THE EFFECTS OF WATER QUALITY ON THE TOXICITY OF PESTICIDES TO THE AUSTRALIAN NON-BITING MIDGE *CHIRONOMUS TEPPERI*.

Submitted by

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ABSTRACT

Environmental toxicology, or ecotoxicology, is the study of the effects of anthropogenic contamination on the environment, from singular organisms to whole ecosystems. Until recently laboratory aquatic environmental toxicology has largely been focused on investigating the effects of one or two contaminants on standard test organisms. But in recent years there has been a push to make ecotoxicology laboratory tests more indicative of the multiple stressors (e.g. salinity, nutrients, pH, temperature, metals, pesticides, pharmaceuticals) than can affect an aquatic ecosystem. There has also been more emphasis on developing ecotoxicity testing models with local species, particularly in countries outside of North America and Europe. This focus on local species with multiple stressors has been useful for regulators in Australia as they can now derive water quality guidelines based on more accurate information.

The aims of this thesis were to contribute to the increasing amount of information of Australian standard ecotoxicology organisms and their response to multiple stressors. Firstly, I investigated the effects of a single stressor (phosphorus) on the Australia standard ecotoxicology species *Chironomus tepperi*. This was in order to characterise the response of *C. tepperi* to elevated concentrations of phosphorus. I conducted laboratory bioassays investigating how both lower and higher concentrations of phosphorus affected the growth, emergence, and energy reserves of *C. tepperi* larvae. I conducted these bioassays in sediment and water to determine the whether there was any moderating effect of the sediment on the response of *C. tepperi* to phosphorus manipulation. In the water-only bioassay, the addition of P decreased emergence time and increased wing length in *C. tepperi* adults. In the sediment tests, the addition of P had a less clear effect on development but resulted in lower lipid concentrations in *C. tepperi* larvae compared to treatments with low P concentrations, suggesting utilisation of energy reserves.

The next chapter was an extension of the previous where I added both varying concentrations of the pesticide permethrin and P in sediment bioassays. I again examined the growth, emergence, and energy reserves response of *C. tepperi* to these exposures. In this chapter, I found that at very high permethrin concentrations, an increased concentration of P did not alter the toxicity and there was still a reduction in survival after 96 hours and the proportion emerged. However, P did alter the effects of permethrin on emergence day and on the energy reserves in *C. tepperi* larvae after 96 hours.

In the last data chapter, I investigated the effects of salinity on the toxicity of a different pesticide, imidacloprid. Salinity was chosen as salinisation is an increasing problem in fresh waters, and imidacloprid is a pesticide still in heavy use in many places around the world. In this chapter I used water-only bioassays to investigate the effects of salinity on imidacloprid toxicity to *C. tepperi*. Overall, increasing salinity and increasing imidacloprid concentrations had a negative effect on the survival and emergence of *C. tepperi*. This effect was shown in both acute (96 hour) and chronic (15 day) bioassays. In energy reserves there was an overall increase in glycogen, lipid, and protein concentrations in response to the combination of stressors.

This thesis overall shows that there is a need to investigate how water quality can affect the toxicity of commonly used pesticides. This is not only important in ecotoxicology to better understand the model organisms and their response, but it is also useful in for regulators as there is a need to understand how local aquatic conditions will increase or decrease the toxicity of a pollution event.

DECLARATION

This is to certify that:

i. The thesis comprises only my original work towards the PhD

ii. Due acknowledgement has been made in the text to all other material used

iii. The thesis is less than 100,000 words in length, excluding tables, maps, bibliographies, and appendices.

Molly N Hoak

April 2019

PREFACE

This thesis comprises one introduction chapter, three manuscripts, one discussion chapter and an appendix.

Chapter 1: General Introduction

Chapter 2:

Molly N. Hoak, Sara M. Long, and Vincent J. Pettigrove (in preparation). The effects of phosphorus on the development of the Australian midge *Chironomus tepperi* in water and sediment toxicity tests.

The major research of this manuscript is my own work. Other co-authors provided scientific advice, training in laboratory and data analysis techniques, and review of the manuscript.

Chapter 3:
Molly N. Hoak, Sara M. Long, and Vincent J. Pettigrove (in preparation). The effects of phosphorus on the toxicity of permethrin to the Australian midge, *Chironomus tepperi*.

