Insecticide contamination of streams in a warming climate

Samuel James Macaulay

A thesis submitted for the degree of Doctor of Philosophy at the University of Otago, Dunedin, New Zealand.

June 2019
Abstract

Feeding a growing global population in the context of a changing climate will bring challenges for food producers and environmental managers seeking to mitigate the impact of intensifying agricultural practices. The control of insect pest populations through insecticide application is an important practice for improving crop yields, yet contamination of the environment with insecticides combined with impacts from climate change are together subjecting ecosystems to novel stressors and stressor combinations. Contamination of surface waters with neonicotinoids, the most widely used insecticides in the world, has become a chronic global problem. Despite a growing body of research investigating the impacts of neonicotinoids in aquatic ecosystems, prior to my research there were no published toxicity data for their effects on aquatic insects in New Zealand. Moreover, international data on chronic, neonicotinoid-mixture, interactive multiple-stressor and community-level effects in stream ecosystems were also lacking. My overall PhD research objective was to investigate the ecological impacts of neonicotinoid insecticides on New Zealand’s freshwater macroinvertebrate communities in a multiple-stressor and climate-change context.

I developed and optimized laboratory procedures for testing chronic toxicities of neonicotinoids and observing their lethal and sublethal effects on stream insect larvae in controlled laboratory experiments. Through a series of 4-week experiments, the chronic toxicities of three commonly used neonicotinoids (imidacloprid, clothianidin and thiamethoxam) to nymphs of the ubiquitous New Zealand mayfly *Deleatidium* spp. were determined (Chapter 2) and possible interactions between the three insecticides were investigated (Chapter 3). In a subsequent 6-week multiple-stressor laboratory experiment, also with *Deleatidium* nymphs, I focused on possible interactions of low-level imidacloprid exposure with simulated heatwaves and a period of food limitation (Chapter 4). I then conducted a stream-side mesocosm experiment in 128 flow-through stream channels to investigate the individual and combined effects of realistic, pulsed exposures of imidacloprid and raised water temperature on stream macroinvertebrate communities characteristic of fast-flowing and slow-flowing microhabitats (Chapter 5). This experiment is the first empirical evaluation of stream macroinvertebrate community dynamics in response to the world’s most widely used agricultural insecticide, increased water temperature and reduced flow velocity (simulating streams subject to reduced flows due to water abstraction and
climate change). Six hundred and forty invertebrate drift and insect emergence samples each were collected throughout the experiment (on five occasions during and after the insecticide pulses), and 128 benthic invertebrate samples were collected after 24 days of heating and insecticide manipulations. In combination, the long-term laboratory experiments using larvae of a sensitive aquatic insect taxon and the field experiment in stream mesocosms allowed assessing neonicotinoid effects on stream macroinvertebrates at the individual, population and community level in a multiple-stressor and climate-change context.

The 28-day concentration-response experiments (Chapter 2) and the neonicotinoid mixture experiment (Chapter 3) revealed imidacloprid as the most toxic of the three neonicotinoids to *Deleatidium* nymphs and also showed its potential for synergistic interactions with the other, comparably less toxic neonicotinoids clothianidin and thiamethoxam. Imidacloprid was therefore chosen for the subsequent multiple-stressor experiments (Chapters 4 and 5). Both of these demonstrated the strong effects of raised water temperatures on stream invertebrates and especially on *Deleatidium* larvae. In the 42-day laboratory experiment, sublethal and lethal effects of exposure to 0.4 µg/L imidacloprid took 24−36 days to manifest and were clearest in the absence of heatwaves and starvation because these stressors alone already strongly reduced *Deleatidium* survival. In the mesocosm experiment, all three manipulated factors strongly affected invertebrate drift community composition, with the first pulse of imidacloprid and increased temperature having a greater impact on communities in fast-flowing channels. Heating and imidacloprid exposure both generally resulted in increased emigration by drift. Increased temperature was the most pervasive stressor for the benthic invertebrate community, negatively affecting 80% of response variables. This was in part due to a natural 10-day heatwave which occurred during the manipulative period, raising temperatures in ambient mesocosms to 29.8°C and in heated channels to 32.9°C. Water temperature in the river reached 31.2°C during a second heatwave seven weeks after the end of the experiment. The snail *Potamopyrgus antipodarum* showed the only positive response to raised water temperatures, also responded positively to slow flow and was unaffected by imidacloprid highlighting the general tolerance of this invasive species to stressors. Monitoring drift and emergence patterns periodically throughout the experiment also provided insights into how invertebrate community composition changed in response to the natural heatwave. Taken together, the findings of my thesis demonstrate the importance of efforts to mitigate climate change and reduce contamination of surface waters with imidacloprid in order to protect our vulnerable freshwater ecosystems.
I dedicate this work to my parents: the ones I’ve had from the very beginning, and the ones I’ve gained along the way.
Acknowledgements

I was told before I started this project that finding a good supervisor is more important than finding a good research topic. I was lucky enough to choose both an outstanding primary supervisor and a stimulating area of research. My heartfelt thanks to Christoph Matthaei for his guidance and support over these years of work. Thank you for always having an open door, for providing such helpful feedback and for showing interest in me as a person as much as wanting to see me develop as a researcher. This journey has been made all the more enjoyable and fulfilling under your guidance and direction. Many thanks too to Jay Piggott and Kimberly Hageman for their co-supervision of this project. I could not have solved many issues along the way without your help. Thank you both for bringing your esteemed and valued perspectives to this work.

I am extremely grateful to the many hands that assisted me both in the field and in the lab; Peranjali Latchoumy, Ludovic Vincent, Johanna Khan, Teresa Be, Jessica and Nils Styger, Quentin Amalric, Pavel Mikheev, Ewan Bakker, Charlotte Patterson, Noel Juvigny-Khenafou, Nat Lim, Nicola McHugh, Stu Borland, and to Rob Alumbaugh and Sean Lyons in the lab at Utah: this would not have been possible without your important contributions. A special thanks to Jeremy Xu for working through all those samples of bugs that you were able to count. I doubt I’ll meet someone who can id. inverts as efficiently as you can!

Thanks to Clyde and Jan Douglas for access to and use of their property at the Kauru. I will always have fond memories of my campsite by the river, especially Jan’s free-range eggs!

My research was supported by a University of Otago Doctoral Scholarship and a research budget from the Department of Zoology.

And to my family and friends—my closest supporters! I am so grateful for having good friends who’ve been an invaluable source of support, laughter and encouragement during this journey.

Mum and Dad, thank you for instilling in me an interest in the outdoors from my earliest days. For carrying me across streams and rivers before I was big enough to wade through them myself or old enough to know what an incredible world existed beneath their surface. Thank you for fostering my passion for ecology, for your continued interest in my work and for your unwavering and unending support and encouragement to see the task through.
Jonathan and Mary, thank you both for your keen interest in my research and for sparking new passion when my own has waned. I will always think it funny how I came to research streams and climate change and gain you two as parents at the same time.

Lastly, to my wonderful Grace, I really am so lucky to have had you by my side every step of the way. I couldn’t have done this without you. You mean the world to me, thank you.
# Table of Contents

Abstract ............................................................................................................................... i

Acknowledgements ........................................................................................................... iv

1 General Introduction ......................................................................................................... 2
  1.1 Global food security, agriculture and climate change ....................................................... 2
  1.2 Impacts of agriculture and climate change in freshwater ecosystems ......................... 5
  1.3 Neonicotinoid Insecticides ............................................................................................ 9
  1.4 Thesis aim and outline ................................................................................................. 12

2 Chronic toxicities of neonicotinoids to nymphs of the common New Zealand mayfly *Deleatidium* spp. ........................................................................................................... 15
  2.1 Summary .................................................................................................................... 15
  2.2 Introduction ................................................................................................................ 15
  2.3 Methods ..................................................................................................................... 19
    2.3.1 Artificial stream water (ASW) ............................................................................... 19
    2.3.2 Experimental design ............................................................................................. 19
    2.3.3 Insecticide application, sampling and quantification ............................................. 20
    2.3.4 Mayfly food supply ............................................................................................... 21
    2.3.5 Test specimen collection and acclimation conditions .......................................... 21
    2.3.6 Recording invertebrate responses ........................................................................ 22
    2.3.7 Data analysis ........................................................................................................ 23
  2.4 Results ....................................................................................................................... 24
    2.4.1 Neonicotinoid exposures ....................................................................................... 24
    2.4.2 Time-to-effect observations and comparative toxicities ..................................... 24
    2.4.3 Effects on moulting propensity ............................................................................. 31
  2.5 Discussion .................................................................................................................. 33
    2.5.1 Comparison of chronic toxicities for mayflies and midges ................................. 33
2.5.2 Effects on moulting propensity ................................................................. 36
2.5.3 Relative toxicities of neonicotinoids ...................................................... 37
2.5.4 Conclusions and further research ........................................................... 37

3 A potent cocktail: Imidacloprid dominates the combined toxicities of neonicotinoid mixtures to Deleatidium spp. nymphs .................................................. 40

3.1 Summary ........................................................................................................ 40
3.2 Introduction .................................................................................................... 40
3.3 Methods .......................................................................................................... 43
  3.3.1 Experimental design .................................................................................. 43
  3.3.2 Insecticide application, sampling and analysis quantification .................. 44
  3.3.3 Test specimen collection, acclimation conditions and reading invertebrate responses .................................................................................. 44
  3.3.4 Data analysis ............................................................................................ 44
3.4 Results ............................................................................................................. 45
  3.4.1 Neonicotinoid exposures .......................................................................... 45
  3.4.2 Individual and combined effects of neonicotinoid exposure ..................... 46
3.5 Discussion ....................................................................................................... 54
  3.5.1 Primary neonicotinoid effects and comparative toxicities ....................... 54
  3.5.2 Neonicotinoid mixture effects and interactions ........................................ 55

4 Delayed effects of food limitation and chronic exposure to imidacloprid interact with strong effects of simulated heatwaves on mayfly nymphs ......................... 60

4.1 Summary ........................................................................................................ 60
4.2 Introduction .................................................................................................... 60
4.3 Methods .......................................................................................................... 65
  4.3.1 Experimental design .................................................................................. 65
  4.3.2 Insecticide application, sampling and analysis ........................................ 66
  4.3.3 Test specimen collection, acclimation conditions and reading invertebrate responses .................................................................................. 66
4.3.4 Data analysis ................................................................. 67
4.4 Results ............................................................................. 67
  4.4.1 Imidacloprid exposure concentrations .................................. 67
  4.4.2 Stressor main effects on mayfly survivorship and impairment .......... 67
  4.4.3 Interaction effects on mayfly survivorship and impairment .......... 71
  4.4.4 Moulting propensity ....................................................... 73
4.5 Discussion ........................................................................ 75
  4.5.1 Heatwave main effects .................................................... 75
  4.5.2 Imidacloprid main effects ............................................... 76
  4.5.3 Starvation main effects .................................................. 79
  4.5.4 Interaction effects and delayed effects of starvation and imidacloprid .... 79
  4.5.5 Conclusions and implications for risk assessment ..................... 81

5 Climate warming and imidacloprid pulses determine stream macroinvertebrate community dynamics ........................................................................... 84

  5.1 Summary ........................................................................... 84
  5.2 Introduction ....................................................................... 85
  5.3 Methods ............................................................................ 87
    5.3.1 Study site ...................................................................... 87
    5.3.2 Experimental system and study design ......................... 88
    5.3.3 Colonisation period ....................................................... 91
    5.3.4 Macroinvertebrate and insect emergence sampling ......... 91
    5.3.5 Invertebrate responses ............................................... 92
    5.3.6 Data Analysis ............................................................. 93
  5.4 Results ................................................................................ 95
    5.4.1 Imidacloprid exposure concentrations ......................... 95
    5.4.2 Temporal community shifts in the drift ......................... 95
    5.4.3 Invertebrate drift responses to experimental manipulations .... 98
Chapter 1

General Introduction
1 General Introduction

1.1 Global food security, agriculture and climate change

One of the greatest challenges of the 21st century will be ensuring that a global population of more than 9 billion people has adequate food and nutrition, while at the same time mitigating anthropogenic environmental impacts (Godfray et al. 2010, Foley et al. 2011). In 2015, the United Nations adopted the 2030 Agenda for Sustainable Development which included 17 Sustainable Development Goals (SDGs). The second of these goals is to end hunger, achieve food security and improved nutrition and promote sustainable agriculture, which can only be achieved in context of Goal 13: to take urgent action on climate change and its impacts (UN 2015). These goals have to be worked towards jointly as they are interdependent—each one directly influences the other (Figure 1.1; Table 1.1). On the one hand, agriculture is a significant contributor to greenhouse-gas emissions (Foley et al. 2005, West et al. 2010). On the other hand, the impacts of climate change on agriculture can be devastating due to increased climate variability (e.g. altered rainfall and temperature patterns) and exposure to more complex, frequent and intense climate extremes (e.g. flooding, heatwaves and droughts; FAO 2018). The Food and Agriculture Organization of the United Nations’ (2018) report on the state of food security and nutrition in the world focused on the need to build climate resilience into food-production systems because hunger is significantly worse in countries where agriculture is highly sensitive to rainfall and temperature variability and severe drought (Figure 1.1. Arrow i.). This considerable challenge of increasing food production while mitigating the impacts of climate change and agriculture will require a strategic, global effort to implement more climate-resilient and sustainable agricultural practices (Godfray et al. 2010, Foley et al. 2011).

There is no simple solution to this dilemma; however, the most sustainable proposed method of providing global food security and meeting the increasing demands for crop production is through closing yield gaps (Godfray et al. 2010, Ray et al. 2013). This means getting more (ideally, the most) out of already-farmed land, which means agriculture must intensify, rather than expand into new areas (Tilman et al. 2011; Figure 1.1. Arrows ii. & iii.). While agricultural intensification brings its own environmental impacts, several authors have proposed these to be relatively less detrimental than converting more natural (native) habitat
Figure 1.1. Thesis context schematic showing the interdependence of the UN’s Sustainable Development Goals to A) improve global food security and B) reduce climate change, which both cause increased C) agricultural intensification (relative to expansion) and D) pesticide use to control pest populations, with the resulting pesticide contamination of the environment contributing to adverse effects on E) the ecological integrity of Earth’s freshwater resources. Positive and negative feedback loops and relationships are represented by arrows i–xiii. Green arrows represent a positive (increasing) effect, whereas red arrows represent a negative (decreasing) effect. My thesis focuses on the impacts of B, C and D (relationships ix–xi) on E) the integrity of Earth’s running-water ecosystems. See Table 1.1 for effect descriptions.
<table>
<thead>
<tr>
<th>Effect</th>
<th>Description</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>i.</td>
<td>Climate change reduces global food security through climate variability and extremes in temperature and rainfall (droughts) and increasing pest prevalence.</td>
<td>(IPCC 2014; Campbell et al. 2016; FAO 2018)</td>
</tr>
<tr>
<td>ii.</td>
<td>Improving global food security requires increased agricultural intensification to close yield gaps.</td>
<td>(Godfray et al. 2010; Foley et al. 2011)</td>
</tr>
<tr>
<td>iii.</td>
<td>Agricultural intensification improves crop yields (food production), which increases global food security.</td>
<td>(Godfray et al. 2010)</td>
</tr>
<tr>
<td>iv.</td>
<td>Climate change increases the need for intensified agriculture to strengthen resilience against increased climate variability and extremes (e.g. irrigation mechanisms to combat rainfall variability and droughts).</td>
<td>(FAO 2018)</td>
</tr>
<tr>
<td>v.</td>
<td>Agricultural intensification (of crop production) rather than agricultural expansion reduces relative carbon emissions, contributing less to climate change than further expansion of agricultural land.</td>
<td>(West et al. 2010; Foley et al. 2011; Tilman et al. 2011)</td>
</tr>
<tr>
<td>vi.</td>
<td>Climate change extends the latitudinal ranges of insect pests, increasing the need for pest control. Climate change causes increased environmental contamination by pesticides through altered precipitation patterns (increased heavy rainfall events).</td>
<td>(Noyes et al. 2009; Kattwinkel et al. 2011)</td>
</tr>
<tr>
<td>vii.</td>
<td>Agricultural intensification typically involves increased pest control through pesticide use (e.g. prophylactic application of neonicotinoids), which causes environmental contamination with pesticides.</td>
<td>(Matson et al. 1997; Tilman 1999; Tilman et al. 2001; Tilman et al. 2002)</td>
</tr>
<tr>
<td>viii.</td>
<td>Prophylactic pesticide use (e.g. neonicotinoids) can result in pesticide resistance in insect pest populations, and environmental contamination with pesticides harms non-target organisms that benefit agriculture (e.g. pollinators, biological control), thereby harming agricultural productivity.</td>
<td>(Matson et al. 1997; Chagnon et al. 2015; Douglas and Tooker 2015; Hladik et al. 2018)</td>
</tr>
<tr>
<td>ix.</td>
<td>Agricultural intensification reduces freshwater resources and degrades their ecological integrity through abstraction for irrigation and contamination with pollutants.</td>
<td>(Tilman 1999; Jackson et al. 2001; Dudgeon et al. 2006)</td>
</tr>
<tr>
<td>x.</td>
<td>Use of water-soluble pesticides causes contamination of freshwaters, harming non-target organisms and reducing the biological integrity of freshwater ecosystems.</td>
<td>Stehle and Schulz 2015; van der Sluijs et al. 2015; Van Lexmond et al. 2015</td>
</tr>
<tr>
<td>xi.</td>
<td>Climate change reduces freshwater resources and degrades their ecological integrity through reduced rainfall patterns causing droughts and increased temperatures and more extreme weather events (heatwaves/floods), which are detrimental to freshwater biota.</td>
<td>(IPCC 2014; Heino et al. 2009; Strayer and Dudgeon 2010; Woodward et al. 2010)</td>
</tr>
<tr>
<td>xii. &amp; xiii.</td>
<td>Freshwater resources and their biological integrity are critical for food production and improving global food security.</td>
<td>(FAO 2018)</td>
</tr>
</tbody>
</table>

1.2 Impacts of agriculture and climate change in freshwater ecosystems

Wherever land is modified for agricultural use, impacts on the surrounding freshwater ecosystems follow (Vorosmarty et al. 2010; Figure 1.1; Arrow ix.). Over half of the world’s accessible freshwater runoff has been appropriated for human use, with abstraction for agriculture being the largest use (Jackson et al. 2001). Water abstraction for irrigation has direct effects on river discharge and flow dynamics (Malmqvist and Rundle 2002). Even some of the largest rivers in the world are subjected to such intense abstraction that there are now periods of the year when they contain no flowing water (Postel 2003). Although river flow regimes have natural fluctuations, including seasonal low flow during periods of low rainfall, human activities cause alterations to these natural patterns by extending or creating new low-flow periods (Smakhtin 2001, Dewson et al. 2007). Moreover, the demand for water for irrigation is greatest when flows are naturally at their lowest, further exacerbating their impact (Dewson et al. 2007). Add to this the consequences of climate change of altered precipitation patterns and increased frequency and duration of droughts (IPCC 2014), and it becomes clear that the impacts of reduced flows on the world’s streams and rivers are only going to become more pervasive and prevalent (Vörösmarty et al. 2000, Vörösmarty et al. 2010) in the future.

In addition to increasing the frequency and severity of droughts, climate change will directly impact freshwater ecosystems through altered patterns of precipitation and increased temperatures (IPCC 2013; Figure 1.1. Arrow xi.). Increasing rainfall variability and extremes
will result in a greater incidence of heavy rainfall events and floods (IPCC 2014). The more severe droughts and floods will impact freshwater biodiversity through losses of species unable to tolerate such changes in natural flow regimes and increases in those species resistant or resilient to these disturbances (Malmqvist and Rundle 2002). With the majority of aquatic organisms being ectotherms, for whom temperature plays an important role in their physiology and performance (Vannote and Sweeney 1980, Huey and Kingsolver 1989, Sokolova and Lannig 2008, Willmer et al. 2009), there will be direct impacts of increasing global temperatures on freshwater biodiversity (Heino et al. 2009, Strayer and Dudgeon 2010, Woodward et al. 2010). Temperature affects growth, metabolism, reproduction, emergence and geographic distribution of aquatic insects (Sweeney and Vannote 1978, Vannote and Sweeney 1980). Unless organisms are able to emigrate, they must face the challenge of acclimating or adapting to the changing local conditions (Stillman 2003, Calosi et al. 2008). For species with low plasticity, however, even small increases in daily mean or maximum water temperatures could cause thermal stress, particularly for those already living in thermal conditions at their ecological optima where such increases will begin to exceed thermal tolerance limits (Portner 2001, Portner and Farrell 2008).

Whether global temperature increases will most strongly affect organisms living near their thermal maxima (Stillman 2003) or minima (Calosi et al. 2008) is debated. On one hand, species with higher thermal-tolerance limits are thought to be most vulnerable to future warming because they already live close to their upper thermal tolerance limits and have the least acclimatory capacity (Stillman 2003, Pörtner et al. 2006). On the other hand, species’ upper thermal-tolerance limits are thought to be positively related to their acclimatory ability and geographical range size, implying the long-term persistence of species with low thermal tolerances and restricted geographies would be most at risk from future warming (Calosi et al. 2008). Increased water temperatures are also likely to strongly affect cold-water species in high latitudes that may not be able to escape novel, stressful thermal conditions (Wrona et al. 2006, Heino et al. 2009). In either case, phenotypic plasticity and migratory ability will be key factors determining species’ longevity in a warming climate. Many species, including marine and freshwater organisms, have already exhibited poleward range-shifts in response to rising global temperatures (Parmesan and Yohe 2003, Hickling et al. 2005, Hickling et al. 2006, Parmesan 2006, Heino et al. 2009). Temperature-mediated range-shifts are also resulting in the increased spread of invasive (Rahel and Olden 2008, Huang et al. 2011) and
pest species (Kattwinkel et al. 2011; Figure 1.1. Arrow vi.), the latter of which further threaten agricultural productivity and food security.

The control of pest populations is essential for maximising crop yields (Matson et al. 1997, Tilman et al. 2002, Godfray et al. 2010; Figure 1.1. Arrow vii.), but agricultural pesticide use has resulted in global contamination of the environment and is contributing to worldwide losses in biodiversity (Gibbs et al. 2009, Geiger et al. 2010, Potts et al. 2010, Stehle and Schulz 2015, Sánchez-Bayo and Wyckhuys 2019). Freshwater ecosystems are particularly susceptible to pesticide pollution as they readily receive contaminated runoff from agricultural land (Liess et al. 1999, Schulz 2004; Figure 1.1. Arrow x.). While fertilisers are typically added to agricultural land in much higher quantities than pesticides, even low quantities of pesticides, particularly insecticides, can cause considerable reductions in freshwater biodiversity due to their efficient mode of action and high toxicities to non-target organisms, especially to freshwater insects (Liess and Schulz 1999, Schulz and Liess 1999, Schulz 2004, Liess and Ohe 2005, Schäfer et al. 2007, Schäfer et al. 2011b, Beketov et al. 2013, Stehle et al. 2013, Stehle and Schulz 2015).

Besides increasing pesticide usage to control novel pests (due to spatial and temporal shifts in distribution), climate change is also predicted to increase pesticide contamination of streams through increased surface runoff during more frequent and intense heavy rain events (Noyes et al. 2009, Kattwinkel et al. 2011; Figure 1.1. Arrow vi.). Moreover, the toxicity of pesticides and other contaminants to aquatic organisms, and the sensitivity of aquatic organisms to toxicants, will also be influenced under climate change (Noyes et al. 2009, Hooper et al. 2013, Moe et al. 2013). For example, it is generally considered that chemicals will become more toxic due to greater uptake rates of toxicants with increased temperatures (Sokolova and Lannig 2008, Holmstrup et al. 2010, Laskowski et al. 2010). Further, organisms already exposed to thermal stress may be more sensitive to additional stress from exposure to toxicants (Moe et al. 2013). The “toxicology of climate change”, as first reviewed by Noyes et al. (2009), is a complex area of research with the potential for many and varied interactions at different levels of biological organisation (Woodward et al. 2010, Hooper et al. 2013, Moe et al. 2013), and there is a need for research from the mechanistic level to the ecosystem level to better inform the development of predictive models for how climate change and contaminants will interact in freshwaters (Stewart et al. 2013).
A growing body of research has investigated multiple-stressor effects, especially in aquatic ecosystems (Jackson et al. 2016, Nõges et al. 2016), because most present-day ecosystems are subjected to multiple stressors acting simultaneously or sequentially (Folt et al. 1999, Christensen et al. 2006, Crain et al. 2008, Ormerod et al. 2010). In running waters, agricultural stressors such as nutrient enrichment, deposited fine sediment and reduced flow have often been shown to interact in complex ways when affecting stream communities (Matthaei et al. 2010, Wagenhoff et al. 2011, Wagenhoff et al. 2012, Wagenhoff et al. 2013, Lange et al. 2014, Bruder et al. 2015). As discussed by Ormerod et al. (2010) (and references therein), the impacts of climate change are predicted to exacerbate, confound and complicate multiple-stressor effects already existing in freshwaters, which in the case of temperature increases has since been investigated and demonstrated (Piggott et al. 2012, Piggott et al. 2015a, Piggott et al. 2015b, Piggott et al. 2015c). There is a need for more studies to provide quantitative evidence on multiple-stressor effects in order to complement and better inform our conceptual knowledge base (Nõges et al. 2016), which is constantly being refined and improved.

As identified by Piggott et al. (2015d) in their re-assessment of Crain et al.’s (2008) meta-analysis of multiple-stressor studies in marine ecosystems, a key challenge in multiple-stressor research is consistency in the interpretation of stressor interactions. In addition to this, Schäfer and Piggott (2018) point out another key limitation being the lack of a theoretical framework for the selection of null-models in analysing stressor interactions. In order to inform better null-model development and selection, an improved understanding of the underlying mechanisms behind stressor interactions is required. For this, highly controlled laboratory experiments are important; however, these also suffer from low ecological realism. Consequently, mesocosm experiments can be a vital tool in achieving higher environmental realism while still maintaining controlled conditions, especially in the context of multiple-stressor and climate-change research (Stewart et al. 2013). My thesis incorporates the use of both forms of experiments in assessing neonicotinoid insecticide effects (introduced below) on stream invertebrate communities in a multiple-stressor and climate-change context.
1.3 Neonicotinoid Insecticides

Neonicotinoids are a class of new-generation insecticides that were introduced to the market in the mid-1990s. Since then, neonicotinoids have over-taken other classes of insecticides including organophosphates, carbamates and pyrethroids to become the most widely used group of insecticides in the world (Jeschke et al. 2011). What has made neonicotinoids so popular? They have several properties which give rise to easy application and high efficacy. First, neonicotinoids are highly water-soluble and exhibit systemic activity, meaning the chemical is distributed throughout a developing plant’s vascular tissues and foliage from the germinating seed. These two properties allow them to be used as seed dressings, a convenient method of application whereby seed is prophylactically coated with the insecticide prior to soil drilling. This has become the most popular method of application (Jeschke et al. 2011).

Another reason for the neonicotinoids’ popularity is their specific mode of action. Neonicotinoids bind agonistically to the post-synaptic membrane of the invertebrate nicotinic acetylcholine receptor (nAChR) and show selective toxicity for insects over vertebrates (Matsuda et al. 2001). This means they are highly effective against sucking, chewing and some biting insect pests but have a very low affinity for vertebrate nAChRs and therefore low vertebrate toxicity (Tomizawa and Casida 2005).

Unfortunately, their prophylactic use as seed dressings has led to increased environmental contamination by neonicotinoids (Bonmatin et al. 2015), and their high affinity for insect nAChRs can cause non-target toxicity to many insect species (Pisa et al. 2015). These negative consequences of prophylactic pesticide use are precisely what integrated pest management strategies intend to avoid (Goulson 2013). These strategies aim to minimise pesticide applications by using a pest-monitoring approach and applying agrochemicals in a targeted method (Metcalf and Luckmann 1994). This targeted approach only treats pest populations when necessary and seeks to use selective agrochemicals with high specificity to the target pest, avoiding broad-spectrum insecticides with high potential for environmental persistence in order to reduce impacts on non-target organisms and likelihood of pesticide resistance developing in pest populations (Goulson 2013). In stark contrast to these goals, prophylactic use of broad-spectrum neonicotinoid-treated seeds has become extremely widespread (Douglas and Tooker 2015), and the literature detailing the ensuing environmental impacts is incontrovertible (Wood and Goulson 2017, Hladik et al. 2018b).
Research on the uptake and translocation in plants of imidacloprid, the most commonly used neonicotinoid worldwide, has shown that only approximately 5% (between 1.6–20%) of the active ingredient is taken up by the crop plant after soil or seed treatment (Sur and Stork 2003). The majority of the pesticide (80-98%) enters the soil and ground water (Goulson 2014). Depending on soil type, neonicotinoids can persist in measurable concentrations in soil for long periods (months to years), though dissipation times vary considerably (Bonmatin et al. 2015). Goulson (2013) reviewed laboratory and field studies reporting neonicotinoid dissipation half-life times (DT_{50S}) in soil and found that these ranged from 28 to 1250 days for imidacloprid, 148 to 6931 days for clothianidin and 7 to 353 days for thiamethoxam. Wood and Goulson (2017) also reviewed field studies conducted since 2013, many of which document neonicotinoids persisting in soils for longer than the annual agricultural cycle, with detectable levels present in agricultural soils more than a year after application by drilling of treated seeds. Thus, repeated application in some soil types can result in neonicotinoid accumulation, which appears to plateau after 2–6 years due to sufficient degradation (preventing indefinite accumulation; Wood and Goulson 2017). Such persistence of neonicotinoids in soils allows for uptake by follow-on crops and field-margin plants where they can affect non-target soil-dwelling invertebrates, herbivorous insects and pollinators exposed in the surrounding vegetation (Goulson 2014, Botías et al. 2015, Botías et al. 2016, Wood and Goulson 2017).

Their toxicity to pollinators, especially honeybees (Apis mellifera), is of particular concern given the worldwide decline of bee populations over recent decades (Potts et al. 2010, Godfray et al. 2014, Lundin et al. 2015, Sánchez-Bayo and Wyckhuys 2019). Bees and other pollinators can be exposed to neonicotinoids through direct contact with contaminated dust clouds generated during the sowing of neonicotinoid-treated seed (Girolami et al. 2012, Tapparo et al. 2012) or (due to neonicotinoids’ systemic activity), through consumption of pollen, nectar and guttation droplets from treated crops (Girolami et al. 2009, Tapparo et al. 2011, Krupke et al. 2012, Stoner and Eitzer 2012, Codling et al. 2016) and wildflowers containing neonicotinoid residues (Krupke et al. 2012, Botías et al. 2015, David et al. 2016). An analysis of honey samples from five continents found neonicotinoids present in honey across the world (Mitchell et al. 2017). Beneficial soil-dwelling invertebrates can also be negatively impacted by neonicotinoids persisting in agricultural soils (Pisa et al. 2015). A study by Douglas et al. (2015) even demonstrated their potential to reduce crop yields in soy-bean fields treated with the neonicotinoid thiamethoxam by disrupting the natural
biological control of pest slugs through higher toxicity to non-target predatory beetles. Thus, the potential for negative effects on pollinators and other non-target organisms that are beneficial to agricultural production highlight how prophylactic use of systemic insecticides such as neonicotinoids, which has become such a widespread practice associated with agricultural intensification, can actually reduce crop production (Figure 1.1. Arrow viii.).

Contamination of surface water with neonicotinoids and their negative effects on non-target aquatic invertebrates has become another major problem associated with neonicotinoid use (Morrissey et al. 2015, Smit et al. 2015, Sánchez-Bayo et al. 2016; Figure 1.1. Arrow x.). Due to their high solubility, low concentrations of neonicotinoids continuously leach into surrounding surface waters from groundwater (Lamers et al. 2011, Huseth and Groves 2014). Neonicotinoids can also enter surface waters through snow melt (Main et al. 2014), spray drift from foliar application, contaminated dust clouds created during seed-drilling (Krupke et al. 2012, Nuyttens et al. 2013), and transport of decaying neonicotinoid-treated plant material into water bodies (Kreutzweiser et al. 2008). However, the major source of surface water contamination by neonicotinoids is from runoff and amplified leaching from groundwater after periods of high rainfall, which cause pulses of higher concentrations to enter waterways (Armbrust and Peeler 2002, Chiovarou and Siewicki 2008, Hladik et al. 2014, Struger et al. 2017).

Douglas and Tooker (2015) reported that 33–44% of soybeans and 79–100% of maize hectares planted in the USA in 2011 were seed-treated with neonicotinoids. Given their high solubility and leaching potential as previously described, the logical prediction is that neonicotinoids are present in surface waters surrounding maize and soybean producing areas. This was first demonstrated by Hladik et al. (2014), who collected water samples during the 2013 growing season from nine agricultural streams in Iowa, the top producer of corn and soybeans in the United States. They found that neonicotinoid occurrence and concentrations in these streams were largely consistent with the total amounts applied across the state. Clothianidin, the most heavily used insecticide in Iowa (about 215,000 kg in 2013), was detected most frequently. It occurred in 75% of the 79 stream samples collected, with a maximum concentration of 0.257 µg/L and a median concentration of 0.0082 µg/L. Thiamethoxam (49,900 kg used) and imidacloprid (70,700 kg) were the next-most commonly detected neonicotinoids, in 47% and 23% of samples, with respective maximum concentrations of 0.185 µg/L and 0.0427 µg/L.
A year later, the same authors published the first national-scale assessment of neonicotinoids in streams in the USA (Hladik and Kolpin 2016). In this study, single grab samples were collected from 38 streams across 24 states plus Puerto Rico, and at least one neonicotinoid was detected at 53% of the sites sampled. Imidacloprid was the most commonly detected (at 37% of sites), with a maximum concentration of 0.14 µg/L. Clothianidin and thiamethoxam were also frequently detected (24% and 21%), with respective maximum concentrations of 0.066 and 0.19 µg/L. Because these values represent a single snapshot in time for each site sampled, they most likely underestimate maximum (peak) concentrations which occur after heavy rainfall events (Sánchez-Bayo and Hyne 2014, Hladik and Kolpin 2016, Wood and Goulson 2017).

The neonicotinoids imidacloprid, clothianidin and thiamethoxam have been registered for use in New Zealand to control a range of pests on a variety of pasture and forage crops since 1994 (imidacloprid; thiamethoxam and clothianidin registered in 2010; Chapman 2010). These neonicotinoids are widely use in orchards, crops and pastures, yet to date there are no monitoring programmes for neonicotinoids (or any regular monitoring programmes for pesticides in general) in running waters in New Zealand. This is despite the New Zealand Environmental Protection Authority (2018a) recently setting environmental exposure limits for imidacloprid and thiamethoxam and identifying these for reassessment review (NZ EPA 2018b). In addition, no published toxicity data for neonicotinoid effects on freshwater organisms in New Zealand existed prior to my research.

1.4 Thesis aim and outline

The central aim of my thesis is to investigate the ecological impacts of the neonicotinoid insecticides widely used in New Zealand agriculture (imidacloprid, clothianidin and thiamethoxam) on stream macroinvertebrate communities in a multiple-stressor and climate-change context.

In Chapters 2–4, I use chronic (≥28-day) laboratory experiments to test the effects of the neonicotinoids of interest on nymphs of a common New Zealand mayfly. The experiments in Chapter 2 determine the chronic toxicities and optimise the laboratory procedures for the experiments in Chapters 3 and 4. Employing a full-factorial experimental design, in Chapter 3 I determine the relative toxicities of the three neonicotinoids and assess their interactive
effects according to an additive null model. In Chapter 4, I investigate the individual and combined effects of low-level imidacloprid exposure and two commonly-occurring stressors in real stream ecosystems, food limitation and simulated heatwaves, in a 6-week laboratory experiment using the same species of mayfly nymphs with a focus on delayed effects of the manipulated stressors. Chapter 2 is currently in revision with *Environmental Toxicology and Chemistry* (revised version submitted 17 June) as a co-authored manuscript and therefore uses the pronouns “we” and “our” rather than “I” and “my” (as in the remaining chapters).

In Chapter 5, I use an outdoor stream mesocosm experiment to address my overall thesis aim by investigating the effects of imidacloprid and raised water temperature (by 3°C) on stream macroinvertebrate community dynamics in fast and slow-flowing streams. Within the broader context of my thesis (Figure 1), this experiment therefore investigates the relationships associated with arrows ix, x and xi in an environmentally realistic, tightly controlled, and statistically powerful experimental stream system.

My thesis concludes with a General Discussion (Chapter 6). This synthesizes the findings from my empirical laboratory and field assessments of neonicotinoids effects on New Zealand stream macroinvertebrates, discusses management implications with respect to neonicotinoid use and the impacts of climate change and identifies some future research needs.
Chapter 2

Chronic toxicities of neonicotinoids to nymphs of the common New Zealand mayfly *Deleatidium* spp.
2 Chronic toxicities of neonicotinoids to nymphs of the common New Zealand mayfly *Deleatidium* spp.

2.1 Summary

Neonicotinoid insecticides have been shown to have high chronic relative to acute toxicity, therefore short-term toxicity tests of $\leq$96 hours in duration may underestimate their environmental risks. Among non-target aquatic invertebrates, insects of the orders Diptera and Ephemeroptera have been found to be the most sensitive to neonicotinoids. To undertake more accurate assessment of the risks posed by neonicotinoids to freshwater ecosystems, more data are needed from long-term tests using the most sensitive taxa. Using nymphs of the common New Zealand mayfly genus *Deleatidium* spp., we performed 28-day static-renewal exposures with the widely used neonicotinoids imidacloprid, clothianidin and thiamethoxam. We monitored survival, immobility, impairment, and mayfly moulting propensity at varying time points throughout the experiment. Imidacloprid and clothianidin exerted strong chronic toxicity to *Deleatidium* nymphs, with respective 28-day LC50s of 0.28 and 1.36 µg/L, while thiamethoxam was the least toxic, with a 28-day LC50 $>$4 µg/L (highest concentration tested). Mayfly moulting propensity was also negatively affected by clothianidin (during 3 of 4 weeks), imidacloprid (2 of 4) and thiamethoxam (1 of 4). Comparisons with published neonicotinoid chronic toxicity data for other mayfly taxa and larvae of the midge genus *Chironomus* showed similar sensitivities for mayflies and midges, suggesting experiments using these taxa provide reliable assessments of the threats of neonicotinoids to the most vulnerable freshwater species.

2.2 Introduction

The rapid rise in global use of neonicotinoids over the last few decades has resulted in their widespread contamination of surface waters where they can pose a considerable threat to non-target aquatic organisms, especially insects. Previous research to investigate the effects of neonicotinoids on non-target insects has consistently shown high toxicity to the insect taxa tested (Wood and Goulson 2017). A comprehensive review of neonicotinoid effects in aquatic ecosystems by Morrissey et al. (2015) demonstrated that there is considerable
variation in toxicity among invertebrate taxa (more than six orders of magnitude in difference within aquatic arthropods; see Figure 2.1) and that aquatic insect taxa are the most sensitive. This is not surprising, given that neonicotinoids were designed to target nicotinic receptors of terrestrial pest insects (Matsuda et al. 2001) and, among aquatic invertebrates, the physiology and nicotinic-receptor binding sites of aquatic insects are most similar to those of terrestrial insects. Consequently, when conducting ecotoxicological studies with neonicotinoids, it is important to consider species belonging to these more sensitive taxa, which exhibit lethal and sublethal effects (e.g. impaired mobility, feeding, reproduction, growth, emergence) at much lower concentrations than commonly used crustacean test species (Anderson et al. 2015).

