.016 µg/L.

Table A2.4 Summary of the MIXTOX analysis output of the acute effects of imidacloprid-clothianidin mixtures on the survival of *Chironomus dilutus.* *

	Concentration Addition				Independent Action			
	Reference	S/A	**DR**	DL	Reference	S/A	DR	DL
μ_{max}	95.5	95.5	**95.4**	95.6	95.2	95.5	95.6	95.3
β_{IMI}	4.99	3.77	**3.58**	2.95	2.55	4.68	4.59	7.49
β_{CLO}	12.13	20.22	**21.66**	21.13	22.03	12.33	13.02	16.98
$LC50_{IMI}$	8.25	6.83	**7.32**	6.29	4.29	7.83	7.73	8.36
$LC50_{CLO}$	5.80	5.61	**5.58**	5.60	5.55	5.65	5.67	5.60
a	-	0.63	**1.22**	1.87	-	- 6.52	-7.64	-10.47
b_{DR}	-	-	**-1.68**	-	-	-	2.41	-
b_{DL}	-	-	**-**	0.45	-	-	-	0.75
RD	49.2	44.8	**42.5**	44.4	61.8	44.5	44.2	41.52
			vs. Reference Model					
χ^2	-	4.36	**6.66**	4.79	-	17.37	17.64	20.31
df	-	1	**2**	2	-	1	2	2
$p(\chi^2)$	-	0.04	**0.04**	0.09	-	< 0.01	< 0.01	< 0.01
			vs. S/A					
χ^2	-	-	**2.30**	0.44	-	-	0.27	2.94
df	-	-	**1**	1	-	-	1	1
$p(\chi^2)$	-	-	**0.13**	0.5	-	-	0.60	0.09
R^2 (%)	90.9	91.7	**92.1**	91.8	87.9	91.3	91.4	91.9

*Bolded column represents the mixture model of best fit.

Table A2.5 Summary of the MIXTOX analysis output of the acute effects of clothianidin-thiamethoxam mixtures on the survival of *Chironomus dilutus.* *

		Concentration Addition			Independent Action			
	Reference	S/A	DR	DL	Reference	S/A	DL	DR
μ_{max}	**93.2**	93.2	93.2	93.2	89.7	89.7	89.7	89.7
β_{CLO}	**15.78**	15.81	15.69	15.81	82.97	82.97	82.97	82.97
β_{TMX}	**30.87**	43.82	9304.50	52.52	96.56	96.56	96.56	96.56
$LC50_{CLO}$	**5.91**	5.92	5.91	5.92	5.30	5.30	5.30	5.30
$LC50_{TMX}$	**37.51**	39.33	45.44	40.19	27.56	27.56	27.56	27.56
a	**n/a**	-0.08	-0.47	-0.00047	n/a	-	-	-
b_{DR}	**n/a**	n/a	0.26	n/a	n/a	-	-	-
b_{DL}	**n/a**	n/a	n/a	-25.02	n/a	-	-	-
RD	**43.0**	43.0	43.0	43.0	77.1	-	-	-
			vs. Reference Model					
χ^2	n/a	0.01	0.07	0.02	n/a	-	-	-
df	n/a	1	2	2	n/a	-	-	-
$p(\chi^2)$	n/a	0.92	0.96	0.99	n/a	-	-	-
			vs. S/A					
χ^2	n/a	n/a	0.06	0.007	n/a	-	-	-
df	n/a	n/a	1	1	n/a	-	-	-
$p(\chi^2)$	n/a	n/a	0.8	0.93	n/a	-	-	-
R^2 (%)	**94.2**	94.2	94.2	94.2	89.7	-	-	-

*Bolded column represents the mixture model of best fit.

Table A2.6 Summary of the MIXTOX analysis output of the acute effects of imidacloprid-thiamethoxam mixtures on the survival of *Chironomus dilutus.* *

	Concentration Addition				Independent Action			
	Reference	S/A	DR	DL	Reference	S/A	**DR**	DL
μ_{max}	89.4	90.3	89.8	90.2	90.0	89.9	**89.9**	89.2
β_{IMI}	4.98	3.47	4.82	3.85	4.32	4.72	**5.03**	4.38
β_{TMX}	6.59	188.24	16.64	376.94	13.13	12.29	**17.63**	9.14
$LC50_{IMI}$	6.31	4.69	6.13	4.77	4.68	5.24	**5.88**	5.36
$LC50_{TMX}$	45.99	36.80	35.40	36.83	38.59	40.41	**36.20**	43.61
a	n/a	1.87	3.20	1.15	n/a	-2.32	**11.99**	-0.01
b_{DR}	n/a	n/a	- 4.59	n/a	n/a	n/a	**-26.41**	n/a
b_{DL}	n/a	n/a	n/a	-0.40	n/a	n/a	**n/a**	-850.9
RD	151.5	121.0	107.3	120.2	121.1	117.0	**100.5**	111.5
				vs. Reference Model				
χ^2	n/a	30.49	44.1	31.28	n/a	4.10	**20.61**	9.55
df	n/a	1	2	2	n/a	1	**2**	2
$p(\chi^2)$	n/a	< 0.01	< 0.01	< 0.01	n/a	0.04	**< 0.01**	< 0.01
				vs. S/A				
χ^2	n/a	n/a	13.66	0.80	n/a	n/a	**16.50**	5.45
df	n/a	n/a	1	1	n/a	n/a	**1**	1
$p(\chi^2)$	n/a	n/a	< 0.01	0.37	n/a	n/a	**< 0.01**	< 0.01
R^2 (%)	75.0	80.0	82.3	80.1	80.0	80.7	**83.4**	81.6

*Bolded column represents the mixture model of best fit.

Table A2.7 Summary of the MIXTOX analysis output of the acute effects of imidacloprid-clothianidin-thiamethoxam mixtures on the survival of *Chironomus dilutus.* *

	Concentration Addition		Independent Action	
	Reference	S/A	Reference	**S/A**
μ_{max}	87.0	90.7	94.1	**92.6**
β_{IMI}	3.07	3.64	3.85	**6.99**
β_{CLO}	307.73	307.60	3.36	**6.79**
β_{TMX}	4.32	7.38	3.89	**7.14**
$LC50_{IMI}$	5.31	3.91	3.44	**3.55**
$LC50_{CLO}$	7.61	5.47	2.92	**3.70**
$LC50_{TMX}$	35.97	43.92	32.05	**41.37**
$a_{IMI,CLO}$	n/a	1.79	n/a	**4.95**
$a_{CLO,TMX}$	n/a	-0.20	n/a	**-6.33**
$a_{IMI,TMX}$	n/a	1.41	n/a	**0.13**
$a_{IMI,CLO,TMX}$	n/a	4.92	n/a	**- 50.34**
RD	718.6	485.8	583.4	**406.3**
		vs. Reference Model		
χ^2	n/a	232.79	n/a	**177.06**
df	n/a	1	n/a	**1**
$p(\chi^2)$	n/a	< 0.01	n/a	**< 0.01**
R^2 (%)	68.5	78.7	74.4	**82.2**

*Bolded column represents the mixture model of best fit.