The major research of this manuscript is my own work. Other co-authors provided scientific advice, training in laboratory and data analysis techniques, and review of the manuscript.

Chapter 4:
Molly N. Hoak, Robin Hale, Sara M. Long, and Vincent J. Pettigrove (in preparation). The effects of salinity on the toxicity of imidacloprid to the growth and development of the Australian midge *Chironomus tepperi*.

The major research of this manuscript is my own work. Some of the results of the conductivity only portion of the exposures, as well as an extra conductivity-only treatment, were reported in "Robin Hale, Valentina Colombo, Molly Hoak, Vin Pettigrove, Stephen E. Swearer. 2019. The influence of potential stressors on oviposition site selection and subsequent growth, survival and emergence of the non-biting midge (Chironomus tepperi). *Ecology and Evolution*. DOI: https://doi.org/10.1002/ece3.5148". Robin Hale assisted with the set up and pack down of the laboratory exposures. Other co-authors provided scientific advice, training in laboratory and data analysis techniques, and review of the manuscript.

Chapter 5: General Discussion

Appendix A: Problems with using Nitrate in chronic toxicity testing with *Chironomus tepperi*
The major research of this appendix is my own work. Vincent Pettigrove and Sara Long provided scientific advice, training in laboratory techniques and data analysis, and review of the writing.

Appendix B: Chemical analysis for sediment used in tests

Appendix C: ANOVA, Kruskal-Wallis, and Tukey Tables

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I dedicate this PhD to my family. Thank you to my extended family for supporting me in whatever way you could and I'm glad you all know what a Chironomid is now. Thank you to my grandparents on both sides of the family. I started this process with three of you and ended it with one, but this whole thesis is the result of your dedication to fostering my love of knowledge and learning as a child. Thank you to my little sister who provided love and support when it was most needed. And thank you most especially to mum and dad who fostered my love of science and biology when I was a kid. I wouldn't have started this process without your support, and I wouldn't have finished it without it either. You bought me my first microscope and it was all downhill from there.

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CHAPTER 1: GENERAL INTRODUCTION

There are large fields of study dedicated to the investigation of human impacts on the environment. When 90% of the human population lives within 10 km of freshwater bodies (Kummu et al. 2011), it is no surprise that many of those studies are often focused on effects on aquatic ecosystems. It has been established that anthropogenic pollution poses a widespread threat to the health of aquatic ecosystems around the world (Foley et al. 2005). In most aquatic environments, the ecosystem undergoes stress from multiple anthropogenic and natural sources. Many different types of pollutants can affect the ecological health of waterways, including pesticides, metals, and pharmaceuticals (Laws 2017; Pascoe et al. 1989; Schäfer et al. 2011; Smith et al. 1999) and features of water quality such as nutrients, salinity, pH, and water temperature can also be detrimental (El-Sheekh 2017; Hasler 1947; Jackson et al. 2015; Laws 2017). This is further complicated by the fact that the movement of water throughout a catchment or river system means that pollutants can be dispersed over dozens or hundreds of kilometres, so that one source of a pollutant has the potential to mix with many others, altering habitats in a myriad of ways (Allan 2004).

The field of environmental toxicology, or ecotoxicology, is the study of the effects of anthropogenic contamination on the environment, from singular organisms to whole ecosystems. Until recently laboratory aquatic environmental toxicology has largely been focused on investigating the effects of one or two contaminants on standard test organisms. The relationship between mixtures of contaminants have been classified into “synergistic” (when exposure causes toxicity greater than the effects of the individual chemicals) and “antagonistic” (when toxicity is less than their individual effects). In recent years there has been a push to make ecotoxicology laboratory tests more indicative of the multiple stressors (e.g. altered flow regimes, salinity, nutrients, pH, temperature, metals, pesticides, pharmaceuticals) than can affect an aquatic ecosystem (Chapman 2002). There has also been more emphasis on developing ecotoxicity testing models with local species, particularly in countries outside of North America and Europe (Lacher Jr and Goldstein 1997; Mehler et al. 2017). The results of ecotoxicity tests are used by governments to inform their regulations for the protection of the environment (e.g. sediment and water quality guidelines).

The mixing of multiple stressors and different pollutants have long posed two major problems for the field of environmental toxicology:

1) From a research perspective, how do we incorporate local conditions into our generalised evaluation of the toxicity of pollutants?