Figure 2.1 (reproduced from Morrissey et al. 2015). Range of neonicotinoid toxicity (L[E]C50: 24–96-h in µmol/L) among all tested aquatic invertebrate orders. For context, three of the most common test species (open bars) for the orders Cladocera (Daphnia magna), Amphipoda (Gammarus pulex) and Diptera (Chironomus dilutus) are shown to illustrate differences in sensitivity by species. Vertical lines within bars represent geometric means of test values. Crustaceans are represented by the taxa above the horizontal line and insect taxa are below it.

Morrissey et al. (2015) reviewed data from toxicity studies conducted with 49 arthropod species, including acute studies with durations of ≤ 96 h (178 tests) and chronic studies with durations between 7 and 39 days (36 tests), and concluded that mayflies (Ephemeroptera) were the most sensitive taxonomic group. Using 24–96-hour median lethal concentrations (LC50s; 137 tests) and median effective concentrations evaluated with sublethal endpoints (EC50s; 77 tests), they calculated an acute geometric mean L[E]C50 of 3.9 µg/L for Ephemeroptera. In contrast, they found that Cladocerans, in particular the most widely used
test species *Daphnia magna*, were the least sensitive group, having the highest acute geometric mean L[E]C50 of 43,926.5 µg/L. This finding is concerning given *D. magna* has traditionally been considered the global industry standard invertebrate test species, having been used for 82% of chemicals tested (Sánchez-Bayo 2006).

Mayflies are used worldwide as biological indicators of stream health because of their high sensitivity to pollutants and their critical role in freshwater food webs (Sánchez-Bayo et al. 2016). For these reasons and because they are among the most sensitive aquatic taxa to neonicotinoids, they are prime study organisms for evaluating environmental risks of neonicotinoids in fluvial ecosystems. In New Zealand, the mayfly genus *Deleatidium* spp. (Leptophlebiidae) is distributed ubiquitously in running waters across the country and comprises 16 described species (Hitchings 2010). The aquatic nymphs of this genus can be determined most reliably by genetic analysis and a study by Macher et al. (2016), who used DNA barcoding to identify *Deleatidium* spp. sampled from streams in the Southland region of New Zealand, found that the majority of their sites contained only 1 or 2 different species. *Deleatidium* nymphs are periphyton grazers that feed on biofilm growing on rock surfaces and are an important food source for fish, other aquatic insects and birds (Winterbourn 1974, Scrimgeour 1991), with a seasonal and temperature-dependent larval cycle resulting in several overlapping generations per year (Winterbourn 1974, Huryn 1996). Nymphs that hatch in spring or early summer can achieve their maximum size in 3 months, but those hatching in late summer can have a larval stage of up to 12 months (Scrimgeour 1991). These long aquatic larval stages are likely to render them vulnerable to exposure to neonicotinoids in streams draining agricultural land.

A consistent pattern observed in the literature concerning the aquatic ecotoxicity effects of neonicotinoids is a high acute-chronic ratio (ACR) indicating considerably lower chronic-effect concentrations than those required to cause acute toxicity (Sánchez-Bayo et al. 2016). In the review by Morrissey et al. (2015), sublethal end-points in chronic studies were frequently an order of magnitude or more below those for acute tests. It has been proposed that irreversible binding of neonicotinoids to the insect nicotinic acetylcholine receptor, causing continual firing of electrical impulses and eventually neuronal death (Tennekes 2010), is the reason for their adverse effects to accumulate with time (Tennekes and Sánchez-Bayo 2013). Consequently, initial toxicity assessments based on acute tests with standard test species underestimated the risks posed by neonicotinoids to aquatic ecosystems (Sánchez-Bayo et al. 2016). However, there is also some evidence of the potential for
reversible binding, with freshwater invertebrate recovery from an impaired state observed following short-term pulses of imidacloprid (Raby et al. 2018a).

To account for ACRs and other uncertainties in environmental risk assessment, ‘uncertainty factors’ are applied to acute LC50s or EC50s determined from highly standardised, short-term laboratory tests (Heugens et al. 2001) run in the first tier of tiered risk assessment schemes (e.g. EFSA 2013). However, when initial acute tests are run with a highly tolerant test organism (e.g. *D. magna* when first assessing neonicotinoids) and when combined with high ACRs, the risks may still be underestimated even after the cautionary uncertainty factors have been applied (Tennekes and Sánchez-Bayo 2011, van Wijngaarden et al. 2015). Due to the higher expense of performing experiments longer than 96 hours (Smith et al. 1991) and the difficulty of maintaining sufficient control survivorship for the exposure duration for some taxa, acute tests still dominate the toxicological literature (83% of those reviewed by Morrissey et al. 2015 spanned ≤96 hours) and there is a lack of chronic neonicotinoid toxicity data, especially for sensitive test species (Anderson et al. 2015). Yet chronic laboratory studies lasting 28 days or longer and field or mesocosm studies are recommended as the primary guides for determining regulatory acceptable concentrations for neonicotinoids, because these studies give more accurate and realistic assessments of the environmental risks (Morrissey et al. 2015).

Currently only three studies have investigated chronic toxicities of neonicotinoids to mayfly larvae (Roessink et al. 2013, Van den Brink et al. 2016, Raby et al. 2018b). The first of these determined acute and chronic toxicities of imidacloprid to the European mayflies *Cloeon dipterum* and *Caenis horaria*, three other aquatic insects and two crustaceans. Large ACRs of at least 10 were found for all species tested (the largest ratio being 336 for *C. dipterum*), leading the authors to conclude that acute toxicity data are inappropriate for assessing effects of long-term exposure to imidacloprid. Several other studies have also reported on chronic effects of neonicotinoids to larvae of the freshwater dipterans *Chironomus riparius* (Langer-Jaesrich et al. 2010, Finnegan et al. 2017, Saraiva et al. 2017), *Chironomus dilutus* (Cavallaro et al. 2017, Maloney et al. 2018a, Raby et al. 2018b), *Chironomus tentans* (Stoughton et al. 2008) and *Chaoborus* sp. (Finnegan et al. 2017; Roessink et al. 2013). However, only four studies have reported chronic laboratory effects of clothianidin on aquatic insects: the mayfly *Neocloeon triangulifer* was investigated by Raby et al. (2018b), *C. dilutus* by Cavallaro et al. (2017), Maloney et al. (2018a) and Raby et al. (2018b), and *C. riparius* by Drottar et al. (2000; presented in Morrissey et al. 2015).
The aim of the present study was to expand the limited knowledge about the effects of chronic exposure of pollution-sensitive freshwater mayflies to neonicotinoids (imidacloprid, clothianidin and thiamethoxam). We used nymphs of the ubiquitous endemic New Zealand mayfly, *Deleatidium* spp., as our test organism, making our study just the second to provide chronic toxicity data for clothianidin to mayfly nymphs. Although *Deleatidium* spp. is a species complex, the test specimens used in all experiments were collected from the same stream reach, therefore we are confident that the species tested would have been consistent throughout. Neonicotinoids have been registered for use to control the pests of a variety of pasture and forage crops throughout New Zealand since 1991 (Chapman 2010), but their presence in surface waters is currently not monitored. Based on the related studies on European mayfly species mentioned above, we predicted all three neonicotinoids would elicit chronic toxicity to *Deleatidium* nymphs with 28-day LC50s < 1 µg/L.

2.3 Methods

2.3.1 Artificial stream water (ASW)

The base water for all laboratory experiments was prepared according to the American Society for Testing Materials artificial soft water (ASW) recipe and consisted of deionised water (Gemini-MB Ultra High Purity Water System; Aries FilterWorks, West Berlin, NJ, U.S.A.) containing (in mM/L): 0.57 NaHCO$_3$, 0.17 CaSO$_4$·2H$_2$O, 0.25 MgSO$_4$·7H$_2$O, and 0.03 KCl. ASW was stored in 25-L carboys at climate room temperature and aerated overnight prior to use.

2.3.2 Experimental design

Three separate 28-day, static-renewal laboratory experiments were performed with clothianidin (May–June 2017), thiamethoxam (June–July 2017) and imidacloprid (May–June 2018). In each experiment, *Deleatidium* nymphs were exposed to ten neonicotinoid concentrations ranging from 0 to 4 µg/L. This range was selected because prior experiments with *Deleatidium* nymphs exposed to imidacloprid had shown strong effects on mayfly survival (partial $\eta^2$ effect size 0.67) and impairment (partial $\eta^2$ effect size 0.76) at concentrations within this range (0.9 and 2.1 µg/L) after 9 days of exposure (Hunn 2016). Therefore, we expected this range to cover a full concentration-response profile.
across 28 days for the three neonicotinoids tested. Treatment concentrations were randomly allocated to 50 × 1.16 L aerated glass chambers (19.9 × 14.4 × 6.3 cm, see Appendix A, Figure A1), with five replicates per concentration and at least 15 Deleatidium nymphs per replicate (see below). ASW containing neonicotinoids (and control ASW with no neonicotinoids) was renewed every 7 days.

2.3.3 Insecticide application, sampling and quantification

Working stock solutions of 10 mg/L imidacloprid, clothianidin and thiamethoxam were prepared using 10 mg/mL each insecticide (PESTANAL® analytical standard grade, Sigma-Aldrich, Castle Hill, NSW, Australia). The ten exposure concentrations (0, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 2.4, 3.2 and 4.0 µg/L) were prepared by dosing 2.5 L of ASW with the required amount of working stock. From the glass chambers, 2-mL water samples were collected for analysis from each of three replicates of the 0.05, 0.4 and 4.0 µg/L concentrations. These samples were collected at the beginning and end of each test week and stored in 4-mL glass vials with Teflon caps in the dark at -20°C until shipping to the analytical laboratory with ice packs, where they were again stored at -20°C until analysis.

All neonicotinoid standards used for neonicotinoid quantitation (including Clothianidin-d3, see below) were PESTANAL® standard analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA). Neonicotinoids were quantified using liquid chromatography tandem mass spectrometry (LC-MS/MS). Analyte separation was achieved using an Agilent 1290 Infinity Binary Pump connected to an Agilent Poroshell 120 EC-C18 column (4.6 mm x 100 mm x 2.7 µm) and a gradient elution method (see Appendix A, Table A1). The column was held at 30.0°C, the total flow rate was 0.600 mL/min and sample injection volumes were 100 µL. Quantification was achieved with an Agilent 6490 Triple Quadrupole Mass Spectrometer in positive electrospray ionization mode, using multiple reaction monitoring. The source temperature was 200°C, the gas flow rate was 14 L/min and the nebulizer pressure was 20 psi. The sheath gas temperature was 325°C, sheath gas flow rate was 11 L/min and the capillary voltage was 3500 V. Internal calibration curves were prepared from the ratio of the peak area of the target analyte to the internal standard (Clothianidin-d3), with target analyte concentrations ranging from 0.05–20 µg/L. Additional analyte quantification details are provided in Appendix A, Table A2. Signal to noise (S/N) ratios >3 at the lowest standard concentration (0.01 µg/L) gave limits of detection <0.01 µg/L for all three
neonicotinoids. Additional S/N ratios at all calibration standards are provided in Appendix A, Table A3.

2.3.4 Mayfly food supply

*Deleatidium* were fed periphyton grown on 10 × 10 × 1 cm ceramic tiles *in situ* in Lindsay Creek, located in North East Valley, Dunedin (45.8420°S, 170.5408°E). This site was chosen for its ease of accessibility, concealment from public view, presence of *Deleatidium*, and because the small stream drained a nature reserve clad in native forest that was unlikely to flood (causing bed disturbance) during high rainfall or have pesticides present. A period of two weeks was deemed sufficient for periphyton growth which was assumed to be consistent in quantity and composition or at least not to vary systematically with treatment type. One randomly chosen periphyton tile was placed in each chamber. This tile also acted as a substratum for the nymphs and was exchanged with a fresh tile from Lindsay Creek every 7 days, at the same time as renewal of the exposure solutions.

2.3.5 Test specimen collection and acclimation conditions

*Deleatidium* specimens were collected on three separate occasions, once prior to each experiment, from Silver Stream, a fourth-order stream located in an unpolluted native forest catchment about 15 km from Dunedin, New Zealand (45.8096°S, 170.4211°E). The same stream reach (ca. 75 m) was sampled on each occasion using a pulsed DC backpack electro-shocker (Kainga EFM300; NIWA, Christchurch, New Zealand). Electric fishing strongly increases invertebrate drift rates and is a fast, efficient method for collecting stream invertebrates including mayflies (Taylor et al. 2001). The technique has been used successfully in New Zealand and North America for ecological experiments where large numbers of live invertebrate specimens were needed (e.g. McIntosh and Townsend 1994, Peckarsky and McIntosh 1998). Specimens drifting downstream were caught in a pole-net (0.9 × 0.7 m, mesh size 3 mm) and transferred to large holding bins for sorting *in situ*. Small to medium-sized nymphs (head-and-body length 5-15 mm) were preferentially targeted because in previous experiments, late instars (especially those with visible wing-pads) had been less tolerant to laboratory conditions and more likely to emerge during experiments than others.

To maximise specimen survival during transport from the collection site to the climate-controlled room, battery-operated air pumps (Aqua One Battery Air 250; Aqua One,
Ingleburn, NSW, Australia) and crushed ice were used to maintain high dissolved oxygen levels. Stream temperatures during collections ranged from 3.7–7.5°C. Nymphs were left in aerated buckets with stream water overnight to allow acclimation to the temperature of the climate-controlled room (12°C). The following morning, all specimens were transported to holding containers (40 L) with aerated ASW that was already at climate-room temperature and contained 15 ceramic tiles pre-colonised with periphyton (see above) for a further 48 hours of acclimation. During all acclimation and test periods, the climate-controlled room was maintained at 12 ± 1°C with a 16:8 hour light:dark regime (with a 1-hour ramp from light–dark and vice versa). A random sample of at least 15 (maximum 17, mean 15.5), healthy nymphs with all limbs and cerci intact, representative of the range of sizes and instars collected, were distributed to each treatment replicate at the start of each exposure period.

2.3.6 Recording invertebrate responses

At the time of each tile exchange and renewal of ASW containing neonicotinoids (and controls), mayfly survival and two sublethal responses indicating mayfly nymph motility were recorded. Nymphs that were still able to walk but unable to right themselves by performing a normal swimming movement (after being gently inverted using forceps) were classified as “impaired”. Nymphs that were unable to move either by swimming or walking and that could only twitch their appendages were classified as “immobile”. These sublethal responses for motility follow the continuum of observed toxicity symptoms described by Camp and Buchwalter (2016) for nymphs of the mayfly Isonychia bicolor exposed to imidacloprid. According to their classification, “righting inability” occurs during the “impairment” phase of responses and “immobility” occurs following onset of muscle spasms and unresponsiveness, immediately preceding mortality. This continuum of toxicity symptoms means, for the purpose of calculating chronic toxicities from our concentration-response experiments, all dead nymphs were assumed to have previously been impaired and immobilised, and all immobile nymphs were assumed to have been impaired prior to immobilisation (e.g. immobile = dead + immobile; impaired = dead + immobile + impaired). Validity of the chronic tests was determined according to control immobilisation out of alive specimens (excluding mortality in this case), following Roessink et al. (2013) who considered tests valid if the proportion of immobilised mayflies out of the alive mayflies did not exceed 10% in controls. In our study, this proportion was always zero throughout the three experiments (see Appendix A, Figures A2 and A3).
Survival, impairment and immobility were also recorded four days after each tile exchange, i.e. on Days 11, 18 and 25. Further, “moulting propensity” was calculated as the number of moults occurring during each experimental week (1−4) out of the number of Deleatidium nymphs that had been alive on the start of each week; this was recorded because acute exposure of imidacloprid to Deleatidium had significantly affected moulting frequency in previous experiments (Macaulay et al. 2019). Dead specimens and shed exuviae were removed daily from the chambers and used to calculate survivorship and moulting propensity. Emerged mayflies were also removed and their final moults recorded as part of the moulting response. Emergence was too infrequent to be considered as a response variable on its own.

2.3.7 Data analysis

Separate log-logistic (binomial) generalized linear regressions were performed in R (Version 3.5.1; R Core Team) to determine the individual effects of imidacloprid, clothianidin and thiamethoxam on the responses of Deleatidium survivorship, impairment, immobility and moulting propensity on Days 7, 11, 14, 18, 21, 25 and 28. The significance level $\alpha$ for all regressions was set conservatively at $p = 0.01$ so that any effects significant at this $\alpha$ would be clearly visible in the data. All regressions were very highly significant ($p < 0.001$) unless stated otherwise. Median lethal concentrations (LC50s), median immobilising concentrations (IC50s) and effective concentrations causing impairment (EC50s) were calculated with 95% confidence intervals (CI) for all significant regressions using the dose.p function in R package MASS (Venables 2002). McFadden’s pseudo-$R^2$ values ($\rho^2$) and dispersion parameters were calculated for each model. McFadden’s $\rho^2$ (McFadden 1974) tend to be considerably lower than those of the linear $R^2$ measure and are therefore to be interpreted differently to linear regression standards of model fit (Domencich 1975). According to McFadden (1977), $\rho^2$ of 0.2–0.4 represents an excellent model fit. Log-logistic regression summaries for survivorship, immobility and impairment regressions are presented in Table 2.1. Summaries for mayfly moulting propensity are presented in Table 2.2.

2.3.7.1 Multiple inference and a priori significance level

Although the significance level $\alpha$ was adjusted in this chapter, in the subsequent data chapters where multiple tests are also performed, there are no further adjustments for multiple inference. This was done in the interests of maintaining statistical power and not increasing the occurrence of Type 2 errors (Nakagawa 2004). In Chapters 3-5, partial $\eta^2$
effect sizes are always provided for effects where \( p < 0.1 \) to allow the reader to assess the biological relevance of results and, when these effect sizes are less than 0.1 or when no clear mechanistic basis for an effect is evident, the corresponding effects are interpreted with caution (see, for example, Section 4.5.1). In Chapter 5, some effects with partial \( \eta^2 \) effect sizes < 0.1 are still interpreted and discussed as being biologically relevant because the high statistical power from this experimental design combined with the complexity of the study design (number of predictor variables) means that small effects can be detected in the data.

2.4 Results

2.4.1 Neonicotinoid exposures

Mean initial and final concentrations (samples taken at the beginning and end of each week) of imidacloprid, clothianidin and thiamethoxam from the lowest, middle and highest treatments (0.05, 0.4 and 4 µg/L, respectively) are presented in Appendix A, Table A4. The mean initial and final concentrations of the middle treatment (0.4 µg/L) for imidacloprid were 0.42 and 0.42 µg/L, 0.30 and 0.29 µg/L for clothianidin, and 0.71 and 0.72 µg/L for thiamethoxam. Given the high consistency in mean initial and final concentrations we were satisfied that there was minimal pesticide degradation over the week-long static-renewal periods. The verified concentrations for imidacloprid, clothianidin, and thiamethoxam deviated on average by –19, –38, and 66% from nominal, respectively. While the verified concentrations for clothianidin and thiamethoxam deviated by >20% from nominal, because we had verified concentrations for only three of the ten treatments, it was more feasible to use the nominal concentrations in our analyses. The differences between nominal and achieved concentrations is most likely due to systematic error, either from the working stock solutions differing from their intended concentration or human and/or instrumentation error when making up the diluted treatments. The implications of the considerably higher achieved concentrations for thiamethoxam compared to the lower achieved concentrations for imidacloprid and clothianidin are addressed in the Discussion (Section 2.5) below.

2.4.2 Time-to-effect observations and comparative toxicities

Overall, the toxicity of each neonicotinoid on survivorship and mayfly mobility (immobility and impairment) increased over time, as indicated by strengthening effects (\( \rho^2 > 0.4 \) can be
considered a strong effect, and $\rho^2 < 0.2$ a weak effect; Domencich 1975; McFadden 1977) and decreasing median lethal, immobilising and effective concentrations (Table 2.1; Figures 2.2–4). When comparing the three insecticides, imidacloprid was the most toxic, causing more than 50% mortality after just 11 days of exposure ($LC_{50} = 1.78 \mu g/L$) and more than 50% immobility and impairment over the first 7 days. Clothianidin was intermediate in toxicity, resulting in over 50% mortality after 18 days of exposure, 50% immobility after 14 days and 50% impairment after 11 days. Compared to imidacloprid and clothianidin, thiamethoxam was less toxic. Although exposure to thiamethoxam caused some significant reductions in *Deleatidium* survival, this did not drop below 50% over the 28-day exposure period and all median effect concentrations were $> 4 \mu g/L$ (Table 2.1). While some control mortality occurred in the imidacloprid experiment, immobilisation or impairment (as a proportion of alive nymphs never exceeded 10% (Figures A2 and A3), therefore this test was still considered valid. Above the 0.4 $\mu g/L$ treatment (where survivorship remained $>75\%$ throughout the entire exposure period), survival dropped sharply due to the insecticide, which can be verified by the lack of an immobilising or impairing effect on the specimens still alive in all imidacloprid treatments below 0.4 $\mu g/L$ (see Figures A2 and A3).

### 2.4.2.1 Week 1

Only imidacloprid had significantly reduced *Deleatidium* survivorship after the first 7 days of exposure (Figure 2.2a). Considering the sublethal response of immobility (in addition to mortality) resulted in a stronger effect of imidacloprid and a significant, albeit weak effect of clothianidin (Figure 2.3a-b). Including the sublethal response of impairment as well showed an even stronger effect of imidacloprid, with almost 100% of *Deleatidium* nymphs impaired at the highest concentration ($4 \mu g/L$) after just 7 days (Figure 2.4a). Therefore, a 7-day EC50 of 1.21 $\mu g/L$ (the concentration at which 50% of *Deleatidium* nymphs were adversely affected, in this case, impaired) could be calculated for imidacloprid. No significant effects of thiamethoxam were observed in the first week.

### 2.4.2.2 Week 2

After 14 days, survivorship had been reduced below 50% at 0.8 $\mu g/L$ imidacloprid (Figure 2.3.2a; $LC_{50} = 0.86 \mu g/L$). Both imidacloprid and clothianidin had strongly increased *Deleatidium* immobility (Figure 2.3a and b) and impairment (Figure 2.4a and b), with respective 14-day EC50s of 0.4 $\mu g/L$ and 2.46 $\mu g/L$. While a significant effect of
thiamethoxam did occur for *Deleatidium* survival ($p = 0.008$; Figure 2.3.2c) and impairment (Figure 2.4c), these effects were not strong enough to be considered biologically relevant.

### 2.4.2.3 Week 3

By Day 21, imidacloprid and clothianidin had both strongly reduced *Deleatidium* survivorship ($\rho^2 > 0.4$). Their respective 21-day LC50s were 0.38 and 2.12 µg/L. The effects of imidacloprid and clothianidin were stronger when considering the sublethal mayfly responses, especially impairment, compared to mortality just alone, with lower median effect concentrations of 0.30 and 1.47 µg/L. As in the second week, the effects of thiamethoxam were still too weak to consider biologically relevant.

### 2.4.2.4 Week 4: final 28-day toxicities

The respective 28-day LC50s of imidacloprid, clothianidin and thiamethoxam were 0.28, 1.36 and > 4 µg/L, corresponding to strong effects of imidacloprid and clothianidin and a weak effect of thiamethoxam on *Deleatidium* survivorship (Figure 2.2). As observed in the first three weeks, including the sublethal mayfly responses marginally increased the strengths of the effects of each neonicotinoid, which was most evident when evaluating the impairment responses (Figure 2.4). The corresponding 28-day EC50s for imidacloprid, clothianidin and thiamethoxam were 0.19, 1.02 and > 4 µg/L, respectively.
Table 2.1. Results summary of log-logistic generalized linear models for *Deleatidium* survivorship, immobility (including mortality) and impairment (including immobility and mortality) responses from 28-day chronic exposures to imidacloprid, clothianidin and thiamethoxam (*p*-values for all regressions are < 0.001 unless stated otherwise).

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Day</th>
<th>LC50 (µg/L)</th>
<th>(95% CI)</th>
<th>Slope&lt;sup&gt;a&lt;/sup&gt;</th>
<th>ρ&lt;sup&gt;2&lt;/sup&gt;</th>
<th>IC50 (µg/L)</th>
<th>(95% CI)</th>
<th>Slope&lt;sup&gt;a&lt;/sup&gt;</th>
<th>ρ&lt;sup&gt;2&lt;/sup&gt;</th>
<th>EC50 (µg/L)</th>
<th>(95% CI)</th>
<th>Slope&lt;sup&gt;a&lt;/sup&gt;</th>
<th>ρ&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Imidacloprid</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>&gt;4</td>
<td>NC</td>
<td>-0.6</td>
<td>0.21</td>
<td>2.60</td>
<td>(2.13–3.13)</td>
<td>1.5</td>
<td>0.62</td>
<td>1.21</td>
<td>(1.06–1.36)</td>
<td>2.8</td>
<td>0.82</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>1.78</td>
<td>(1.36–2.29)</td>
<td>-1.0</td>
<td>0.25</td>
<td>1.09</td>
<td>(0.89–1.30)</td>
<td>1.6</td>
<td>0.49</td>
<td>0.53</td>
<td>(0.46–0.61)</td>
<td>4.0</td>
<td>0.74</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>0.86</td>
<td>(0.66–1.07)</td>
<td>-1.4</td>
<td>0.32</td>
<td>0.68</td>
<td>(0.54–0.84)</td>
<td>1.8</td>
<td>0.44</td>
<td>0.40</td>
<td>(0.34–0.47)</td>
<td>4.0</td>
<td>0.68</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>0.46</td>
<td>(0.36–0.57)</td>
<td>-2.4</td>
<td>0.52</td>
<td>0.41</td>
<td>(0.33–0.5)</td>
<td>2.9</td>
<td>0.59</td>
<td>0.34</td>
<td>(0.27–0.4)</td>
<td>4.0</td>
<td>0.66</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>0.38</td>
<td>(0.29–0.47)</td>
<td>-2.7</td>
<td>0.55</td>
<td>0.36</td>
<td>(0.28–0.44)</td>
<td>3.0</td>
<td>0.59</td>
<td>0.30</td>
<td>(0.24–0.36)</td>
<td>4.2</td>
<td>0.66</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>0.33</td>
<td>(0.25–0.41)</td>
<td>-2.9</td>
<td>0.66</td>
<td>0.31</td>
<td>(0.24–0.38)</td>
<td>3.4</td>
<td>0.61</td>
<td>0.22</td>
<td>(0.17–0.28)</td>
<td>4.6</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>0.28</td>
<td>(0.21–0.36)</td>
<td>-3.1</td>
<td>0.59</td>
<td>0.26</td>
<td>(0.2–0.33)</td>
<td>3.5</td>
<td>0.66</td>
<td>0.19</td>
<td>(0.14–0.25)</td>
<td>4.7</td>
<td>0.74</td>
<td></td>
</tr>
<tr>
<td><strong>Clothianidin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>&gt;4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>NC</td>
<td>-0.7</td>
<td>0.07</td>
<td>&gt;4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>NC</td>
<td>1.0</td>
<td>0.13</td>
<td>&gt;4</td>
<td>NC</td>
<td>1.4</td>
<td>0.23</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>&gt;4</td>
<td>NC</td>
<td>-1.6</td>
<td>0.35</td>
<td>&gt;4</td>
<td>NC</td>
<td>2.2</td>
<td>0.49</td>
<td>3.48</td>
<td>(3.11–3.89)</td>
<td>3.1</td>
<td>0.63</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>&gt;4</td>
<td>NC</td>
<td>-2.2</td>
<td>0.49</td>
<td>3.07</td>
<td>(2.74–3.44)</td>
<td>2.9</td>
<td>0.59</td>
<td>2.46</td>
<td>(2.24–2.69)</td>
<td>3.5</td>
<td>0.67</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>2.75</td>
<td>(2.46–3.06)</td>
<td>-2.9</td>
<td>0.64</td>
<td>2.25</td>
<td>(2.05–2.47)</td>
<td>3.4</td>
<td>0.71</td>
<td>1.83</td>
<td>(1.68–2.00)</td>
<td>4.2</td>
<td>0.76</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>2.12</td>
<td>(1.91–2.34)</td>
<td>-3.1</td>
<td>0.66</td>
<td>1.80</td>
<td>(1.63–1.97)</td>
<td>3.6</td>
<td>0.73</td>
<td>1.47</td>
<td>(1.33–1.62)</td>
<td>4.1</td>
<td>0.77</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>1.56</td>
<td>(1.41–1.72)</td>
<td>-3.6</td>
<td>0.7</td>
<td>1.45</td>
<td>(1.31–1.60)</td>
<td>3.8</td>
<td>0.72</td>
<td>1.21</td>
<td>(1.09–1.34)</td>
<td>4.2</td>
<td>0.76</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>1.36</td>
<td>(1.22–1.50)</td>
<td>-3.8</td>
<td>0.71</td>
<td>1.24</td>
<td>(1.12–1.38)</td>
<td>4.0</td>
<td>0.73</td>
<td>1.02</td>
<td>(0.91–1.13)</td>
<td>4.6</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td><strong>Thiamethoxam&lt;sup&gt;d&lt;/sup&gt;</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>&gt;4&lt;sup&gt;e&lt;/sup&gt;</td>
<td>NC</td>
<td>-0.6</td>
<td>0.06</td>
<td>&gt;4&lt;sup&gt;e&lt;/sup&gt;</td>
<td>NC</td>
<td>0.6</td>
<td>0.06</td>
<td>&gt;4</td>
<td>NC</td>
<td>0.8</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>&gt;4</td>
<td>NC</td>
<td>-0.6</td>
<td>0.07</td>
<td>&gt;4</td>
<td>NC</td>
<td>0.6</td>
<td>0.07</td>
<td>&gt;4</td>
<td>NC</td>
<td>0.7</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>&gt;4</td>
<td>NC</td>
<td>-0.8</td>
<td>0.1</td>
<td>&gt;4</td>
<td>NC</td>
<td>0.8</td>
<td>0.11</td>
<td>&gt;4</td>
<td>NC</td>
<td>0.8</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>&gt;4</td>
<td>NC</td>
<td>-0.7</td>
<td>0.11</td>
<td>&gt;4</td>
<td>NC</td>
<td>0.7</td>
<td>0.11</td>
<td>&gt;4</td>
<td>NC</td>
<td>0.8</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>&gt;4</td>
<td>NC</td>
<td>-0.8</td>
<td>0.14</td>
<td>&gt;4</td>
<td>NC</td>
<td>0.9</td>
<td>0.16</td>
<td>&gt;4</td>
<td>NC</td>
<td>1</td>
<td>0.23</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Slope estimates of the log-logistic regression curves
<sup>b</sup>p = 0.04
<sup>c</sup>p = 0.001
<sup>d</sup>Regressions for thiamethoxam before day 14 were not significant to p = 0.01 so are not presented
<sup>e</sup>p = 0.008

NC = not calculated
Figure 2.2. Mean mayfly survivorship over 28 days of exposure to a) imidacloprid b) clothianidin, and c) thiamethoxam. Error bars are ± standard error (n = 5). Solid red lines represent slopes of the log-logistic regressions; dotted blue lines are 95% CIs.
Figure 2.3. Mean mayfly immobility (including mortality) over 28 days of exposure to a) imidacloprid b) clothianidin, and c) thiamethoxam. Error bars are ± standard error ($n = 5$). Solid red lines represent slopes of the log-logistic regressions; dotted blue lines are 95% CIs.
Figure 2.4. Mean mayfly impairment (including immobility and mortality) over 28 days of exposure to a) imidacloprid b) clothianidin, and c) thiamethoxam. Error bars are ± standard error (n = 5). Solid red lines represent slopes of the log-logistic regressions; dotted blue lines are 95% CIs.
2.4.3 Effects on moulting propensity

Overall, when neonicotinoid effects on *Deleatidium* moulting propensity were observed, they followed an inverse pattern to those observed for neonicotinoid effects on impairment and immobility. During the first week of exposure, there were no observable effects of the neonicotinoids on moulting propensity (Table 2.2; Figure 2.5). During the second week, moulting propensity decreased significantly with increasing concentrations of imidacloprid and clothianidin while thiamethoxam had no effect. These effects mirrored the strong increases in immobility and impairment with exposure to imidacloprid and clothianidin (and lack of effect for thiamethoxam) that had also occurred by this stage (Figures 2.3 and 2.4). Similar patterns were observed in the third week when reductions in moulting propensity occurred with increased concentrations of imidacloprid and clothianidin (Figure 2.5a-b), but now a weak reduction in moulting propensity with increasing concentration of thiamethoxam was also observed (Figure 2.5c). In the fourth week, a significant reduction in moulting propensity was observed for clothianidin only (Figure 2.5b) whereas neither thiamethoxam nor imidacloprid had an effect.

Table 2.2. Results summary of log-logistic generalized linear models for *Deleatidium* mayfly moulting propensity from 28-day chronic exposures to imidacloprid, clothianidin and thiamethoxam (*p*-values are bolded where *p* < 0.01)

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Day</th>
<th>df&lt;sup&gt;a&lt;/sup&gt;</th>
<th>p-Value</th>
<th>Slope&lt;sup&gt;b&lt;/sup&gt;</th>
<th>ρ²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imidacloprid</td>
<td>7</td>
<td>49</td>
<td>0.5</td>
<td>0.68</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>49</td>
<td>&lt;0.001</td>
<td>-1.05</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>49</td>
<td>&lt;0.001</td>
<td>-1.36</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>37</td>
<td>0.8</td>
<td>-0.11</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Clothianidin</td>
<td>7</td>
<td>49</td>
<td>0.4</td>
<td>-0.11</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>49</td>
<td>&lt;0.001</td>
<td>-0.59</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>49</td>
<td>&lt;0.001</td>
<td>-0.79</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>45</td>
<td>&lt;0.001</td>
<td>-1.18</td>
<td>0.34</td>
</tr>
<tr>
<td>Thiamethoxam</td>
<td>7</td>
<td>49</td>
<td>0.04</td>
<td>-0.25</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>49</td>
<td>0.2</td>
<td>-0.19</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>49</td>
<td>0.007</td>
<td>-0.37</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>49</td>
<td>0.5</td>
<td>-0.09</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

<sup>a</sup>Degrees of freedom

<sup>b</sup>Slope estimates of the log-logistic regression curves
Figure 2.5. Mean mayfly moulting propensity (proportion of moulted *Deleatidium* nymphs that were alive at the start of each week) over 28 days of exposure to a) imidacloprid b) clothianidin, and c) thiamethoxam. Error bars are ± standard error (n = 5). Solid red lines represent slopes of the log-logistic regressions; dotted blue lines are 95% CIs.
2.5 Discussion

This study assessed the chronic effects of three neonicotinoids (imidacloprid, clothianidin and thiamethoxam) on the winter nymphs of the ubiquitous New Zealand mayfly Deleatidium spp. Over the 28-day exposure period, all three neonicotinoids elicited some degree of chronic toxicity, but this occurred to different degrees. Imidacloprid, the most toxic, was the only neonicotinoid to cause effects consistent with those predicted, resulting in a 28-day LC50 below 1 µg/L (0.28 µg/L; 0.21–0.36). Clothianidin was slightly less toxic than imidacloprid, with a 28-day LC50 of 1.36 µg/L (1.22–1.5). Compared to imidacloprid and clothianidin, thiamethoxam was considerably less toxic to Deleatidium nymphs; there were no cases in which it led to more than 50% mortality, immobility or impairment over the entire exposure period, resulting in a 28-day LC50 which was outside the range of concentrations tested (> 4 µg/L). Although the achieved concentrations for thiamethoxam were considerably higher than the nominal concentrations, this only strengthens our findings that thiamethoxam was less toxic than the other two neonicotinoids (for which their achieved concentrations were lower than the nominal concentrations).

2.5.1 Comparison of chronic toxicities for mayflies and midges

Table 2.3 summarizes all published results of imidacloprid, clothianidin and thiamethoxam chronic toxicities to the mayfly (Ephemeroptera) and midge (Diptera) species tested. To our knowledge, only three previous studies reported chronic toxicities of imidacloprid to mayfly nymphs. Our findings for Deleatidium were closely matched by the observations of Roessink et al. (2013) for chronic toxicities of imidacloprid to two European mayflies, Cloeon dipterum and Caenis horaria, with 28-day LC50s of 0.2 and 0.32 µg/L, respectively. Van den Brink et al. (2016) found that the winter generation of C. dipterum nymphs were more tolerant to imidacloprid than the summer generation of the same species used by Roessink et al. (2013), calculating 28-day LC50s and EC50s 4–6 times higher (0.85 and 0.68 µg/L). Given we used an overwintering population of Deleatidium nymphs this difference suggests imidacloprid, and potentially other neonicotinoids, could be even more toxic if tested on a summer generation of Deleatidium nymphs. When testing the chronic toxicities of six neonicotinoids to the mayfly Neocloeon triangulifer, Raby et al. (2018b) calculated an LC50 for imidacloprid higher than those already mentioned (1.75 µg/L). Contrary to our findings, their LC50 for clothianidin was lower than for imidacloprid (0.91 µg/L).
### Table 2.3. Published chronic (≥ 28 day) median lethal (LC50; when available) and median effect (EC50; sublethal responses vary between studies, see text for details) concentrations (µg/L) with 95% confidence intervals (CI; when available) for the mayfly (Ephemeroptera) and midge (Diptera) species tested.

