

ASSESSING THE EFFECTS OF CHRONIC NEONICOTINOID INSECTICIDE EXPOSURE
ON AQUATIC INSECTS USING MULTIPLE EXPERIMENTAL APPROACHES

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By

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ABSTRACT

Neonicotinoid insecticides are among the most widely used plant protection products in industrialized agriculture, and are hypothesized to be contributing to losses in non-target insect biodiversity. The lack of toxicological data evaluating the effects of neonicotinoids restricts the ability of regulators to adequately protect sensitive insects particularly those with aquatic larval stages. Many Prairie wetlands are in regions of intensive agriculture and are directly at risk of neonicotinoid contamination and require special considerations to best protect these ecologically important areas. Under laboratory conditions, I compared the effects of imidacloprid, clothianidin, thiamethoxam on the model aquatic insect, *Chironomus dilutus*. Reduced emergence success, advanced emergence timing, and male-biased sex ratios were observed across all compounds tested. Imidacloprid and clothianidin reduced emergence success at similar concentrations whereas thiamethoxam required a concentration an order of magnitude greater to observe similar toxicity. Normalizing the clothianidin and thiamethoxam toxic responses to imidacloprid, I calculated acute (lethality) 14-day toxic equivalency factors (TEFs) for imidacloprid, clothianidin and thiamethoxam as 1.00, 1.05 and 0.14, respectively, and chronic (emergence inhibition) 40-day TEFs as 1.00, 1.62 and 0.11, respectively. To expand upon these single-species laboratory assessments, *in situ* limnocorrals were used to determine the chronic effects of the three neonicotinoids to emerging aquatic insect communities in a Prairie wetland. Imidacloprid and clothianidin treatments had similar community responses and non-biting midge (Diptera: Chironomidae) and damselfly (Odonata: Zygoptera) emerged significantly earlier than the controls (18 to 25 days earlier). An additional limnocorral study with clothianidin was inconclusive. While laboratory and limnocorral studies were useful to isolate neonicotinoid effects, multiple anthropogenic stressors were hypothesized to cumulatively influence insect emergence from natural Prairie wetlands surrounded by neonicotinoid-treated canola fields. Multivariate analysis showed neonicotinoid concentration, turbidity, vegetation disturbance and continuity of grasses were significant factors modifying the abundance and composition of emerging insects. Total insect abundance was negatively affected by neonicotinoids ($\beta \pm \text{S.E.} = -0.61 \pm 0.14$, $P < 0.001$) but positively affected by vegetation disturbance ($\beta \pm \text{S.E.} = 0.34 \pm 0.11$, $P < 0.001$). Collectively, these data suggest more rigorous water quality guidelines and agricultural management strategies are needed to protect aquatic insects and the higher trophic organisms that rely on this important food source.

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DEDICATION

I dedicate this dissertation to my first academic mentor and entomology professor, who opened my mind to the world of insects and taught me how to “think like a bug”, Dr. Chris Tipping.

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LIST OF ABBREVIATIONS

ACT	acetamiprid
AIC	Akaike information criterion
CCME	Canadian Council of Ministers of the Environment
CLO	clothianidin
df	degrees of freedom
EC50	50% effect concentration
EPA	Environmental Protection Agency
EPSP	excitatory post-synaptic potential
IMI	imidacloprid
kJ	kilojoule
LC50	50% lethal concentration
LOD	limits of detection
LOQ	limits of quantification
nAChR	nicotinic acetylcholine receptor
NOEC	no observed effect concentration
NWA	National Wildlife Area
PCA	Principal component analysis
PMRA	Pesticide Management Regulatory Agency
PPR	Prairie Pothole Region
PRC	Principal Response Curve
QA/QC	quality assurance/quality control
RC	recovery correction
RDA	Redundancy analysis
TEF	toxic equivalence factor
TEQ	toxic equivalency quotient
THI	thiacloprid
THX	thiamethoxam
TWA	time-weighted average

CHAPTER 1: INTRODUCTION

1.1 Research purpose

Contemporary shifts in agricultural practices have substantially altered agrochemical inputs since the mid-2000s such as the increased use of prophylactic insecticide seed treatments, predominantly coated in neonicotinoid insecticides (Douglas and Tooker 2015). Seven neonicotinoid active ingredients are used commercially worldwide (Jeschke and Nauen 2008). The most commonly applied neonicotinoids in North America include imidacloprid, clothianidin, and thiamethoxam. Favored for their systemic properties, simplified application methods (i.e., seed treatments), and high pest insect toxicity, neonicotinoids were designed to control defoliating pest insects and prevent damage to crop seedlings (Elbert et al. 2008, Goulson 2013). Neonicotinoids demonstrate time-dependent toxicity where increased exposure duration requires lower concentrations to induce an effect at a common endpoint (Tennekes and Sánchez-Bayo 2011).

Neonicotinoids display prolonged environmental persistence in soils and high water solubility, entering aquatic ecosystems readily from precipitation and snowmelt runoff (Jones et al. 2014, Main et al. 2016). Due to their proximity to productive agricultural land, wetlands located in the Prairie Pothole Region (PPR) of North America are at enhanced risk to neonicotinoid contaminated runoff (Main et al. 2014). Ecological and landscape-level factors dictate the magnitude and frequency of contamination events, and water sampling efforts have confirmed measurable concentrations of neonicotinoids in PPR wetlands (Main et al. 2014, 2015). Often this equates to a chronic, low-level exposure profile to aquatic organisms occupying freshwater habitats near farming operations using neonicotinoid-treated seeds. Among the most sensitive aquatic taxa at risk to neonicotinoid contamination are immature stages of aquatic insects (Morrissey et al. 2015).

Immature stages of aquatic insects are well-cited indicators of environmental change and are sensitive to chemical stressors, especially insecticides (Catallo et al. 1993). Neonicotinoids exhibit comparable or greater toxicity to sensitive target and non-target insects (Simon-Delso et al. 2015). Model aquatic insect taxa (e.g., the non-biting midge, *Chironomus dilutus*; Diptera: Chironomidae) are practical sentinel organisms for characterizing the risks of water-borne insecticide contamination to aquatic insect populations under varying experimental venues, exposure durations, and common endpoints. Due to the fate and persistence of neonicotinoids in

the environment, aquatic insects are at enhanced risk to chronic toxicity where exposure could continue throughout each immature insect stage until adult emergence. Emergence inhibition is reported as a sensitive endpoint for chronic neonicotinoid exposure (Stoughton et al. 2008). To date, most neonicotinoid toxicity tests address the risks of acute exposure scenarios under controlled laboratory settings using a single active ingredient, imidacloprid.

Aquatic insects are fundamental components of aquatic biological communities and nearby terrestrial habitats. Imagos, or mature winged adults, exiting the aquatic environment form a key energy link between primary production and secondary consumers, terrestrial and aquatic alike (Gratton and Vander Zanden 2009). Certain taxa will also provide pollination services or act as predatory biocontrol agents (Sathe and Shinde 2008, Stewart et al. 2017). Cross-habitat linkages exist for many aquatic insects occurring as seasonal fluxes. Synchronous emergence and predictable phenologies are imperative for a number of wetland dependent fauna and adjacent wetland plant communities. The purpose of this ecotoxicological study was to fill existing knowledge gaps focusing on chronic toxicity endpoints, community assessments, and environmentally realistic exposure scenarios using multiple approaches in controlled, semi-controlled, and natural settings.

1.2 Research objectives

Incorporating a diversified approach to assessing the risk of neonicotinoid exposure to non-target aquatic insects, I investigated the comparative chronic toxicity of three neonicotinoid compounds, imidacloprid, clothianidin, and thiamethoxam, to non-target aquatic insect taxa using laboratory toxicity tests, limnocorral experiments, and wetland biomonitoring to specifically characterize insecticide effects on adult insect emergence. Therefore, my main research objectives were to:

- (1) compare the chronic toxicity of imidacloprid, clothianidin, and thiamethoxam to the model benthic macroinvertebrate species, *Chironomus dilutus*, focusing on sub-lethal (e.g., biomass, sex ratios, and emergence timing) and chronic endpoints (e.g., emergence inhibition) and to develop chronic toxic equivalency factors for clothianidin and thiamethoxam to characterize their relative toxic potency compared to the well-studied neonicotinoid compound, imidacloprid (Chapter 2).
- (2) assess the effects of low-level imidacloprid, clothianidin, and thiamethoxam exposure to aquatic insect community composition, population abundances, and emergence timing,

and to expand on the potential chronic effects of clothianidin at less frequent but increased dose range using *in situ* limnocorrals in a model Prairie wetland (Chapter 3 and 4).

- (3) measure neonicotinoid concentrations repeatedly in wetlands surrounded by farm fields planted with neonicotinoid seed-treated canola and determine the relative impacts of key water quality parameters, wetland habitat characteristics, and neonicotinoid contamination on aquatic insect community composition and abundance at different sampling periods over a growing season (Chapter 5).

1.3 Review of literature

The following sections provide an overview of key topics which helped inform the set of objectives outlined above. Generally, I will discuss agroecosystems and Prairie wetlands, focusing on the biology that may be affected by agriculture. Then, I highlight the importance of aquatic insect emergence as a product of healthy, productive wetland systems. Thereafter, I describe the prevalence and fate of pesticides in the aquatic environment which has direct and indirect effects on aquatic insect production and adjacent habitats. This leads into a background on neonicotinoid insecticides, including use, fate, persistence, and toxicity. Lastly, I discuss the utility of mesocosms in ecotoxicology.

1.3.1 Agroecosystems and Prairie wetlands

A common trait among all ecological systems is the varying level of feedbacks between components, and agroecosystems are no different. Depending on the region, agroecosystems are viewed as a component of a conventional ecosystem (Gliessman 1990). Agroecosystems are largely manipulated by human activities, and their effects are not restricted to the immediately planted cropland. Effects from farm production can impact a substantial radius of the surrounding landscape and watershed. Management of these systems directly affects the wellbeing of humans and adjacent ecosystems (Waltner-Toews 1996). Certain farming practices (e.g., intensive tillage, heavy irrigation, synthetic chemical inputs) can seriously degrade and exploit both aquatic and terrestrial agroecosystems (Alteri 1999).

Worldwide agricultural development is predicted to double by 2050 due to food demand by a rapidly increasing global population (Tilman et al. 2002). Conventional farming is known to negatively impact the structure and function of agroecosystems. Field cultivation methods can drastically influence the rate and magnitude of soil erosion and compromise the viability of

wetland ecosystem function (Gleason et al. 2003). Rapid soil erosion causes sedimentation to surrounding water bodies and degrading of the nutrient quality of the cultivated soil. The misuse or repeated use of agrochemicals to control pests can lead to a loss in (plant and animal) species diversity and poor soil quality (Oldeman 1991). Runoff from conventional agriculture fields can cause nonpoint source contamination by pesticides and fertilizers (Wightwick and Allison 2007). As one of the key drivers of biodiversity loss in agroecosystems, agrochemicals can affect multiple trophic levels (McLaughlin and Mineau 1995, Alteri 1999, Newbold et al. 2015). Excess nutrients can harm aquatic communities and induce toxic algal blooms that may compromise livestock drinking water sources (Sarre 2009). Among the ecosystems most affected by conventional agricultural practices are Prairie wetlands. Changes in precipitation patterns onset by climate change will further compound the collective effects of these stressors (Skagen et al. 2016).

The Prairie landscape encompasses the most ecologically diverse grassland-wetland ecosystem in North America (Baldassarre and Bolden 2006). The PPR is a vast area extending from central Iowa to central Alberta containing millions of glaciated depressions (Driver 1971, Guntenspergen et al. 2002, Johnson et al. 2008). Due to their biophysical features, Prairie wetland systems provide a number of critical environmental and societal services (Johnson et al. 2008, Hentges and Stewart 2010, Dias and Belcher 2015); these ecosystems harbor high plant and animal biodiversity, improve water quality through phytoremediation, reduce flooding, and sequester carbon (Zedler and Kercher 2005). There is also a significant reliance on wetlands by higher trophic level organisms such as waterfowl, insectivorous birds, amphibians, bats, and semi-aquatic rodents which in some cases exclusively use Prairie wetlands (Batt et al. 1989, Steen and Powell 2012, Forcey et al. 2015, Baecher et al. 2017, Bartzen et al. 2017). For example, this region represents only 10% of the range used by the continental breeding duck population, but produces 50% of the total annual recruitment (Smith et al. 1964, Klett et al. 1988). In addition to providing important habitat structure, Prairie wetlands generate high invertebrate secondary production.

1.3.2 Importance of aquatic insect emergence in freshwater ecosystems

Adult insects emerging from aquatic environments enter their final stage of metamorphosis as flying adults, or imagos. Adults are short-lived and responsible for reproduction and dispersal. Most aquatic insects as adults are highly susceptible to predation

from aquatic and terrestrial secondary consumers and have a brief window to locate a mate. Reproductive strategies include synchronous and asynchronous emergence effectively saturating predation mechanisms or allowing more time to find a mate, respectively. Emigration of biomass from aquatic ecosystems in the form of insect secondary production forms a critical energy link from aquatic to terrestrial habitats, and vice versa (Jackson and Fisher 1986). That is, adults contributing to the terrestrial food web and returning to the aquatic environment to lay eggs play important roles in the cycling of nutrients and forming cross-habitat linkages.

Secondary production measures the number of individuals and reproductive output of a population and acts as a functional measure of energy flow through that population (Benke et al. 1988). For example, individuals from the dipteran family, Chironomidae, act as important food resources to aquatic and terrestrial predators at all stages of their life cycle (Pinder 1995). Some surface water habitats can harbor densities of >100,000 individuals per square meter during peak adult emergence (Parker 1992). Pupae and adults are especially susceptible to predation due to their immobility or limited mobility; as adults, the caloric content can range between 19.1-25.3 kJ per dry mass (gram) (Encarnação and Dietz 2006). This can account for millions of kJ of available energy per square meter with Prairie ponds ranging in size from less than a hectare to several hectares in surface area. Foraging in certain transitional habitats near water bodies is more efficient and often harbors greater food resources (Fukui et al. 2006). Since many secondary consumers will overlap key life cycle events (e.g., egg-laying, offspring-rearing, spring migration) with insect emergence, the timing and abundance of these calories can be critical.

Synchronous and asynchronous insect emergence events are often reliable food resources and can be timed from seasonal changes in the environment (e.g., photo period, growing degree days, crossing temperature threshold value); however, shifts in phenologies from higher/lower temperatures or contaminants might accelerate or slow growth, disrupting the timing of metamorphosis. For example, waterfowl chicks primarily feed on aquatic insects and depend heavily on this resource for successful growth and development. By incrementally increasing spring temperatures in temperate surface waters by 3° C, Hansson et al. (2014) observed a 2-week advancement in *Chironomus sp.* emergence which would have led to a mismatch in insect abundance with chick food needs. Abundant food resources originating from the aquatic environment are also important to migrating birds who need to refuel along the way (MacDade et

al. 2011). Moreover, these food sources from the aquatic environment are considered high-quality food items which contain a suite of essential nutrients and are linked to improved immune function in terrestrial consumers (Twining et al. 2016, Fritz et al. 2017).

1.3.3 Prevalence and consequence of pesticides in aquatic ecosystems

The majority of pesticides used in large-scale conventional agriculture are synthetic, produced through industrial processes. Broad-spectrum pesticides (i.e., targeted for a wide range of pest species) are applied at different rates that depend on manufacturer's recommendations on product labels. At the global scale, approximately 4×10^6 tons of pesticides are applied to cropland annually (Sánchez-Bayo 2011). Roughly, 40% of the non-ice land cover on earth is agricultural cropland or tamed pasture (Foley et al. 2005). The risk and likelihood of pesticide exposure to a range of terrestrial (Mineau and Whiteside 2013) and aquatic agroecosystems is thus very high (Ippolito et al. 2015). Pesticide application methods are based on the type of pesticide applied (e.g., systemic vs. contact) in agricultural systems. Some examples are soil drenching, foliar spray, and seed treatments. All of these methods have some susceptibility to run-off into field margins or adjacent water bodies.

Several factors may promote the entry and persistence of pesticide contamination in surface waters such as local rainfall, water chemistry, topography, chemical properties of the pesticide, microbial communities, rate of application, etc. This movement may also be influenced by atmospheric deposition, plant uptake (and subsequent senescence), sorption (adsorption or desorption), or infiltration through soil (Levanon et al. 1993, Kreutzweiser et al. 2008, Majewski et al. 2014). Once in the aqueous phase, pesticides will photo-degrade, hydrolyze or undergo microbial breakdown into metabolites that may potentially exhibit some toxicity (Burden et al. 2016). Ultimately, these properties can affect the exposure profile to non-target organisms (Norton et al. 1992).

The impacts of pesticide contamination to aquatic ecosystems are frequently discussed at varying geographic scales (regional to global). Regional land-use patterns (i.e., increased agricultural activities) can directly impact nearby surface waters through overspray and run off of pesticides. Over 2,000 water samples collected over 20 years from surface and ground water sources in Hungary identified recurrent and frequent detection of herbicide compounds (e.g., glyphosate) that consistently reflected regional usage (Székács et al. 2015). Surface water sampling efforts surrounding the Mekong Delta in Vietnam detected pesticides (e.g., buprofezin,

cypermethrin, isoprothiolane, profenofos) at levels up to 17 times greater near areas of heavy agriculture (Van Toan et al. 2013). In Saskatchewan, Canada, Main et al. (2014) detected neonicotinoid insecticide residues in 17-91% of Prairie wetlands sampled; wetlands surrounded by canola contained the highest neonicotinoid water concentration during the summer sampling (post-application). Scaling up from the regional level, Ippolito et al. (2015) created the first global risk map on insecticide runoff. Their analysis found that water bodies contained within 40% of global land surface may be exposed to concentrations of insecticides that cause adverse effects due to their proximity to conventional agriculture operations. Using 4,000 water monitoring sites around Europe, Malaj et al. (2014) identified pesticides as the greatest contributor of organic pollution.

The regulation and risk assessment of pesticides are evaluated by toxicity to non-target organisms (including humans), persistence (biotic and abiotic), fate in the environment, and degradation by-products. To protect aquatic ecosystems, government regulators use a decision-making process for regulating pesticide products and derive aquatic life benchmarks from toxicity endpoints (e.g., EC10, NOEC) available in the scientific literature (e.g., ECOTOX data base). For example, Health Canada's Pest Management Regulatory Agency (PMRA) regulates the use of pesticides in Canada, where currently over 6,800 different pesticide products are registered with over 74% originating from agricultural sector sales (PMRA 2014). Current or pending pesticide active ingredients amount to 1,135 (Health Canada 2018). Establishing science-based protocols for developing numerical concentration limits to protect environmental quality is critical. For example, in the reevaluation of imidacloprid the environmental risk assessment framework followed this step-by-step process: Identify hazards (e.g., human health, terrestrial and aquatic life), identify exposure (e.g., exposure modeling and field data screening), characterize the magnitude of risk (i.e., tiered approach), evaluate the options to mitigate the risk, select a risk management strategy, and, lastly, make a regulatory decision.

Exceeding aquatic life benchmarks (acute or chronic) can imperil aquatic ecosystem structure and function. Laboratory and field-based studies, sometimes in combination, are critical to the regulation and evaluation of pesticide-use and to risk assessment. Mitigation strategies to reduce movement of pesticides through water, air, and soil beyond the site of application include increased buffer strip width (Borin et al. 2010, Nuyttens et al. 2011), crop scouting (Walker et al. 2012), planting wind breaks to reduce spray drift (Ucar and Hall 2001), and banning of dust seed

lubricants (PMRA 2015). Despite regulations, contamination by pesticides and other agrochemicals remains one of the most challenging global threats to maintaining the quality of freshwater resources (Schultz 2004, Green et al. 2005).

1.3.4 Neonicotinoid insecticide use, fate, and persistence

Neonicotinoid insecticides are the most abundant chemical-form of invertebrate pest control on the market (Goulson 2013). Of the seven-recognized neonicotinoid active ingredients available, imidacloprid is the most accepted and versatile compound for crop protection; it is registered for use in 120 different countries and is applied to roughly 140 different crops (Jeschke and Nauen 2008). There is a total of approximately 500 neonicotinoid-based products on the market for agricultural, household, and urban pest control (Stevens and Jenkins 2014).

Neonicotinoids were first developed in 1970 by Shell Development Company in California. The decades following the creation of its basic chemical structure involved molecular development to improve photosensitivity and efficacy towards target pests. Test subjects included house flies, pea aphids, and corn earworm larvae (Tomizawa and Casida 2005). The following list outlines the patent dates for neonicotinoids and related compounds (oldest to newest): heterocyclics nithiazine (1977), imidacloprid (1985), thiacloprid (1985), the acyclics nitenpyram (1988), acetamiprid (1989), clothianidin (1989), thiamethoxam (1992), and dinotefuran (1994). Recently, two active substances of insecticides that display similar modes of action to neonicotinoids, sulfoxaflor and flupyradifurone, were registered for use in North America and the European Union. The companies producing sulfoxaflor and flupyradifurone have classified these compounds as sulfoximines and butenolides, respectively, although there is some debate on whether these classifications are appropriate (Sparks et al. 2013, Nauen et al. 2015).

Favored for their various methods of application, neonicotinoids can be applied as a seed dressing, foliar spray, stem injection, or soil drenching (Jeschke and Nauen 2008, Žabar et al. 2012). The most recent controversy regarding neonicotinoids includes the broad use of seed treatment application (Gontijo et al. 2014). This is a preventative application method that protects seedlings and mature plants from phytophagous invertebrate pests. Jeschke and Nauen (2008) report that nearly 80% of all seed treatments (e.g., canola, corn, lentils, cereals, etc.) are coated with a neonicotinoid-based insecticide. Because of this high demand, neonicotinoids constitute 24% of global insecticide market share (Jeschke et al. 2011). High water solubility,

versatile application methods, and improved rain fastness are all qualities specifically targeted by manufacturers to increase the bioavailability of the toxicant within the plant tissue (Elbert et al. 2008). Systemic insecticides are characterized by having a low octanol-water partition coefficient—e.g., below 40 (Cloyd and Bethke 2011). Neonicotinoids are incredibly water soluble with thiamethoxam being the most water soluble. These qualities make the compounds incredibly mobile in the aquatic environment.

Depending on their physical and chemical properties, pesticide mobility can vary between environmental mediums and pesticides can often move beyond the site of application (Pimentel 1995). For example, Surr and Stork (2003) tested the systemic activity and seed treatment effectiveness of the neonicotinoid insecticide imidacloprid on five different crops. Plant uptake by the targeted crop ranged between 1.6-20%, leaving 80-98.4% in the soil. The remaining active ingredient in the soil body is susceptible to precipitation run-off, entering adjacent water bodies (Morrissey et al. 2015). Neonicotinoids show substantial environmental persistence in soils depending on soil type and aeration (Goulson 2013). For example, clothianidin can persist in certain soil types for as long as 6,931 days (19 years) after application (Goulson 2013). A field study conducted in Saskatchewan, Canada, discovered negligible degradation after 25 months (De Cant and Barrett 2010); silty loam soils tend to extend the compound half-life (Goulson 2013). Prairie soils are also ideal for arable land, being high in nutrients and providing efficient crop substrate. Therefore, extended longevity and stability within soil is likely to occur in many agricultural regions. Once in water, neonicotinoid compounds are highly photosensitive, breaking down quickly in the water column with adequate light penetration. Field studies document first-order degradation to be the greatest with 64-75% loss within 24-48 hr post-application (Armbrust and Peeler 2002). Neonicotinoids are relatively stable to hydrolysis at a neutral or acidic pH (Pesticide Products Database). Additional variables such as the presence/absence of certain wetland plant species and vegetative buffer width also influence the magnitude and frequency of neonicotinoid detection in surface waters (Main et al. 2015).

Parent neonicotinoid compounds will break down at multiple molecular sites in the environment into various metabolites depending on the active ingredient. It is important to note, thiamethoxam is readily converted to clothianidin, also an applied active ingredient, by ring methylene hydroxylation, whereas clothianidin undergoes N-demethylation to form subsequent

metabolites (Tomizawa and Casida 2005). Unconjugated metabolites of compounds such as imidacloprid will retain insecticidal activity.

1.3.5 Toxicity of imidacloprid, clothianidin, and thiamethoxam to non-target aquatic insects

Due to the conserved nature of insect neurophysiology, both pest and non-target insects are impacted by neonicotinoid exposure (Sánchez-Bayo, 2012). Neonicotinoids were designed to be ingested by sucking, boring, and root feeding pest insects (Goulson 2013). Each compound varies in molecular structure but displays a similar mode of action. Neonicotinoids are agonists of the nicotinic acetylcholine receptors (nAChRs) in insect nerve synapses (Tomizawa and Casida, 2005). These receptors are found throughout the insect and concentrated along the ventral nerve cord which constitutes the insect central nervous system. Due to their hydrophobicity, neonicotinoids can enter nerve tissues and bind to the post-synaptic nAChRs (single or multiple sites) mimicking acetylcholine; this binding causes the nerve to fire continuously that eventually debilitates neural activity (Jeschke and Nauen, 2008). Some studies have observed potential recovery and partial dissociation at the binding site (Liu and Casida 1993). When the neonicotinoid compound reaches the nAChR, subsequent activation causes an increase in sodium ion conductance, followed by a depolarization of the post-synaptic membrane. These compounds consequentially produce constant neuronal activation, which leads to hyper-excitation of the insect nervous system, followed by convulsions, paralysis, and ultimately death (Tomizawa and Casida 2005).

The available toxicity data overwhelmingly indicates that neonicotinoids are most toxic to aquatic insect species, over crustaceans, with most tests focusing on imidacloprid. Mortality from acute neonicotinoid exposure is reported in over 49 species of aquatic insects with mayflies exhibiting the greatest sensitivity (reviewed by Morrissey et al. 2015). For example, the reported 24-hr LC50 for *Epeorus longimanus* (Ephemeroptera: Heptageniidae) and 96-h LC50 for *Caenis horaria* (Ephemeroptera: Caenidae) is 2.1 µg/L and 6.7 µg/L, respectively (Alexander et al. 2007, Roessink et al. 2013). Aquatic dipterans make up a large proportion of the neonicotinoid toxicity data and display a wide range in sensitivity. The non-biting midge, *Chironomus dilutus*, had reported 96-h LC50 values between 5.4 to 5.8 µg/L for formulated and technical grade imidacloprid, respectively (Stoughton et al. 2008); whereas, the phantom midge species *Chaoborus obscuripes* had a reported 96-h LC50 of 294.0 µg/L (Roessink et al. 2013). Chronic

toxicity data suggest time-dependent effects occur at lower concentrations than required to induce adverse lethal and sub-lethal effects (Tennekes and Sánchez-Bayo 2011). For example, the mayfly species *C. horaria* displayed greater sensitivity to chronic imidacloprid exposure with a 28-d LC50 of 0.3 µg/L. This pattern was also observed in *C. dilutus* with a 28-d LC50 of 0.9 µg/L (Stoughton et al. 2008). Some of the most recent literature is shifting focus to the effects of water quality parameters on toxicity, specifically temperature. Camp and Buchwalter (2016) found that after a series of 96-h exposures to imidacloprid at temperatures ranging between 15-24° C, the mayfly species *Isonychia bicolor* (Ephemeroptera: Isonychiidae) experienced greater uptake and increased toxicity (i.e., sub-lethal impairment and immobility) at higher temperatures.

Different neonicotinoids display a range of molecular selectivity profiles and a high affinity for insect nAChRs; this binding potential is what causes their insecticidal activity. A range of model test insects have been used to specifically determine the IC50 (inhibition concentration, 50% effect) which include *Musca domestica*, *Myzus persicae*, and *Drosophila sp.* These studies have focused on structure activity correlations and electrophysiological responses, while other tests use common toxicological endpoints to determine the relative toxicity of each compound.

Comparative studies focusing on the neonicotinoid compounds, imidacloprid, clothianidin, and thiamethoxam, can vary from species to species and endpoint to endpoint. Based on IC50 (nM) values, selectivity profiles for these three compounds (most selective to least selective) are clothianidin (2.2 nM), imidacloprid (4.6 nM), and thiamethoxam (5000 nM), suggesting that clothianidin and imidacloprid display neurological effects at similar doses, whereas thiamethoxam requires concentrations an order of magnitude greater. Vehovszky et al. (2015) documents the excitatory post-synaptic potential (EPSP) inhibition of the VD4-RPeD1 synaptic connection (acetylcholine-evoked membrane) in the central nervous system of the pond snail, *Lymnaea stagnalis*, across five neonicotinoid active ingredients. Imidacloprid and clothianidin were found to significantly inhibit the EPSP amplitudes at identical doses, whereas thiamethoxam exhibited negligible effects. Acetylcholine in *L. stagnalis* controls both excitatory and inhibitory neurotransmission; comparing responses between imidacloprid and clothianidin indicate similar mechanistic effects. Recent reviews of the neonicotinoid toxicity literature have identified similar patterns in the comparative toxicity of these three compounds to aquatic insects (Whiteside et al. 2008; Morrissey et al. 2015). Whiteside et al. (2008) conducted a rank-based

risk assessment focusing on the adverse effects of agrochemicals on aquatic communities, including algae, invertebrates, fish, and other aquatic organisms. Of the 206 compounds evaluated, imidacloprid ranked the highest of the three neonicotinoids, followed by clothianidin and thiamethoxam (Whiteside et al., 2008). Imidacloprid, clothianidin, and thiamethoxam also display similar acute toxicity geometric means (LC50 data from 24-h to 96-h tests), but the lack of clothianidin and thiamethoxam toxicity values in the primary literature do not allow for transparent interpretation across compounds (Morrissey et al. 2015). It is important to recognize this when assessing the risk of neonicotinoids to aquatic communities and developing research hypotheses.

1.3.6 Utility of mesocosms in ecotoxicology

Single-species laboratory experiments remain a mainstay in the regulation of contaminants and assessing the potential risks to non-target organisms (van den Brink 2005). These single-species toxicity data are extrapolated to predict no-effect concentrations for the environment, relying on links between observed laboratory effects and anticipated effects to natural ecosystems. Most regulatory frameworks use tiered approaches or risk screening on simplistic experimental systems (Solomon et al. 2008, Clements et al. 2012). Effects on survival, growth, and/or reproduction determined by lower-tier (single-species) or higher-tier (multi-species) laboratory toxicity tests merit further evaluation of environmental risk in more environmentally relevant settings. An intermediate test system between controlled laboratory and natural field exposures is known as a “-cosm” (e.g., nano-, micro-, meso-). The origin of mesocosm experiments extends back to Odum (1984), who defined mesocosms as “bounded and partially enclosed outdoor experimental setups... falling between laboratory and microcosms and the large, complex, real world macrocosms.” Mesocosms are extremely valuable in risk assessment when lower-tier laboratory studies show possible risks.

These experimental enclosures often test specific hypotheses informed by previous stages of the risk assessment and methods for execution may include limnocorrals, littoral enclosures, artificial ponds, etc. Mesocosm research has focused on several aspects of contaminants and their impacts on the environment. This may include: 1) determining the fate and distribution (Farmer et al. 1995, Sanderson et al. 2007); 2) assessing the direct effects on specific populations or communities (Alexander et al. 2007, Columbo et al. 2013); 3) confirming predictive models of fate and effects (Iwasaki et al. 2013); 4) estimating indirect effects to higher trophic level

organisms (Sánchez-Bayo and Goka 2006, Kasai et al. 2016); and 5) evaluating the effects on ecosystem function (Pestana et al. 2009a).

Often mesocosms are differentiated by other ‘cosm-type tests based on size, number of replicates, and the endpoints measured (OECD 2006). For example, mesocosms will range in size from 1 to 10^4 m³ and in time from days to several months, whereas microcosms tend to be smaller in size (10^{-3} to 10 m³), contain fewer species and test shorter intervals (hours to weeks). Due to their larger scale, mesocosm tests may feature a minimum of two replicates per treatment and three or more test concentrations, while it is recommended that microcosm tests have three or more replicates per treatment and five or more test concentrations (OECD 2006). This downsizing or compartmentalizing of entire ecosystems allows for replication and concentration-response, where full field studies would not be logistically possible (Culp et al. 2000).

Mesocosms give community ecotoxicology researchers a unique ability to determine the effects of a contaminant over a range of concentrations and a diverse range of taxa, while collecting data on interspecific and intraspecific interactions (Caquet et al. 2000, Rohr and Crumrine 2005, Relyea and Hoverman 2006). The inclusion of biological complexity (i.e., community interactions) achieved in mesocosm-based experiments can influence the sensitivity of responses from exposure to a contaminant. Typically measured by conventional biotic indices (e.g., Shannon, Simpson, Bray-Curtis), structural endpoints are often more sensitive in complex systems than functional endpoints such as community metabolism, respiration, or decomposition (i.e., redundancy; Pratt and Cairns 1996). It is critical to clearly and adequately design mesocosm experiments to maximize data collection based on the endpoints of interest.

Physical mesocosm designs can augment the mass of biotic components and the method of colonizing the experimental units can be either natural (e.g., allowing adult aquatic insects to oviposit) or inoculated (e.g., transplanting immature aquatic insects from a field collection site). Other designs to bolster the community (especially invertebrates) include *in situ* experimental units; *in situ* mesocosms (e.g., limnocorrals) are self-contained, experimental systems which act as a subdivision (enclosures) of a natural ecosystem colonized with indigenous organisms (Liber et al. 1998). *In situ* experimental systems typically target the endogenous biotic community and are nearing complete ecosystem manipulation along the continuum of field-based assessments.

One key reason to conduct a mesocosm study is to evaluate the ecological relevance of effects observed under laboratory conditions. This strength also challenges mesocosm

experiments and can limit their interpretation. The chief tradeoff is the increased variability of the measured biotic responses and the ability to detect effects with any level of confidence (Caquet et al. 2000). Due to natural environmental variation, impacts of different contaminants on biotic communities are often difficult to detect and can vary from region to region (Maltby et al. 2000). Variables such as taxonomic group, life history strategy, physiology, body size, historic exposure, and geography compound the uncertainty of values extrapolated from a collection of single-species laboratory tests (Selck et al. 2002). RIVM's *A guidance document of the Dutch Platform for the Assessment of Higher Tier Studies* attempts to standardize the data generated from mesocosm-based experiments, setting a systematic set of criteria to cope with the levels of biological organization and described variance (De Jong et al. 2008). Moreover, the Society of Environmental Toxicology and Chemistry (SETAC) published a similar document titled *Community-level Aquatic System Studies—Interpretation Criteria* that outlines recommendations on experimental design, ecological interpretation, and placement into risk assessment from a group of mesocosm experts.

1.3.7 Synthesis

Potential adverse effects of insecticides on ecological subsidies (e.g., aquatic insect emergence) is a core ecotoxicology research question, containing the principles of ecology and toxicology (Walters et al. 2008, Paetzold et al. 2011, Kraus et al. 2014). Understanding the impact of neonicotinoids at different levels of biological organization is critical to improve protections to aquatic life and the organisms that depend on productive, timely secondary production (Van der Sluijs et al. 2015). The neonicotinoid toxicity literature shows that aquatic insects are among the most sensitive invertebrate species compared to most freshwater crustaceans (Morrissey et al. 2015). Given the known spatial prevalence and environmental persistence of neonicotinoids, more toxicological data specifically addressing chronic toxicity are needed to fully understand risk to aquatic insects. There is a lack of chronic toxicity data for most neonicotinoid compounds (e.g., clothianidin and thiamethoxam) such that toxicity is difficult to compare to the more well-studied neonicotinoid active ingredient, imidacloprid. I hypothesize that *C. dilutus*, a model benthic macroinvertebrate, will be more sensitive to chronic exposure to neonicotinoid insecticides exposure than to acute exposure, and, under similar laboratory conditions, clothianidin and thiamethoxam will be more toxic than imidacloprid. Furthermore, there are limited data available on the effects of neonicotinoids on natural aquatic

insect communities. Emergence inhibition is a reliable, ecologically important endpoint used to assess chronic toxicity (Stoughton et al. 2008). I hypothesize that *in situ* wetland limnocorrals treated with imidacloprid, clothianidin, and thiamethoxam will display similar effects on emergence patterns consistent with those seen in the laboratory study; moreover, to expand upon the potential effects observed in *in situ* wetland limnocorrals, I hypothesized that chronic clothianidin exposure will elicit a dose-response on the emerging aquatic insect community composition and abundance. In regions with higher broad-spectrum use of neonicotinoids, community-level risk assessments with low-level, environmentally relevant exposure profiles are especially needed. The Canadian Prairie landscape is a prime example of an area subjected to intense agricultural practices and heavy neonicotinoid use (Main et al. 2014). Moreover, Prairie wetlands situated in the PPR are often in areas under agricultural development and at risk to neonicotinoid contamination (Main et al. 2014, Smalling et al. 2015, Evelsizer and Skopec 2016). I hypothesized that wetlands on the Canadian Prairies receiving runoff from neonicotinoid seed-treated canola fields may experience reduced abundance or altered communities of emerging adult aquatic insects and that neonicotinoid concentrations will have a greater influence than water quality parameters and wetland habitat characteristics. Here, I explored and addressed some of these key knowledge gaps.