2) From a regulatory perspective, how do we create environmental water and sediment quality guidelines when there are multiple stressors that can affect each other and the ecosystem?

1.1 How do we incorporate local conditions into laboratory ecotoxicology research?

The question of how to make environmental toxicity research both controlled and as environmentally relevant as possible has been asked for almost twenty years (Chapman 2002; Newman and Clements 2008). How do we model natural phenomenon as closely as possible while also being able to control enough variables to draw a reasonable conclusion? This is particularly important in environmental toxicology research as human impacted ecosystems are always affected by more than one problem. Additionally, the results of these studies are used as a basis for regional regulation of water and sediment quality, and so should reflect the real world as much as possible.

With climate change already altering the environment our need to fully understand how multiple stressors will affect the response of ecosystems is vital for predicting future changes and impacts. Freshwater ecosystems seem to be facing an increasing extinction risk (Koehn and Lintermans 2012; Revenga et al. 2005; Strayer and Dudgeon 2010) and as a result we must better understand how the toxicity of what are traditionally thought of as pollutants (i.e. chemicals, such as pesticides, metals, pharmaceuticals) are affected by other water quality characteristics (e.g. temperature, salinity and nutrients). In much of the literature on multiple stressors a wider definition of "toxicity" is used than in traditional ecotoxicity testing. Under traditional definitions "toxicity" refers to two stressors interacting to affect an organism; however the multiple stressor literature uses "toxicity" to refer to the effect of the pollutant on the organism, which can be heightened or lessened by aspects of water quality.

Much of the work that investigates how the toxicity of pollutants is affected by water and sediment quality has been based on models that extrapolate from relatively simple laboratory exposures in order to predict the complex exposures found in the field (Warne et al. 2018). There has been work into how water quality parameters affect metal toxicity primarily using Biotic Ligand Models (BLM) although other toxicokinetic and mechanistic effect modelling have been investigated (Paquin et al. 2002; Preuss et al. 2009). Much of the research in this area has been around metals, however, with much less attention paid towards pesticides and other classes of pollutants. These models are generally used in risk assessments to predict the toxicity of a metal to the ecosystem being investigated. They incorporate the physiology of the organism being considered as well as the chemistry of the metal and its interaction with other aspects of water quality (i.e. pH, hardness, alkalinity), which all result in a toxicity value to be used for regulatory or research purposes.
Additionally, there have been many studies investigating the effects of temperature and/or pH on the toxicity of pesticides or other pollutants (Brown 1987; Cheng and Chen 2000; DeLorenzo et al. 2009; Harwood et al. 2009; Sarkar et al. 1999; Valles et al. 1998; Weston et al. 2009b). It is unsurprising that these aspects of water quality are researched more often as they are some of the more obvious effects of global climate change, and some of the easiest parameters to measure and manipulate.
There have also been some studies that have examined how multiple stressors affect aquatic ecosystems on a global scale (Jackson et al. 2015). Unsurprisingly, these general studies highlight the need for research into multiple stressors at the local scale. The meta-analysis from Jackson et al. (2015) found that a large portion of studies show an antagonistic relationship between pairs of stressors (41%) indicating that the multiple stressors in freshwater systems are not necessarily increasing toxicity to aquatic ecosystems. They compared a broad range of stressors including acidification, contamination, habitat alteration, invasion, eutrophication, ultra-violet radiation, and global warming. However, a large limitation with this study is that the research and supplementary information from Jackson et al. (2015) do not mention a geographic bias. The majority of the research into multiple stressors has been carried out in Europe and North America, and therefore the methods are standardised with locally endemic species (OECD 2010; USEPA 2002). This makes research into the effects of multiple stressors on Australian species all the more relevant.
Incorporating local conditions into ecotoxicology research has become increasingly important. Australia's environment is unique as it is the driest inhabited continent on earth (Khan 2008) and the research methods and assumptions applied overseas (and largely in the Northern Hemisphere) cannot necessarily be applied in Australia. The evolutionary history and adaptation ability of a species can create a difference in their response to environmental stressors. For instance, in a review of marine species response to metals, Chapman et al. (2006) found that there were significant differences in