<table>
<thead>
<tr>
<th>Neonicotinoid</th>
<th>Order</th>
<th>Species/genus</th>
<th>Duration</th>
<th>LC50</th>
<th>CI</th>
<th>EC50</th>
<th>CI</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imidacloprid</td>
<td>Ephemeroptera</td>
<td>Deleatidium spp.</td>
<td>28 d</td>
<td>0.28</td>
<td>(0.21-0.36)</td>
<td>0.19</td>
<td>(0.14-0.25)</td>
<td>Present Study</td>
</tr>
<tr>
<td></td>
<td>Ephemeroptera</td>
<td>Neocloeon triangulifer</td>
<td>32 d</td>
<td>1.75</td>
<td></td>
<td>1.02</td>
<td>(0.91-1.13)</td>
<td>Raby et al. (2018b)</td>
</tr>
<tr>
<td></td>
<td>Ephemeroptera</td>
<td>Cloeon dipterum</td>
<td>28 d</td>
<td>0.85</td>
<td>NA</td>
<td>0.68</td>
<td>(0.45-1)</td>
<td>Van den Brink et al. (2016)</td>
</tr>
<tr>
<td></td>
<td>Ephemeroptera</td>
<td>Cloeon dipterum</td>
<td>28 d</td>
<td>0.2</td>
<td>(0.11-0.34)</td>
<td>0.12</td>
<td>(0.08-0.20)</td>
<td>Roessink et al. (2013)</td>
</tr>
<tr>
<td></td>
<td>Ephemeroptera</td>
<td>Caenis horaria</td>
<td>28 d</td>
<td>0.32</td>
<td>NA</td>
<td>0.13</td>
<td>(0.07-0.23)</td>
<td>Roessink et al. (2013)</td>
</tr>
<tr>
<td></td>
<td>Diptera</td>
<td>Chironomus dilutus</td>
<td>56 d</td>
<td>0.24</td>
<td></td>
<td>0.28</td>
<td>(0.22-0.27)</td>
<td>Raby et al. (2018b)</td>
</tr>
<tr>
<td></td>
<td>Diptera</td>
<td>Chironomus dilutus</td>
<td>28 d</td>
<td>0.5</td>
<td></td>
<td>0.39</td>
<td>(0.31-0.42)</td>
<td>Maloney et al. (2018a)</td>
</tr>
<tr>
<td></td>
<td>Diptera</td>
<td>Chironomus dilutus</td>
<td>40 d</td>
<td>0.91</td>
<td></td>
<td>0.91</td>
<td>(0.73-1.12)</td>
<td>Cavallaro et al. (2017)</td>
</tr>
<tr>
<td></td>
<td>Diptera</td>
<td>Chironomus tentans</td>
<td>28 d</td>
<td></td>
<td></td>
<td>0.125, 0.625 (NOEC, LOEC)</td>
<td>NA</td>
<td>See Morrissey et al. (2015)</td>
</tr>
<tr>
<td></td>
<td>Diptera</td>
<td>Chironomus riparius</td>
<td>28 d</td>
<td>12.6</td>
<td>(7.33-21.6)</td>
<td>11.8</td>
<td>(8.17-17.1)</td>
<td>Roessink et al. (2013)</td>
</tr>
<tr>
<td>Clothianidin</td>
<td>Ephemeroptera</td>
<td>Deleatidium spp.</td>
<td>28 d</td>
<td>1.36</td>
<td>(1.22-1.5)</td>
<td>1.02</td>
<td>(0.91-1.13)</td>
<td>Present Study</td>
</tr>
<tr>
<td></td>
<td>Ephemeroptera</td>
<td>Neocloeon triangulifer</td>
<td>32 d</td>
<td>0.91</td>
<td></td>
<td>0.39</td>
<td>(0.39-1.43)</td>
<td>Raby et al. (2018b)</td>
</tr>
<tr>
<td></td>
<td>Diptera</td>
<td>Chironomus dilutus</td>
<td>56 d</td>
<td>0.68</td>
<td></td>
<td>0.68</td>
<td>(0.60-0.77)</td>
<td>Raby et al. (2018b)</td>
</tr>
<tr>
<td></td>
<td>Diptera</td>
<td>Chironomus dilutus</td>
<td>28 d</td>
<td>0.71</td>
<td></td>
<td>0.71</td>
<td>(0.50-0.85)</td>
<td>Maloney et al. (2018a)</td>
</tr>
<tr>
<td></td>
<td>Diptera</td>
<td>Chironomus dilutus</td>
<td>40 d</td>
<td>0.28</td>
<td></td>
<td>0.28</td>
<td>(0.20-0.33)</td>
<td>Cavallaro et al. (2017)</td>
</tr>
<tr>
<td></td>
<td>Diptera</td>
<td>Chironomus riparius</td>
<td>28 d</td>
<td>1</td>
<td></td>
<td>NA</td>
<td></td>
<td>NA</td>
</tr>
<tr>
<td>Thiamethoxam</td>
<td>Ephemeroptera</td>
<td>Deleatidium spp.</td>
<td>28 d</td>
<td>&gt; 4</td>
<td>NA</td>
<td>&gt; 4</td>
<td>NA</td>
<td>Present Study</td>
</tr>
<tr>
<td></td>
<td>Ephemeroptera</td>
<td>Neocloeon triangulifer</td>
<td>32 d</td>
<td>2.18</td>
<td></td>
<td>2.18</td>
<td>(1.60-3.20)</td>
<td>Raby et al. (2018b)</td>
</tr>
<tr>
<td></td>
<td>Ephemeroptera</td>
<td>Cloeon dipterum</td>
<td>28 d</td>
<td>0.68</td>
<td></td>
<td>0.68</td>
<td>(0.38-1.2)</td>
<td>Van den Brink et al. (2016)</td>
</tr>
<tr>
<td></td>
<td>Diptera</td>
<td>Chironomus dilutus</td>
<td>56 d</td>
<td>12.95</td>
<td></td>
<td>12.95</td>
<td>(8.54-17.35)</td>
<td>Raby et al. (2018b)</td>
</tr>
<tr>
<td></td>
<td>Diptera</td>
<td>Chironomus dilutus</td>
<td>28 d</td>
<td>8.91</td>
<td></td>
<td>8.91</td>
<td>(5.79-12.37)</td>
<td>Maloney et al. (2018a)</td>
</tr>
<tr>
<td></td>
<td>Diptera</td>
<td>Chironomus dilutus</td>
<td>40 d</td>
<td>4.13</td>
<td></td>
<td>4.13</td>
<td>(3.53-4.76)</td>
<td>Cavallaro et al. (2017)</td>
</tr>
<tr>
<td></td>
<td>Diptera</td>
<td>Chironomus riparius</td>
<td>30 d</td>
<td>11.4</td>
<td></td>
<td>NA</td>
<td></td>
<td>Finnegan et al. (2017)</td>
</tr>
<tr>
<td></td>
<td>Diptera</td>
<td>Chironomus riparius</td>
<td>28 d</td>
<td>6.5, 10.5 (NOEC, LOEC)</td>
<td>NA</td>
<td></td>
<td>Saraiva et al. (2017)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Diptera</td>
<td>Chaoborus sp.</td>
<td>34 d</td>
<td>480</td>
<td></td>
<td>480</td>
<td></td>
<td>Finnegan et al. (2017)</td>
</tr>
</tbody>
</table>

NA = not available; NOEC = no observed effect concentration; LOEC = lowest observed effect concentration.
Raby et al. (2018b) is the only other study to test the chronic toxicity of clothianidin to mayfly larvae. They also determined chronic neonicotinoid toxicities to larvae of the freshwater midge, *Chironomus dilutus* (see below). Three further studies have assessed the chronic toxicity of clothianidin to *Chironomus* spp. The first of these, Drottar et al. (2000), is no longer available but was referenced in Morrissey et al. (2015, Supplemental Data Table A.2), with a 28-day EC50 of 1 µg/L for *Chironomus riparius* (using successful emergence), which is equal to the 28-day EC50 for clothianidin we calculated using the endpoint of impairment for *Deleatidium* nymphs. Cavallaro et al. (2017), Maloney et al. (2018a) and Raby et al. (2018b) assessed chronic toxicities of imidacloprid, clothianidin and thiamethoxam to *C. dilutus* and, measuring emergence rates, calculated 40-day, 28-day and 56-day EC50s. Across the three studies, these were between 0.24–0.5 µg/L for imidacloprid, 0.28–0.71 µg/L for clothianidin and 4.13–12.95 µg/L for thiamethoxam, results which are remarkably consistent with the chronic median lethal and effective concentrations we calculated for *Deleatidium* nymphs (Table 2.3).

Chronic toxicity of thiamethoxam to mayfly nymphs was also assessed by Van den Brink et al. (2016), for *C. dipterum*. In this case, the over-wintering generation had the same 28-day EC50 as for imidacloprid (0.68 µg/L). Finnegan et al. (2017) summarised the acute and chronic effects of thiamethoxam to a wide range of aquatic organisms, including three freshwater insect larvae, *Chaoborus* sp., *Chironomus riparius* and *Chironomus dilutus*. They observed that *C. riparius* was the most sensitive, with a 30-day EC50 (emergence) of 11.4 µg/L. Saraiva et al. (2017) also evaluated chronic effects of thiamethoxam exposure to larvae of *C. riparius*. After 28 days they observed a significantly lower emergence rate in treatments above 6.5 µg/L compared to controls, with successful emergence being only 12.5% at 10.5 µg/L compared to a 77.5% control emergence success. This led to their generation of a No Observable Effect Concentration (NOEC) of 6.5 µg/L and a Lowest Observable Effect Concentration (LOEC) of 10.5 µg/L. Median effect concentrations (EC50 using emergence) were not presented.

Based on these studies with *C. riparius* and *C. dilutus*, it appears that imidacloprid, clothianidin and thiamethoxam have comparable sublethal toxicities to midge larvae (assessed using emergence rates) as to *Deleatidium* nymphs (assessed using mortality, impairment and immobility) and perhaps to those of other mayfly nymphs, although more data are needed to test this possibility. Regardless, the common finding that aquatic insect taxa are most sensitive to neonicotinoids among aquatic invertebrates is supported, with
chronic effect concentrations for imidacloprid and clothianidin being frequently <1 µg/L and ~10 µg/L (an order of magnitude higher) for thiamethoxam. These findings are encouraging for risk assessment procedures employing *Chironomus* spp. as test organisms. Given mayflies have been found to be the most sensitive taxa to neonicotinoids (Morrissey et al. 2015), it would seem that neonicotinoid risk assessments using *Chironomus* spp. may also be protective for the most vulnerable freshwater taxa.

### 2.5.2 Effects on moulting propensity

*Deleatidium* moulting propensity decreased with increasing exposure to imidacloprid and clothianidin in the second and third weeks of exposure, which was likely related to increased impairment and immobility of the mayflies. In week 4, mortality in the imidacloprid treatments with concentrations above 0.4 µg/L was probably so high that moulting was too rare to allow detecting a significant imidacloprid impact. Because moulting is controlled by the neuroendocrine system, neonicotinoid exposure could have directly disrupted moulting processes, though only a handful of studies have investigated this phenomenon and mechanistic explanations remain unstudied. For example, Song et al. (1997) observed a reduction in successful moulting and increased moulting-related mortality in Yellow-fever mosquito larvae, *Aedes aegypti*, with increasing concentration of imidacloprid over a 48-h exposure. They hypothesised that neurological disruption induced by imidacloprid may interrupt the moulting process, causing death during moulting. Further, in the abovementioned chronic experiments with *C. dilutus*, Cavallaro et al. (2017) found that imidacloprid, clothianidin and thiamethoxam all caused moulting-related mortality during emergence. Similarly, Stoughton et al. (2008), who tested the effects of imidacloprid on the life-cycle of another midge, *Chironomus tentans*, observed a 55% reduction in emergence (final moult) under chronic exposure to 1.14 µg/L imidacloprid compared to controls after 28 days. These findings suggest there may indeed be the potential for neonicotinoid-induced moulting disruption, although the specific mechanisms behind this remain unclear.

During our experiments, *Deleatidium* nymph mortality associated with incomplete moulting was irregularly observed but was not recorded. Regardless, the trend toward reduced moulting frequency with exposure to increasing concentration of imidacloprid and clothianidin matches a pattern observed in 96-h exposures to imidacloprid with *Deleatidium* nymphs (Macaulay et al. 2019). In this experiment, moulting was significantly reduced compared to controls with acute exposure to 8 µg/L imidacloprid and further reduced at 40
µg/L. Whether there is more to these patterns than a simple reflection of the overall effect of impaired mayfly mobility caused by these insecticides requires further investigation.

2.5.3 Relative toxicities of neonicotinoids

The relative toxicities of imidacloprid, clothianidin and thiamethoxam to non-target aquatic insect taxa discussed above contrast with the findings of Van den Brink et al. (2016), who found thiamethoxam to be just as toxic to C. dipterum nymphs as imidacloprid. Their findings were supported by one comparison in Morrissey et al.’s (2015) review, where the latter authors calculated the geometric mean of all available acute L[E]C50s for aquatic insects and found no difference between imidacloprid and thiamethoxam. However, Morrissey et al. (2015) also ranked the available acute toxicity data for C. riparius by comparing the acute LC50 for imidacloprid (20 µg/L; 0.08 µmol/L) to the acute EC50s for clothianidin (22 µg/L; 0.09 µmol/L) and thiamethoxam (35 µg/L; 0.12 µmol/L), a ranking which supports the trends observed for larvae of C. dilutus and mayfly nymphs in the present experiment and several other recent studies (Cavallaro et al. 2017, Bartlett et al. 2018, Maloney et al. 2018a, Raby et al. 2018b, Raby et al. 2018c). In all these studies, imidacloprid and clothianidin were more toxic than thiamethoxam, with imidacloprid also regularly displaying a higher toxicity than clothianidin. Hazardous concentrations for 5% of species (HC5s) were determined using toxicity data for crustaceans by Whiteside et al. (2008). Although their assessment was not based on neonicotinoid toxicities to aquatic insects (due to data lacking for some neonicotinoids), they also concluded imidacloprid presented the greatest risk to crustaceans with an HC5 of 0.70 µg/L, followed by clothianidin (HC5 = 39 µg/L) and thiamethoxam was the least toxic with an HC5 of 430 µg/L.

2.5.4 Conclusions and further research

More toxicity data are needed to elucidate the relative toxicities of different neonicotinoids to non-target invertebrates, and Morrissey et al. (2015) especially highlighted a lack of toxicity data for clothianidin (particularly for insect taxa). While the observed differences in toxicity among neonicotinoids are relatively minor compared to the differences in toxicity among taxonomic groups, when we consider the environmentally relevant concentrations and the most sensitive aquatic insect taxa, they are still relevant. For instance, the geometric mean for average global surface water concentrations of 0.73 µg/L for imidacloprid calculated by Sánchez-Bayo et al. (2016) is higher than the 28-day LC50 and EC50 for
imidacloprid we calculated for Deleatidium (0.28 and 0.19 µg/L, respectively). In contrast, the 28-day LC50s we calculated for clothianidin and thiamethoxam (1.36 and >4 µg/L, respectively) are well above this concentration (Sánchez-Bayo et al. 2016 do not report the specific geometric means for these compounds in their review). In New Zealand, neonicotinoids are registered for use to control pests of a variety of pasture and forage crops (Chapman 2010), however their usage and concentrations in surface waters are currently unmonitored. Very little is therefore known about the detection frequencies, concentrations and potential ecological impacts of neonicotinoids and other pesticides in New Zealand streams.

Full-factorial mixture experiments would allow more accurate evaluation of the comparative toxicities between the three neonicotinoids. Such experiments would also elucidate the interactive effects of multiple neonicotinoids acting simultaneously—a scenario which has been shown to occur in surveys of surface waters in many locations (Main et al. 2014, Sánchez-Bayo and Hyne 2014, Hladik and Kolpin 2016). These interactions were predicted to be additive rather than synergistic because of their shared modes of action, which suggests a concentration-addition model of combined toxicity (Rodney et al. 2013, Morrissey et al. 2015). However, several studies have found that this prediction does not always hold (Loureiro et al. 2010, Pavlaki et al. 2011, Maloney et al. 2017, Maloney et al. 2018a) with varying antagonistic and synergistic effects observed, though these rarely met accepted thresholds of model deviations, and mainly additive effects have been observed in field trials (Maloney et al. 2018b, Rico et al. 2018). More tests with sensitive freshwater species such as Deleatidium spp. mayflies in environmentally realistic experiments would be beneficial to improve our understanding of these multiple-stressor scenarios.
Chapter 3

A potent cocktail: Imidacloprid dominates the combined toxicities of neonicotinoid mixtures to *Deleatidium* spp. nymphs
3 A potent cocktail: Imidacloprid dominates the combined toxicities of neonicotinoid mixtures to *Deleatidium* spp. nymphs

3.1 Summary

Mixtures of multiple neonicotinoid insecticides are being detected in surface waters around the world as more monitoring data become available. Combinations of imidacloprid, clothianidin and thiamethoxam are most common, but until recently there were no empirical studies testing their combined toxicities to freshwater invertebrates. Employing a full-factorial ANOVA design, I tested the individual and combined chronic toxicities of these three neonicotinoids in a 28-day laboratory experiment using *Deleatidium* spp. mayfly nymphs. In this experiment, imidacloprid reduced mayfly survival and mobility much more strongly than clothianidin and thiamethoxam and interacted synergistically with both other neonicotinoids to cause greater than additive toxicity when combined. Though not based on the standard method for analysing combined toxicities of pesticides in ecotoxicology, these results provide a proof-of-principle that synergistic interactions between neonicotinoids can occur and need to be investigated further. These findings also emphasise the higher toxicity of imidacloprid to non-target freshwater insects compared to clothianidin and thiamethoxam, implying that stricter regulation to control the use of imidacloprid may need to be prioritised.

3.2 Introduction

Monitoring of contaminants in the aquatic environment has demonstrated that when present, multiple chemicals usually occur together at any one time (Lydy et al. 2004, Rodney et al. 2013). In urban areas, this cocktail of chemicals typically consists of pharmaceuticals and personal care products, whereas mixtures of pesticides are more common in agricultural environments. It is unrealistic to test the effects of all possible mixtures of pesticides and other chemicals that are found in the environment (Lydy et al. 2004, Spurgeon et al. 2010). Therefore, there are two basic models used to predict the combined effects of pesticide mixtures based on their chemical properties and modes of action; concentration addition (CA) and independent action (IA). According to Lydy et al. (2004), the mode of toxic action
of a chemical consists of a series of processes. These begin with the interaction of the chemical with a receptor site in an organism and proceed through operational and anatomical changes in the organism to the resulting effect of a sublethal or lethal response. Where two or more chemicals in a mixture elicit similar modes of action, the CA model is applied because the observed effects are often additive in nature. By contrast, when compounds exhibit completely different modes of action, the IA model is used which does not assume similar toxicokinetics (Lydy et al. 2004). It is therefore generally easier to predict the combined effects of pesticides from the same class than mixtures crossing chemical classes. However, testing the combined effects of pesticides from the same class is important to investigate the potential for deviations from expected toxic outcomes, i.e. lesser than additive toxicity (antagonism) or greater than additive toxicity (synergism).

Thirteen surveys of surface waters across six countries have tested for the presence of more than one neonicotinoid insecticide (Sánchez-Bayo et al. 2016). Multiple neonicotinoids have often been detected at the same sites in both urban environments and areas of intensive agricultural use (Morrissey et al. 2015). In their survey of 136 wetlands in the Saskatchewan Province of Canada, Main et al. (2014) frequently detected more than one neonicotinoid, contributing to maximum total neonicotinoid concentrations of 3.11 µg/L. Schaafsma et al. (2015) found both clothianidin and thiamethoxam in all but one of their 76 samples from maize field surface waters in Ontario. In their survey of 38 streams across the United States, Hladik and Kolpin (2016) found two or more neonicotinoids in 26% of samples and three or more in 11% of samples. Subsequently, Hladik et al. (2018a) sampled 10 tributaries of the Great Lakes every month for 12 months. They detected more than one neonicotinoid in 38% of 120 samples and 10% of samples had three neonicotinoids present. In Sydney, Australia, Sánchez-Bayo and Hyne (2014) detected two or more neonicotinoids in 93% of samples collected from 13 river sites immediately after high rainfall, with concentrations ranging from 0.2 to 4.56 µg/L. At two of the sites sampled, one in a residential park and the other in an orchard-draining creek, residues of five neonicotinoids were detected. This finding shows that, while wetlands and rivers receiving runoff from agricultural regions (especially croplands) appear to be most susceptible to neonicotinoid contamination, surface waters in urban environments can still contain neonicotinoids at similar concentrations (Morrissey et al. 2015).

Across multiple surveys of surface waters in North America and Canada, the three most commonly detected neonicotinoids have been imidacloprid, clothianidin and thiamethoxam.
Another common trend to emerge from these studies has been a correlation between detection frequencies and certain land use types. Thus, detections and concentrations of clothianidin and thiamethoxam increased as the percent of cultivated crops in the catchment increased, whereas imidacloprid detections and maximum concentrations increased as the percentage of urbanisation increased (Hladik and Kolpin 2016, Struger et al. 2017, Hladik et al. 2018a). Due to the broad variety of uses of imidacloprid including agricultural applications (seed treatments, soil drenches and foliar applicants) and uses more common in urban areas such as lawn care, tree drenches and flea treatments for pets (Jeschke et al. 2011, Simon-Delso et al. 2015, Hladik et al. 2018b) imidacloprid is the most commonly used (Jeschke et al. 2011, Goulson 2013) and most commonly detected (Morrissey et al. 2015) neonicotinoid in the world. Over the last 15 years, however, the highest rates of increased average residue levels have been for clothianidin and thiamethoxam (Sánchez-Bayo et al. 2016), reflecting their increased usage worldwide (Simon-Delso et al. 2015).

In summary, the available data show that the three neonicotinoids most frequently found as mixtures in the environment with the potential to exert cumulative toxicity are imidacloprid, thiamethoxam and clothianidin (Wood and Goulson 2017, Hladik et al. 2018a). Despite their prevalence, however, relatively few studies have investigated the toxicity of neonicotinoid mixtures to non-target aquatic organisms (Maloney et al. 2018b) and until recently, their combined toxicities had not been formally tested (Morrissey et al. 2015). According to conventional mixture-toxicity models, neonicotinoid mixtures would be predicted to follow a CA model of cumulative toxicity given their common mode of action. Though this has been observed in the majority of cases (Rico et al. 2018), there have also been several studies to show this is not always the case. Gomez-Eyles et al. (2009) investigated toxicities of mixtures of two neonicotinoids, imidacloprid and thiacloprid, to the earthworm *Eisenia fetida* and the nematode *Caenorhabditis elegans*. Effects of the binary mixtures on *C. elegans* deviated significantly from the assumptions of CA whereas effects on *E. fetida* were consistent with CA model predictions, indicating species-dependent responses to neonicotinoid mixtures. Loureiro et al. (2010) and Pavlaki et al. (2011) investigated effects of binary mixtures of imidacloprid and thiamethoxam on *Daphnia magna*, also observing deviations from CA model predictions (though at concentrations far exceeding environmentally relevant levels). The first two studies to investigate the effects of neonicotinoid mixtures (imidacloprid, clothianidin, thiamethoxam) on an aquatic insect, the
larvae of *Chironomus dilutus*, also demonstrated the potential for deviation from the default CA model (Maloney et al. 2017, Maloney et al. 2018a). To date, the effects of neonicotinoid mixtures on the aquatic larvae of mayflies, one of the most pesticide-sensitive groups of freshwater invertebrates (Morrissey et al., 2015; Sánchez-Bayo et al., 2016), have not been studied in a controlled, laboratory setting.

To address this knowledge gap, the present study investigated the individual and combined effects of the three most commonly used neonicotinoids, imidacloprid, clothianidin and thiamethoxam, on nymphs of the mayfly *Deleatidium* spp., by employing a full-factorial experimental design to determine the relative toxicities of each insecticide and the interactions between them. Despite some recent evidence for deviation from additive effects when combined, given the lack of neonicotinoid mixture toxicity data for mayflies, I hypothesised the neonicotinoids would display additive combined toxicity (tested by full-factorial ANOVA, see below). In addition, based on the results of prior chronic concentration-response experiments with these three insecticides using *Deleatidium* nymphs (see Chapter 2) and other toxicological data from pesticide-sensitive aquatic insects (*Chironomus dilutus*; Cavallaro et al. 2017, Maloney et al. 2017, Maloney et al. 2018a, Raby et al. 2018b and *C. riparius*; reviewed in Morrissey et al. 2015), I tested the hypothesis that chronic toxicity of the three neonicotinoids tested would follow the order imidacloprid > clothianidin > thiamethoxam.

### 3.3 Methods

#### 3.3.1 Experimental design

A 28-day static-renewal laboratory experiment was performed with imidacloprid, clothianidin and thiamethoxam using nymphs of *Deleatidium* spp. as a model organism. *Deleatidium* is endemic to New Zealand, common and widespread in running waters throughout the country, and well-known to be sensitive to a wide range of pollutants (see Chapter 2 Introduction for more information). Two levels of each insecticide were tested in a full-factorial design (presence/absence), with the artificial soft water (ASW; see Chapter 2) containing neonicotinoids renewed every 7 days. The same target concentration was chosen for each insecticide (1.4 µg/L) to allow comparisons of relative toxicities (for testing Hypothesis 2). This concentration was chosen based on the results of prior chronic
concentration-response experiments with imidacloprid, clothianidin and thiamethoxam and was equivalent to the 28-day LC50 for clothianidin which was intermediate in toxicity (see Chapter 2). Treatments were randomly assigned to 48 aerated glass chambers (volume: 1.16 L; 19.9 × 14.4 × 6.3 cm), with 6 replicates of each treatment combination and at least 15 mayfly nymphs (maximum 17, mean 15.6) per chamber.

3.3.2 Insecticide application, sampling and analysis quantification

Working stock solutions of 10 mg/L imidacloprid, clothianidin and thiamethoxam were prepared using 10 mg/mL each insecticide (PESTANAL® analytical standard grade. Sigma-Aldrich; Castle Hill, NSW, Australia). Each pesticide treatment was prepared by dosing 3 L ASW with the required amount of each 10 mg/L working stock. From the glass chambers, 2-mL water samples were collected for analysis from three replicates of the imidacloprid, clothianidin, thiamethoxam only treatments and the mixture of all three (“cocktail” treatment). These samples were collected at the beginning and end of each test week and stored in 4-mL glass vials with Teflon caps in the dark at -20°C until shipping to the analytical laboratory with ice packs, where they were again stored at -20°C until analysis. Neonicotinoids were quantified using liquid chromatography tandem mass spectrometry (LC-MS/MS) following the same procedure described in Chapter 2 (see Section 2.3.3 and Appendix A, Tables A1–A3).

3.3.3 Test specimen collection, acclimation conditions and reading invertebrate responses

*Deleatidium* specimens were collected prior to the experiment employing the same method as described for the previous concentration-response experiments (Chapter 2). All transport, acclimation and experimental conditions (including feeding, tile exchange, pesticide-medium renewal and the coinciding readings of invertebrate responses) were performed in identical procedures to those described in the previous chapter.

3.3.4 Data analysis

Separate three-way ANOVAs were performed in R (Version 3.5.3; R Core Team) to test the individual and combined effects of the three neonicotinoids (categorical predictors) on *Deleatidium* survivorship, immobility, impairment and moulting propensity on each day when the readings of sub-lethal responses were recorded (Day 7, 11, 14, 18, 21, 25 and 28).
The three categorical factors in each model were imidacloprid, clothianidin and thiamethoxam with two levels each (controls and 1.4 µg/L). The significance level $\alpha$ for all tests was $p = 0.05$. Standardised effect sizes (partial $\eta^2$ values) are presented for all results with $p < 0.10$ to allow assessing the biological relevance of results (Nakagawa and Cuthill 2007). Where significant higher-order interactions were present, the recommendation of Quinn and Keough (2002) for interpreting lower-order interactions and main effects was followed. In such situations, lower-order interactions and main effects should be interpreted only where the effect size of the higher-order interaction is smaller than the size of the corresponding lower-order interactions or main effects. If this condition is not met, the higher-order interaction overrides the lower-order interactions or main effects.

ANOVA models were used rather than binomial generalized linear models (GLMs) because overall there was sufficient homogeneity of variances and even distribution of data between 0–1 for the responses measured that employing ANOVAs could accurately test for main and interaction effects and enable calculation of partial $\eta^2$ effect sizes (which were required for determining which significant main effects and lower-order interactions should be interpreted; see above). Partial $\eta^2$ effect sizes cannot be calculated from binomial GLMs, and standardised regression coefficients, which can be computed for logistic regression, are unreliable for assessing relative predictor effect importance (Azen and Traxel 2009). While there have been some efforts to develop dominance analysis methods which allow determining the relative importance of predictors in log-logistic regression, binomial GLMs (which were run for comparison in R) proved unreliable as they did not provide accurate results when determining significant interactions.

### 3.4 Results

#### 3.4.1 Neonicotinoid exposures

Initial concentrations (taken at the start of each 7-day exposure period) of imidacloprid, clothianidin and thiamethoxam were, on average $88.9 \pm 2.8\%$, $78.2 \pm 2.3\%$ and $196.5 \pm 14.6\%$ of the nominal concentrations, respectively (Table 3.1). Final concentrations (taken at the end of each 7-day exposure period) were $89.0 \pm 1.6\%$, $80.8 \pm 2.1\%$ and $210.6 \pm 12.2\%$ of nominal, respectively. Averaged over initial and final samples, achieved concentrations for imidacloprid were $1.24 \mu g/L$, $1.11 \mu g/L$ for clothianidin and $2.85 \mu g/L$ for thiamethoxam.
All results are subsequently described according to the average achieved concentrations for each neonicotinoid. Implications for the considerably higher achieved concentrations of thiamethoxam are addressed below.

<table>
<thead>
<tr>
<th>Neonicotinoid Treatment</th>
<th>Initial (µg/L)</th>
<th>Final (µg/L)</th>
<th>Overall (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imidacloprid (µg/L)</td>
<td>1.24 ± 0.04</td>
<td>1.25 ± 0.02</td>
<td>1.24 ± 0.02</td>
</tr>
<tr>
<td>1.4 µg/L (%)</td>
<td>88.9% ± 2.8%</td>
<td>89.0% ± 1.6%</td>
<td>88.9% ± 1.6%</td>
</tr>
<tr>
<td>Clothianidin (µg/L)</td>
<td>1.10 ± 0.20</td>
<td>1.13 ± 0.03</td>
<td>1.11 ± 0.02</td>
</tr>
<tr>
<td>1.4 µg/L (%)</td>
<td>78.2% ± 2.3%</td>
<td>80.8% ± 2.1%</td>
<td>79.5% ± 1.6%</td>
</tr>
<tr>
<td>Thiamethoxam (µg/L)</td>
<td>2.75 ± 0.20</td>
<td>2.95 ± 0.17</td>
<td>2.85 ± 0.13</td>
</tr>
<tr>
<td>1.4 µg/L (%)</td>
<td>196.5% ± 14.6%</td>
<td>210.6% ± 12.2%</td>
<td>203.5% ± 9.5%</td>
</tr>
</tbody>
</table>

### 3.4.2 Individual and combined effects of neonicotinoid exposure

All results described below were significant at \( \alpha = 0.05 \) (see Table 3.2 and 3.3 and for individual \( p \)-values and effect sizes; partial \( \eta^2 \) values). To aid interpretation of the response patterns described and shown in Figures 3.1–3.6, additional plots for all significant individual factor main effects and two-way interaction effects (Figures B1–B11) are provided in Appendix B.

#### 3.4.2.1 Week 1

There were no effects on *Deleatidium* survival in any of the neonicotinoid treatments after the first 7 days of exposure (Figure 3.1a). Exposure to 1.11 µg/L of clothianidin caused a slight increase in mayfly immobility and impairment (Figure 3.1b and c). Exposure to all three insecticides in combination also caused a small increase in impairment, whereas there were no significant increases in impairment with exposure to 1.24 µg/L imidacloprid or 2.85 µg/L thiamethoxam on their own (three-way interaction; Figure 3.2). Only exposure to imidacloprid caused a small reduction in mayfly moulting during the first week of the exposure period (Table 3.3; Figure 3.3; see also Figure B1).
Table 3.2. Results summary (p-values and effect sizes where $p < 0.1$) of three-way ANOVAs for *Deleatidium* mayfly survivorship, immobility and impairment over 28 days of exposure to the neonicotinoids imidacloprid (IMI), clothianidin (CLO) and thiamethoxam (TMX). P-values are bolded where $p < 0.05$, and effect sizes (partial $\eta^2$ values; range 0–1) are in parentheses wherever $p < 0.1$. Effect size categories are: < 0.10 ‘trivial’, ≥ 0.10 ‘small’, > 0.30 ‘medium’, > 0.50 ‘large’ (Nakagawa and Cuthill 2007). Interactions that overrode one or more main effects or lower-order interactions are underlined.

<table>
<thead>
<tr>
<th>Day</th>
<th>Response</th>
<th>IMI</th>
<th>CLO</th>
<th>TMX</th>
<th>CLO × TMX</th>
<th>IMI × TMX</th>
<th>IMI × CLO</th>
<th>IMI × CLO × TMX</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>Survivorship</td>
<td>0.002 (0.22)</td>
<td>0.045 (0.10)</td>
<td>0.02 (0.13)</td>
<td>0.002 (0.13)</td>
<td>0.09 (0.07)</td>
<td>0.04 (0.10)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Immobility</td>
<td>&lt;0.001 (0.33)</td>
<td>&lt;0.001 (0.34)</td>
<td>0.05 (0.09)</td>
<td>0.008 (0.16)</td>
<td>0.004 (0.18)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Impairment</td>
<td>&lt;0.001 (0.85)</td>
<td>&lt;0.001 (0.61)</td>
<td>&lt;0.001 (0.36)</td>
<td>&lt;0.001 (0.39)</td>
<td>&lt;0.001 (0.54)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Survivorship</td>
<td>0.001 (0.47)</td>
<td>&lt;0.001 (0.33)</td>
<td>0.002 (0.22)</td>
<td></td>
<td></td>
<td></td>
<td>0.04 (0.10)</td>
</tr>
<tr>
<td></td>
<td>Immobility</td>
<td>&lt;0.001 (0.75)</td>
<td>&lt;0.001 (0.58)</td>
<td>&lt;0.001 (0.29)</td>
<td>&lt;0.001 (0.29)</td>
<td>&lt;0.001 (0.47)</td>
<td>0.04 (0.10)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Impairment</td>
<td>&lt;0.001 (0.94)</td>
<td>&lt;0.001 (0.58)</td>
<td>&lt;0.001 (0.36)</td>
<td>&lt;0.001 (0.36)</td>
<td>&lt;0.001 (0.48)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Survivorship</td>
<td>&lt;0.001 (0.77)</td>
<td>&lt;0.001 (0.49)</td>
<td>0.008 (0.16)</td>
<td>0.03 (0.11)</td>
<td>&lt;0.001 (0.31)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Immobility</td>
<td>&lt;0.001 (0.87)</td>
<td>&lt;0.001 (0.61)</td>
<td>&lt;0.001 (0.29)</td>
<td>&lt;0.001 (0.25)</td>
<td>&lt;0.001 (0.50)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Impairment</td>
<td>&lt;0.001 (0.99)</td>
<td>&lt;0.001 (0.41)</td>
<td>&lt;0.001 (0.26)</td>
<td>0.005 (0.18)</td>
<td>0.006 (0.18)</td>
<td>0.03 (0.11)</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>Survivorship</td>
<td>&lt;0.001 (0.80)</td>
<td>&lt;0.001 (0.53)</td>
<td>0.002 (0.22)</td>
<td></td>
<td></td>
<td>&lt;0.001 (0.32)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Immobility</td>
<td>&lt;0.001 (0.89)</td>
<td>&lt;0.001 (0.58)</td>
<td>&lt;0.001 (0.38)</td>
<td>0.003 (0.21)</td>
<td>&lt;0.001 (0.37)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Impairment</td>
<td>&lt;0.001 (0.98)</td>
<td>&lt;0.001 (0.38)</td>
<td>0.002 (0.21)</td>
<td></td>
<td></td>
<td>0.09 (0.07)</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>Survivorship</td>
<td>&lt;0.001 (0.84)</td>
<td>&lt;0.001 (0.44)</td>
<td>0.02 (0.12)</td>
<td></td>
<td></td>
<td></td>
<td>0.03 (0.11)</td>
</tr>
<tr>
<td></td>
<td>Immobility</td>
<td>&lt;0.001 (0.94)</td>
<td>&lt;0.001 (0.61)</td>
<td>&lt;0.001 (0.32)</td>
<td>0.01 (0.15)</td>
<td>0.04 (0.10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Impairment</td>
<td>&lt;0.001 (0.98)</td>
<td>&lt;0.001 (0.45)</td>
<td>0.002 (0.24)</td>
<td>0.09 (0.07)</td>
<td>&lt;0.001 (0.27)</td>
<td>0.02 (0.12)</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>Survivorship</td>
<td>&lt;0.001 (0.87)</td>
<td>&lt;0.001 (0.59)</td>
<td>0.001 (0.24)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Immobility</td>
<td>&lt;0.001 (0.93)</td>
<td>&lt;0.001 (0.65)</td>
<td>&lt;0.001 (0.29)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Impairment</td>
<td>&lt;0.001 (0.98)</td>
<td>&lt;0.001 (0.70)</td>
<td>&lt;0.001 (0.36)</td>
<td>0.02 (0.14)</td>
<td>0.006 (0.27)</td>
<td>&lt;0.001 (0.66)</td>
<td>0.001 (0.23)</td>
</tr>
</tbody>
</table>

47
Figure 3.1. Mean Deleatidium nymph (a) survivorship (b) immobility and (c) impairment over 28 days of exposure to the neonicotinoids imidacloprid (IMI), clothianidin (CLO) and thiamethoxam (TMX), individually, in binary mixtures and in a ternary mixture (error bars ± SE).
Figure 3.2. Day 7 three-way interaction effect between imidacloprid, clothianidin and thiamethoxam on *Deleatidium* impairment. Error bars ± SE.

Table 3.3. Results summary (*p*-values and effect sizes where *p* < 0.1) of three-way ANOVAs for *Deleatidium* mayfly moulting propensity in each week of the 28-day exposure to imidacloprid (IMI), clothianidin (CLO) and thiamethoxam (TMX). *P*-values are bolded where *p* < 0.05, and effect sizes (partial η² values; range 0-1) are in parentheses below wherever *p* < 0.1. Effect size categories are: < 0.10 ‘trivial’, ≥ 0.10 ‘small’, > 0.30 ‘medium’, > 0.50 ‘large’ (Nakagawa and Cuthill 2007).

<table>
<thead>
<tr>
<th>Week</th>
<th>IMI</th>
<th>CLO</th>
<th>TMX</th>
<th>CLO × TMX</th>
<th>IMI × TMX</th>
<th>IMI × CLO</th>
<th>IMI × CLO × TMX</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.009</td>
<td>0.09</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Day 0-7)</td>
<td>(0.16)</td>
<td>(0.07)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.1</td>
<td>0.09</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Day 8-14)</td>
<td>(0.16)</td>
<td>(0.07)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>&lt;0.001</td>
<td>0.02</td>
<td>0.001</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Day 15-21)</td>
<td>(0.39)</td>
<td>(0.13)</td>
<td>(0.23)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>&lt;0.001</td>
<td>0.08</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Day 22-28)</td>
<td>(0.36)</td>
<td>(0.07)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 3.3. Mean *Deleatidium* nymph moulting propensity during each week of a 28-day exposure to the neonicotinoids imidacloprid (IMI), clothianidin (CLO) and thiamethoxam (TMX), individually, in binary mixtures and in a ternary mixture (error bars ± SE).
3.4.2.2 Week 2

Over the second week of the exposure period, both imidacloprid and clothianidin caused weak to moderate reductions of *Deleatidium* survival compared to controls, and strong sublethal effects on immobility and impairment (especially imidacloprid; Figure 3.1, Figures B2–B3). These two insecticides also interacted synergistically, having a greater combined effect on reduced survivorship (on Day 14) and increased immobility and impairment than the sum of their individual effects on these responses (Figure B4). The strongest interactions were observed for the sublethal responses, especially impairment. Similar two-way interactions also occurred between imidacloprid and thiamethoxam for the sublethal responses on Days 11 (impairment) and 14 (impairment and immobility; Figure B4). However, in these cases the interactions overrode the main effects of thiamethoxam (see effect sizes in Table 3.2), meaning thiamethoxam mainly caused an increase in mayfly impairment and immobility when combined with imidacloprid.

On Day 11, weak three-way interactions were observed for survivorship and immobility, where thiamethoxam mainly caused a reduction in *Deleatidium* survival and an increase in immobility when combined with both imidacloprid and clothianidin (Figure 3.4). In the absence of imidacloprid, however, the combined effect of clothianidin and thiamethoxam on survival and immobility was antagonistic, with the effect of clothianidin on these responses being reversed when combined with thiamethoxam. A similar three-way interaction (with toxicity being highest when all three neonicotinoids were applied together) also occurred on Day 14 for immobility (Figure 3.5). In this instance, the three-way interaction was much weaker than the imidacloprid × clothianidin interaction (see Table 3.2), indicating that for this response, imidacloprid and clothianidin were also synergistically more toxic in combination than on their own.