1.4 Thesis structure

This thesis contains four manuscripts following this chapter, in addition to a concluding chapter. In the first manuscript (Chapter 2), I report the results of a laboratory investigation comparing the relative toxicity of three common neonicotinoid compounds to a model insect species, *Chironomus dilutus*. Imidacloprid, thiamethoxam, and clothianidin have frequently been detected in surface water bodies around the globe and represent some of the most water-soluble insecticides ever used on a large scale. Toxicity tests followed field-relevant exposure durations and identical laboratory test conditions, where similar toxicity endpoints were evaluated on day 14 and day 40. From these data, I report the first attempt to derive toxic equivalency factors (TEFs), relative to imidacloprid, for both clothianidin and thiamethoxam.

In Chapter 3, I used *in situ* limnocorrals to compare the relative chronic toxicity of three neonicotinoid compounds to an endogenous aquatic insect community in a local wetland, assessing community structure and phenology. These data offer insights into the effects of neonicotinoids on emerging aquatic insects under semi-controlled conditions.

Using the same methodology in Chapter 4, I expanded the concentration range previously used in Chapter 3, and I focused on a single active ingredient, clothianidin. Here, a 7-week study investigated the effects of clothianidin on emergent aquatic insect abundance and community composition with an emphasis on interpreting the advantages and disadvantages of mesocosm studies.

In Chapter 5, I aimed to elucidate the relative effects of water quality parameters, habitat characteristics, and mean neonicotinoid concentrations on aquatic insect community composition and abundance in natural wetlands. Following a biennial canola-cereal crop rotation schedule (2013 and 2015), I sampled 22 semi-permanent wetlands for 10 weeks over two growing seasons (11 wetlands per year) in central Saskatchewan, Canada for aquatic insect production, water quality parameters, and neonicotinoid concentrations.

The concluding Chapter 6 summarizes the key lessons learned from the study, outlines the greater implications to sustaining biodiversity in agroecosystems, and future directions.

1.5 Authorship

All data chapters are written in manuscript style and are either published, in review, or in preparation to be submitted to targeted journals. I am the primary author of each chapter. I also collected and analyzed the data presented in each main chapter, then took the lead on writing and completing the final manuscripts. My co-supervisors, Drs. Christy Morrissey and Karsten Liber, secured research funding, offered advice and guidance on experimental design and execution, and edited each chapter; they are listed as co-authors for chapters 2, 3, 4, and 5. Other co-authors included Drs. Anson Main, Iain Phillips, and John Headley, and Mr. Kerry Peru, contributing statistical guidance, taxonomic expertise, or chemical analyses. A number of other colleagues offered insight and aided in experimental design and methodology. These individuals are acknowledged at the end of each chapter where appropriate.

PREFACE TO CHAPTER 2

The potential threat of neonicotinoid insecticides to aquatic invertebrates, specifically immature aquatic insects, is a major issue. Few chronic toxicity studies provide data on the neonicotinoids clothianidin and thiamethoxam. Moreover, there is limited data comparing these compounds to the well-studied neonicotinoid, imidacloprid. The paucity of chronic single-species toxicity data impairs our ability to best protect sensitive insect taxa. The objective of this chapter was to: 1) compare the chronic toxicity of technical grade imidacloprid, clothianidin, and thiamethoxam to the model benthic macroinvertebrate species, *C. dilutus* under field-relevant exposure durations and identical laboratory test conditions for three different neonicotinoids; and 2) calculate toxic equivalency factors for these three common neonicotinoids to advance the cumulative risk assessment of neonicotinoid insecticides and inform protective aquatic life benchmarks for clothianidin and thiamethoxam.

Chapter 2 is published in the journal *Environmental Toxicology and Chemistry**. See citation: Cavallaro, M.C., Morrissey, C.A., Headley, J.V., Peru, K.M. and Liber, K., 2017. Comparative chronic toxicity of imidacloprid, clothianidin, and thiamethoxam to *Chironomus dilutus* and estimation of toxic equivalency factors. *Environmental Toxicology and Chemistry*, 36(2):372-382.

*Minimal changes have been made to the original published manuscript text including clarification of the methods and reference formatting.

CHAPTER 2: COMPARATIVE CHRONIC TOXICITY OF IMIDACLOPRID, CLOTHIANIDIN, AND THIAMETHOXAM TO *CHIRONOMUS DILUTUS* AND ESTIMATION OF TOXIC EQUIVALENCY FACTORS

2.1 Abstract

Non-target aquatic insects are susceptible to chronic neonicotinoid insecticide exposure during the early stages of development from repeated run-off events and prolonged persistence of these chemicals. Investigations on the chronic toxicity of neonicotinoids to aquatic invertebrates have been limited to a few species, under different laboratory conditions that often preclude direct comparisons of the relative toxicity of different compounds. Here, full life-cycle toxicity tests using *Chironomus dilutus* were performed to compare the toxicity of three commonly used neonicotinoids: imidacloprid, clothianidin, and thiamethoxam. Test conditions followed a static-renewal exposure protocol where lethal and sub-lethal endpoints were assessed on days 14 and 40. Reduced emergence success, advanced emergence timing, and male-biased sex ratios were sensitive responses to low-level neonicotinoid exposure. The 14-day LC50 values for imidacloprid, clothianidin, and thiamethoxam were 1.52 µg/L, 2.41 µg/L, and 23.60 µg/L, respectively. The 40-day EC50 (emergence) values for imidacloprid, clothianidin, and thiamethoxam were 0.39 µg/L, 0.28 µg/L, and 4.13 µg/L, respectively. Toxic equivalence, relative to imidacloprid, was estimated through a three-point response average at L(E)C(20, 50, 90) and plotted concentration-response curves. Relative to imidacloprid (TEF=1.0), chronic (lethality) 14-day TEFs for clothianidin and thiamethoxam were 1.05 and 0.14, respectively, and chronic (emergence inhibition) 40-day TEFs were 1.62 and 0.11, respectively. These population-relevant endpoints and TEFs suggest that imidacloprid and clothianidin exert comparable chronic toxicity to *C. dilutus*, whereas thiamethoxam induced comparable effects only at concentrations an order of magnitude higher. However, I caution that under field conditions thiamethoxam readily degrades to clothianidin, thereby likely enhancing toxicity.

2.2 Introduction

Chemical input into aquatic environments from agricultural run-off remains one of the most challenging global threats to the quality of freshwater resources (Stehle and Schulz 2015), and extensive contamination of both lotic and lentic systems is well-documented (Main et al. 2014, Ippolito et al. 2015). Aquatic arthropods inhabiting watersheds dominated by conventional agriculture operations can be at risk from both lethal and sub-lethal exposure to insecticides. In

particular, systemic insecticides, which typically feature a low octanol-water partition coefficient (Cloyd and Bethke 2011), are particularly susceptible to leaching and run-off into aquatic environments. Growing concern over the loss of biodiversity from the intensification of agricultural operations necessitates further assessment of the threats systemic insecticides pose to aquatic invertebrate populations and associated ecosystem structure and function (Tilman et al. 2002, Chagnon et al. 2015).

Neuroactive, systemic insecticides are currently the most abundant form of arthropod pest-control globally (Casida and Durkin 2013). Among the principal classes of insecticides used in crop protection are the neonicotinoids. Neonicotinoids are broadly applied on a suite of crop types worldwide and over a variety of landscapes where environmental conditions, active ingredient, and application rates can differ substantially. One of the most recent controversies regarding neonicotinoids concerns the broad use of seed treatment application (van der Sluijs et al. 2015). This is a prophylactic application method that protects seedlings and mature plants from phytophagous invertebrate pests by translocating and incorporating the insecticide throughout the plant during its development (Elbert et al. 2008). Jeschke and Nauen (2008) report that nearly 80% of all seed treatments (e.g., canola, corn, lentils, cereals) are coated with a neonicotinoid-based insecticide. In North America, imidacloprid, clothianidin, and thiamethoxam have frequently been detected in surface water bodies; not surprisingly since they represent some of the most water-soluble insecticides ever used on a large scale (Main et al. 2014, Schaafsma et al. 2015, Smalling et al. 2015). Once in water, neonicotinoid compounds break down at multiple molecular sites into various metabolites; a characteristic important in this study as thiamethoxam can be transformed into clothianidin by ring methylene hydroxylation. Furthermore, clothianidin undergoes *N*-demethylation to form subsequent metabolites which retain insecticidal properties (Tomizawa and Casida 2005).

Neonicotinoids are agonists of the nicotinic acetylcholine receptors (nAChRs) in insect nerve synapses (Tomizawa and Casida 2005). They disrupt neural activity in invertebrates by binding to the post-synaptic nAChRs, functionally interfering with normal neural activity (Žabar et al. 2012). When the neonicotinoid compound reaches the nAChR, subsequent activation causes an increase in sodium ion conductance, followed by a depolarization of the post-synaptic membrane. Unlike acetylcholine, the activity of neonicotinoids is not limited by acetylcholinesterase; neonicotinoids consequentially produce prolonged neuronal activation,

which leads to hyper-excitation of the insect nervous system, followed by convulsions, paralysis, and ultimately death. The binding of neonicotinoids to the nAChRs is believed to be largely irreversible and to some extent cumulative over time (Tennekes and Sánchez-Bayo 2011). Even low doses over time can promote adverse effects in invertebrates, such as inhibited growth and development (Stoughton et al. 2008), altered behavior (Azevedo-Pereira et al. 2011), limited mobility (Roessink et al. 2013), decreased adult emergence (Alexander et al. 2008, Mohr et al. 2012), and reduced feeding (Alexander et al. 2007, Agatz et al. 2014). Moreover, due to the conserved nature of insect neurophysiology, both pest and non-target species are affected by neonicotinoids (Sánchez-Bayo 2012), albeit to varying degrees among species.

To date, the majority of the aquatic invertebrate toxicity studies conducted with neonicotinoids have focused on imidacloprid using single-species under acute exposure scenarios. Of the 214 single-species aquatic invertebrate laboratory studies on neonicotinoids reviewed by Morrissey et al. (2015), 178 (83%) were acute whereas only 36 (17%) were chronic. Given their environmental persistence and high water solubility, chronic studies on sensitive aquatic taxa are still needed. Several studies have shown a direct relationship between more persistent neonicotinoid exposure and increased mortality or other sub-lethal effects (Tennekes and Sánchez-Bayo 2011). Furthermore, some studies have found that repeated, short-term exposure to neonicotinoids may have a delayed lethal and sub-lethal effect on freshwater invertebrates (Mohr et al. 2012, Beketov and Liess 2008a).

Chironomidae (non-biting midges) are ideal model organisms for freshwater toxicity tests, especially for insecticides. However, acute and chronic neonicotinoid toxicity tests conducted with *Chironomus dilutus* (previously *C. tentans*; taxonomic description found in Shobanov et al. (1999)) only exist for imidacloprid and clothianidin in two separate studies. Toxicity tests with clothianidin and thiamethoxam have been conducted using different study species, durations, and inter-laboratory methodologies, which creates comparing the toxic potency of individual compounds difficult (Rico and van den Brink 2015). Other model *Chironomus spp.* used in neonicotinoid research include *C. riparius* and *C. tepperi*. Previous toxicity studies comparing *C. dilutus* and *C. riparius* have shown that *C. dilutus* is typically more sensitive to a number of toxicants, including the legacy insecticide lindane (Watts and Pascoe 2000).

World-wide, regulatory aquatic life benchmarks have only been established for imidacloprid, which is only one of seven common neonicotinoid active ingredients. Toxicity of the different neonicotinoids has been assumed equivalent for the different compounds (Morrissey et al. 2015), however this assumption has not been formally tested. The maximum allowed levels of imidacloprid in freshwater ecosystems for the protection of aquatic life range from 0.0083 µg/L in the Netherlands (RIVM 2014) to 1.05 µg/L in the United States (U.S. EPA 2015). The Canadian Council of Ministers of the Environment (CCME) lists the interim Canadian Water Quality Guidelines for the Protection of Aquatic Life for imidacloprid as 0.23 µg/L (CCME 2007). Morrissey et al. (2015) documented that 66% of all the neonicotinoid laboratory toxicity tests reviewed tested imidacloprid, while clothianidin and thiamethoxam accounted for only 3.7% and 4.2% of published studies, respectively. Given the prevalence of clothianidin and thiamethoxam in aquatic environments, it remains unclear whether these benchmarks for imidacloprid are appropriate for all neonicotinoid compounds (Morrissey et al. 2015).

While the evaluation of lethality during an aquatic insect larval stage is important for evaluating toxicity, these organisms rarely experience the level of insecticide exposure in the field necessary to invoke a lethal response restrictive to a portion of their life span. However, exposure to sub-lethal contamination throughout their immature life stages is more common and of greater environmental relevance. This research aimed to compare the chronic toxicity of technical grade imidacloprid, clothianidin, and thiamethoxam to the model benthic macroinvertebrate species, *C. dilutus*. The chronic toxicity was evaluated under field-relevant exposure durations and identical laboratory test conditions for three different neonicotinoids. Data generated from this study allowed for the calculation of toxic equivalency factors for these three common neonicotinoids thus helping improve the cumulative risk assessment of neonicotinoid insecticides and inform protective aquatic life benchmarks for clothianidin and thiamethoxam. I hypothesize that *C. dilutus* will be more sensitive to chronic exposure to neonicotinoid insecticides than acute exposure. Furthermore, clothianidin and thiamethoxam will be more toxic than imidacloprid.

2.3 Materials and Methods

2.3.1 Experimental animals

A population of *C. dilutus* was cultured in environmental chambers at the Toxicology Centre, University of Saskatchewan, Saskatoon, SK at $23.0 \pm 1.0^\circ$ C with a 16:8 (L:D)

photoperiod following the modified protocol outlined by Environment Canada (1997) and Benoit et al. (1997). Briefly, adult *C. dilutus* were collected with an aspirator into a 500-mL Erlenmeyer flask. Adults were transferred to 1-L glass mason jars each containing a small (5x5 cm) piece of Parafilm[®], two plastic screens (5x12 cm) for mating surfaces, and approximately 150-mL of water; adults were given three days to produce egg masses or discarded. To avoid disturbance, breeding jars were isolated in cardboard boxes and checked for egg masses daily. New egg masses ≤ 24 h old were transferred to new 18.9-L tanks with culture water and 2.5 cm of washed silica sand. The culture water used in all experiments was carbon-filtered, biofiltered City of Saskatoon municipal water. Water quality parameters (mean \pm SD) were as follows: pH 8.2 ± 0.3 , conductivity 475 ± 63 μ S/cm, total hardness 137 ± 7 as mg/L CaCO₃, and alkalinity 85 ± 9 as mg/L CaCO₃. Rearing tanks were fed with 5-mL of macerated fish food (Tetramin[®]) every other day. After 7 days, larvae were removed from the rearing tank and placed into test beakers with their cases to reduce transfer stress.

2.3.2 Chronic tests

All toxicity tests were performed at the Toxicology Centre, University of Saskatchewan, under conditions similar to those used for culturing the test animals. Technical grade imidacloprid (98.8% pure; 1-[(6-Chloro-3-pyridinyl)methyl]-*N*-nitro-2-imidazolidinimine) and clothianidin (99.6% pure; [*C(E)*]-*N*-[(2-Chloro-5-thiazolyl)methyl]-*N*'-methyl-*N*''-nitroguanidine) were obtained from Bayer CropScience (Mississauga, ON, Canada); technical grade thiamethoxam (98.9% pure; 3-(2-chloro-thiazol-5-ylmethyl)-5-methyl-[1,3,5]oxadiazinan-4-ylidene-*N*-nitro-amine) was acquired from Syngenta Crop Protection, LLC (Guelph, ON, Canada). Stock solutions were prepared in 1-L volumetric flasks with reverse osmosis water (Barnstead[®] Diamond[™] NANOpure, 18.2 megohm/cm; Barnstead International, Dubuque, IA, USA) and then diluted to the desired test concentrations using culture water.

Chronic, static-renewal toxicity tests were 40 days in duration and used eight replicate beakers, each containing 10 second-instar *C. dilutus* larvae (6-7 days old), 50-mL of washed silica sand, and 200-mL of treatment water. Beakers were continuously aerated gently to maintain adequate oxygen saturation ($\geq 80\%$) and roughly 150-mL of water from each beaker were changed every third day. Larvae were fed daily by adding 60- μ L of macerated fish food (50 g d.w. Tetramin[®]/500-mL Barnstead[®] water) into each beaker. To prevent photo-degradation of test compounds, borosilicate glass was placed on top of the beakers. Larvae were exposed to

nominal concentrations of 0 µg/L (control), 0.1 µg/L, 0.3 µg/L, 1.0 µg/L, 3.3 µg/L, and 10.0 µg/L of each insecticide. On day 14, four replicates from each treatment were removed, organisms counted to assess survival, and surviving larvae weighed. The remaining four replicates were maintained for an additional 26 days to allow larvae to emerge as adults. Emerging adults were collected daily, their sex determined, and weighed. Larvae and adults were dried at 60° C to a constant weight. A cumulative total of emerged males and females for each beaker ensured that all individuals were accounted for and served to determine emergence synchrony across treatments. Emergence synchrony was defined as an emergence event representing the greatest proportion of the cumulative total of adults emerging within a two-day span. Adult chironomids were considered to have successfully emerged when the adult completely dissociated from its pupal exuvia and exited the water (Benoit et al. 1997).

2.3.3 Water quality

A water sample (10 mL per beaker) was removed from four beakers in each treatment and pooled for water quality analysis before and after each partial water change. Water changes were conducted every third day to maintain static test concentrations and prevent significant ammonia buildup related to feeding. Temperature and dissolved oxygen (DO) were measured with an ORION[®] dissolved oxygen meter (model 835; ORION Research, Beverly, MA, USA). Water hardness and alkalinity were calculated with a Hach Digital Titrator (model 16900; Hach Company, Loveland, CO, USA), pH was measured with an ORION[®] PerpHect LogR meter (model 370; ORION Research, Beverly, MA, USA), and ammonia levels were assessed with a YSI Photometer (model 9300; YSI, Inc, Yellow Springs, OH, USA).

2.3.4 Neonicotinoid analysis

Water (60 mL) was sampled from four randomly selected replicate beakers per treatment, pooled into a 250-mL amber bottle, and stored at 4° C until analyzed. Both old and new water samples (every third day) were collected, and a subset of samples were analyzed to determine insecticide exposure and determine whether any degradation had occurred. Water samples were analyzed at the National Hydrology Research Centre, Environment Canada, Saskatoon, SK using analytical methods previously described by Main et al. (2014). Analytical standards of imidacloprid, thiamethoxam, and clothianidin were acquired from Chem Service (West Chester, PA, USA) and the internal standard from CDN Isotopes (Pointe-Claire, QC, Canada). Water samples were solid phase extracted using Oasis HLB cartridges (Waters, Mississauga, ON,

Canada). Neonicotinoid analytes within the sorbent bed were reconstituted in de-ionized water with the addition of the internal standard. Neonicotinoid residues were quantified using a Waters® Model 2695, high performance liquid chromatograph interfaced with a Micromass Quattro Premier mass-spectrometer with a stainless steel column (100 x 2.2 mm; Waters® MS Xterra C-8) (Waters Corp., Milford, MA, USA). Water samples were injected into the LC/MS/MS system; the average flow through run time was 10 min, with an injection volume of 20 µL (16 µL of 99.9% water and 0.1% formic acid, and 4 µL of 90% acetonitrile, 9.9% water, and 0.1% formic acid). Limits of quantification (LOQ) in water samples were as follows: imidacloprid, 0.0038 ± 0.002 µg/L; clothianidin, 0.004 ± 0.001 µg/L; and thiamethoxam, 0.011 ± 0.001 µg/L. Mean recoveries from MilliQ and river water spiked at 500, 100, and 0.005 µg/L were as follows: imidacloprid, $91.3 \pm 6.7\%$; clothianidin, $78.97 \pm 4.0\%$; and thiamethoxam, $86.3 \pm 4.2\%$; (mean \pm SD). Controls and laboratory blanks were all below the limits of detection, and all water concentration data were recovery corrected to allow for comparison among runs. Data and calculated endpoint values are reported on measured concentrations.

2.3.5 Data analysis

Survival data were used to calculate 14-day (mortality) LC50 values and 40-day (emergence) EC50 values (median lethal effective concentrations) using the trimmed Spearman-Kärber method (Benoit et al. 1997, Hamilton et al. 1977). Dry weights of larvae (day 14) and successfully emerged adults (day 40) were used to estimate EC20, EC50, and EC90 values (observed 20%, 50%, and 90% effect) using the U.S. EPA ICp program (U.S. EPA 1990, U.S. EPA 1993).

All other statistical analyses were performed using SigmaPlot™ Version 13.0 (Systat Software, Inc., San Jose, CA, USA) with a 95% ($\alpha = 0.05$) level of confidence. Significant differences among treatments within an individual test (compound) for day 14 and day 40 survival and biomass endpoints were assessed using one-way analysis of variance (F statistic) followed by a Tukey post-hoc test for all multiple pairwise comparisons. To determine significant differences in adult emergence relative to the controls, a one-way analysis of variance (ANOVA) followed by a Dunnett's post-hoc test was performed on the mean cumulative proportion emerged on the day where 50% of the controls had successfully emerged. If data did not fit a normal distribution, a non-parametric Kruskal-Wallis test (H statistic) was used to determine significance. Degrees of freedom (df) varied between tests and among compounds due

to mortality in some treatments. For the purposes of comparing similar endpoints that include dose-response relationships, independent of dose choice, an EC20 was calculated as an appropriate derivation in favor over the more controversial NOEC and LOEC estimates (Laskowski 1995, Landis and Chapman 2011).

Toxic equivalency factors (TEFs) were estimated through a three-point response average at L(E)C(20, 50, 90) for both day 14 (lethality) and day 40 (emergence inhibition) endpoints. The relative potency of both clothianidin and thiamethoxam were compared to imidacloprid (TEF=1). Acute and chronic endpoints were graphed on a Probit scale to visually verify assumptions of parallelism of slopes for the three compounds. This was followed by a one-way analysis of covariance (ANCOVA) to statistically validate the assumption of equal slopes for both the day 14 and day 40 endpoints.

2.4 Results

2.4.1 Water chemistry

Active ingredient concentrations in old and new water confirmed that neonicotinoid exposures remained constant throughout the experimental period of each test. Mean measured concentrations for imidacloprid, clothianidin, and thiamethoxam were 83.1%, 51.4%, and 59.7% of the target nominal doses (Table 2.1). All calculated toxicity endpoints were based on analyzed neonicotinoid water concentrations. Additionally, at no time during the thiamethoxam tests was clothianidin detected in any water sample, indicating that no degradation had occurred, an observation corroborated by Nauen et al. (2003). Therefore, each test evaluated the toxic effects of a single active ingredient.

Routine water quality parameters were measured during each experiment and all mean values represent an average across treatment means. There was no difference in the water quality means among treatments. Mean values (\pm SE) for the three chronic tests were as follows: DO 7.2 ± 0.09 mg/L, temperature $23.0 \pm 0.1^\circ$ C, pH 8.2 ± 0.02 , conductivity 459 ± 14 μ S/cm, total hardness 136 ± 1.2 as mg/L CaCO₃, and alkalinity 86 ± 3.5 as mg/L CaCO₃. Dissolved oxygen remained >7.0 mg/L throughout each test. Ammonia concentration, food consumption, and waste production increased over time as larval growth increased.

Table 2.1 Calculated (mean ± SE) neonicotinoid exposure concentrations (µg/L) measured in water over 3-day intervals during separate full *Chironomus dilutus* larval static-renewal lifecycle tests with imidacloprid, clothianidin and thiamethoxam.

Test period	Control	Nominal concentrations (µg/L)						
		0.1	0.3	1	3.3	10	20	40
<u>Imidacloprid concentrations</u>								
Days 1-14 ^a	<LOQ ^c	0.108 ± 0.010	0.25 ± 0.02	0.78 ± 0.08	2.62 ± 0.45	7.82 ± 0.93	NA	NA
Days 1-40 ^b	<LOQ	0.100 ± 0.010	0.26 ± 0.01	0.79 ± 0.06	2.82 ± 0.30	8.24 ± 0.80	NA	NA
<u>Clothianidin concentrations</u>								
Days 1-14	<LOQ	0.043 ± 0.010	0.21 ± 0.06	0.42 ± 0.05	1.86 ± 0.12	4.67 ± 0.40	NA	NA
Days 1-40	<LOQ	0.046 ± 0.010	0.20 ± 0.04	0.48 ± 0.06	1.87 ± 0.10	4.97 ± 0.46	NA	NA
<u>Thiamethoxam concentrations</u>								
Days 1-14	<LOQ	0.061 ± 0.004	0.27 ± 0.01	0.74 ± 0.03	2.23 ± 0.12	5.90 ± 0.45	11.99 ± 2.00 ^d	33.76 ± 4.60 ^d
Days 1-40	<LOQ	0.066 ± 0.004	0.24 ± 0.01	0.68 ± 0.04	2.11 ± 0.12	5.69 ± 0.36	NA	NA

^aMean neonicotinoid concentration calculated for the initial 14 days of exposure

^bMean neonicotinoid concentration calculated for the entire 40-day study

^cLOQ = limit of quantification

^dThiamethoxam treatments >10.0 µg/L were required to calculate L(E)C50 values

Table 2.2 Calculated lethal (LC) and sub-lethal (EC) toxicity values (µg/L (95% CI)) for *Chironomus dilutus* larvae exposed to technical grade imidacloprid, clothianidin, or thiamethoxam over periods of 14 and 40 days.

LC _x	Day 14 (larvae survival)			Day 40 (emergence)			
	Imidacloprid	Clothianidin	Thiamethoxam	EC _{xx}	Imidacloprid	Clothianidin	Thiamethoxam
20 ^a	0.47 (0.29-0.98)	0.34 (0.12-1.27)	1.94 (1.58-7.42)	20	0.06 (0.05-0.17)	0.02 (0.019-0.036)	0.48 (0.05-2.76)
50 ^b	1.52 (0.99-1.82)	2.41 (1.73-2.83)	23.60 (20.36-26.89)	50	0.39 (0.31-0.42)	0.28 (0.20-0.33)	4.13 (3.53-4.76)
90 ^c	4.83 (2.48-7.03)	4.21 (4.76-5.07)	>33.76 (± 4.6) ^d	90	0.71 (0.81-0.83)	1.48 (1.32-1.74)	>5.69 (± 0.36) ^d

^aConcentrations estimated to produce a 20% effect ± confidence intervals (α = 0.05) using the ICp method U.S. EPA

^bMedian lethal (or effect) concentration calculated with the trimmed Spearman-Kärber method Hamilton et al. (1977)

^cConcentrations estimated to produce a 90% effect ± confidence intervals (α = 0.05) using the ICp method U.S. EPA

^dCalculation extrapolated due to <90% effect observed at highest treatment; used extrapolated EC90 for calculation of TEF

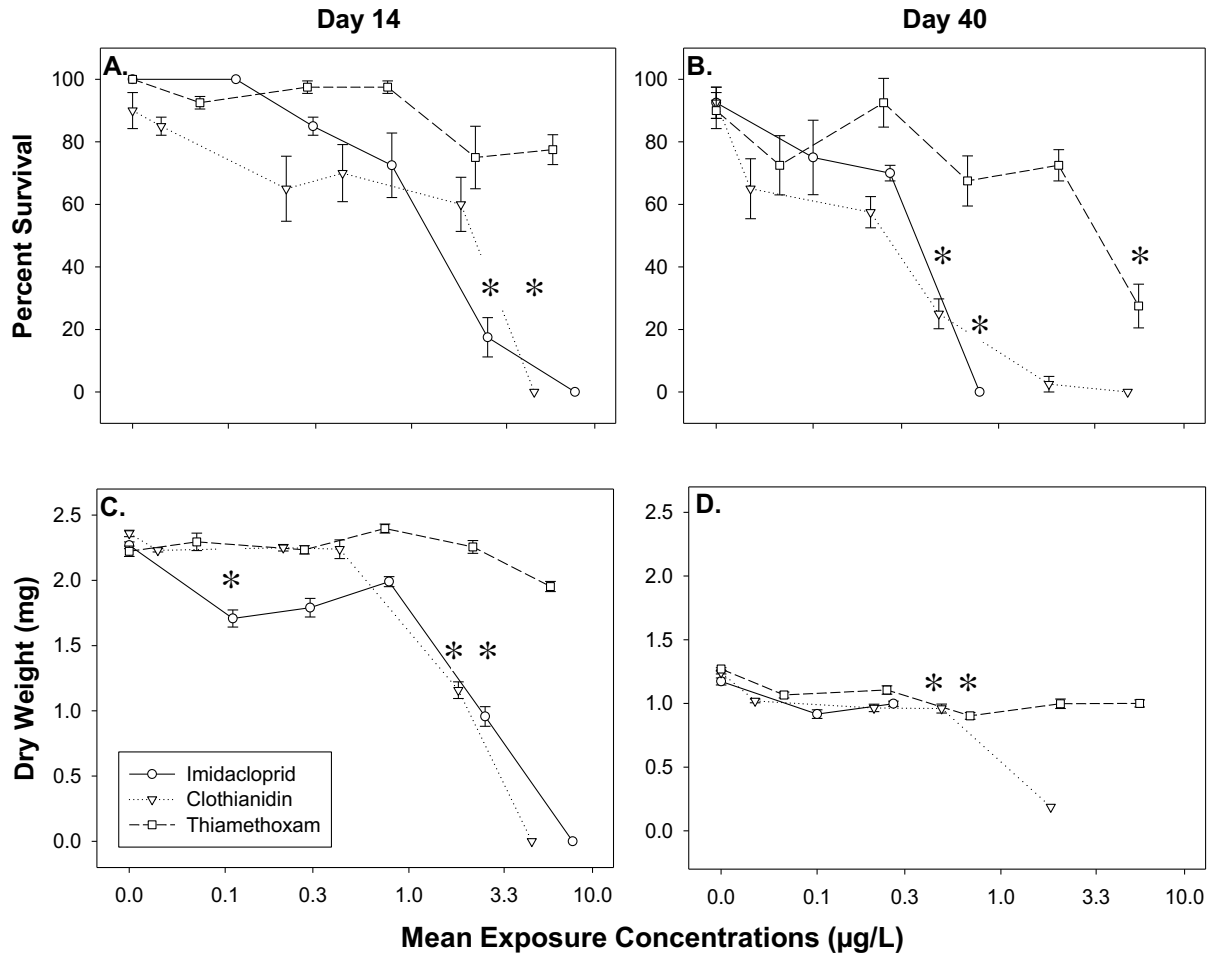


Figure 2.1 Percent survival or emergence (mean \pm SD) and dry weight (mean \pm SD) of *Chironomus dilutus* larvae on day 14 (A, C) and adults on day 40 (B, D) plotted against nominal treatments (See Table 1 for mean exposure concentrations for each compound tested).

* Significantly different from the control as determined by a Dunnett's post-hoc test ($p \leq 0.05$).

On average, I observed lower ammonia concentrations in new water samples (0.42 ± 0.07 mg/L) when compared to old water samples (1.4 ± 0.2 mg/L), but mean and peak concentrations were well below the reported ammonia LC50 value of 121.9 mg/L for *C. dilutus* (Whiteman et al. 1996).

2.4.2 Larval chronic toxicity endpoints

After 14 days of exposure, imidacloprid was the most toxic and thiamethoxam the least toxic of the three compounds to *C. dilutus* larvae (Table 2.2). Fourteen-day LC50 values for imidacloprid, clothianidin, and thiamethoxam were 1.52, 2.41, and 23.60 $\mu\text{g/L}$, respectively. A significant decrease in survival relative to the controls was observed at mean concentrations >2.62 $\mu\text{g/L}$ ($H=17.799$, $df=4$, $p=0.001$) for imidacloprid. At the same nominal dose group, a

nearly significant decrease in survival was observed in clothianidin test ($F=3.04$, $df=4$, $n=20$, $p=0.051$). Larval biomass was reduced by 50% at mean concentrations of 2.23, 1.83, and 21.39 $\mu\text{g/L}$ for imidacloprid, clothianidin, and thiamethoxam, respectively (Table 2.3). Larval dry weight was consistent in the thiamethoxam and clothianidin treatments at nominal test concentrations of 0, 0.1, 0.3, and 1.0 $\mu\text{g/L}$. Both imidacloprid and clothianidin caused a statistically significant reduction in average larval dry weight at the mean exposure concentrations of 2.62 $\mu\text{g/L}$ ($H=17.00$, $df=4$, $n=20$, $p=0.002$) and 1.86 $\mu\text{g/L}$ ($F=117.7$, $df=4$, $n=20$, $p<0.001$), respectively. Interestingly, only imidacloprid treatments caused significant decreases in survival at concentrations greater than $2.82 \pm 0.30 \mu\text{g/L}$ in addition to decreases in mean dry weight. Larvae exposed to thiamethoxam displayed significant reduction in mean dry weight at 5.69 $\mu\text{g/L}$ ($F=10.87$, $df=5$, $n=24$, $p<0.05$).

2.4.3 Adult chronic toxicity endpoints

The EC50 (emergence) values for imidacloprid, clothianidin, and thiamethoxam were 0.39, 0.28, and 4.13 $\mu\text{g/L}$, respectively (Table 2.2). Both imidacloprid and clothianidin displayed significant decreases in percent survival at mean exposure concentrations of 0.80 $\mu\text{g/L}$ ($H=16.94$, $df=4$, $n=20$, $p=0.002$) and 0.48 $\mu\text{g/L}$ ($H=12.31$, $df=3$, $n=16$, $p=0.006$), respectively (Figure 2.1). Thiamethoxam emergence success was significantly higher than for imidacloprid and clothianidin ($H=10.05$, $df=2$, $n=72$, $p=0.007$), which was consistent with the other measured endpoints. A significant decrease in emergence in the thiamethoxam test was only observed at a mean concentration of 5.75 $\mu\text{g/L}$. Developmental complications were apparent in some surviving individuals in nominal treatments $> 3.3 \mu\text{g/L}$ across all active ingredients tested. For example, individuals attempting to complete metamorphosis would occasionally become fixed to the resulting pupal exuvia. This led to adults drowning, unable to breach the surface of the water and were counted as mortalities.

Emergence timing and cumulative emergence were consistent among control treatments in the three tests, with the greatest production occurring between days 21 and 22 (Figure 2.2). For each test, I compared the mean proportion of adults emerged on the day where 50% of the controls had successfully emerged (day 22 for imidacloprid and thiamethoxam and day 21 for clothianidin). Emergence timing of adults was similar in the clothianidin and thiamethoxam tests; however, the 0.53 $\mu\text{g/L}$ clothianidin treatment was significantly earlier than the controls ($H=15.74$, $df=5$, $n=24$, $p=0.008$; Figure 2.2D). The rate with which adults emerged in the

clothianidin and thiamethoxam treatments, relative to the cumulative total, increased with exposure concentration (Figure 2.2C and 2.2F) although the thiamethoxam test did not yield a statistically significant response ($H=6.27$, $df=5$, $n=24$, $p=0.28$). Although not statistically significant, the most pronounced delay in emergence relative to the control was observed in the imidacloprid test ($H=4.20$, $df=2$, $n=12$, $p=0.145$), where a comparable proportion of adults (50%) emerged on days 26-27 (Figure 2.2B). Adult dry weight was significantly reduced in clothianidin and thiamethoxam treatments $>0.20 \mu\text{g/L}$ ($H=10.19$, $df=3$, $n=16$, $p=0.017$) and $>0.68 \mu\text{g/L}$ ($H=18.14$, $df=5$, $n=24$, $p=0.003$), respectively; adults emerging from the $0.10 \mu\text{g/L}$ imidacloprid treatment were also significantly lower in weight than adults emerging from the controls ($H=8.80$, $df=2$, $n=12$, $p=0.001$).