response between species from different regions, and they concluded that universal guideline values are not necessarily appropriate for all species in all climates. They also found that temperature was not the explanatory factor for these differences in species response, but differences in water quality that altered metal toxicity. This difference in response was supported by King and Riddle (2001) who exposed an Antarctic sea urchin (*Sterechinus neumayeri*) to copper, cadmium, zinc, and lead and compared the response to tropical and temperate species. They found that when using comparative endpoints for a test, rather than time limits, the Antarctic species was more sensitive to copper and cadmium than temperate or tropical species. However, to date there have been no broad comparisons of toxicity data from Australia to Northern Hemisphere species. This lack of assessment of local taxa toxicity has been identified as a priority research question in Latin America (Furley et al. 2018) where they face similar problems with a paucity of local species toxicity data especially from testing under local conditions.

Much of the aquatic research in Australia has focused on marine environments. This is not surprising considering approximately 80% of the Australian population live within 25 km of the coast (Chen and McAneney 2006). However, all of Australia's major cities are built around freshwater environments, particularly major rivers, and there has been less focus on how an increasingly urbanised population is changing those freshwater resources. Freshwater ecotoxicology using Australian test species has only become standardised in the last 20 years (Batley and Simpson 2016; Gagliardi et al. 2015; Mehler et al. 2017; Stevens et al. 2002; Vu et al. 2015) and as a result we are just starting to get a better understanding of the effects of multiple stressors on the Australian environment.
In regard to multiple stressors there are issues in Australian waters that have been identified as concerning, particularly nutrients and salinity (Matthaei and Piggott 2019). When combined with an increasing population, high concentrations of nutrients and salinity are likely to occur with contaminants associated with human population and urbanisation (i.e. pesticides, metals, PAHs, and pharmaceuticals). The work outlined in this thesis is an attempt to establish whether these stressors should be incorporated into toxicity tests in Australia, and particularly how they alter the response of organisms to toxicants. For this thesis, phosphorus and salinity were chosen as examples of 'natural stressors' likely to interact with anthropogenic stressors in Australian waterways; however there are many other combinations of stressors that could be examined in future.

1.2 How do we create guidelines with multiple stressors?
This second problem for the development of water and sediment quality guidelines and regulations is largely informed by how we incorporate local conditions into ecotoxicity testing. As scientists we can only give governments and regulatory agencies the research we have conducted, and as a result we need to first incorporate multiple stressors into our research. Having a better understanding of the effects of water quality on contaminant toxicity will lead to more realistic guidelines.
Some governments have acknowledged that multiple stressors and local conditions need to be considered in assessing contamination and its impacts on aquatic environments. In the European Union Environmental Quality Standards (EQS) water quality parameters such as water hardness and, pH, need to be measured when assessing metal contamination (SCHEER 2018). This is because there has been extensive research into how water quality affects the toxicity of metals (Paquin et al. 2002). In Australia, most of the national and state waterway management standards and allowable toxicant concentrations are based on values from European or North American tests with species endemic to those regions, which are not necessarily relevant in an Australian context (ANZECC and ARMCANZ 2018).

The Australian and New Zealand Environmental and Conservation Council (ANZECC) water quality guidelines have only recently been updated to include more local data and species (ANZECC and ARMCANZ 2018). Because of this lack of local data there has been a concerted effort by Australian researchers and government agencies to standardise Australian ecotoxicology model species and methods that are applicable to local environments (Batley and Simpson 2016). There have been some attempts to account for diverse ecosystem specific water quality measures (SEPP(Waters) 2018), however these are often quite generalised and only include a few measures of water and sediment quality. In the above State Environment Protection Policy (Waters) the Australian state of Victoria has divided the state into five inland freshwater areas, each with different protection and water quality requirements. This has allowed for finer granularity in the way water quality and pollution objectives are measured and regulated. However, these are not a replacement for site-specific understandings and there is a clear need for better information on how water quality measures affect the toxicity of frequently detected pesticides and other common contaminants in Australian waters.

1.3 Energy Reserves

There is a consistent need in aquatic ecotoxicology and biological monitoring for tests that are relatively cheap and relatively quick. This is particularly true for regulatory purposes where budgetary constraints are a large factor. In order to get a sense of the environmental impact of a pollutant it's best to assess the invertebrate community, however, in an assessment of the invertebrate community the primary stressors affecting them cannot be identified and other tests to establish causality are needed. From a purely scientific perspective it is also essential to understand how stressors affect the organisms that are being monitored or that are used in standard ecotoxicity testing. One way environmental toxicology researchers have come up with to answer these questions is through measuring energy reserves. These are biomarkers chosen as a way of assessing how much stress the organism is experiencing as a result of a toxicant or altered water quality.