3.4.2.3 Week 3

In the third week of exposure, there were significant main effects of all three neonicotinoids on all mayfly responses (Figure 3.1; Figures B5–B6). A clear order of toxicity was also evident on Days 18 and 21. The strongest effects observed were caused by exposure to 1.24 µg/L imidacloprid. By Day 18, nearly 100% of the increase in *Deleatidium* impairment could be explained by the main effect of imidacloprid (Table 3.2; Figure B5). Clothianidin (at 1.11 µg/L) was not as toxic as imidacloprid but, by Day 21, had also strongly reduced survival and increased immobility as well as moderately increasing impairment. Thiamethoxam (at
2.85 µg/L) was the least toxic but still exerted weak main effects, reducing survival and increasing immobility and impairment compared to controls. From Days 15–21 all three neonicotinoids reduced *Deleatidium* moulting propensity, with weak effects of clothianidin and thiamethoxam and a moderate reduction caused by imidacloprid (Table 3.3; Figure 3.3).

On Day 18, all responses (except for moulting) were affected by two-way interactions between imidacloprid and the other two neonicotinoids. The effects on reduced survival, increased impairment and increased immobility of exposure to imidacloprid in combination with either clothianidin or thiamethoxam were generally larger than the sums of their individual effects (Figure 3.1; Figure B7). The strongest of these synergistic interactions occurred for imidacloprid and clothianidin when increasing immobility, followed by a moderate interaction of the same two insecticides when reducing survival. On Day 21, the same synergistic two-way interactions for survival and immobility occurred for imidacloprid and clothianidin (both of moderate strength), but synergistic interactions for impairment could no longer be observed. Between imidacloprid and thiamethoxam, there was just one two-way interaction on Day 21, a weak synergistic effect on increased immobility (Figure B8).

![Figure 3.4](image.png)

Figure 3.4. Day 11 three-way interaction effects between imidacloprid, clothianidin and thiamethoxam on (a) survivorship and (b) immobility of *Deleatidium* nymphs. Error bars ± SE.
In the final week of the exposure period, all three neonicotinoids reduced *Deleatidium* survival and increased immobility and impairment (Figures B9–B10). Imidacloprid was clearly the most toxic, eliciting very strong effects on all three responses. Consistent with previous weeks, the main effect of imidacloprid on impairment was the strongest effect observed, causing almost 100% impairment by itself. Clothianidin also exerted strong effects on all responses (apart from moulting), though not quite as strong as imidacloprid, while thiamethoxam weakly reduced survivorship and weakly to moderately increased immobility and impairment. *Deleatidium* moulting propensity was only reduced with exposure to imidacloprid in the last week of exposure (Table 3.3; Figure 3.3), reflecting the generally high degree of impairment in these mayflies.

On Day 25, the synergistic two-way interactions between imidacloprid and clothianidin for *Deleatidium* survival and immobility that had been observed in the second and third weeks were still present, but they had become weak (Figure B10). On Day 28, they had disappeared completely. Instead, the strongest interaction on both Days 25 and 28 was an antagonistic two-way interaction between imidacloprid and clothianidin for impairment. This interaction occurred because of the already extremely high level of impairment caused due to the main effect of imidacloprid alone, so when combined with other neonicotinoids (mainly clothianidin, but also thiamethoxam on Day 28), it appeared these reduced the effect of imidacloprid (Figure B10–B11). However, this only occurred because impairment cannot increase above 100% and, as the main effect of imidacloprid on impairment was already nearly 100%, the combined effects with the other insecticides had to be less than additive.

![Figure 3.5. Day 14 three-way interaction effect between imidacloprid, clothianidin and thiamethoxam on *Deleatidium* immobility. Error bars ± SE.](image)

### 3.4.2.4 Week 4

In the final week of the exposure period, all three neonicotinoids reduced *Deleatidium* survival and increased immobility and impairment (Figures B9–B10). Imidacloprid was clearly the most toxic, eliciting very strong effects on all three responses. Consistent with previous weeks, the main effect of imidacloprid on impairment was the strongest effect observed, causing almost 100% impairment by itself. Clothianidin also exerted strong effects on all responses (apart from moulting), though not quite as strong as imidacloprid, while thiamethoxam weakly reduced survivorship and weakly to moderately increased immobility and impairment. *Deleatidium* moulting propensity was only reduced with exposure to imidacloprid in the last week of exposure (Table 3.3; Figure 3.3), reflecting the generally high degree of impairment in these mayflies.

On Day 25, the synergistic two-way interactions between imidacloprid and clothianidin for *Deleatidium* survival and immobility that had been observed in the second and third weeks were still present, but they had become weak (Figure B10). On Day 28, they had disappeared completely. Instead, the strongest interaction on both Days 25 and 28 was an antagonistic two-way interaction between imidacloprid and clothianidin for impairment. This interaction occurred because of the already extremely high level of impairment caused due to the main effect of imidacloprid alone, so when combined with other neonicotinoids (mainly clothianidin, but also thiamethoxam on Day 28), it appeared these reduced the effect of imidacloprid (Figure B10–B11). However, this only occurred because impairment cannot increase above 100% and, as the main effect of imidacloprid on impairment was already nearly 100%, the combined effects with the other insecticides had to be less than additive.
Similar antagonistic effects were observed for the mixture of all three neonicotinoids in weak three-way interactions for immobility and impairment on Day 25 (Figure 3.6) and for impairment on Day 28 (Figure 3.7). However, embedded within these three-way interactions were still synergistic effects between two neonicotinoids that were then absent in the combination of all three. In the absence of clothianidin, imidacloprid interacted synergistically with thiamethoxam to cause a small increase in *Deleatidium* immobility on Day 25 (Figure 3.6a). Similarly, in the absence of imidacloprid, clothianidin and thiamethoxam interacted synergistically to increase *Deleatidium* immobility and impairment on Day 25 (Figure 3.6) and only impairment on Day 28 (Figure 3.7).

![Figure 3.6](image.png)

Figure 3.6. Day 25 three-way interaction effect between imidacloprid, clothianidin and thiamethoxam on (a) *Deleatidium* immobility and (b) *Deleatidium* impairment. Error bars ± SE.
3.5 Discussion

3.5.1 Primary neonicotinoid effects and comparative toxicities

The results of this full-factorial, 28-day experiment with imidacloprid, clothianidin and thiamethoxam validated the observations of relative toxicities from prior single-pesticide concentration-response experiments with these three compounds lasting for the same exposure time and using the same study organism (Chapter 2). Thus, my second hypothesis was confirmed whereby imidacloprid was clearly the most toxic neonicotinoid, followed by clothianidin, while thiamethoxam was the least toxic. From Day 14 onward (when this pattern was first evident, particularly for the impairment response), this order of toxicity was demonstrated by all responses. By the end of the four-week exposure period, it was clear that at 1.24 µg/L, imidacloprid was severely toxic to Deleatidium mayfly nymphs, while at a similar concentration (1.11 µg/L) clothianidin was less toxic but still caused strong lethal effects and some impairment of mobility, whereas the effects of thiamethoxam were still relatively weak at more than double these concentrations (2.85 µg/L). As also occurred in Chapter 2 (see Section 2.5), although the achieved concentrations for thiamethoxam were considerably higher than the nominal concentrations, this only strengthens our findings that thiamethoxam was less toxic than the other two neonicotinoids (for which their achieved concentrations were slightly lower than the nominal concentrations). These results confirm the results of the 28-day single-pesticide experiments described in Chapter 2 and I refer to the relevant discussion of the relative toxicities of these three neonicotinoids from this chapter (see Section 2.5.3).
Briefly, while some previous research had found no strong differences in toxicity between neonicotinoids when testing aquatic insects (Morrissey et al. 2015, Van den Brink et al. 2016), there is mounting evidence for an order of toxicity consistent with that found in my experiment. For the aquatic midge *Chironomus dilutus*, Cavallaro et al. (2017), Raby et al. (2018b) and Maloney et al. (2018a) demonstrated the same order of toxicity using EC50s for emerging adults (see Table 2.3), with one exception where the 40-day EC50 for imidacloprid (0.39 μg/L) calculated by Cavallaro et al. (2017) was not lower than clothianidin (0.28 μg/L). The acute data for *Chironomus riparius* (as reviewed by Morrissey et al. 2015) were imidacloprid (LC50 = 20 μg/L; 0.08 μmol/L) ≥ clothianidin (EC50 = 22 μg/L; 0.09 μmol/L) > thiamethoxam (EC50 = 35 μg/L; 0.12 μmol/L) comparing of the LC50 for imidacloprid with the EC50s for clothianidin and thiamethoxam. When combined, this evidence suggests that pesticide-sensitive aquatic insect taxa (in these cases of the orders Ephemeroptera and Diptera) have differing sensitivities to the most commonly used neonicotinoids, following the consistent pattern of increasing toxicity in the order imidacloprid > clothianidin > thiamethoxam.

### 3.5.2 Neonicotinoid mixture effects and interactions

According to Morrissey et al.’s (2015) global-scale review, neonicotinoids are known to be additively or synergistically toxic when they occur together, with several binary mixtures having demonstrated synergistic insecticidal activity. There is growing evidence from experiments with non-target aquatic species also demonstrating the potential for deviations from the concentration-addition (CA) model of cumulative toxicity. Adding to this, I observed a range of additive, synergistic and antagonistic cumulative effects of imidacloprid, clothianidin and thiamethoxam on nymphs of the New Zealand mayfly *Deleatidium* spp. (using a non-standard method of testing combined toxicities, discussed later). While these results are broadly consistent with recent findings for *Chironomus dilutus* larvae exposed to mixtures of the same insecticides (the only other chronic laboratory experiment to investigate neonicotinoid mixture effects on aquatic insects; Maloney et al. 2018a), the specific synergisms observed were not always the same in the two studies. The strongest synergistic interactions in my experiment occurred in binary mixtures with imidacloprid, especially the combination of imidacloprid with clothianidin, whereas Maloney et al. (2018a) observed no deviation from the CA model for this particular mixture. The only synergistic effects that were consistent between the two studies were for the imidacloprid-
thiamethoxam mixtures where Maloney et al. (2018a) observed dose-dependent synergistic cumulative toxicity, primarily in mixtures with higher concentrations of thiamethoxam. The additive effects in their clothianidin-thiamethoxam mixtures also mainly followed the patterns I observed.

In the ternary neonicotinoid mixture, I found significant three-way interactions in the final week of exposure for the sublethal responses (impairment on Days 18 and 25, immobility on Day 25). In these, clothianidin and thiamethoxam interacted synergistically in the absence of imidacloprid to cause greater-than-additive increases in impairment of Deleatidium mobility when combined. It is possible a synergistic cumulative effect of all three neonicotinoids would have also occurred at this point had a lower concentration of imidacloprid been used. However, because of the aim to compare the relative toxicities between the three neonicotinoids in this experiment, the 28-day EC50 of clothianidin (1.4 µg/L) was chosen as the target concentration for all treatments. This concentration clearly demonstrated that imidacloprid is more toxic than clothianidin, resulting in severe sublethal effects in the imidacloprid treatments after just 14 days of exposure. Therefore, antagonistic interactions between all three neonicotinoids were observed toward the end of the exposure duration because exposure to imidacloprid alone had already caused almost 100% impairment. Nevertheless, synergistic cumulative effects of all three neonicotinoids were observed earlier in the experiment on Days 7–14 while sublethal responses were still overall < 50%. Thus, the combined effects of all three neonicotinoids were greater than the sums of their individual effects for Deleatidium impairment on Day 7, survivorship and immobility on Day 11, and immobility on Day 14.

In contrast to my findings, Maloney et al. (2018a) observed no deviation from concentration-additive toxicity in their ternary mixtures over a 28-day exposure. In an acute (96-hour) exposure experiment, however, the same authors had observed weak synergistic reductions in C. dilutus survival in imidacloprid-clothianidin-thiamethoxam mixtures (Maloney et al. 2017). Moreover, all mixture treatments exerted greater-than-additive toxicity in the acute experiment, suggesting that synergistic interactions in neonicotinoid mixtures might be more likely at higher exposure concentrations.

Speculating about the mechanistic responses causing synergistic toxicity of neonicotinoid mixtures to larvae of C. dilutus, Maloney et al. (2017) discussed the potential for the presence of multiple nicotinic acetylcholine receptor (nAChR) subtypes in C. dilutus larvae to allow
slightly different toxicological actions between neonicotinoids. Different neuronal functional responses to and binding affinities for nAChR subtypes among imidacloprid, clothianidin and thiamethoxam have been demonstrated with cockroaches, *Periplaneta americana* (Salgado and Saar 2004, Ihara et al. 2006, Thany 2009, 2011). It is therefore a plausible hypothesis that, although an overall agonistic mode of action is shared between neonicotinoid insecticides, the presence of multiple nAChR subtypes in *C. dilutus* larvae and *Deleatidium* nymphs (with different functional responses to specific neonicotinoid agonists) could be the mechanism underlying deviations from concentration-additive responses to neonicotinoid mixtures. However, molecular and functional characterisation of the nAChRs would need to be investigated in order to verify such mechanisms in these aquatic insects.

There are several key differences in method between my experiment and that of Maloney et al. (2018a), the first being differing study species. However, despite belonging to different insect orders, mayfly nymphs and midge larvae have been found to be among the three most sensitive aquatic taxa to neonicotinoids (along with caddisfly larvae; Morrissey et al. 2015). Based on the results of my experiments with *Deleatidium* mayfly nymphs and those performed with the midge *C. dilutus*, the neonicotinoids imidacloprid, clothianidin and thiamethoxam appear to be similarly toxic to the two organisms across chronic 28-day exposure durations.

The most significant differences between the studies are in the study design. In my experiment, employing a balanced full-factorial design with two levels of each neonicotinoid and six replicates of each treatment combination allowed direct comparison of relative neonicotinoid toxicities and testing for deviations from additive interactive toxicities using ANOVA. By contrast, Maloney et al. (2018a) used a fixed-ray experimental design with five toxic-unit dose ratios. They observed a dose-dependent synergism in their binary imidacloprid-thiamethoxam mixture over a chronic exposure and had previously demonstrated dose-dependent responses in all binary and ternary mixtures of imidacloprid, clothianidin and thiamethoxam in acute exposures (at higher, less environmentally relevant concentrations; Maloney et al. 2017). They also used the equivalent toxic unit concept of determining exposure concentrations (where a toxic unit is defined as the actual concentration of a chemical divided by its toxicity threshold) to standardise for the different toxicities of the three neonicotinoids. Employing a fixed-ray design with five toxic-unit dose ratios necessitated reduced replication (only two replicates per treatment) to allow for a greater number of exposure combinations. While permissible in their regression-based
analyses, adequate replication is fundamental to maintaining statistical power in the full-factorial design I employed and was also necessary given this is just the third study performed using these methods (Hunn 2016; Chapter 2). Therefore, including more treatment levels (neonicotinoid concentrations) was not feasible. This may limit extrapolation of my findings to real-world exposure scenarios at even lower concentrations (i.e. in the order of ng/L).

Given the use of non-standard methods and analyses for testing deviation from concentration-addition, there are limitations to the implications that can be drawn from my findings and comparisons with those of Maloney et al. (2018a). Nevertheless, despite the differences in study design, there is proof-of-principle from these two chronic laboratory experiments using sensitive aquatic insect larvae that neonicotinoids can interact synergistically in mixtures. In particular, I demonstrated that imidacloprid can interact with both clothianidin and thiamethoxam to cause greater-than-additive toxicity than would be predicted based on their individual effects alone. It was also clear how much more toxic imidacloprid is to Deleatidium nymphs than clothianidin and especially thiamethoxam. Given these joint findings of higher toxicity and the potential to strongly interact synergistically with clothianidin and thiamethoxam, there is particular ground for concern over the contamination of aquatic ecosystems with imidacloprid—the most widely-used and commonly detected neonicotinoid (Sánchez-Bayo et al. 2016). Moreover, as discussed in the chapter introduction, detections of imidacloprid-clothianidin-thiamethoxam mixtures in surface waters are becoming more common as more surveys test for multiple neonicotinoids. The potential for greater-than-additive cumulative effects demonstrated in my experiment highlights the serious environmental problem that contamination of waterways with multiple neonicotinoids could be causing. Future studies should extend the research discussed here by testing the potential for synergistic toxicity of neonicotinoid mixtures to Deleatidium mayflies employing standard methods for testing deviation from concentration-addition.
Chapter 4

Delayed effects of food limitation and chronic exposure to imidacloprid interact with strong effects of simulated heatwaves on mayfly nymphs
4 Delayed effects of food limitation and chronic exposure to imidacloprid interact with strong effects of simulated heatwaves on mayfly nymphs

4.1 Summary

The global intensification of agriculture means agrochemicals such as pesticides are having an increasingly important role as anthropogenic stressors and drivers of environmental change. There is also an increased need to understand how these contaminants will interact with other environmental stressors, especially with those that are predicted to become more severe with climate change. In the present study, I performed a 6-week laboratory experiment with *Deleatidium* spp. mayfly nymphs to investigate the individual and combined effects of the world’s most widely used insecticide, imidacloprid, and two stressors naturally occurring in running waters, heatwaves and food limitation, both of which are predicted to occur more often and with greater severity under climate change. Two 6-day heatwaves were simulated, one during a starvation period prior to imidacloprid addition and one during the first 6 days of imidacloprid exposure, to investigate the potential for direct and delayed interactive effects of the stressors over the 6-week exposure period. The simulated heatwaves alone caused such drastic negative effects on *Deleatidium* survival and mobility that mainly antagonistic interactions were observed with the other stressors. This meant lethal effects of imidacloprid could only be detected in the absence of heatwaves or starvation. Exposure to 0.4 μg/L imidacloprid took 24 days to have a significant effect but eventually impaired *Deleatidium* mobility as strongly as the heatwave main effects, highlighting the potential environmental impacts of chronic exposure to low concentrations of imidacloprid on freshwater insects.

4.2 Introduction

In recent decades, research in ecotoxicology has shown that interactions between toxicants (toxic chemicals) and natural stressors are a problem that has traditionally been overlooked in ecological risk assessment (Heugens et al. 2001, Bednarska et al. 2013). Reviews by Holmstrup et al. (2010) and Laskowski et al. (2010) found that such interactions were not
uncommon. For example, Holmstrup et al. (2010) reviewed over 150 studies that evaluated the effects of a variety of pollutants combined with other environmental stressors such as temperature, desiccation, hypoxia, pathogens and immunomodulatory factors, and found that more than 50% of these studies reported synergistic interactions between stressors. Similarly, Laskowski et al. (2010) performed a meta-analysis on 61 studies investigating the effects of temperature, humidity and dissolved oxygen on the toxicity of a range of chemicals and also found significant interactions in 62% of all cases. Their meta-analysis showed that the null hypothesis assuming no interaction between chemicals and natural environmental factors should be rejected at a very high significance level. In particular, temperature and food limitation are two environmental factors which have been shown to alter the toxicity of a variety of contaminants to a range of aquatic organisms (Heugens et al. 2001, Bednarska et al. 2013). Nevertheless, studies investigating interactive effects of temperature or food limitation with toxicants are uncommon, and experiments incorporating all three stressors in combination are even rarer (but see Cooney et al. 1983, Janssens et al. 2014 and Dinh et al. 2016).

Insect populations are declining worldwide (Potts et al. 2010, Hallmann et al. 2017, Sánchez-Bayo and Wyckhuys 2019), and two key drivers of this decline are increased temperatures as a result of climate change and use of agricultural insecticides (Tilman et al. 2001, Benton et al. 2002, Hickling et al. 2006, Beketov et al. 2013, Ollerton et al. 2014, Stehle and Schulz 2015, Van Lexmond et al. 2015, Sánchez-Bayo and Wyckhuys 2019). As global mean surface temperatures rise with climate change, the frequency and duration of hot temperature extremes will increase at daily and seasonal timescales (IPCC 2014). Heat waves can cause acute physiological stress in ectotherms, for whom temperature plays a key role in their physiology and performance (Vannote and Sweeney 1980, Sokolova and Lannig 2008), by increasing metabolic demands associated with thermal tolerance protection mechanisms (Feder and Hofmann 1999, Cherkesov et al. 2006). Though the effects of mean temperature increases have traditionally received more attention in global change research, the ability to cope with temperature extremes is thought to be more relevant for the persistence of populations under changing climates (Thompson et al. 2013). Aquatic ecosystems are particularly vulnerable to the effects of exposure to extreme temperatures and contaminants because most aquatic species are ectotherms and freshwaters receive a myriad of contaminants from their surrounding catchment (Schäfer et al. 2011a, Schäfer et al. 2011b, Stehle and Schulz 2015).
Imidacloprid, the most widely used insecticide in the world, was the first of the neonicotinoid insecticides to be introduced to the market (Jeschke et al. 2011). A major reason for its rapid rise to success is its solubility, allowing its prophylactic use as a seed treatment (the preferred method of application for many crops). However, high solubility also means imidacloprid readily leaches into groundwater and, from there, enters surface waters in low but chronically present concentrations. A review of monitoring data from 33 surveys of inland surface waters conducted in 11 countries found that imidacloprid was commonly present, with an average concentration of 0.73 µg/L (Sánchez-Bayo et al. 2016). Imidacloprid present at this concentration would far exceed regulatory threshold levels in the USA (EPA 2018), Canada (CCME 2018) and Europe (Smit et al. 2015, Hladik et al. 2018b). Moreover, it would negatively impact the larvae of sensitive freshwater insects such as mayflies and midges, which have been shown to have median lethal and sublethal concentrations (determined through emergence, mobility, feeding, growth and other developmental rates in ecotoxicological experiments) below 0.73 µg/L (Alexander et al. 2007, Roessink et al. 2013, Van den Brink et al. 2016, Cavallaro et al. 2017, Maloney et al. 2018a, Naveen et al. 2018, Raby et al. 2018b).

These eight studies have all assessed the effects of imidacloprid in isolation under laboratory conditions that included optimal temperatures and food supply. By contrast, in real freshwater ecosystems organisms regularly face temperature extremes and periods of food shortage, and the latter may be even more prominent during heatwaves (Adamo et al. 2012). It is therefore important to investigate how combinations of these three stressors may affect non-target organisms in freshwater ecosystems. The negative effects of exposure to imidacloprid on nymphs of pollution-sensitive mayfly taxa have been shown to be worsened by exposure to a prior 5-day starvation period during a 14-day experiment (Hunn 2016) or with raised water temperatures over acute (≤96 hr) time periods (Camp and Buchwalter 2016, Van den Brink et al. 2016, Macaulay et al. 2019). However, the combined effects of all three stressors (imidacloprid, food limitation and raised temperature) are yet to be studied, especially over chronic exposure periods (>2 weeks).

Besides the realisation that we have to improve our understanding of how heatwaves and food limitation interact with toxicants, there is a growing awareness of the need to understand the potential for delayed interactive effects of these stressors and how the timing of these periods can affect stressor interactions (Arambourou and Stoks 2015, Dinh et al. 2016, Janssens et al. 2017). Studies involving multiple stressors typically expose organisms
to all stressors simultaneously, but the order of exposure can change the outcome of stressor interactions (Segner 2011). Moreover, exposure to stressors such as resource limitation and heat stress in one stage of an organism’s life history have been shown to have delayed effects in later life-stages (Janssens and Stoks 2013, Janssens et al. 2014, Janssens et al. 2017). For example, Arambourou and Stoks (2015) investigated whether a heatwave prior to insecticide (chlorpyrifos) exposure influenced its toxicity to larvae of the damselfly *Ischnura elegans*. The authors observed immediate positive sublethal effects of the heatwave, but these were followed by a delayed negative synergistic effect with chlorpyrifos exposure. Similarly, Dinh et al. (2016) found that chlorpyrifos exposure to larvae of another damselfly only caused considerable mortality in larvae that had previously been exposed to the combination of a prior starvation period and simulated heatwave. These studies highlight the need to consider the order of exposure to these common stressors in freshwater ecosystems to improve our understanding of the potential for them to interact in a direct or delayed manner.

In Chapter 3, I demonstrated that the neonicotinoid insecticides imidacloprid, clothianidin and thiamethoxam can interact synergistically with each other in mixtures to cause greater toxicity to *Deleatidium* spp. mayfly nymphs when combined than would be predicted based on the sum of their individual effects. Imidacloprid was particularly toxic and interacted synergistically with both clothianidin and thiamethoxam. To help address the two knowledge gaps introduced above, the present chapter focuses on the interaction of exposure to imidacloprid—the most widely used, most commonly detected, and seemingly most toxic neonicotinoid—with two natural stressors commonly faced by freshwater organisms; periods of extreme high temperatures (heatwaves) and food limitation (Heugens et al. 2001, Metcalfe and Monaghan 2001, Bednarska et al. 2013). To investigate how the interactive effects of imidacloprid (and food limitation) with heatwaves might vary depending on the order of exposure, this experiment included two successive 6-day heatwaves; one corresponding with a 6-day starvation period prior to imidacloprid exposure, and the second occurring after the starvation period, simultaneous to the first six days of imidacloprid exposure. The stressors were investigated in a full-factorial design to determine the potential for starvation periods to have delayed interactive effects and heatwaves to have direct and delayed interactive effects with chronic imidacloprid exposure to *Deleatidium* nymphs.

I tested eight hypotheses in total (Table 4.1) assuming an additive null-model for multiple-stressor interaction effects (Piggott et al. 2015d, Schäfer and Piggott 2018): three each for...
individual stressor effects (H1–H3), two-way interactions between imidacloprid and the other stressors (H4–H6), and three-way interactions between all stressors (H7–H8).

Table 4.1. Hypothesised effects of stressors and stressor interactions with rationale based on literature cited in the introduction.

<table>
<thead>
<tr>
<th>Stressors</th>
<th>Hypothesised effects</th>
<th>Supporting Reference/Rationale</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1 Heatwaves</td>
<td>Increased mortality, impaired mobility and increased mayfly moulting frequency</td>
<td>(Macaulay et al. 2019)</td>
</tr>
<tr>
<td>H2 Imidacloprid (0.4 µg/L)</td>
<td>Increased mortality, impaired mobility and reduced moulting frequency with increasing exposure time</td>
<td>(Macaulay et al. 2019), (Chapter 2)</td>
</tr>
<tr>
<td>H3 Starvation</td>
<td>Increased mortality and reduced moulting frequency with no direct effect on mayfly mobility</td>
<td>In line with observations by Hunn (2016)</td>
</tr>
<tr>
<td>H4 Prior starvation period × imidacloprid</td>
<td>Delayed, amplifying effect on sublethal and lethal effects of imidacloprid exposure</td>
<td>In line with observations by Hunn (2016)</td>
</tr>
<tr>
<td>H5 Prior heatwave × imidacloprid</td>
<td>Delayed, amplifying effect of imidacloprid on increased mortality and impairment of mayfly mobility</td>
<td>As in Dinh et al. (2016)</td>
</tr>
<tr>
<td>H6 Simultaneous heatwave × imidacloprid</td>
<td>Direct, amplifying effect of imidacloprid on increased mayfly mortality and impairment</td>
<td>Acute increases in temperature have been shown to enhance the toxicity of imidacloprid to mayfly nymphs (Camp and Buchwalter 2016, Macaulay et al. 2019)</td>
</tr>
<tr>
<td>H7 Prior starvation period × prior heatwave × imidacloprid</td>
<td>Amplifying effect on mayfly mortality and impaired mobility in a delayed manner.</td>
<td>Similar to observations of chlorpyrifos exposure to damselfly larvae (Dinh et al. 2016)</td>
</tr>
<tr>
<td>H8 Prior starvation period × simultaneous heatwave × imidacloprid</td>
<td>Both direct (due to the simultaneous heatwave) and delayed (due to the earlier starvation period) amplifying effects on mayfly mortality and impairment.</td>
<td>Combining the findings of Hunn (2016), Camp and Buchwalter (2016), Macaulay et al. (2019)</td>
</tr>
</tbody>
</table>
4.3 Methods

4.3.1 Experimental design

A static-renewal exposure experiment was performed over 42 days in the laboratory from 21 June to 2 August 2018. The experiment used nymphs of the mayfly *Deleatidium* spp. as a model organism (as in Chapters 2 & 3) in a full-factorial design with three categorical predictors: the neonicotinoid insecticide imidacloprid, a starvation period prior to imidacloprid exposure, and simulated heatwaves. The experiment was run at a baseline temperature of 12°C because previous related experiments involving Austral/Early winter generation *Deleatidium* nymphs had shown very low control mortality (Macaulay et al. 2019) and longevity in the laboratory at this temperature (Chapters 2 & 3).

There were two levels of imidacloprid: 0 and 0.4 µg/L. The latter concentration was chosen because chronic effects were observed at a similar concentration after 28 days of exposure in a prior concentration-response experiment with imidacloprid (Chapter 2). There were two starvation treatments: fed and starved for six days prior to imidacloprid exposure. The six-day starvation period was selected to match the duration of the simulated heatwaves, and a prior experiment with a 5-day starvation period had observed significant effects of starvation on nymph mortality and synergistic interactive effects with imidacloprid exposure (Hunn 2016). There were three heatwave treatments: no heatwave, a six-day heatwave prior to imidacloprid exposure (during the starvation period), and a six-day heatwave simultaneous to the first six days of imidacloprid exposure (following the starvation period). Both heatwaves were graduated in temperature change, with an increase from 12 to 16°C for the first 24 hours, followed by 96 hours at 20°C and a graduated decrease with another 24 hours at 16°C before returning to 12°C for the rest of the experiment. Imidacloprid exposure began on Day 6, coinciding with the start of the second heatwave.

The ASW (see Chapter 2) containing neonicotinoids (and control ASW with no neonicotinoids) was renewed every 6 days for 36 days. Treatments were randomly allocated to 48 aerated glass chambers (19.9 × 14.4 × 6.3 cm, volume 1.16 L) with four replicates of each treatment combination and at least 20 mayfly nymphs per chamber. Figure 4.1 shows a schematic representation of the experimental period and the timing of treatment applications.
Figure 4.1. Schematic representation of the experiment timeline showing the three heatwave treatments (heatwave prior to imidacloprid exposure, heatwave simultaneous to the first six days of imidacloprid exposure, and no heatwave), two starvation treatments (exposed to a prior starvation period, fed throughout) and two imidacloprid treatments (exposed to 0.4 µg/L from day 6 onwards, control). Imidacloprid was renewed every 6 days coinciding with the exchange of tile substrata (containing periphyton biofilm) and the recording of invertebrate responses.

4.3.2 Insecticide application, sampling and analysis

A working stock solution of 10 mg/L imidacloprid was prepared using 10 g/L PESTANAL® analytical standard grade imidacloprid (Sigma-Aldrich; Castle Hill, NSW, Australia). Each pesticide treatment was prepared by dosing 2 L ASW with the required amount of each 10 mg/L working stock solution, mixed using a magnetic stir plate and distributed between 4 x 500 mL replicates of each treatment concentration. From the glass chambers, 2-mL water samples were collected from the same two replicates of each treatment combination at the start, during the middle and at the end of each six-day period and stored in 4-mL glass vials with Teflon caps in the dark at -20°C until shipping to the analytical laboratory with ice packs (where they were again stored at -20°C until analysis). Imidacloprid was quantified using liquid chromatography tandem mass spectrometry (LC-MS/MS) according to the same methods as described in Chapter 2 (see Section 2.3.3 and Appendix A, Tables A1−A3).

4.3.3 Test specimen collection, acclimation conditions and reading invertebrate responses

Deleatidium specimens were collected three days prior to the experiment (to allow time for the 48-hour acclimation period pre-manipulation), employing the same method as described for the previous concentration-response experiments (Chapter 2). All transport, acclimation and experimental conditions (including feeding, tile exchange, pesticide-medium renewal and the coinciding readings of invertebrate responses) were performed in identical procedures to those described in Chapter 2.
4.3.4 Data analysis

Separate three-way ANOVAs were performed in R (Version 3.5.3; R Core Team) to test the individual and combined effects of the three categorical predictors on Deleatidium survivorship, impairment and moulting propensity on each day when the readings of sub-lethal responses were recorded (Day 6, 12, 18, 24, 30, 36 and 42). The three categorical factors in each model were imidacloprid (0, 0.4 µg/L), starvation (fed, starved) and heatwaves (no heatwave, prior heatwave to imidacloprid exposure, simultaneous heatwave to imidacloprid exposure). An additive multiple-stressor null model was used when determining stressor interactions (Folt et al. 1999, Piggott et al. 2015d). The significance level α for all tests was $p = 0.05$. Standardised effect sizes (partial $\eta^2$ values) are presented for all results with $p < 0.1$ to allow assessing the biological relevance of results (Nakagawa and Cuthill 2007). Where significant higher-order interactions were present, the recommendation of Quinn and Keough (2002) for interpreting lower-order interactions and main effects was followed. In such situations, lower-order interactions and main effects should be interpreted only where the effect size of the higher-order interaction is smaller than the size of the corresponding lower-order interactions or main effects. If this condition is not met, the higher-order interaction overrides the lower-order interactions or main effects. $P$-values and effect sizes (partial $\eta^2$ values) for all results are provided in Table 4.2 and all results described below are significant to $p = 0.05$.

4.4 Results

4.4.1 Imidacloprid exposure concentrations

Initial (taken at the start of each 6-day exposure period) and final (taken at the end of each period) concentrations of imidacloprid were, on average, $94.7 \pm 3.6\%$ ($0.38 \pm 0.01 \mu g/L$) and $92.4 \pm 3.4\%$ ($0.37 \pm 0.01 \mu g/L$) of the intended concentrations ($0.4 \mu g/L$), respectively. The nominal concentration is therefore used in all results descriptions and interpretations.

4.4.2 Stressor main effects on mayfly survivorship and impairment

From Days 6 to 36 of the experiment, by far the strongest effects observed were the main effects of the heatwaves (Table 4.2; Figure 4.2). Apart from Day 6 (when there had only been the prior heatwave period), there were no differences between the effects of the two
heatwaves on *Deleatidium* survival and impairment. Both heatwaves consistently reduced survival and increased impairment. The strongest heatwave main effect occurred on Day 30 for *Deleatidium* impairment (Figure 4.2a).

It took 24 days of exposure to 0.4 µg/L imidacloprid to cause a significant effect on impairment or survival. On Day 30, there was a weak increase in mayfly impairment with exposure to imidacloprid. This strengthened to a moderate increase by Day 36 and, on Day 42, became equally as strong an effect as the strongest heatwave effect observed during the entire experimental period (Figure 4.2b).

On Day 42, there was a weak reduction in *Deleatidium* survival with exposure to imidacloprid which was overridden by a stronger three-way interaction (described below). On Days 36 and 42, there were weak main effects of starvation on *Deleatidium* impairment which were also overridden by stronger interactions with the other stressors (see below).
Table 4.2. Results summary (p-values and effect sizes where \( p < 0.1 \)) of three-way ANOVAs for Deleatidium mayfly survivorship, impairment and moulting propensity during the 42-day experiment. \( P \)-values are bolded where \( p < 0.05 \), and effect sizes (partial \( \eta^2 \) values; range 0–1) are in parentheses wherever \( p < 0.1 \). Effect size categories are: < 0.10 ‘trivial’, \( \geq 0.10 \) ‘small’, > 0.30 ‘medium’, > 0.50 ‘large’ (Nakagawa and Cuthill 2007). Interactions that overrode one or more main effects or lower-order interactions are underlined. Tukey HSD symbols correspond to the three heatwave treatments: N = ‘no heatwave’, P = ‘prior heatwave’ (during days 0-6, prior to imidacloprid exposure) and S = second heatwave’ (during days 6-12 and the first six days of imidacloprid exposure).

<table>
<thead>
<tr>
<th>Day</th>
<th>Response</th>
<th>Heatwaves</th>
<th>Tukey HSD</th>
<th>Imidacloprid × Starvation</th>
<th>Imidacloprid × Heatwaves</th>
<th>Imidacloprid × Starvation × Heatwaves</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>Survivorship</td>
<td>&lt;0.001 (0.4)</td>
<td>N=S&gt;P</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Impairment</td>
<td>&lt;0.001 (0.44)</td>
<td>N=S&lt;P</td>
<td>0.04 (0.12)</td>
<td>&lt;0.001 (0.35)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Moulting</td>
<td>&lt;0.001 (0.41)</td>
<td>N=S&lt;P</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Survivorship</td>
<td>0.005 (0.25)</td>
<td>N=P&gt;S</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Impairment</td>
<td>0.002 (0.30)</td>
<td>N&lt;P=S</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Moulting</td>
<td>0.004 (0.26)</td>
<td>N=S&gt;P</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>Survivorship</td>
<td>&lt;0.001 (0.42)</td>
<td>N=P&gt;S</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Impairment</td>
<td>&lt;0.001 (0.46)</td>
<td>N&lt;P=S</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Moulting</td>
<td>&lt;0.001 (0.32)</td>
<td>N=P&lt;S</td>
<td>0.03 (0.13)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>Survivorship</td>
<td>&lt;0.001 (0.55)</td>
<td>N=P&gt;S</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Impairment</td>
<td>&lt;0.001 (0.61)</td>
<td>N&lt;P=S</td>
<td>0.051 (0.10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Moulting</td>
<td></td>
<td></td>
<td>0.049 (0.11)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>Survivorship</td>
<td>&lt;0.001 (0.62)</td>
<td>N=P&gt;S</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Impairment</td>
<td>&lt;0.001 (0.68)</td>
<td>N&lt;P=S</td>
<td>0.007 (0.18)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Moulting</td>
<td></td>
<td></td>
<td>0.08 (0.08)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>Survivorship</td>
<td>&lt;0.001 (0.61)</td>
<td>N=P&gt;S</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Impairment</td>
<td>&lt;0.001 (0.59)</td>
<td>N&lt;P=S</td>
<td>&lt;0.001 (0.44)</td>
<td>0.02 (0.15)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Moulting</td>
<td>0.006 (0.26)</td>
<td>S=N&lt;P=S</td>
<td>0.07 (0.09)</td>
<td>0.02 (0.14)</td>
<td></td>
</tr>
<tr>
<td>42</td>
<td>Survivorship</td>
<td>&lt;0.001 (0.39)</td>
<td>N=P&gt;S</td>
<td>0.01 (0.16)</td>
<td>0.07 (0.09)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Impairment</td>
<td>0.003 (0.28)</td>
<td>S=N=P</td>
<td>&lt;0.001 (0.68)</td>
<td>0.005 (0.20)</td>
<td>0.02 (0.14)</td>
</tr>
<tr>
<td></td>
<td>Moulting</td>
<td>0.02 (0.21)</td>
<td>P=N&lt;S=P</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.2. Mean *Deleatidium* nymph (a) survivorship, (b) impairment and (c) moulting propensity (during the previous 6-days) throughout the experiment (error bars ± SE; n = 4). Note: Control treatment groups are the left-most bar within each plot (Fed, No heatwave and 0 µg/L imidacloprid treatment on each sampling date).
4.4.3 Interaction effects on mayfly survivorship and impairment

Several two and three-way interaction effects were observed among the stressors from Days 30 to 42. On Day 30, there was a weak starvation × heatwave interaction where starvation increased *Deleatidium* impairment only in the absence of both heatwaves (Figure 4.3a). On Day 36 this antagonistic interaction had strengthened, overriding the main effect of starvation on impairment (Figure 4.3b), with the same pattern now also occurring for survivorship (starvation reduced survival in the absence of the heatwave treatments).