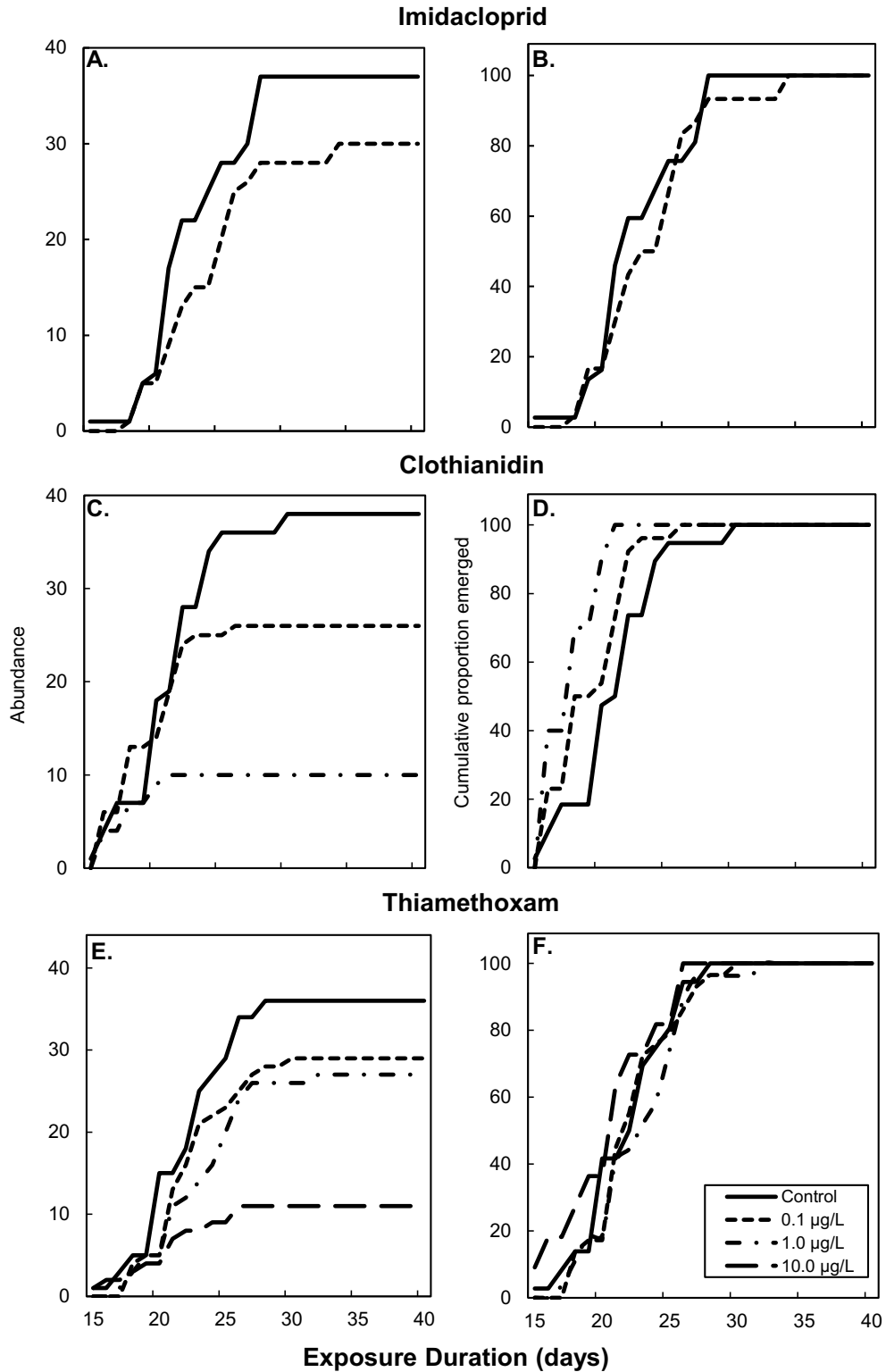


Figure 2.2 Total emergence (A, C, E) and proportion of surviving individuals that emerged (B, D, F) of *Chironomus dilutus* adults from days 15 to 40 exposed to aqueous solutions of thiamethoxam, clothianidin or imidacloprid at one of four nominal test concentrations (0, 0.1, 1.0, 10.0 µg/L).

2.4.4 Sex ratios

Adult *C. dilutus* are sexually dimorphic and exhibit protrandry, where males emerge before females (Pinder 1995). Sex ratios were evaluated as a proportion average among each replicate after each 40-day test. Sex ratios were skewed in favor of a male dominant population at mean concentrations of 0.17, 0.46, and 3.60 µg/L for imidacloprid, clothianidin, and thiamethoxam, respectively (Table 2.3). EC50 (sex ratio) values for imidacloprid and thiamethoxam were lower than their respective EC50 (emergence) values, suggesting that skewed sex ratio may be an even more sensitive population endpoint than survivorship to emergence. Although differences between EC50 (sex ratio) values and EC50 (emergence) values were not statistically significant (US EPA ICp; $\alpha = 0.05$), they may be ecologically important.

Table 2.3 Calculated sub-lethal toxicity endpoints of biomass and sex ratio (EC20 and EC50 µg/L; 95% CI) for *Chironomus dilutus* larvae exposed to technical grade imidacloprid, clothianidin, or thiamethoxam over periods of 14 and 40 days.

	<u>Imidacloprid</u>	<u>Clothianidin</u>	<u>Thiamethoxam</u>
<u>Biomass^a</u>			
EC20	0.81 (0.10-0.94)	0.89 (0.74-0.98)	10.17 (7.38-14.58)
EC50	2.23 (2.09-2.54)	1.83 (1.74-2.08)	21.39 (17.38-28.65)
<u>Sex Ratio^b</u>			
EC20	0.11 (0.02-0.14)	0.15 ^c	0.31 (0.12-0.75)
EC50	0.17 (0.05-0.19)	0.46 (0.29-1.17)	3.60 ^c

^aDry weight of larvae (Day 14)

^bAdult males to females M:F (Day 40)

^cNo confidence intervals could be calculated with the provided data

2.4.5 Toxic equivalency factors

Toxic equivalency factors, relative to imidacloprid, were estimated through a three-point response average at LC/EC(20, 50, 90) and plotted as concentration-response curves (Figure 2.3). Relative to imidacloprid (TEF=1.0), acute (lethality) 14-day TEFs for clothianidin and thiamethoxam were 1.05 and 0.14, respectively. Chronic (emergence) 40-day TEFs were 1.62 and 0.11, respectively. Slopes for both the 14-day lethality ($F=0.26$, $df=2$, $n=9$, $p=0.785$) and 40-day emergence ($F=0.35$, $df=2$, $n=9$, $p=0.731$) endpoints passed the equality of slopes ANCOVA test, thus meeting the assumption of parallelism.

Table 2.4 Toxic equivalency factors (TEFs) for clothianidin and thiamethoxam relative to imidacloprid.

	<u>Imidacloprid</u>	<u>Clothianidin</u>	<u>Thiamethoxam</u>
<u>Day 14^a</u>			
20	1.0	1.38	0.24
50	1.0	0.63	0.06
90	1.0	1.15	0.12
Mean ±			
SD	-	1.05 ± 0.38	0.14 ± 0.09
<u>Day 40^b</u>			
20	1.0	3.0	0.13
50	1.0	1.39	0.09
90	1.0	0.48	0.11
Mean ±			
SD	-	1.62 ± 1.28	0.11 ± 0.02

^aLarvae survival; LC_{XX}: lethal concentration data for day 14

^bAdult emergence; EC_{XX}: effective concentration data for day 40

2.5 Discussion

This is the first study I am aware of that has compared the chronic toxicity of three different neonicotinoid active ingredients, including the well-studied imidacloprid against the second-generation compounds, clothianidin and thiamethoxam, under identical laboratory conditions. Compared to imidacloprid, both clothianidin and thiamethoxam have been largely overlooked in the peer-reviewed literature and in setting regulatory guidelines. Due to the widespread use of imidacloprid and the associated rise in pest insect tolerance, second generation neonicotinoids were developed to improve crop protection (Goulson 2013). Together, clothianidin and thiamethoxam are now the most heavily applied neonicotinoid active ingredients in both North America and the United Kingdom, but concerns remain around their prolonged environmental persistence in soil and their high water solubility which may lead to adverse effects on aquatic biota (Goulson 2013, Simon-Delso et al. 2014, Douglas and Tooker 2015). Furthermore, recent studies have identified ecological and abiotic variables, such as the presence/absence of specific plant species and communities (Main et al. 2015), or runoff of cold spring snow meltwater (Main et al. 2016), as factors that may extend neonicotinoid exposure to aquatic organisms.

Previous literature reviews of neonicotinoid toxicity data have identified imidacloprid as the most toxic neonicotinoid active ingredient, or as equally toxic as some other neonicotinoid

compounds to aquatic invertebrates, followed by clothianidin and thiamethoxam (Whiteside et al. 2008, Morrissey et al. 2015). Whiteside et al. (2008) conducted a rank-based risk assessment focusing on the adverse effects of agrochemicals on aquatic communities, including algae, invertebrates, fish, and other aquatic organisms. Of the 206 compounds evaluated, imidacloprid ranked the highest of the three neonicotinoids at number 51, followed by clothianidin at 143 and thiamethoxam at 190. A similar pattern surfaced in a review by Morrissey et al. (2015), with imidacloprid, clothianidin, and thiamethoxam displaying similar acute toxicity geometric means (LC50 data from 24 h to 96 h tests), but the lack of clothianidin and thiamethoxam toxicity data in the primary literature precluded accurate comparison across compounds. From the identical test concentrations and endpoints evaluated in this study, I confirmed the order of toxicity to be similar to previous reports, with imidacloprid having the lowest L(E)C50 values for every endpoint except day 40 (emergence) value, where clothianidin was marginally lower (0.28 vs. 0.39 µg/L). In general, however, imidacloprid and clothianidin displayed similar toxicity to *C. dilutus* larvae, while thiamethoxam was approximately one order of magnitude less toxic.

In addition, the toxicity thresholds for imidacloprid were within the range of values reported from other studies. Stoughton et al. (2008) described both the acute and chronic toxicity of imidacloprid to *C. dilutus*, and reported chronic L(E)C50 values of 3.17 µg/L at day 10 and 0.91 µg/L at day 28. Similar chronic studies investigating the sensitivity of *C. riparius* to second generation neonicotinoids have reported 1.0 µg/L (EC50) for clothianidin and 10.0 µg/L (NOEC) for thiamethoxam after a 28-day exposure period (EU 2006). However, details on the methodology for both chronic tests were not disclosed in the original documents. Additionally, the endpoint values appear to be based on nominal exposures with no analytical validation. Acute (96 h LC50) values of 2.32 µg/L and 35 µg/L, for clothianidin and thiamethoxam, respectively, were reported for *C. dilutus* and *C. riparius* exposed to technical grade active ingredients (>98% pure) (PMRA 2007, de Perre et al. 2015).

Recent comparative neurophysiological studies offer further insight to the mechanism of action for imidacloprid, clothianidin, and thiamethoxam; Vehovsky et al. (2015) documented the excitatory post-synaptic potential (EPSP) inhibition of the VD4-RPeD1 synaptic connection (acetylcholine-evoked membrane) in the central nervous system of the pond snail, *Lymnaea stagnalis*, for five neonicotinoid active ingredients. Imidacloprid and clothianidin significantly inhibited the EPSP amplitudes at identical dose ranges (10.0-100.0 mg/L), whereas

thiamethoxam exhibited negligible effects. Acetylcholine in *L. stagnalis* controls both excitatory and inhibitory neurotransmission, suggesting a similar neural response between imidacloprid and clothianidin.

The family Chironomidae is one of the most sensitive taxa to neonicotinoids and my results corroborate this; only species of Ephemeroptera and Trichoptera appear to be more sensitive (Beketov and Liess 2008a, Roessink et al. 2013, Morrissey et al. 2015). The ephemeropteran species *Cloeon dipterum* and *Caenis horaria* have estimated 28-day imidacloprid LC50 values of 0.195 $\mu\text{g/L}$ and 0.316 $\mu\text{g/L}$, respectively (Roessink et al. 2013). A recent study by van den Brink et al. (2015) comparing the toxicity of imidacloprid, thiacloprid, and thiamethoxam to the mayfly, *Cloeon dipterum*, found comparable 28-day LC50 values for imidacloprid (0.85 $\mu\text{g/L}$) and thiamethoxam (0.94 $\mu\text{g/L}$). Similar LC50 values for imidacloprid and thiamethoxam indicate a parallel conclusion that these neonicotinoids, albeit possibly acting on different nAChR receptor subunits (Taillebois et al. 2014), exert similar toxic effects on this species. Differences in LC50 or EC50 values between species are likely attributed to the metabolic biotransformation rate of thiamethoxam to clothianidin. Given that van den Brink et al. (2015) conducted less intensive but adequate water changes, some thiamethoxam may have degraded into clothianidin in the aqueous solution or *in vivo* which, in the present study, displayed similar toxicity to imidacloprid. Limnephilidae (caddisflies) are also sensitive to imidacloprid with a 96 h (immobilization) EC50 value of 1.79 $\mu\text{g/L}$ (Roessink et al. 2013). Phylogenetically related taxa to the Chironomidae include members from the genera *Chaoborus* and *Culex*. *Chaoborus obscuripes* has an estimated 28-day imidacloprid LC50 value of 12.6 $\mu\text{g/L}$ (Roessink et al. 2013) and *Culex pipiens* a 14-day thiacloprid LC50 value of 6.04 $\mu\text{g/L}$ (Beketov and Liess 2008a).

In the present study, there is a direct relationship between duration of exposure and the concentration required to induce an adverse effect, emphasizing the importance of chronic neonicotinoid toxicity tests with aquatic insects. Furthermore, day 14 LC50 and day 40 EC50 values both represent a measure of lethality (i.e., LC50 larval mortality and EC50 adult emergence inhibition). My data demonstrate a relationship well established in the literature where toxicity increases with exposure duration (i.e., incipient LC50s were not reached in 14 days). Day 14 LC50 values were 3.9, 8.6, and 5.7 times higher than day 40 EC50 values for imidacloprid, clothianidin, and thiamethoxam, respectively. This trend was similarly observed by

Roessink et al. (2013) and van den Brink (2015). Compared to previous chronic studies, I evaluated toxicity at day 14, not day 10 (Stoughton et al. 2008). This difference allowed for exposure to occur for >80% of the larvae's life-cycle, to within a day from pupation in some instances.

Differences in sensitivity among taxa, especially at low exposure concentrations, can, at least partly, be broadly explained by environmental conditions, physiological status, and life history traits (Liess and Beketov 2013, Taillebois et al. 2014), and by inherent differences in sensitivity among different taxa. Life history traits that are associated with increased sensitivity include generations per year, mobility in the aquatic environment, and reactions to biotic or abiotic stress (Liess and Beketov 2013). A recent study by Rico and van den Brink (2015) calculated the mode-specific sensitivity of synthetic insecticide classes to aquatic invertebrates using traits such as potential maximum size, mode of respiration, lifecycle length, temperature preference, and exoskeleton sclerotization. Members from the family Chironomidae were among the best represented taxa in the analysis due to their popularity as test species. The relative sensitivity of chironomids to all five insecticide classes from most toxic to least toxic were as follows: organophosphates, carbamates, organochlorines, neonicotinoids, and pyrethroids. Available neonicotinoid toxicity data for the Chironomidae are summarized in Table 2.5.

Insect metabolism, and associated growth and development, is largely governed by environmental conditions and available resources. Alexander et al. (2007) found a decrease in feeding rates of the mayfly, *Epeorus longimanus*, when exposed to 0.5 µg/L of imidacloprid for 24 h. A reduction in feeding rate was also observed at a mean 24 h exposure concentration of 1.0 µg/L, well below the LC50 of 2.1 µg/L (Alexander et al. 2007). In my study, fourth-instar chironomid larvae were observed to be excessively active on the substrate surface at nominal concentrations below 1.0 µg/L, but larval dry weights decreased only at concentrations >1.0 µg/L; EC50 (biomass) estimates were 1.83 to 2.23 µg/L for clothianidin and imidacloprid, respectively.

All toxicity tests in the present study started with second-instar larvae. Numerous studies have shown that earlier instars are more sensitive to contaminants than prepupal larvae (Williams et al. 1986). The most important immature stages for growth and development are instars second to fourth (Goulson 2013), which completely overlaps with the exposure scenario used in the present study. In addition to exhibiting excessive locomotive activity, surviving fourth-instar

larvae in nominal treatments >3.3 µg/L of clothianidin and thiamethoxam were periodically observed to build elongated silk cases, in some instances measuring 6-7 cm (M. Cavallaro, pers. obs.). This extra case building activity may have negatively influenced emergence success in higher concentrations, promoting vulnerability during pupal development. Unnecessarily utilizing resources to build an uncharacteristically long case may have both energetic and physical consequences during this critical stage in development.

Table 2.5 Available neonicotinoid active ingredient (A.I.) toxicity data for Chironomidae species (L[E]C50=lethal [effect] concentration to 50% of test population).

Species	A.I.	Study Duration	Study Type	Endpoint	Conc. (µg/L)	Reference
<i>Chironomus riparius</i>	ACT	28 d	Chronic	NOEC ^a	5	EU 2006
	CLO	48 h	Acute	EC50	22	CCME 2007
	CLO	48 h	Acute	EC50	29	EU 2006
	CLO	28 d	Chronic	EC50 ^c	1	EU 2006
	CLO	28 d	Chronic	EC15 ^c	0.72	PMRA 2011
	IMI	24 h	Acute	LC50	55.2	EFSA 2008
	IMI	48 h	Acute	LC50	19.9	Azevedo-Pereira et al. 2011
	IMI	96 h	Acute	EC50 ^b	12.94	Pestana et al. 2009a
	IMI	28 d	Chronic	EC50 ^c	3.11	EFSA 2008
	IMI	28 d	Chronic	EC50 ^c	3.6	EFSA 2008
	THI	28 d	Chronic	EC50	1.8	EU 2006
	THX	N.A.	Acute	EC50	35	PMRA 2011
	THX	30 d	Chronic	NOEC	10	EU 2006
<i>Chironomus dilutus</i>	CLO	96 h	Acute	LC50	2.32	de Perre et al. 2015
	IMI	48 h	Acute	EC50	69	CCME 2007
	IMI	96 h	Acute	LOEC ^a	3.39	CCME 2007
	IMI	96 h	Acute	LC50	10.5	CCME 2007
	IMI	96 h	Acute	LC50	10.5	Stoughton et al. 2008
	IMI	96 h	Acute	LC50	5.4	Stoughton et al. 2008
	IMI	96 h	Acute	LC50	5.75	Stoughton et al. 2008
	IMI	96 h	Acute	LC50	2.65	LeBlanc et al. 2012
	IMI	10 d	Chronic	LC50	3.17	Stoughton et al. 2008
	IMI	28 d	Chronic	EC50 ^c	0.91	Stoughton et al. 2008
<i>Chironomus tepperi</i>	THI	24 h	Acute	LC50	1.58	Beketov and Liess 2008

^aN(L)OEC = no (low) observed effect concentration; ^bImmobilization; ^cEmergence

By altering feeding habits, body size, and emergence timing, pesticide exposure can dictate the success and speed of metamorphosis. High treatments of imidacloprid, clothianidin, and thiamethoxam caused lower emergence success of *C. dilutus*. In some cases, the leg sheaths of individuals attempting to emerge would become tangled in the pupal exuvia, causing the pharate adult to sink and drown. Song et al. (1997) found similar molt related mortality with the IGH inhibitor tebufenozide and imidacloprid. Premolt-related mortality was displayed in the mosquito species, *Aedes aegypti*, after exposure to imidacloprid in a 48 h acute test with molting difficulties increasing with concentration (Song et al. 1997). In the present study, all three of the active ingredients tested caused molt-related mortality of *C. dilutus* during emergence. Previous studies have shown neonicotinoids to influence aquatic insect emergence across several taxonomic groups. Although I recognize the substantial variation that can occur among species sensitivities within similar taxonomic groups, the taxon shown to be most affected by a single pulse exposure of neonicotinoids is Trichoptera, whereas Diptera and Ephemeroptera were most affected by repeated exposure (Mohr et al. 2012). Similar to the present study, imidacloprid was found to reduce successful emergence of *C. dilutus* by 55% at 1.14 µg/L (EC50) during a 28-day full life-cycle toxicity test (Stoughton et al. 2008). Full life-cycle tests therefore contribute more robust data to population-level risk assessments than short-term tests, particularly for holometabolous insect species.

In swarming dipteran species, changes in sex ratio can influence swarming success and subsequent egg mass fertility. Large chironomid swarms with even sex ratios are documented as having more fertile egg masses (Pinder 1995). I found that relative to the other neonicotinoids tested, imidacloprid exerted the greatest effects on adult *C. dilutus* sex ratios (Table 2.3). Imidacloprid, clothianidin, and thiamethoxam shifted sex ratios in favor of male-dominant populations with increasing exposure concentrations. This observation is consistent with results from full life-cycle toxicity tests using dichlorodiphenyltrichloroethane (DDT) (Rakotondravelo et al. 2006). Female adults require more time to develop than males, and most adult females carry fully-formed ovary follicles and other egg mass constituents (Pinder 1995). Longer developmental times prolong exposure to aquatic contaminants, which may help explain their increased sensitivity. Another hypothesis includes the greater physiological demand on females during transition from pupae to adult. Since the sex of *C. dilutus* is genetically predetermined, female sensitivity appears to be attributed to complications during development. Compared to

the control treatment, the greater proportional loss of female adults at higher insecticide concentrations may have contributed to the lower EC50 values and significant decreases in adult dry weight. Adult female *Chironomus sp.* can weigh up to 57.3% more than males (Day et al. 1994). This proportional loss of female adults could compromise wild chironomid populations and should be further explored. Studies focused on the intergenerational effects of chronic neonicotinoid exposure may shed further light on long-term population viability.

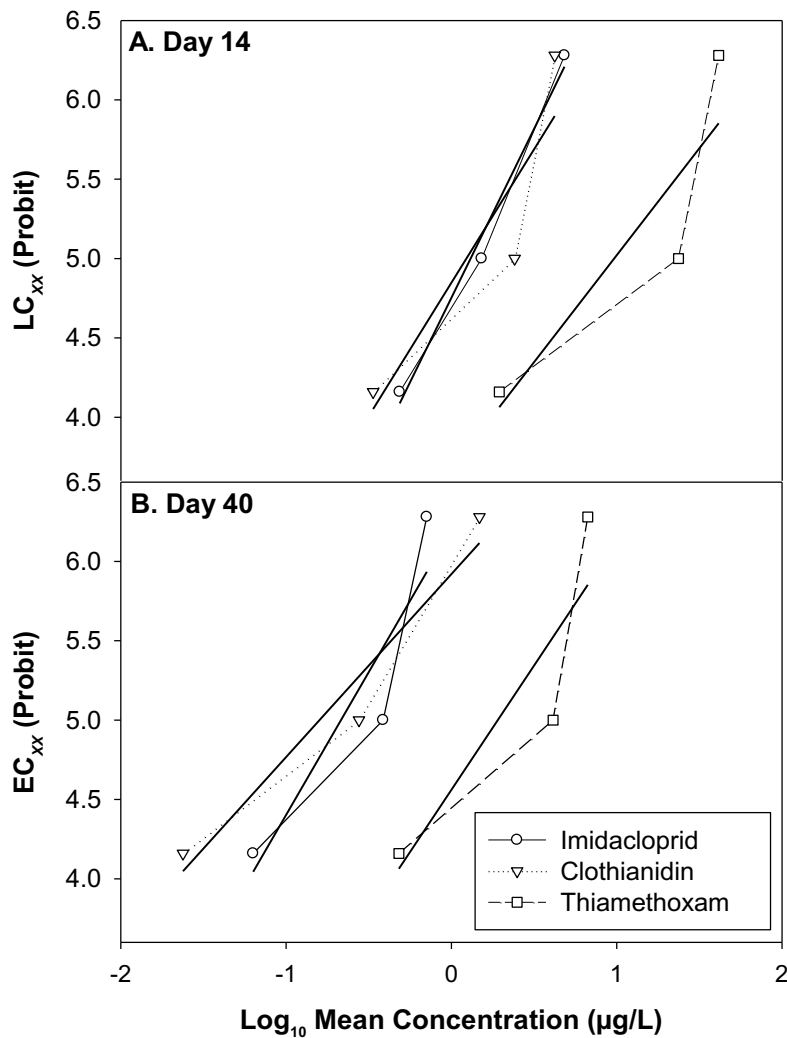


Figure 2.3 Concentration-response curves for imidacloprid, clothianidin, and thiamethoxam to *Chironomus dilutus* based on (A) 14-day lethality and (B) 40-day emergence inhibition. The y-axis response plots the individual LC/EC 20/50/90 estimates for the three compounds on a probit scale.

While individual current-use pesticides continue to receive the most research attention, in the environment these pesticides are often found as mixtures of similar or different active ingredients. The toxicity of neonicotinoid mixtures to aquatic life is still largely speculative with studies estimating cumulative environmental exposure by summing total neonicotinoid concentrations (Main et al. 2014), or standardizing among compounds by molecular weight (Morrissey et al. 2015). The data presented here provide a first opportunity to better describe the relative toxicity of three common neonicotinoids, an essential step towards calculating appropriate toxic equivalency factors (TEFs) of multiple neonicotinoids (Table 2.4). When plotting the concentration-response curves for the three insecticides used to estimate LC_{XX} and EC_{XX} values, the slopes of the lethality and the sub-lethal effect lines are reasonably parallel. From these lines, I was able to calculate the TEF for clothianidin and thiamethoxam relative to imidacloprid. For each compound, the LC_{XX} and EC_{XX} estimates were plotted to create a three point-estimate curve (Figure 2.3). Acknowledging the limited data used to calculate these TEFs, and the slight deviation of the curves from being truly parallel, these TEFs do provide a first attempt at appropriately standardizing and summing the estimated toxicity from mixtures of imidacloprid, clothianidin, and thiamethoxam. Based on the relative potencies described here, mixture toxicity of these three neonicotinoids can be approximated by equations (1) and (2):

14-day neonicotinoid exposure (lethality)

$$[\text{Imidacloprid toxic equivalence}] = [\text{IMI conc.}] + 1.05[\text{CLO conc.}] + 0.14[\text{THX conc.}] \quad (1)$$

40-day neonicotinoid exposure (emergence inhibition)

$$[\text{Imidacloprid toxic equivalence}] = [\text{IMI conc.}] + 1.62[\text{CLO conc.}] + 0.11[\text{THX conc.}] \quad (2)$$

These imidacloprid toxic equivalence values could subsequently be used where multiple neonicotinoids are found in water in order to compare summed equivalence to existing regulatory water quality benchmarks for imidacloprid, such as the Canadian water quality guideline for the protection of aquatic life of 0.23 $\mu\text{g/L}$ (CCME 2007). Future research could aim to strengthen the dataset on which these TEFs are calculated, but in the interim the TEFs

presented here could be used to provide a reasonable, or at least improved, estimate of the hazard of imidacloprid, clothianidin, and thiamethoxam mixtures to non-target insects in aquatic ecosystems.

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PREFACE TO CHAPTER 3

Controlled laboratory toxicity tests have determined neonicotinoids pose certain risks to aquatic insects under chronic scenarios. However, there is a lack of semi-controlled, field data exploring the risks to aquatic insect communities in wetlands. Prairie wetlands surrounded by neonicotinoid-treated crops are at advanced risk to waterborne neonicotinoid residues. Further, previous work has demonstrated aquatic insect emergence to be a sensitive measure to neonicotinoid exposure. The objective of this chapter was to determine the comparative community-level and phenological effects of imidacloprid, clothianidin, and thiamethoxam at chronic, environmentally relevant concentrations on emerging aquatic insects within a typical Prairie wetland using custom designed *in situ* limnocorrals. I acknowledge co-authors Dr. John Headley and Mr. Kerry Peru, contributing neonicotinoid chemical analyses.

Chapter 3 is in review with the journal Environmental Toxicology and Chemistry authored by Cavallaro, M.C., Liber, K., Headley, J.V., Peru, K.M. and Morrissey, C.A. and entitled “Community-level and phenological responses of emerging aquatic insects exposed to three neonicotinoid insecticides: an *in situ* wetland limnocorral approach.”

CHAPTER 3: COMMUNITY-LEVEL AND PHENOLOGICAL RESPONSES OF EMERGING AQUATIC INSECTS EXPOSED TO THREE NEONICOTINOID INSECTICIDES: AN *IN SITU* WETLAND LIMNOCORRAL APPROACH

3.1 Abstract

Seasonal aquatic insect emergence represents a critical subsidy link between aquatic and terrestrial ecosystems. Early and late instar larvae developing in wetlands near neonicotinoid-treated cropland are at risk of chronic insecticide exposure. An *in situ* wetland limnocorral experiment compared emergent insect community responses to imidacloprid, clothianidin, and thiamethoxam. Over 15 weeks, 21 limnocorrals were dosed weekly for 9 weeks to target peak nominal doses of 0.0, 0.05 or 0.5 $\mu\text{g/L}$, followed by a 6-week recovery period. Thirty-nine aquatic insect taxa were recorded but 11 taxa groups made up 97% of the community composition. Principal response curves indicated that during the dosing period, community composition among the treatments resembled the controls. During the 6-week recovery period, significant deviance was observed in the high imidacloprid treatment with similar trends in the clothianidin treatment, suggesting that community effects from neonicotinoid exposure can be delayed. Non-biting midges (Diptera: Chironomidae) and damselflies (Odonata: Zygoptera) also emerged 18 to 25 days earlier in the imidacloprid and clothianidin neonicotinoid treatments, relative to controls. These data suggest that phenology and subtle community effects can occur at measured neonicotinoid concentrations of 0.045 $\mu\text{g/L}$ (imidacloprid) and 0.038 $\mu\text{g/L}$ (clothianidin) under chronic repeated exposure conditions. Synchronization and community dynamics are critical to aquatic insects and consumers; thus, neonicotinoids may have broad implications for wetland ecosystem function.

3.2 Introduction

Species-specific timing of life cycle events (e.g., metamorphosis, emergence, oviposition) inherently drives community dynamics (Nakazawa 2012), especially for insects emigrating from aquatic to terrestrial ecosystems. Aquatic insects transfer energy and nutrients between aquatic and terrestrial environments, supplying timely, abundant resources and ecosystem services (Batzer and Wissinger 1996). These linkages are paramount to numerous wetland dependent consumers. Aquatic insect life cycles are regulated by abiotic and biotic environmental variables, specifically temperature, photoperiod, food resources, and competition (Moore and Schlinder 2010), which dictate phenological synchronization of events. However, these environmental

variables can be disrupted in areas of intensive agricultural production where there is an increased risk of sedimentation, drainage, and agrochemical contamination, including exposure to insecticides (Bartzen et al. 2010).

Among the many insecticides commonly applied over vast spatial scales are the neonicotinoids (Main et al. 2014). Neonicotinoid insecticides, specifically imidacloprid, clothianidin, and thiamethoxam, constitute some of the most heavily applied insecticides in the agricultural sector worldwide (Douglas and Tooker 2015; Simon-Delso et al. 2015). As nicotine agonists, neonicotinoids stimulate nicotinic acetylcholine receptor action and are highly toxic to pest and non-target insects alike (Tomizawa and Casida 2005). Simplified insecticide application methods (i.e., seed treatments) have greatly increased the amount of residual neonicotinoid active ingredient in the environment, particularly within soils (Jones et al. 2014) and surface waters (Morrissey et al. 2015). Episodic heavy rainfall events that promote insecticide runoff can shift and, in some cases, permanently alter aquatic insect community structure (Liess and Schulz 1999, Liess and Beketov 2011).

Numerous single-species laboratory studies have shown that aquatic insects are extremely sensitive to neonicotinoid insecticides (Morrissey et al. 2015), especially under chronic exposure conditions. However, field-based studies examining the effects of neonicotinoids on aquatic insect communities are less common (van Dijk et al. 2013). The use of aquatic invertebrate mesocosm studies offer promise for evaluating the community-level risks of neonicotinoid exposure under more ecologically relevant conditions (Sánchez-Bayo et al. 2016). Based on lab studies, concentrations required to induce lethal effects to immature aquatic insect communities range from 1.0 µg/L (Sánchez-Bayo and Goka 2006; Hayasaka et al. 2012a; Hayasaka et al. 2012b) to 3.2 µg/L (Colombo et al. 2013). Studies of sub-lethal exposures under more natural conditions are required to fill the gap between species-specific laboratory and community-level field studies. Additionally, population endpoints such as aquatic insect emergence are more sensitive to neonicotinoid exposure than lethality and may cause indirect ecological impacts (Chapter 2).

Prairie wetlands are characterized as having unique hydrologic regimes and distinct aquatic insect assemblages associated with variable water retention time (van der Kamp 1995; Euliss et al. 2004), as well as frequent and chronic neonicotinoid detections (Main et al. 2014; Evelsizer and Skopec 2016). Long term contamination of Prairie wetlands is largely driven by

extended persistence in frozen soils and mobilization from snow melt into receiving wetlands (Main et al 2016) combined with recurrent use and persistence in surface waters (Main et al. 2015). Here, I conducted a 107-day (dosing and recovery) study to determine the comparative community-level and phenological effects of imidacloprid, clothianidin, and thiamethoxam on emerging aquatic insects. Exposures were at chronic, environmentally relevant concentrations within a typical Prairie wetland using custom designed in situ limnocorrals. The effects of imidacloprid to laboratory organisms are well-represented in the aquatic toxicology literature, but data are lacking on other neonicotinoids and the responses of communities exposed under more natural conditions (Morrisey et al. 2015). Given the lack of field data on effects of imidacloprid, clothianidin and thiamethoxam relative to their wide-spread use in the agricultural sector, more data on those chemicals will better inform the current regulatory reviews and future policy decisions on their environmental safety.

3.3 Materials and Methods

3.3.1 Study site

The experiment was conducted in a single class 5 (permanent) Prairie wetland (Stewart and Kantrud 1971) at the St. Denis National Wildlife Area (NWA) roughly 40 km east of Saskatoon, SK, Canada. The St. Denis NWA serves as a model Prairie wetland complex inhabited by representative species of waterfowl and wetland dependent fauna. The St. Denis NWA is under the management of Environment and Climate Change Canada where the surrounding land-use consists primarily of native Prairie and hay fields. Intensive studies on hydrology and avian ecology over the past three decades provided an extensive, reliable dataset for selecting an experimental Prairie wetland. The wetland was selected for its consistent inter-annual central water depth (1.0-1.3 m), characteristic Prairie wetland water physicochemical parameters (e.g., pH, conductivity, dissolved oxygen), inflow from lands not under conventional production, and high insect secondary production. Emergent vegetation primarily consisted of *Typha latifolia* and *Alisma triviale*. Floating and submersed aquatic macrophyte composition within the study pond included a patchy distribution of *Lemna sp.* and *Ceratophyllum demersum*. Water quality testing prior to the study revealed no traces of neonicotinoid residues in the selected study wetland.

3.3.2 Experimental design

Twenty-one custom-built limnocorrals (1.0 x 1.0 x 1.5 m) fitted with aquatic insect emergence traps were purchased from Curry Industries Ltd. (Winnipeg, MB, Canada; Figure 3.1). Limnocorrals were fixed to Styrofoam floats encased with polyvinyl, and at the corners of each float, a nylon rope secured to a ring of ABS plumbers pipe (4" in diameter) was placed over the wooden stake; this allowed the floats secured to the limnocorrals to rise and fall with the water level throughout the experiment (extra material in the walls allowed for expansion without pulling the bottom out of the sediment). Due to seasonal strong wind gusts and storms customary to the region, wooden stakes (8.0' x 1.0" x 1.0") anchored the floats and galvanized steel chains weighted the open bottoms of the polyethylene sleeves after firmly pressing them 12-15 cm into the sediment. The sediment seal along the sleeves was visually confirmed with the aid of an Aqua Scope II™. Emergence traps covered the entire limnocorral and featured a removable acrylic collection chamber which led to a polypropylene jar containing 70% ethanol. Glycol was added when evaporation rates accelerated in mid-summer from prolonged sun exposure and higher temperatures. Given the potential for biotic heterogeneity and seasonal changes in water depth, treatments were randomized across 3x7 blocks of limnocorrals located in the center of the experimental wetland at a water depth of approximately 1 m.

3.3.3 Abiotic measurements

Temperature was monitored hourly inside and outside limnocorrals throughout the dosing period with a HOBO Onset® temperature data logger (Bourne, MA, USA) and every two weeks during the recovery period. Physicochemical water quality parameters were also measured during the dosing and recovery periods. Dissolved oxygen (mg/L), conductivity (µS/cm), and pH were measured with a YSI ProPlus (YSI Inc., Yellow Springs, OH, USA) water monitoring meter.

3.3.4 Water and insect sampling

All dosing and sampling were conducted by boat throughout the 107-day long (9-week dosing and 6-week recovery) experiment to avoid disturbing the limnocorral sleeves and surrounding sediments. Water samples were taken 4 days before the start of the experiment (day -4), day 0 and immediately before and after each insecticide application during the dosing period, and then weekly during the recovery period. Adult insects were collected from the polypropylene jars every 3 to 4 days. To improve realism, and due to the small size of the limnocorrals, long

duration of the experiment, and potential for patchy larval distribution, emergence traps were removed for 4 days every 12 days to allow for recolonization. Insects were collected by changing the entire collection chamber and preserved and stored in 70% ethanol until identified and counted. Adult Chironomidae were identified to at least the subfamily-level and all other insects to the lowest possible taxonomic-level using dichotomous keys (Pinder 1995; Merritt and Cummins 1996).