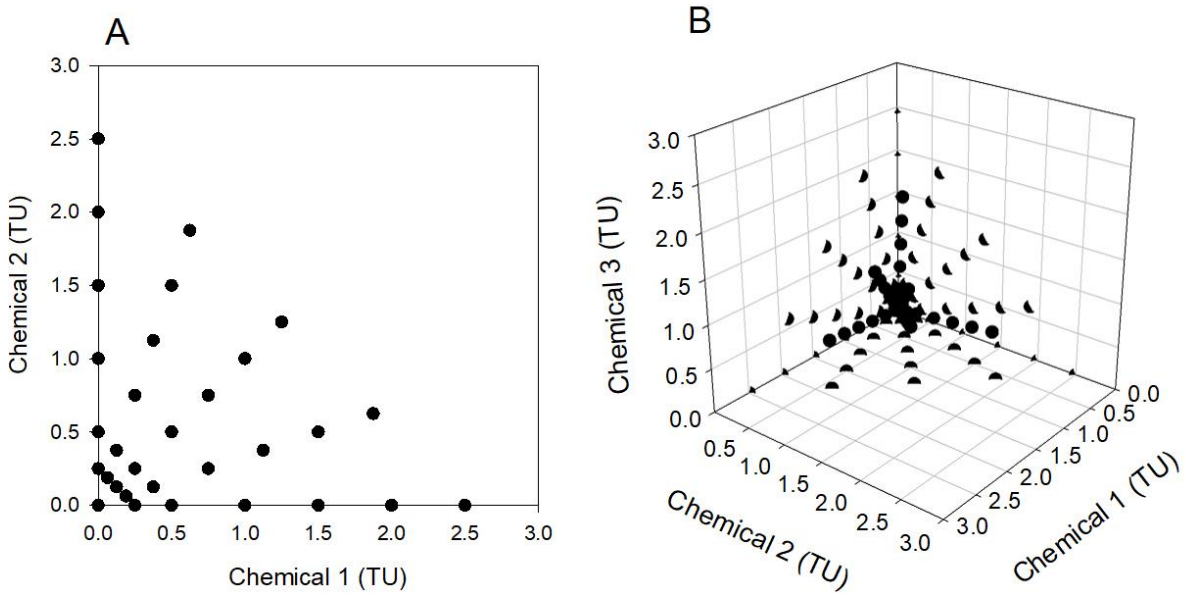

Figure A3.1 Fixed-ray experimental design applied in binary (A) and ternary (B) mixture toxicity tests.

Table A3.1 Mean (± SE) measured neonicotinoid concentrations (µg/L) for single-compound 28-day *Chironomus dilutus* toxicity tests.

Imidacloprid		**Clothianidin**		**Thiamethoxam**	
Nominal	*Measured*	*Nominal*	*Measured*	*Nominal*	*Measured*
Control	< LOQ[a]	Control	< LOQ[a]	Control	< LOQ[a]
0.16	0.18 ± 0.01	0.10	0.09 ± 0.01	1.25	1.10 ± 0.04
0.31	0.33 ± 0.02	0.25	0.21 ± 0.04	2.50	2.10 ± 0.09
0.625	0.68 ± 0.05	0.63	0.59 ± 0.05	5.00	4.34 ± 0.18
1.25	1.26 ± 0.07	1.56	1.47 ± 0.17	10.00	8.05 ± 0.69
2.50	2.67 ± 0.15	3.91	3.77 ± 0.38	20.00	19.23 ± 0.52
5.00	5.49 ± 0.36	9.77	11.58 ± 1.64	40.00	40.69 ± 1.62

[a] Limits of quantification (LOQ); imidacloprid = 0.004 - 0.006 µg/L, clothianidin = 0.0007 - 0.002 µg/L, thiamethoxam = 0.007 - 0.02 µg/L.

Table A3.2 Mean (±SD) measured neonicotinoid concentrations (µg/L) for binary and ternary mixture 28-day *Chironomus dilutus* toxicity tests.

Imidacloprid		**Clothianidin**		**Thiamethoxam**	
Nominal	*Measured*	*Nominal*	*Measured*	*Nominal*	*Measured*
		<u>Imidacloprid-Clothianidin</u>			
Control	< LOQ[a]	Control	< LOQ[a]		
0.12	0.13 ± 0.01	0	< LOQ	-	-
0.25	0.26 ± 0.02	0	< LOQ	-	-
0.5	0.42 ± 0.16	0	< LOQ	-	-
0.75	0.75 ± 0.13	0	< LOQ	-	-
1.0	1.10 ± 0.14	0	< LOQ	-	-
1.49	1.56 ± 0.06	0	< LOQ	-	-
0	< LOQ	0.18	0.18 ± 0.06	-	-
0	< LOQ	0.35	0.39 ± 0.06	-	-
0	< LOQ	0.71	0.68 ± 0.06	-	-
0	< LOQ	1.06	1.04 ± 0.13	-	-
0	< LOQ	1.41	1.47 ± 0.09	-	-
0	< LOQ	2.12	2.11 ± 0.38	-	-
0.09	0.10 ± 0.01	0.04	0.05 ± 0.16	-	-
0.19	0.20 ± 0.02	0.09	0.08 ± 0.13	-	-
0.37	0.40 ± 0.04	0.18	0.16 ± 0.02	-	-
0.56	0.55 ± 0.03	0.2	0.26 ± 0.04	-	-
0.75	0.76 ± 0.09	0.35	0.36 ± 0.05	-	-
1.12	1.20 ± 0.09	0.53	0.53 ± 0.04	-	-
0.06	0.07 ± 0.01	0.09	0.09 ± 0.03	-	-
0.12	0.13 ± 0.02	0.18	0.17 ± 0.02	-	-
0.25	0.27 ± 0.02	0.35	0.35 ± 0.04	-	-
0.37	0.43 ± 0.03	0.53	0.54 ± 0.03	-	-

0.5	0.53 ± 0.05	0.7	0.76 ± 0.09	-	-
0.75	0.83 ± 0.05	1.06	1.04 ± 0.08	-	-
0.03	0.04 ± 0.01	0.13	0.13 ± 0.02	-	-
0.06	0.07 ± 0.01	0.26	0.22 ± 0.01	-	-
0.12	0.14 ± 0.02	0.53	0.50 ± 0.07	-	-
0.19	0.20 ± 0.02	0.79	0.71 ± 0.20	-	-
0.25	0.13 ± 0.14	1.06	1.06 ± 0.08	-	-
0.37	0.39 ± 0.02	1.59	1.50 ± 0.07	-	-