Another antagonistic interaction occurred on Day 36 between imidacloprid and the heatwave treatments where exposure to imidacloprid caused a stronger increase in *Deleatidium* impairment in the absence of heatwaves (Figure 4.4a). This interaction strengthened over the final six days, becoming the strongest interaction effect observed during the experiment and overriding the heatwave main effect on impairment on Day 42 (Table 4.2; Figure 4.4b). On Day 42, this two-way interaction was overlaid by a slightly weaker three-way interaction, in which the effect of increased impairment due to imidacloprid was clearest in the absence of the heatwaves and starvation (Figure 4.5b).

On Day 42, a significant three-way interaction also occurred for *Deleatidium* survivorship. This interaction overrode the main effect of imidacloprid and the starvation × heatwave interaction effect (Table 4.2). Exposure to imidacloprid only caused a reduction in survivorship in the absence of both heatwaves and starvation (Figure 4.5a).

Figure 4.3. Starvation × heatwave interaction effects on *Deleatidium* impairment on (a) Day 30 and (b) Day 36. Error bars ± SE.
Figure 4.4. Imidacloprid × heatwave interaction effects on *Deletidium* impairment on (a) Day 36 and (b) Day 42. Error bars ± SE.

Figure 4.5. Day 42 imidacloprid × starvation × heatwave interaction effects on (a) *Deletidium* survivorship and (b) *Deletidium* impairment. Error bars ± SE.
4.4.4 Moulting propensity

4.4.4.1 Heatwave main effects
The strongest and most consistent patterns for *Deleatidium* moulting propensity were in response to the heatwave treatments over the first 18 days of the experiment. During the first six days (the heatwave and starvation period prior to imidacloprid exposure), there was significantly higher moulting in the prior heatwave treatments compared to the other two (Table 4.2; Figure 4.2). Then during the second heatwave (simultaneous to the first six days of imidacloprid exposure; Days 7–12), there was higher moulting propensity for mayflies exposed to this heatwave than those that had been exposed to the first heatwave (or none at all). The reverse pattern was then observed during Days 13–18; nymphs that had either not been exposed to a heatwave or were exposed to the first heatwave had higher moulting propensity than those exposed to the second heatwave. No effects of the heatwaves on moulting propensity were observed for the next twelve days. Then on Day 36, *Deleatidium* moulting propensity was higher in the prior heatwave than the no-heatwave treatment and on Day 42, moulting propensity was higher in the simultaneous than no-heatwave treatment.

4.4.4.2 Starvation and imidacloprid main effects and interaction effects
During the starvation period, mayfly moulting propensity was higher in the starved than in the fed treatment. Though imidacloprid exposure had not started yet, moulting propensity was also slightly higher in treatments that had imidacloprid added later on. During the second heatwave (simultaneous to the first six days of imidacloprid exposure), a complex three-way interaction for moulting occurred. In the non-heatwave treatment, moulting propensity increased with imidacloprid for fed nymphs, whereas the opposite pattern occurred for previously-starved nymphs (Figure 4.6a).

During the second six days of imidacloprid addition (Days 13–18), moulting propensity was again higher overall in the imidacloprid treatment, but this was overridden by a stronger three-way interaction. In this interaction, the opposite pattern to that observed in the non-heatwave treatment in the previous six days occurred: moulting propensity decreased with imidacloprid for nymphs that had neither been exposed to a heatwave nor starved, whereas it increased with imidacloprid for nymphs not exposed to a heatwave but starved previously (Figure 4.6b).
This pattern was again reversed during the next six days where, on Day 24, a weak imidacloprid × starvation interaction occurred that saw moulting propensity increase with imidacloprid for non-starved nymphs and decrease with imidacloprid for previously-starved nymphs (Figure 4.6c). The only other significant effect on moulting occurred on Day 36 when moulting propensity was lower overall in the starvation treatment.

Figure 4.6. Interactions for Deleatidium moulting propensity on (a) Day 12 and (b) Day 18 and (c) Day 24. Error bars ± SE.
4.5 Discussion

4.5.1 Heatwave main effects

The simulated heatwaves in my experiment (a graded increase in temperature from 12 to 20°C for 96 hours) clearly demonstrated the sensitivity of *Deleatidium* mayfly nymphs to short-term heat stress. Throughout the 42-day experiment, the heatwaves had the strongest effects on all three mayfly responses (survivorship, impairment and moulting) until the effect of imidacloprid on *Deleatidium* impairment on Day 42, which matched the strongest effect of the heatwaves, also on impairment (on Day 30). These findings support my hypotheses H1 and H2 that exposure to heatwaves and imidacloprid would negatively affect *Deleatidium* survivorship and mobility as individual stressor main effects. Both of these effects were observed in previous acute laboratory experiments investigating the individual and combined effects of increased temperature and exposure to imidacloprid on *Deleatidium* and *Coloburiscus humeralis* nymphs (Macaulay et al. 2019). In this earlier study, survival of *Deleatidium* nymphs after 96 hours was strongly reduced as temperatures increased from 9 to 24°C. A non-linear effect on survival was observed, where *Deleatidium* survivorship decreased most strongly at the highest temperature, reducing from ~75% at 21 °C to ~10% at 24°C (in the absence of imidacloprid). The heatwave effects observed in the present experiment are consistent with these earlier findings, with *Deleatidium* survivorship being reduced by at least 25% after both 6-day heatwaves.

Two previous studies had also investigated the thermal tolerance of *Deleatidium* spp. nymphs, but without any pesticide addition (Quinn et al. 1994, Cox and Rutherford 2000a). Of the twelve invertebrate taxa tested by Quinn et al. (1994), *Deleatidium* were the most sensitive to 96-hour increases in temperature, with a median lethal temperature of 22.6 ± 0.8°C. The negative impact of increased temperatures on *Deleatidium* spp. in real stream ecosystems can also be inferred from the findings of Quinn and Hickey (1990), who observed a lower abundance of Ephemeroptera in rivers where summer temperatures exceeded 21°C.

The increase in *Deleatidium* moulting propensity in response to the first heatwave also supports my first hypothesis. Further, this response parallels previous observations of increased moulting by *Deleatidium* nymphs at higher temperatures (Macaulay et al. 2019) and observations of increased moulting frequency by the North American mayfly *Cloeon dipterum* when reared at higher temperatures (Camp et al. 2014). In the acute (96-hour)
experiment by Macaulay et al. (2019), *Deleatidium* moulting frequency increased as temperature increased from 9–21°C in the absence of imidacloprid, whereas the opposite response occurred in the presence of 8 µg/L imidacloprid. The considerably lower concentration of imidacloprid applied in the present, chronic experiment (0.4 µg/L) meant this interaction was not observed during the second heatwave (simultaneous to the first six days of imidacloprid exposure). During this period, moulting propensity in the simultaneous and no-heatwave treatments was higher than in the prior heatwave treatment. Therefore, during the second heatwave, my prediction of increased moulting in response to increased temperature was not supported. However, the significantly lower moulting propensity in the prior heatwave treatment during the second heatwave period may simply reflect a temporal dynamic in mayfly nymph moulting activity. That is, nymphs that had already moulted during the previous six-day period did not need to moult again during the following six-day period and therefore exhibited lower moulting propensity, and vice versa. This would also explain the significant heatwave effect on moulting propensity during Days 13–18 (when all treatments were again at 12°C), which was the reverse of the pattern observed during the previous six days.

The other significant main effects of the heatwaves on moulting propensity occurred on Days 36 and 42 where there were fewer moults in the non-heatwave treatment than in the prior (Day 36) or simultaneous (Day 42) heatwave treatments. This result is surprising, given both heatwaves had ended by Day 12 and there were no effects of the heatwaves on moulting propensity between Days 18–30. Perhaps the strong increase in mayfly impairment with exposure to imidacloprid in the no-heatwave treatment contributed to the overall reduction in moulting propensity in this treatment during the final twelve days of the experiment (as impaired mayflies exhibited reduced moulting; Fig 2.). However, as I also mention below when addressing the weak main effects of imidacloprid on moulting propensity, many of the relatively weak moulting patterns significant at $p < 0.05$ may not represent true responses to the stressors but instead reflect temporal variations in moulting propensity. For this reason, I only discuss the main effects on moulting propensity (none of the complex interaction effects for moulting propensity are therefore discussed).

### 4.5.2 Imidacloprid main effects

Supporting H2, imidacloprid negatively affected *Deleatidium* survivorship and mobility as an individual stressor main effect from Day 30 onwards. The effects of chronic exposure to
0.4 µg/L imidacloprid were initially somewhat weaker than would have been expected given the L/EC50s calculated in the 28-day concentration-response experiment with imidacloprid described in Chapter 2. The achieved imidacloprid concentration in the present experiment was equivalent to the 21-day LC50 for Deleatidium from Chapter 2 (0.38 µg/L) and to the 14-day EC50 (50% impairment; 0.4 µg/L). Given this, one would have expected imidacloprid to affect impairment by 14 days of exposure, but it took 24 days before a significant effect of imidacloprid on impairment was observed (though a trend of increased impairment with imidacloprid was visible on Day 24 after 18 days of imidacloprid exposure; Table 4.2). However, closer inspection of the data from Chapter 2 reveals that the effects of imidacloprid observed in both experiments are quite consistent. This is because, in the concentration-response experiment, there was a considerable jump in reduced mayfly survival and increased impairment between the 0.4 and 0.8 µg/L treatments. At 0.4 µg/L, survivorship was still 75% and impairment 50% after 28 days of exposure (Chapter 2, Figures 2.2 and 2.4). These numbers are consistent with the effects of exposure to 0.4 µg/L imidacloprid observed in the present experiment, where impairment with imidacloprid in the non-heatwave treatments was above 50% on Day 36 (after 30 days of imidacloprid exposure). Over the final six days, this then became the equal strongest effect observed in the entire experiment, causing ~100% impairment by 36 days of imidacloprid exposure. Importantly, it was the long-term nature of the experiment that allowed detecting the strong delayed effects of imidacloprid that would have been missed had the experiment been 1–2 weeks shorter. Exposures of such duration (>28 days) are still rare in ecotoxicology (Sánchez-Bayo and Tennekes 2015, Sánchez-Bayo et al. 2016, Hladik et al. 2018b).

The importance of evaluating sublethal responses such as impaired mobility of test organisms in ecotoxicological experiments (Desneux et al. 2007, Brooks et al. 2009, Roessink et al. 2013) is also highlighted by the strength of the imidacloprid effects on mayfly nymph impairment compared to survivorship. Had the lethality of imidacloprid alone been evaluated, its chronic effects would have been considerably underestimated. Mobility is very important for macroinvertebrates living in the stream benthos, especially for mayfly taxa which actively drift to find more suitable microhabitats or avoid predation (Brittain and Eikeland 1988, Beketov and Liess 2008, Hammock et al. 2012). Impairment of their swimming ability, as was the assessment of impairment in the present experiment (righting ability by performing a normal, swimming motion), would dramatically reduce their ability to drift in a controlled manner, rendering them more susceptible to predation by fish (Brooks
et al. 2009). Therefore, even if concentrations sufficient to cause mortality are not encountered in the environment, lower concentrations of imidacloprid (or other contaminants) causing impairment would still be functionally effective in terms of the viability of aquatic insect populations. Other sublethal impairments caused by neonicotinoids which are likely to negatively impact survival or reproduction include feeding (Alexander et al. 2007, Pestana et al. 2009b, Loureiro et al. 2010, Nyman et al. 2013), growth (Pestana et al. 2009b, Bartlett et al. 2018, Naveen et al. 2018) and developmental processes such as moulting and emergence (Song et al. 1997, Cavallaro et al. 2017, Finnegon et al. 2017, Saraiva et al. 2017, Maloney et al. 2018a, Naveen et al. 2018, Raby et al. 2018b).

Sánchez-Bayo et al. (2016) conducted a meta-analysis of neonicotinoid concentrations in surface waters from 11 countries and found imidacloprid to have an average concentration of 0.73 µg/L (geometric mean calculated using data from 33 surveys). The findings of the present experiment imply that, if such average levels were present in streams containing *Deleatidium* spp. or other aquatic insects with similar sensitivity to imidacloprid, there could be strong impacts on the stream macroinvertebrate community. Other mayfly taxa and dipterans of the genus *Chironomus* have been found to have similar chronic sensitivities to the neonicotinoids imidacloprid, clothianidin and thiamethoxam (Chapter 2, Table 2.3). Mayflies and chironomids are vital components of stream food webs as primary consumers and food sources for higher-trophic level consumers. They also provide an important link to terrestrial food webs through metamorphosis into their adult life-stage (Benton et al. 2002, Cavallaro et al. 2018). Therefore, neonicotinoids (especially imidacloprid), have the potential to not only impact higher-trophic levels in aquatic food networks (Hayasaka et al. 2012a, Hayasaka et al. 2012b, Bruder et al. 2019), but to span both terrestrial and aquatic ecosystems (Chagnon et al. 2015) – a phenomenon for which some evidence from the Netherlands already exists (Goulson 2014, Hallmann et al. 2014).

In contrast with H2, the only observed effect of imidacloprid on moulting propensity of *Deleatidium* nymphs was a weak positive response on Day 18. However, given there was also higher moulting during the first 6-day period when no imidacloprid was present, this response may only represent random or temporal patterns rather than actual responses to the stressors (see related discussion on heat wave effects on moulting above). Consequently, moulting frequency may not be a very informative response metric for detecting imidacloprid effects on *Deleatidium*. 
4.5.3 Starvation main effects

Rejecting H3, mayflies starved during the first six days of the experiment did not suffer increased mortality or reduced moulting as a direct result of starvation. Rather, there was a moderate increase in moulting propensity during the starvation period. This increase contrasted with the predicted effect that periods of nutritional deficit are often associated with reduced metabolism, immune function, growth and development (Metcalfe and Monaghan 2001, De Block and Stoks 2008, Storey 2015). A previous experiment with Deleatidium spp. nymphs involving a 5-day starvation period had resulted in increased mortality of starved nymphs whereas no effect of starvation on mayfly moulting frequency was observed (Hunn 2016). In other experiments investigating the effects of starvation on a variety of European damselfly larvae, developmental rate of Enallagma cyathigerum (Janssens and Stoks 2013), immune function of Lestes viridus (De Block and Stoks 2008) and growth rate, energy storage and immune function of Coenagrion puella (Dinh et al. 2016) were all reduced by food limitation. The short-term increase in moulting propensity under starvation observed in my experiment therefore diverges from these findings. It is possible, however, that increased moulting under starvation could be explained as a stress response to nutritional limitation. Research into the development of Drosophila melanogaster has shown that individuals that have attained a critical weight can respond to starvation by increasing moulting frequency in order to reach metamorphosis faster than if they are able to continue feeding (Beadle et al. 1938, Mirth et al. 2005, Stieper et al. 2008). In this way, starvation-triggered moulting could be an adaptive response to food limitation to accelerate development to metamorphosis (Callier and Nijhout 2013), though further investigation of this response is required for D. melanogaster and it is yet undemonstrated for Deleatidium mayflies.

4.5.4 Interaction effects and delayed effects of starvation and imidacloriprid

In contrast to the hypothesised synergisms between the three stressors (H4–H8), all significant interaction effects on Deleatidium mayfly nymph survivorship and impairment were antagonistic in nature. This result is probably mainly due to the strong heatwave effects and the additive multiple-stressor model used when determining stressor interactions because additive models do not allow detecting synergisms when the sum of individual stressor effects exceeds 100% (Folt et al. 1999, Lange et al. 2018, Schäfer and Piggott 2018). In other words, the main effects of the heatwaves on survivorship and impairment were
already so severe on their own that delayed detrimental effects of starvation and imidacloprid exposure could only be detected in the absence of the heatwaves. For example, prior starvation resulted in increased impairment in the no-heatwave treatment on Days 30 and 36, and on reduced survivorship in the same treatment on Day 36. In a related 14-day experiment (Hunn 2016), a 5-day starvation period increased mortality of *Deleatidium* nymphs nine days after the starvation period. Moreover, prior starvation synergistically amplified imidacloprid-induced increases in mayfly impairment and mortality.

The lack of such synergisms in my experiment may be a result of using a low concentration of imidacloprid (0.4 µg/L) to observe chronic effects over ≥ 4 weeks exposure, whereas the imidacloprid treatments used by Hunn (2016) were two to five times as high (0.9 and 2.1 µg/L). Moreover, Hunn (2016) used a different definition of impairment (which did not include mortality, as in my experiment), therefore impairment values were often clearly below 100%, and this may have made it easier to detect synergisms (Folt et al. 1999, Lange et al. 2018). The antagonistic interactions in my experiment are also likely due to the timing of exposure to the heatwaves relative to imidacloprid exposure. Future experimentation could manipulate a heatwave after several weeks of *Deleatidium* nymph exposure to imidacloprid (when sublethal effects of the insecticide are evident), rather than at the beginning of the exposure period, to test the findings of Camp and Buchwalter (2016) and Macaulay et al. (2019) for increased temperature to directly enhance the toxicity of chronic, low-level exposure to imidacloprid.

Immediate and delayed negative effects of starvation in combination with exposure to the organophosphate insecticide chlorpyrifos have also been found in studies involving several species of European damselfly larvae. The above-mentioned study by Dinh et al. (2016) observed a mixture of immediate and delayed negative sublethal effects of starvation on *Coenagrion puella* growth rate and physiology. In their experiment testing the interactive effects of a prior starvation period and a heatwave with chlorpyrifos exposure (3 µg/L), the authors also observed delayed synergistic three-way interactions on damselfly mortality. Chlorpyrifos only caused considerable mortality in larvae that had previously been exposed to the combination of a heatwave and starvation. This delayed synergism for mortality differs from the delayed three-way antagonistic interactions in my experiment where imidacloprid only caused an effect on mayfly impairment and survival in the absence of heatwaves and starvation. Whereas the delayed synergism in Dinh et al. (2016) may be explained by cumulative metabolic depression caused by the three stressors, the delayed antagonism I
observed can be explained by the severe effects of the heatwaves prior to the onset of delayed imidacloprid and starvation effects (see above).

In two separate experiments focusing on a range of responses in the damselfly *E. cyathigerum* (see also earlier in discussion), Janssens and Stoks (2013) also observed synergistic interactions between chlorpyrifos (1 µg/L) and food level (high and low), and between chlorpyrifos and temperature (18 and 24 °C). Both larval development time and mass at emergence were only negatively affected by chlorpyrifos at the low food level. Likewise, increased temperature and chlorpyrifos exposure (2 µg/L) had no effect on damselfly mortality alone, but chlorpyrifos reduced survival by more than 50 % at the higher temperature. In a related study using *Ischnura elegans* damselflies, Janssens et al. (2014) tested the combined effects of larval food stress, chlorpyrifos exposure and adult heat stress exposure and found that delayed effects of larval chlorpyrifos exposure on damselfly physiology depended on subsequent adult heat exposure, thus interacting across metamorphosis. While I observed some adult emergence in the heatwave treatments in my experiment using the winter generation of *Deleatidium* nymphs, this was too rare to consider as a response. However, future experiments with *Deleatidium* nymphs in spring or summer could consider the potential for interactive effects of pesticides and natural stressors to bridge metamorphosis, where larval exposure causes delayed effects observed in adults (i.e. as in Janssens and Stoks 2013).

### 4.5.5 Conclusions and implications for risk assessment

My study adds further evidence that the interactive effects of pesticides with natural stressors such as food limitation and temperature can vary considerably depending on the chemical, the study species, and the order and timing of exposure. For example, in stark contrast to the drastic detrimental impact of heatwaves on *Deleatidium* nymphs in my experiment, heatwaves have also been shown to increase performance of damselfly larvae (e.g. in an experiment with chlorpyrifos exposure Arambourou and Stoks 2015). While Van Dievel et al. (2017) found that positive effects of heatwave exposure only occurred when sufficient nutrition was available to allow an increase in food intake higher than increased metabolic rate under the heatwave.

This particular combination of stressors (contaminants, temperature and food availability) has long been of interest to ecotoxicologists. The ability of nutritional state to alter the
toxicity of contaminants to aquatic organisms was demonstrated by Cooney et al. (1983), who studied effects of temperature and nutritional state on the toxicity of acridine to the copepod *Diaptomus clavipes*. Yet, to this day the challenge remains to more thoroughly understand their interactive effects, and indeed the interactive effects of contaminants with other natural or anthropogenic stressors (Holmstrup et al. 2010), particularly in the context of rapid global warming and the increasing prevalence of extreme temperature events with climate change (Noyes et al. 2009, Hooper et al. 2013, Noyes and Lema 2015). Considering the effects of natural stressors such as temperature and nutritional state in more ecotoxicological experiments will help develop predictive frameworks of their interactions and improve our understanding of how exposure to contaminants might affect organisms facing these conditions in real life (Bednarska et al. 2013, Gessner and Tlili 2016). As ecologists and ecotoxicologists continue to integrate principles and methods to develop a more thorough understanding of these multiple stressor effects and the mechanisms driving them, our ability to select null models on mechanistic bases will also improve—a vital step in moving toward the ultimate goal of predicting and explaining multiple stressor effects (Schäfer and Piggott 2018).

The key finding from my multiple-stressor experiment, that chronic (36 days) exposure to low, environmentally relevant concentrations of imidacloprid eventually caused equally as strong detrimental effects as the strong negative effects of heatwaves on *Deleatidium* nymphs, also highlights the need for further similar long-term experiments, because even 21–28-day long experiments would have missed the delayed effects of pesticide exposure. Moreover, while heatwaves of increasing severity will clearly pose a major threat to freshwater biodiversity, my findings show that we cannot ignore the additional impacts of pesticide use on freshwater ecosystems in the face of global climate change.
Chapter 5

Climate warming and imidacloprid pulses determine stream macroinvertebrate community dynamics
5 Climate warming and imidacloprid pulses determine stream macroinvertebrate community dynamics

5.1 Summary

Water abstraction for irrigation and pesticide application are commonly associated with intensive agriculture and are known to negatively affect freshwater ecosystems. Pesticide contamination of streams that have already experienced reduced flows may affect these stream communities differently to those from unimpacted streams. In the context of a changing climate, it is also important to understand how exposure to emerging contaminants such as new generation insecticides will affect stream communities under future scenarios of increased global temperatures. In a seven-week experiment in 128 streamside mesocosms, I investigated the effects of exposure to 48-hour pulses of the neonicotinoid insecticide imidacloprid (at three environmentally relevant levels; 0.1, 0.48 and 4.6 μg/L, plus controls) and water temperature (ambient and 3°C above) on stream macroinvertebrate communities from fast-flowing and slow-flowing microhabitats. Invertebrate drift and insect emergence were monitored during three pesticide pulses (ten days apart) and during the 48 hours immediately following the first two pulses. This repeated sampling allowed observing shifts in community composition in response to the manipulated and natural stressors (an intense 10-day heatwave occurred during the manipulative period). Benthic invertebrate communities in the entire mesocosms were sampled after 24 days of heating and pesticide manipulations.

All three stressors significantly affected benthic invertebrate community composition, with the largest effects overall from increased water temperature and reduced flow velocity. Imidacloprid pulses and increased temperature strongly affected drift community composition, especially in fast-flow communities. The strongest taxon-specific effects of increased temperature and imidacloprid addition (both negative) occurred for Deleatidium spp. mayflies, while Chydoridae (Cladocera) responded positively to imidacloprid addition, and Potamopyrgus antipodarum to increased temperature and reduced flow. Further, the combined effect of stressor manipulations and the natural heatwave drastically reduced relative abundances of EPT (mayfly, stonefly and caddisfly) and insect overall and caused a shift to oligochaete, crustacean and gastropod-dominated communities. My findings
demonstrate the potential impacts of heatwaves on freshwater ecosystems under future climate scenarios and reveal which invertebrate taxa will be most at risk from the combined effects of water abstraction, insecticide contamination and increased temperatures.

5.2 Introduction

Mesocosm experiments are a valuable tool for assessing the impacts of contaminants in realistic experimental systems, especially in combination with other stressors that are not easily manipulated *in situ* (Stewart et al. 2013). They allow replication of a model ecosystem in a controlled, semi-field environment that still achieves environmentally realistic physical and biotic conditions by simulating natural densities of ecosystem biota. In environmental risk assessment, they provide an opportunity to perform ecosystem-level research by manipulating toxic substances in a realistic yet controlled environment that can complement the even more highly controlled, but much less realistic, laboratory bioassays (Pestana et al. 2009a, Sánchez-Bayo et al. 2016). With the relative ease of manipulating additional factors compared to field experiments and including species interactions that are ignored in laboratory tests, mesocosms represent an ideal method for integrating ecological principles into ecotoxicological research and environmental risk assessment (Gessner and Tlili 2016).

Several reviews have identified a need for more realistic assessment of neonicotinoid impacts on freshwater ecosystems using field or semi-field experiments (Anderson et al. 2015, Morrissey et al. 2015, Hladik et al. 2018b), and the need for investigating the potential for multiple stressors, whether environmental or anthropogenic in origin, to interact with neonicotinoids (Goulson 2013, Moe et al. 2013, van der Sluijs et al. 2015). In the last decade, a number of studies have performed stream or pond mesocosm experiments to investigate neonicotinoid effects on aquatic invertebrate communities (see discussion in Section 5.5.3). However, no studies have involved open mesocosm systems that allow natural immigration and emigration of stream organisms into and out of the experimental units. Moreover, interactions with additional stressors are rarely investigated (but see Alexander et al. 2016, who investigated effects of imidacloprid exposure, nutrient addition and predation pressure on stream invertebrate communities in closed mesocosms).

Average global surface temperatures are rising as a result of anthropogenic activities (IPCC 2014, 2018). Increases in global mean surface temperatures of just 2°C are predicted to
negatively impact terrestrial, freshwater and coastal ecosystems, and to increase intensity and frequency of extreme weather events including heatwaves, droughts and heavy rainfall events (IPCC 2018). Understanding how climate change will affect the vulnerability of ecosystems to chemical pollutants is a challenge for ecotoxicologists requiring more attention (Noyes et al. 2009, Hooper et al. 2013, Noyes and Lema 2015). For example, increased toxicity of chemical contaminants with increasing temperatures has been regularly demonstrated in laboratory experiments (Holmstrup et al. 2010, Laskowski et al. 2010), but the extrapolation of these findings to the community or ecosystem level remains a challenge to be addressed (Moe et al. 2013). Specifically, the most widely used neonicotinoid insecticide imidacloprid (Jeschke et al. 2011) has been shown to cause increased toxicity to mayfly larvae at higher temperatures in laboratory experiments (Camp and Buchwalter 2016, Van den Brink et al. 2016, Macaulay et al. 2019). However, it has not been tested whether such interactions are also observed in more environmentally realistic scenarios (i.e. at the community or ecosystem level) and at environmentally relevant concentrations.

In Chapters 2–4, I employed long-term (4–6 weeks) laboratory experiments to investigate the individual and combined chronic toxicities of three widely used insecticides, the neonicotinoids imidacloprid, clothianidin and thiamethoxam, to larvae of the ubiquitous New Zealand mayfly Deleatidium spp. In Chapter 4, I also investigated the potential for exposure to low concentrations of imidacloprid to interact with food limitation and heatwaves and mainly observed antagonistic interactions due to the severe effects of the heatwaves on mayfly nymph survival in the laboratory. In the present chapter, I address some of the knowledge gaps highlighted above by employing stream mesocosms to investigate the individual and combined effects of pulsed imidacloprid exposure and increased water temperature on stream macroinvertebrate communities from fast- and slow-flow habitats. This 45-day experiment (21 days of colonization at fast or slow flow, 24 days of temperature and pesticide manipulations) provides the first empirical evaluation of stream macroinvertebrate dynamics in response to the world’s most widely used agricultural insecticide imidacloprid and increased water temperature.

The following hypotheses were investigated:

1. Flow velocity conditions throughout the experiment shape benthic invertebrate community composition according to the microhabitat preferences of individual taxa, and this overarching influence of flow is reflected in different drift and
emergence patterns between fast and slow-flowing mesocosms during the manipulative period (Dewson et al. 2007, Rolls et al. 2012).

2. Macroinvertebrate communities from fast and slow-flowing habitats respond differently to insecticide and climatic stress, with fast-flowing communities being more severely affected because they contain more sensitive taxa that prefer fast-flow.

3. Pulses of imidacloprid increase insect drift during the 48 hours of imidacloprid addition, but not in the 48 hours following the pulse. The effect of the three imidacloprid pulses is reflected in the benthos by a reduction in insect abundance and richness (Morrissey et al. 2015).

4. Increased water temperature causes increased invertebrate drift and insect emergence and changes benthic invertebrate community composition according to the thermal tolerances of individual taxa (Piggott et al. 2015c).

5. Increased temperature interacts with imidacloprid exposure to further increase insects drift and emergence responses, leading to greater reductions in benthic densities of these taxa (increased temperature has been shown to enhance the toxicity of imidacloprid to freshwater insect in laboratory experiments; Camp and Buchwalter 2016, Macaulay et al. 2019; and has been shown to interact with other agricultural stressors to worsen their negative effects on stream macroinvertebrate communities; Piggott et al. 2012, Piggott et al. 2015c).

5.3 Methods

5.3.1 Study site

The experiment was conducted during Austral spring/summer from 25 October to 9 December 2017 in 128 circular stream mesocosms installed on the bank of the Kauru River, a fifth-order stream in the Otago province of New Zealand. The Kauru catchment (124 km²) lies in the rain shadow of the Southern Alps, ranges from 55 to 1273 m above sea level and receives a mean annual rainfall of 817 mm. Mean annual discharge, measured 300 m upstream of the stream channel site, is 1.29 m³/s (ORC 2003). The vegetation in the catchment upstream consists predominantly of native tussock grass and exotic pasture. Land use is mainly sheep and beef grazing at low stock densities (0.1–3 animals per hectare). The
river water is relatively nutrient-poor. Nutrient concentrations during my experiment were 16.8 ± 0.7 µg/L for nitrate (NO$_3$), 26.6 ± 1.1 µg/L for ammonia (NH$_3$), and 1.9 ± 0.08 µg/L for dissolved reactive phosphorus (DRP) (± SE, n = 128, 50 mL water samples taken from each mesocosm on Day 9 of the experiment). The river contains diverse and abundant periphyton (Lange et al. 2011, Piggott et al. 2015b) and invertebrate communities (Herrmann, 2009).

5.3.2 Experimental system and study design

The experimental stream mesocosm system (*ExStream System*) comprised a 4.1 m high, 20-m long two-level scaffold erected near the bank of the river. The upper level supported eight 135-L polythene header tanks. Adjacent to the scaffold, a 1-m high, 1.2-m wide wooden bench supported the circular stream mesocosms, each with an external diameter of 25 cm and an inner outflow ring of 6 cm (volume 3.5L; Microwave Ring Moulds, Interworld, Auckland). Dual centrifugal pumps (Onga 415, capacity 300 L/min; Onga Limited, Auckland, NZ) supplied the entire system with water from the river (243 L/min) through dual intakes placed in a fast-flowing riffle section, protected by cylindrical metal-mesh coverings (mesh size 4.5 mm) and secured close to the stream bed by a rubber cord attached to a steel stake driven into the bed.

Figure 5.1. Schematic of the Kauru experimental stream system (*ExStream System*; modified from Piggott et al. 2015b): River water is pumped to 8 header tanks where gas-fired heaters add heated water to half of the tanks (+ temp). Imidacloprid is dripped directly into the mesocosms in three concentrations (+ imidacloprid) and flow velocity is manipulated by adding an inflow jet (+) or altering the inflow angle (−) in the mesocosm (± flow velocity).
River water was transported from the pump intakes to the pumps themselves along dual 20-m long, 50-mm polythene pipes, and from there along dual 80-m long, 50-mm pipes to a central manifold connected to eight 25-mm polythene pipes which fed the stream water to the eight header tanks (each controlled by a ballcock valve). Four additional 25-mm polythene pipes passed through separate inline filters (0.5 mm), three to individually supply the three pesticide lines (see below), and one which passed through a second inline filter to supply four gas-fired water heaters (model VT26; Rinnai, Germany) mounted parallel to the lower scaffold level. Each heater had an outflow pipe connecting to regulating inflow taps on one of the four heated header tanks. Each header tank gravity-fed stream water to its block of mesocosms at a constant flow rate of 1.9 L/min per mesocosm (calibrated daily), via a further 4 m of 13-mm polythene piping controlled by a tap regulator (see Figure 5.1 for a schematic diagram of the experimental system).

Each mesocosm contained 500 mL of small to medium-sized gravels collected from the river floodplain and sieved to remove particles < 2 mm, with 12 randomly selected 40–50 mm flat stones placed on top. This mixture simulated the natural substratum composition of small New Zealand farmland streams (low intensity sheep/beef farming in the Otago region; Matthaei et al. 2006).

I manipulated flow velocity at two levels (fast and slow), water temperature at two levels (ambient and heated) and imidacloprid at four levels (0, 0.1, 0.48 and 4.61 µg/L; actual concentrations), in a full-factorial design with eight replicates of each treatment combination. To allow investigating the effect of raised temperature and imidacloprid exposure on stream communities from fast and slow-flowing microhabitats, flow velocity was already manipulated during the colonisation period, beginning 21 days prior to the start of the heating and imidacloprid-pulse manipulations (beginning on Day 0). Flow velocity was manipulated by installing an inflow jet to half of the channels to increase flow velocity (fast-flow treatment) and altering the inflow angle to the other half of the channels (with no jet) to reduce velocity (slow-flow treatment). This resulted in fast- and slow-flow velocities of 20.5 ± 0.2 cm/s and 1.4 ± 0.1 cm/s, respectively (means ± SD measured in 64 channels across 4 dates, see below) which represent realistic values for pool or run habitats in small agricultural streams (see e.g. Matthaei et al. 2006, Lange et al. 2014). Water inflow into each mesocosm was calibrated daily and flow velocity was measured on four occasions during the colonisation (and flow-manipulation) phase of the experiment (days -17, -13, -9, -5) using a hand-held flow meter (MiniWater20; Schiltknecht, Industriestrasse, Goussau,
Switzerland). Temperature treatments were assigned randomly at the header-tank level within four spatial blocks (each consisting of two header tanks with 16 mesocosms each).

Raised water temperature began on Day 0 and was applied continuously for 24 days, with an average heating treatment of 3.0 ± 0.86°C (SD) above ambient (measured at 5-minute intervals from Days 0–24 using HOBO® Pendant Temperature/Light Data Loggers; Onset Computer Corporation, Bourne, MA, U.S.A). This corresponds to the projected increase in mean temperatures for New Zealand by 2090 (2081–2100, relative to 1986–2005) with climate-change under the high emissions scenario (Representative concentration pathway 8.5 IPCC 2014, Ministry for the Environment 2016). Water temperatures in the Kauru River where the system’s intakes were placed were, on average, 0.2 ± 0.007°C cooler than ambient stream channels).

Imidacloprid, applied in the commercial formulation CONFIDOR® (Confidor SC 350; Bayer CropScience Bayer New Zealand Limited, New Zealand), was supplied to three quarters of the channels in solution in three 48-hour pulses (on Days 0–2, 10–12 and 20–22) by individually attached pressure compensating drippers (model RXLD2SC; RX Plastics, New Zealand) at a rate of 2 L/hour from three 13-mm pipes. The pipe ran the length of the wooden bench beside the mesocosms and was connected to three separate supply lines fed filtered stream water from the central manifold. Three concentrated imidacloprid solutions were continuously injected into each pipe by three fluid-metering pumps during each pesticide pulse (FMI CERAMPUMP® Lab Pump Model QBG, Fluid Metering Inc., Syosset, NY, USA) from three adjacent 240-L supply barrels. Stock solutions were prepared prior to each pulse by dosing 150 L of filtered stream water with the required amount of CONFIDOR® (350 g/L) to achieve the target stock concentration. The nominal concentrations of imidacloprid used (0.1, 0.5 and 5 µg/L) are within the ranges of concentrations detected in stream surveys internationally (Morrissey et al. 2015, Sánchez-Bayo et al. 2016). The three-pulse series was designed to simulate realistic exposure scenarios of pesticide runoff during heavy rain events which cause the highest concentrations of contaminant (and neonicotinoid) input into streams and surface waters (Chiovarou and Siewicki 2008, Hladik et al. 2014, Morrissey et al. 2015). Three periods of heavy rainfall within a 24-day period is a realistic scenario in New Zealand’s oceanic climate, which is often highly changeable and can have regular heavy rain events in spring and early summer. Several previous studies have also investigated the effects of an imidacloprid pulse-series of

Water samples were taken during and after each pesticide pulse to quantify imidacloprid concentrations and confirm the pulsed-exposure regime. Fifty-millilitre filtered samples were taken from half the channels on each sampling occasion and stored in 50 mL Falcon tubes in black plastic bags and refrigerated until transport back to the laboratory (usually on the same day). Samples were kept frozen in the dark at -20°C until shipping to the analytical laboratory (one-day travel time, with ice packs) where they were again stored at -20 ºC until analysis. Imidacloprid was quantified using liquid chromatography tandem mass spectrometry (LC-MS/MS) according to the same methodology as described in Chapter 2 (see Section 2.3.3 and Appendix A, Tables A1–A3).

5.3.3 Colonisation period

Flow began on 25 October 2017 (Day -21), with three weeks of invertebrate colonisation of fast- and slow-flow mesocosms before the start of the heating and pesticide-pulse manipulations on Day 0. Natural colonisation of the mesocosms in the system by invertebrates and algae drifting into the channels has been shown to be highly effective in past experiments. For example, Wagenhoff et al. (2012) observed approximately 1000 invertebrates per 100 m³ drifting into each mesocosm in 49 hours. On 11 November (Day -2), natural colonisation was augmented by adding one standard load of invertebrates to each mesocosm, to supplement the community with taxa naturally underrepresented in the drift. This standard load was a randomly selected eighth of a kick-net sample (3 min duration; frame 60 × 40 cm; mesh size 200 µm), which had been collected from the adjacent river in fast (riffle) or slow (run/pool) bed patches of 0.36 m² each and divided using an automated subsampler. Kick-net samples were collected sequentially from downstream to upstream, with sample eighths from riffle kick samples randomly assigned to fast-flowing mesocosms and sample eights from slow-flowing run/pool kick samples to slow-flowing mesocosms.

5.3.4 Macroinvertebrate and insect emergence sampling

The experimental stream system (ExStream System) is an open mesocosm system which has stream water constantly flowing into and out of the stream channels. This is advantageous in ensuring realistic stream conditions with the same light regime, water temperature and
chemistry as in the source river (Wagenhoff et al. 2012, Wagenhoff et al. 2013). In addition, this permits continuous, natural immigration and emigration of stream organisms (drifting invertebrates, algae and microbes) which can be sampled throughout the experiment. Drifting invertebrates and emerging insects were caught in drift nets (nylon, mesh size 250 µm) and emergence hoods (polyester, mesh size 50 µm) fitted to the channels during the 48-hour pesticide pulses and the 48 hours immediately following the first two pesticide pulses. With nets fitted, all water leaving the mesocosms (approx. 1.5 min residence time) through the inner circular outflow passed through the nylon net, trapping all drifting material and biota > 250 µm. At the end of each monitoring period, drift nets and emergence hoods containing invertebrates were removed and preserved in 70% ethanol for transport to the laboratory. On the final day of heating manipulation (Day 24), flow to each mesocosm was stopped and benthic invertebrate communities in each mesocosm were sampled by elutriating all substratum using a 250-µm sieve and filtered stream water. Benthic invertebrates were preserved in 90% ethanol in the field. In the laboratory, drifting invertebrates and emerged insects were removed from drift nets and emergence hoods and retrieved in a 250-µm mesh sieve. Benthic invertebrates were divided into ¼ and ¾ subsamples using an automated subsampler. All samples were stained with Rose Bengal and stored in 90% ethanol prior to identification.