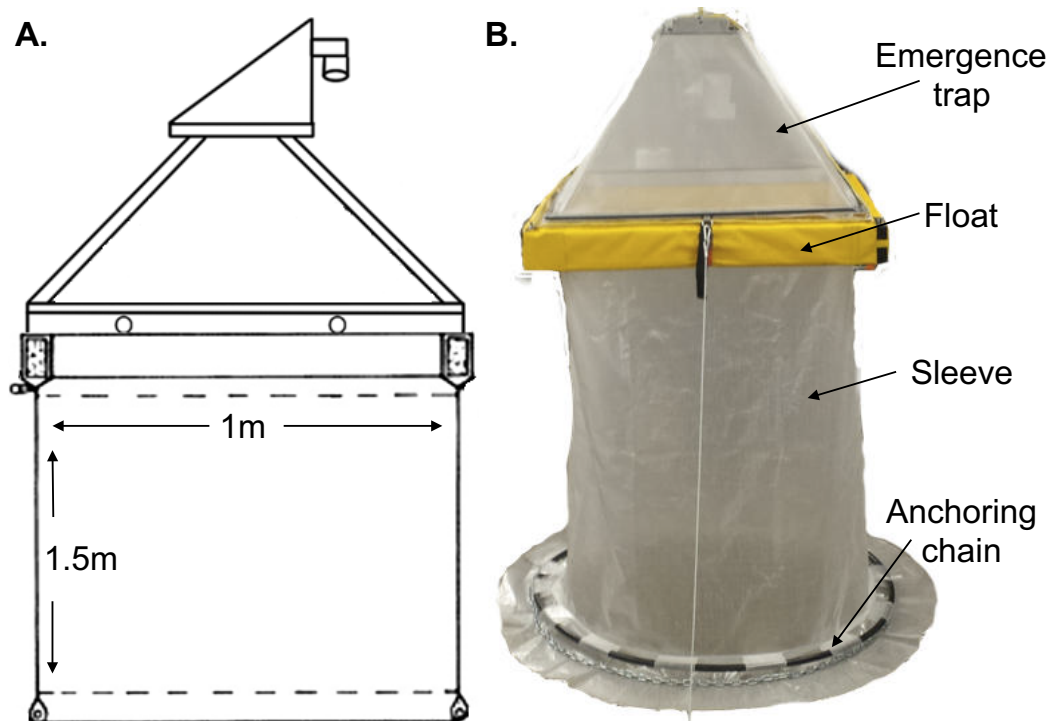


Figure 3.1 Schematic (A) and photograph (B) of an individual, custom-built limnocorral unit manufactured by Curry Industries Ltd. (Winnipeg, MB, Canada) with an emergence trap.

3.3.5 Neonicotinoid application and analysis

Limnocorrals were treated with imidacloprid, clothianidin, or thiamethoxam once weekly during the exposure period to reach target nominal concentrations of 0.05 $\mu\text{g/L}$ (low) or 0.5 $\mu\text{g/L}$ (high) relative to controls. Each neonicotinoid treatment had three replicate limnocorrals with the addition of three controls, for a total of 21 limnocorrals. Several water sampling surveys have identified the recurrent and prevalent distribution of neonicotinoids in Prairie wetlands (Main et al. 2014, 2015, 2016). The two exposure concentrations were selected to represent

environmentally relevant concentrations detected in the region and were within the range of recently proposed and interim acute and chronic regulatory aquatic life guidelines (CCME 2007; RIVM 2014; Morrissey et al. 2015). For example, a seasonally structured water sampling effort in the Canadian Prairies (2012-2013) found that the average annual summed neonicotinoid concentrations (i.e., total imidacloprid, clothianidin, thiamethoxam, and acetamiprid concentrations) measured in wetland water ranged between 0.04 to 0.77 µg/L with peak concentrations up to 3.1 µg/L (Main et al. 2014). Similar water monitoring studies in Iowa, USA, found that mean clothianidin concentrations ranged between 0.17 to 0.52 µg/L in tile-drained wetlands over three sampling periods between May and June in 2014 (Evelsizer and Skopec 2016). Morrissey et al. (2015) also reported geometric means of 0.13 µg/L (averages) and 0.63 µg/L (maxima) in 29 surface water monitoring studies from 9 countries.

Imidacloprid (98.8% pure) and clothianidin (99.6% pure) were acquired from Bayer CropScience (Mississauga, ON, Canada); thiamethoxam (98.9% pure) was acquired from Syngenta Crop Protection (Guelph, ON, Canada). Stock solutions were prepared in 1-L volumetric flasks with reverse osmosis water (Barnstead® Diamond™ NANOpure, 18.2 MV/cm), then the appropriate volume was transferred into 250-mL bottles, transported in coolers to the study site, and poured directly into the appropriate limnocorral at each dosing event. Water in the limnocorral was gently stirred with a paddle to aid mixing. Further mixing was achieved through natural turnover within the pond from temperature-aided water density changes. The volume of each limnocorral was calculated by measuring the pond depth (i.e., $L \times W \times H$) and estimated to contain 1,000-1,300-L of wetland water. Individual volume measurements combined with predicted degradation rates, were used to adjust weekly dosing accordingly. In order to maintain the target exposures over the entire study period, I measured actual concentrations in the limnocorrals immediately post dosing, then 3 and 7 days later to obtain representative 7-day degradation rates for each neonicotinoid compound (Appendix A: S.I. Table 1). This information allowed us to calculate the remaining concentration in the limnocorrals and subsequently dose with the appropriate amount to reach my target nominal dose each week (Figure 3.2 and Appendix A: S.I. Table 1).

Limnocorral water samples for chemical analysis were collected by grab-style sampling at >10 cm below the surface of the water in the center of each limnocorral with 250-mL amber bottles and stored at 4° C. All water samples were analyzed at the National Hydrology Research

Centre, Environment and Climate Change Canada in Saskatoon, SK. Methods adapted from Xie et al. (2011) allowed for simultaneous extraction of imidacloprid, clothianidin, and thiamethoxam when present through aqueous sample extraction, solid-phase extraction, and LC-MS/MS and are fully described in Main et al. (2014). Analytical standards of imidacloprid, clothianidin, and thiamethoxam were purchased from Chem Service (West Chester, PA, USA). Quality assurance/quality control (QA/QC) results provided recovery correction factors (RC %), limits of quantification (LOQ), and limits of detection (LOD). Mean (\pm SE) QA/QC were as follows: imidacloprid (RC=83.9 \pm 4.4%; LOQ=6.5 \pm 0.5 ng/L; LOD=2.2 \pm 0.1 ng/L), clothianidin (RC=78.9 \pm 6.6%; LOQ=10.0 \pm 1.0 ng/L; LOD=3.3 \pm 0.4 ng/L), and thiamethoxam (RC=91.3 \pm 0.3%; LOQ=12.5 \pm 1.5 ng/L; LOD=4.2 \pm 0.5 ng/L). All concentrations were batch recovery corrected.

3.3.6 Data analysis

Univariate statistical tests compared all treatments and blocks within wetlands for physicochemical water chemistry parameters using a one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. To account for the potential influence by predatory taxa on emergence, total Zygoptera was also compared among treatments using Chi-square tests, respectively ($\alpha = 0.05$ level of confidence). Tests were performed using SigmaPlot™ Version 13.0 (Systat Software, Inc., San Jose, CA, USA).

The effects of the three neonicotinoids relative to controls on emerging adult insects were analyzed by the Principal Response Curves (PRC) method developed by van den Brink and Ter Braak (1999). These multivariate models are designed to evaluate community effects in experiments with repeated measures over time where the emphasis is on species composition. The PRC method executes a time-series partial redundancy analysis (pRDA) where the interaction between sampling time and treatment act as explanatory variables and sampling times as covariates (van den Brink et al. 2009). PRC results are interpreted by graphical representation with the sampling time on the x-axis and the first Principal Component (PRC1) of the treatment effects on the y-axis. In this format, spatial deviations from the mean of controls (center line) can be compared over repeated temporal sampling to determine community response, thus quantifying the effects of the insecticide treatment across multiple species simultaneously. Significance of the PRC among treatments was evaluated by random Monte Carlo method (999 permutations) using the limnocorral taxa counts ($\log(x + 1)$ -transformed), obtaining an *F*-type

test statistic based on the eigenvalue of the first Principal Component. The PRC method identifies the variance partitioning by reporting the explanatory content (in %) of first canonical axis (van den Brink and Ter Braak 1999). PRC scores for all species can be found in the supplementary file (Appendix A: S.I. Table 2) and those species with scores + or – 0.15 are displayed. The PRC analysis was performed using R version 3.4.1 (R Core Team 2017) package “vegan” (Oksanen et al. 2011), and a Dunnett’s test of the first principal component was used to determine whether significant differences between treatments and controls occurred at any sampling time points using package “multcomp” (Hothorn et al. 2008).

In order to determine how timing of emergence varied among treatments, the cumulative proportional emergence of all taxa and for each of the 4 major taxa: Chironomidae, Zygoptera, Chaoboridae, and Limnephilidae was calculated as the number of insects emerging on each sampling day divided by the relative total abundance. Data were analyzed by fitting cumulative proportion curves with 95% confidence intervals for each treatment (control, 0.05, 0.5 $\mu\text{g/L}$ imidacloprid, clothianidin, thiamethoxam) over time using nonlinear curve fits. Model selection (i.e., logistic, cubic, quadric, exponential) of curve fits was based on AIC_c weight and parameter estimates are shown for each best fit curve by treatment. The curve mean inflection point indicates the day which separates the curve into two equal regions of opposite concavity (i.e., 50% cumulative proportion). Parameter estimates of curves were statistically compared between treatments and controls using an analysis of means (ANOM). Significance was indicated by upper (later emergence) or lower (earlier emergence) exceedance of the generated 95% C.I.s ($\alpha = 0.05$). An equivalence ratio was generated by comparing the rate of emergence in the control limnocorrals to each treatment ($\alpha = 0.05$; default setting of a 25% change compared to the controls) where the control equivalence ratio is 1.00 and the C.I.s are 0.8-1.25 (i.e., 25% change from the control). Curve fitting and derivation of parameter estimates were performed in JMP[®], Version 11.0 (SAS Institute Inc., Cary, NC, USA).

3.4 Results

3.4.1 Physicochemical water quality parameters

On average, temperatures (mean minimum and maximum; °C) inside and outside the limnocorrals during the dosing period ranged from 13.9-27.0°C and 14.2-25.9°C, respectively. Mean differences in temperatures between the inside and outside of the limnocorrals were not significant (Student’s *t*-test, $p > 0.05$), indicating that the sleeves had minimal insulating effects.

Temperature and depth decreased during the recovery period (Table 1). Mean (\pm SE) water physicochemistry measurements across all limnocorrals during dosing were characteristic of a typical Prairie wetland: dissolved oxygen (6.36 ± 0.17 mg/L), conductivity (2090.2 ± 19.83 μ S/cm), pH (8.07 ± 0.02), and depth (1.27 ± 0.01 m), which were similar among treatments (Table 1). Water physicochemical parameters were also similar among the 3 experimental blocks (ANOVA; $p > 0.05$) during both the dosing and recovery period.

Table 3.1 Mean (\pm SE) measured water quality parameters in limnocorrals among treatments.

Parameter	Control	Imidacloprid	Clothianidin	Thiamethoxam
Dissolved oxygen (mg/L)	5.8 (\pm 0.4)	5.9 (\pm 0.2)	6.0 (\pm 0.3)	5.7 (\pm 0.3)
Conductivity (μ S/cm)	2209 (\pm 50)	2210 (\pm 40)	2252 (\pm 43)	2225 (\pm 43)
pH	8.14 (\pm 0.05)	8.10 (\pm 0.04)	8.13 (\pm 0.03)	8.10 (\pm 0.03)
	Dosing Period	Recovery Period		
Depth (m) ^a	1.27 (\pm 0.0)	1.04 (\pm 0.0)		
Temperature ($^{\circ}$ C) ^a				
Inside limnocorrals	20.46 (\pm 0.7)	15.54 (\pm 0.4)		
Outside limnocorrals	20.04 (\pm 0.7)	15.20 (\pm 2.2)		

^aRecorded on the inner and outer regions of the 3X7 limnocorral block design during the dosing and recovery period

3.4.2 Neonicotinoid exposure and degradation

Mean (\pm SE) measured high dose concentrations of neonicotinoids in water immediately after weekly dosing were $0.436 (\pm 0.062)$ μ g/L imidacloprid, $0.384 (\pm 0.048)$ μ g/L clothianidin, and $0.386 (\pm 0.106)$ μ g/L thiamethoxam which was 87%, 77%, and 77% of the target nominal high dose (0.5 μ g/L). Low dose concentrations were measured as $0.045 (\pm 0.006)$ μ g/L imidacloprid, $0.038 (\pm 0.015)$ μ g/L clothianidin, and $0.045 (\pm 0.018)$ μ g/L thiamethoxam which was 90%, 76%, and 90% of the target nominal low dose (0.05 μ g/L), respectively (Figure 3.2, Appendix A: S.I. Table 1). The control limnocorrals remained below detection throughout the study. Although thiamethoxam can degrade to clothianidin, I only found 2 clothianidin detections in a 0.5 μ g/L thiamethoxam treatment on day 49 and 56 and both were $<$ LOQ (LOQ= 10.0 ± 1.0 ng/L). To estimate dissipation between weekly insecticide applications, I calculated the mean percent active ingredient remaining seven days after the initial dosing (week 1) for a subset of imidacloprid, clothianidin, and thiamethoxam limnocorrals; this was repeated for the final week of the exposure period (Figure 3.2). On average, seven days post-dosing,

measured concentrations of the three neonicotinoids decreased by 37.6%, 35.7%, and 75.3% for imidacloprid, clothianidin, and thiamethoxam, respectively.

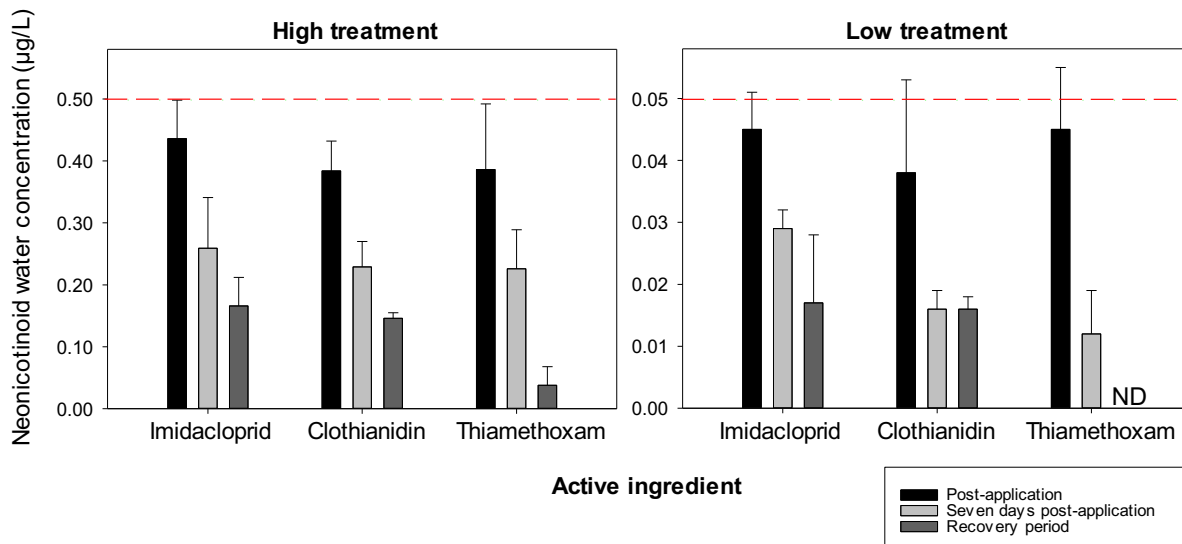


Figure 3.2 Mean (\pm SE) measured concentrations for the high and low treatment for each active ingredient immediately post-application, seven days post-application (AI degradation), and recovery period. The dashed red line denotes the target concentration for each treatment post-application.

3.4.3 Insect abundance and diversity

Eleven of the 39 insect taxa identified accounted for 97% of the total number of emerging adult insects collected and include the following: *Limnephilus infernalis* (27%), *Chironomus sp.* (26%), Tanytarsini sp. (13%), Chironomini sp. (8%), *Cricotopus sp.* (5%), *Ablabesmyia peleensis* (5%), *Chaoborus americanus* (4%), Orthoclaadiinae (4%), *Psectrocladius sp.* (3%), *Lestes disjunctus* (1.3%), and *Enallagma annexum* (0.7%). To characterize the potential role of predatory pressure on emergence, I determined the total proportion of predatory damselfly taxa among treatments ranged from 1.6% to 6.9%. One treatment (low thiamethoxam) had a greater proportion of predators observed ($\chi^2=20.65$, d.f.= 6, $p=0.002$) with a single limnocorral outlier of 15.1%.

The PRC analyses for imidacloprid treated limnocorrals showed that 59.8% of the overall variance in the community composition dataset was explained by time (days) and 11.9% by treatment (neonicotinoid concentration in $\mu\text{g/L}$). The first canonical axis (PRC1) of the PRC explained 34.1% of the variance, whereas the variance in taxa composition between limnocorral replicates was 7.1%. PRC1 captured a significant portion of the variance in the insect community

composition by the imidacloprid treatment (Monte Carlo permutation test, 999 permutations, F-ratio=22.55, p=0.046). Positive taxa weights were found for the hard-bodied flies. These taxa significantly increased in abundance in the high imidacloprid treatment during the recovery period relative to the controls (Dunnett's test; p=0.03) at sampling day 71 (Figure 3.3; Monte Carlo permutation test, 999 permutations, F-ratio=2.70, p=0.015). The highest species weight was calculated for Ephydriidae (0.2) followed by Canacidae and Musicidae. Negative taxon weights were found for primarily multivoltine (≥ 1 generation per year) groups (e.g., *Chironomus sp.*, *Ablabesmyia peleensis*, *Procladius sp.*, *C. americanus*, and *Tantarsini sp.*).

The PRC analyses for clothianidin treated limnocorral showed that 57.1% of the overall variance in the community composition dataset was explained by time (days) and 9.4 % by treatment (neonicotinoid concentration in $\mu\text{g/L}$). The first canonical axis of the PRC explained 29.3% of the variance (Monte Carlo permutation test, 999 permutations, F-ratio=12.74, p=0.74), whereas the variance in taxa composition between limnocorral replicates was 11.7%. Among the taxa with positive weights were three of the semi-aquatic parasitic wasp families, Diapriidae (0.06), Eulophidae (0.09), and Scelionidae (0.04). The highest positive taxon weight was calculated for Orthoclaadiinae (0.5). Similar to the imidacloprid treatments, the negative taxon weights were found for the multivoltine taxa with the highest negative taxon weight calculated for *Chironomus sp.* (-3.4).

The PRC analyses for thiamethoxam showed that 55.5% of the overall variance in the community composition dataset was explained by time (days) and 12.3 % by treatment (neonicotinoid concentration in $\mu\text{g/L}$). The first canonical axis of the PRC explained 32.3% of the variance (Monte Carlo permutation test, 999 permutations, F-ratio=19.22, p=0.26), whereas the variance in taxa composition between limnocorral replicates was 10.7%. Tanypodinae showed the highest negative taxon weight (-0.06). Many taxa weights in the thiamethoxam treatments were positive with the highest taxon calculated for *Chironomus sp.* (2.8). This was followed by the caddisfly, *Limnephilus infernalis* (2.1), and the chironomid subfamily Orthoclaadiinae (1.3).

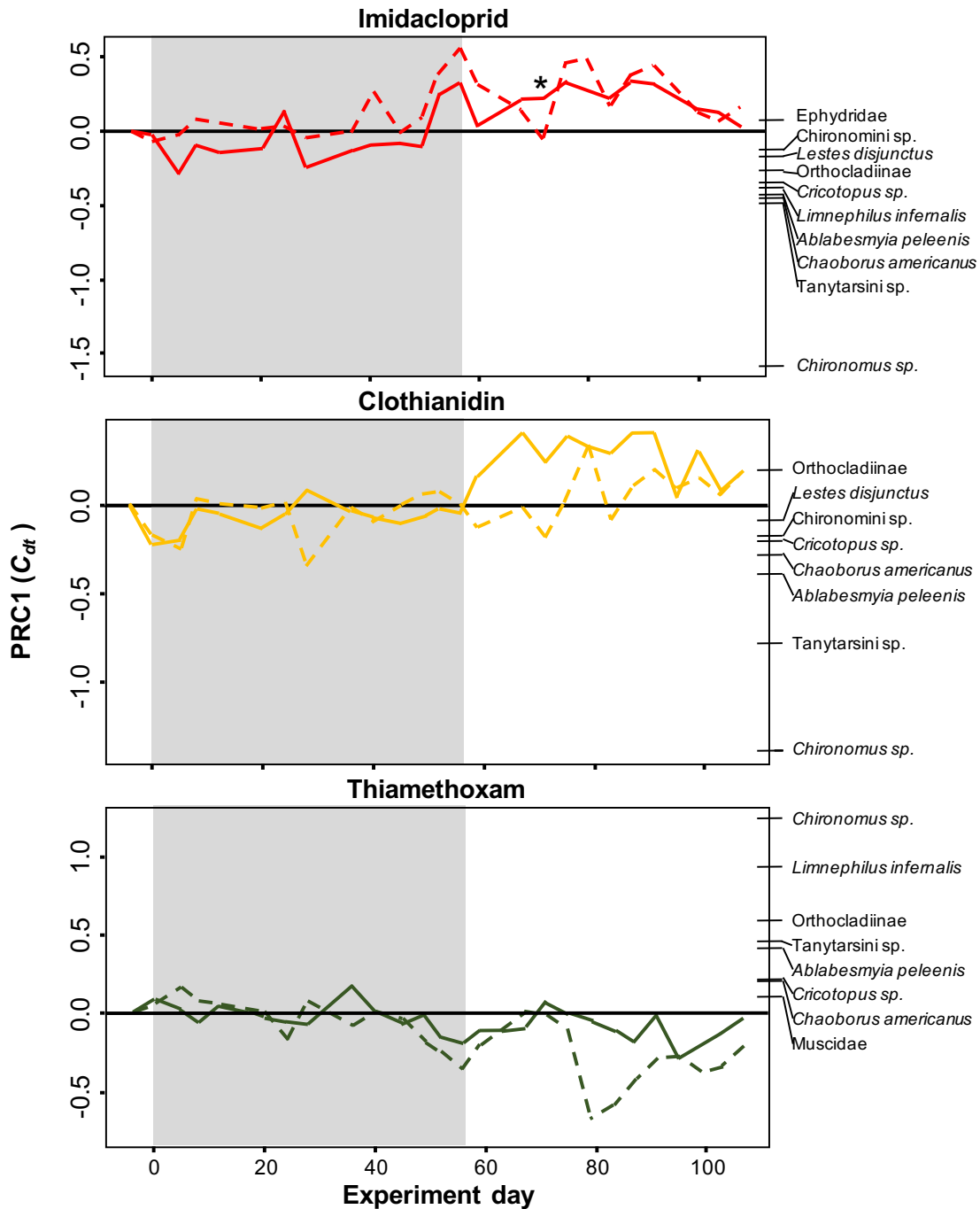


Figure 3.3 Principal response curves (PRC1) displaying the emerging insect community response of replicated limnocorrals (Y-axis determined by canonical coefficients C_{dt}) over time for three neonicotinoid insecticides, relative to the control limnocorrals (“0” line). Solid and dashed lines represent 0.5 µg/L (high) and 0.05 µg/L (low) treatments, respectively. The scores above 0.15 or below -0.15 on the species weight axis (right) are displayed for clarity. Values near “0” are considered more similar to the control community (i.e., taxa with positive values represent an increase in abundance relative to the controls, whereas negative values represent a decrease in abundance relative to controls). Grey shaded area indicates the 9-week pulsed-dosing period. * Significantly different from the control as determined by a Dunnett’s test ($p \leq 0.05$).

3.4.4 Proportion and timing of emergence

A nonlinear curve fit function was performed to compare the emergence timing of all taxa combined, and the 4 most common taxa Chironomidae, Zygoptera, Chaoboridae, and Limnephilidae (Figure 3.4, Table 3.2). Equivalence ratios indicated significant differences in curve slope (i.e., >25% different from the set α of 0.05) for Chironomidae (imidacloprid high and low; clothianidin high) and Zygoptera (imidacloprid high; clothianidin high and low). The mean inflection point for all taxa was day 59; Chironomidae was day 44; Zygoptera was day 47; Chaoboridae was day 57, and Limnephilidae was day 92. Exceedance of the decision limits ($\alpha = 0.05$ level of confidence) indicated Chironomidae and Zygoptera exhibited significantly earlier (Lower) emergence with neonicotinoid exposure (Table 3.2). For example, Chironomidae, representing 64% of the total insect secondary production, emerged 18-19 days earlier in imidacloprid (high = day 38 and low = day 39) treatments relative to the controls (day 56) and 9-15 days earlier in the clothianidin treatments (high = day 41 and low = day 47). Zygoptera emerged 19-25 days earlier in clothianidin (high = day 36 and low = day 42) treatments relative to the controls (61) and 17 days earlier in the low imidacloprid treatment compared to the controls. Thiamethoxam was not observed to affect timing of emergence for any of the common taxa (Figure 3.4).

Table 3.2 Emergence timing from major taxa groups identified in limnocorrals treated with high or low concentrations of imidacloprid, clothianidin, or thiamethoxam neonicotinoids relative to controls. Shown are the best estimates of the mean inflection point in experimental day (i.e., day when 50% cumulative proportion emergence was achieved) and the upper and lower decision limits of the 95% confidence interval (C.I.) around those best estimates. Curve fits were determined by greatest AIC_c weight; all curves were 2-parameter logistic curves. Equivalence ratios are relative to the rate (i.e., slope) of emergence displayed in control limnocorrals. Bold text indicates significantly earlier than the mean inflection point ($\alpha = 0.05$ level of confidence).

Taxa group	Curve-fit parameters	Control	Imidacloprid		Clothianidin		Thiamethoxam	
			High	Low	High	Low	High	Low
All taxa								
	Decision limits ^e	(51-66)	(55-63)	(53-64)	(54-63)	(54-63)	(53-65)	(54-63)
	Inflection point estimate	72	54	61	57	59	67	54
	Limit exceeded	Upper	Lower	–	–	–	Upper	–
	Equivalence ratio	1.00 ^j	0.74	0.84	0.79	0.82	0.93	0.75
Chironomidae ^a								
	Decision limits ^f	(40-48)	(41-48)	(41-48)	(41-48)	(41-48)	(41-48)	(41-48)
	Estimate	56	38	39	41	47	52	42
	Limit exceeded	Upper	Lower	Lower	Lower	–	Upper	–
	Equivalence ratio	1.00	0.68	0.69	0.73	0.84	0.94	0.76
Zygoptera ^b								
	Decision limits ^g	(41-54)	(43-52)	(43-52)	(43-51)	(43-52)	(43-52)	(41-54)
	Inflection point estimate	61	47	44	36	42	60	57
	Limit exceeded	Upper	–	–	Lower	Lower	Upper	Upper
	Equivalence ratio	1.00	0.76	0.72	0.59	0.68	0.98	0.94
Chaoboridae ^c								
	Decision limits ^h	(54-60)	(55-59)	(54-60)	(54-60)	(55-59)	(54-60)	(54-60)
	Inflection point estimate	60	54	53	57	58	63	64
	Limit exceeded	–	Lower	Lower	–	–	Upper	Upper
	Equivalence ratio	1.00	0.89	0.88	0.95	0.96	1.05	1.07

Limnephilidae^d

Decision limits ⁱ	(91-92)	(91-92)	(91-92)	(91-92)	(91-92)	(91-92)	(91-92)
Inflection point estimate	91	92	93	91	92	92	90
Limit exceeded	–	–	Upper	–	–	–	Lower
Equivalence ratio	1.00	0.99	1.01	0.99	0.99	0.99	0.98

^a*Chironomus sp.*, *Ablabesmyia peleensis*, *Psectrocladius sp.*, *Cricotopus sp.*, Tanytarsini sp., Tanypodinae and Orthocladiinae

^b*Enallagma annexum* and *Lestes disjunctus*

^c*Chaoborus americanus*

^d*Limnephilus infernalis* and *Philarctus quaeris*

^eAIC_c weight = 0.962; Mean inflection point = 58.76

^fAIC_c weight = 0.998; Mean inflection point = 44.39

^gAIC_c weight = 0.931; Mean inflection point = 47.43

^hAIC_c weight = 0.513; Mean inflection point = 57.08

ⁱAIC_c weight = 0.994; Mean inflection point = 91.69

^jDefault decision limits = (0.80-1.25); 25% difference from reference curve (i.e., controls)

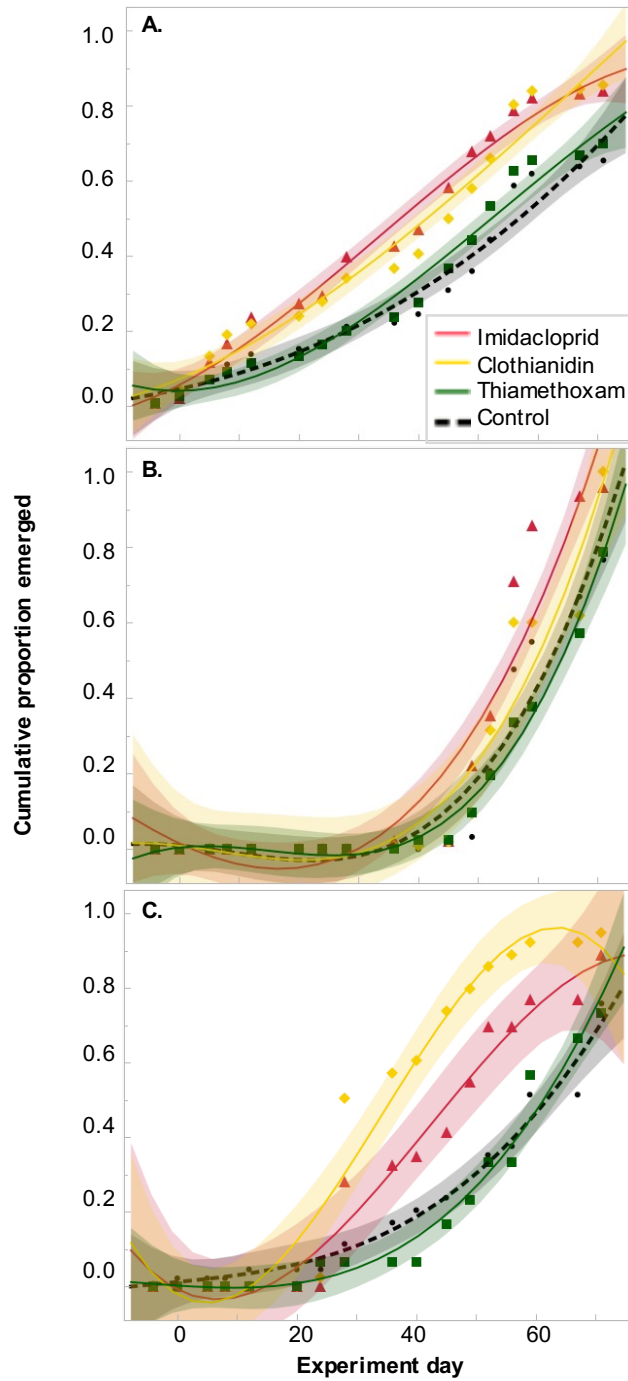


Figure 3.4 Nonlinear curve-fits of the cumulative relative proportion emerged during the 9 week dosing period of the total 15 week (107-day) limnocorral experiment. Effects from imidacloprid, clothianidin, and thiamethoxam are displayed for three affected taxa; Chironomidae (A.), Chaoboridae (B.), and Zygoptera (C.). Solid lines represent the high treatment for each active ingredient and the dashed line represents the control treatment. Low treatments were removed for graph clarity.

3.5 Discussion

The results of this study indicate that low doses of imidacloprid and clothianidin, though not thiamethoxam, can have subtle but important effects on emerging aquatic insect community phenology and to a lesser extent community composition within a wetland. In this complex experiment involving *in situ* limnocorrals, I attempted to maintain nominal concentrations at 0.5 µg/L and 0.05 µg/L under semi-natural conditions – levels that are within the range of proposed water quality guidelines for imidacloprid and, based on water sampling data, represent observed concentrations in natural Prairie wetlands (Main et al. 2014). Since the neonicotinoids naturally dissipated during the 7 days between dosing, average exposures were lower and thus these results represent a conservative but realistic scenario. Recent preliminary risk assessments by the U.S. EPA (0.01 - 0.39 µg/L) and Health Canada (0.041 - 0.36 µg/L) are comparable to the water quality criteria proposed by earlier reviews (0.035 - 0.2 µg/L) based on all neonicotinoid compounds (Morrissey et al. 2015; U.S. EPA 2017). My data suggest that phenological effects can occur at mean peak measured concentrations of 0.045 µg/L (imidacloprid) or 0.038 µg/L (clothianidin) and effects on aquatic insect communities emerging from wetlands may persist over time. No concentration-response was observed during the dosing period across all compounds tested. Once insecticide application ceased, aquatic insect communities displayed high variance among treatments during the recovery period with an observed significant change in community composition on Day 71 only in the high imidacloprid treatment (0.436 µg/L). Latent effects of chronic neonicotinoid exposure may alter the recovery trajectory of aquatic insect communities in Prairie wetlands, particularly where recolonization from uncontaminated areas may be less accessible.

3.5.1 Community alterations

Aquatic insects found in wetlands function as ecological generalists and can often occupy a range of wetland types (Wrubleski and Ross 2011). These wetlands can be characterized by varying hydroperiods, salinity levels, and plant communities (Stewart and Kantrud 1971) as well as aquatic insect assemblages (Driver 1977). Depending on climatic conditions, geographic location, surrounding land-use, and other landscape variables, aquatic insect communities can change drastically, or shift gradually, year-to-year even in the same wetland (Batzer 2013). This variability produces high diversity in wetland ecosystems with species inventories reporting greater than 400 species in the Prairie Pothole Region (Euliss et al. 1999). Advanced tolerance

for environmental fluctuation exhibited by wetland insect species, from daily to annual variability, has challenged wetland scientists, hence the lack of studies demonstrating clear ecological patterns influencing aquatic insect communities (Batzer 2013). A subtle decreasing trend in multivoltine species such as *Chironomus sp.*, *Ablabesmyia peleensis*, *Procladius sp.*, *Chaoborus americanus*, and *Tantarsini sp.* in the imidacloprid and clothianidin treatments was observed during the weekly pulsed-dosing period. These taxa are considered eurytopic and cosmopolitan due to their tolerance to a range of aquatic habitats (Pinder 1995). Specifically, *Chironomus sp.* were more abundant in the control limnocorrals, suggesting that imidacloprid and clothianidin potentially caused similar effects on emergence success. Similar to recent laboratory tests with *Chironomus dilutus* that describe the relatively equivalent toxicity of imidacloprid and clothianidin (Chapter 2; Maloney et al. 2017; Raby et al. 2018), the present study demonstrates that these two neonicotinoids may affect wild populations of *Chironomus sp.* under field conditions.

Separating the relative influence of a chemical stressor and normal environmental variation is a notable challenge in mesocosm studies. Some case studies cite habitat and physicochemical water parameters as having a greater impact on invertebrate communities than chemical stressors (Rico et al. 2016). An interesting aspect of pesticide sensitivity is the role of nutrients, specifically if nutrients (e.g., total nitrate or phosphate) may reduce or enhance larval insect sensitivity. Resource demand and sensitivity to pesticides have varying implications to biological trade-offs in larval insect depending on their rate of growth and size (Alexander and Culp 2008). Similar challenges exist in studies involving Prairie wetlands. Chipps et al. (2006) reported greater prevalence of dominant multivoltine species (i.e., Culicidae and Chironomidae) in Prairie wetlands under increased agricultural disturbance, suggesting concomitant effects from runoff and changes in physicochemical water parameters (Chipps et al. 2006). In the present study, experimental units were isolated within the same Prairie wetland, thus creating a more uniform baseline and generally lower environmental variation. This indicates that the impacts of imidacloprid and clothianidin on emerging aquatic insect communities are chemically-induced and not from environmental disturbance or other physicochemical differences.