Clothianidin-Thiamethoxam

-	-	Control	< LOQ[a]	Control	< LOQ[a]
-	-	0.18	0.14 ± 0.01	0	< LOQ
-	-	0.35	0.37 ± 0.08	0	< LOQ
-	-	0.71	0.83 ± 0.05	0	< LOQ
-	-	1.06	1.17 ± 0.08	0	< LOQ
-	-	1.41	1.53 ± 0.13	0	< LOQ
-	-	2.12	2.21 ± 0.11	0	< LOQ
-	-	0	< LOQ	2.23	2.75 ± 0.30
-	-	0	< LOQ	4.46	5.11 ± 0.32
-	-	0	< LOQ	8.91	9.27 ± 0.68
-	-	0	< LOQ	13.37	14.91 ± 0.34
-	-	0	< LOQ	17.82	19.95 ± 1.58
-	-	0	< LOQ	26.73	32.52 ± 2.51
-	-	0.04	0.04 ± 0.00	1.67	2.05 ± 0.37
-	-	0.09	0.08 ± 0.00	3.34	3.79 ± 0.53
-	-	0.18	0.15 ± 0.01	6.68	8.01 ± 0.52
-	-	0.26	0.23 ± 0.01	10.02	11.29 ± 0.88
-	-	0.35	0.33 ± 0.03	13.37	16.87 ± 1.67
-	-	0.53	0.55 ± 0.06	20.05	27.36 ± 5.50

-	-	0.09	0.07 ± 0.01	1.11	1.28 ± 0.17
-	-	0.18	0.14 ± 0.02	2.23	2.92 ± 0.22
-	-	0.35	0.31 ± 0.02	4.46	6.07 ± 0.67
-	-	0.53	0.48 ± 0.03	6.68	8.44 ± 0.91
-	-	0.71	0.63 ± 0.05	8.91	10.16 ± 1.30
-	-	1.06	0.99 ± 0.06	13.37	16.47 ± 1.13
-	-	0.13	0.11 ± 0.01	0.56	0.72 ± 0.07
-	-	0.26	0.22 ± 0.02	1.11	1.48 ± 0.11
-	-	0.53	0.46 ± 0.03	2.23	3.25 ± 0.39
-	-	0.79	0.69 ± 0.04	3.34	4.55 ± 0.21
-	-	1.06	1.00 ± 0.04	4.46	5.67 ± 0.25
-	-	1.59	1.55 ± 0.05	6.68	10.14 ± 0.47

Imidacloprid-Thiamethoxam

Control	< LOQ[a]	-	-	Control	< LOQ[a]
0.12	0.11 ± 0.02	-	-	0	< LOQ
0.50	0.46 ± 0.03	-	-	0	< LOQ
0.75	0.91 ± 0.33	-	-	0	< LOQ
1.49	1.72 ± 0.07	-	-	0	< LOQ
0	< LOQ	-	-	2.23	1.92 ± 0.41
0	< LOQ	-	-	4.46	4.57 ± 0.65
0	< LOQ	-	-	8.91	8.78 ± 0.81
0	< LOQ	-	-	13.40	15.21 ± 0.09
0	< LOQ	-	-	17.80	23.01 ± 3.72
0	< LOQ	-	-	26.70	38.87 ± 1.51
0.09	0.13 ± 0.07	-	-	0.56	0.95 ± 0.86
0.19	0.13 ± 0.06	-	-	1.11	0.92 ± 0.15
0.37	0.38 ± 0.02	-	-	2.23	3.36 ± 0.34
0.56	0.60 ± 0.02	-	-	3.34	4.68 ± 1.11

0.75	0.85 ± 0.07	-	-	4.46	5.84 ± 0.59
1.12	1.23 ± 0.04	-	-	6.68	9.75 ± 1.01
0.06	0.04 ± 0.00	-	-	1.11	1.14 ± 0.34
0.12	0.11 ± 0.00	-	-	2.23	2.68 ± 0.19
0.25	0.27 ± 0.00	-	-	4.46	4.94 ± 1.04
0.37	0.38 ± 0.02	-	-	6.68	8.16 ± 0.16
0.50	0.58 ± 0.12	-	-	8.91	10.72 ± 0.82
0.75	0.82 ± 0.01	-	-	13.40	17.82 ± 0.53
0.03	0.03 ± 0.00	-	-	1.67	1.98 ± 0.60
0.06	0.06 ± 0.00	-	-	3.34	3.91 ± 0.29
0.12	0.12 ± 0.02	-	-	6.68	8.09 ± 1.25
0.19	0.19 ± 0.01	-	-	10.0	11.55 ± 1.93
0.25	0.25 ± 0.00	-	-	13.4	18.00 ± 1.15
0.37	0.44 ± 0.02	-	-	20.0	26.60 ± 0.17

Imidacloprid-Clothianidin-Thiamethoxam

Control	< LOQ[a]	Control	< LOQ[a]	Control	< LOQ[a]
0.12	0.14 ± 0.02	0	< LOQ	0	< LOQ
0.25	0.26 ± 0.13	0	< LOQ	0	< LOQ
0.5	0.52 ± 0.07	0	< LOQ	0	< LOQ
0.75	0.85 ± 0.09	0	< LOQ	0	< LOQ
0.99	1.20 ± 0.08	0	< LOQ	0	< LOQ
1.49	1.55 ± 0.40	0	< LOQ	0	< LOQ
0	< LOQ	0.36	0.20 ± 0.03	0	< LOQ
0	< LOQ	0.71	0.72 ± 0.06	0	< LOQ
0	< LOQ	1.07	1.47 ± 0.47	0	< LOQ
0	< LOQ	1.42	1.96 ± 0.57	0	< LOQ
0	< LOQ	2.13	2.58 ± 1.26	0	< LOQ
0	< LOQ	0	< LOQ	2.23	3.81 ± 0.53
0	< LOQ	0	< LOQ	4.46	7.45 ± 3.08

0	< LOQ	0	< LOQ	8.91	14.92 ± 6.07
0	< LOQ	0	< LOQ	13.37	24.02 ± 9.69
0	< LOQ	0	< LOQ	17.82	33.24 ± 15.38
0	< LOQ	0	< LOQ	26.73	49.87 ± 15.70
0.04	0.05 ± 0.00	0.06	0.07 ± 0.03	0.74	1.49 ± 0.52
0.08	0.09 ± 0.01	0.12	0.13 ± 0.03	1.49	2.66 ± 0.99
0.17	0.19 ± 0.02	0.24	0.24 ± 0.03	2.97	3.63 ± 0.58
0.25	0.43 ± 0.18	0.36	0.33 ± 0.03	4.46	6.13 ± 0.49
0.33	0.38 ± 0.07	0.47	0.44 ± 0.08	5.94	12.07 ± 4.60
0.497	0.57 ± 0.07	0.71	0.84 ± 0.28	8.91	15.31 ± 4.78
0.06	0.09 ± 0.01	0.04	0.05 ± 0.02	0.56	1.23 ± 0.32
0.12	0.12 ± 0.09	0.09	0.10 ± 0.03	1.11	2.42 ± 0.91
0.25	0.28 ± 0.05	0.18	0.21 ± 0.08	2.23	4.18 ± 1.39
0.38	0.42 ± 0.04	0.27	0.31 ± 0.05	3.34	4.18 ± 0.07
0.50	0.50 ± 0.07	0.36	0.37 ± 0.05	4.46	7.78 ± 3.34
0.75	0.69 ± 0.08	0.53	0.48 ± 0.02	6.68	11.64 ± 3.83
0.03	0.04 ± 0.01	0.09	0.07 ± 0.03	0.56	0.89 ± 0.11
0.06	0.07 ± 0.01	0.18	0.19 ± 0.01	1.11	1.58 ± 0.23
0.12	0.16 ± 0.03	0.36	0.34 ± 0.08	2.23	2.93 ± 0.32
0.19	0.20 ± 0.02	0.53	0.52 ± 0.08	3.34	4.61 ± 0.31
0.25	0.29 ± 0.01	0.71	0.70 ± 0.12	4.46	6.03 ± 0.45
0.03	0.06 ± 0.02	0.04	0.07 ± 0.06	0.45	0.72 ± 0.11
0.06	0.07 ± 0.01	0.09	0.12 ± 0.05	0.89	1.02 ± 0.07
0.12	0.18 ± 0.08	0.18	0.19 ± 0.04	1.78	2.15 ± 0.39
0.19	0.21 ± 0.02	0.27	0.28 ± 0.04	2.67	3.45 ± 0.47
0.25	0.29 ± 0.02	0.36	0.38 ± 0.06	3.56	4.94 ± 0.57
0.03	0.04 ± 0.01	0.07	0.08 ± 0.03	0.89	1.19 ± 0.15
0.05	0.07 ± 0.02	0.14	0.15 ± 0.04	1.78	2.53 ± 0.23
0.10	0.12 ± 0.02	0.28	0.29 ± 0.03	3.56	4.41 ± 1.07