Most insects store their energy in the form of protein, lipid, or glycogen and energy is used and moves between these forms depending on development, stress, or species (Arrese and Soulages 2010). For instance, in some insects that must remain airborne for many hours (e.g. locusts) lipids are the preferred energy source for flight, however in other species with shorter flight times carbohydrates are used as the energy source (Beenakkers et al. 1985). In ecotoxicology, energy reserves have been used in many different invertebrates to assess the stress and potential growth and development of the organism (De Coen and Janssen 1997; Hahn 2005; Sak et al. 2006). The measurement of energy reserves and energy use has primarily been developed as a short-term toxicity bioassay that allows researchers to gain a better understanding of how an organism responds to exposure to a toxicant.

This method has so far mostly been used in ecotoxicology in testing either pollutants such as pesticides or metals (Choi et al. 2001; Nunez-Nogueira et al. 2010; Sak et al. 2006) or for other stressors such as salinity, temperature, and nutrition (Hahn 2005; Kleinman et al. 1996; Lee et al. 2017). The measurement of energy reserves have been found to be useful for predicting longer-term toxicity (De Coen and Janssen 1997; Smolders et al. 2004), however, they are more effective as an early indicator of stress when used in conjunction with other biomonitoring tools (Sokolova et al. 2012).

Most of the work on energy reserves has looked at a singular stressor (Encomio and Chu 2000; Hamburger et al. 1995; Nyman et al. 2013). For instance, Sak et al. (2006) showed that wasps (*Pimpla turionellae*) raised on moth larvae dosed with cypermethrin had much lower glycogen, lipid, and protein levels compared to the controls. This result was more prevalent in female glycogen levels and that earlier life stages were more susceptible to changes in energy levels than later life stages. These

sorts of results are not unusual when looking at a single stressor. However, there is comparatively less work on effects of multiple stressors on energy reserves. Work from Muyssen et al. (2010) looked at the effects of temperature and cadmium on *Daphnia magna* using a variety of endpoints and found increased temperature and cadmium concentration decreased energy reserves. However, they did not find a synergistic reaction and there were slight differences in responses between lipid, protein, and glycogen. These lack of simplicity in these responses are possibly why there has been less research into multiple stressor effects on energy reserves, but also why this research is sorely needed.

1.4 Thesis aims and overview

Aims

This thesis had several aims:

To assess the effects of phosphorus on the survival, development, and energy reserves of *Chironomus tepperi*

To investigate how phosphorus alters the toxicity of permethrin to *Chironomus tepperi*

To examine the effects of salinity on *Chironomus tepperi* and whether salinity alters the toxicity of the insecticide imidacloprid.

Thesis summary

Chapter 1: General Introduction

A literature review of water quality effects on the toxicity of pesticides that identifies gaps in the literature.

Chapter 2

Phosphorous and nitrogen were chosen as water quality parameters to be investigated initially. There is not much information on the effects of nutrients on *Chironomus tepperi (*Australian non-biting midge) therefore, this chapter was designed to gain a better understanding of how phosphorus affects its lifecycle and energy reserves. Phosphorus was chosen because it is naturally found in low concentrations throughout Australia (Harris 2001) but is elevated through a variety of anthropogenic activities. Phosphorus is also the main contributor to eutrophication, a problem in aquatic environments throughout the world.

Chapter 3

This chapter extends the phosphorus research in Chapter 2 by introducing a pesticide. The pesticide chosen for these experiments was permethrin, a synthetic pyrethroid insecticide commonly used in urban and agricultural areas both within Australia and throughout the world (Marshall et al. 2016; Wang et al. 2012; Weston et al. 2009a). Synthetic pyrethroids are increasingly detected in areas where there are also likely to be water quality issues (such as high salinity or nutrients). The purpose of this chapter was also to extend the knowledge of previous work using permethrin and other synthetic pyrethroids, which have been found throughout the Melbourne, Australia region and are known to cause developmental effects in *C. tepperi* (Boyle et al. 2016).