Previous neonicotinoid mesocosm studies feature exposure profiles varying from single to multiple pulses in lentic and lotic aquatic habitats (Sánchez-Bayo et al. 2016). Commonly faced with prolonged or repeated pesticide exposures throughout the growing season, previous

studies indicate immature aquatic insect abundance and community structure are impacted by mean peak neonicotinoid concentrations greater than 1.0 µg/L (Sánchez-Bayo and Goka 2006; Beketov et al. 2008; Hayasaka et al. 2012b; Kattwinkel et al. 2016). Sub-lethal effects on feeding performance, leaf litter decomposition, and body length are documented at concentrations below 1.0 µg/L (Alexander et al. 2007, Alexander et al. 2008, Englert et al. 2012). In a similar lentic mesocosm study, a pulsed imidacloprid exposure scenario, ranging in concentration between 0.6 to 40.0 µg/L TWA (time weighted average), researchers found significant community effects at 5.2 µg/L TWA where Chironomidae represented the greatest abundance and diversity (Colombo et al. 2013). Colombo et al. (2013) also detected significant negative effects at much higher concentrations than this study at 12.0 µg/L TWA for abundance and 5.2 µg/L TWA for diversity. However, high variation in abundance among treatments occurred at concentrations ranging from 0.2 to 0.4 µg/L TWA, suggesting low-level concentrations acted as a stochastic stressor to the community. Similar variability was also observed in stream mesocosms after three weekly applications of imidacloprid at 1.63 µg/L where total benthic insect and dipteran density were slightly greater than controls (Pestana et al. 2009a). In the present study, high variation in abundance was observed at lower concentrations (<0.5 µg/L) suggesting general community stress. In an outdoor pond study, Ratte and Memmert (2003) observed Chironomidae and Baetidae as the most sensitive taxa after two applications of Confidor 200 SL (a.i. imidacloprid) with a NOEC (no-observed effect concentration) of 0.6 µg imidacloprid/L. In contrast, three single pulses of Admire at 2.0 and 20.0 µg imidacloprid/L revealed no effects on the Chironomidae community nor reductions in EPT taxa (Ephemeroptera, Plecoptera, and Trichoptera) (Pestana et al. 2009a). Differences in habitat, initial taxa composition, exposure duration, and concentration are some of the critical components to consider when interpreting variation in mesocosm responses.

Dispersal and recolonization are key components to external recovery of aquatic insect communities from pesticide exposure (Trekels et al. 2011). Due to the physical design of my limnocorrals and routine removal of the emergence traps, recolonization was occurring throughout the study. This approach may have influenced the overall sensitivity to neonicotinoid treatments and contributed to variable community responses among treatments. Previous studies have highlighted and quantified the value of recolonization to aquatic insect communities recovering from environmental stressors related to agriculture (Galic et al. 2013). The

imidacloprid treatment was the only compound that showed a significantly different community composition from the controls, but only during the recovery period (Day 71). Given the patterns exhibited among all treatments, this suggests that the trends observed in community composition during the recovery period may be due to a combination of latent effects from neonicotinoid exposure or altered recolonization potential from nearby wetlands.

Imidacloprid is the most widely used neonicotinoid in both laboratory and mesocosm studies (Morrissey et al. 2015; Sánchez-Bayo et al. 2016). However, the paucity of available aquatic toxicity data (laboratory and field) for clothianidin and thiamethoxam has contributed to assumptions of different neonicotinoids having similar toxicity. Here, imidacloprid and clothianidin appeared to have a slightly stronger effect on the emergent insect community structure and emergence timing than thiamethoxam. The more rapid dissipation of thiamethoxam over the 7 days between reapplication may have contributed to the difference. However, comparative studies of thiamethoxam to other neonicotinoids similarly report lower toxicity in terrestrial and aquatic insect species (Chapter 2, Stamm et al. 2001, Jones et al. 2012, Raby et al. 2018). In my laboratory studies on the model aquatic insect, *Chironomus dilutus*, 40-d (emergence inhibition) toxic equivalency factors for clothianidin ($TEF = 1.62 \pm 1.28$) were similar to imidacloprid ($TEF = 1.0$) but thiamethoxam was approximately an order of magnitude less toxic ($TEF = 0.11 \pm 0.02$). To date, there are no other published microcosm or mesocosm studies of thiamethoxam on aquatic insect communities. Though adverse community effects of clothianidin have been reported such as reduced abundance, diversity, and evenness, with a NOEC of $1.0 \mu\text{g/L}$ (dose range, measured concentrations, and LOEC unreported) (U.S. EPA 2011). As a known metabolic breakdown product of clothianidin, thiamethoxam is assessed under a surrogate approach by using clothianidin data (registered degradate CGA-322704) (U.S. EPA 2011). I found clothianidin and thiamethoxam displayed different community-level trajectories during the application and recovery period, and effects on emergence timing were apparent only for clothianidin. In contrast, imidacloprid and clothianidin had unique community and emergence patterns which suggests that effects vary by neonicotinoid compound and assumptions of equivalent toxicity may under or overestimate community responses.

3.5.2 Impacts on aquatic insect phenology

Emergence phenology represents a balance to optimize growth (i.e., biomass) and the probability of survival. To my knowledge, few studies have documented effects on emergence

timing following neonicotinoid exposure. In one pulsed imidacloprid exposure study, mayfly emergence (*Epeorus sp.* and *Baetis sp.*) increased in response to water concentrations of approximately 0.1 µg/L (Alexander et al. 2008). Although I did not measure biomass or size of the insects in my study, reductions in size compared to control treatment have been previously observed (Alexander et al. 2008), which may affect fitness, female reproductive output, and overall survivorship (Sibley et al. 2001). Similar pulsed applications of 100 µg thiacloprid/L to stream mesocosms caused two surges in chironomid emergence immediately after the two applications which the authors attributed to abundant food resources (Kattwinkel et al. 2016). While food supply can alter growth and development, ultimately leading to earlier emergence, it is unlikely that the seasonal availability of food sources would be solely responsible. Alternatively, chronic exposure to low levels of neonicotinoids as larvae may induce a stress response to accelerate development but may simultaneously disrupt processes related to optimal growth, metamorphosis, and survival.

In wetland environments that are highly variable, insects are adapted to increase the rate of emergence when environmental stressors are high in order to enhance survival probability. Enhanced predation threats can also affect the timing and synchrony of emergence events. A laboratory study specifically addressing the relative influence of predation risk and the neonicotinoid imidacloprid found adult *Chironomus riparius* emerged later in all treatments exposed to a high predation risk (Pestana et al. 2009b). The predatory taxa sampled from the emergence traps were restricted to a few odonate species, *Chaoborus americanus*, dytiscid beetles, and, to some extent, Tanypodinae chironomids. The taxa considered as prey items for *Chaoborus americanus* and Tanypodinae chironomids include copepods, cladocerans, and *Tubifex sp.* worms (Swift and Fedorenko 1975; Baker and McLachlan 1979). Dytiscid beetle larvae are cited as aggressive predators, but adult beetles were rare. Among all the limnocostracans, damselflies were the most abundant predatory taxa (ranging between 0.37 and 15.1% of the total abundance) but are known to select primarily microcrustaceans though some damselfly species can consume chironomid larvae (Thompson 1978).

In some insect mating systems, swarming is a critical lifecycle event that increases the probability of reproduction. Low-level neonicotinoid exposure may induce timing alterations to swarming events (i.e., premature swarming or futile searching for mates), adding stress to this time-sensitive mating tactic. Among the insect taxa that utilize swarms and exhibit proterandry

(males emerge slightly before females) are the Chironomidae. On average, neonicotinoid treatments were 13-days (± 2 S.E.) earlier in emergence compared to controls. Chironomid adults are short-lived and benefit from specific timing emergence events to enhance the probability of finding a mate. Natural and pesticide induced constraints on synchrony, paired with recent findings demonstrating skewed sex ratios from neonicotinoid exposure (Chapter 2), may reduce the chance of finding a mate thus exacerbating reproductive stress, potentially affecting population stability. Factors like wind (i.e., increased adult dispersal) and proximity to unaffected wetlands may be important to mitigate these risks.

One potential mechanism for the observed effects on emergence timing is that neonicotinoids may disrupt insect growth hormone homeostasis. Insect hormones regulate a number of functions critical to growth and development including molt and metamorphosis and successful transitioning from larva to adult (termed “ecdysis”) (Soin and Smagghe 2007). Failure to complete ecdysis and premature adult emergence are two common sub-lethal effects induced by insect growth regulating insecticides. For example, mosquito larvicides which mimic ecdysteroid agonists target mosquito larvae undergoing metamorphosis where, upon ecdysis, become trapped in their exuvia (Beckage et al. 2004). Similar effects were observed in chironomids and culicids exposed to imidacloprid under controlled conditions (Chapter 2, Song et al. 1997), suggesting similar physiological complications arising from chronic imidacloprid exposure. It is also possible that neonicotinoid-induced acceleration of the developmental period may interfere with chitin synthesis. Cytochrome P-450, the main detoxification enzymes for neonicotinoids, plays a critical role during hydroxylation in the biosynthesis of ecdysteroids during the final immature larval instar stage (Rewitz et al. 2006).

Phenological plasticity is a fundamental trait for sustaining community-level interactions and supporting biodiversity (Ovaskainen et al. 2013). Changes in insect phenology can have consequences for insectivorous breeding birds in temperate regions, despite some adaptability of northern population to these changes in food resource availability (Charmantier et al. 2008; Gurney et al. 2017). However, a mismatch in peak insect abundance and the avian chick-rearing period may have detrimental long-term impacts (Hansson et al. 2014). Birds, especially long distance migrants, are likely most sensitive to alterations in insect emergence, such that incongruence of phenological events between insect and avian taxa (Both et al. 2009) may be further exasperated by pesticides. Climate change and the neonicotinoid data presented in the

current study indicate that both drivers can simultaneously advance the timing of insect development to the potential detriment of insectivorous consumers that may be unable to adjust their timing of breeding.

3.5.3 Concluding remarks

Collectively, the limnocorral data presented here indicate that emerging wetland insect communities are influenced by low-level, chronic imidacloprid and clothianidin exposure, albeit in a subtle manner. Like other studies ranking the relative toxic effects of neonicotinoid active ingredients, imidacloprid appeared to exert greater effects than clothianidin and thiamethoxam. Phenology and community structure may be compromised by imidacloprid and clothianidin concentrations that exceed current chronic aquatic life benchmarks. The neonicotinoid mesocosm literature suggests that the threshold for significant lethal and severe sub-lethal impairment is approximately 1.0 µg/L. Here, I observed significantly advanced emergence events by Chironomidae and Zygoptera in imidacloprid and clothianidin treatments at concentrations between 0.038-0.44 µg/L. Significant variation was explained by PRC1 in imidacloprid-treated limnocorrals during the recovery period; suggesting effects on community composition may be delayed or persist after the chemical has degraded. Future studies should investigate the physiological relationship between the rate of aquatic insect emergence and sub-lethal neonicotinoid exposure as it relates to ecdysteroid hydroxylation, chitin synthesis, and metamorphosis. In addition, well-designed field studies are still needed to explore effects of widespread contamination of surface waters with neonicotinoid insecticides in combination with other agricultural and climatic stressors which may alter community dynamics across the wider ecosystem including aquatic and terrestrial insectivorous consumers.

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PREFACE TO CHAPTER 4

Existing aquatic mesocosm experiments for neonicotinoids primarily test for effect on aquatic communities after exposure to imidacloprid. Clothianidin is one of the most widely used insecticides in North America, and there is a need for more data on its effects to aquatic insect communities. To further advance our knowledge on the community effects of clothianidin and gain greater insight into the applications of wetland limnocorrals, the objectives were to: 1) determine the effects of the neonicotinoid insecticide, clothianidin on aquatic insect abundance and composition during the exposure and during recovery where the dosing regime and concentration range mirrored environmental monitoring data collected in the region; and 2) to highlight the scope and limitations of mesocosm assessments for interpreting community-level effects in the field. Other co-authors include Dr. John Headley and Mr. Kerry Peru, who contributed resources for neonicotinoid chemical analyses.

CHAPTER 4: CAPTURING ENVIRONMENTAL REALISM IN HIGHER-TIER INSECTICIDE ASSESSMENTS: A CASE STUDY WITH CLOTHIANIDIN APPLICATION TO WETLAND LIMNOCORRALS

4.1 Abstract

Limnocorrals, or *in situ* aquatic enclosures, are self-contained, experimental units within a natural aquatic ecosystem and are useful for testing community responses to pesticides under field conditions. Here, a 7-week study was implemented to investigate the effects of clothianidin, a neonicotinoid insecticide, on emergent aquatic insect abundance and community composition. Limnocorrals were dosed with four pulses at weekly intervals with environmentally relevant clothianidin concentrations: 0.00, 0.05, 0.17, 0.50, and 1.70 $\mu\text{g/L}$ followed by a 3-week recovery period. Members of the family Chironomidae (Order Diptera) were dominant in all limnocorrals (83% of the total emergence). Principal response curves showed a shift in community composition in the highest nominal dose group approximately 7 to 10 days post-treatment, primarily driven by the greater relative abundance of *Psectrocladius sp.*, *Ablabesmyia peleensis*, *Chironomus sp.*, and *Cricotopus sylvestris*. Further analyses using the *SPEAR_{mesocosm}* indicator system detected a greater abundance of sensitive aquatic insect taxa during the recovery period – likely due to seasonal changes in phenology. All treatment limnocorrals resembled the controls two weeks into the recovery period. These data add to a limited body of literature available on the chronic effects of clothianidin to aquatic insects using more environmentally relevant field conditions. Moreover, I highlight the value of these higher-tier risk assessment approaches, but also the design limitations associated with the need for greater replication due to spatial and temporal heterogeneity which challenge all mesocosm studies.

4.2 Introduction

Pesticides and other organic pollutants continue to threaten the integrity of aquatic ecosystems worldwide (Malaj et al. 2012). Ecotoxicologists can employ multiple approaches to determine the environmental risk of synthetic pesticides that include laboratory and field-based techniques (Connon et al. 2012). One of the most common tools, single-species laboratory tests, are frequently criticized for their lack of relevance to natural environmental conditions which may influence the fate, persistence, and bioavailability of a contaminant to natural communities (Cairns 1983). Semi-controlled experimental systems such as mesocosms are commonly used to counter this lack of environmental realism and have been widely used to evaluate contaminant

effects at higher levels of biological organization (Urban 1994). Along the continuum of experimental designs used in pesticide ecotoxicology, mesocosms provide greater ecological realism, allow for replication, and provide accessible experimental units for measuring detailed ecological and/or toxicological endpoints compared to alternative techniques (Rohr and Hoverman 2006, Caquet 2013).

One recent concern in the risk assessment for newer neonicotinoid insecticides, is the lack of higher tiered community assessments. Clothianidin is a second-generation neonicotinoid which is under-studied compared to other neonicotinoid compounds and displays similar toxicity as the older imidacloprid to non-target aquatic insects under laboratory settings (Chapter 2). To date, only two studies report laboratory chronic clothianidin EC50 (emergence) values for insect species, *Chironomus dilutus* having a 40-day EC50 of 0.28 µg/L and *Chironomus riparius* having a 28-day EC50 of 1.0 µg/L (Chapter 2, PMRA 2011). A preliminary report by the U.S. EPA cites a clothianidin NOEC concentration of 1.0 µg/L; the most apparent adverse effects to the invertebrate species assemblage, specifically chironomid larvae, occurred 7 days post-application with recovery displayed after 28-days (US EPA 2013). A microcosm study conducted by the European Health & Consumer Protection Directorate reports an ecologically acceptable concentration (EAC) for clothianidin of 3.1 µg/L a.i. (EU 2005). Field surveys of neonicotinoids in natural surface waters have detected mean clothianidin concentrations from the Canadian Prairies and agricultural fields in Ontario, Canada, ranging from 0.142 to 4.18 µg/L (Main et al. 2014, Schaafsma et al. 2015, Struger et al. 2017). These studies collectively indicate the potential risk to aquatic insect populations at concentrations frequently detected in surface water bodies and suggest a need for greater attention to more ecologically meaningful endpoints and tests under more natural conditions.

Here, I describe a replicated outdoor mesocosm experiment using limnocorrals, which isolate columns of water and sediment in a natural Prairie wetland with indigenous aquatic insects. The *in situ* limnocorral approach allowed for semi-controlled baseline environmental variation and uniform changes in water physicochemical properties. My objective was to investigate the effects of the neonicotinoid insecticide, clothianidin, on aquatic insect abundance and composition during both exposure and recovery periods. The dosing regime and concentration range was modelled after environmental monitoring data collected in the region (Main et al. 2014), and the biotic responses, aquatic insect emergence (i.e., abundance and

diversity), reflect endpoints identified in the literature as ecologically relevant and sensitive to neonicotinoid exposure (Chapter 2). In addition, I discuss the scope and limitations of mesocosm assessments for interpreting community-level effects of chemical stressors, such as pesticides. I hypothesized that chronic clothianidin exposure will elicit a dose-response on the emerging aquatic insect community composition and abundance.

4.3 Materials and Methods

Fifteen custom-built limnocorrals (1.0 x 1.0 x 1.5 m) were purchased from Curry Industries Ltd. (Winnipeg, MB, Canada). Limnocorrals were installed over a period of several days in early May 2015 within a single Prairie wetland located on St. Denis National Wildlife Area (NWA) in central Saskatchewan, Canada and arranged in a completely randomized block design (designated as Block 1, Block 2, or Block 3). Similar to the methods outlined in Chapter 3, limnocorrals featured fitted aquatic insect emergence traps and Styrofoam floats encased with polyvinyl to maintain buoyancy. Each unit's float was tied to four wooden stakes and the sleeves were anchored and buried in the sediment with galvanized steel chains. The cylindrical sleeve attached to the Styrofoam floats could rise and fall with the gradual changes in water depth throughout the experiment.

4.3.1 Collection of water physicochemical parameters and insects

Neonicotinoid application, water sampling, and insect collecting activities were conducted by canoe. Physicochemical parameters of the water were measured weekly during the 7-week experiment. Dissolved oxygen (mg/L), conductivity ($\mu\text{S}/\text{cm}$), and pH were measured with a YSI (YSI Inc., Yellow Springs, Ohio, USA) electronic water monitoring meter. Mean (\pm SE) measurements among the three experimental blocks for dissolved oxygen (mg/L), conductivity ($\mu\text{S}/\text{cm}$), pH, and depth (m) for the dosing and recovery periods are provide in Table 4.1. Temperature was monitored hourly inside limnocorrals with a HOBO Onset[®] temperature data logger (Bourne, Massachusetts, USA).

Table 4.1 Mean (\pm S.E.) measured water quality parameters in each treatment ($n=3$ limnocorrals per treatment) during the dosing and recovery period.

Parameter ^a	Treatment ($\mu\text{g/L}$)				
	0.00	0.05	0.17	0.50	1.70
Dissolved oxygen ^b	6.2 \pm 0.3	6.4 \pm 0.3	6.8 \pm 0.3	6.2 \pm 0.4	6.0 \pm 0.4
Conductivity ^c	2178 \pm 35.8	2148 \pm 24.5	2231 \pm 42.9	2138 \pm 22.5	2163 \pm 50.6
pH	8.1 \pm 0.05	8.0 \pm 0.07	8.0 \pm 0.05	7.9 \pm 0.06	7.9 \pm 0.04

^aWetland water temperature ($^{\circ}\text{C}$) was 20.8 \pm 0.2 (mean \pm S.E.) during the dosing period and 23.9 \pm 0.2 during the recovery period. On average, wetland measured wetland depth (m) surrounding the limnocorrals ranged from 1.01 to 1.18 during the entire experiment.

^b units in mg/L

^c units in $\mu\text{S/cm}$

Limnocorrals were installed in the study wetland two weeks prior to insecticide application to allow the sediment to fully settle and the invertebrate community to recover from the initial disturbance. The same wetland was used in the previous study (Chapter 3). Emergence traps were fixed to the limnocorrals on day -4 (prior to first application) and remained in place throughout the 7-week experiment. Emerging adult insects were collected every 3 to 4 days. Insects were preserved and stored in 70% ethanol until identified and counted. Adult Chironomidae were identified to the subfamily-level with the taxa of interest (*Ablabesmyia peleensis*, *Chironomus sp.*, *Psectrocladius sp.*, *Cricotopus sylvestris*) identified to at least genus and verified by AquaTax Consulting (D. Parker, pers. comm.).

4.4.2 Neonicotinoid application and analysis

Technical grade clothianidin (99.6% pure) was acquired from Bayer CropScience. Clothianidin was applied to limnocorrals at 0.0 (controls), 0.05, 0.17, 0.50, and 1.70 $\mu\text{g/L}$. The array of doses represent the low-range of concentrations observed in surface water bodies globally and are representative of concentrations detected in Prairie wetlands (Main et al. 2014, 2015). Each of the four clothianidin treatments had three replicates, with the addition of three controls, totaling 15 limnocorrals. Water samples (250 mL) for determination of actual neonicotinoid concentrations were taken immediately before and after each weekly application from all limnocorrals during the 4-week dosing period, then stored in amber bottles at 4 $^{\circ}$ C until analysis. To maintain the target concentrations over the 4-week exposure period, I measured actual concentrations in the limnocorrals immediately post dosing and at 3, 12, 24, and 72 hours post-application during Week 0 and Week 3 to determine clothianidin dissipation rates (Appendix B: S.I. Figure 1). Stock solutions (1.0 mg/L) were prepared in 1-L volumetric flasks

with reverse osmosis water (Barnstead® Diamond™ NANOpure, 18.2 MV/cm), transferred into 250-mL bottles, transported in coolers to the study site, and poured directly into the appropriate limnocorral during each dosing event. Water in the limnocorrals was then gently stirred to aid the mixing of the compound within the water column. The volume of each limnocorral was calculated by measuring the pond depth (i.e., $L \times W \times H$) and estimated to contain 1,000-L. Individual volume measurements combined with predicted degradation rates were used to adjust weekly dosing regimens.

Water samples were analyzed at the National Hydrology Research Centre, Environment and Climate Change Canada in Saskatoon, SK, Canada. Chemical analysis included clothianidin and three other neonicotinoid insecticides: imidacloprid, thiamethoxam and acetamiprid. The latter two neonicotinoids were not detected in any samples, but trace amounts of imidacloprid were detected (see data analysis). Full sample extraction, solid-phase extraction, and LC-MS/MS methodologies have been previously described in Main et al. (2014). Quality assurance/quality control (QA/QC) data included recoveries of internal standards (%), limits of quantification (LOQ), and limits of detection (LOD). Mean (SEM) QA/QC were as follows: imidacloprid (Recovery = 85.7 ± 3.2 %; LOQ = 6.5 ± 0.9 ng/L; LOD = 2.2 ± 0.3 ng/L), clothianidin (Recovery = 74.6 ± 2.2 %; LOQ = 7.8 ± 1.0 ng/L; LOD = 2.6 ± 0.3 ng/L), thiamethoxam (Recovery = 86.8 ± 2.4 %; LOQ = 11.5 ± 1.0 ng/L; LOD = 3.8 ± 0.3 ng/L), and acetamiprid (Recovery = 89.4 ± 3.0 %; LOQ = 3.3 ± 0.4 ng/L; LOD = 1.1 ± 0.1 ng/L). Blank samples were all below detection and all neonicotinoid data are reported as recovery corrected.

4.3.3 Data analysis

A one-way analysis of variance compared water physicochemical properties across experimental blocks and treatments ($\alpha = 0.05$ level of confidence). Normality was assessed by Shapiro-Wilk test. Tests were performed using SigmaPlot™ Version 13.0 (Systat Software, Inc., San Jose, California, USA).

After the start of the experiment, unexpected trace amounts of imidacloprid (0.01 to 0.10 $\mu\text{g/L}$) were detected in the wetland and in limnocorrals (Table 4.2), presumably residues from the previous year's experiment (Chapter 3). The presence of imidacloprid and clothianidin required standardizing the exposures to total neonicotinoid concentration of the mixture. Therefore, toxic equivalence factors (TEFs) relative to imidacloprid (i.e., TEF imidacloprid = 1) were used to calculate the combined toxic equivalency quotients (TEQs) following the methods

outlined in Cavallaro et al. (2017). Neonicotinoid concentrations (as TEQ in $\mu\text{g/L}$ of imidacloprid; Equation 1) were log-transformed prior to statistical analysis.

$$\begin{aligned} \text{IMI toxic equivalence} = & 1.0[\text{IMI conc.}] + 1.62[\text{CLO conc.}] \\ & + 0.11[\text{THX conc.}] \end{aligned} \quad (1)$$

Descriptive aquatic insect biotic responses included total emergence abundance, taxa richness, and Shannon-Wiener's diversity index (H' ; Equation 2) which integrates proportional diversity based on taxa richness and abundance.

$$H' = - \sum_{i=1}^s p_i \ln p_i \quad (2)$$

; where s is the total number of species (i.e., distinct taxa for the present study) and p_i is the proportion of s made up of the i th species. The effects of the clothianidin treatments relative to controls on emerging adult insects were analyzed by the Principal Response Curves (PRC) method developed by van den Brink and Ter Braak (1999). PRC models are used to assess community effects by discerning the fraction of variance between the factors time and treatment (repeated-measures). With the primary focus on shifts in community composition, the PRC method performs a time-series partial redundancy analysis (pRDA); the interaction between sampling time and treatment act as explanatory variables and sampling times as covariates (van den Brink et al. 2009). One of the advantages of the PRC method is the readability of the graphical representation, discerning treatment effects from the first Principal Component (PRC1; y-axis) over time (x-axis). The canonical coefficients (C_{dt}) are plotted over time so the spatial deviations from the control mean (center line) can be compared. To assess the statistical significance of the PRC, a Monte Carlo permutation test (999 permutations) was performed using the limnocorral taxa counts ($\log(x + 1)$ -transformed). The PRC method identifies the variance partitioning by reporting the explanatory content (in %) of the first canonical axis (van den Brink and Ter Braak 1999). The PRC analysis was performed using R version 3.4.1 (R Core Team 2017) package “vegan” (Oksanen et al. 2011).

Further calculation of TUs (toxic units) allowed for the use of the indicator system: $SPEAR_{mesocosm}$ (Liess and von der Ohe 2005). Individual TUs are commonly derived from acute

(e.g., 48 h) LC50 values for a standard test organism, e.g., *Daphnia sp.* (Beketov and Liess 2008a). However, *Daphnia sp.* are particularly insensitive to neonicotinoid insecticides (See, Morrissey et al. 2015), and more appropriate model species such as *Chironomus sp.* are recommended (Schäfer et al. 2013, Münze et al. 2015). The present study used the standard test organism *Chironomus dilutus* and endpoints generated from the 28-day toxicity tests of Stoughton et al. (2008). $TU_{C. dilutus}$ was calculated with the TEQ $\mu\text{g/L}$ of imidacloprid. Following the formula (Equation 3) provided in Liess and von der Ohe (2005):

$$TU_{(C. dilutus)} = \max_{i=1}^n \left[\log \left(\frac{c_i}{LC_{50i}} \right) \right] \quad (3)$$

where $TU_{C. dilutus}$ is the highest value of n neonicotinoid detected in each mesocosm, c_i is the measured concentration (log(TEQ $\mu\text{g/L}$ of imidacloprid)), and LC50 is the corresponding median lethal concentration for *C. dilutus* for imidacloprid. $SPEAR_{mesocosm}$ is a modified calculation derived from $SPEAR_{pesticide}$ (Liess and von der Ohe 2005). Based on the dichotomy of ‘species at risk’ and ‘species not at risk’, the SPEAR indices utilize trait-based approaches to assign taxa sensitivity to a toxicant. Selected biological traits considered in the calculation (see, SPEAR Calculator application; UFZ 2014) are generation time, reproduction mode, dispersal capacity, and physiological sensitivity (Schäfer and Liess 2013).

$$SPEAR_{mesocosm} = \frac{\sum_{i=1}^n \log(x_i + 1) * y_i}{\sum_{i=1}^n \log(x_i + 1)} * 100 \quad (4)$$

; where n is the number of taxa, x_i is the abundance of taxon i , and y is 1 if taxon i is classified as ‘species at risk’ and 0 if it is not. $SPEAR_{mesocosm}$ values were compared to TU values from each limnocorral, respectively. Using the SPEAR Calculator desktop application (UFZ 2014), relative abundances of ‘species at risk’ were determined (Equation 4) and tested using linear regression analysis.

4.4 Results

4.4.1 Physicochemical water quality parameters

Wetland water temperature (°C) averaged 20.8 ± 0.2 (mean \pm S.E.) during the dosing period and 23.9 ± 0.2 during the recovery period. Measured water depth (m) surrounding the limnocorrals ranged from 1.01 to 1.18 during the entire experiment. Temperature inside the limnocorrals during the dosing period ranged from 4.3° C (May) to 39.8° C (July) with a mean of 20.8° C. Mean water quality parameters among the five treatments (including controls) did not differ significantly for dissolved oxygen ($F=0.90$, d.f.=4, $P=0.468$), conductivity ($H=2.92$, d.f.=4, $P=0.572$), and pH ($F=2.35$, d.f.=4, $P=0.671$) (Table 4.1). The three experimental blocks were not significantly different.

4.4.2 Neonicotinoid concentrations

The measured neonicotinoid concentrations exceeded the nominal target concentrations of 0.05 $\mu\text{g/L}$, 0.17 $\mu\text{g/L}$, 0.50 $\mu\text{g/L}$, and 1.70 $\mu\text{g/L}$ clothianidin for the four weeks of application, and were consistently over by 35-60%. Measured peak clothianidin concentrations immediately after dosing averaged 0.08, 0.23, 0.70, and 2.37 $\mu\text{g/L}$, for the low to high treatments (Table 4.2). These concentrations of clothianidin declined rapidly after application (Appendix B: S.I. Figure 1). The highest treatment group, 2.37 $\mu\text{g/L}$, experienced the greatest initial loss of $40.5 \pm 4.5\%$ within 3 hours post-application during Week 0. Mean degradation at 24 hours post-dosing for the same treatment was $30.4 \pm 23.0\%$ (Week 0) and $38.7 \pm 17.1\%$ (Week 3).

Table 4.2 Summary of mean (\pm S.E.) measured neonicotinoid concentrations ($\mu\text{g/L}$) immediately post-application of clothianidin (CLO; $n=4$ weeks) at four concentrations. Exposures were standardized by toxic equivalency (TEQs) (See, Chapter 2) due to presence of trace amounts of imidacloprid (IMI) in the clothianidin-treated limnocorrals.

Nominal CLO treatment ($\mu\text{g/L}$)	Mean measured CLO concentration ($\mu\text{g/L}$)	CLO TEF ^a	Mean measured IMI concentration ($\mu\text{g/L}$)	TEQ ^b ($\mu\text{g/L}$ of IMI)	Limnocorral number
0.00	0.02 (\pm 0.02)	1.62	0.01 (\pm 0.01)	0.04	4
	0.00 (\pm 0.00)	1.62	0.01 (\pm 0.01)	0.01	8
	0.00 (\pm 0.00)	1.62	0.01 (\pm 0.01)	0.01	15
0.05	0.09 (\pm 0.02)	1.62	0.01 (\pm 0.02)	0.16	7
	0.08 (\pm 0.04)	1.62	0.01 (\pm 0.01)	0.14	11
	0.08 (\pm 0.02)	1.62	0.10 (\pm 0.04)	0.22	1
0.17	0.14 (\pm 0.04)	1.62	0.04 (\pm 0.02)	0.27	2
	0.37 (\pm 0.18)	1.62	0.05 (\pm 0.03)	0.65	10
	0.19 (\pm 0.07)	1.62	0.01 (\pm 0.01)	0.32	12
0.50	0.52 (\pm 0.15)	1.62	0.03 (\pm 0.02)	0.87	5
	0.69 (\pm 0.26)	1.62	0.02 (\pm 0.01)	1.14	9
	0.91 (\pm 0.35)	1.62	0.03 (\pm 0.02)	1.50	14
1.70	2.57 (\pm 0.32)	1.62	0.08 (\pm 0.06)	4.24	3
	1.70 (\pm 0.52)	1.62	0.03 (\pm 0.01)	2.79	6
	2.85 (\pm 0.49)	1.62	0.02 (\pm 0.01)	4.64	13

^aTEF=toxic equivalency factor

^bTEQ=toxic equivalency quotient

4.4.3 Insect abundance and community structure

Insect emergence in limnocorrals was dominated by Chironomidae which accounted for 83% of the total community composition. Eight distinct taxa represented 96% of the total abundance in the limnocorrals and included Orthoclaadiinae (38%), Chironominae (33%), Tanypodinae (12%), Lestidae (3.3%), Coenagrionidae (3.2%), Chaoboridae (2.6%), Muscidae (2.1%), and Scelionidae (1.6%). The PRC analyses showed that 42.3% of the overall variance to the total dataset was explained by time (days). Treatment accounted for 16.4% of the variance, whereas the variance in taxa composition between limnocorral replicates was 14.3%. Total emerged insects and diversity of insect taxa did not significantly differ between the control and neonicotinoid treatments (PRC1 Monte Carlo permutation tests, F-ratio=19.9, p=0.449). Taxa with a species weight score above 0.3 or below -0.3 are represented in Figure 4.1.

A shift in PRC1 was observed in the highest treatment of 2.37 µg/L of clothianidin approximately 7 to 10 days into the dosing period. This trend was influenced by the positive species weights (species scores) exhibited by the species *Psectrocladius sp.* (2.77), *Ablabesmyia peleensis* (1.81), *Chironomus sp.* (1.49), *Cricotopus sylvestris* (1.00), and other Orthoclaadiinae (0.32) and Tantarsini (1.48) taxa. All treatments exhibited a general departure for the control line between days 10 to 20 after the third application. The damselfly *Lestes disjunctus* was the most negatively affected taxon with a species score of -0.34; the remaining odonate taxa species scores were negligible (range from *Enallagma annexum* -0.02 to *Aeshna interrupta* 0.001). The community composition for lower treatment concentrations mostly followed a similar pattern throughout the dosing and recovery periods. The only exception was observed in the 0.70 µg/L treatment which saw a surge in chironomids 21-28 days into the experiment. Two weeks into the recovery period, the community composition resembled the controls with the canonical coefficients (C_{dt}) alternating slightly above and below the “0” PRC1 line (control).

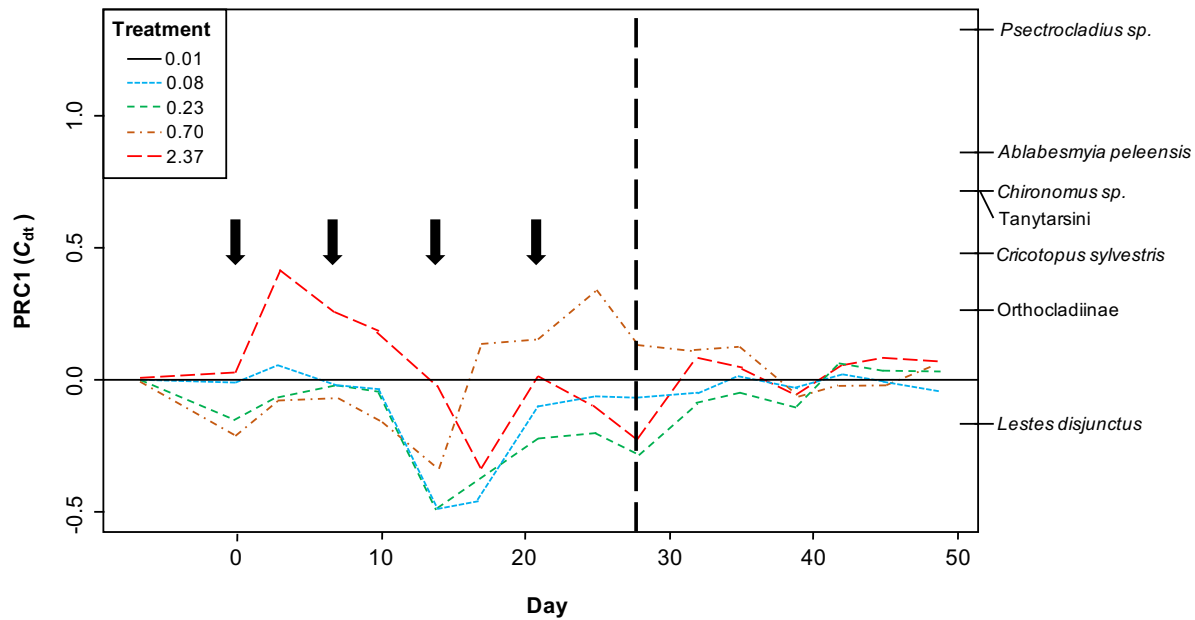


Figure 4.1 Principal response curves (PRC1) showing the emerging insect community response of replicated limnocorrals (Y-axis determined by canonical coefficients C_{dt}) over time for clothianidin at four different measured peaked doses of 0.08 $\mu\text{g/L}$, 0.23 $\mu\text{g/L}$, 0.70 $\mu\text{g/L}$, and 2.37 $\mu\text{g/L}$, relative to the control limnocorrals (“0” line). The scores represented on the species weight axis (right) are above 0.3 or below -0.3; species between these values were omitted for clarity. Values near “0” are considered more similar to the control community (i.e., taxa with positive values represent an increase in abundance relative to the controls, whereas negative values represent a decrease in abundance relative to controls). The black arrows represent the four times clothianidin was applied to the treated limnocorrals and the vertical dashed line indicates the start of the recovery period.