0.15	0.16 ± 0.02	0.43	0.30 ± 0.24	5.35	7.04 ± 1.39
0.20	0.24 ± 0.01	0.57	0.62 ± 0.06	7.13	9.66 ± 1.21
0.05	0.06 ± 0.02	0.04	0.06 ± 0.04	0.89	0.86 ± 0.49
0.10	0.11 ± 0.01	0.07	0.11 ± 0.04	1.78	2.30 ± 0.49
0.20	0.21 ± 0.02	0.14	0.14 ± 0.02	3.56	4.36 ± 0.55
0.30	0.30 ± 0.02	0.21	0.25 ± 0.02	5.35	6.31 ± 0.57
0.40	0.48 ± 0.02	0.28	0.32 ± 0.01	7.13	9.15 ± 1.42
0.60	1.17 ± 0.10	0.43	0.49 ± 0.07	10.70	13.68 ± 3.23
0.05	0.06 ± 0.00	0.07	0.08 ± 0.02	0.45	0.87 ± 0.39
0.10	0.12 ± 0.01	0.14	0.15 ± 0.04	0.89	1.16 ± 0.12
0.20	0.22 ± 0.02	0.28	0.20 ± 0.12	1.78	2.18 ± 0.10
0.30	0.25 ± 0.02	0.43	0.37 ± 0.04	2.67	3.62 ± 0.37
0.40	0.39 ± 0.08	0.57	0.51 ± 0.02	3.56	4.24 ± 0.58
0.60	0.65 ± 0.09	0.85	0.70 ± 0.16	5.35	8.25 ± 1.49

[a] Limits of quantification (LOQ); imidacloprid = 0.003 – 0.008 µg/L, clothianidin = 0.003 – 0.011 µg/L, thiamethoxam = 0.007 – 0.027 µg/L.

Table A3.3 Median effective concentrations (28-d EC_{50}; µg/L) and slopes (β) for successful emergence of *Chironomus dilutus* exposed to single-compound positive controls in chronic mixture toxicity tests. [a]

Statistical Method	Mixture test	**Imidacloprid**		**Clothianidin**		**Thiamethoxam**	
		EC_{50}	β	EC_{50}	β	EC_{50}	β
Log-logistic curve	-	0.43	2.25	1.34	33.95	8.58	2.97
MIXTOX modeling	IMI-CLO	0.25	2.69	0.48	6.43	-	-
	CLO-TMX	-	-	0.82	428.8	9.65	223.2
	IMI-TMX	0.81	5.81	-	-	10.31	4.59
	IMI-CLO-TMX	0.66	3.21	0.66	18.40	11.70	5.51

[a] Calculated via maximum likelihood estimation through MIXTOX analysis (Jonker *et al.*, 2005).

Table A3.4 Summary of the MIXTOX analysis output of the chronic effects of imidacloprid-clothianidin (IMI-CLO) mixtures on the successful emergence of *Chironomus dilutus*.*

	Concentration Addition				Independent Action			
	Reference	S/A	**DR**	DL	Reference	S/A	**DL**	**DR**
μ_{max}	47.9	55.2	**60.4**	55.0	34.9	34.9	**28.9**	**28.9**
β_{IMI}	3.20	12.00	**2.69**	15.52	2.25	2.25	**2.35**	**2.35**
β_{CLO}	44.86	2.67	**6.43**	2.81	33.95	33.95	**33.95**	**33.95**
$EC_{50\ IMI}$	0.48	0.69	**0.25**	0.71	0.33	0.33	**0.30**	**0.31**
$EC_{50\ CLO}$	0.65	0.28	**0.48**	0.29	1.34	1.34	**0.95**	**0.95**
a	-	0.53	**-9.97**	0.004	-	0	**-0.12**	**-0.12**
b_{DR}	-	-	**17.82**	-	-	-	**-0.06**	**-**
b_{DL}	-	-	**-**	-85.42	-	-	**-**	**-0.06**
SS	177.9	164.6	**139.3**	164.4	314.8	314.8	**284.7**	**284.7**
				vs. Reference Model				
χ^2	-	13.34	**38.61**	13.56	-	9.17	**30.08**	**30.08**
df	-	1	**2**	2	-	1	**2**	**2**
$p(\chi^2)$	-	< 0.05	**< 0.05**	0.001	-	< 0.05	**< 0.05**	**< 0.05**
				vs. S/A				
χ^2	-	-	**25.26**	0.21	-	-	**30.08**	**30.08**
df	-	-	**1**	1	-	-	**1**	**1**
$p(\chi^2)$	-	-	**< 0.05**	0.64	-	-	**< 0.05**	**< 0.05**
R^2 *(%)*	60.3	63.3	**68.9**	63.3	29.8	29.8	**36.5**	**36.5**

*Bolded columns represent the mixture models of best fit (best correlation between modeled and measured data) for each reference model (CA and IA).

Table A3.5 Summary of the MIXTOX analysis output for the chronic effects of clothianidin-thiamethoxam **(**CLO-TMX) mixtures on the successful emergence of *Chironomus dilutus.* *

	Concentration Addition				Independent Action			
	Reference	S/A	DR	DL	Reference	**S/A**	DR	DL
μ_{max}	55.8	60.6	**60.5**	60.6	60.4	**60.6**	60.6	60.6
β_{TMX}	12.3	58.0	**22.1**	59.3	5.84	**13.0**	13.0	13.0
β_{CLO}	67.8	9.24	**8.4**	10.0	189.0	**189.0**	189.0	189.0
$EC_{50\ TMX}$	8.29	8.10	**8.20**	8.10	6.97	**8.32**	8.31	8.30
$EC_{50\ CLO}$	1.09	0.63	**0.57**	0.64	0.46	**0.47**	0.47	0.47
a	-	0.84	**2.15**	0.01	-	**-9.13**	-9.13	-9.20
b_{DR}	-	-	**-2.15**	-	-	-	0.09	-
b_{DL}	-	-	-	-54.5	-	-	-	1.29
RSS	140.1	93.9	**91.6**	92.8	114.0	**96.4**	96.4	96.3
				vs. Reference Model				
χ^2	-	46.2	**48.5**	47.2	-	**17.6**	17.6	17.6
df	-	1	**2**	2	-	**1**	1	2
$p(\chi^2)$	-	< 0.01	**< 0.01**	< 0.01	-	**< 0.01**	< 0.01	< 0.01
				vs. S/A				
χ^2	-	-	**2.26**	1.05	-	-	0.001	0.02
df	-	-	**1**	1	-	-	1	1
$p(\chi^2)$	-	-	**0.13**	0.30	-	-	0.97	0.88
R^2 *(%)*	75.5	83.6	**84.0**	83.8	80.0	**83.1**	83.1	83.1

*Bolded columns represent the mixture models of best fit (best correlation between modeled and measured data) for each reference model (CA and IA).