Chapter 4

This chapter moves the focus from nutrients to another important water quality parameter likely to impact aquatic environments, salinity, and investigates how this can affect the toxicity of another common pesticide, imidacloprid. Imidacloprid was chosen largely for practical reasons as it is water soluble and salinity is much easier to manipulate in water and can be altered by the addition of sediment to the test system. Salinisation is a known issue throughout the world, and particularly in

Australia (Nielsen et al. 2003). With increasing salinity there is greater uncertainty as to how ecosystems and organisms will adapt to this changing environment. Most organisms have an ideal osmotic range outside of which they experience stress (Kefford et al. 2012). This has been shown with white-leg shrimp (*Litopenaeus vannamei*), that had a much higher acute toxicity to a pesticide when salinity was low (Wang et al. 2013) and are likely to be shown in other invertebrates. This is an indication that when an organism is dealing with stress from one stressor, such as high or low salinity, it may be less able to deal with stress from a pesticide or other stressor. With a warming climate Australia's water resources will become more prone to salinisation (Nielsen and Brock 2009). Therefore, is it important to understand whether increased salinisation of water bodies will affect the toxicity of pesticides and other substances.

Imidacloprid (a neonicotinoid insecticide) is used widely and it is still in use throughout Australia (Smith et al. 2012)**.** Although neonicotinoid insecticide use is being either banned or severely restricted in the EU (Commission 2013) they are still commonly detected in water quality assessments within Australia and North America (Smith et al. 2012; Starner and Goh 2012) Imidacloprid has previously been tested on *C. tepperi* before under various conditions (Gagliardi 2017; Stevens et al. 2002) and it has been found to be relatively toxic with an LC50 of 1.60 μg/L. The research from Gagliardi (2017) and Stevens et al. (2002) formed the basis for the imidacloprid tests in this chapter.

Chapter 5: General Discussion
The general discussion provides an overview of the main findings and recommends further discussion.

Appendix A
This thesis originally planned to include a chapter on nitrogen and its effects on life cycle responses in *C. tepperi*, however the nitrogen was unstable over the period of testing. A constant nitrogen concentration could not be maintained, which meant that the larvae were only being exposed to the appropriate nitrogen concentrations at the beginning of the experiment and not throughout the whole exposure. Although this work was done, it was not written into a full chapter as the results were not statistically analysed. However, the methods, results and the rationale for ending the experiments can be found in Appendix A.

Appendix B
The chemical analysis of the sediment from Bittern, Victoria, Australia used in the sediment bioassays throughout this thesis.

Appendix C
ANOVA, Kruskal-Wallis, and Tukey test tables for all statistical tests reported.

CHAPTER 2: THE EFFECTS OF PHOSPHORUS ON THE DEVELOPMENT OF THE AUSTRALIAN MIDGE *CHIRONOMUS TEPPERI* IN WATER AND SEDIMENT TOXICITY TESTS

2.1 Introduction

Nutrients (such as nitrogen, phosphorus and carbon) are a vital part of aquatic ecosystems, however, excessive levels of nutrients in aquatic environments around the world from anthropogenic activities including from agriculture, urban areas and wastewater are causing environmental harm (Allan 2004). Fertilizers are a major source of anthropogenic nutrient pollution (Vitousek et al. 1997) and phosphorus-based fertilizers have been found to cause eutrophication on a large scale in waterways throughout the world (Smith 2003).

High phosphorus (P) concentrations in freshwaters, together with sufficient concentrations of other nutrients, will lead to excessive plant growth or algal blooms, which deplete dissolved oxygen concentrations and can create an anoxic environment leading to fish kills and general ecological degradation (Anderson et al. 2005; Elser et al. 2009; Elser et al. 2001; Schindler 1974). Conversely, having little P present in a freshwater system can limit plant growth and so the flora can change, and the fish biomass can be reduced, especially when transitioning from a formerly eutrophic system (Anderson et al. (2005).

Phosphorus and other nutrients are often very low in Australian waters and soils compared to elsewhere in the world (Harris 2001), and as a result, much of the agricultural land has been fertilised with P fertilisers to improve the productivity of pastures and crops. Williams and Hang Fong (1972) found that P was present at elevated concentrations in most human-impacted lakes in Australia and more recent data indicates that many freshwater sources in south-eastern Australia still exceed current guidelines (2015). This makes it particularly important to examine how local Australian species respond to changes in P concentrations.