Invertebrates were identified to the lowest practicable taxonomic level (typically to genus or, where early instars were common, to family; Winterbourn et al. 2006). All invertebrates and adult insects in the drift and emergence samples and ¼ benthic samples were identified and counted and body length was measured to the nearest 1 mm (maximum length without cerci and with cases removed when present) using a dissecting microscope (Olympus SZ51, 8-409, Tokyo, Japan). Body lengths were not measured for Oligochaeta and Nematoda, which tend to fragment, and for the common taxa Cladocera and Copepoda, which were only counted. The remaining ¾ benthic samples were scanned for the presence of rare taxa which were included in all benthic taxon richness measures.

5.3.5 Invertebrate responses

Seventeen invertebrate drift responses were calculated separately for each period when drift was monitored (the 48-hour periods ending on Days 2, 4, 12, 14 and 22): (i) total number of invertebrates, (ii) invertebrate taxon richness, (iii) total number of EPT (larval mayflies, stoneflies and caddisflies) present, (iv) EPT taxon richness, (v) drift community composition
based on the 12 most common drifting taxa on each date, and (vi–xvii) the individual abundances of these common taxa (representing, on average, 98.9% of drifting invertebrates; Table 5.1).

Six insect emergence responses were calculated: (i) total number of emergers, (ii) emergence taxon richness, (iii) mean emerged insect body size (mm), (iv) mean emerged Chironomidae body size (the dominant taxon), (v) abundance of small emergers (<5 mm) and (vi) abundance of large emergers (>5 mm).

Twenty-four benthic invertebrate responses variables were calculated: the same first four community-level responses as for invertebrate drift, plus: (v) Simpson's diversity index, (vi) mean invertebrate body size (mm; excluding the taxa not measured, see above), (vii) mean larval Chironomidae body size, (viii) mean larval *Deleatidium* body size, (ix) abundance of small invertebrates (<1 mm), (x) abundance of medium invertebrates (1–5 mm), (xi) abundance of large invertebrates (>5 mm), (xii) benthic invertebrate community composition based on the 12 most common taxa and (xiii–xxiv) the individual abundances of these taxa (representing 99.2% of all individuals; Table 5.2).

### 5.3.6 Data Analysis

General linear models (GLMs) were performed in R (Version 3.5.3; R Core Team). Temperature and flow velocity were fixed categorical predictors, while imidacloprid was a continuous predictor variable with four levels. Variance inflation factors were calculated to test for collinearity problems that might arise from inclusion of the two and three-way interaction terms of temperature and flow velocity with the continuous predictor, imidacloprid, in the same model. No collinearity problems were detected, therefore imidacloprid was not centred. A block factor with four levels was included to account for background variation occurring between the four temperature blocks (containing sixteen heated and sixteen non-heated mesocosms per block). The resulting model was intercept (d.f. 1) + temperature (1) + imidacloprid (1) + flow velocity (1) + block (3) + temperature × imidacloprid (1) + temperature × flow velocity (1) + imidacloprid × flow velocity (1) + temperature × imidacloprid × flow velocity (1) + error (d.f. = 117; n = 128). Invertebrate drift and emergence response variables on Day 12 had a reduced total sample size of n = 127 (error d.f. 116) due to one missing sample.
To assess effects on the drifting and benthic invertebrate communities using the 12 most common taxa, I performed GLMMs (with the multivariate equivalent of the model above) and also examined the between-subjects effects for each individual taxon. Significance level $\alpha$ for all tests was 0.05. Standardised effect sizes (partial $\eta^2$ values, range 0–1; Garson 2012) are presented for all findings with $p < 0.10$ to allow readers to evaluate the biological importance of results (Nakagawa and Cuthill 2007). Non-metric multidimensional scaling (NMDS) analyses based on Bray-Curtis dissimilarity distances (using the same 12 most common taxa as the GLMMs for the drifting and benthic communities) were also plotted as a way to graphically illustrate differences in invertebrate community composition between the three predictors.

Significant main effects were coded (+ or −) based on the response direction of manipulated vs. control (ambient) levels. Where significant interactions of the categorical predictor variables flow velocity and temperature occurred, these were classified as antagonistic (A) or synergistic (S) according to a directional classification system by comparison of the individual and combined stressor effects with the control treatment (ambient conditions) level (Piggott et al. 2015d). Significant interactions involving the continuous predictor imidacloprid were coded directionally in a similar way as the temperature × flow velocity interaction (+ or − effect of imidacloprid) using the slope of the GLM regression and classified as antagonistic (<) or synergistic (>) not in absolute terms, but according to the change in strength or direction of the imidacloprid effect in the presence of the second stressor; i.e. stronger (>), weaker (<) or opposing (±) positive or negative effect of imidacloprid when combined with the second stressor. A significant three-way interaction implies that the strength (and presence) or direction of an imidacloprid effect changed in the presence or absence of (is dependent on) flow velocity and temperature.

Where a significant interaction was present, the recommendation of Quinn and Keough (2002) for interpreting lower-order interactions and main effects was followed. In such situations, lower-order interactions and main effects should be interpreted only where the effect size of the higher-order interaction is smaller than the size of the lower-order interactions or main effects. If this condition is not met, the higher-order interaction overrides the lower-order interactions or main effects.
5.4 Results

5.4.1 Imidacloprid exposure concentrations

Achieved average concentrations of imidacloprid sampled during each of the three pesticide pulses were $0.102 \pm 0.005 \mu g/L$, $0.48 \pm 0.02 \mu g/L$ and $4.61 \pm 0.2 \mu g/L$, corresponding to $102 \pm 4.9\%$, $95.4 \pm 4.8\%$ and $92.2 \pm 4.3\%$ of the nominal concentrations, respectively. For accuracy, the average achieved concentrations are used in all analyses, results descriptions and interpretations.

5.4.2 Temporal community shifts in the drift

The drifting invertebrate community changed considerably over the 24 days of heating and pesticide manipulations which coincided with a natural heatwave (see stream water temperature dynamics in ambient and heated mesocosms in Figure 5.2). In the first four days (drift sampled at the end of Day 2 and 4), EPT taxa collectively accounted for 14.5 and 14.3 % of the total number of drifting invertebrates (Table 5.1). These relative abundances decreased to 5.3 and 4.1 % on Day 12 and 14 and remained at this lower level (4.5%) on Day 22. The reduced relative abundances of EPT taxa were mainly due to a strong increase in total invertebrate drift, especially of the taxa that increased in proportion of total drifting invertebrates during the experiment (Figure 5.3). Of the 12 most common taxa in the drift across all five sampling occasions, the proportions of oligochaetes, cladocerans (family Chydoridae), copepods, Aoteapsyche spp. and nematodes increased during the experimental period. By contrast, the proportions of chironomids, Deleatidium spp., Austrosimulium sp., Pycnocentrodes spp. Hydrobiosidae, Oxyethira albiceps and Olinga spp. in the drift decreased.
Figure 5.2. Water temperatures in ambient and heated experimental stream channels over the 24-day heating and imidacloprid manipulation period. Coloured boxes and vertical lines show the timing of the three 48-hour pesticide pulses and when invertebrate drift and insect emergence was sampled. Benthic invertebrate communities from the entire mesocosms were sampled at the end of the manipulative period on December 9th 2017 (Day 24). Note: flow velocity manipulation began 3 weeks prior (Day -21) on October 25th 2017. The 10-day natural heatwave occurred between Days 11–20 when the maximum water temperatures in ambient channels was >25°C (November 26th – December 5th).
Figure 5.3. Shifts in drift community composition illustrated through changing relative abundances of the 12 most common drifting invertebrate taxa (averaged across all monitoring periods) in mesocosms with fast (top) or slow flow velocity (bottom) without temperature or imidacloprid manipulation. Error bars ± SE ($n = 8$).
5.4.3  Invertebrate drift responses to experimental manipulations

5.4.3.1  Drift community-level metrics

*Total number of invertebrates*
Consistently fewer invertebrates drifted from slow-flow than fast-flow velocity channels, though the strength of this effect weakened from Day 2 to Day 22 (see Table 5.1 for all invertebrate drift responses described below). Raised water temperature caused an increase in the numbers of drifting invertebrates on Days 2, 4 and 12, whereas imidacloprid addition alone had no effect on total invertebrate drift abundance (Figure 5.4a). On Day 4, however, a two-way interaction occurred between imidacloprid and flow velocity, where the total number of drifting invertebrates decreased with increasing imidacloprid concentrations in fast-flowing channels, while the opposite pattern occurred in slow-flowing channels.

*Total number of EPT*
Fewer EPT individuals drifted from slow-flowing channels on Days 2, 4 and 14, and from heated channels on Day 4 (Figure 5.4b). Drifting EPT increased with imidacloprid addition during the first pulse (Day 2), and this response was stronger at fast flow (imidacloprid × flow velocity interaction). The opposite pattern occurred after the first pulse on Day 4; reduced drift with imidacloprid addition was observed only in fast-flowing channels. Likewise, EPT drift was lower with imidacloprid addition during the third pulse (Day 22).

*Total and EPT taxon richness*
During the first pulse (Day 2), the total number of taxa represented in the drift was higher in heated and fast-flowing channels and increased with imidacloprid addition (Figure 5.5a). The same patterns occurred for the number of EPT taxa (Figure 5.5b). The only other effects on overall taxon richness and EPT taxon richness occurred in the 48 hours immediately following the first imidacloprid pulse (Day 4). There was a weak imidacloprid × flow velocity interaction, where taxon richness decreased with imidacloprid in fast-flowing channels but increased with imidacloprid in slow-flowing channels.
Figure 5.4. Average total number of drifting (a) invertebrates overall and (b) EPT on all five sampling dates in all experimental treatments. (Error bars ± SE; n = 8 per treatment combination). Significant effects are indicated by the predictor terms embedded in each plot.
5.4.3.2 Drift community composition

Multivariate results of GLMMs with the 12 most common taxa on each drift sampling date showed that all three factors affected drift community composition during the three pesticide pulses (sampled on Days 2, 12 and 22), whereas community composition did not change significantly with imidacloprid in the two post-pulse monitoring periods (Days 4 and 14; Table 5.1). Flow velocity generally affected the drift community most strongly across all dates (NMDS plots for the drifting invertebrate community on each sampling date are...
presented in Appendix C, Figures C1–C3). During the first and third pulses, imidacloprid also strongly affected the drift community (Figure C2), exerting an effect of equal strength as flow velocity on Day 22. During the second pulse, temperature had a strong effect on drift community composition; (Figure C3; as did flow velocity). During and immediately following the first imidacloprid pulse, the drifting invertebrate community (sampled on Day 2 and 4) was also affected by a two-way imidacloprid × flow velocity interaction and a temperature × flow velocity interaction affected drifting community composition during the final pesticide pulse (sampled on Day 22).

5.4.3.3 Taxon-specific drift responses

Detailed statistical results for all 12 common drifting taxa are provided in Table 5.1, and Figures 5.6–8 show plots of the six taxa that responded to at least one factor during each drift sampling period. Fewer oligochaetes drifted from slow-flow channels on all dates measured. On Days 2, 4 and 12, raised water temperature increased the number of drifting oligochaetes, and during the second and third imidacloprid pulses (Days 12 and 22) imidacloprid addition reduced oligochaete drift (Figure 5.6). Fewer Chironomidae drifted from slow-flowing channels on all dates except for Day 14 when the opposite response occurred. During and immediately following the first pesticide pulse, raised water temperature increased numbers of drifting chironomids (Day 2 and 4), but by the second pesticide pulse (Day 12) the opposite effect was observed. During each of the three imidacloprid pulses, fewer chironomids drifted at higher levels of imidacloprid, whereas directly following the first two pulses there was no effect of imidacloprid on Chironomidae drift.

Chydoridae (Cladocera) drift (no figure) was mostly unaffected by the stressors, though there were some effects of heating and flow velocity during the second pesticide pulse on Day 12: more Chydoridae drifted from heated than non-heated channels and fewer drifted at slow compared to fast flow. During the final pesticide pulse (Day 22), there was also a temperature × imidacloprid interaction where Chydoridae drift increased with imidacloprid addition in heated channels.
Table 5.1. Summary (p-values and effect sizes where \( p < 0.1 \)) of GLM(M) results comparing invertebrate drift responses (based on absolute abundances) between experimental treatments on all five sampling days. GLMM p-values are for the Pillai’s Trace statistic. Relative abundances of total EPT taxa and individual common taxa are given in %. For the manipulated factors flow velocity (F), imidacloprid (I) and temperature (T), main effects are classified directionally as positive (+) or negative (−) based on the response direction of manipulated vs. control (ambient) levels. Combined (C) two-way interactions with imidacloprid are also classified directionally (+ or − effect of imidacloprid) and as stronger (>), weaker (<) or opposing (±) effects in the presence of the second stressor. Combined (C) temperature \( \times \) flow velocity interactions are classified as directional (+ or −) antagonistic (A) or synergistic (S) interaction effects. \( P \)-values are bolded where \( p < 0.05 \). Effect sizes are in parentheses (partial \( \eta^2 \) values; range 0–1). Interactions that overrode one or more main effects or lower-order interactions are underlined. Community comp. = multivariate results of the GLMM (effects on drift community composition), followed by the univariate results for the 12 most common taxa on average over all sampling days (average relative abundance in parentheses below taxon names).

<table>
<thead>
<tr>
<th>Response</th>
<th>Day</th>
<th>Pulse Period</th>
<th>%</th>
<th>Flow Velocity</th>
<th>F</th>
<th>Imidacloprid</th>
<th>I</th>
<th>Temperature</th>
<th>T</th>
<th>Imidacloprid × Flow Velocity</th>
<th>C</th>
<th>Temperature × Flow Velocity</th>
<th>C</th>
<th>Temperature × Imidacloprid</th>
<th>C</th>
<th>Temperature × Imidacloprid × Flow Velocity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Number of Invertebrates</td>
<td>2</td>
<td>During</td>
<td>&lt;0.001 (0.50)</td>
<td>−</td>
<td>&lt;0.001 (0.17)</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Post</td>
<td>&lt;0.001 (0.37)</td>
<td>−</td>
<td>0.003 (0.07)</td>
<td>+</td>
<td>0.002 (0.08)</td>
<td>±</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>During</td>
<td>&lt;0.001 (0.39)</td>
<td>−</td>
<td>&lt;0.001 (0.11)</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.06 (0.03)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>Post</td>
<td>0.03 (0.04)</td>
<td>−</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>During</td>
<td>0.03 (0.04)</td>
<td>−</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Invertebrate Taxon Richness</td>
<td>2</td>
<td>During</td>
<td>&lt;0.001 (0.2)</td>
<td>−</td>
<td>0.002 (0.08)</td>
<td>+</td>
<td>0.003 (0.07)</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Post</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.02 (0.04)</td>
<td>±</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>During</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>Post</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>During</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Number of EPT</td>
<td>2</td>
<td>During</td>
<td>14.47</td>
<td>&lt;0.001 (0.41)</td>
<td>−</td>
<td>&lt;0.001 (0.36)</td>
<td>+</td>
<td>&lt;0.001 (0.32)</td>
<td>+ &lt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Post</td>
<td>14.34</td>
<td>&lt;0.001 (0.23)</td>
<td>−</td>
<td>0.01 (0.05)</td>
<td>−</td>
<td>0.03 (0.04)</td>
<td>−</td>
<td>0.001 (0.10)</td>
<td>±</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>During</td>
<td>5.32</td>
<td>0.06 (0.03)</td>
<td>−</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>Post</td>
<td>4.11</td>
<td>0.048 (0.03)</td>
<td>−</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>During</td>
<td>4.45</td>
<td>0.096 (0.02)</td>
<td>0.01 (0.05)</td>
<td>−</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EPT Taxon richness</td>
<td>2</td>
<td>During</td>
<td>&lt;0.001 (0.27)</td>
<td>−</td>
<td>0.048 (0.03)</td>
<td>+</td>
<td>0.002 (0.08)</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Post</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.04 (0.04)</td>
<td>±</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>During</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>Post</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>During</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Community comp.</td>
<td>2</td>
<td>During</td>
<td>98.18</td>
<td>&lt;0.001 (0.72)</td>
<td>−</td>
<td>&lt;0.001 (0.54)</td>
<td>&lt;0.001 (0.33)</td>
<td>&lt;0.001 (0.39)</td>
<td>−</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(98.87%)</td>
<td>4</td>
<td>Post</td>
<td>98.42</td>
<td>&lt;0.001 (0.62)</td>
<td>−</td>
<td>&lt;0.001 (0.32)</td>
<td>0.001 (0.25)</td>
<td>&lt;0.001 (0.32)</td>
<td>−</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>During</td>
<td>99.06</td>
<td>&lt;0.001 (0.53)</td>
<td>−</td>
<td>0.01 (0.21)</td>
<td>&lt;0.001 (0.5)</td>
<td>0.09 (0.16)</td>
<td>−</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.02 (0.19)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>Post</td>
<td>99.27</td>
<td>&lt;0.001 (0.45)</td>
<td>−</td>
<td>&lt;0.001 (0.36)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>During</td>
<td>99.44</td>
<td>&lt;0.001 (0.42)</td>
<td>−</td>
<td>&lt;0.001 (0.42)</td>
<td>0.002 (0.24)</td>
<td>0.01 (0.21)</td>
<td>−</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 5.1 continued

<table>
<thead>
<tr>
<th>Response</th>
<th>Day</th>
<th>Pulse Period</th>
<th>%</th>
<th>Flow Velocity</th>
<th>F</th>
<th>Imidacloprid</th>
<th>I</th>
<th>Temperature</th>
<th>T</th>
<th>Temperature × Flow Velocity</th>
<th>C</th>
<th>Temperature × Imidacloprid</th>
<th>C</th>
<th>Temperature × Temperature × Imidacloprid</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oligochaeta</td>
<td>2</td>
<td>During</td>
<td>35.26</td>
<td>&lt;0.001 (0.29)</td>
<td>–</td>
<td>&lt;0.001</td>
<td>(0.11)</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(40.04%)</td>
<td>4</td>
<td>Post</td>
<td>22.16</td>
<td>&lt;0.001 (0.16)</td>
<td>–</td>
<td>&lt;0.001</td>
<td>(0.14)</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>During</td>
<td>61.11</td>
<td>&lt;0.001 (0.44)</td>
<td>–</td>
<td>0.004</td>
<td>(0.07)</td>
<td>–</td>
<td>&lt;0.001</td>
<td>(0.12)</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>Post</td>
<td>43.28</td>
<td>0.005 (0.07)</td>
<td>–</td>
<td>0.09</td>
<td>(0.02)</td>
<td>–</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>During</td>
<td>38.40</td>
<td>&lt;0.001 (0.09)</td>
<td>–</td>
<td>0.006</td>
<td>(0.06)</td>
<td>–</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chironomidae</td>
<td>2</td>
<td>During</td>
<td>43.29</td>
<td>&lt;0.001 (0.55)</td>
<td>–</td>
<td>&lt;0.001</td>
<td>(0.23)</td>
<td>–</td>
<td>&lt;0.001</td>
<td>(0.2)</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(29.91%)</td>
<td>4</td>
<td>Post</td>
<td>52.09</td>
<td>&lt;0.001 (0.35)</td>
<td>–</td>
<td>0.002</td>
<td>(0.08)</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>During</td>
<td>19.06</td>
<td>0.02 (0.05)</td>
<td>–</td>
<td>&lt;0.001</td>
<td>(0.11)</td>
<td>–</td>
<td>0.02</td>
<td>(0.04)</td>
<td>–</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>Post</td>
<td>18.64</td>
<td>0.02 (0.05)</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>During</td>
<td>16.48</td>
<td>0.02 (0.04)</td>
<td>–</td>
<td>&lt;0.001</td>
<td>(0.24)</td>
<td>–</td>
<td>0.097</td>
<td>(0.02)</td>
<td>–</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cladocera</td>
<td>2</td>
<td>During</td>
<td>0.37</td>
<td>0.06 (0.03)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(9.77%)</td>
<td>4</td>
<td>Post</td>
<td>0.54</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>During</td>
<td>3.44</td>
<td>0.03 (0.04)</td>
<td></td>
<td>&lt;0.001</td>
<td>(0.16)</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>Post</td>
<td>18.12</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>During</td>
<td>26.35</td>
<td></td>
<td>0.05</td>
<td>(0.02)</td>
<td></td>
<td>0.04</td>
<td>(0.04)</td>
<td>+&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Copepoda</td>
<td>2</td>
<td>During</td>
<td>0.76</td>
<td>0.02 (0.05)</td>
<td>+</td>
<td>0.09</td>
<td>(0.02)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(6.59%)</td>
<td>4</td>
<td>Post</td>
<td>0.89</td>
<td>0.02 (0.05)</td>
<td>+</td>
<td>0.03</td>
<td>(0.04)</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>During</td>
<td>7.55</td>
<td></td>
<td></td>
<td>&lt;0.001</td>
<td>(0.27)</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>Post</td>
<td>11.51</td>
<td></td>
<td></td>
<td>0.03</td>
<td>(0.04)</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>During</td>
<td>12.24</td>
<td></td>
<td>&lt;0.001 (0.22)</td>
<td>+</td>
<td>0.007</td>
<td>(0.06)</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deleatidium spp.</td>
<td>2</td>
<td>During</td>
<td>7.10</td>
<td>&lt;0.001 (0.56)</td>
<td>–</td>
<td>&lt;0.001</td>
<td>(0.26)</td>
<td>+</td>
<td>&lt;0.001</td>
<td>(0.24)</td>
<td>+&lt;</td>
<td>0.049 (0.03)</td>
<td>+&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(3.55%)</td>
<td>4</td>
<td>Post</td>
<td>7.58</td>
<td>&lt;0.001 (0.38)</td>
<td>–</td>
<td>0.002</td>
<td>(0.08)</td>
<td>–</td>
<td>&lt;0.001</td>
<td>(0.13)</td>
<td>±</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>During</td>
<td>1.74</td>
<td>&lt;0.001 (0.10)</td>
<td>–</td>
<td>0.08</td>
<td>(0.03)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>Post</td>
<td>1.01</td>
<td>0.01 (0.05)</td>
<td>–</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>During</td>
<td>0.35</td>
<td></td>
<td>0.03</td>
<td>(0.04)</td>
<td></td>
<td>0.01</td>
<td>(0.05)</td>
<td>+A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Austrosimulium</td>
<td>2</td>
<td>During</td>
<td>3.85</td>
<td>0.01 (0.05)</td>
<td>–</td>
<td>0.01</td>
<td>(0.05)</td>
<td>+</td>
<td>0.01</td>
<td>(0.06)</td>
<td>+&lt;</td>
<td>0.009 (0.06)</td>
<td>+&lt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>spp.</td>
<td>4</td>
<td>Post</td>
<td>8.21</td>
<td></td>
<td></td>
<td>0.002</td>
<td>(0.08)</td>
<td>–</td>
<td>0.03</td>
<td>(0.04)</td>
<td>±</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(3.35%)</td>
<td>12</td>
<td>During</td>
<td>2.11</td>
<td></td>
<td></td>
<td>&lt;0.001</td>
<td>(0.11)</td>
<td>–</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>Post</td>
<td>2.05</td>
<td>0.006 (0.06)</td>
<td>–</td>
<td>&lt;0.001</td>
<td>(0.21)</td>
<td>–</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>During</td>
<td>0.54</td>
<td></td>
<td>0.02</td>
<td>(0.04)</td>
<td></td>
<td>0.02</td>
<td>(0.05)</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pycnocentrodes sp.</td>
<td>2</td>
<td>During</td>
<td>2.48</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1.61%)</td>
<td>4</td>
<td>Post</td>
<td>2.18</td>
<td>0.03 (0.04)</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>During</td>
<td>1.88</td>
<td></td>
<td>0.04</td>
<td>(0.03)</td>
<td></td>
<td>0.06</td>
<td>(0.03)</td>
<td>–</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>Post</td>
<td>1.15</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 5.1 continued

<table>
<thead>
<tr>
<th>Response</th>
<th>Day</th>
<th>Pulse Period</th>
<th>%</th>
<th>Flow Velocity</th>
<th>F</th>
<th>Imidacloprid</th>
<th>I</th>
<th>Temperature</th>
<th>T</th>
<th>Temperature × Flow Velocity</th>
<th>C</th>
<th>Temperature × Imidacloprid</th>
<th>C</th>
<th>Temperature × Imidacloprid × Flow Velocity</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pycnocentrodes sp.</td>
<td>22</td>
<td>During</td>
<td>0.35</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aoteapsyche spp.</td>
<td>2</td>
<td>During</td>
<td>0.26</td>
<td>0.06 (0.03)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.03 (0.04) ±</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1.27%)</td>
<td>4</td>
<td>Post</td>
<td>1.15</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>During</td>
<td>0.95</td>
<td>0.07 (0.03)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>Post</td>
<td>1.10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>During</td>
<td>2.90</td>
<td>0.01 (0.05)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.01 (0.06) −</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydrobiosidae</td>
<td>2</td>
<td>During</td>
<td>2.34</td>
<td>&lt;0.001 (0.15)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.01 (0.09) + &lt;0.001 (0.14) + 0.002 (0.08) +&lt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1.01%)</td>
<td>4</td>
<td>Post</td>
<td>1.97</td>
<td>0.06 (0.03)</td>
<td></td>
<td>0.02 (0.05)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>During</td>
<td>0.29</td>
<td>0.04 (0.04)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.05 (0.03)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>Post</td>
<td>0.20</td>
<td>0.01 (0.05)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>During</td>
<td>0.26</td>
<td>0.09 (0.02)</td>
<td></td>
<td>0.02 (0.05)</td>
<td></td>
<td></td>
<td></td>
<td>0.03 (0.04) − 0.07 (0.03)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nematoda</td>
<td>2</td>
<td>During</td>
<td>0.29</td>
<td></td>
<td></td>
<td>0.01 (0.06)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(0.73%)</td>
<td>4</td>
<td>Post</td>
<td>0.36</td>
<td></td>
<td></td>
<td>0.07 (0.03)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>During</td>
<td>0.36</td>
<td></td>
<td></td>
<td>0.06 (0.03)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>Post</td>
<td>1.72</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>During</td>
<td>0.94</td>
<td></td>
<td></td>
<td>0.008 (0.06)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxyethira albiceps</td>
<td>2</td>
<td>During</td>
<td>1.28</td>
<td>&lt;0.001 (0.12)</td>
<td></td>
<td>0.04 (0.04)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(0.51%)</td>
<td>4</td>
<td>Post</td>
<td>0.69</td>
<td>0.009 (0.06)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.09 (0.03)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>During</td>
<td>0.02</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.08 (0.03)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>Post</td>
<td>0.14</td>
<td>0.051 (0.03)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.02 (0.05) ±</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>During</td>
<td>0.43</td>
<td>0.05 (0.03)</td>
<td></td>
<td>0.04 (0.03)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Olinga spp.</td>
<td>2</td>
<td>During</td>
<td>0.72</td>
<td></td>
<td></td>
<td>0.07 (0.03)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(0.41%)</td>
<td>4</td>
<td>Post</td>
<td>0.53</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>During</td>
<td>0.35</td>
<td></td>
<td></td>
<td>0.02 (0.05)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>Post</td>
<td>0.36</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>During</td>
<td>0.10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
On Days 2, 4 and 22, more copepods drifted from slow-flowing channels (Figure 5.7). Apart from Day 2, copepod drift was also higher with increased temperature on all sampling dates. As for Chydoridae, there were no main effects of imidacloprid addition on copepod drift but on Day 22, more copepods drifted with imidacloprid addition in heated channels (temperature × imidacloprid interaction). There were consistently fewer Deleatidium spp. drifting from slow-flowing channels until Day 22. On that date, reduced drift at slow flows was only observed in heated channels, while heating increased drift at fast flows (positive-antagonistic temperature × flow velocity interaction). Imidacloprid addition caused an overall increase in Deleatidium drift during the first and third pulses (Day 2 and 22). During the first pulse, this was overlaid by an interaction with flow velocity where there was a much stronger increase in drift with imidacloprid addition in fast-flowing channels, which was again overlaid by a weaker three-way interaction where this effect was yet more pronounced at ambient temperatures. Following the first imidacloprid pulse (Day 4), the imidacloprid × flow velocity interaction was the reverse from that observed on Day 2, with fewer Deleatidium drifting from fast-flowing channels with imidacloprid added during the first pulse. On Day 4, fewer Deleatidium also drifted from heated channels.

The same imidacloprid × flow velocity interaction pattern as for Deleatidium drift on Days 2 and 4 affected the number of drifting Austrosimulium spp., in this case overriding the imidacloprid and flow velocity main effects on Day 2 (Figure 5.8). There was also a three-way interaction on Day 2, with a different pattern to that observed for Deleatidium. In this case, imidacloprid addition increased Austrosimulium drift in all treatment combinations except for the heated, slow-flowing channels. During the first two days of heating, raised water temperature caused increased Austrosimulium drift. The opposite effect was then observed until Day 22 when a temperature × imidacloprid interaction overrode the main effect of temperature: Austrosimulium drift increased with imidacloprid addition in heated channels but decreased with imidacloprid in non-heated channels. Hydrobiosidae drift was affected by all three factors on Day 2; more hydrobiosids drifted from heated channels and with imidacloprid addition, and fewer from slow-flowing channels. An imidacloprid × flow velocity interaction indicated that the increased drift in response to imidacloprid addition mainly occurred in fast-flowing channels. During and following the second pulse of imidacloprid (Days 12 and 14), the only effect was a reduction in drifting Hydrobiosidae from slow-flowing channels. During the third pulse (Day 22), there was no longer a main effect of flow velocity, but an imidacloprid × flow velocity interaction indicated a reduction
in Hydrobiosidae drift with imidacloprid addition at slow but not at fast flow. On Day 22, heating reduced Hydrobiosidae drift.

Only two effects on numbers of drifting *Pycnocentrodes* spp. (no figure) were observed across all five sampling dates: an increase in numbers drifting from slow-flowing channels on Day 4 and a decrease in drift with imidacloprid addition during the second pulse on Day 12. The only effect on numbers of drifting *Aoteapsyche* spp. (no figure) prior to the final pesticide pulse was a two-way interaction between imidacloprid and flow velocity on Day 2, where imidacloprid addition reduced the number of *Aoteapsyche* drifting from fast-flowing channels but increased the number drifting from slow-flowing channels. On Day 22, *Aoteapsyche* drift was negatively affected by imidacloprid addition and slow flow velocity.

Raised water temperature increased the number of nematodes drifting (no figure) during the first 48 hours of heating (Day 2). No other effects on nematode drift were observed until the final pesticide pulse (Day 22) when a temperature \(\times\) imidacloprid interaction indicated that nematode drift decreased with imidacloprid addition in non-heated channels but increased with imidacloprid in heated channels. *Oxyethira albiceps* drift (no figure) increased with imidacloprid addition during the first pulse (Day 2) and was lower at slow flow, with the latter pattern also occurring on Day 4. The only effect on *Olinga* spp. drift (no figure) was an imidacloprid \(\times\) flow velocity interaction on Day 12. *Olinga* drift increased slightly with imidacloprid addition in slow-flowing channels but not at fast flow.
Figure 5.6. Average number of drifting (a) Oligochaeta and (b) Chironomidae on all five sampling dates in all experimental treatments. (Error bars ± SE; n = 8 per treatment combination). Significant effects are indicated by the predictor terms embedded in each plot.
Figure 5.7. Average number of drifting (a) Copepoda and (b) Deleatidium spp. on all five sampling dates in all experimental treatments. (Error bars ± SE; n = 8 per treatment combination). Significant effects are indicated by the predictor terms embedded in each plot.
Figure 5.8. Average number of drifting (a) Austrosimulium spp. and (b) Hydrobiosidae on all five sampling dates in all experimental treatments. (Error bars ± SE; n = 8 per treatment combination). Significant effects are indicated by the predictor terms embedded in each plot.
5.4.4 Insect emergence responses

5.4.4.1 Total number of emerged insects

On all sampling dates apart from Day 14 (the 48-hour period after the second pesticide pulse), fewer insects emerged from slow-flowing than fast-flowing channels (Figure 5.9a). This effect was strongest during the first pesticide pulse (Day 2) and subsequently became weaker. A positive effect of heating on insect emergence occurred on Days 2 and 4 (Figure 5.9b). On Day 14, this positive effect had been reversed, with fewer insects emerging from heated channels. This pattern had strengthened considerably by Day 22, representing the strongest effect on insect emergence abundance during the experiment. On Day 4, the main effect of heating on increased emergence was overlaid by a temperature × imidacloprid interaction, which saw fewer insects emerging from heated channels with imidacloprid addition (Figure 5.10a). On Day 12 during the second pulse, another main effect of imidacloprid was overlaid by an interaction with flow velocity where fewer insects emerged from fast-flowing channels with imidacloprid addition (Figure 5.10b-c).

Figure 5.9. Main effects of (a) flow velocity and (b) temperature on the mean total number of emerged insects per channel on all five sampling dates. *P*-values are bolded where *p* < 0.05, and partial η² effect sizes are presented in parentheses where *p* < 0.1. Error bars ± SE (*n* = 64 per treatment).
5.4.4.2 Emergence taxon richness

None of the manipulated factors strongly affected emergence taxon richness, which was generally low (Figure 5.11). The only repeated pattern was a lower number of insect taxa emerging from slow-flowing channels during the first and last imidacloprid pulses (Days 2 and 22). During the first pulse, this pattern was overlaid by an interaction with imidacloprid where the number of emerged taxa decreased with imidacloprid addition in slow-flowing but not in fast-flowing channels (Figure 5.12a). Other effects on emergence taxon richness were a positive effect of heating on Day 4, followed by the opposite effect of heating on Day 22 (Figure 5.12b), and a negative effect of imidacloprid on Day 14 (Figure 5.12c).
5.4.4.3 Emergence body size metrics

On all days except Day 12, mean body size of insects emerging from fast-flowing channels was larger than those emerging from slow-flowing channels (Figure 5.13). Similarly, Chironomidae emerging from fast-flowing channels were larger than those emerging from slow-flowing channels on all days except Day 14 (Figure 5.14). Mean insect body size and mean Chironomidae size also showed a negative relationship with increasing imidacloprid concentration on Day 14 (Figure 5.15). The only interaction effect for emerging insect body size occurred on Day 12, where the mean body size of emerging Chironomidae decreased with imidacloprid only in heated, slow-flowing channels (three-way interaction; Figure 5.16).

Figure 5.12. (a) Flow velocity × imidacloprid interaction effect on Day 4, (b) main effects of temperature on Day 4 and 22, and (c) main effect of imidacloprid on Day 14 on emergence taxon richness. See Figure 5.9 for details. Error bars ± SE (n = 16, 64 and 32 per treatment, respectively).

Figure 5.13. Main effect of flow velocity on overall mean emerged insect body size on all five sampling dates. See Figure 5.9 for details. Error bars ± SE (n = 64 per treatment).
Figure 5.14. Main effect of flow velocity on mean emerged Chironomidae body size on all five sampling dates. See Figure 5.9 for details. Error bars ± SE (n = 64 per treatment).

Figure 5.15. Day 14 main effects of imidacloprid on (a) overall mean emerged insect body size, and (b) mean emerged Chironomidae body size. See Figure 5.9 for details. Error bars ± SE (n = 32 per treatment).

Figure 5.16. Day 12 three-way interaction effect on mean emerged Chironomidae body size. $P = 0.04$, partial $\eta^2 = 0.04$, error bars ± SE (n = 8 per treatment combination).
Most emerging insects were less than 5 mm in body length, thus the responses for small emerging insects closely reflected the patterns observed for the total number of emerged insects (Table C1). Large emerging insects (>5 mm body length), however, showed several distinct patterns. The only significant effect of heating on these insects was a reduction in emergence during the final imidacloprid pulse (Day 22; Figure 5.17a). On Day 14, fewer large insects emerged from channels with added imidacloprid (Figure 5.17b). On all dates, more large insects emerged from fast- than from slow-flowing channels (Figure 5.18). On Day 12, this pattern was overlaid by a temperature × flow velocity interaction where this effect occurred with heating but not at ambient stream temperatures (Figure 5.17c).

Figure 5.17. (a) Main effect of temperature on Day 22, and (b) main effect of imidacloprid on Day 14 and (c) temperature × flow velocity interaction effect on Day 12 on the mean number of large emerging insects (>5 mm). See Figure 5.9 for details. Error bars ± SE (n = 64, 32 and 32 per treatment, respectively).

Figure 5.18. Main effect of flow velocity on the mean number of large emerging insects (>5 mm) on all five sampling dates. See Figure 5.9 for details. Error bars ± SE (n = 64 per treatment).
5.4.5  Benthic invertebrate community responses

5.4.5.1  Community-level metrics
Detailed statistical results for all benthic invertebrate responses described in Section 5.4.5 are presented in Table 5.2, and the corresponding response patterns for all community-level metrics are illustrated in Figure 5.19. Increased temperature reduced total number of invertebrates per channel, strongly reduced the number of EPT individuals present, caused moderate reductions in total taxon richness and EPT taxon richness, and a weak reduction in diversity (Simpson’s). Pulsed imidacloprid addition weakly reduced total invertebrate abundance and EPT abundance and caused a weak increase in diversity. Diversity was also lower at slow flow velocity, whereas slow flow caused a weak increase in EPT taxon richness. An imidacloprid × flow velocity interaction affected total taxon richness; imidacloprid caused reduced richness in fast-flowing channels, but increased richness in slow-flowing channels. Total number of EPT was affected by an antagonistic temperature × imidacloprid interaction and a temperature × imidacloprid × flow velocity interaction. The reduction in EPT abundance with imidacloprid was stronger in non-heated channels, and this effect was clearest in fast-flowing, non-heated channels.

5.4.5.2  Benthic invertebrate body size metrics
Benthic invertebrate body length (averaged across all measured individuals per mesocosm) was smaller with increased temperature and imidacloprid addition. A temperature × imidacloprid interaction indicated that the reduction in body size with imidacloprid was stronger in non-heated channels (Figure 5.20). The same main effect patterns with temperature and imidacloprid also occurred for larval chironomid size. In this case, a flow velocity × imidacloprid interaction indicated that the reduction in body size with imidacloprid was slightly stronger in fast-flowing channels. While there were no significant main effects on larval Deleatidium spp. size, a flow velocity × imidacloprid interaction indicated that body size increased with imidacloprid in fast-flowing channels but decreased with imidacloprid in slow-flowing channels.

Numbers of invertebrates from all body size classes (small, medium and large) were lower in heated channels. Only large invertebrates (>5 mm) were affected (negatively) by the imidacloprid pulses. This response pattern was overlaid by a temperature × imidacloprid
Table 5.2. Summary (p-values and effect sizes) of GLMMs comparing benthic invertebrate responses between experimental treatments. GLMM p-values are for Pillai’s Trace statistic. Relative abundances are given in %. P-values are bolded where p < 0.05. Effect sizes (partial $\eta^2$ values; range 0–1) are shown in parentheses for all cases where $p < 0.1$. Interactions that overrode one or more main effects or lower-order interactions are underlined. Community composition is the multivariate result for the GLMM with the 12 most common taxa. See Table 5.1 for effect classification details.