Table A3.6 Summary of the MIXTOX analysis output for the chronic effects of imidacloprid-thiamethoxam (IMI-TMX) mixtures on the successful emergence of *Chironomus dilutus*.*

	Concentration Addition				Independent Action			
	Reference	S/A	**DR**	DL	Reference	**S/A**	DR	DL
μ_{max}	63.0	62.3	**61.9**	62.3	64.3	**62.0**	62.0	62.0
β_{TMX}	3.23	3.82	**4.59**	3.86	2.58	**10.64**	10.55	10.98
β_{CLO}	6.25	6.27	**5.81**	6.31	4.90	**5.05**	5.04	5.45
$EC_{50\ IMI}$	0.83	0.80	**0.81**	0.80	0.63	**0.92**	0.92	0.92
$EC_{50\ TMX}$	10.40	9.55	**10.31**	9.56	8.35	**10.15**	10.16	10.13
a	-	0.68	**-0.95**	0.61	-	**-5.79**	-5.89	-6.52
b_{DR}	-	-	**3.10**	-	-	**-**	0.26	-
b_{DL}	-	-	**-**	-0.07	-	**-**	-	0.32
SS	175.5	172.6	**168.7**	172.6	177.9	**168.0**	168.0	167.9
				vs. Reference Model				
χ^2	**-**	2.94	**6.8**	2.94	-	**9.90**	9.90	10.09
df	**-**	1	**1**	2	-	**1**	2	2
$p(\chi^2)$	**-**	0.09	**< 0.05**	0.23	-	**< 0.05**	< 0.05	< 0.05
				vs. S/A				
χ^2	-	-	**3.86**	0.002	-	-	0.002	0.19
df	-	-	**1**	1	-	-	1	1
$p(\chi^2)$	-	-	**< 0.05**	0.96	-	-	0.97	0.66
R^2	65.8	66.4	**67.1**	66.3	65.3	**67.3**	67.3	65.9

* Bolded columns represent the mixture models of best fit (best correlation between modeled and measured data) for each reference model (CA and IA).

Table A3.7 Summary of the MIXTOX analysis output for the chronic effects of imidacloprid-clothianidin-thiamethoxam (IMI-CLO-TMX) mixtures on the successful emergence of *Chironomus dilutus*.*

	Concentration Addition		Independent Action	
	Reference	**S/A**	Reference	**S/A**
μ_{max}	54.2	**54.8**	49.7	**58.1**
β_{IMI}	3.21	**3.21**	1.91	**3.95**
β_{CLO}	18.40	**18.40**	33.94	**5.37**
β_{TMX}	6.51	**5.51**	3.43	**4.63**
$EC_{50\ IMI}$ (µg/L)	0.71	**0.71**	0.34	**0.63**
$EC_{50\ CLO}$ (µg/L)	0.65	**0.66**	0.62	**0.61**
$EC_{50\ TMX}$ (µg/L)	11.70	**11.70**	8.52	**7.66**
$a_{IMI,CLO}$	-	**-1.24**	-	**-6.19**
$a_{CLO,TMX}$	-	**-0.18**	-	**-2.23**
$a_{IMI,TMX}$	-	**0.54**	-	**0.73**
$a_{IMI,CLO,TMX}$	-	**0.20**	-	**-38.9**
SS	618.0	**588.1**	793.1	**564.4**
	vs. Reference Model			
χ^2	-	**29.9**	-	**228.7**
df	-	**1**	-	**1**
$p(\chi^2)$	-	**< 0.05**	-	**<0.05**
R^2 (%)	63.9	**65.6**	53.6	**67.0**

*Bolded columns represent the mixture models of best fit (best correlation between modeled and measured data) for each reference model (CA and IA).

Table A4.1 Mean (± standard deviation) cumulative total insect and Chironomidae emergence from experimental limnocorrals prior to neonicotinoid treatment (n = 3 days of sampling over 8 days prior to neonicotinoid treatment) in field study.

Treatment	Total Insect Abundance	Chironomidae Abundance
Control	29 ± 24	22 ± 25
IMI	10 ± 7	6 ± 5
IMI-CLO	36 ± 25	31 ± 26
CLO	47 ± 42	43 ± 42
CLO-TMX	31 ± 3	22 ± 5
TMX	12 ± 14	8 ± 12
IMI-TMX	23 ± 17	18 ± 16

[a] Significantly different from control (one-way ANOVA, $p < 0.05$).

Table A4.2 Mean (± standard deviation) physicochemical water quality parameters in control and neonicotinoid treated limnocorrals over the 56-d field exposure period.

Water Quality Parameter	Treatment						
	Control	**IMI**	**IMI-CLO**	**CLO**	**CLO-TMX**	**TMX**	**IMI-TMX**
Dissolved Oxygen (mg/L)	4.5 ± 0.7	4.7 ± 0.8	6.2 ± 3.0	5.2 ± 1.2	5.1 ± 1.4	4.8 ± 0.7	6.1 ± 1.6
Temperature (°C)	19.7 ± 0.8	20.3 ± 0.2	20.5 ± 0.1	20.6 ± 0.1	20.7 ± 0.2	20.9 ± 0.2	20.8 ± 0.9
Ammonia (mg/L)	0.4 ± 0.1	0.6 ± 0.0	1.4 ± 0.7	0.7 ± 0.0[a]	0.6 ± 0.2	0.6 ± 0.1	0.6 ± 0.1
pH	8.1 ± 0.0	8.1 ± 0.0	8.2 ± 0.1	8.1 ± 0.1	8.2 ± 0.2	8.0 ± 0.1	8.1 ± 0.1
Conductivity (mS/cm^3)	2937 ± 120	2935 ± 86	2959 ± 125	2899 ± 90	3021 ± 85	2850 ± 23	2936 ± 58
Hardness (mg/L as $CaCO_3$)	1773 ± 87	1771 ± 82	1741 ± 73	1709 ± 21	1677 ± 50	1609 ± 57	1678 ± 27
Alkalinity (mg/L as $CaCO_3$)	419 ± 9	405 ± 20	411 ± 39	413 ± 15	393 ± 36	392 ± 10	469 ± 72
Nitrate (mg/L)	3.5 ± 0.4	3.7 ± 0.7	3.8 ± 0.6	3.0 ± 0.3	4.7 ± 0.1	4.0 ± 0.2	4.9 ± 0.4
Phosphate (mg/L)	1.1 ± 0.4	1.7 ± 0.6	1.3 ± 0.7	0.7 ± 0.5	0.8 ± 0.4	1.0 ± 0.2	2.2 ± 1.6
Dissolved Organic Carbon (mg/L)	31.5 ± 1.6	31.6 ± 2.3	34.5 ± 5.3	31.9 ± 1.6	30.9 ± 1.1	31.2 ± 1.2	32.5 ± 1.6

[a] Significantly different from control (One-way ANOVA, $p<0.05$).