The data revealed a low abundance of SPEAR “species at risk”, or taxa vulnerable to neonicotinoid exposure, during the dosing period, with high variability in community composition among limnocorrals (Table 4.3). $SPEAR_{mesocosm}$ values during the dosing ($r^2 < 0.001$, $P = 0.453$) and recovery periods ($r^2 < 0.001$, $P = 0.502$) were not related to measured TU (log(TEQ $\mu\text{g/L}$ of imidacloprid)). The recovery period on average displayed a greater relative abundance of sensitive taxa compared to the dosing period. However, this effect was present in all limnocorrals indicating this was likely due to seasonal emergence patterns of several sensitive taxa (Table 4.3).

Table 4.3 Comparison of emerging aquatic insect community metrics (mean \pm S.E.) after exposure to and recovery from different clothianidin treatments. This included $SPEAR_{mesocosm}$ values (i.e., proportion of species at risk) from each treatment during the dosing and recovery period.

Nominal	Measured ^a	TU _(<i>C. dilutus</i>) ^{b,c}	Period	$SPEAR_{mesocosm}$	Cumulative abundance	Shannon's Diversity
0	0.01	-1.78 ± 0.12	Dosing	9.71 ± 5.85	303 ± 124	1.52 ± 0.08
			Recovery	30.60 ± 5.47	94 ± 33	2.21 ± 0.01
0.05	0.08	-0.83 ± 0.10	Dosing	15.84 ± 4.60	160 ± 47	1.84 ± 0.03
			Recovery	25.98 ± 3.73	79 ± 24	2.01 ± 0.04
0.17	0.23	-0.46 ± 0.09	Dosing	10.40 ± 1.65	140 ± 28	1.75 ± 0.07
			Recovery	25.39 ± 1.60	74 ± 7	2.26 ± 0.04
0.5	0.7	-0.02 ± 0.12	Dosing	12.89 ± 2.50	220 ± 55	1.68 ± 0.12
			Recovery	34.72 ± 5.46	101 ± 25	2.37 ± 0.06
1.17	2.37	0.58 ± 0.07	Dosing	14.98 ± 1.15	273 ± 54	1.76 ± 0.06
			Recovery	21.06 ± 10.81	85 ± 31	2.29 ± 0.14

^aMean post-application clothianidin concentration ($n=4$ weeks of clothianidin application)

^bGenerated using the 28-day EC50 from Stoughton et al. (2008)

^cSee Equation 2

4.5 Discussion

Results from single-species laboratory toxicity tests indicating the potential risks of non-target aquatic insects to neonicotinoids has led to several mesocosm-based studies that continue to increase in number (Sanchez-Bayo et al. 2016). Here, I present replicated limnocorral data conducted in a natural wetland on the Canadian Prairies – a critical region of the continent to a number of transient and resident wetland-dependent species (Doherty et al. 2016). Observations contrasted from a limnocorral study conducted one year prior (Chapter 3), as clothianidin treatments demonstrated no significant effects to emerging insect community structure and abundance. Similarly, apart from a surge in Orthoclaadiinae chironomid emergence after two applications of clothianidin at 2.37 $\mu\text{g/L}$, the data presented here describe no substantial effects at the tested concentrations. Since clothianidin is one of the primary neonicotinoids used on the Canadian Prairies on the most economically viable crops, such as canola and cereals (Main et al. 2014), a more detailed understanding of the effects of this neonicotinoid to aquatic insect

communities was needed. A recent laboratory comparison between imidacloprid and clothianidin reported similar chronic toxicity values for *Chironomus dilutus* (Chapter 2). Taxonomic data from wetland habitat degradation studies show that chironomids are tolerant of the effects of intensive agricultural practices and make up a major proportion of the insect taxa in Prairie wetlands (Chipps et al. 2006, Campbell et al. 2009, Wrubleski and Ross 2011). My experimental design used an *in situ* limnocorral approach within a single wetland which offers greater control and consistency in water physicochemistry and temperature among treatments. Moreover, this strategy allowed for replication and testing of a range of clothianidin exposures held reasonably constant over time to measure important biotic responses to wetland invertebrate communities.

4.5.1 Clothianidin laboratory and field mesocosm toxicity data

Relative to other neonicotinoids, data on clothianidin toxicity to aquatic organisms has largely been overlooked in the primary literature and lacking detailed methodology in government reports. Reports from the European Commission Health & Consumer Protection Directorate and the U.S. EPA on clothianidin mesocosm tests do not provide comprehensive context or details on specific experimental approaches (i.e., study system, dose ranges or regimes, sampling techniques), making it a challenge to evaluate (EU 2005, US EPA 2013). Fortunately, other mesocosm studies give detailed descriptions of methodology which allows for more appropriate comparison and/or discussion (See, Kasai et al. 2016, Miles et al. 2017, Balsey and Goulson 2018). Moreover, other pesticide studies directly examining the differences between laboratory toxicity tests and mesocosm results are limited in the literature with only a few sentinel studies providing a comprehensive comparison (e.g., Wijngaarden et al. 1996, Schroer et al. 2004, Hose and van den Brink 2004).

Although scarce for most aquatic organisms, clothianidin toxicity data do exist for chironomids in both laboratory and field settings. *Chironomus dilutus* and *Chironomus riparius*, two cosmopolitan chironomid species, are commonly used in single-species toxicity tests and represent appropriate model organisms for Prairie wetlands due to their frequent occurrence in the region. *C. riparius* is generally more tolerant to insecticide contamination than *C. dilutus* (Watts and Pascoe 2000). Chronic clothianidin toxicity endpoints (EC50 [emergence inhibition]) for *C. dilutus* and *C. riparius* are 0.28 and 1.0 µg/L, respectively (Chapter 2, PMRA 2011). Acute (48-96 hr) *C. riparius* toxicity tests report LC50 values between 2.41 µg/L to 29.0 µg/L (EU 2005, De Perre et al. 2015). Laboratory exposures are often held constant during the entire

test, whereas field studies observe dissipation. In the present study, pulsed, weekly clothianidin applications and subsequent degradation led to lower cumulative exposure (Appendix B: S.I. Figure 1). A separate microcosm assessment found that clothianidin had adverse effects on the chironomid population at nominal concentrations exceeding 10 µg/L, conservatively an order of magnitude higher than the highest dose group used here (Balsey and Goulson 2018). Kasai et al. (2016) observed slight increases in chironomid larvae relative to the controls after planting clothianidin-treated rice plant seedlings (2.69 µg/L two hours post-seeding). Overall, the collection of semi-controlled field studies support that under field conditions chironomids appear to be more tolerant to clothianidin than under laboratory conditions.

Studies using 'cosm-type experimental units are limited for clothianidin, and have used relatively high exposure concentrations to induce adverse effects. For example, an outdoor mesocosm test using inoculated cattle tanks found significant effects on the invertebrate community at 352 µg/L, specifically on the predaceous taxa: *Notonecta undulata* and *Belostoma flumineum*. Sub-lethal effects on predation rates were dose-dependent and negative effects occurred at approximately 5 µg/L (Miles et al. 2017). Due to their high mobility (strong flying capabilities), *N. undulata* and *B. flumineum* populations are unlikely to be adversely affected by neonicotinoid contamination.

The inclusion of natural stressors, discontinuous exposure, interspecific interactions and behavioral effects are elements of environmental realism often omitted in laboratory tests in favour of obtaining precise toxicity values. Mesocosm studies on the other hand offer greater biological complexity and realism. Here, I aimed to determine the community-level effects of clothianidin on emerging aquatic insects. There were no significant community effects indicated by PRCs or the $SPEAR_{mesocosm}$ approach at the range of exposures tested. After the third clothianidin application, all treatments exhibited a general negative trend in the canonical coefficients; this was driven by a relative decrease in damselfly *Lestes disjunctus* abundance (species score = -0.34). Collectively, the field data presented here reinforce the value of differentiating experimental approaches (i.e., laboratory vs. field) to fully realize the potential ecotoxicological effects of a contaminant and the stochasticity associated with the natural environment.

The two approaches used in the present study to analyze insect community data, PRC and $SPEAR_{mesocosm}$, are highly debated in the ecotoxicology literature (Liess and Beketov 2012, van

den Brink and ter Brack 2012). *SPEAR_{mesocosm}* is a univariate analysis that solely relies on a set of *a priori* designations. The responses to species traits (e.g., generation time, reproduction mode, dispersal capacity, and physiological sensitivity) and the stressor in question can vary considerably. PRC in a multivariate approach that considers the community variance captured in the species data by the treatment, without *a priori* sensitivity considerations. Both approaches did not detect significant treatment effects; however, there were appreciable differences regarding sensitivity in the Chironomidae data. For example, the PRC data show Orthocladiinae and Chironominae display deviations in sensitivity to neonicotinoid exposure with Orthocladiinae being more tolerant. The *SPEAR* characterization grades these taxa with similar scores (i.e., similar sensitivity to insecticides), contradicting the observed differences in the PRC analyses. In combination, these two approaches shed light on the complexities on interpreting and disseminating community data in an ecotoxicological context.

4.5.2 Application of wetland limnocorrals

Deciding on the type of mesocosm to use for specific research questions can be an arduous task, and some approaches may be given preference due to budget constraints, existing equipment, or consideration of the complexity of statistical analyses. Inoculated mesocosms are stocked artificially with a known number of test organisms and a finite number of species, depending on the goals of the study, and pseudo-inoculated techniques aim to examine the natural communities and stocked test organisms. In the present study, *in situ* limnocorrals were used to isolate columns of water in a wetland ecosystem representing those frequently exposed to neonicotinoid contamination over longer repeated exposures (chronic). Limnocorrals within a single water body are impermeable and only connected to the larger wetland via sediment pore water, permitting each limnocorral to maintain similar physicochemical water parameters, temperature fluctuations, and indigenous benthic macroinvertebrates while allowing for different pesticide exposure concentrations. One variable that challenged uniformity among limnocorrals was the heterogeneity of the macroinvertebrate community within the Prairie wetland. Spatial distribution of macrophytes can also challenge mesocosm systems (Beketov and Liess 2008b).

Patchy distribution of overwintering larvae and macrophytes, and preferential sediment microhabitats may influence biotic responses even when precautionary measures are taken (i.e., experimental block design). I also used a closed system without recolonization by keeping emergence traps on the enclosures throughout the experiment. During the previous summer, a

similar study was conducted where every 12 days the emergence traps, normally fixed to the top of the experimental units, were removed to allow for adult insects to oviposit in the limnocorrals. Anecdotally, this appeared advantageous to earlier emerging *Chironomus sp.* (Chironomidae: Chironominae); the subfamily Chironominae constituted the greatest proportion of adult chironomids in 2014. In the present study, recolonization was not permitted and the emergence traps remained fixed to the experimental units for the dosing and recovery periods. In this experiment, the subfamily Orthoclaadiinae made up the majority of the chironomid community with one species, *Cricotopus sylvestris*, representing 36.5% of the Orthoclaadiinae emergence. Previous accounts of the *Cricotopus* genus describe a reliance on epiphytic pupation sites on submerged aquatic vegetation such as *Myriophyllum spicatum* and *Ceratophyllum sp.* (Menzie 1981, Tarkowska-Kukuryk 2010).

Since there was variability in the vegetation structure and presence of invertebrate species within each limnocorral, dynamic interactions may lead to greater variance and lower statistical power when compared to more controlled mesocosms experiments (e.g., inoculated tanks) or multi-species laboratory tests (Winkler and Van Buskirk 2012). As a consequence, I saw a few important trends driven by the abundance of chironomids but individual limnocorral variance suggests the need for greater numbers of replicates and more expansive concentration range. Control limnocorrals produced greater overall cumulative abundance and the highest treatment demonstrated a surge in chironomids early during the dosing period. On average, the coefficient of variation (CV), or relative standard deviation (i.e., standard deviation divided by the mean) of chironomid abundance during the dosing period among treatments was 52%, whereas the mean CV for Shannon's diversity index and taxa richness were 4% and 12%, respectively. Increased replication and higher doses may reduce this variability in abundance.

The ability to discern treatment effects (i.e., signal-to-noise ratio) largely relies on the strength of the treatment, experimental design (i.e., replicates), and variance not explained by the tested treatment (de Castro et al. 2017). Power analyses are often ignored when designing a mesocosm experiment due to overriding logistical and budgetary constraints. Standard inferential statistics are fundamentally impractical in mesocosm studies, generally forecasting a need for high replication per treatment. Considering the present study with three replicates per treatment, the mean effect size between the controls and clothianidin treatments was 0.64 (i.e., [mean of experimental group – mean of control group]/standard deviation) with a range between 0.1 (weak

effects) to 1.0 (strong effects). If we target a statistical power of 0.8 (i.e., 80% chance of observing a statistically significant response with that experimental design) with the same effect size and set α to 0.05, the required number of replicates per treatment would be 11, which would have been impossible for this limnocorral experiment.

4.5.3 Regulatory implications

Mesocosm studies, if well designed and testing the range of effect concentrations seen in laboratory settings, have the potential to validate effects under more relevant and complex field exposures scenarios. Clements et al. (2012) articulates the importance of context dependence when interpreting data derived from more complex experimental scenarios. The 2014 (Chapter 3) and 2015 limnocorral experiments displayed different responses among clothianidin treatments. In practice, the results of field-mesocosm experiments may potentially display effects which lead to under- or over-estimation of field effects based on how representative the experimental system is compared to the natural system of concern (De Jong et al. 2008). I speculate that the inability to allow for recolonization in the present study may have been one of the reasons for the lack of congruent responses. In both years, I observed a predominantly chironomid community emerging from the limnocorrals; however, Chironominae was more abundant in 2014 and Orthocladiinae in 2015. While abiotic variables are well-represented in these condensed experimental systems (Dzialowski et al. 2014), one of the more challenging aspects of using aquatic mesocosms is the potential for high biological variability between replicates (as seen in this study), trading statistical power for environmental realism (Liber et al. 1998). These well-accepted trade-offs are recognized and will continue to be topical in ecology and ecotoxicology studies utilizing mesocosm systems (Winkler and Van Buskirk 2012, Miko et al. 2015).

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PREFACE TO CHAPTER 5

Prairie wetlands surrounded by intensive agriculture are subjected to a variety of anthropogenic stressors. Attempting to characterize their effects on wetland biota is compounded by the natural hydrologic disturbance patterns exhibited by wetlands. Semi-controlled, wetland limnocorrals (i.e., experimental units in a single wetland) offer a greater understanding to community-level impacts; however, I was interested in how neonicotinoids and other abiotic factors affect the invertebrate community at the wetland-level (i.e., entire wetland). Therefore, the objectives of the research described in this chapter were to: 1) conduct a set of 10-week biomonitoring studies to determine the impact of neonicotinoid exposure in combination with water and habitat quality variables on emerging wetland insect communities; 2) assess multiple factors that may influence insect emergence; and 3) assess natural and anthropogenic-induced fluctuations in aquatic insect community composition and abundance. Other co-authors of a yet to be submitted manuscript include Drs. Anson Main, Iain Phillips, and John Headley, and Mr. Kerry Peru. I acknowledge Dr. Anson Main for aiding in data collection and experimental design, Dr. Iain Phillips for contributing taxonomic and insect trapping expertise, and Dr. John Headley, and Mr. Kerry Peru for neonicotinoid chemical analyses.

CHAPTER 5: FACTORS INFLUENCING AQUATIC INSECT EMERGENCE IN PRAIRIE WETLANDS SURROUNDED BY NEONICOTINOID-TREATED CROPLAND

5.1 Abstract

Threats to wetland water quality and secondary insect production in agricultural landscapes are multifaceted and responses are known to vary spatially and temporally. Wetlands on the Canadian Prairies are exposed to multiple water quality and habitat stressors including runoff from neonicotinoid treated seeds. I hypothesized that wetlands on the Canadian Prairies receiving runoff from neonicotinoid seed-treated canola fields may experience reduced abundance or altered communities of emerging adult aquatic insect emergence. Following a biennial canola-cereal crop rotation schedule (2013 and 2015), I sampled 22 semi-permanent wetlands over two growing seasons (11 wetlands per year) in central Saskatchewan, Canada. Over the two sampling years, dipterans from the families Chironomidae, Muscidae and Ceratopogonidae made up the majority of emergent taxa, representing 83-94% of the total emergence. Multivariate analysis of eight water quality and nine wetland habitat variables, revealed that neonicotinoid concentration, turbidity, vegetation disturbance, and continuity of grasses were significant factors influencing the insect community composition. Generalized linear mixed effects models (GLMMs) indicated total insect emergence was correlated to neonicotinoids and vegetation disturbance. Neonicotinoid concentrations displayed an adverse effect over time ($\beta \pm \text{S.E.} = -0.61 \pm 0.14$, $P < 0.001$), whereas greater vegetation disturbance increased total emergence ($\beta \pm \text{S.E.} = 0.34 \pm 0.11$, $P < 0.001$). Overall, I observed consistency between both water and habitat quality GLMMs due to tolerance characteristics of the dominant taxa and highly correlated indicators of wetland degradation (e.g., pesticides, turbidity, nutrients, and vegetation disturbance). Collectively, these multivariate field data provide a better understanding of how complex agricultural management practices, including neonicotinoid use, interact to shape wetland aquatic insect communities.

5.2 Introduction

The North American Prairie landscape encompasses one of the most ecologically diverse grassland-wetland ecosystems on the globe with some localities exceeding 70 wetlands per km² (Baldassarre and Bolden 2006, Ducks Unlimited 2017). Prairie wetland ecosystems are well-adapted to periodic natural disturbance regimes, primarily dictated by the regional hydrology (Euliss et al. 2004). Intensive agricultural practices (e.g., mechanization and synthetic chemical

pest control) impose additional stressors to natural wetland disturbance patterns. Agricultural intensification in the region has led to elevated sedimentation and increased agrochemical contamination (e.g., pesticides and fertilizers) exerting stress to Prairie wetlands (Guntenspergen et al. 2002, Foote and Hornung 2005, Bartzen et al. 2010). Physical habitat alteration (i.e., wetland drainage and/or vegetative buffer zone removal) to expand arable land and plant more crops can degrade the ecosystem services provided by those habitats and can severely impact biodiversity (Dias and Belcher 2015).

Cumulative impacts on surface water quality from agricultural intensification are currently compounded by the extensive presence and persistence of neonicotinoid insecticides (Main et al. 2014, Sánchez-Bayo and Hynes 2014, Székács et al. 2015, Hladik and Kolpin 2016). Neonicotinoids can persist in soils and enter aquatic ecosystems readily from the leaching of seeds and contaminated soils (Jones et al. 2014, Main et al. 2016). Several water sampling surveys in the Prairie region of North America have identified the recurrent and widespread contamination of neonicotinoids in Prairie wetlands (Main et al. 2014, 2015, 2016, Evelsizer and Skopec 2016). Abiotic conditions that prolong the residence of neonicotinoids in the aquatic environment include increased turbidity and low pH (Guzsvany et al. 2006, Lu et al. 2015). Photolysis and alkaline waters will accelerate degradation; however, seasonal changes in light intensity may create a 7- to 8-fold variation in the rate of degradation (Lu et al. 2015). Wetland habitat characteristics also play an important role in the frequency and magnitude of neonicotinoid detection. Neonicotinoid concentrations are higher in wetlands without vegetative buffer zones, and plant community structure strongly reflects the detection frequency and concentration of neonicotinoids in water (Main et al. 2015, 2016).

Among the most abundant invertebrate taxa represented in wetlands, aquatic insects are fundamental components of most aquatic biological communities and nearby terrestrial habitats. During their immature stages, aquatic insects are commonly used as early indicators of environmental quality, specifically responding to the effects of organic pollution and nutrient enrichment in aquatic ecosystems (Catallo et al. 1993, Feio et al. 2007). Imagos, or mature winged adults, exiting the aquatic environment form a key energy link between primary production and secondary consumers (Gratton and Vander Zanden 2009). In a region like the Prairies, the distribution and abundance of aquatic insect populations are greatly influenced by habitat ephemerality and natural habitat gradients (e.g., hydroperiod, primary production)

(Wissinger 1997, Euliss et al. 2004). Consequently, these food subsidies can impact the success of higher trophic level organisms (Johnson et al. 2008). Shorebirds, breeding ducks, amphibians, and semiaquatic rodents will associate with different wetland classes (e.g., seasonal, temporary, semi-permanent) throughout the year which often align with availability of food resources (Kantrud et al. 1989, Niemuth et al. 2006, Bartzen et al. 2017). Aquatic insect emergence inhibition is a sensitive toxicological endpoint used to measure the chronic impacts of neonicotinoids (Chapter 2, Stoughton et al. 2008).

Here, I conducted a 10-week biomonitoring study to determine the impact of chronic neonicotinoid exposure in combination with other agricultural stressors (measured using water quality and wetland habitat variables) on emerging wetland insect communities. Since Prairie wetlands are known to have extreme variations in physical structure, and physicochemical and biological composition (Euliss et al. 2004), I designed the study to assess multiple factors that may influence insect emergence. To our knowledge, this is the first study to track seasonal insect emergence alongside neonicotinoid concentrations in agricultural wetlands to better understand natural and anthropogenic-induced fluctuations in aquatic insect community composition and abundance. I hypothesized that neonicotinoid concentrations will have a greater influence than water quality parameters and wetland habitat characteristics on aquatic insect community composition and abundance.

5.3 Materials and Methods

5.3.1 Site selection, wetland assessments, and physicochemical water parameters

Following a biennial canola-cereal crop rotation schedule, I sampled 22 semi-permanent wetlands in 2013 and 2015 ($n = 11$ per year) situated in clothianidin-treated canola fields (Prosper[®] Bayer CropScience) near Alvena, Saskatchewan, Canada (2.5167° N, 106.0167° W). In 2015, canola fields adjacent to my study fields were also planted with thiamethoxam-treated canola (Helix Xtra[®] Syngenta); however, none directly surrounded any of the monitored wetlands. Based on landowner seeding records, no known application of other neonicotinoid (i.e., imidacloprid or acetamiprid) or other insecticide products had occurred over five years prior to the study. To reduce potential variation from farming practices (i.e., synchronous seeding time, application method and rate), I carried out this study on a single large-scale farming operation. I targeted primarily semi-permanent wetlands of similar size (approximately 0.5 ha) and depth (75 to 100 cm).

In both years, rapid wetland assessments were conducted based on a modified method of Main et al. (2015). Variables recorded included central wetland depth (cm), basin fill (1 [0-25%], 2 [25-50%], 3 [50-75%], 4 [75-100%], 5 [>100%]), vegetative buffer zone width (m), wetland surface area (ha), dominant plant species per wetland zone, concentric vegetation continuity (%), algal cover (%), surface macrophyte cover (%), and vegetation disturbance (%). These variables were selected because of their suitability for predicting the frequency of neonicotinoid detection and concentration (Main et al. 2015).

5.3.2 Neonicotinoid water sampling and physicochemical water quality parameters

One-litre grab water samples were taken biweekly (Weeks 0, 2, 4, 6, 8, 10) at 10 cm below the water surface in the center of the wetland with an amber bottle for neonicotinoid insecticide analysis. Each water sample was immediately placed in a cooler, transported to the laboratory at the end of the day, and stored at 4° C until analysis. All water samples were analyzed at the National Hydrology Research Centre, Environment and Climate Change Canada in Saskatoon, SK. Following the methods outlined in Main et al. (2014), imidacloprid, clothianidin, thiamethoxam, and acetamiprid were analyzed using aqueous solid-phase extraction and LC-MS/MS (adapted from Xie et al. 2011). Analytical standards of imidacloprid, clothianidin, thiamethoxam, and acetamiprid were purchased from Chem Service (West Chester, PA, USA). Quality assurance/quality control (QA/QC) included batch % recoveries (RC %), limits of quantification (LOQ), and limits of detection (LOD). All concentrations presented have been batch recovery corrected (Appendix C: S.I. Table 1).

Dissolved oxygen (mg/L; % saturation), conductivity ($\mu\text{S}/\text{cm}$), pH, and temperature ($^{\circ}\text{C}$) were measured directly within each wetland with a handheld YSI ProPlus meter (YSI Inc., Yellow Springs, OH, USA). Additional water samples (500 mL) were collected in polypropylene bottles and stored refrigerated at 4°C for basic water quality analysis. Total nitrate (mg/L; NO_3), phosphate (mg/L; PO_4), ammonia (mg/L; N), and turbidity (FTU) were measured using a YSI 9500 Photometer (YSI Inc., Yellow Springs, OH, USA).

5.3.3 Emerging aquatic insect trapping

Using floating pyramidal emergence traps (1 m x 1 m), adult aquatic insects were collected every three to four days. Two emergence traps were placed on each of the 22 wetlands with one in the open water and one in the emergent vegetation zone to capture the range of taxa in those habitats (Adamus and Hairston 1996). Emergence traps were inspected and repaired

from any damage cause by the local muskrat population (See, Cavallaro et al. 2014). Emergent vegetation was cut approximately 30 cm above the water surface to facilitate placement of each emergence trap and to maintain the physical structure for insects requiring substrate to emerge. The collection of the emerged aquatic insects started approximately two to three weeks after ice-off to ensure emergence traps were securely anchored in the sediment. Emergence traps were secured on one corner using a piece of 3 m rebar which allowed the trap to remain fixed in place, but to pivot with the wind, preventing the potential effects of substrate shading. At the top of the trap, insects accumulated in a collection jar containing 70% ethanol (with ~5% glycerol to slow evaporation rate). Insects were subsequently transferred and preserved in 70% ethanol until identified and counted. The emerging aquatic insect samples (open water and emergence vegetation zone) were pooled at each sampling event. Adult Chironomidae were identified to the subfamily-level with a Zeiss Stemi SV 11 stereomicroscope (Carl Zeiss Microscopy, LLC. Thornwood, NY, USA) and Nikon Labophot-2 phase contrast compound microscope (Nikon Instruments Inc., Melville, NY, USA) and all other insects to the family-level using dichotomous keys (Clifford 1991, Merritt et al. 2008). Incidental capture of terrestrial insect taxa (e.g., Aphidoidea, Cicadidae, Curculionoidea) and a single female stonefly (*Isoperla transmarina*) were removed and not included in the resulting analyses.

5.3.4 Data analyses

Descriptive statistics and commonly used community metrics were used to summarize differences between years. Percent taxa composition and richness were enumerated to determine the relative proportion of taxa emerged and the number of unique taxa present. Shannon's Diversity Index (H' ; Equation 1) was calculated to describe proportional diversity based on taxa richness and abundance; where s is the total number of species (i.e., distinct taxa for the present study) and p_i is the proportion of s made up of the i th species.

$$H' = - \sum_{i=1}^s p_i \ln p_i \quad (1)$$

Neonicotinoid compounds are frequently detected in mixtures within the same wetland (Main et al. 2015) and cumulative effects can be described using compound specific toxicity. Therefore, toxic equivalency quotients (TEQs) were calculated using the chironomid chronic toxicity (EC50 for emergence inhibition) values reported in Cavallaro et al. (2017) where

concentrations are normalized to imidacloprid TEQ = 1.0 µg/L before summing. The active ingredient acetamiprid (ACT) was assumed to have a multiplier of 1.0 as the TEF is not known for this compound (See, Equation 2).

$$\begin{aligned} \text{IMI toxic equivalence} = & 1.0[\text{IMI conc.}] + 1.62[\text{CLO conc.}] \\ & + 0.11[\text{THX conc.}] + 1.0[\text{ACT conc.}] \end{aligned} \quad (2)$$

I aimed to characterize aquatic insect community responses from differences in a) eight wetland water quality variables and b) nine wetland habitat quality variables in combination with neonicotinoid concentrations which were the main variable of interest. Using a series of multivariate ordination techniques, I assessed the trends and relationships in the insect community data and the water quality and habitat variables in a low dimensional space. I selected linear Principal Components Analysis (PCA) and Redundancy Analysis (RDA) models over a unimodal analysis by running an unconstrained, detrended correspondence analysis (DCA) on $\log_{10}(x+1)$ -transformed taxa abundance data to ascertain data gradient lengths. Data gradient lengths (i.e., total beta diversity) are defined as a byproduct from detrending and rescaling of species data segments. The DCA estimated a gradient length of <2.0, which indicated that a linear method would best fit the data (ter Braak and Šmilauer 2002). PCA was used to broadly describe the insect community structure. A geometric reduction of the data (principal component axes; PC) allowed for the visual evaluation of trends and patterns among environmental (water quality and habitat quality) variables (i.e., unconstrained ordination). To assess the explanatory power (r^2) of neonicotinoid concentration on taxa composition, a RDA was performed with all water quality variables + mean TEQ concentration (µg/L). A second RDA was performed with quantitative wetland habitat variables + mean neonicotinoid TEQ concentration (µg/L). Significance of the environmental variable axes was assessed by a Monte Carlo permutation test (999 unrestricted iterations). Ordinations were conducted in Canoco version 4.5 for Windows (Biometris, Wageningen, Netherlands; ter Braak and Šmilauer 2002).

The resulting multivariate analyses (e.g., significance of RDA1 and RDA2) informed the inclusion of the most important environmental variables to build models to explicitly test the hypothesis of whether neonicotinoids or other measures of wetland water quality and/or habitat quality significantly affected the total abundance of insects that emerged over time. I used

generalized linear mixed models (package “lme4”; Bates et al. 2014) in R version 3.4.1 (R Core Team 2017) with total insect emergence as the response variable. I generated two model sets with combinations of fixed effect covariates which were significant in the ordination analyses. A model for the water quality measures included mean neonicotinoid TEQ concentration ($\mu\text{g/L}$), time (week [linear and quadratic]), study year, and turbidity (FTU) as fixed effects, and a model for the wetland habitat characteristics included mean neonicotinoid TEQ concentration ($\mu\text{g/L}$), time (week), study year, vegetation disturbance (%), and continuous grass buffer zone (%) as fixed effects. Interaction terms time \times neonicotinoid TEQ concentration were included to identify conditional effects. All model sets also accounted for wetland number (i.e., wetland identity) as a random effect as insect abundance and the environmental variables were measured repeatedly. Neonicotinoid TEQ concentration and environmental covariates (e.g., vegetation disturbance, continuous grasses/sedges, and turbidity) were scaled and centered before running models. I assessed normality of the residuals by a Shapiro-Wilk test and model fit distributions were initially evaluated using visual graphing tools in package “car” and further confirmed by a goodness-of-fit with a chi-square test based on residual deviance which yielded a negative binomial distribution. Using the dredge function in package “MuMIn” (Bartoń 2016), I compared the set of top models ranked by corrected Akaike's Information Criterion (AIC_c) values.

5.4 Results

5.4.1 Wetland water quality

I sampled water six times (2-week intervals) from each of the 22 wetlands from May to July in 2013 and 2015 for a total of 132 water samples ($n=66$ per year). Neonicotinoid insecticides were detected in 100% of samples in 2013 (max. concentration = $0.572 \mu\text{g/L}$) and 64% of samples in 2015 (max. concentration = $0.113 \mu\text{g/L}$) (Table 5.1). The frequency of individual neonicotinoid compounds detected for both years ranked as follows: 96% clothianidin > 49% thiamethoxam > 18% imidacloprid > 7% acetamiprid (Table 5.1). Neonicotinoid mixtures (≥ 2 active ingredients) were detected in 48/66 (73%) of water samples in 2013 and 10/66 (16%) of samples in 2015. Wetlands sampled in 2013 were characterized by higher nutrient concentrations than 2015 (S.I. Table 5.2). In 2013, nitrate ($t_{72}=-5.68$, $P<0.001$), phosphate ($t_{129}=5.05$, $P<0.001$), and ammonia ($t_{130}=2.52$, $P=0.013$) concentrations were $+0.5 \text{ mg/L}$, $+2.17 \text{ mg/L}$, and $+0.13 \text{ mg/L}$ higher than in 2015, respectively. Likely a product of excess nutrient

loads, algal growth was more developed in 2013 and covered an average of $19.1 \pm 5.5\%$ of the water surface with one wetland displaying 50% coverage. Water quality variables including conductivity and pH were lower in 2013 compared to 2015. Some of the differences in water quality may have been due to higher average rainfall in 2013 with 30.6 ± 3.45 cm during the 10-week monitoring period compared to 11.53 ± 1.45 cm in 2015.

5.4.2 Rapid wetland assessments of habitat characteristics

Wetlands sampled in 2013 were characterized by greater water depth, basin fill, and algal cover (S.I. Table 2). Wetlands sampled in 2013 were also significantly more isolated with a mean nearest wetland distance of 32.6 ± 6.0 meters ($t_{13}=3.04$, $P=0.010$) compared to 12.7 ± 2.2 meters in 2015. Overall, habitat structure and vegetative continuity were more intact in the 2015 wetlands, than 2013. For example, wetlands sampled in 2013 were typically cropped to the edge and did not feature a vegetative buffer zone (=100% vegetation disturbance), whereas 2015 wetland disturbance scores averaged much lower ($23.2 \pm 5.9\%$). On average, the vegetative buffer margin was 7x wider in 2015 with a mean measure of 8.4 ± 1.6 m. Continuity scores of reeds/rushes and grasses was $81.4 \pm 8.6\%$ and $22.7 \pm 5.5\%$, respectively; which was 2x and 10x greater in 2015 than 2013, respectively.

Table 5.1 Summary of $\mu\text{g/L}$ means (\pm S.E.), $\mu\text{g/L}$ maximum, and detection frequency (%) of neonicotinoids in wetland water sampled over 10 weeks from May to July 2013 and 2015 in Alvena, Saskatchewan ($n = 11$ wetlands per year). No wetlands sampled in 2013 were sampled in 2015.

Neonicotinoid	2013			2015		
	Mean (\pm S.E.)	Max.	Frequency (%)	Mean (\pm S.E.)	Max.	Frequency (%)
Imidacloprid	0.010 (\pm 0.001)	0.024	10 (15)	0.023 (\pm 0.002)	0.03	8 (12)
Clothianidin	0.077 (\pm 0.010)	0.353	65 (98)	0.020 (\pm 0.001)	0.048	31 (47)
Thiamethoxam	0.060 (\pm 0.008)	0.23	41 (62)	0.031 (\pm 0.006)	0.052	8 (12)
Acetamiprid	0.106	0.106	1 (2)	0.016 (\pm 0.004)	0.038	6 (9)
Number of a.i. detected ^a	2 (1)	3	---	1 (1)	3	---
Neonicotinoid TEQ ^b	0.131 (\pm 0.020)	0.597	66 (100)	0.031 (\pm 0.003)	0.116	42 (64)
Summed neonicotinoids ^c	0.117 (\pm 0.010)	0.572	66 (100)	0.027 (\pm 0.004)	0.113	42 (64)

^aNumber of active ingredients (a.i.) detected in any sample

^bConcentrations corrected to imidacloprid toxic equivalents (TEQs) using equation 2 (see methods)

^cConcentrations summed (concentration-addition) and not corrected by TEQs

5.4.3 Aquatic insect community responses

Taxa from the order Diptera were the most abundant in both study years and were largely dominated by non-biting midges from the subfamily Chironominae (Figure 5.1). During the sampling periods for both years a total of 36 distinct taxa were identified with six unique taxa each year; this equated to a taxa richness score of 30 for both 2013 and 2015. On average, Shannon's Diversity indices (\pm S.E.) were 1.31 (\pm 0.08) and 1.58 (\pm 0.15) in 2013 and 2015, respectively. Overall, just 8 of 36 identified taxa comprised 96.8% of the total secondary production in 2013 and 13 of 36 identified taxa accounted for 96.5% of the total secondary production in 2015.

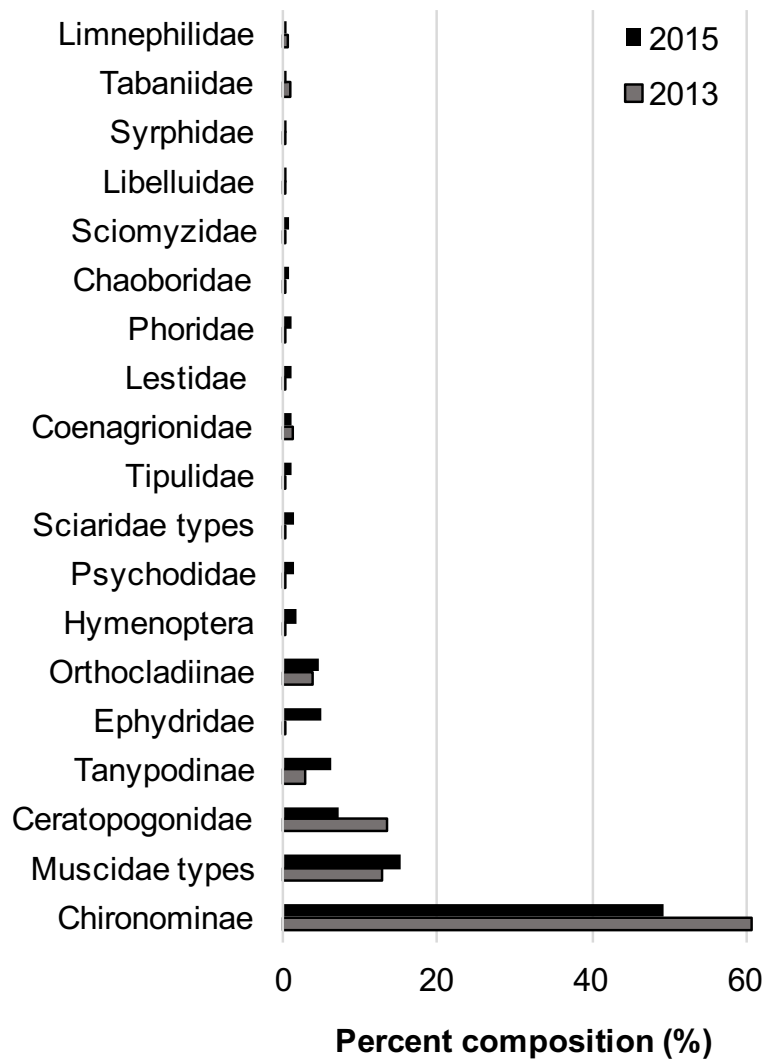


Figure 5.1 Composition of the emerging insect community for 2013 and 2015. Composition data are presented when the percentage was greater than 0.5% in at least one of the two years.