<table>
<thead>
<tr>
<th>Response Variable</th>
<th>%</th>
<th>Flow Velocity</th>
<th>F</th>
<th>Imidacloprid</th>
<th>Temperature $\times$ F</th>
<th>Temperature $\times$ Flow Velocity</th>
<th>Temperature $\times$ Imidacloprid</th>
<th>Temperature $\times$ Flow Velocity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Number of Invertebrates</td>
<td>0.17</td>
<td>0.04 (0.03)</td>
<td></td>
<td></td>
<td>$&lt;0.001$ (0.43)</td>
<td>0.57</td>
<td>0.11</td>
<td>0.46 (0.09)</td>
</tr>
<tr>
<td>Invertebrate Taxon Richness</td>
<td>0.23</td>
<td>0.34</td>
<td></td>
<td></td>
<td>$&lt;0.001$ (0.30)</td>
<td>$0.004$ (0.07)</td>
<td>$\pm$ 0.6</td>
<td>0.25 (0.21)</td>
</tr>
<tr>
<td>Simpson’s Diversity Index</td>
<td>0.03</td>
<td>(0.04)</td>
<td></td>
<td></td>
<td>$&lt;0.001$ (0.08)</td>
<td>$0.01$ (0.05)</td>
<td>0.58</td>
<td>0.9 (0.41)</td>
</tr>
<tr>
<td>Total Number of EPT</td>
<td>2.3</td>
<td>0.35</td>
<td></td>
<td></td>
<td>$&lt;0.001$ (0.11)</td>
<td>$0.01$ (0.05)</td>
<td>0.1</td>
<td>$0.002$ (0.08)</td>
</tr>
<tr>
<td>Larval Chironomidae Body Size</td>
<td>0.78</td>
<td>$&lt;0.001$ (0.15)</td>
<td></td>
<td></td>
<td>$&lt;0.001$ (0.13)</td>
<td>$0.04$ (0.04)</td>
<td>$&lt;0.001$</td>
<td>0.58</td>
</tr>
<tr>
<td>Larval Deleatidium spp. Body Size</td>
<td>0.43</td>
<td>0.72</td>
<td></td>
<td></td>
<td>0.32</td>
<td>$0.01$ (0.08)</td>
<td>$\pm$ 0.53</td>
<td>0.35</td>
</tr>
<tr>
<td>Small Invertebrates (&lt;1 mm)</td>
<td>10.1</td>
<td>0.88</td>
<td></td>
<td></td>
<td>$&lt;0.001$ (0.09)</td>
<td>0.86</td>
<td>0.53</td>
<td>0.35</td>
</tr>
<tr>
<td>Medium Invertebrates (1-5 mm)</td>
<td>82.8</td>
<td>0.17</td>
<td></td>
<td></td>
<td>$&lt;0.001$ (0.51)</td>
<td>0.17</td>
<td>$&lt;0.001$ (0.10)</td>
<td>$-A$ 0.55</td>
</tr>
<tr>
<td>Large Invertebrates (&gt;5 mm)</td>
<td>7.1</td>
<td>0.37</td>
<td></td>
<td></td>
<td>$&lt;0.001$ (0.12)</td>
<td>$&lt;0.001$ (0.5)</td>
<td>0.12</td>
<td>0.17</td>
</tr>
<tr>
<td>Community Composition</td>
<td>99.2</td>
<td>$&lt;0.001$ (0.58)</td>
<td></td>
<td></td>
<td>$&lt;0.001$ (0.31)</td>
<td>$0.04$ (0.18)</td>
<td>$0.03$ (0.19)</td>
<td>0.14</td>
</tr>
<tr>
<td>Oligochaeta</td>
<td>39.4</td>
<td>0.21</td>
<td></td>
<td></td>
<td>$&lt;0.001$ (0.38)</td>
<td>0.18</td>
<td>0.15</td>
<td>0.38</td>
</tr>
<tr>
<td>Copepoda</td>
<td>33.5</td>
<td>$&lt;0.001$ (0.33)</td>
<td></td>
<td></td>
<td>$&lt;0.001$ (0.16)</td>
<td>$0.02$ (0.05)</td>
<td>$\pm$ 0.64</td>
<td>0.69</td>
</tr>
<tr>
<td>Chironomidae</td>
<td>9.5</td>
<td>$&lt;0.001$ (0.09)</td>
<td></td>
<td></td>
<td>0.34</td>
<td>0.21</td>
<td>$&lt;0.001$ (0.10)</td>
<td>$-A$ 0.16</td>
</tr>
<tr>
<td>Chydriidae (Cladocera)</td>
<td>8.8</td>
<td>0.12</td>
<td>$0.008$ (0.06)</td>
<td></td>
<td>$&lt;0.001$ (0.48)</td>
<td>0.49</td>
<td>$0.005$ (0.07)</td>
<td>$-A$ 0.56</td>
</tr>
<tr>
<td>Potamopyrgus antipodarum</td>
<td>2.3</td>
<td>$0.02$ (0.05)</td>
<td></td>
<td></td>
<td>$0.03$ (0.04)</td>
<td>0.47</td>
<td>0.33</td>
<td>0.34</td>
</tr>
<tr>
<td>Nematoda</td>
<td>1.4</td>
<td>0.33</td>
<td></td>
<td></td>
<td>0.35</td>
<td>0.7</td>
<td>0.52</td>
<td>0.93</td>
</tr>
<tr>
<td>Ostracoda</td>
<td>1.4</td>
<td>0.3</td>
<td></td>
<td></td>
<td>0.25</td>
<td>0.39</td>
<td>0.72</td>
<td>0.36</td>
</tr>
<tr>
<td>Austrosimulium spp.</td>
<td>0.7</td>
<td>0.36</td>
<td></td>
<td></td>
<td>$&lt;0.001$ (0.21)</td>
<td>0.94</td>
<td>0.7</td>
<td>0.39</td>
</tr>
<tr>
<td>Aoteapsyche spp.</td>
<td>0.7</td>
<td>$&lt;0.001$ (0.11)</td>
<td></td>
<td></td>
<td>0.88</td>
<td>0.052 (0.03)</td>
<td>0.85</td>
<td>0.91</td>
</tr>
<tr>
<td>Deleatidium spp.</td>
<td>0.6</td>
<td>0.72</td>
<td>$&lt;0.001$ (0.15)</td>
<td></td>
<td>$&lt;0.001$ (0.71)</td>
<td>$0.06$ (0.03)</td>
<td>0.28</td>
<td>$0.001$ (0.11)</td>
</tr>
<tr>
<td>Oxyethira albiceps</td>
<td>0.5</td>
<td>$0.003$ (0.07)</td>
<td></td>
<td></td>
<td>$&lt;0.001$ (0.61)</td>
<td>0.62</td>
<td>0.26</td>
<td>0.9</td>
</tr>
<tr>
<td>Hydrobiosidae</td>
<td>0.2</td>
<td>0.17</td>
<td>$0.02$ (0.05)</td>
<td></td>
<td>$&lt;0.001$ (0.29)</td>
<td>0.31</td>
<td>0.72</td>
<td>0.08 (0.03)</td>
</tr>
</tbody>
</table>
interaction, where the decrease of large invertebrates with increasing concentration of imidacloprid was stronger in non-heated channels. Finally, numbers of medium-sized benthic invertebrates were affected by a negative-antagonistic temperature × flow velocity interaction.
interaction, where there was a less-than-additive combined effect of increased temperature and slow flow on this size class.

Figure 5.20. Averages of benthic invertebrate body size metrics and the common taxa Chironomidae and Deleatidium spp. across the experimental treatments (sampled on Day 24; Error bars ± SE; n = 8 per treatment combination). Significant effects are indicated by the predictor terms embedded in each plot.
5.4.5.3 Benthic invertebrate community composition

Multivariate results of GLMMs with the 12 most common taxa showed that all three factors affected benthic community composition. The strongest factor main effect was for temperature, followed by flow velocity (with another large effect) and imidacloprid (with a moderate effect) (Figure 5.21). Weak temperature × flow velocity and imidacloprid × flow velocity interactions also affected community composition.

![NMDS dissimilarity plots](image)

Figure 5.21. NMDS dissimilarity plots of the benthic invertebrate community composition with the twelve most common taxa grouped according to (a) flow velocity, (b) temperature and (c) imidacloprid treatments. 2D Stress = 0.19.
The most common taxon in the benthos was Oligochaeta, making up almost 40% of the total number of invertebrates counted. Increased temperature caused a moderate reduction and imidacloprid addition a weak reduction in oligochaete abundance (Figure 5.22). Copepods were the second-most abundant taxon (33.5% of total benthic count). Their numbers were weakly reduced by increased temperature and increased in response to slow flow. A flow velocity × imidacloprid interaction, which overrode a weaker main effect of imidacloprid, indicated that imidacloprid reduced copepod numbers only in slow-flow channels. Chironomidae (9.5% of total benthic count) were strongly negatively affected by increased temperature. A negative antagonistic interaction between temperature and flow velocity overrode a weaker main effect of flow velocity. In this interaction, Chironomidae abundance declined at slow flow only in non-heated channels. This pattern was overlaid by a three-way interaction where chironomid numbers generally declined with imidacloprid addition but increased slightly in slow-flowing, heated channels. Chydoridae (8.8% of total benthic count) were the only taxon that responded positively to imidacloprid. Further, Chydoridae were affected by increased temperature (negatively) and an antagonistic interaction between temperature and flow velocity, where numbers were reduced at slow flow only in non-heated channels. Potamopyrgus antipodarum (2.3% of total count) was the only taxon to respond positively to increased temperature, and also showed a positive response to slow flow.

Austrosimulium spp., Aoteapysche spp., Deleatidium spp., Oxyethira albiceps and Hydrobiosidae each contributed less than 1% of the total invertebrate count. Deleatidium was the most strongly affected of all 12 common benthic taxa by increased temperature and imidacloprid addition, both of which decreased Deleatidium abundance (Figure 5.23). These stressor main effects were overlaid by a temperature × imidacloprid interaction where the negative response to imidacloprid was clearest in the absence of heating (because Deleatidium was generally almost absent from heated channels). Hydrobiosidae responded negatively to increased temperature and imidacloprid addition, while Oxyethira was reduced by increased temperature and by slow flow. Austrosimulium was only affected by a reduction in heated channels (Figure 5.22) and Aoteapysche only showed a weak increase at slow flow velocity. Finally, Nematoda and Ostracoda (each 1.4% of total count) were unaffected by any of the stressors (no figures).
Figure 5.22. Average abundances of common invertebrate taxa in the mesocosm benthos (sampled on Day 24; Error bars ± SE; n = 8 per treatment combination). Significant effects are indicated by the predictor terms embedded in each plot.
5.5 Discussion

5.5.1 Differential macroinvertebrate colonisation due to flow velocity

Manipulating stream channel current velocity throughout the 7-week experiment achieved differing macroinvertebrate communities between the two flow treatments, supporting my first hypothesis. This is evident by the strong effects of flow velocity on the drifting invertebrate community composition from Day 2 onwards (multivariate GLM and NMDS analyses with the 12 most common taxa). Flow velocity effects on the drift community were strongest during the first pesticide pulse (first 48 hours of heating and imidacloprid manipulations; partial $\eta^2$ of 0.72) and became progressively weaker on later sampling dates (partial $\eta^2$ of 0.42 on Day 22, during the final pesticide pulse). However, flow velocity still

Figure 5.23. Average abundances of common invertebrate taxa in the mesocosm benthos (sampled on Day 24; Error bars ± SE; $n = 8$ per treatment combination). Significant effects are indicated by the predictor terms embedded in each plot.
represented the strongest effect of the manipulated factors on drift community composition on each monitoring date (equal strength as the imidacloprid main effect on Day 22).

The pervasive effect of flow velocity on invertebrate colonisation of the stream channels is evident in the multivariate GLM and in the NMDS plot displaying the 12 most common taxa that drifted during the first pesticide pulse (Day 2; Figure C1), separated by flow velocity. EPT taxa were more closely associated with fast-flow velocity channels, with *Deleatidium* spp., *Oxyethira albiceps*, Hydrobiosidae and *Olinga* spp. all present in greater numbers, as well as the dipterans *Aphrophila* spp. and *Austrosimulium* spp. By contrast, Copepoda and *Potamopyrgus antipodarum* were associated with slow flow. While there is still some overlap in the community assemblages, this would be expected given some taxa will be tolerant of a range of habitat types (James and Suren 2009). For example, Chironomidae, Oligochaeta and the stony-cased caddis *Pycnocentrodes* spp. overlapped both regions of flow velocity, though generally more oligochaetes and chironomids were found in the drift from fast-flowing channels. Moreover, the broad taxonomic classification for groups such as the diverse dipteran family Chironomidae limit our inference of macroinvertebrate community responses to flow velocity, which may be clearer—or more complex—at lower taxonomic resolutions (Beermann et al. 2018b).

The relatively distinct community assemblages in fast- and slow-flow channels on Day 2 are further supported by the GLM results for the invertebrate community metrics and also the taxon-specific (univariate) results of the GLMM. There were consistently fewer invertebrates overall and EPT individuals drifting from slow-flow velocity channels during all five drift sampling dates, effects which were strongest during the first pulse of imidacloprid and in the 48 hours after (Figure 5.4). Invertebrate taxon richness and EPT richness were also reduced in slow-flowing channels on Day 2, patterns which are generally associated with reduced flow due to a reduction in habitat diversity (Dewson et al. 2007). The effects of flow velocity observed in the drift were also reflected in the emerged insect responses with insect emergence, emerged taxon richness and emerged insect body size consistently lower in the slow flow velocity channels. Again, these effects became weaker as the experiment progressed. The reduction in mean body size of emerged insects overall and of emerged Chironomidae (the most abundant emerging taxon but comprising a range of different species) at slow flow could show that larger insect larvae prefer fast flow, or that smaller insect larvae are more tolerant of slower flow. Several studies have found that rivers with naturally occurring low flows tend to favour macroinvertebrate taxa with shorter life
spans, smaller body sizes, lower fecundity and multivoltine reproduction (Arscott et al. 2010, Rolls et al. 2012), patterns which are also associated with intermittent streams (Bonada et al. 2007, Chakona et al. 2008). The effect of slow flow on body size of emerged insects overall will also be influenced by the consistently higher number of large emerging insects from fast-flowing channels during all monitoring periods. This result makes sense given *Deleatidium* spp., the most common large emerging insect, was consistently associated with fast flow (e.g. drifting more from fast-flow channels).

These combined results show macroinvertebrates colonising the channels preferentially selected for favourable stream microhabitats according to taxon-specific flow velocity tolerances. I had predicted this outcome as flow velocity affects a variety of stream habitat characteristics and taxon-specific responses to changes in habitat suitability regularly cause alterations in stream invertebrate community composition (Dewson et al. 2007, Rolls et al. 2012), and this has also been consistently observed in experiments manipulating flow velocity in stream channels (Matthaei et al. 2010, Elbrecht et al. 2016, Beermann et al. 2018a, Ward 2018).

In support of my second hypothesis, the different macroinvertebrate communities in slow- and fast-flowing channels resulted in several interesting interactions between flow velocity and the other manipulated stressors (temperature and imidacloprid pulses), particularly during the first 4 days of heating and imidacloprid manipulations, which will be discussed below. At the start of the temperature and imidacloprid manipulations, most drift and insect emergence responses pointed to higher invertebrate colonisation of fast-flowing channels with more insects, EPT individuals and higher invertebrate and EPT richness. However, the weakening strengths of all flow velocity effects between Days 2 and 24 show how the heating and imidacloprid-pulse manipulations, as well as the natural heatwave that occurred between Days 11–20, increasingly impacted the invertebrate communities in the stream channels.

This temporal shift in the importance of the three manipulated stressors was reflected in the final benthic invertebrate results which, in comparison to the drift and insect emergence results (which showed 44 of 85 possible and 23 of 30 main effects of flow, respectively), were less often affected by flow velocity (7/24 main effects). However, consistent with the previous studies that manipulated flow velocity in stream channels cited above, there was still a strong effect of flow velocity on multivariate benthic invertebrate community
composition. The strongest taxon-specific response to slow flow velocity in the benthos was a positive response of moderate strength by Copepoda, the second-most abundant taxon. Copepoda comprised less than 1% of the drifting invertebrates on Day 2 and 4, but by the end of the experiment they contributed more than 12% of drifting invertebrates and 33% of benthic invertebrates. Their affinity for slow flow is emphasised by an antagonistic imidacloprid × flow velocity interaction in the benthos, where a negative effect of imidacloprid was observed only in slow-flowing channels where copepods were most abundant. The preference of Copepoda for slow flows is supported by the findings of Matthaei et al. (2010) and Ward (2018), where, in both studies, they were the only taxon to respond positively to reduced flow velocity. Other taxa to respond positively to slow flow velocity in my experiment were the freshwater snail *Potamopyrgus antipodarum* and larvae of the caddisfly *Aoteapsyche* spp. Unexpectedly, EPT taxon richness increased at slow flow, a finding that contrasts with previous studies manipulating flow velocity in stream mesocosms in which EPT richness either responded negatively to flow (Elbrecht et al. 2016) or was unaffected (Beermann et al. 2018a, Ward 2018). Benthic invertebrate diversity was slightly reduced in slow-flowing channels, a result consistent with previous observations (Matthaei et al. 2010, Beermann et al. 2018a, Ward 2018). This response pattern is likely due to an increased dominance in these channels of relatively abundant taxa that either favoured slow flow (Copepoda and *P. antipodarum*) or were not reduced by slow flow (Oligochaeta and Chydoridae).

Monitoring invertebrate drift and emergence throughout the 24-day manipulations of temperature and imidacloprid allowed observing temporal shifts in the stream macroinvertebrate assemblage that would have gone unnoticed had I only sampled the stream community on the final day. Because the manipulative period encompassed an intense heatwave, the effects of the manipulated factors cannot be decoupled from this natural event. The drift and emergence sampling occasions during and after the three pesticide pulses occurred at three different points relative to the heatwave (before, during and after). Therefore, given the dramatic fluctuation in ambient temperature with the heatwave over these three sampling periods, the remaining discussion sections will discuss the impacts of my pulsed imidacloprid exposure and a continual 3°C temperature manipulation in combination with the effects of the natural heatwave that led to changes in the imidacloprid and temperature effects with time.
5.5.2 Imidacloprid-induced drift

Consistent with my third hypothesis, the environmentally relevant concentrations of imidacloprid applied in three 48h-pulses significantly affected the composition of the drifting invertebrate community. Imidacloprid exposure strongly affected drift community composition during the first pulse and had moderate effects during the second and third pulses but had no effect in the 48 hours immediately after the first and second pulses.

The first imidacloprid pulse caused an increase in the total number of drifting taxa and in the number of drifting EPT taxa. Likewise, the total number of drifting EPT individuals increased with imidacloprid addition, especially in fast-flowing channels (imidacloprid × flow velocity interaction). These responses are consistent with the predicted pattern that imidacloprid addition would increase insect drift due to its specific mode of neuronal action targeting insect acetylcholine receptors (Matsuda et al. 2001) and the high toxicity to aquatic insect larvae observed in ecotoxicological studies with imidacloprid (see reviews by Morrissey et al. 2015, Smit et al. 2015 and Sánchez-Bayo et al. 2016).

During the 48 hours immediately following the first pulse, antagonistic interactions between imidacloprid and flow velocity affected all community-level invertebrate metrics, with total number of drifting invertebrates, total invertebrate taxa, EPT individuals and EPT taxa all being reduced in fast-flowing channels where imidacloprid had been added in the previous 48 hours, but not in slow-flowing channels. These patterns likely reflect the high level of drift that had occurred during the preceding imidacloprid pulse. Where imidacloprid-induced emigration by drift had been highest, competition for space and resources would then be lowest following the pulse. Such short-term reversals in behavioural invertebrate drift patterns provide interesting insights into links between pesticide-induced drift and drift in response to natural biotic drivers, which would have been missed without monitoring drift over both periods.

Imidacloprid-induced drift of freshwater invertebrates has been observed in previous experiments involving pulsed-exposure scenarios (Beketov and Liess 2008, Berghahn et al. 2012). In single-species drift tests using stream microcosms, Beketov and Liess (2008) observed increased drift of the European mayfly *Baetis rhodani* after just 2-4 hours of imidacloprid exposure (0.97 µg/L). In another stream mesocosm experiment, 12-hour pulses of 12 µg/L imidacloprid caused increased drift of insect larvae and gammarids (Berghahn et
In my experiment, the strongest taxon-specific effects of imidacloprid on drift were for *Deleatidium* spp. and Chironomidae. Interestingly, these two taxa showed opposite patterns. Whereas imidacloprid exposure increased *Deleatidium* drift during the first and final pesticide pulses, chironomid drift decreased with imidacloprid addition during all three pulses. The responsiveness of *Deleatidium* to pulsed imidacloprid addition observed through increased drift supports previous observations of their high sensitivity to neonicotinoid exposure in single-taxon laboratory experiments (Chapter 2, Macaulay et al. 2019). By contrast, the relative tolerance of Chironomidae seems to contradict previous research that has found *Chironomus* species to be highly sensitive to neonicotinoids (Cavallaro et al. 2017, Finnegan et al. 2017, Saraiva et al. 2017, Maloney et al. 2018a, Naveen et al. 2018, Raby et al. 2018b, Raby et al. 2018c). However, as noted by Beermann et al. (2018a) when interpreting unexpected responses of Chironomidae to stressors in their experiment (salinity, fine sediment and flow velocity), this taxon is a highly diverse family that comprises a range of pollution-sensitive and -tolerant species. Due to the prohibitive effort of identifying all individuals within this family to genus or species (Beermann et al. 2018a), DNA barcoding analysis (as done successfully by Beermann et al. 2018b) would be a necessary further step to delineate the complex stressor responses of individual midge genera or species in my experiment. The reduced Chironomidae drift in channels with imidacloprid addition is likely due to tolerant midge taxa being able to better exploit the available habitat and resources left by the not-so tolerant taxa. This phenomenon has been observed in previous mesocosm experiments involving imidacloprid pulses for other tolerant taxa including gastropods (Colombo et al. 2013) and will be discussed further in combination with the results for the mesocosm benthos below (Section 5.5.3). In my experiment, reduced drift also occurred for Oligochaeta during the second and third imidacloprid pulses, *Pycnocentrodes* spp. in the second pulse, and *Aoteapsyche* spp. and *Oxyethira albiceps* in the final pulse (although effects were much weaker for these taxa than for Chironomidae).

Besides *Deleatidium* spp., two other common taxa demonstrated increased drift during the imidacloprid pulses. Both *Austrosimulium* spp. and Hydrobiosidae drifted more in response to imidacloprid addition during the first pulse, but only from fast-flowing channels. This pattern was the same as for *Deleatidium* drift that, together with *Austrosimulium*, was overlaid by a three-way interaction. For these taxa, the drift increase in response to
imidacloprid during the first pulse was strongest in non-heated, fast-flow channels. By contrast, the reduced Chironomidae drift during the first pulse showed the opposite imidacloprid × flow velocity interaction to the imidacloprid-sensitive taxa, with the reduction in chironomid drift being most pronounced in slow-flow channels. Together with the significant imidacloprid × flow velocity effect on drift community composition detected by the GLMM on Days 2 and 4, these results show that stream invertebrate communities from fast- and slow-flowing environments can respond differently to insecticide exposure, and that imidacloprid contamination of surface waters may impact healthier, EPT-rich communities in fast-flowing streams more strongly than those already subjected to reduced flows.

5.5.3 Imidacloprid effects on benthic invertebrates and insect emergence

The invertebrate drift dynamics results were largely supported by the effects of the imidacloprid pulses on the final benthic invertebrate community, sampled on Day 24. As for the drift results, imidacloprid strongly impacted benthic invertebrate community composition, overlaid by an imidacloprid × flow velocity interaction indicating that imidacloprid affected the benthic communities from slow and fast-flowing channels differently. Providing further support for my second hypothesis, invertebrate taxon richness was reduced by imidacloprid only in fast-flowing channels. While total drift abundance was unaffected by the imidacloprid pulses, total benthic invertebrate abundance was reduced slightly by imidacloprid. In line with the imidacloprid effects on increased drift, imidacloprid reduced benthic EPT abundance most strongly in fast-flowing, non-heated channels where these taxa were most abundant in the absence of imidacloprid.

The strongest taxon-specific benthic response to imidacloprid was a reduction in *Deleatidium* spp., which only occurred at ambient water temperatures because the heating treatment (and also the natural heatwave) had already strongly reduced *Deleatidium* abundance (discussed further in Section 5.5.4). Benthic abundances of four more of the 12 common taxa were negatively affected by imidacloprid: Hydrobiosidae, Oligochaeta and Copepoda, all in slow-flowing channels and benthic Chironomidae declined with imidacloprid addition in fast-flowing, non-heated channels (three-way interaction). The negative effects on *Deleatidium* and Hydrobiosidae are in line with findings from three
previous stream mesocosm experiments that observed negative effects on EPT taxa following three 24-hour imidacloprid pulses (1.63 and 17.6 µg/L; Pestana et al. 2009a), mayflies, caddisflies and dipterans following three 12-hour imidacloprid pulses (12 µg/L; Mohr et al. 2012) and the mayfly Baetis following 21 days of continuous imidacloprid exposure at a concentration range similar to the pulse concentrations in my experiment (1.23–5.97 µg/L; Alexander et al. 2016). Pestana et al. (2009a) also observed a reduction in Oligochaeta by 75% in their highest imidacloprid pulse treatment (17.6 µg/L). Although this concentration is higher than those applied in my experiment (0.1–4.61 µg/L), the response parallels the negative effect of imidacloprid on benthic oligochaetes that I observed. Negative effects of imidacloprid on freshwater oligochaetes have also been observed in laboratory studies, where 96-hour exposure to 6.2 ± 1.4 µg/L imidacloprid caused 50% oligochaete immobility (Alexander et al. 2007).

Five recent studies have investigated the impacts of imidacloprid on aquatic invertebrate communities in pond mesocosms, also termed ‘limnocorals’. The first involved three pulses of imidacloprid over 21 days and assessed impacts on insect emergence and the aquatic macroinvertebrate community (Colombo et al. 2013), discussed below. Cavallaro et al. (2018) tested toxicities of imidacloprid, clothianidin and thiamethoxam individually, and Maloney et al. (2018b) tested them individually and in binary mixtures. Both studies assessed effects on insect community emergence responses and observed delayed shifts in the emerging community composition with exposure to imidacloprid and clothianidin. Sumon et al. (2018) and Rico et al. (2018) assessed effects of imidacloprid on zooplankton and macroinvertebrate communities in sub-tropical and Mediterranean conditions, respectively, and both found results consistent with the effects on stream macroinvertebrate communities in warm, temperate conditions in my experiment. In both these earlier studies, zooplankton and macroinvertebrate communities were significantly affected by imidacloprid, with the strongest taxon-specific effects on Cloeon sp. mayflies. In their zooplankton communities, several Copepoda taxa were found to be highly sensitive to imidacloprid, paralleling the negative effect of imidacloprid on Copepoda I observed.

In my experiment, only one taxon in the benthos responded positively to the imidacloprid pulses: the cladoceran family Chydoridae. This result for a benthic stream cladoceran supports the common finding that cladocerans are much more tolerant of neonicotinoids than many insect taxa, with LC50 values up to six orders of magnitude higher than the most sensitive aquatic insects tested (Morrissey et al. 2015, Sánchez-Bayo et al. 2016) and
highlights the potential problems with using such tolerant test organisms in the first stages of ecological risk assessments (Sánchez-Bayo and Tennekes 2015, Sánchez-Bayo et al. 2016). Moreover, the contrasting responses of two crustacean taxa, Copepoda and Cladocera, to imidacloprid in my experiment—one with high sensitivity, the other with high tolerance—also shows that even crustaceans can have markedly different tolerances to neonicotinoids.

The subtle negative effects of pulsed imidacloprid addition on insect emergence patterns in my study are broadly consistent with the findings of Maloney et al. (2018b) who observed reduced emergence of adult Chironomidae after continuous exposure of larvae to imidacloprid (42% lower than controls after 28 days and 71–90% lower after 56 days). Colombo et al. (2013) also found reduced insect emergence in response to increasing concentrations of three imidacloprid pulses (0.2–12 µg/L, 7 days apart), especially for Caenis sp. mayflies, which failed to emerge from mesocosms receiving more than 0.4 µg/L imidacloprid. By contrast, Cavallaro et al. (2018) observed no significant effects on Chironomidae emergence abundance but found they emerged at least 18 days earlier in imidacloprid treatments than controls. I also observed weak reductions in mean body size of emerged insects (and Chironomidae) following exposure to the second imidacloprid pulse that could imply earlier emergence after exposure to imidacloprid, though these results needs to be interpreted cautiously given they include a range of different species with varying adult body sizes. Alexander et al. (2008) found that 12-hour pulses of imidacloprid at just 0.1 µg/L were enough to reduce body sizes of emerging mayflies (reduced head length in Baetis spp. and thorax length in Epeorus spp.). However, these findings were also accompanied by strong increases in mayfly emergence with imidacloprid exposure (implying imidacloprid-induced emergence), which contrasts with the findings of my mesocosm study and those of Colombo et al. (2013) and Maloney et al. (2018b).

5.5.4 Effects of heating manipulations and the natural heatwave

My temperature manipulation of 3°C above ambient temperatures affected the drifting macroinvertebrate community during all five drift monitoring periods. Moderate temperature effects were observed on Days 2 and 4. The strongest effects of temperature on the drift community occurred after 10–12 days of heating manipulations, during the second
pesticide pulse (Day 12). This 48-hour period also coincided with the beginning of the natural 10-day heatwave. At this time in the experiment, maximum ambient water temperatures rose by approximately 10°C, from 17°C (on Day 9) to 27°C. On Day 12, the heating treatment reached a maximum of 29.9°C, slightly hotter than the highest temperature reached in ambient stream channels (29.8°C), and in the river itself (29.0°C) during the entire experiment as a result of the heatwave.

The beginning of the heating manipulation saw an increase in drifting Chironomidae (over the first 4 days) and oligochaetes (until Day 12). As these taxa contributed more than 70% of the total number of individuals in the drift on Days 2 and 4, their response also resulted in increased total invertebrate drift, supporting my fourth hypothesis for invertebrate drift. Although Chironomidae showed the opposite response to temperature on Day 12, Oligochaetes were still the dominant drifting taxon. Together with Copepoda and Chydoridae (which were both also more abundant in the drift with increased temperature on Day 12), they still contributed to an overall positive effect of increased temperature on invertebrate drift. These findings generally agree with those observed for the drift propensity measures by Piggott et al. (2015c) who also investigated the effect of raised water temperature on macroinvertebrate community drift dynamics (individually and in combination with nutrient and sediment addition). In their study, invertebrate drift was higher overall with increased temperature at ambient nutrient levels, but lower with increased temperature under nutrient enrichment. The findings of both studies are therefore consistent, given I had no nutrient treatment in my experiment (all treatments were under ambient, low nutrient conditions).

There is, however, an important distinction between the measurement and calculation of invertebrate drift and insect emergence to be made between the two studies. Piggott et al. (2015c) sampled drift and emergence immediately prior to the final benthic sampling and were therefore able to calculate these responses in terms of propensities, that is, the relative proportions of the benthic invertebrates that drifted or emerged. However, being interested in the immediate drift and emergence responses of the stream community to pulses of imidacloprid, I sampled these responses repeatedly across 3.5 weeks. Given the dramatic changes in macroinvertebrate community composition that occurred during this period (Figure 5.3), it made more sense to interpret drift (and emergence) as absolute abundances rather than in relative terms (compared to benthic abundances) at the end of the experiment.
This made interpreting shifts in drift and emergence patterns of individual taxa across the experimental period more intuitive.

The latter is illustrated well for the temperature main effects on insect emergence. During the first 4 days of heating manipulations, insect emergence increased in mesocosms where temperature had been raised by 3°C. This pattern was consistent with previous findings for this temperature increase (Piggott et al. 2015c) and supported my fourth hypothesis for insect emergence. On Day 12, however, temperature no longer caused an increase in insect emergence, and by Day 14 there was a negative effect of heating on insect emergence. Despite having just one raised temperature treatment in my experiment, due to the natural increase in water temperature over this 12-day period, these results show how temperature can have non-linear effects on insect metabolism and development. From Days 12–14, the temperatures in the heated channels had already surpassed 30°C (max. temp 30.2°C). This temperature was hotter than the highest temperature (~28.5°C) achieved by Piggott et al. (2015c), who raised water temperatures by up to 6°C above ambient and observed unimodal nonlinear responses of Chironomidae and total emergence propensity to rising temperature, indicating a temperature threshold where positive effects on behaviour and physiology declined as temperatures continued to rise. The contention that this threshold was also exceeded in my experiment is further supported by the strong reduction in insect emergence with increased temperature during the final monitoring period (Day 22), which began three days after the highest temperatures (32.9°C) had been reached during the heatwave (and following maximum temperatures of 32.6°C the day before). This reduction in insect emergence in the heated channels also reflects the combined effects of the natural heatwave and raised water temperature on the benthic insect abundances.

The effect of increased temperature was by far the most pervasive stressor in this experiment, supporting my fourth hypothesis for benthic invertebrates by strongly affecting benthic invertebrate community composition and over 80% of the benthic response variables. All benthic community-level metrics were negatively affected by the heating treatment, including mean invertebrate body size. These results agree with the generally negative effects of temperature on the benthic response variables observed by Piggott et al. (2015c) and conform to the ‘temperature-size rule’ widely observed for aquatic ectotherms (Forster et al. 2012).
The strongest benthic taxon-specific reductions in response to increased temperature were for *Deleatidium*, Chironomidae and *Oxyethira albiceps*. An antagonistic interaction with flow velocity also affected Chironomidae, which were more strongly reduced by raised temperature in fast-flowing channels. For *Deleatidium*, the reduction with imidacloprid (described in the previous section) was only clear in non-heated channels because abundance was already strongly reduced by temperature alone in heated channels. This antagonistic temperature × imidacloprid interaction highlights the sensitivity of *Deleatidium* both to increased temperatures and to imidacloprid exposure (Macaulay et al. 2019) and contradicts my fifth hypothesis. While I had predicted that raised water temperature would enhance the negative effects of imidacloprid exposure, the antagonistic interaction observed instead highlights the limitations of using an additive null model when assessing stressor interactions for response variables with a fixed boundary such as invertebrate abundance, which cannot be less than zero (Folt et al. 1999). In this case, because the effect of raised water temperature alone already reduced *Deleatidium* abundance so strongly, additive or synergistic interactions between the heating and imidacloprid treatments could no longer be observed. The drastic effect of the natural heatwave on *Deleatidium* can be inferred by comparing their benthic abundances in non-heated, fast-flowing channels with no imidacloprid addition in my experiment with those from the ‘control channels’ in recent experiments by Piggott et al. (2015c) and Ward (2018) when no such heatwave occurred. In these experiments, benthic *Deleatidium* numbers were approximately 200 and between 250–300 individuals per channel, respectively (at ambient temperatures and with no added nutrients, sediment or reduced flow), whereas average benthic *Deleatidium* abundances in the ‘control’ channels in my experiment were at least four times lower (50 individuals per channel ± 6; SE).

Despite an overall negative effect of temperature on benthic copepod abundance, their large increase in absolute drift abundance (and proportion of total drifting invertebrates) over the heatwave period suggests they are more tolerant to strong temperature rises than other benthic taxa. The same is true for Chydoridae, which also increased considerably in relative and absolute drift abundance from before to after the heatwave, despite showing reduced benthic abundance in heated channels on the final day of the experiment. Nematoda were unaffected by temperature in the benthos but their prevalence in the drift community also increased during the experiment. These results are generally consistent with the taxon-specific responses to increased temperature observed by Piggott et al. (2015c), where these taxa either responded positively to or were unaffected by temperature. In my experiment, the
only taxon to show a positive response to the heating treatment, and the only positive benthic response overall, was the freshwater snail *Potamopyrgus antipodarum*, which weakly increased in number with increased temperature. *Potamopyrgus* also responded positively to reduced flow velocity and was unaffected by imidacloprid pulses, consistent with the common finding that molluscs are relatively tolerant to neonicotinoids compared to other organisms (Morrissey et al. 2015, Smit et al. 2015). For example, increased abundance of another freshwater snail, *Radix* sp., was observed by Colombo et al. (2013) in their pond mesocosm study involving three imidacloprid pulses over 21 days (concentrations ranging from 0.2–12 µg/L; see also earlier in discussion). These increases in the populations of tolerant taxa such as Gastropoda and Crustacea represent indirect effects of pollution or habitat modification and illustrate the importance of considering how biotic interactions can influence community and ecosystem responses to chemical and climatic stressors (Stoks et al. 2017, Bray et al. 2018).

5.5.5 Conclusions and management implications

Monitoring drift and emergence patterns of stream macroinvertebrate communities from fast and slow-flowing microhabitats on five separate occasions during 24 days of heating and pesticide pulse manipulations has provided several unique insights. Firstly, I found that macroinvertebrate communities from fast-flowing habitats are likely to be more negatively affected by exposure to imidacloprid than communities characteristic of slow flows. This has important implications for management, as streams with fast flows should receive protection from water abstraction as well as pesticide contamination in order to maintain their biological integrity. Conversely, surface waters draining agricultural land that have already been heavily modified and experience reduced flows from water abstraction may also be somewhat resistant to pesticide exposure. Conservation efforts may therefore be most effective when allocated to protecting healthy systems from exposure to contaminants, as well as other forms of degradation that can alter flow regimes including channel modification and abstraction for irrigation.

Secondly, I observed strong temporal shifts in macroinvertebrate species assemblages according to naturally varying conditions in climate, in this case an intense 10-day heatwave. Insects became relatively less abundant, as indicated by reductions in the proportions of
drifting EPT and dipterans, while oligochaetes, crustaceans (Cladocera and Copepoda) and gastropods (*Potamopyrgus antipodarum*) became more abundant and dominated the benthic community on the final sampling day.

Thirdly, the coinciding of my experimental heating by 3°C above ambient with a natural heatwave allowed for novel insights into how future extreme temperature events under predicted climate change scenarios (IPCC 2014) might impact freshwater macroinvertebrate communities. The changing pattern of insect emergence with heating over the course of the heatwave accurately illustrated a non-linear, unimodal response pattern of ectotherm physiology and associated behaviour to increased temperature (Portner and Farrell 2008, Piggott et al. 2015c). At temperatures within the insects’ thermal tolerance limits, heating by 3°C positively affected insect development and emergence. However, where temperatures continued to increase beyond a threshold, this effect became negative. The sensitivity of EPT insect taxa, especially nymphs of the ubiquitous New Zealand mayfly *Deleatidium* spp., to temperature extremes was clear in the benthic response patterns of these taxa. *Deleatidium* were still present at ambient temperatures at the end of the experimental period, even after the heatwave had naturally heated the river to 29°C (and the experimental channels to 29.8°C). However, in the heating treatment 3°C above this (32.9°C) there were virtually no *Deleatidium* remaining, and few EPT individuals overall. Depending on the plasticity of these organisms to adapt to rising temperatures, the vulnerability of these ecologically important taxa to climate change poses a serious risk to the biological integrity of the world’s freshwater ecosystems.
Chapter 6

General Discussion
6 General Discussion

6.1 Synthesis and novel contributions

My thesis is the first empirical assessment of the impacts of neonicotinoid insecticides on New Zealand’s running freshwater ecosystems, and the first such assessment worldwide in the context of climate change. There are several novel aspects to my research which represent an original contribution to applied ecology and ecotoxicology, especially to the toxicological literature of neonicotinoid insecticides in aquatic ecosystems and their combined effects with climate change.