Because the indirect effects of P are widely known, many of the studies on the effects of P on macroinvertebrates are field-based, where macroinvertebrate assemblages and water quality parameters are compared (Struijs et al. 2011; Vermonden et al. 2009). These studies highlight that some organisms are better adapted to P-enriched waters than others as the presence of some macroinvertebrates changes with increasing or decreasing P concentrations.

It is important, however, to examine the effect of P on individual organisms as P is associated with many important processes such as cell signalling, DNA and protein synthesis, and energy and metabolism (Nation 2008). Lipids, carbohydrates (in the form of glycogen) and protein provide the major energy resources in insects. Excess P may cause toxic effects by shifting the molecular balance in certain energy reactions. For instance, in insects organic P (as α-glycerophosphate) is a crucial component in the production of phospholipids (a component of cell membranes) and triacylglycerols (the storage form of lipid) (Nation 2008); too much or too little will result in an imbalance of either molecule, which may have detrimental effects to the organism.

Much of the work on the effects of P enrichment on aquatic invertebrates has been through altering the diet. Elser et al. (2001) enriched the food of *Daphnia dentifera* and found that the higher concentrations of dietary P increased growth rates. Similarly, Meng et al. (2014) examined the effects of dietary P on *Daphnia similoides* and *Moina irrasa* and found that population density in both species increased with increasing P. In addition, growth and development positively correlated with increasing P in food. This pattern of increased growth rate with increasing P was also found in a field study by Ramírez and Pringle (2006) who reported faster growth rates in larval Chironomidae in streams that were either artificially enriched or had naturally high P concentrations. Phosphorus limitation in the diet of *Potamopyrgus antipodarum*, a freshwater mud snail, resulted in a decreased growth rate, and the authors suggested that adaptation to P can affect the resilience of populations to a subsequent change in P (Krist et al. 2014). Therefore, an increase in P should increase insect growth rates (Arrese and Soulages 2010.

Our laboratory bioassays were conducted using *Chironomus tepperi,* a native Australian freshwater midge species. Chironomid species, particularly those from the genus *Chironomus*, are commonly used as test species in aquatic ecotoxicology worldwide as they have a cosmopolitan distribution, are highly abundant, can be cultured in the laboratory, and – importantly for ecology – are a food source for many organisms (Maher and Carpenter 1984). The use of *Chironomus tepperi* in ecotoxicology has recently become standardised for both acute and chronic water and sediment toxicity tests (Batley and Simpson 2016; Boyle et al. 2016; Kellar et al. 2014; Townsend et al. 2012). Northern hemisphere species such as *Chironomus riparius* and *Chironomus dilutus* (formerly known as *C. tentans*) are fully standardised and are routinely used in OECD and US EPA guidelines (OECD ; USEPA 2002).

Martin and Porter (1978) described the laboratory biology of *C. tepperi*, but there are relatively few papers that have examined the ecology and field biology of this species. Stevens et al. (2006) reported that *C. tepperi* were usually the first chironomid species to colonise newly flooded rice fields in southern New South Wales, but often only for one generation and would not continue to breed in the same field. This is an indication that this species is largely ephemeral and opportunistic, and so maybe more tolerant of changes in water quality. Because *C. tepperi* capitalise on newly inundated areas, they should respond to differences in nutrient levels, and therefore the amount of available food. However, even though there are standard water and sediment tests for *C. tepperi,* there has been no investigation into how nutrients in sediments or water may affect toxicity. Based on studies of growth in other insects, we hypothesize that there will be more energy available for chironomids that have been exposed to higher concentrations of P. This increase in energy availability may increase their growth rate and decrease the development time from larvae to adults. However, it is clear that there is little understanding of the effects of P when it is introduced in a controlled laboratory system through spiking the water to mimic a pulse event, rather than manipulation of the diet.

This study aims to assess the effects of P addition to water and sediment on the development of *C. tepperi*. The secondary aim of this study is to compare *C. tepperi* responses between water-only and sediment/water bioassays. We expect to find that P will decrease the development time of *C. tepperi* but have a limited effect on survival or energy reserves. The investigation of the effects of P on the development time as well as the energy resources of the organism will add to the current understanding of this nutrient on this species.