Table A4.3 Mean measured neonicotinoid concentrations (µg/L) in limnocorrals on each dosing day (post-dose) over the 56-d exposure period. Nominal doses of neonicotinoids were as follows: IMI (single compound = 0.50 µg/L; in binary mixtures = 0.25 µg/L), CLO (single compound = 0.71 µg/L; in binary mixtures = 0.36), and TMX (single compound = 8.91 µg/L; in binary mixtures = 4.46 µg/L).

Day	Treatment (µg/L)											
	Control (0 µg/L)			IMI (0.50 µg/L)			CLO (0.70 µg/L)			TMX (8.91 µg/L)		
	IMI	CLO	TMX	IMI	CLO	TMX	IMI	CLO	TMX	IMI	CLO	TMX
0	-	-	-	-	-	-	-	-	-	-	-	0.04
4	-	-	<0.01	0.46	-	-	-	0.53	<0.01	-	0.02	8.41
8	-	-	-	0.94	-	-	-	1.28	0.21	-	-	17.00
12	-	-	-	0.73	-	-	-	0.97	0.04	-	0.04	16.31
16	-	-	0.002	0.61	-	0.03	-	0.83	0.12	-	0.07	12.78
20	-	-	-	0.54	-	-	-	0.76	-	-	0.07	10.60
24	<0.01	-	-	0.49	-	0.07	-	0.63	-	-	-	8.17
28	<0.01	-	-	0.51	-	<0.01	-	0.71	-	<0.01	0.07	7.48
32	-	-	-	0.42	-	-	-	0.51	-	-	-	6.50
36	-	-	-	0.52	-	-	-	0.84	-	-	-	5.72
40	-	-	<0.01	0.48	-	-	-	0.74	0.03	-	-	6.15
44	<0.01	-	-	0.45	-	-	-	0.65	-	-	-	6.50
48	-	-	-	0.37	-	-	-	0.56	-	-	-	7.10
52	-	-	<0.01	0.39	-	-	-	0.60	-	-	-	10.68
56	-	-	-	0.32	-	-	-	0.59	-	-	0.07	6.87

Day	Treatment (µg/L)								
	IMI-CLO (0.25 µg/L, 0.35 µg/L)			**CLO-TMX (0.35 µg/L, 4.45 µg/L)**			**IMI-TMX (0.25 µg/L, 4.45 µg/L)**		
	IMI	CLO	TMX	IMI	CLO	TMX	IMI	CLO	TMX
0	-	-	-	-	-	-	-	-	0.02
4	0.25	0.30	-	-	0.30	5.64	0.22	<0.01	4.72
8	0.43	0.65	0.14	-	0.66	11.33	0.41	0.02	9.74
12	0.32	0.48	0.01	-	0.49	9.41	0.33	0.03	8.92
16	0.27	0.34	0.02	-	0.43	8.38	0.24	0.03	7.18
20	0.26	0.33	-	-	0.43	6.07	0.24	0.03	5.78
24	0.20	0.25	-	-	0.33	4.78	0.18	0.03	4.89
28	0.15	0.19	-	-	0.38	3.68	0.16	0.04	3.29
32	0.17	0.19	0.02	-	0.33	3.89	0.15	0.03	2.86
36	0.26	0.41	-	-	0.45	3.50	0.25	0.02	3.17
40	0.27	0.39	-	-	0.37	3.71	0.29	0.02	3.91
44	0.25	0.37	-	-	0.36	4.47	0.25	0.02	3.81
48	0.23	0.28	-	-	0.28	4.55	0.24	0.03	4.27
52	0.21	0.30	-	-	0.34	6.81	0.20	0.04	5.99
56	0.17	0.29	-	-	0.33	4.60	0.20	0.03	3.83

* All unreported concentrations were lower than the limits of quantification (LOQ): IMI: 0.0028 ± 0.0006 µg/L, CLO: 0.0034 ± 0.0008 µg/L, TMX: 0.0058 ± 0.0009 µg/L.

Table A4.4 Mean measured neonicotinoid concentrations expressed as toxic units (TU) in limnocorrals at each dosing day over the 56-d exposure period. Nominal TU of neonicotinoids were as follows: IMI (single compound = 1; in binary mixtures = 0.5), CLO (single compound = 1; in binary mixtures = 0.5), and TMX (single compound = 1; in binary mixtures = 0.5).

	Treatment (TU)											
Day	**Control (0 TU)**			**IMI (1.0 TU)**			**CLO (1.0 TU)**			**TMX (1.0 TU)**		
	IMI	CLO	TMX	IMI	CLO	TMX	IMI	CLO	TMX	IMI	CLO	TMX
0	-	-	-	-	-	-	-	-	-	-	-	<0.01
4	-	-	<0.01	0.93	-	-	-	0.75	<0.01	-	0.03	0.94
8	-	-	-	1.89	-	-	-	1.80	0.02	-	-	1.91
12	-	-	-	1.46	-	-	-	1.37	<0.01	-	0.06	1.83
16	-	-	<0.01	1.21	-	<0.01	-	1.17	0.01	-	0.10	1.43
20	-	-	-	1.08	-	-	-	1.07	-	-	0.01	1.19
24	<0.01	-	-	0.98	-	<0.01	-	0.88	-	-	-	0.92
28	<0.01	-	-	1.03	-	<0.01	-	1.0	-	<0.01	0.10	0.84
32	-	-	-	0.84	-	-	-	0.72	-	-	-	0.73
36	-	-	-	1.03	-	-	-	1.19	-	-	-	0.64
40	-	-	<0.01	0.97	-	-	-	1.04	<0.01	-	-	0.69
44	<0.01	-	-	0.90	-	-	-	0.92	-	-	-	0.73
48	-	-	-	0.73	-	-	-	0.80	-	-	-	0.80
52	-	-	<0.01	0.78	-	-	-	0.85	-	-	-	1.20
56	-	-	-	0.63	-	-	-	0.84	-	-	0.09	0.77