5.4.4 Effect of water quality on aquatic insects

The first two axes for the water quality unconstrained PCA explained over half of the variation in the taxa composition (50.5%) with principal component one (PC1) accounting for 37.0% of the variation. The water quality PC1 separated mean neonicotinoid TEQ and nutrients from other variables such as turbidity, conductivity, and pH, whereas PC2 appeared to be mostly driven by temperature and dissolved oxygen concentration (Figure 5.2A). Overlapping vector arrows for mean neonicotinoid TEQ and phosphate and ammonia suggested correlated agronomic sources. Constrained RDA analysis extracted two significant factors (axes), mean neonicotinoid TEQ concentration ($F=4.89$, $P=0.001$) and turbidity ($F=3.39$, $P=0.002$), that were significant variables influencing the emerging insect community composition (Table 5.2). The dominant dipteran families (e.g., Chironominae, Muscidae types, and Ceratopogonidae) were associated with higher neonicotinoid TEQ concentrations (Figure 5.2B). This was not true of all dipteran families; a diverse range of taxa were associated with lower neonicotinoid TEQ concentrations (e.g., Psychodidae, Syrphidae, Tipulidae).

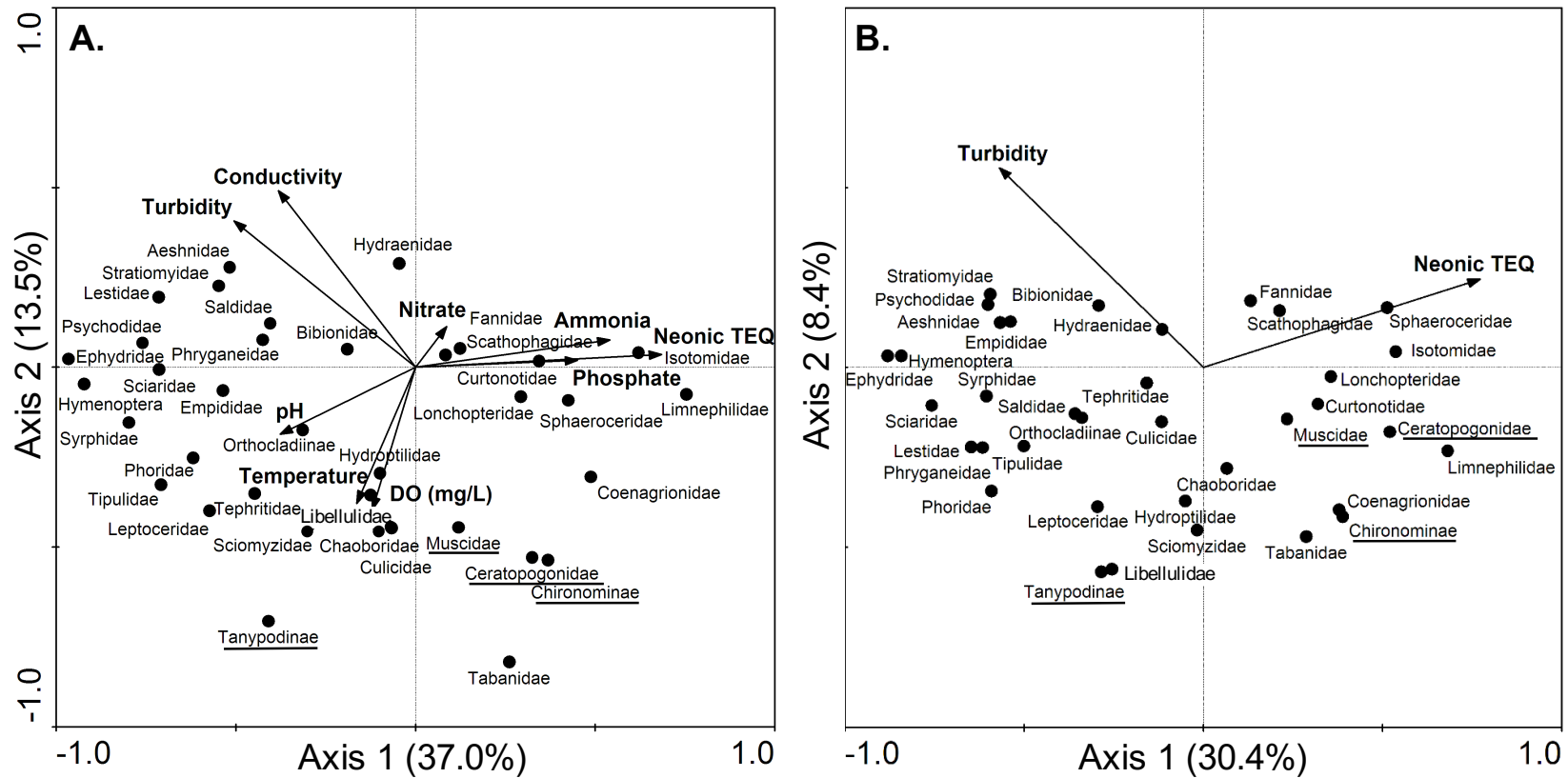


Figure 5.2 (A) Principal components analysis (PCA) and (B) Redundancy analysis (RDA) ordination biplots showing aquatic insect community and water quality variables of interest collected from 22 wetlands sampled in 2013 and 2015 ($n=11$ per year). Points represent insect families and vectors (arrow length) indicate strength of the environmental variables. Neonicotinoid concentration is presented as toxic equivalency quotient [TEQ] $\mu\text{g/L}$ of imidacloprid (See, Appendix C: S.I. Table 2 for remaining vector units). Of the 8 environmental variables, the RDA revealed that mean neonicotinoid TEQ and turbidity were significant vectors in the overall solution (Monte Carlo permutation test $P < 0.05$). Dominant taxa are underlined.

5.4.5 Effect of habitat quality on aquatic insects

Similar to the water quality PCA, the first two PCA gradients for the wetland habitat quality ordination explained over half of the variation in the insect taxa composition (50.5%) with principal component one (PC1) accounting for 37.0% of the variation. The wetland habitat quality PC1 clearly separated the influences of zonal wetland vegetation continuity (e.g., vegetation buffer zone width, continuity of reeds/rushes, and continuity of grasses/sedges) and vegetation disturbance (Figure 5.3A). Neonicotinoid TEQs were positively correlated with vegetation disturbance and negatively correlated with the buffer width and continuity of the wetland vegetation. Increasing continuity of wetland vegetation was associated with a greater taxa diversity and included aquatic Hymenoptera (e.g., Braconidae, Scelionidae, Trichogrammatidae), and other families (e.g., Ephydriidae, Syrphidae, Lestidae, Aeshnidae, Psychodidae, and Stratiomyidae). Taxa that appeared to be more tolerant of vegetation disturbance included some of the most abundant families (e.g., Chironominae, Muscidae, Scathophagidae, Anthomyiidae, and Ceratopogonidae). The RDA further confirmed the importance of two habitat variables (vectors), vegetation disturbance ($F=7.85$, $P=0.001$) and continuity of the grass/sedge community ($F=1.85$, $P=0.048$), though neonicotinoid TEQ was not retained as a significant vector (Table 5.2; Figure 5.3B). Overall, I observed parallelism between the water and habitat quality ordination plots due to tolerance of the various taxa and common indicators of wetland degradation (e.g., pesticides, turbidity, nutrients and vegetation disturbance) which were all correlated.

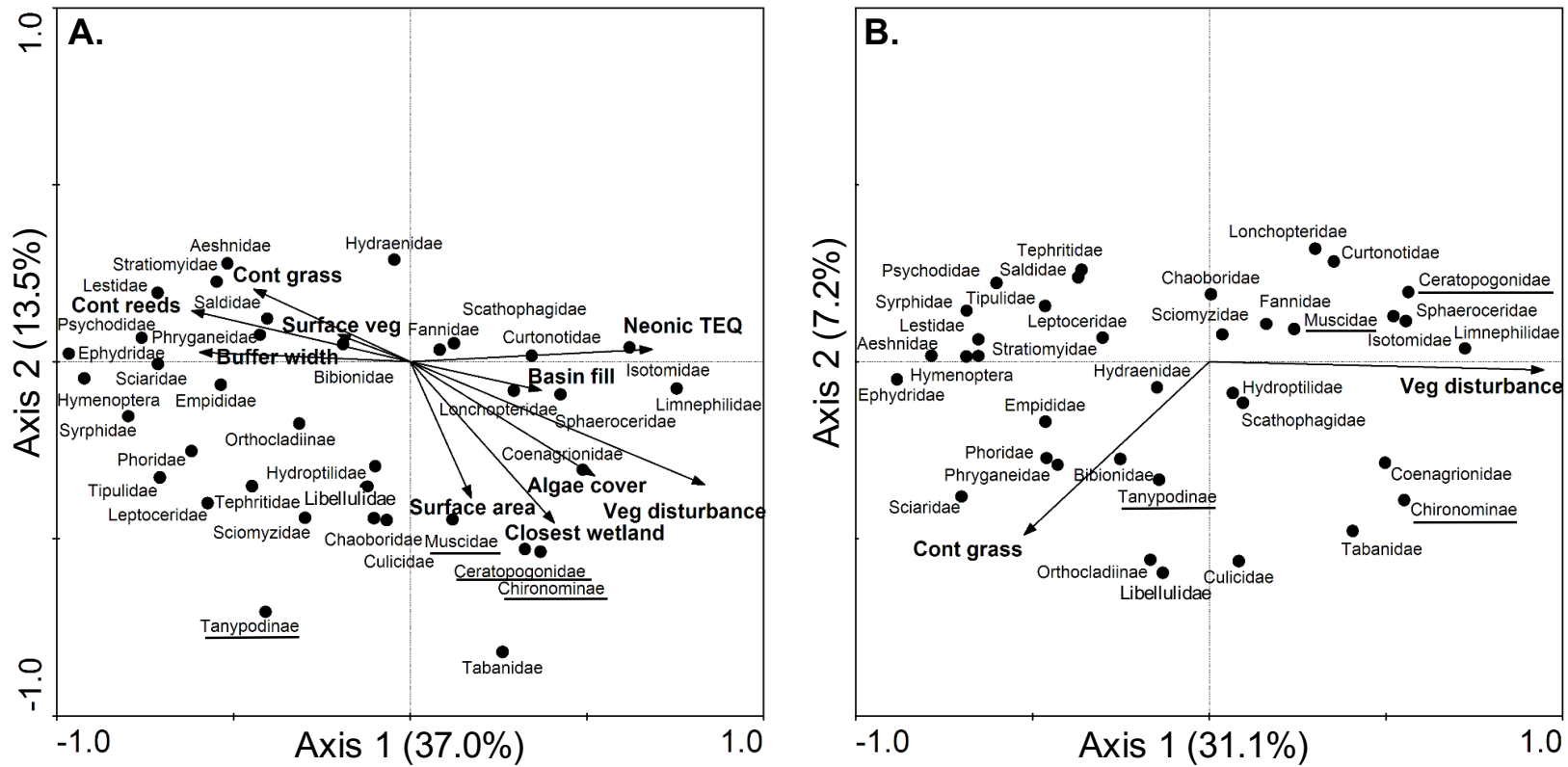


Figure 5.3 (A) Principal components analysis (PCA) and (B) Redundancy analysis (RDA) ordination biplots showing the relative impact of wetland characteristics on emerging insect communities. Points represent insect families and vectors (arrow length) indicate increasing values for the environmental variables. Data are from emergence traps set in 22 wetlands sampled in 2013 and 2015 ($n=11$ per year). Neonicotinoid concentration is presented as toxic equivalency quotient [TEQ] $\mu\text{g/L}$ of imidacloprid (See, Appendix C: S.I. Table 2 for remaining vector units). Of the 9 environmental variables, the RDA revealed that vegetation disturbance and continuous grass buffer were significant axes in the overall solution (Monte Carlo permutation test $P < 0.05$). Dominant taxa are underlined.

Table 5.2 Conditional effects of the environmental variables determined by forward selection in multivariate constrained Redundancy Analysis. Bolded values indicate statistical significance ($P < 0.05$; *) of the axes determined using Monte Carlo permutation tests.

Wetland habitat quality variables	Conditional Effects			
	Eigenvalue	VIF ^a	<i>F</i>	<i>P</i>
Vegetation disturbance (%)	0.28	10.14	7.85	*0.001
Continuous grass buffer (%)	0.07	3.86	1.83	*0.048
Neonicotinoid TEQ ^b	0.05	2.18	1.75	0.073
Basin fill ^c	0.05	1.83	1.56	0.112
Closest wetland (m)	0.04	4.51	1.22	0.266
Algae cover (%)	0.03	1.82	0.97	0.445
Buffer width (m)	0.03	8.33	0.95	0.495
Continuous reed buffer (%)	0.03	2.94	0.90	0.525
Surface area (ha)	0.02	1.32	0.63	0.729
Surface vegetation (%)	0.02	4.11	0.38	0.955
Water quality variables				
Neonicotinoid TEQ^b	0.20	2.79	4.89	*0.001
Turbidity (FTU)^d	0.12	1.42	3.39	*0.002
Dissolved oxygen (mg/L)	0.06	2.30	1.79	0.061
Conductivity (μS/cm)	0.05	1.86	1.60	0.078
Total nitrate (mg/L; NO ₃)	0.05	1.73	1.36	0.184
pH	0.03	3.31	0.95	0.490
Total ammonia (mg/L; N)	0.03	1.60	0.95	0.467
Temperature (°C)	0.03	2.24	0.97	0.477
Total phosphate (mg/L; PO ₄)	0.02	1.84	0.46	0.933

^aVariation inflation factors

^bMean neonicotinoid concentration (toxic equivalency quotient [TEQ] μg/L of imidacloprid)

^cBasin fill (1-5; see Appendix C: S.I. Table 2)

^dFTU = Formazin Turbidity Units

5.4.6 Models of aquatic insect abundance over time

Generalized linear mixed effects models were used to compare the relative importance of water quality and habitat variables together on total insect abundance at different sampling periods. Although year was clearly important for influencing the environmental variables, it was not a strong predictor of emergent insect abundance and was excluded. The inclusion of a quadratic time term (week²) significantly improved the model-fit for both model sets. Variation explained by wetland identity was also minimal ($R^2 < 0.001$).

The top water quality model for total aquatic insect abundance included only the interaction term time \times neonicotinoid TEQ (neonicotinoid concentration in TEQ $\mu\text{g/L}$ of imidacloprid) (Table 5.3). Emergence significantly increased over time ($\beta \pm \text{S.E.} = 1.16 \pm 0.10$, $P < 0.001$); however, I observed a significant decrease in insect emergence in wetlands that had higher neonicotinoid TEQ concentrations ($\beta \pm \text{S.E.} = -0.35 \pm 0.11$, $P = 0.002$).

The best habitat quality model for total insect emergence included the interaction term, time \times neonicotinoid TEQ and wetland vegetation disturbance (Table 5.3). Like the water quality top model, emergence significantly increased over time ($\beta \pm \text{S.E.} = 1.18 \pm 0.10$, $P < 0.001$), whereas neonicotinoid TEQ concentration negatively impacted insect abundance ($\beta \pm \text{S.E.} = -0.61 \pm 0.14$, $P < 0.001$). Interestingly, higher vegetation disturbance had a positive influence on total emergence over time ($\beta \pm \text{S.E.} = 0.34 \pm 0.11$, $P = 0.002$). Tolerant dipteran taxa made up the greatest portion of emerging insects throughout each monitoring period and were positively influenced by vegetation disturbance.

A direct comparison with likelihood ratio tests (LRT) between the two top models determined that the wetland habitat quality model which included time \times neonicotinoids and vegetation disturbance was superior to the water quality model (LRT; $\chi^2(1) = 8.45$, $P = 0.004$). Consistent in all models, higher neonicotinoid TEQ correlated negatively with emerging insects. Furthermore, the removal of the neonicotinoid term resulted in a poorer model fit for the water quality model (LRT; $\chi^2(2) = 14.31$, $P < 0.001$) and the wetland habitat quality model (LRT; $\chi^2(2) = 22.62$, $P < 0.001$), suggesting neonicotinoid TEQ contributed significant explanatory power.

Table 5.3 Results from top models (i.e., ranked AIC_c) assessed by water quality measures and by wetland characteristics measured during wetland water sampling for change in total emergence at different sampling periods in Prairie wetlands. Models with an $AIC_{wt} > 0.001$ are listed.

Model structure	Model parameters ^a			
	logLik	AIC_c	ΔAIC_c	AIC_{wt}
Water quality				
Time × Neonic TEQ	-787.95	1590.8	0	0.990
Time + Neonic TEQ	-794.32	1601.3	10.52	0.005
Time	-795.43	1601.3	10.53	0.005
Intercept-only (null)	-841.26	1688.7	97.91	<0.001
Wetland habitat quality				
Time × Neonic TEQ + Veg. Disturbance	-783.72	1584.6	0	0.960
Time × Neonic TEQ	-788.04	1591.0	6.38	0.040
Intercept-only (null)	-843.99	1694.2	109.57	<0.001

^aModel parameters are listed by column: logLik ($-2 \times \log$ likelihood), corrected Akaike's Information Criterion (AIC_c), change in AIC_c (ΔAIC_c), and model weights (AIC_{wt}). Covariates for the full model included mean neonicotinoid TEQ (toxic equivalency quotient [TEQ] $\mu\text{g/L}$ of imidacloprid) and time (weeks 0, 2, 4, 6, 8, 10) which performed better as an interaction term. Turbidity (FTU) was used in the water quality models. Continuous grass buffer (%) and vegetation disturbance (%) were used in the wetland habitat quality models.

5.5 Discussion

Cross-habitat linkages created by aquatic insects occur in seasonal fluxes where available resources occur at different times. Exposure of immature aquatic insects to neonicotinoids can alter emergence patterns and overall abundance. Moreover, the structure of emerging aquatic insect communities can be shifted by intensive agricultural activity (Wrubleski and Ross 2011); however, elucidating the specific causes can be complex. This study was conducted on a large-scale farming operation (>20,000 ha) employing conventional agronomic practices that can degrade wetlands (e.g. cropping into wetland margins, high fertilizer inputs, and clothianidin-seed treatments). Redundancy analyses of eight water quality and nine habitat variables showed that neonicotinoid concentration, turbidity, vegetation disturbance and continuity of buffer zone grasses were significant drivers of emerging insect community composition. Total insect abundance over time was best predicted by wetland disturbance and neonicotinoid TEQ × time interaction. Higher neonicotinoid concentrations were significantly associated with lower total insect emergence, while vegetation disturbance had the opposite effect. These data suggest that

collateral effects from agricultural intensification (including aqueous neonicotinoid exposure) may potentially impact aquatic insect emergence and diversity in Prairie wetlands.

5.5.1 Factors influencing aquatic insect community responses

Wetland habitat characteristics, water quality parameters, and occurrence of resident biota (e.g., fish, amphibians, and birds) are interrelated (Sundberg et al. 2016). Due to increased relative habitat disturbance and the overabundance of habitat generalists, clear relationships between land use, water quality and invertebrate assemblages are regularly debated in the literature (Tangen et al. 2002, Batzer 2013, Gleason and Rooney 2017). The explicit relationships from pesticide contamination are more consistent, but cautiously discussed (e.g., Gibbs et al. 2009). Through separate ordination analyses, I determined that mean neonicotinoid concentration and vegetation disturbance significantly influenced the emerging aquatic insect community. Increased runoff potential from under-developed or removed vegetative buffer zones can augment sediment loads and enhance contamination events (Gleason and Euliss 1998, Skagen et al. 2008). This was reflected in this study by a simplified community dominated by tolerant dipteran families, primarily chironomids.

Chironomid communities are not normally monotypic and are often one of the most diverse taxonomic groups (Wrubleski and Ross 2011). Chironomid diversity is strongly related with wetland class and surrounding seasonal farming practices, with row crops and reduced/greater water permanency shown to significantly decrease chironomid taxa richness (Driver 1971). My study yielded over 18,000 chironomids (64% of the total production) with the subfamily Chironominae making up the majority of the community. Studies conducted throughout the Prairie Pothole Region (PPR) report similar results and identify chironomids as a critical component of secondary production (Wrubleski and Rosenberg 1990). Parker (1992) documented chironomids representing 66-71% of the emerging insect community in a single wetland in central Saskatchewan, whereas some inventories range from 60% in Prairie wetlands located in North Dakota to 78% in three wetlands in Manitoba (Bataille and Baldassarre 1993). Campbell et al. (2009) reported greater abundances of *Chironomus* and *Glyptotendipes* larvae (Chironomidae: Chironominae) in ponds located on Minnesota farms which had higher total nitrogen. Wetlands found within 10 meters of agricultural fields (i.e., high habitat disturbance) in central North Dakota exhibited more multivoltine taxa, primarily chironomids and culicids (Chippis et al. 2006). Degradation of water quality parameters in Prairie wetlands (i.e., anoxia

and eutrophication) create conditions that favour chironomids, especially those surrounded by agriculture.

To my knowledge, *Chironomus sp.* are the best represented aquatic insect species in the neonicotinoid toxicology literature with 15 studies evaluating various lethal and sub-lethal endpoints (Chapter 2). Under chronic exposure conditions, such as those likely experienced in the field, full life-cycle tests using *C. dilutus* reported EC50s (emergence inhibition) of 0.39 µg/L (0.31-1.42 µg/L; 95% C.I.), 0.28 µg/L (0.20-0.33 µg/L; 95% C.I.), and 4.13 µg/L (3.53-4.76 µg/L; 95% C.I.) for imidacloprid, clothianidin, and thiamethoxam, respectively (Chapter 2). In the current study, measured mean water concentrations of neonicotinoids (TEQ µg/L of imidacloprid) in wetland water did not exceed 0.131 µg/L in either year. In 2013, maximum measured concentrations for a single compound or mixture were as high as 0.353 µg/L (clothianidin) and 0.597 µg/L (neonicotinoid TEQ), respectively. A general decrease in chironomid emergence was observed at mean measured imidacloprid and clothianidin concentrations of 0.436 and 0.385 µg/L, respectively, following exposure to the neonicotinoids imidacloprid, clothianidin, and thiamethoxam under *in situ* wetland mesocosm (See, Chapter 3). Different sensitivities of chironomid species to pesticide exposure and wetland characteristics have been reported and highlight the potential variation within taxa (Watts and Pascoe 2000, Campbell et al. 2009).

Dragonflies and damselflies (Odonata) are among the few taxa for which there are neonicotinoid toxicity data and field data associated with agricultural intensification. During chronic exposure to clothianidin in mesocosms, libellulid dragonflies showed a significant increase in nymphal mortality and reductions in emergence at a mean measured clothianidin concentration < 2.69 µg/L (Kasai et al. 2016). When exposed to the neonicotinoid thiacloprid in mesocosms, the dragonfly nymph *Sympetrum striolatum* Charpentier (Odonata: Libellulidae) had an LC50 of 31.19 µg/L 11-days post-application (Beketov and Liess 2008a). More controlled laboratory experiments suggest Odonata nymphs, *Anax junius* Drury (Odonata: Aeshnidae), are more tolerant than other odonates to neonicotinoid exposure with an LC50 of 1.0 mg a.i./L Arena 0.25%; clothianidin (Miles et al. 2017). Mixtures of insecticides and varying levels of nutrients can further complicate predicted responses. Alexander et al. (2013) found *Gomphus* dragonfly nymph (Odonata: Gomphidae) responses to vary significantly between oligotrophic and

mesotrophic nutrient treatments at insecticide mixtures measuring 0.6 TU (toxic units; equitoxic mixture of chlorpyrifos, dimethoate, and imidacloprid). Therefore, confounding stressors could play an important role in odonate community responses in agricultural wetlands.

My ordination plots showed that some Odonata taxa (e.g., Aeshnidae, Lestidae, and Libellulidae) were positively associated with larger buffer zone widths and higher dissolved oxygen concentrations, while other families (e.g., Coenagrionidae) were more correlated with wetland surface and vegetation disturbance. The most conclusive effects on odonate species from agricultural practices are discussed in studies examining macroinvertebrate communities in wetlands surrounded by grazed and ungrazed pasture. Loss of wetland vegetation was associated with a significant decrease in odonate species richness, specifically affected by lower vegetation species richness and percentage of stems grazed (Hornung and Rice 2003). These trends were also observed in my dataset; however, overall odonate taxa richness was too low to discern any clear patterns. I would expect severe habitat alterations to compound the effects on aquatic insect communities. Moreover, the presence of an intact vegetated buffer zone has been correlated with lower neonicotinoid concentrations, and wetland plant species have been documented to partially ameliorate neonicotinoid contamination (Main et al. 2017). Therefore, preservation of wetland vegetation may be the key management strategy to reduce neonicotinoid contamination and produce complex habitat structure.

5.5.2 Potential food web implications

Aquatic and terrestrial consumers rely on seasonally abundant food resources exported from aquatic ecosystems. Some migrating songbirds rely on food items of an aquatic origin to refuel (MacDade et al. 2011). Studies in the PPR also indicate that aerial insectivores such as Tree Swallows (*Tachycineta bicolor*) increase foraging efforts in areas of intense agriculture planted with neonicotinoid-treated crops (Stanton et al. 2016). From 2003 to 2010, farmland bird populations in the Netherlands experienced a significant decline in abundance near surface waters containing greater than 0.02 µg/L of imidacloprid (Hallmann et al. 2014). Moreover, subsidies originating from aquatic environments are considered high-quality food items that contain a suite of essential nutrients and are linked to improved immune function and growth performance in terrestrial consumers (Twining et al. 2016, Fritz et al. 2017). Measurable amounts of pesticides, including neonicotinoids, can be detected in emergent insects which may directly affect consumers (Haroune et al. 2015). Mason et al. (2013) discusses the potential for

compromised immune function in vertebrates associated with sub-lethal neonicotinoid exposure. Ultimately, lower food item quality and quantity may indirectly create cascading trophic effects in ecosystems.

5.5.3 Wetland contamination and neonicotinoid exposure profile

Few studies have repeated within-year sampling to track changes in neonicotinoid concentrations in surface waters over time. In the present study, water was sampled from two sets of wetlands over a span of 10 weeks encompassing pre- and post-seeding periods (after neonicotinoid-treated seeds were planted). Neonicotinoid concentrations were detected in all wetlands during both periods supporting the mounting evidence that neonicotinoids persist in the environment. A seasonally structured sampling of 136 wetlands in Saskatchewan, Canada, from 2012-2013 found mean summed neonicotinoid (imidacloprid, clothianidin, thiamethoxam, and acetamiprid) concentrations ranging from 0.004 µg/L to 0.077 µg/L (Main et al. 2014). Peak concentrations occurred during the growing season, reaching 3.11 µg/L; however, spring (pre-seeding) sampling efforts found neonicotinoid concentrations of up to 0.184 µg/L (2012) and 0.212 µg/L (2013). A similar study in Iowa, USA, detected imidacloprid, clothianidin, and thiamethoxam in wetland water samples with concentrations as high as 3.5 µg/L for clothianidin and 6.9 µg/L for thiamethoxam (Evelsizer and Skopec 2016). The latter study sampled drained wetlands where higher neonicotinoid concentrations were more probable. In addition to contamination from runoff events, transport of neonicotinoids in surface waters can occur through the leaching of senesced vegetation (Englert et al. 2017). Moreover, the ingestion of leaves contaminated with neonicotinoid compounds can adversely impact immature larvae of shredder taxa (Kreutzweiser et al. 2009). Wet meadow and emergent vegetation in the PPR accumulate quantifiable amounts of imidacloprid, clothianidin, and acetamiprid ranging from 0.30 µg/kg (LOQ) to 8.44 µg/kg (Max; thiamethoxam) and may constitute an important means of entry into and cycling within aquatic ecosystems (Main et al. 2017). The ability of neonicotinoids to move from the site of application continues to be of concern.

Neonicotinoid mixtures have been reported in several environmental field monitoring studies (Anderson et al. 2013, Hladik et al. 2014, Sánchez-Bayo and Hyne 2014). I found a high percentage of water samples with neonicotinoid mixtures in 2013 (72%) and markedly fewer in 2015 (15%). Since my study wetlands were all surrounded by clothianidin treated canola, neonicotinoid mixtures are likely the result of persistence of previously used neonicotinoids in

agricultural soils and wetland sediments, and transport in surface water (DeCant and Barrett 2010, Starner and Goh 2012). Interactions of mixture compounds may pose greater threats to non-target invertebrates due to nicotinic receptor specificity and diversity (Jones and Satelle 2010, Darriet and Chandre 2013). Previous studies have also demonstrated significant deviations from the predicted concentration-addition mixture model for imidacloprid, clothianidin, and thiamethoxam (Maloney et al. 2017). For instance, the joint toxicity of clothianidin and thiamethoxam, two neonicotinoids frequently detected as binary mixtures in this study, displayed concentration-additive synergism to the non-biting midge species *C. dilutus* under acute laboratory exposure (96-h). Even though neonicotinoid seed treatments often include multiple compounds and cover over 80 million hectares annually (Stevens and Jenkins 2014), there are limited studies that have investigated the potential enhanced toxicity of mixtures in field studies.

5.5.4 Importance of biomonitoring

Over the past few decades, the causes and implications of observed global declines in insect populations are yet to be fully understood; however, it is hypothesized that agricultural intensification plays a major role (Hallmann et al 2017). The current literature supports a strong relationship between increasing agricultural intensification and decreasing biodiversity with non-target invertebrate taxa being among the most affected organisms (Benton et al. 2003). A limited number of studies capture the historic and spatial extent of such trends to adequately assess populations, especially for non-charismatic species (Dirzo et al. 2014). To date, high neonicotinoid use is cited as one of the primary causes for reduced regional aquatic biodiversity and ecosystem function (Beketov et al. 2013, Chagnon et al. 2015).

Shifts in abundance and community composition of emerging aquatic insects may pose serious direct and indirect effects to ecosystem function in parts of the Prairies where neonicotinoid uses are concentrated. Many invertebrates are capable of recovery from pesticide contamination in the terrestrial and aquatic environment (Kattwinkel et al. 2015), but habitat variables identified in this study may further facilitate neonicotinoid transport and contamination (i.e., vegetation disturbance) which may challenge or slow population recovery. Ecological management strategies at the farm or rural municipality level may help improve wetland habitat, increase insect diversity, and mitigate neonicotinoid contamination through reduced vegetation disturbance.

Despite their widespread use in agriculture and their frequent detection in surface waters, insecticides in surface and ground water are not sufficiently monitored and lack long-term data (Stehle and Schulz 2015). Monitoring programs for pesticides in surface waters, especially in ecologically important wetland habitats such as the Prairies, are needed to protect aquatic life in areas at risk from water-borne neonicotinoid and other pesticide exposure. The current regulatory thresholds of chronic imidacloprid toxicity by Health Canada's Pest Management Regulatory Agency (PMRA) is 0.041 µg/L (Health Canada 2016).

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CHAPTER 6: SYNTHESIS AND CONCLUSIONS

6.1 Conclusions, synthesis, and limitations

The prophylactic use of neonicotinoids in industrialized agriculture poses certain risks to non-target aquatic insects (Alexander et al. 2008, Beketov and Liess 2011, Beketov et al. 2013, Morrissey et al. 2015). The goal of this study was to compare the chronic toxicity of imidacloprid, clothianidin, and thiamethoxam to aquatic insects using controlled standard single-species methods (i.e., population-level) and semi-controlled *in situ* limnocorral units (i.e., community-level). Moreover, I sought to advance our knowledge on the effects of neonicotinoid exposure under true field conditions by monitoring wetlands directly at risk from contamination. This research was prompted by the enhanced risks of aquatic insects in Prairie wetlands from neonicotinoid contamination and the direct/indirect effects to wetland dependent biota residing in an ecologically significant area, the Prairie Pothole Region.

When my thesis research started, chronic neonicotinoid toxicity data for different active ingredients were sparse, with most studies in the primary literature focusing on acute imidacloprid exposures. The need to expand the literature and contribute more data on the risks from clothianidin and thiamethoxam was considered a high priority by Health Canada's Pesticide Management Regulatory Agency and the U.S. EPA. During my thesis research, several literature reviews were published describing the risks of neonicotinoids to non-target insects, such as pollinators and aquatic insects (Anderson et al. 2015, Lundin et al. 2015, Morrissey et al. 2015). Identifying research gaps caused a surge in studies focusing on neonicotinoid exposure to non-target insects. The work presented in this thesis has contributed to this body of literature and to better understand risks to non-target aquatic insects from chronic neonicotinoid exposure.

6.1.1 Comparative chronic toxicity of imidacloprid, clothianidin, and thiamethoxam to *Chironomus dilutus* and estimation of toxic equivalency factors

Standardization by using single-species laboratory studies is intrinsic to deriving aquatic life benchmarks and water quality criteria. Consistency in the use of model species used for single-species toxicity tests has resulted in only a handful of sentinel species providing the bulk of the toxicity data, representing the constituents of entire taxonomic group(s). For example, with over 20,000 described species, non-biting midges (Diptera: Chironomidae) are represented by only a few commonly used species, such as *C. dilutus* and *C. riparius*, in laboratory single-species toxicity tests. Prior to the body of research presented in this thesis, few studies in the

primary literature investigated the effects of clothianidin and thiamethoxam to aquatic insects, with imidacloprid representing 66% of the total aquatic toxicity data (Morrissey et al. 2015). Few single-species data existed on sensitive aquatic insects, and there was no science-supported, quantitative method to determine the relative risks of clothianidin and thiamethoxam to imidacloprid. Comparing other commonly used active ingredients to imidacloprid was critical since all aquatic life benchmarks and water quality criteria in existence today are for imidacloprid only.

To improve the risk assessment of neonicotinoid single compounds and mixtures, I developed TEFs to determine the relative potency of clothianidin, thiamethoxam, and their mixtures relative to imidacloprid using the model organism, *Chironomus dilutus* (Chapter 2). The standardized protocol and identical test endpoints allowed for a direct comparison of sub-lethal and lethal toxicity endpoints. As an added advantage, *C. dilutus* is a cosmopolitan species with a vast geographic distribution. The goal was to calculate acute and chronic toxicity endpoints (i.e., LC50 and EC50) and define clothianidin and thiamethoxam multipliers relative to imidacloprid endpoints. Using these data, I developed 14-day and 40-day TEFs for clothianidin and thiamethoxam relative to imidacloprid (i.e., 1.0 TEF = 1.0 imidacloprid concentration unit). In this study, I confirmed that aquatic insects are at risk from chronic neonicotinoid exposure, including chronic clothianidin and thiamethoxam exposure. For context, the mean field clothianidin concentrations range from 0.142 to 4.18 $\mu\text{g/L}$ in Canadian surface waters (Main et al. 2014, Schaafsma et al. 2015, Struger et al. 2017), well within the calculated 40-day EC50 of 0.28 $\mu\text{g/L}$ ($\pm 0.20\text{-}0.33$). The chronic 40-day endpoint (emergence inhibition) was 3.9X, 8.6X, and 5.7X more sensitive than the 14-day endpoint (lethality) for imidacloprid, clothianidin, and thiamethoxam, respectively. Since the development of imidacloprid TEFs, many published studies (2015-current day) corroborate my findings and note the demonstrated risks of clothianidin and thiamethoxam, albeit with varying risks among taxa and compounds (de Perre et al. 2015, van den Brink et al. 2016, Finnegan et al. 2017, Maloney et al. 2017, Saraiva et al. 2017, Raby et al. 2018).