In Chapter 2, I tested the chronic toxicities of the three most commonly used neonicotinoid insecticides in 28-day long laboratory exposures to nymphs of the ubiquitous New Zealand mayfly, *Deleatidium* spp. This provided previously unknown information of the toxicity of thiamethoxam and clothianidin to a sensitive freshwater insect in New Zealand, and a valuable contribution to the general paucity of toxicity data for these two widely-used insecticides (Anderson et al. 2015, Morrissey et al. 2015, Wood and Goulson 2017). An important insight was the observation that imidacloprid was relatively more toxic than the other two neonicotinoids, especially compared to thiamethoxam. These observations were consistent with the growing body of research investigating neonicotinoid effects on aquatic insects, including larvae of the aquatic midges *Chironomus* spp. which often have toxicity endpoint values (usually EC50s assessed by emergence success) in a very similar range to the toxicities to mayfly nymphs—the aquatic taxon consistently found to be most sensitive to neonicotinoids (Morrissey et al. 2015, Smit et al. 2015, Raby et al. 2018c).

In order to empirically verify the finding from Chapter 2 that imidacloprid was relatively more toxic than clothianidin and thiamethoxam, in Chapter 3 I performed a 28-day “neonicotinoid cocktail” experiment, which also aimed to test the effects of mixtures of the three commonly-used neonicotinoids and whether their combined toxicities would deviate from additivity according to analysis by full-factorial ANOVAs. While not the standard null model for testing additive combined toxicities of chemical mixtures, this method allowed examining the relative toxicities of the three neonicotinoids and testing several hypotheses developed based on the findings of the previous chapter. Consistent with these hypotheses, imidacloprid induced much stronger lethal and sublethal effects on *Deleatidium* impairment.
and immobility than clothianidin and thiamethoxam, and the effect of thiamethoxam overall was relatively benign (even at a concentration >2 times that of the imidacloprid and clothianidin treatments). In combination with clothianidin and imidacloprid, however, thiamethoxam exerted some synergistic interactive effects. Imidacloprid also interacted synergistically with clothianidin to cause greater than additive toxicity (according to the additive model tested by ANOVAs). These findings further emphasised the potency of imidacloprid in comparison with the other two neonicotinoids.

In Chapter 4, I addressed the second part of my overall thesis aim to investigate the influence of multiple stressors on the toxicity of neonicotinoids to non-target aquatic invertebrates in a 42-day laboratory experiment. Using the methodology developed and refined in Chapters 2 and 3 with Deleatidium nymphs as a model organism, I tested how two stressors naturally occurring in real stream ecosystems, food limitation and exposure to extreme high temperatures in the form of simulated heatwaves, can affect chronic low-level exposure to imidacloprid (0.4 µg/L). Using an additive model for determining interaction effects (ANOVA), I mainly observed antagonistic interactions between the three stressors because the effects of the simulated heatwaves in the first twelve days of the experiment were so severe. These severe heatwave effects highlighted the sensitivity of Deleatidium nymphs to short-term increases in temperature (by 8°C), which were also observed in my field experiment—both under the heating manipulation of just 3°C, and the natural heatwave (see Chapter 5 below). The experiment described in Chapter 4 also demonstrated that chronic exposure to imidacloprid at an environmentally relevant concentration can have delayed and initially subtle effects that can eventually result in considerable impairment of mayfly nymphs—equally as severe as the simulated heatwaves after 4–6 weeks of exposure—findings that highlighted the need for more long-term laboratory experiments with pesticides at environmentally relevant concentrations.

In Chapter 5, I performed an ambitious 7-week streamside channel experiment using 128 flow-through circular mesocosms to test the effects of pulsed imidacloprid exposure and raised water temperature on stream macroinvertebrate communities representative of fast and slow-flowing habitats. The mesocosms were open systems, naturally colonised by drifting stream biota from the river feeding the setup throughout the experiment. In this experiment, 5 sets of 128 invertebrate drift and insect emergence samples were collected during the heating and imidacloprid pulse manipulative period, and 128 benthic invertebrate
samples at the end of the experiment. By monitoring invertebrate drift and insect emergence patterns regularly throughout the manipulations, important insights into macroinvertebrate responses to imidacloprid exposure and raised water temperature were gained. Of particular interest was the way some responses changed during the experiment as a 10-day natural heatwave strongly altered drift invertebrate community composition (discussed further in Section 6.3). These community changes would have been missed had the regular drift monitoring not taken place.

6.2 Chronic exposures with regular sampling

A consistent theme throughout my evaluation of neonicotinoid effects on stream macroinvertebrate communities in a multiple-stressor and climate-change context was the implementation of experiments lasting much longer than the traditional acute, 24–96-hour exposures. These still represent the majority of tests in the field of ecotoxicology (Sánchez-Bayo and Tennekes 2015, Sánchez-Bayo et al. 2016); 83 % of the tests involving neonicotinoids reviewed by Morrissey et al. (2015) were ≤96-h in duration). In all of my experiments, recording responses repeatedly throughout the exposures also allowed gaining a fuller picture of the effects of the neonicotinoids and other manipulated stressors than would have been achieved by only assessing responses at the end of the experiments. Implementing the method of regular monitoring of responses during chronic experiments is especially important for the assessment of neonicotinoid toxicity, as the acute-to-chronic ratio (ACR) for this class of chemicals has been shown to be particularly high (Roessink et al. 2013, Morrissey et al. 2015). For example, the ACRs for imidacloprid calculated by Roessink et al. (2013) were >10 for all seven freshwater invertebrate taxa tested (including crustaceans and insects), with the highest ACR (336) recorded for the mayfly Cloeon dipterum. Using the 96-hour LC50 for imidacloprid calculated by Macaulay et al. (2019) for Deleatidium (40.6 µg/L) and the respective EC50 from my Chapter 2 (0.19 µg/L) (as LC/EC10s could not be calculated) derives an ACR for Deleatidium of 214. Therefore, the chronic toxicities for Deleatidium mayfly nymphs (derived from 28-day long exposures) are more than two orders of magnitude lower than the acute toxicities calculated after 4-day exposure durations. For mayfly nymphs such as Deleatidium that have larval stages spanning several months or longer, chronic exposure to low levels (<1 µg/L) of imidacloprid therefore presents a serious risk.
As well as providing a thorough picture of the delayed, time-cumulative toxicity of chronic neonicotinoid exposure, the regular monitoring of *Deleatidium* responses in my laboratory experiments (Chapters 2–4) led to the observation of several notable stressor interactions that would otherwise have gone undetected. For example, the synergistic interactions between the three neonicotinoids imidacloprid, clothianidin and thiamethoxam observed in Chapter 3 would have been missed without regularly reading mayfly responses from Days 7–25 and only determining responses at the end of the exposure period. By this point in the experiment, the effect of imidacloprid exposure alone, the most toxic neonicotinoid, was so strong that antagonistic interactions dominated.

In my stream mesocosm experiment (Chapter 5), regularly monitoring invertebrate drift and insect emergence during and following the imidacloprid pulses was particularly important, not just for repeatedly detecting the effect that imidacloprid addition increased macroinvertebrate drift, but also because of the marked temporal changes in drift community composition due to the intense 10-day heatwave that occurred during the main experimental period. The reduction in the proportion of drifting EPT from 14.5% to 4.5% of the total drift community from the first drift sampling period (Day 2) to the last (Day 22) highlights the detrimental impact of the heatwave on these sensitive aquatic insects. Conversely, less sensitive invertebrate taxa including oligochaetes, cladocerans, copepods, nematodes and gastropods were able to tolerate the heatwave conditions and exploit the habitat and resources made available by the loss of sensitive insect taxa. Consequently, these taxa strongly increased in relative and absolute abundance from before to after the heatwave. These drift community shifts were also reflected in the changing presence or strength of the drift and emergence responses to all three stressors. In general, the effects of flow velocity, pulsed imidacloprid addition and heating on drift and emergence responses became weaker from the first to the third imidacloprid pulse, reflecting the shift to a generally more stress-tolerant macroinvertebrate community that developed during the natural heatwave.

### 6.3 Macroinvertebrate thermal tolerances and climate change implications

A key observation of my research was the strong influence of temperature on stream macroinvertebrate communities. *Deleatidium* mayfly nymphs were particularly sensitive, being strongly negatively affected in both experiments where temperature was manipulated
(Chapters 4 and 5). Both simulated heatwaves in the 42-day laboratory experiment (conducted during Austral winter) caused direct mortality in the nymphs tested by up to 50% during the heatwave period, effects which became even stronger in the days after each heatwave. In the stream mesocosm experiment (conducted during spring/early summer), the reduction in numbers of benthic *Deleatidium* was the strongest response to heating. Interestingly, *Deleatidium* were still present in the ambient temperature stream channels that had reached much warmer temperatures (28.9°C) than the simulated heatwaves that caused such drastic effects in my laboratory experiment (temperature increase from 12–20°C).

One likely explanation for this difference is that the summer and winter generations of *Deleatidium* have differing thermal tolerances, with the faster-growing summer generation being able to tolerate higher temperatures than the slower-growing winter generation (Winterbourn 1974, Scrimgeour 1991, Huryn 1996). It is well-established that temperature is a major driver of structure and function of stream macroinvertebrate communities and aquatic insect development (Sweeney and Vannote 1978, Vannote and Sweeney 1980, Ward and Stanford 1982), including mayflies (Sweeney 1978, Sweeney et al. 1986). Huryn (1996) found temperature to be the strongest factor determining growth rate of *Deleatidium* spp. nymphs in two Otago streams and several acute laboratory studies have observed overwintering generations of *Deleatidium* spp. to be adversely affected by water temperatures ranging from 21–27°C (depending on whether temperatures were kept constant or fluctuated diurnally; Quinn et al. 1994, Cox and Rutherford 2000a, Macaulay et al. 2019). Notably, the specimens tested in these experiments were all acclimated at 15–16°C prior to the experiments. By contrast, the mayflies used in my chronic laboratory experiment in Chapter 4 were acclimated at 12°C and were strongly affected by temperature raised to just 20°C. This difference suggests it is not only absolute temperatures that determine thermal tolerance but also relative increases in temperature and the rate of increase which can cause thermal (and metabolic) stress to the detriment of the organisms tested. A similar acclimation to warmer water temperatures could therefore explain why summer-generation *Deleatidium* nymphs were able to tolerate higher temperatures in the stream channels. This potential explanation gains further support from the findings of Cox and Rutherford (2000b) who found evidence for higher thermal tolerances of *Deleatidium* nymphs collected in summer and acclimated at 21–22°C than winter generations acclimated at 16–17°C. Future research could further investigate the role of acclimation to increased temperatures for the thermal tolerances of New Zealand aquatic invertebrates.
As demonstrated by the benthic responses of *Deleatidium* spp. and other EPT taxa (including the caddisflies *Oxyethira albiceps* and Hydrobiosidae) to the temperature treatment in my stream channel experiment (Chapter 5), almost none of these taxa remained in heated channels at the end of the experiment but were still present in ambient-temperature channels. Thus, a heatwave reaching temperatures just 3°C hotter than what occurred naturally was enough to cause the loss of these temperature-sensitive taxa. The implications for climate change are clear: unless these organisms are able to adapt to rising temperatures by acclimatising to even hotter summer temperatures as heatwaves become more frequent and severe (IPCC 2014, Ministry for the Environment 2016), the viability of future populations of these taxa may be under real threat. Further increasing the realism of my stream channel experiment, the simulated heatwave scenario is actually not far off realistic summer-temperature scenarios in the Kauru River feeding the experimental setup. Later in the same summer of my experiment, an 18-day heatwave in mid-late January resulted in even hotter temperatures in the Kauru River than during my experiment, with river temperatures about 0.5 km upstream of the stream channel site reaching >31°C (Figure 6.1). Monitoring of the benthic invertebrate community in the river during future extreme temperature events would help reveal if the macroinvertebrate responses I observed in the experimental stream channels are consistent with real in-stream community effects. Moreover, future experiments and in-stream monitoring could investigate the ability of aquatic insects such as *Deleatidium* spp. to adapt to rising temperatures.
Figure 6.1. Water temperatures in the Kauru River during the Austral summer of 2017/18 from the start of the Stream Channel Experiment (25 Oct) showing the first 10-day heatwave (26 November – 5 December) and the second, even hotter heatwave from 14–31 January (18 days). Source: ORC Hilltop server (http://gisdata.orc.govt.nz/hilltop/Global.hts). The ORC temperature data logging site is located about 0.5 km upstream of the stream channel site.
Notably, all benthic invertebrate responses that were significantly affected by the raised water temperature treatment (including all invertebrate community metrics and body size metrics) were negative effects except for the benthic abundance of *Potamopyrgus antipodarum*. Despite reaching over 32°C in the heated channels, *P. antipodarum* increased in number in these channels. *Potamopyrgus* snails are known to be pollutant-tolerant (Stark and Maxted 2007) and have been found to thrive in streams affected by multiple stressors such as nutrients and sediment (Matthaei et al. 2010, Wagenhoff et al. 2011). The positive responses of this taxon to high temperatures and reduced flow velocity are further stressors that could be added to this list. These findings for *P. antipodarum* are also supported by the experiments of Piggott et al. (2015c) and Ward (2018), as this species showed no response at all to raised water temperature or reduced flow velocity in these two studies, respectively. In the acute laboratory tests by Quinn et al. (1994) and Cox and Rutherford (2000a), *P. antipodarum* was the most tolerant invertebrate taxon to increased water temperature, with respective 96-hour median lethal temperatures of 32.4 ± 2.5°C (constant temperature) and 33.6 ± 0.4°C (maximum fluctuating temperature). The tolerance of *P. antipodarum* to so many stressors helps to explain why it has been such a successful worldwide invader of both lotic and lentic habitats (Alonso and Castro-Díez 2008, Alonso and Castro-Díez 2012, Alonso 2019). Due to its high tolerance to raised temperature, heatwaves and reduced streamflow velocities as demonstrated in my experiment, the invasiveness of *P. antipodarum* will likely increase even further under future global climate change scenarios (Daufresne et al. 2004, Früh et al. 2012, Hesselschwerdt and Wantzen 2018).

### 6.4 Implications for neonicotinoid insecticide management

While the strength of the temperature effects I observed have profound implications in the context of climate change, my research also shows clearly that the effects of neonicotinoids cannot be ignored either. The toxicity of imidacloprid and its potential to interact synergistically with clothianidin and thiamethoxam is of particular cause for concern. In the first assessment of chronic neonicotinoid toxicities to aquatic insects in New Zealand, *Deleatidium* spp. nymphs were adversely affected (i.e. were unable to swim) after 4 weeks of exposure to <0.5 µg/L of imidacloprid and <1 µg/L of clothianidin. While thiamethoxam was comparatively less toxic at the concentrations tested, further experimentation at a higher
concentration range is required to determine chronic median lethal concentrations for *Deleatidium*. I discussed in Chapters 2 and 3 the growing evidence to support my findings that thiamethoxam is less toxic than imidacloprid and clothianidin, however, this does not mean that its use poses no threat to non-target organisms. For example, a study investigating the effects of thiamethoxam and clothianidin on macroinvertebrate colonisation of pond mesocosms found that thiamethoxam caused stronger negative effects than clothianidin (Basley and Goulson 2018). Further, thiamethoxam treatment of soybeans has been shown to reduce crop yields through disruption of biological control by impacting non-target predatory beetles more than pest slugs (Douglas et al. 2015). These two studies from outside running waters further highlight the need for community- and ecosystem-level research into neonicotinoid impacts so that we can determine to which extent the findings of laboratory experiments can be extrapolated to environmentally realistic scenarios (Sánchez-Bayo and Tennekes 2015).

My stream mesocosm experiment (Chapter 5) complemented the findings of my several chronic laboratory experiments on *Deleatidium* by demonstrating the ability for imidacloprid to adversely affect entire stream macroinvertebrate communities. These effects were more prominent in communities from fast-flowing habitats and, for several benthic responses, were also stronger in the absence of raised water temperature (+3°C). The sensitivity of *Deleatidium* to imidacloprid observed under laboratory exposures was confirmed in the mesocosm experiment, where *Deleatidium* was the invertebrate taxon most strongly affected by imidacloprid addition both in the drift and in the benthos (and one of the benthic taxa affected by an antagonistic temperature × imidacloprid interaction). Another intriguing observation in the mesocosm experiment was the positive response of benthic cladocerans (Chydoridae) to imidacloprid addition. This result further emphasises the high tolerance of Cladocera to neonicotinoids—a common observation from laboratory studies—and stresses the importance of using more sensitive taxa in ecotoxicity assessments (Anderson et al. 2015, Raby et al. 2018c).

Comparisons of neonicotinoid toxicity data for mayflies and midges of the genus *Chironomus* (Chapter 1) which are now a standard test organism in many environmental risk assessments showed that these two insect groups share similar sensitivities to neonicotinoids. While not entirely supported in Chapter 5 by imidacloprid effects on drift dynamics of the family Chironomidae (which drifted less during the imidacloprid pulses), benthic Chironomidae were still reduced with imidacloprid in fast-flowing, non-heated channels.
(temperature × imidacloprid × flow velocity interaction). Because this taxon contains many genera and species with highly diverse feeding modes and ecologies, chironomid community composition most likely differed considerably among my experimental treatments. Consequently, the above interaction suggests that the chironomid species that were sensitive to flow-velocity reduction and temperature increases were also more sensitive to imidacloprid addition, supporting my general conclusions that macroinvertebrate communities from fast-flowing (and in this case, cooler) habitats are more sensitive to contamination by imidacloprid. In future research, employing state-of-the-art DNA barcoding techniques to identify this family to the species level, combined with obtaining quantitative abundance data for individual species (as first done by Beermann et al. 2018b) may reveal hidden patterns within the Chironomidae, which encompass species wide-ranging in pollution-tolerances (Beermann et al. 2018a).

The antagonistic interactions of imidacloprid with temperature that affected several invertebrate responses in Chapter 5, including benthic Deleatidium, were a finding consistent with the heatwave × imidacloprid interaction effects in my multiple-stressor laboratory experiment (Chapter 4) because, in both cases, the negative effects of heating alone were already so strong (discussed above). The resulting antagonisms with imidacloprid contrasted with my predicted synergistic interactions and highlight a limitation of the additive multiple-stressor model being most commonly used when assessing stressor interactions for metrics with a fixed boundary, such as survivorship (in Chapter 4) or invertebrate abundance (in Chapter 5), both of which cannot be less than zero (Folt et al. 1999, Lange et al. 2018). Due to the fixed-boundary constraints of these responses, stressor interactions are forced toward antagonisms which become the only possible interaction when a single stressor has a dominant effect (e.g. the imidacloprid effects in Chapter 3 and the heatwave effects in Chapters 4 and 5). In such situations, using a multiplicative model for assessing stressor interactions might be a more appropriate null model (Schäfer and Piggott 2018), by reducing the effect of fixed boundary-constraints on stressor interactions (Folt et al. 1999). Future meta-analyses could compare stressor interaction effect outcomes when using additive and multiplicative multiple-stressor null models.

Despite these limitations of additive null-models for many of my response variables measured, there are still important insights to be gained from the antagonistic interactions I observed between temperature (heatwaves) and imidacloprid exposure. For example, my results imply that, while heatwaves might negatively impact stream macroinvertebrate
communities more noticeably than imidacloprid in the short-term, failing to monitor neonicotinoid use and contamination of surface waters may have as equally severe detrimental effects in the longer term if their rates of application and presence in the environment are left unchecked.

New Zealand currently lacks any regular monitoring programme for neonicotinoids or any other pesticides in streams (while regular pesticide monitoring in groundwater does occur). However, several pesticides have recently been identified for reassessment review by the New Zealand Environmental Protection Authority (2018b). The NZ EPA (2018a) has also calculated Environmental Exposure Limits (EELs) for a number of hazardous substances, including the neonicotinoids imidacloprid (0.038 µg/L) and thiamethoxam (0.35 µg/L). In my experiments, chronic effects of imidacloprid occurred after 24–36 days of exposure to ten times the EEL for imidacloprid (0.38 µg/L; Chapter 4), and thiamethoxam caused only weak effects at a concentration above 4 µg/L. Therefore, these EELs would seem to be relatively protective of the most sensitive freshwater insect taxa in New Zealand streams. However, monitoring data of neonicotinoid concentrations in surface waters is urgently needed in New Zealand to show that these limits are not being exceeded, especially during periods of rain-driven surface runoff when pesticide concentrations in streams are often several orders of magnitude higher than during periods of stable flows (Chiovarou and Siewicki 2008, Hladik et al. 2014). The recent banning of imidacloprid, clothianidin and thiamethoxam application outside of greenhouses in Europe (Butler 2018) and in Ontario, Canada (Government of Ontario 2019), where data for their presence and concentrations in surface waters have been collected for over a decade—highlights the need for this information in New Zealand and other countries where it is also lacking at present.

6.5 Conclusions and outlook

Pesticide application has become standard farming practice associated with the intensification of agriculture as farmers endeavour to maximise yield production (Matson et al. 1997, Tilman et al. 2001, Tilman et al. 2002). However, prophylactic use of systemic insecticides such as the neonicotinoids has resulted in their widespread contamination of the environment where they can have harmful effects on non-target organisms, especially insects (Bonmatin et al. 2015, Pisa et al. 2015, Wood and Goulson 2017). My PhD research has
added to the growing global body of evidence for high neonicotinoid toxicity in non-target aquatic insects, by providing the first chronic toxicity data for freshwater taxa from New Zealand. This research showed that the world’s most widely used insecticide, imidacloprid, causes negative sublethal and lethal effects to mayfly nymphs at concentrations below those regularly found in international surveys of surface waters (Sánchez-Bayo et al. 2016). Comparisons with chronic toxicity data for the midges Chironomus dilutus and Chironomus riparius, which are now standard test species in many ecological risk assessment procedures, revealed that neonicotinoid toxicities to these taxa (and other mayfly species or genera that have been tested) are very similar, a result which is encouraging for ecotoxicological risk assessments utilising these test species. These comparisons and the results from my full-factorial neonicotinoid-mixture experiment also added to the evidence that imidacloprid and clothianidin are relatively more toxic than thiamethoxam, though this conclusion is yet to be supported by the results of more environmentally realistic mesocosm experiments.

The sustainable management of insecticide use in the context of climate change is an important issue for environmental managers to address. My research has demonstrated that temperature extremes associated with climate change will have significant detrimental effects on stream macroinvertebrate communities, especially those in healthy, unmodified environments with high proportions of sensitive insect taxa. Moreover, these ecosystems will also be negatively impacted by contamination of imidacloprid if its prophylactic use remains uncontrolled and contamination of the environment unmonitored. While crop protection methods will always be needed to reduce yield losses from insect pests, sustainable pest-control procedures must be implemented to reduce the environmental harm and potential reduction in ecosystem function and services from harmful pesticides (Chagnon et al. 2015). The world is acutely aware of the growing impacts of climate change and global efforts are underway to reduce their severity. However, despite a global call from scientists to restrict the use of neonicotinoids (Goulson 2018), especially their coating of seeds for prophylactic use (Sánchez-Bayo 2014, Tooker et al. 2017, Hladik et al. 2018b), the majority of countries outside of the EU and Canada have as yet failed to take any action on mitigating their use. The biodiversity, ecological integrity and long-term sustainability of many vulnerable freshwater ecosystems will depend on continued global action to reduce anthropogenic greenhouse gas emissions and similar global action being taken to reduce the environmental impacts of neonicotinoid insecticides.
References


FAO. 2018. The state of food security and nutrition in the world 2018. Building climate resilience for food security and nutrition. FAO, Rome, Italy.


Janssens, L., and R. Stoks. 2013. Fitness effects of chlorpyrifos in the damselfly Enallagma cyathigerum strongly depend upon temperature and food level and can bridge metamorphosis. PLOS ONE 8:e68107.


Winterbourn, M. 1974. The life histories, trophic relations and production of *Stenoperla prasina* (Plecoptera) and *Deleatidium* spp. (Ephemeroptera) in a New Zealand river. Freshwater Biology **4**:507-524.


Appendices
Appendix A. Supporting Tables and Figures for Chapter 2.

Table A1. Gradient elution method used to achieve analyte separation by LC-MS/MS.

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>%A (5 mM formic acid)</th>
<th>%B (HPLC-grade acetonitrile)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td>30</td>
<td>70</td>
</tr>
<tr>
<td>7</td>
<td>30</td>
<td>70</td>
</tr>
<tr>
<td>7.5</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>10</td>
<td>80</td>
<td>20</td>
</tr>
</tbody>
</table>

Table A2. Analyte quantification details including M/z transition, fragmentor voltage (Frag), collision energy (CE), and retention time details for each analyte.

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>Precursor ion</th>
<th>Product ion</th>
<th>Frag (V)</th>
<th>CE (V)</th>
<th>Ret. Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clothianidin (Q)</td>
<td>250.0</td>
<td>169.0</td>
<td>380</td>
<td>8</td>
<td>5.218</td>
</tr>
<tr>
<td>Clothianidin (q)</td>
<td>250.0</td>
<td>132.0</td>
<td>380</td>
<td>12</td>
<td>5.218</td>
</tr>
<tr>
<td>Clothianidin-d3 (IS) (Q)</td>
<td>253.0</td>
<td>172.0</td>
<td>380</td>
<td>8</td>
<td>5.206</td>
</tr>
<tr>
<td>Clothianidin-d3 (IS) (q)</td>
<td>253.0</td>
<td>113.0</td>
<td>380</td>
<td>30</td>
<td>5.206</td>
</tr>
<tr>
<td>Imidaclorpid (Q)</td>
<td>256.0</td>
<td>209.0</td>
<td>380</td>
<td>12</td>
<td>5.399</td>
</tr>
<tr>
<td>Imidaclorpid (q)</td>
<td>256.0</td>
<td>175.0</td>
<td>380</td>
<td>12</td>
<td>5.399</td>
</tr>
<tr>
<td>Imidaclorpid (q)</td>
<td>256.0</td>
<td>84.0</td>
<td>380</td>
<td>12</td>
<td>5.399</td>
</tr>
<tr>
<td>Thiamethoxam (Q)</td>
<td>292.0</td>
<td>211.0</td>
<td>380</td>
<td>8</td>
<td>4.702</td>
</tr>
<tr>
<td>Thiamethoxam (q)</td>
<td>292.0</td>
<td>181.0</td>
<td>380</td>
<td>20</td>
<td>4.702</td>
</tr>
</tbody>
</table>

Q=quantification ion; q=qualifier ion; IS=internal standard

Table A3. Signal to noise ratios (S/N) for each compound at calibration standard concentrations (µg/L).

<table>
<thead>
<tr>
<th>Calibration Standard</th>
<th>Imidaclorpid</th>
<th>Clothianidin</th>
<th>Thiamethoxam</th>
</tr>
</thead>
<tbody>
<tr>
<td>ppb</td>
<td>Conc. (µg/L)</td>
<td>S/N</td>
<td>Conc. (µg/L)</td>
</tr>
<tr>
<td>0.01</td>
<td>0.005</td>
<td>9</td>
<td>0.008</td>
</tr>
<tr>
<td>0.02</td>
<td>0.016</td>
<td>17</td>
<td>0.020</td>
</tr>
<tr>
<td>0.04</td>
<td>0.028</td>
<td>10</td>
<td>0.030</td>
</tr>
<tr>
<td>0.05</td>
<td>0.04</td>
<td>17</td>
<td>0.048</td>
</tr>
<tr>
<td>0.06</td>
<td>0.062</td>
<td>31</td>
<td>0.070</td>
</tr>
<tr>
<td>0.08</td>
<td>0.079</td>
<td>38</td>
<td>0.090</td>
</tr>
<tr>
<td>0.1</td>
<td>0.091</td>
<td>17</td>
<td>0.095</td>
</tr>
<tr>
<td>0.2</td>
<td>0.227</td>
<td>49</td>
<td>0.240</td>
</tr>
<tr>
<td>0.5</td>
<td>0.494</td>
<td>156</td>
<td>0.500</td>
</tr>
</tbody>
</table>
Table A4. Mean neonicotinoid concentrations (µg/L ± standard errors) for initial (taken at the start of each week) and final (taken at the end of each week) samples from 28-day experiments.

<table>
<thead>
<tr>
<th>Neonicotinoid</th>
<th>Treatment (µg/L)</th>
<th>Initial</th>
<th>Final</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imidacloprid</td>
<td>0.05</td>
<td>0.04 ± 0.003</td>
<td>0.03 ± 0.001</td>
<td>0.03 ± 0.001</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>0.42 ± 0.042</td>
<td>0.42 ± 0.053</td>
<td>0.41 ± 0.031</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>2.94 ± 0.101</td>
<td>2.89 ± 0.094</td>
<td>2.92 ± 0.068</td>
</tr>
<tr>
<td>Clothianidin</td>
<td>0.05</td>
<td>0.02 ± 0.003</td>
<td>0.02 ± 0.001</td>
<td>0.02 ± 0.002</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>0.30 ± 0.005</td>
<td>0.29 ± 0.007</td>
<td>0.29 ± 0.004</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>2.79 ± 0.104</td>
<td>2.82 ± 0.057</td>
<td>2.79 ± 0.050</td>
</tr>
<tr>
<td>Thiamethoxam</td>
<td>0.05</td>
<td>0.07 ± 0.007</td>
<td>0.07 ± 0.006</td>
<td>0.07 ± 0.004</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>0.71 ± 0.017</td>
<td>0.72 ± 0.014</td>
<td>0.71 ± 0.011</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>7.40 ± 0.257</td>
<td>7.01 ± 0.221</td>
<td>7.21 ± 0.170</td>
</tr>
</tbody>
</table>

Figure A1. Aerated glass chamber containing a 10 × 10 × 1 cm ceramic tile colonized by periphyton biofilm, Deleatidium mayfly nymphs and artificial stream water/pesticide solution (one mayfly nymph and shed exuvia visible).
Figure A2. Mean proportion of immobilised mayflies out of the number of larvae alive at the start of each 7-day period over 28 days of exposure to a) imidacloprid b) clothianidin and c) thiamethoxam. Error bars are +/- SE (n = 5).
Figure A3. Mean proportion of impaired mayflies out of the number of larvae alive at the start of each 7-day period over 28 days of exposure to a) imidacloprid b) clothianidin and c) thiamethoxam. Error bars are +/- SE (n = 5).
Appendix B. Supporting Figures for Chapter 3.

Figure B1. Main effects of clothianidin on immobility and impairment, and main effect of imidacloprid on moulting propensity on Day 7.
Figure B2. Main effects of imidacloprid and clothianidin on Day 11.
Figure B3. Main effects of imidacloprid and clothianidin on Day 14.
Figure B4. Two-way interaction effects on Day 11 and 14.
Figure B5. Main effects of imidacloprid, clothianidin and thiamethoxam on Day 18.
Figure B6. Main effects of imidacloprid, clothianidin and thiamethoxam on Day 21.
Figure B7. Two-way interaction effects on Day 18.
Figure B8. Two-way interaction effects on Day 21
Figure B9. Main effects of imidacloprid, clothianidin and thiamethoxam on Day 25.
Figure B10. Main effects of imidacloprid, clothianidin and thiamethoxam on Day 28.
Figure B11. Two-way interaction effects on Day 25 and 28.
Appendix C. Supporting Tables and Figures for Chapter 5.

Figure C1. NMDS dissimilarity plots of the drifting invertebrate community during each monitoring period grouped according to flow velocity treatment. 2D Stress from Days 2–22 = 0.24, 0.25, 0.23, 0.17 and 0.22, respectively.
Figure C2. NMDS dissimilarity plots of the drifting invertebrate community during each monitoring period grouped according to imidacloprid treatment. 2D Stress from Days 2–22 = 0.24, 0.25, 0.23, 0.17 and 0.22, respectively.
Figure C3. NMDS dissimilarity plots of the drifting invertebrate community during each monitoring period grouped according to temperature treatment. 2D Stress from Days 2–22 = 0.24, 0.25, 0.23, 0.17 and 0.22, respectively.
Table C1. Summary (p-values and effect sizes) of GLM results comparing insect emergence responses between experimental treatments on all sampling days. See Table 5.1 for further details.

<table>
<thead>
<tr>
<th>Response</th>
<th>Day</th>
<th>Pulse Period</th>
<th>Flow Velocity</th>
<th>Imidacloprid</th>
<th>Temperature × Imidacloprid</th>
<th>Temperature × Flow Velocity</th>
<th>Temperature × Imidacloprid</th>
<th>Temperature × Flow Velocity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Number of Emergers</td>
<td>2</td>
<td>During</td>
<td>&lt;0.001 (0.22)</td>
<td>0.48</td>
<td>0.02 (0.05)</td>
<td>0.18</td>
<td>0.4</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Post</td>
<td>&lt;0.001 (0.16)</td>
<td>0.11</td>
<td>&lt;0.001 (0.09)</td>
<td>+ 0.63</td>
<td>0.91</td>
<td>0.02 (0.05) ± 0.36</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>During</td>
<td>0.004 (0.07)</td>
<td>0.02 (0.04)</td>
<td>0.28</td>
<td>0.03 (0.04) ± &lt; 0.61</td>
<td>0.92</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>Post</td>
<td>0.91</td>
<td>0.07 (0.03)</td>
<td>0.006 (0.06)</td>
<td>0.08 (0.03) ± 0.23</td>
<td>0.17</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>During</td>
<td>0.01 (0.05)</td>
<td>0.45</td>
<td>&lt;0.001 (0.42)</td>
<td>- 0.49</td>
<td>0.17</td>
<td>0.23</td>
</tr>
<tr>
<td>Emergence Taxon Richness</td>
<td>2</td>
<td>During</td>
<td>0.01 (0.05)</td>
<td>0.1</td>
<td>0.03 (0.04) ± 0.88</td>
<td>0.79</td>
<td>0.93</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Post</td>
<td>0.15</td>
<td>0.31</td>
<td>0.04 (0.03)</td>
<td>+ 0.59</td>
<td>0.23</td>
<td>0.09 (0.02) ± 0.76</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>During</td>
<td>0.06 (0.03)</td>
<td>0.18</td>
<td>0.26</td>
<td>0.22</td>
<td>0.38</td>
<td>0.92 ± 0.07 (0.03)</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>Post</td>
<td>0.19</td>
<td>0.01 (0.05)</td>
<td>- 0.41</td>
<td>0.6</td>
<td>0.72</td>
<td>0.18 ± 0.58</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>During</td>
<td>0.03 (0.04)</td>
<td>0.8</td>
<td>0.01 (0.05)</td>
<td>- 0.51</td>
<td>0.27</td>
<td>0.71 ± 0.99</td>
</tr>
<tr>
<td>Mean Body Size</td>
<td>2</td>
<td>During</td>
<td>&lt;0.001 (0.25)</td>
<td>0.89</td>
<td>0.94</td>
<td>0.2</td>
<td>0.84</td>
<td>0.25 ± 0.63</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Post</td>
<td>&lt;0.001 (0.16)</td>
<td>0.55</td>
<td>0.09 (0.02)</td>
<td>0.59</td>
<td>0.47</td>
<td>0.65 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>During</td>
<td>0.13</td>
<td>0.27</td>
<td>0.07 (0.03)</td>
<td>0.94</td>
<td>0.14</td>
<td>0.19 ± 0.33</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>Post</td>
<td>0.02 (0.05)</td>
<td>0.003 (0.07)</td>
<td>0.31</td>
<td>0.16</td>
<td>0.61</td>
<td>0.19 ± 0.56</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>During</td>
<td>&lt;0.001 (0.12)</td>
<td>0.89</td>
<td>0.99</td>
<td>0.71 ± 0.86</td>
<td>0.6</td>
<td>0.74</td>
</tr>
<tr>
<td>Mean Chironomidae Body</td>
<td>2</td>
<td>During</td>
<td>&lt;0.001 (0.18)</td>
<td>0.54</td>
<td>0.69</td>
<td>0.42</td>
<td>0.75 ± 0.29</td>
<td>0.89 ± 0.89</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Post</td>
<td>&lt;0.001 (0.12)</td>
<td>0.95</td>
<td>0.3</td>
<td>0.51</td>
<td>0.44</td>
<td>0.56 ± 0.63</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>During</td>
<td>0.02 (0.05)</td>
<td>0.91</td>
<td>0.11</td>
<td>0.15</td>
<td>0.47</td>
<td>0.63 ± 0.04 (0.04)</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>Post</td>
<td>0.32</td>
<td>0.03 (0.04)</td>
<td>0.99</td>
<td>0.22</td>
<td>0.96 ± 0.53</td>
<td>0.72 ± 0.72</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>During</td>
<td>0.002 (0.08)</td>
<td>0.94</td>
<td>0.86</td>
<td>0.78</td>
<td>0.51 ± 0.63</td>
<td>0.63 ± 0.39</td>
</tr>
<tr>
<td>Small Emergers (&lt;5 mm)</td>
<td>2</td>
<td>During</td>
<td>&lt;0.001 (0.18)</td>
<td>0.44</td>
<td>0.02 (0.05)</td>
<td>+ 0.19</td>
<td>0.41</td>
<td>0.14 ± 0.67</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Post</td>
<td>&lt;0.001 (0.14)</td>
<td>0.11</td>
<td>0.001 (0.08)</td>
<td>+ 0.62</td>
<td>0.91 ± 0.02 (0.05)</td>
<td>± 0.37</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>During</td>
<td>0.01 (0.05)</td>
<td>0.048 (0.03)</td>
<td>0.16</td>
<td>0.05 (0.03) ± 0.34</td>
<td>0.73 ± 0.16</td>
<td>0.16 ± 0.16</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>Post</td>
<td>0.71</td>
<td>0.19</td>
<td>0.005 (0.06)</td>
<td>0.13</td>
<td>0.28</td>
<td>0.21 ± 0.52</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>During</td>
<td>0.04 (0.04)</td>
<td>0.55</td>
<td>&lt;0.001 (0.40)</td>
<td>- 0.64</td>
<td>0.1</td>
<td>0.21 ± 0.21</td>
</tr>
<tr>
<td>Large Emergers (&gt;5 mm)</td>
<td>2</td>
<td>During</td>
<td>&lt;0.001 (0.18)</td>
<td>0.69</td>
<td>0.88</td>
<td>0.79 ± 0.88</td>
<td>0.65 ± 0.76</td>
<td>0.76 ± 0.76</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Post</td>
<td>0.001 (0.09)</td>
<td>0.89</td>
<td>0.23</td>
<td>0.89 ± 1</td>
<td>0.96 ± 0.96</td>
<td>0.96 ± 0.96</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>During</td>
<td>0.04 (0.04)</td>
<td>0.06 (0.03)</td>
<td>0.14</td>
<td>0.14 ± 0.04 (0.04)</td>
<td>± 0.23</td>
<td>0.42 ± 0.42</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>Post</td>
<td>0.005 (0.06)</td>
<td>0.007 (0.06)</td>
<td>0.8</td>
<td>0.21</td>
<td>0.61 ± 0.59</td>
<td>0.96 ± 0.96</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>During</td>
<td>&lt;0.001 (0.10)</td>
<td>0.23</td>
<td>0.003 (0.07)</td>
<td>- 0.11</td>
<td>0.21 ± 0.35</td>
<td>0.79 ± 0.79</td>
</tr>
</tbody>
</table>