Day	Treatment (TU)								
	IMI-CLO (1.0 TU)			CLO-TMX (1.0 TU)			IMI-TMX (1.0 TU)		
	IMI	CLO	TMX	IMI	CLO	TMX	IMI	CLO	TMX
0	-	-	-	-	-	-	-	-	<0.01
4	0.49	0.42	-	-	0.43	0.63	0.43	0.01	0.53
8	0.86	0.92	0.02	-	0.93	1.3	0.82	0.03	1.09
12	0.65	0.67	<0.01	-	0.68	1.06	0.66	0.04	1.00
16	0.54	0.48	<0.01	-	0.61	0.94	0.47	0.04	0.81
20	0.52	0.46	-	-	0.61	0.68	0.47	0.05	0.65
24	0.40	0.35	-	-	0.47	0.54	0.36	0.04	0.55
28	0.30	0.27	-	-	0.53	0.41	0.31	0.05	0.37
32	0.35	0.27	<0.01	-	0.47	0.44	0.29	0.04	0.32
36	0.52	0.58	-	-	0.63	0.39	0.50	0.03	0.36
40	0.55	0.54	-	-	0.51	0.42	0.46	0.02	0.44
44	0.50	0.52	-	-	0.51	0.50	0.50	0.03	0.43
48	0.46	0.39	-	-	0.39	0.51	0.49	0.04	0.48
52	0.41	0.42	-	-	0.48	0.77	0.40	0.06	0.67
56	0.34	0.32	-	-	0.46	0.52	0.40	0.04	0.43

* All unreported toxic units were measured at concentrations lower than the limits of quantification (LOQ): IMI: 0.0028 ± 0.0006 µg/L, CLO: 0.0034 ± 0.0008 µg/L, TMX: 0.0058 ± 0.0009 µg/L.

Table A4.5 Mean (± standard deviation) abundances of emerged insect taxa in untreated limnocorrals (controls) compared to limnocorrals treated with single compounds or binary neonicotinoid mixtures for 56 days (cumulative), (n = 3 limnocorrals/treatment).

Treatment	**Diptera**		**Trichoptera**	**Hymenoptera**	**Odonata**	**Coleoptera**	**Ephemeroptera**
	Total	*Chironomidae*					
Control	1972 ± 773	1962 ± 782	6 ± 3	2 ± 0	20 ± 12	3 ± 2	0 ± 1
IMI	201 ± 31	197 ± 28	4 ± 1	1 ± 1	24 ± 38	0 ± 1	0 ± 0
IMI-CLO	653 ± 364	638 ± 364	8 ± 6	1 ± 2	25 ± 17	2 ± 3	0 ± 0
CLO	284 ± 109	282 ± 103	8 ± 5	2 ± 1	16 ± 10	2 ± 2	0 ± 1
CLO-TMX	284 ± 258	284 ± 249	7 ± 6	2 ± 3	14 ± 19	3 ± 1	0 ± 0
TMX	579 ± 156	572 ± 156	9 ± 4	3 ± 1	12 ± 11	1 ± 1	0 ± 1
IMI-TMX	765 ± 853	735 ± 823	12 ± 4	2 ± 2	13 ± 11	2 ± 1	0 ± 0

* Significantly different from the control (One-way ANOVA, $p < 0.05$).

Table A4.6 Coefficients of variation (%) for cumulative emergence and biomass of emerged Chironomidae in neonicotinoid treated limnocorrals after 56 days of exposure (n = 3 replicates/treatment).

Treatment	Coefficient of Variation for Emergence (%)	Coefficient of Variation for Biomass (% CV)
Control	39.9	52.4
IMI	14.2	29.7
CLO	36.6	46.3
TMX	27.4	14.0
IMI-CLO	57.1	38.1
CLO-TMX	87.8	67.4
IMI-TMX	111.9	82.4

Table A5.1 Comparative efficiencies of five different experimental methodologies for the extraction of membrane protein from larval Chironomidae (spp. unknown; mixed culture). Bolded method indicates most efficacious, and thus was used for all membrane protein extraction.

Method	Homogenization Instrument	Dissociation Medium	Quantity of Chironomidae used in Extraction (g)	Concentration of Extracted Membrane Protein (µg/mL)	Quantity of Membrane Protein Extracted (g)	Membrane Protein Extraction Efficiency (%) *
1	Manual Homogenization (Mortar + Pestle)	Fly [a]	5.2	1436	0.0029	0.06
2	Automatic Blender	Fly [a]	5.4	2254	0.005	0.08
3	Automatic Blender	Aphid [b]	5.9	1322	0.005	0.09
4	Automatic Pestle Motor	Fly [a]	2.7	1819	0.003	0.09
5	**Automatic Pestle Motor**	**Aphid [b]**	**4.0**	**2056**	**0.004**	**0.15**

* Membrane extraction efficiency (%) $= \frac{Quantity\ of\ membrane\ protein\ extracted\ (g)}{Quantity\ of\ Chironomidae\ (g)}\ x\ 100\ \%$

[a] Fly dissociation medium (/6 mL of media, dissolved in reverse-osmosis water, pH = 7.0): 100 mM Na_2HPO_4, 0.32 mM sucrose, 0.1 mM EDTA, 100 µM PMSF, and 1 µM of each leupeptin (dissolved in water) and chymostatin (dissolved in DMSO). Adapted from Liu and Casida (1993).

[b] Aphid dissociation medium (/6 mL of media, dissolved in reverse-osmosis water, pH = 7.4): 20 mM sodium phosphate, 150 mM sodium chloride, 1 mM EDTA, 0.1 mM PMSF, and 2 µg of each pepstatin (dissolved in methanol), chymostatin (dissolved in DMSO), and leupeptin (dissolved in water). Adapted from Taillebois et al., (2014) and Wiesner and Kayser (2000).

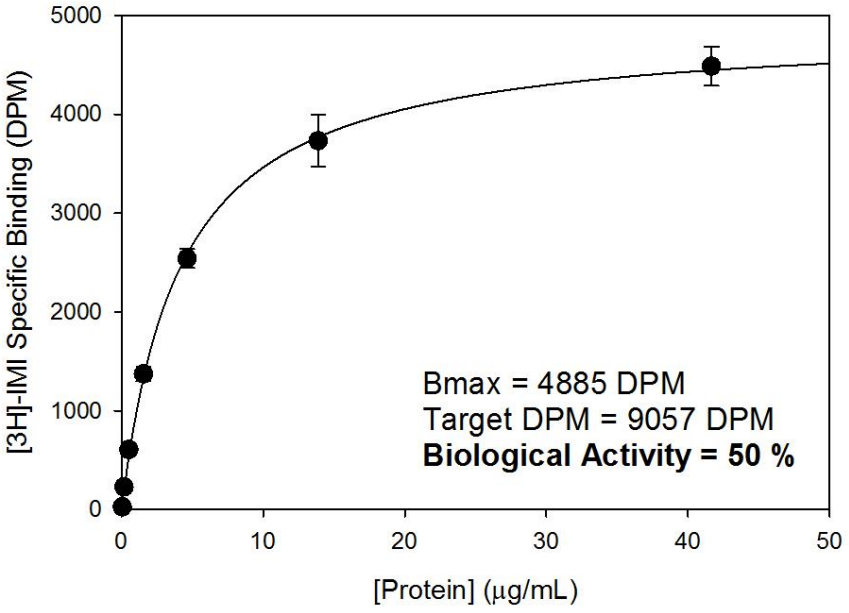

Figure A5.1 Specific binding (disintegrations per minute, DPM) of ^{3}H-imidacloprid ([3H]-IMI) to membrane protein isolated from *C. riparius* larvae. Biological activity of [^{3}H]-IMI was evaluated by comparing the maximal binding (B_{max} = 4885 DPM) to the target [3H]-IMI concentration (9057 DPM) (i.e. $Biological\ Activity = Bmax/Target\ [[3H] - IMI]$).