Beyond the standard measures of mortality and emergence inhibition, I also assessed the effects of the three neonicotinoid compounds on *C. dilutus* sex ratios, emergence insect timing, and biomass (larvae and adults). I demonstrated that under chronic conditions the concentration needed to induce an adverse effect was significantly lower than for acute exposure, highlighting

the risk of environmentally relevant, chronic exposure scenarios. This pattern was true for all compounds tested and endpoints measured. Thiamethoxam proved to be an order of magnitude less toxic than clothianidin and imidacloprid; roughly one-tenth the toxicity for 14-day and 40-day endpoints. Interestingly, across all compounds an increasing neonicotinoid concentration affected the sex ratio in favor of a male-dominant population. I offer further insights on these data in the future work section. The strengths and weaknesses of using single-species toxicity tests as validation tools have been discussed and challenged for decades (Cairns 1983). Some researchers suggest laboratory confinement reduces overall fitness and test performance (Moore et al. 2016), but most variation in single-species data typically derives from inter-laboratory methodology (Rico et al. 2016). Single-species toxicity tests will continue to be an important tool for assessing aquatic contaminants, and, like any tool, it is important to understand how to best apply and interpret data from such tests. Exposure to the same compound under similar controlled laboratory settings using the same species can yield results that differ significantly, sometimes by a factor of three (Baird et al. 1989). Compiling such data and providing context to its implications for understanding risks to higher levels of biological organization is achievable and further governs the direction research should take (Maltby et al. 2000).

In addition to providing important and much needed toxicity data for clothianidin and thiamethoxam, I offer an improved method to determine the risk of neonicotinoid mixtures to aquatic insects by developing a formula to normalize clothianidin and thiamethoxam to imidacloprid. However, this approach is acknowledged as somewhat limited by its simplicity. Currently, five insect nAChR gene families are identified and they feature seven subunits with varying degrees of divergence, attesting to the range in sensitivity within and across insect taxa to different active ingredients (Jones and Satelle 2010). Neonicotinoid mixtures may trigger activation of different subunits which are not observed when exposed to a single active ingredient. Current risk assessments are limited by available toxicity data on clothianidin and thiamethoxam with recent reviews treating mixtures as additive by summing total neonicotinoid concentration or normalizing concentrations by molecular weight (Morrissey et al. 2015). Studies with a mechanistic approach to the toxicity of neonicotinoid mixtures are largely absent in the primary literature; however, a few recent studies utilizing systematic deviations from a set of standard models has been used to determine synergism, antagonism, dose level-dependence,

or dose ratio-dependence in the toxicity of binary mixtures (Gomez-Eyles et al. 2009, Maloney et al. 2017).

6.1.2 Responses of emerging aquatic insects exposed to neonicotinoid insecticides using *in situ* wetland limnocorrals

Acknowledging the demonstrated adverse effects neonicotinoids have under controlled laboratory conditions, I addressed the lack of community-level clothianidin and thiamethoxam assessments using *in situ* limnocorrals. Mesocosm studies simulate natural ecosystems and allow for experiments that incorporate multiple species interactions and competition to understand mechanisms driving ecosystem processes. Based on data from water monitoring and aquatic life benchmarks (CCME 2007, Main et al. 2014, Morrissey et al 2015), I performed a series of limnocorral experiments simulating neonicotinoid exposure in Prairie wetland in spring and early-summer. Considered here in combination, the results of Chapters 3 and 4 demonstrated the use of aquatic mesocosm experiments as a risk assessment tool. Prior to these studies, no mesocosm data on clothianidin and thiamethoxam existed in the primary literature with 76% of the neonicotinoid mesocosm experiments focusing on imidacloprid (Morrissey et al. 2015). Before these studies, there were a limited number of mesocosm experiments testing the effects of clothianidin and thiamethoxam, three in total (Sánchez-Bayo et al. 2016).

Most of the emerged insect taxa in both limnocorral studies (Chapters 3 and 4) consisted of non-biting midges (Diptera: Chironomidae). In 2014 (Chapter 3), I found that chronic exposure (9-weeks) to imidacloprid and clothianidin can cause subtle alterations in the aquatic insect community, resulting in a decrease in multivoltine taxa. Exposure to clothianidin and thiamethoxam did not significantly alter the emerging aquatic insect community relative to the controls. However, significant deviance in community composition was observed in the imidacloprid treatments post dosing (recovery period). While clothianidin and thiamethoxam exposure did not significantly affect the emerging aquatic insect community, emergence of chironomids and zygopteran were significantly advanced by clothianidin application. Early emergence events of chironomids and zygopteran were also observed in imidacloprid treatments, 18 to 25 days earlier than controls, respectively. These changes in emergence patterns and complications with metamorphosis have previously been described in the neonicotinoid literature (Song et al. 1997, Alexander et al. 2008, Kattwinkel et al. 2016), however, the cause is still unclear. Under chronic exposure, at concentrations as low as 0.045 µg/L (imidacloprid) and

0.038 µg/L (clothianidin), aquatic insects may experience shifts in timing of biological processes (e.g., emergence).

In 2015 (Chapter 4), I attempted to repeat the previous years' experiment using only clothianidin at a wider dose range and fewer limnocorrals. Ultimately, this study provided many “lessons learned” as it was challenged by an unexpected contamination by imidacloprid likely from the previous year's experiment. Using the toxic equivalency factors derived in Chapter 2, I calculated toxic equivalency quotients (TEQs) to characterize the combined neonicotinoid exposure. Mean TEQ concentration displayed a nonsignificant negative effect on the insect community composition and showed that time had a greater influence on abundance and diversity. The limitations of mesocosm studies are discussed in Chapter 4, outlining the key challenges presented by increased environmental realism and replicate variability.

Complications can also arise from extrapolating mesocosm data to larger scales. Among the limitations of ‘cosm-type experiments are low replication, reduced experimental control, wall-effects, and high replicate variation. Even with this greater complexity, mesocosms are often criticized as over-simplifying natural ecosystems with scale acting as one of the greatest issues (Spivak et al. 2011). Here, the limitations of high replicate variation were apparent during both studies which was likely driven by a combination of scale (i.e., limnocorral volume) and wetland heterogeneity. The interpretation of these results are discussed within the context of the study and its implications to wetland ecosystems. Moreover, there are clear challenges in using mesocosm experimental units, but overall, they provide insight into the protection offered by aquatic life benchmarks. Semi-controlled, natural conditions allow us to determine if additional safety factors are required or if rare species necessitate special considerations—context is imperative to any mesocosm assessment (Clements et al. 2012).

6.1.3 Factors influencing aquatic insect emergence in Prairie wetlands exposed to neonicotinoid contamination

As described in Chapters 3 and 4, I found that aqueous neonicotinoid contamination affected total aquatic insect emergence and slightly shifted its community composition. Based on this, I selected 22 wetlands surrounded by neonicotinoid-treated canola and monitored them for water quality (including neonicotinoids) and habitat quality, while enumerating aquatic insect abundance and emergence diversity over time. To my knowledge, my study on the factors driving aquatic insect emergence in wetlands was the first to continuously monitor emergence

and measure a suite of water quality variables and habitat characteristics alongside neonicotinoid residue concentrations. Disentangling the relative influence of water quality and habitat characteristics on aquatic insect communities in wetlands is often discussed among ecologists and entomologists (Tangen et al. 2002, Batzer 2013, Gleason and Rooney 2017). This offered a glimpse of what aquatic insect communities in the PPR are exposed to under real-world conditions in areas planted with neonicotinoid-treated crops.

Based on the farmer's seeding records and previous water sampling efforts, I focused my monitoring efforts from late-spring to mid-summer. Seasonally, it captured a window with important life cycle events for higher trophic level organisms, and it overlapped with water sampling times identified as "high risk" for contamination (i.e., post-seeding runoff). Similar to observations from my limnocorral studies, chironomids were the most abundant insect taxon followed by hard-bodied flies (Muscidae types) and biting midges (Ceratopogonidae). Using a sequence of ordination techniques, I found these dipteran taxa to be associated with higher neonicotinoid concentrations and greater vegetation disturbance which indicate wetland habitat degradation. Models on insect abundance over time containing neonicotinoid concentration and vegetation disturbance were also found to offer the greatest explanatory power. Neonicotinoid concentration had a significant negative effect on total emergence over time whereas vegetation disturbance significantly increased total emergence. Together, these field data further support the need for better protections against potentially harmful intensive agricultural management practices on wetland quality and habitat structure. However, recognizing the limitations of the study, the measurement used to estimate exposure was derived from discrete grab samples (two weeks between samples). As a snapshot in time, grab samples fail to capture the variable or episodic nature of contaminant concentrations which may have over or under estimated exposure. Precautions to best-represent exposure were taken to reduce this variation. By selecting wetlands surrounded by the same crop on a single conventional farming operation maintained a level of baseline control (i.e., same seeding and application rate, similar topography and soil type). This was reinforced by the concentration data which over time displayed increases in neonicotinoid concentration among all wetlands sampled. Passive water samplers would be an improved method to estimating exposure (Sánchez-Bayo and Hyne 2014). Despite this, these data could improve the risk assessment of neonicotinoids in aquatic ecosystems and better illustrate the possible largescale impacts of prophylactic neonicotinoid use.

Table 6.1 Summary of methods and findings by chapter.

Chapter	Compounds tested/detected ^a	Concentration range (µg/L)	Experimental approach	Experimental conditions	Duration (days)	Endpoint(s)	Findings
2	IMI ^b , CLO ^c , THX ^d	0.1-10.0	Single-species toxicity test	Controlled	40	Lethality, emergence inhibition, biomass, sex ratios, emergence timing	Comparative lethal and sub-lethal effects on <i>Chironomus dilutus</i> from IMI and CLO exposure, not THX.
3	IMI, CLO, THX	0.05-0.5	Wetland limnocorrals	Semi-controlled	107	Community composition, abundance, emergence timing	Comparative effects on community composition and emergence timing on emerging aquatic insects from IMI and CLO exposure, not THX.
4	CLO	0.05-1.7	Wetland limnocorrals	Semi-controlled	49	Community composition, abundance of rare taxa	No concentration-response on aquatic insect community from CLO exposure.
5	IMI, CLO, THX, ACT ^e	0.0-0.353 ^f	Biomonitoring	Natural setting	70	Community composition, abundance	Cumulative effects of agricultural intensification on emerging aquatic insect community composition and abundance.

^aActive ingredients detected in Chapter 5

^bImidacloprid

^cClothianidin

^dThiamethoxam

^eAcetamiprid

^fMaximum single-compound concentration measured

6.2 Importance to sustaining agroecosystem biodiversity

There is a strong, well-established relationship between increasing agricultural intensification and decreasing biodiversity and ecosystem services, with holistic, ecologically-based farming practices largely being underappreciated (Tschardt et al. 2008; Lundgren and Fausti 2015). Since the 1500s, changes in land-use, such as agricultural development, has triggered an average global species reduction of 10.7% (total abundance) and 13.6% (within-region species richness) (Newbold et al. 2015). In more heavily affected areas, some habitats displayed a 39.5% decreased total abundance and 76.6% reduction in species richness, with agricultural intensification and urban development identified as the key causes to this decline. Perhaps, the most troubling decreases observed in an agricultural setting, reduction in pollinator biodiversity. Similar reductions are being displayed in various taxa including invertebrates as a result of direct and indirect effects of anthropogenic disturbance to the landscape (Dirzo et al. 2014) such as extensive monoculture cropping and chemical inputs (e.g., pesticides and fertilizers). The loss of 20% of local-scale, or regional-scale, species diversity is likely to impair ecosystem services, ultimately affecting human wellbeing. The future of agriculture will require a paradigm shift towards sustainable practices and more environmentally responsible use of pesticides (Cui et al. 2018).

Agricultural pesticides are typically applied as broad-spectrum foliar spray or seed treatments over an already simplified landscape. Chemical control of invertebrate pests is currently dominated by neurotoxic neonicotinoid insecticides. Within the last two decades, pesticide use has followed this path, mostly through the adoption of neonicotinoid seed-treatment applications which now constitute 80% of the neonicotinoid insecticide sales (Jeschke and Nauen 2008). This prophylactic application method typically comes in the form of a mixed coating of insecticide and fungicide. Due to the limited availability of untreated seeds, farmers are reliant and often obligated to purchase treated seeds to maintain their operations (Howard et al. 2009). Neonicotinoid seed treatments are one of the greatest contributors to non-target ecosystem contamination (Chagnon et al. 2014; Ippilto et al. 2015). Furthermore, the implementation of seed treatments as a pest management strategy has drastically increased the amount of neonicotinoid active ingredient used, especially in soybean and maize (Douglas and Tooker 2015).

Contrary to long-standing sustainability practices such as integrated pest management (IPM), current agricultural insecticide use is largely preventative, justifying the use of seed treatments based on virtually no monitoring data. The ecological harm of these compounds has been well-documented in the literature (See, van der Sluijs et al. 2015) and builds a strong case for IPM practices. The premise of IPM was developed from both observations of resistance by targeted pest insects and the destruction of non-target beneficial insects. IPM primarily focuses on arthropod pests but include weeds, pathogens, and some vertebrates (Kogan 1998). Before traditional IPM practices, biologists used basic pest biology and cultural practices to reduce crop damage. At its core, IPM is an effective, sustainable ecosystem-based strategy to maintain long-term control of pests and their damage to crops. This is where biological control, habitat manipulations, different cultural practices, and pest resistant dilution are used as treatments to regulate pest populations before chemical control. Chemical application is dictated by monitoring crop health. In a recent reevaluation note from Canada's PMRA, the agency states, "...identifying pest pressure poses considerable challenges for growers." This is fundamentally due to the departure from crop scouting practices. Crop scouting is one of the most important aspects of IPM; pesticides are applied only when monitoring by scouts indicates they are required based on a set of crop risk guidelines. Overall, IPM intends to reduce risks to non-target wildlife, the environment, as well as human health (Kogan 1998).

Beyond IPM, sustainable land-use planning can take on many forms. Diversification of cropland can enhance the landscape, even if only small land changes are implemented. For example, cropland in the state of Iowa, USA, occupies 63% of the total land cover. A land management technique called STRIPS (Science-based Trials of Row-crops Integrated with Prairie Strips) aims to increase ecological health by incorporating native prairie in corn and soybean operations. By replacing 10% of the planted crop area with native prairie plants, researchers increased bird abundance, reduced sediment runoff, and drastically improved native insect-host plant interactions (Liebman and Schulte 2015). Promotion of birds and beneficial insect predators reduces pest numbers and the need for chemical control. Crop production did decrease by 10% as a result of losing the maximum amount of potential cropland area; however, ecological intensification significantly improved biodiversity and promoted soil conservation. Land sharing allows for cropland habitat diversification, where strips of land within agricultural fields are set aside for colonization by indigenous flora and fauna. A quantitative meta-analysis

of 54 studies in 20 different countries revealed land sharing and land sparing restoration strategies led to an average 68% biodiversity recovery (Barral et al. 2015). In addition, biodiversity recovery was collinear with ecosystem service recovery. Furthermore, crop rotation schedules and tillage, although not always active in potential biodiversity conservation strategies, are valuable tools for farmers to alleviate the pressures of pest infestation and subsequent pesticide use. In practice, proper crop rotations prevent the establishment of pests, balance soil fertility, and promote advantageous soil microbial communities. Davis et al. (2012) compared several key environmental and economic variables between 2-year (corn, soybean), 3-year (corn, soybean, red clover), and 4-year (corn, soybean, red clover, oat) crop rotations. Due to the current economic value of corn and soybeans, many farmers may plant these crops out of rotation to maximize economic yields. Rotations over two years significantly reduced the amount of mineral fertilizers and pesticide use which led to less labor and fuel requirements to operate machinery. In addition, corn and soybean yields were greater on fields rotated every 3-4 years, increasing by 30-60 lbs. per acre (Davis et al. 2012).

A number of recent studies have addressed the current progression of global, prophylactic neonicotinoid use (van der Sluijs et al. 2015). Research outcomes on biodiversity of pollinators (Gill and Raine 2014), aquatic invertebrates (Morrissey et al. 2015), amphibians (Mason et al. 2013), and insectivorous birds (Hallmann et al. 2014), as well as ecosystem function of aquatic and terrestrial environments (Chagnon et al. 2014) is mounting in favor of alternative pest management strategies. Historically, greater understanding of pesticides and their effects on human and ecosystem wellbeing has translated to beneficial policy and management changes, ranging from small management policies (Cole et al. 2011) to the formation of regulatory governing bodies (Williams 1993). Conservation of biodiversity in agroecosystems is posed to remain a challenge until better management and policy frameworks are implemented. Environmental, societal, and economic barriers will necessitate a transdisciplinary approach from multiple stakeholders including agronomists, ecologists, toxicologists, regulators, industry representatives, and farmers.

Beyond the natural variability of agroecosystems, there are important socio-economic considerations to crop production and future pesticide use. The concept of a technological treadmill was first introduced by Willard Cochrane in 1958. In the context of agriculture, the technological treadmill refers to the reliance of farmers on synthetic pesticides and other

chemical inputs to maintain production and their ability to manage profits while saving seeds for the following year (Howard et al. 2009). As regional food supplies meet their demands, the price of the crop will decrease. If a farmer cannot maintain production and economic gain during this crop price instability, they fall off the technological treadmill and are typically bought out by their neighbor who continues to expand production. This requires large-scale farming operations to have almost obligate ties to the seed industry and chemical companies (Howard et al. 2009). Revisiting biological conservation, the situation ultimately becomes an evaluation of livelihood and human wellbeing versus species conservation and a sustainable environment. There are a plethora of positive feedbacks farmers acquire from investments in ecological intensification, but the economic risk and the need for high, profitable yields are still their reality. Without accessible alternative information, transparency from seed companies, available untreated seed products, or educational resources to manage their operations in a more holistic manner, the system is at a stand-still. The preventive application of neonicotinoids reduces this economic gamble, and, even in a bad year, frequent rotations of pretreated economically viable crops appear to reduce the risk of low profit.

Over twenty years ago, McLaughlin and Mineau (1995 p.208) directly stated in a review on the impact of agricultural practices on biodiversity, “Agriculture has repeatedly been identified as one of the largest contributors to the loss of biodiversity world-wide.” Until we can better manage and reduce our reliance on chemical inputs, accept crop yield loss from natural variation (i.e., poor weather conditions), increase landscape complexity, and offer farmers economic, holistic options, society will continue to misuse pesticides. Sustainable farming operations need to be funded, created, and managed as resilient agroecosystems with ecological principles dictating pest prevention and minimizing pest tolerance to last resort, well-informed insecticide applications. In the closing chapter of *Silent Spring*, Rachel Carson submits:

“The choice after all, is ours to make. If, having endured much, we have at last asserted our “right to know,” and if, knowing, we have concluded that we have been asked to take senseless and frightening risks, then we should no longer accept the counsel of those who tell us that we must fill our world with poisonous chemicals; we should look about and see what other course is open to us.” (Carson 1962 p.321)

6.3 Future research directions

The research presented in this thesis contributed significant single-species toxicity data and field assessments addressing the effects of chronic neonicotinoid exposure to aquatic insects, specifically to insect emergence. Even with the surge in neonicotinoid research over the last few years, there are still many questions that can be explored and some that are more paramount than others. More questions arose from my research findings, and there are a number of laboratory and field studies which, if implemented, could advance our knowledge. Here are several recommendations for future work.

During my laboratory experiments, I made several observations relating to insect behavior and physiology. These were specifically related to chironomid larval activity (e.g., an increase in locomotion and case-building activities), a male-dominant sex ratio bias, and an inability of adult chironomids to successfully disassociate from their pupal exuvia. Insect hormones govern a number of functions critical to growth and development including molting and metamorphosis (Soin and Smagghe 2007). Insects are described as possessing several peptide hormones primarily originating from the central nervous system (CNS) and outer lining of the midgut (Yin et al. 1994). Protein precursors through cleavage and other post-transcription processes produce active peptides such as prothoracicotrophic hormones (PTTH). PTTHs are responsible for prothoracic gland stimulation which produces ecdysone and adipokinetic hormone (Soin and Smagghe 2007). Ecdysone is a prohormone which is converted by cytochrome P-450 to the active hormone, 20-hydroxyecdysone (20E). Cytochrome P-450 plays a critical role during the last sequences of hydroxylation in the biosynthesis of ecdysteroids to 20E during the final immature larval instar (Rewitz et al. 2006). Through a macromolecular complex consisting of an ecdysteroid receptor (EcR) and ultraspiracle (USP), 20E exerts effects on an ecdysone response element which targets genes responsible for molting and metamorphosis. To adjust, or regulate this development, insects produce juvenile hormones (JHs), which counter ecdysteroid action (e.g., ecdysone) (Riddiford et al. 2003). The combination of JHs and 20E dictates immature insect development until pupal ecdysis (breaking from pupal exuvium). Neonicotinoids interact with biological target sites found in the CNS and gut of aquatic macroinvertebrates (Nyman et al. 2014), the same organ systems which produce peptide hormones. Insecticide metabolism is mediated by the expression of various isozymes, such as cytochrome P-450 monooxygenases, carboxylesterases, and glutathione-S-transferases (Casida and

Durkin 2013). Biotransformation and subsequent activation or detoxification of parent insecticide compounds ultimately dictates toxicity (Casida and Quistad 2004). Repeated exposure can induce over expression of P-450 alleles, where elevated levels can indicate insecticide resistance (Daborn et al. 2002). Transgenic up-regulation of P-450 genes by insecticides is described in a number of species. Daborn et al. (2002) describes the overexpression of the P-450 gene, *Cyp6g1*, in *Drosophila melanogaster* from developed resistance to DDT, imidacloprid, nitenpyram, and lufenuron. In the presence of the neonicotinoid, nitenpyram, up-regulation of the P-450 genes, *Cyp6g1* and *Cyp6g2* increased *D. melanogaster* survival (Daborn et al. 2007); perhaps, influencing the incentive to continue overexpression to survive exposure in future generations. Future studies should characterize the gene expression patterns of a model benthic macroinvertebrate (e.g., *Chironomus riparius*) as it relates to 20E regulation and cuticle development among immature instars. Similar studies have been conducted with BPA-substitutes, antibacterial agents, and IGRs evaluated by ecdysone linked biomarkers (Planelló et al. 2015, Martínez-Paz et al. 2017, Herrero et al. 2018).

Higher trophic level organisms rely on aquatic insect emergence for nutrients, and previous work suggests contaminated subsidies could pose a threat to secondary consumers. As a primary component of freshwater and terrestrial food webs, aquatic insects are known to accumulate neonicotinoids in their tissues (Haroune et al. 2015). Kraus et al. (2014) characterized the flow of contaminants in aquatic insect prey subsidies through aquatic and terrestrial food webs using stable isotope analyses. Many lipophilic insecticides display biomagnification through the food chain and negatively impact terrestrial and aquatic consumers. Partition coefficients (i.e., $\log K_{OW}$) dictate the ability of a contaminant to bioaccumulate within an organism. Neonicotinoids have a high affinity for insect nicotinic acetylcholine receptors and bind irreversibly to the post-synaptic connections (Tomizawa, and Casida 2005). Within arthropod bodies, neonicotinoids will concentrate along the ventral nerve cord and/or the midgut depending on the route of exposure (i.e., cuticular vs. ingestion). Measureable concentrations of neonicotinoids have been detected in the tissues of freshwater crustaceans (Nyman et al. 2014), non-target adult insects (Haroune et al. 2015), and pest insect species (Nauen et al. 2003). Ecosystems yielding lower quality prey items, or an overall reduction in insect productivity, may potentially impact higher trophic level organisms and long-term population success. To my knowledge, no study has quantified the magnitude of neonicotinoid-laden prey subsidies to

secondary consumers and their potential impacts to sensitive fish and wildlife species. Recent studies have suggested that global pathogen and parasite outbreaks in vertebrate populations are correlated with increased pesticide use, specifically neonicotinoids (Mason et al. 2013). Research on the consumption of neonicotinoid-laden prey by predatory insects suggests significantly reduction in predator mobility and immune system function (Bredeson et al. 2015, Pisa et al. 2015). Evaluating the potential risk to vertebrate species ingesting neonicotinoid-contaminated prey items is critically important to implement best management practices to protect at-risk populations.

Wetland habitat features such as water permanency can significantly alter the emerging aquatic insect community (Driver 1971, Wrubleski and Ross 2011, Batzer 2013). In a region already subjected to intensive agricultural practices including wetland drainage, it would be useful to determine how impactful habitat remediation strategies (e.g., wetland restoration, phytoremediation, mycoremediation) are in areas using pesticide-treated crops, not limited to neonicotinoid use (Zedler 2003, Cundy et al. 2013). Main et al. (2017) detected trace amounts of neonicotinoid active ingredients in wetland plants (e.g., *Typha sp.*, *Alisma sp.*, and *Equisetum sp.*) at concentrations ranging from 1.0-8.4 $\mu\text{g}/\text{kg}$, and buffered wetlands have been shown to mitigate the negative effects of direct runoff from cropland (Riens et al. 2013). Data presented in Chapter 5 indicated greater insect diversity with increasing buffer zone width; I collected more predatory taxa and some important pollinators in wetlands surrounded by larger vegetative buffers that provide beneficial ecosystem services (Sathe and Shinde 2008, Stewart et al. 2017). Pesticides will continue to be a widely-used tool to curb pest damage and increase crop yields. Data testing the appropriate use of pesticides with more ecologically resilient tactics (i.e., more wetlands and larger vegetative buffers) may provide practical, farm-level solutions to reduce broad-spectrum pesticide use. Supporting farmers with strategies they can implement on their personal operations may be an influential, grassroots resolution to our modern agricultural dilemma.

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APPENDIX A: SUPPLEMENTAL INFORMATION FOR CHAPTER 3

S.I. Table 1. Mean (\pm SE) measured neonicotinoid concentrations immediately post-dosing, 7 days post-dose, and during the recovery period (n = number of weeks samples were analyzed/total number of weeks).

Treatment	Nominal dose ($\mu\text{g/L}$)	Mean concentration (\pm SE $\mu\text{g/L}$)		
		Post-dose ($n = 7/9$)	7 days post-dose ($n = 4/9$)	Recovery period ($n = 3/6$)
Control	0.00	ND ^a	ND	ND
Imidacloprid	0.05	0.045 (\pm 0.006)	0.029 (\pm 0.003)	0.017 (\pm 0.011)
	0.50	0.436 (\pm 0.062)	0.259 (\pm 0.082)	0.166 (\pm 0.046)
Clothianidin	0.05	0.038 (\pm 0.015)	0.016 (\pm 0.003)	0.016 (\pm 0.002)
	0.50	0.384 (\pm 0.048)	0.229 (\pm 0.041)	0.146 (\pm 0.009)
Thiamethoxam	0.05	0.045 (\pm 0.018)	0.012 (\pm 0.007)	0.000 (\pm 0.000)
	0.50	0.386 (\pm 0.106)	0.226 (\pm 0.063) ^b	0.038 (\pm 0.030)

^aND=No detection (imidacloprid LOD=2.2 \pm 0.1 ng/L; clothianidin LOD=3.3 \pm 0.4 ng/L thiamethoxam LOD=4.2 \pm 0.5 ng/L)

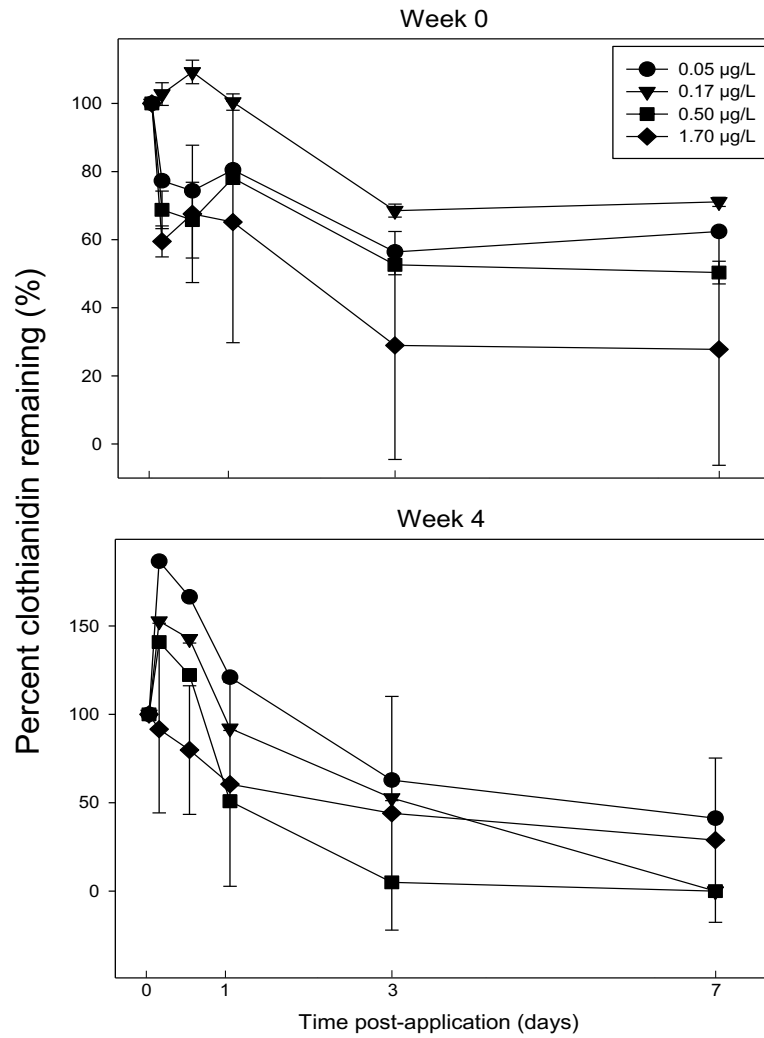
^b<LOQ concentration of clothianidin was detected twice in a single limnocorral.

S.I. Table 2. Taxa list, corresponding species axis labels, and species scores on the Principal Response Curves.

Label	Lowest taxonomic ID	Family-level ID	Species scores (PRC1)		
			IMI	CLO	THX
CMINI1	<i>Chironomus sp.</i>	Chironomidae	-3.50	-3.41	2.79
CMINI2	Chironomini sp.	Chironomidae	-0.26	-0.44	0.00
TSINI	Tanytarsini sp.	Chironomidae	-1.03	-1.91	1.04
ORT1	<i>Psectrocladius sp.</i>	Chironomidae	-0.12	0.03	0.13
TANY1	<i>Ablabesmyia peleensis</i>	Chironomidae	-0.91	-0.95	0.95
ORT2	<i>Cricotopus sp.</i>	Chironomidae	-0.75	-0.48	0.51
TANY2	Tanypodinae	Chironomidae	0.00	0.00	-0.06
ORT3	Orthocladiinae	Chironomidae	-0.56	0.50	1.35
HYDRO	<i>Agraylea multipunctata</i>	Hydroptilidae	0.03	0.06	0.04
LIM1	<i>Philarctus quaeris</i>	Limnephilidae	-0.14	0.00	0.13
LIM2	<i>Limnephilus infernalis</i>	Limnephilidae	-0.83	-0.18	2.11
CHAO	<i>Chaoborus americanus</i>	Chaoboridae	-0.97	-0.67	0.48
BAE	<i>Callibaetis ferrungis</i>	Baetidae	0.00	-0.01	0.01
PSYC	<i>Psychoda sp.</i>	Psychodidae	-0.03	-0.10	0.06
COE	<i>Enallagma annexum</i>	Coenagrionidae	0.04	0.04	0.08
LES	<i>Lestes disjunctus</i>	Lestidae	-0.34	-0.23	0.14
BRAC	Braconidae	---	0.00	-0.02	-0.02

CANA	Canacidae	---	0.17	0.08	-0.02
CERATO	Ceratopogonidae	---	-0.05	-0.18	0.18
CORE	Corethrellidae	---	0.00	0.00	0.00
DIAP	Diapriidae	---	-0.01	0.06	-0.01
DOLI	Dolichopodidae	---	-0.07	-0.12	0.05
DRYO	Dryomyzidae	---	-0.01	-0.07	0.06
DYST	Dysticidae	---	0.02	0.04	-0.01
EMP	Empididae	---	-0.01	0.00	0.00
EPHY	Ephydriidae	---	0.20	-0.08	0.19
EULO	Eulophidae	---	0.00	0.09	-0.01
FIGI	Figitidae	---	-0.02	0.00	0.00
MUS	Muscidae	---	0.08	-0.04	0.27
MYCE	Mycetophilidae	---	0.00	-0.02	0.00
MYM	Mymaridae	---	0.00	-0.03	0.01
NEMA	Nematocera	---	-0.08	0.12	-0.02
SCAT	Scathophagidae	---	-0.05	-0.04	0.01
SCEL	Scelionidae	---	-0.04	0.06	0.04
SCIO	Sciomyzidae	---	0.00	0.04	-0.05
SIMU	Simuliidae	---	0.01	0.01	-0.01
SYRP	Syrphidae	---	-0.01	-0.04	0.04
TABA	Tabanidae	---	0.00	0.06	0.00
TIPU	Tipulidae	---	-0.01	0.00	0.01
TRICH	Trichogrammatidae	---	0.00	0.00	0.01

APPENDIX B: SUPPLEMENTAL INFORMATION FOR CHAPTER 4



S.I. Figure 1. Degradation of clothianidin (\pm SE) during the start of the exposure period (Week 0) and the end of the exposure period (Week 3) for each of the four treatments. Clothianidin application occurred four times (weekly) over the 28-day exposure.

APPENDIX C: SUPPLEMENTAL INFORMATION FOR CHAPTER 5

S.I. Table 1. Summary of limits of quantification (LOQ) and limits of detection (LOD) for wetland water. Concentrations for LOQ and LOD are in ng/L.

Method limits - QA/QC ^a			
Active ingredient	% Recovery correction	LOQ	LOD
Imidacloprid	83.5 (± 2.1)	3.2 (± 1.1)	1.0 (± 0.3)
Clothianidin	67.9 (± 6.9)	4.0 (± 1.2)	1.3 (± 0.4)
Thiamethoxam	80.1 (± 3.9)	6.4 (± 1.7)	2.1 (± 0.6)
Acetamiprid	92.6 (± 2.5)	1.3 (± 0.3)	0.4 (± 0.1)

^aLab blanks (D.I. H₂O; *n*= 17).

S.I. Table 2. Summary of physicochemical water quality parameters, qualitative and quantitative wetland characteristics (mean \pm S.E.) of Prairie agricultural wetlands sampled in each of 2013 ($n=11$) and 2015 ($n=11$). Values in bold are statistically significant differences between study years (t-test; $P<0.05$).

Measurement	2013	2015
<u>Water chemistry</u>		
Dissolved oxygen (%)	72.05 (\pm 4.61)	78.18 (\pm 8.09)
Dissolved oxygen (mg/L)	6.59 (\pm 0.43)	7.66 (\pm 0.79)
Conductivity (μ S/cm)	331.04 (\pm 20.7)	980.56 (\pm 92.0)
pH	7.70 (\pm 0.06)	8.04 (\pm 0.07)
Temperature ($^{\circ}$ C)	17.15 (\pm 0.34)	17.52 (\pm 0.52)
Nitrate mg/L (NO ₃)	5.22 (\pm 1.31)	4.72 (\pm 0.25)
Phosphate mg/L (PO ₄)	3.93 (\pm 0.35)	1.76 (\pm 0.13)
Ammonia mg/L (N)	0.27 (\pm 0.05)	0.14 (\pm 0.03)
Turbidity (FTU)	11.40 (\pm 2.06)	21.5 (\pm 3.65)
<u>Wetland assessment parameters</u>		
Precipitation (mm)	30.6 (\pm 3.45)	11.53 (\pm 1.45)
Wetland depth (cm)	81.52 (\pm 2.24)	67.78 (\pm 2.85)
Basin fill ^a	4.1 ^b	3.8 ^c
Algae cover	19.09 (\pm 5.5)	3.18 (\pm 1.4)
Surface vegetation	38.64 (\pm 9.3)	52.73 (\pm 10.9)
Vegetation disturbance	100 ^d	23.18 (\pm 5.9)
Continuity: Reeds/Rushes (%)	38.65 (\pm 9.8)	81.36 (\pm 8.6)
Continuity: Grasses (%)	2.27 (\pm 1.6)	22.73 (\pm 5.5)
Vegetative buffer width (m)	1.2 (\pm 0.7)	8.4 (\pm 1.6)
Distance to nearest wetland (m)	32.3 (\pm 6.0)	12.7 (\pm 2.2)
Dominant species: Open water	NA	<i>Lemna turionifera</i> <i>Potamogeton sp.</i>
Dominant species: Emergent	<i>Alisma triviale</i> <i>Typha latifolia</i>	NA
Dominant species: Shallow marsh	<i>Alisma triviale</i>	<i>Alisma triviale</i> <i>Carex atherodes</i>
Dominant species: Wet meadow	<i>Phalaris arundinacea</i>	<i>Bromus inermis</i> <i>Calamagrostis canadensis</i> <i>Equisetum fluviatile</i> <i>Hordeum jubatum</i> <i>Phalaris arundinacea</i>

^aBasin fill codes: 1 (0-25%), 2 (25-50%), 3 (50-75%), 4 (75-100%), 5 (>100%)

^bRanged from 3 to 5

^cRanged from 1 to 5

^dEvery wetland assessed scored 100% vegetation disturbance