*Data is presented as mean ± standard error (SE) of three experimental replicates.

Table A5.2 Binding affinity of [^{3}H]-imidacloprid ([^{3}H]-IMI) (K_D; mean ± SE), nicotinic acetylcholine receptor density (B_{max}; mean ± SE), and acute imidacloprid (IMI) toxicity (48 - 96 h LC/EC_{50}; minimum reported value) for larval Chironomidae compared to that previously characterized for other insects.*

Insect Species	Classification	Taxonomic Order	Binding Site (Affinity)	*n*	K_D (nM)	B_{max} (fmol/mg)	Acute Toxicity	Ref*
Chironomus dilutus	**Aquatic Insect**	**Diptera**	**-**	**1**	**0.24**	**5098**	**5 µg/L**	**[1 - 2]**
Chironomus riparius	**Aquatic Insect**	**Diptera**	**-**	**1**	**0.20**	**6522**	**13 µg/L**	**[3 - 4]**
Drosophila melanogaster	Standard Test Species	Diptera	-	4	3.0 ± 0.9	1018 ± 325	195 µg/L	[5 - 6]
Musca domestica	Standard Test Species	Diptera	-	8	4.0 ± 1.0	523 ± 74	6700 µg/L	[5, 7 - 9]
Myzus persicae	Agricultural Pest	Hemiptera	Low	6	4.8 ± 1.8	696 ± 191	73 µg/L	[5, 10 - 12]
			High	3	0.3 ± 0.2	196 ± 53		
Acyrthosiphon pisum	Agricultural Pest	Hemiptera	Low	1	41.70	0.43	38 µg/L	[13]
			High	1	0.16	0.051		
Aphis craccivora	Agricultural Pest	Hemiptera	Low	4	13.2 ± 4.3	1151 ± 125	1160 µg/L	[5, 12, 14 - 15]
			High	3	0.9 ± 0.3	123 ± 53		
Nephotettix cincticeps	Agricultural Pest	Hemiptera	Low	1	1.2	179	30 µg/L	[5, 12, 16]
			High	1	0.004	33		
Nilaparvata lugens	Agricultural Pest	Hemiptera	Low	2	1.5 ± 0.0	18 ± 1	110 µg/L	[5, – 12, 17]
			High	1	0.0035	4		
Locusta migratoria	Agricultural Pest	Orthoptera	Low	2	9.6 ± 0.7	290 ± 46	3 µg/g insect	[5, 12, 17 - 18]
			High	2	0.2 ± 0.0	131 ± 22		
Manduca sexta	Agricultural Pest	Lepidoptera	-	1	1.3	150.0	976 µg/L [a]	[19 - 20]
Heliothis virescens	Agricultural Pest	Lepidoptera	-	1	1.51	134	821 µg/g feed [b]	[19, 21]

*Bolded data was generated in this study.

[a] Toxicity datum is for technical product. [b] Toxicity datum is derived from a feeding assay.

***** [1] Maloney et al. 2017; [2] Raby et al. 2018a; [3] European Food Safety Authority (EFSA), 2014; [4] Posthuma-Doodeman 2008; [5] Crossthwaite et al., 2017; [6] Frantzois et al., 2008; [7] Kaufman et al. 2006; [8] White et al. 2007 ; [9] Abbas et al. 2015; [10] Nauen et al., 1998; [11] Nauen et al. 1998b; [12] Taillebois et al., 2018**;** [13] Taillebois et al., 2014; [14] Tang et al. 2013; [15] Abd-Ella, 2014; [16] Jairin et al. 2005; [17] Matsuda et al., 2009**;** [18] Parkinson et al., 2017; [19] Ohkawara et al., 2002; [20] Eure et al., 2018; [21] Lagadic et al., 1993.

Table A5.3 Competitive binding of [^{3}H]-imidacloprid and unlabelled neonicotinoids, imidacloprid (IMI), clothianidin (CLO), and thiamethoxam (TMX) and acute toxicities (48 - 96 h LC/EC_{50} values) for larval Chironomidae (*C. dilutus* and *C. riparius*) compared to that previously characterized for agricultural pests and other experimental insects.*

Bolded data was generated in this study.

Insect Species	Common Name	Classification	Taxonomic Order	Compound	K_i (nM)	IC_{50} (nM)	Acute L/EC_{50}	Ref**
Chironomus dilutus	**Non-Biting Midge**	**Aquatic Insect**	**Diptera**	**IMI**	**0.42**	**1.29**	**4.63 µg/L**	**[1 - 4]**
				CLO	**0.21**	**0.63**	**3.30 µg/L**	
				TMX	**45.54**	**140.00**	**45.0 µg/L**	
Chironomus riparius	**Non-Biting Midge**	**Aquatic Insect**	**Diptera**	**IMI**	**0.50**	**1.78**	**12.94 µg/L**	**[2 - 4]**
				CLO	**0.43**	**1.53**	**21.80 µg/L**	
				TMX	**43.36**	**153.90**	**55.50 µg/L**	
Drosophila melanogaster	Fruit Fly	Experimental Organism	Diptera	IMI	3000	2.3 - 5.8	6827 - 19407 µg/L	[5 – 7]
				CLO	1800	2.2	0.56 µg/g insect	
				TMX	-	-	-	
Musca domestica	House Fly	Experimental Organism	Diptera	IMI	-	2.6	31.4 µg/g feed	[5, 8]
				CLO	-	1.8	-	
				TMX	-	-	-	
Myzus persicae	Green Peach Aphid	Agricultural Pest	Hemiptera	IMI	2700 – 24200	7.2 - 9.2	470 - 1600 µg/L	[5, 9 - 10]
				CLO	550 - 3100	2.3 - 3.1	1303 - 2341 µg/L	
				TMX	12000 - 2.9 x 10^6	2800 - 4800	4020 µg/L	
Aphis craccivora	Cotton Aphid	Agricultural Pest	Hemiptera	IMI	-	2.3 - 25.2	1160 - 6330 µg/L	[5, 11 - 12]

				CLO	-	2.3 - 8.6	-	
				TMX	-	270 - 2300	600 µg/L	
Acyrthosiphon pisum	Pea Aphid	Agricultural Pest	Hemiptera	IMI	38140	0.061	38 µg/L	[5, 13]
				CLO	126900	0.203	34 µg/L	
				TMX	1047200	1.675	118 µg/L	

* Modified from Taillebois et al., (2018).

** [1] Maloney et al., 2017; [2] Pest Management Regulatory Agency, 2018; [3] Pest Management Regulatory Agency, 2018b; [4] European Food Safety Authority (EFSA), 2014; [5] Taillebois et al., 2018; [6] Frantzois et al., 2008; [7] Arain et al., 2014; [8] Burgess and King, 2015; [9] XueXiang et al., 2011; [10] Cho et al., 2011; [11] Tang et al., 2013; [12] Abd-Ella, 2014; [13] Taillebois et al., 2014.