2.2 Methods

2.2.1 Chironomid Culture and Preparation

Chironomus tepperi were cultured to 5 days old in a modified artificial water solution (as described in Jeppe et al. (2014)), with ethanol-rinsed toilet paper as substrate. Larvae were fed tropical fish flakes (Tetramin®) three times per week, with 0.25 mg per tank provided at each time point.

2.2.2 Preparation of phosphorus solutions

Phosphorus treatments were prepared using a modified artificial water solution without KH_2PO_4, which is usually present at a concentration of between 0.15 and 0.5 mg/L P. Therefore, this concentration was the Control treatment. Phosphorus as KH_2PO_4 was added to create a range of P concentrations in the treatments. The concentrations of P used for the emergence water-only bioassay were 0, 0.0.17 (Control), 1.6, 3.62, 6.75, 13.1, and 25 mg/L. For the 96-hour and emergence sediment bioassays, the concentrations used were 0, 0.17 (Control), 1.6, 3.62, and 13.1 mg/L. These concentrations were chosen based on an examination of the literature and of known concentrations of phosphate present in waters and sediments in south-eastern Australia (Water 2015). The purpose of the highest concentration was to include a potentially toxic concentration of P to *C. tepperi*. These solutions were measured for Total Phosphorus (TP) as P and Total Reactive Phosphorus (TRP) as P by an external commercial laboratory (ALS Environmental, Ringwood, Victoria, Australia).

ALS Methods and QA/QC

TP and TRP in water and sediment were determined by a method based on APHA 21st Ed. 4500-P (APHA 2005) with some modifications taken from Jirka et al. (1976) and Zhang and Kovar (2009). Briefly, a sample aliquot was digested with sulphuric acid to form orthophosphate, then ammonium molybdate and potassium antimonyl tartrate were reacted in an acid medium with orthophosphate to form phosphomolybdic acid which is reduced to molybdenum blue by ascorbic acid. For TP in water, this was measured at 880nm using a discrete analyser. For TRP in all mediums and TP in soil, molybdenum blue was quantified by flow injection analysis. QA/QC procedures involved one laboratory control sample, one method blank, one matrix spike and two duplicates for each group of 20 samples. Standard solutions were prepared with KH_2PO_4. Matrix spike recovery was between 74 and 100% which was within the recovery limits.

2.2.3 Sediment collection and preparation

Whole sediment was collected from a wetland in Bittern, Victoria, Australia—an unpolluted site approximately 100 km south-east of Melbourne (Appendix B). The top 2 cm of sediment was collected by shovel and brought back to the University of Melbourne where it was filtered with tap water through a 63 µm nylon net into a clean 10 L plastic bucket, as described in Boyle et al. (2016). Sediment was sent to ALS Environmental (Ringwood, Victoria, Australia) for measurement of pesticides, metals, and nutrients concentrations (Appendix B).

2.2.4 Exposures

Water-only bioassay

Each 600 mL beaker contained 400 mL of treatment or Control water, with 8 replicates per treatment. One sheet of 2-ply toilet paper rinsed in ethanol was added as substrate for the larvae. Ten 2nd instar *C. tepperi* larvae were randomly added to each beaker with two drops of Tetramin® food preparation (100 mg tetramin per 100 ml reverse osmosis water), which was given twice a week and one drop after emergence began. The treatments were maintained in a temperature-controlled room at 21^{o}C

with 16:8hr L:D cycle with aeration provided and fine mesh stockings placed over each beaker to contain emerged adults. The water level was topped up three times a week with deionized water to counteract evaporation and ensure the salts in the artificial water did not become too concentrated. Emerged adults were collected daily. The numbers of males and females were recorded and preserved in Eppendorf tubes in 70% ethanol. At the end of the experiment water was filtered through a 250 µm sieve and any surviving larvae or pupae were collected. Sex and proportion of adults emerged were recorded, and mean emergence day was calculated.

The water was pooled by treatment and water quality parameters of pH, temperature, and conductivity were measured (Table 1). A sample of this water was sent to a commercial laboratory for analysis of TP and TRP (ALS Environmental, Australia).

Emerged adult wing lengths were measured using a microscope, with a graticule, from the arculus (near wing base) to the tip of the wing, following the methods of Boyle et al. (2016) and Frouz et al. (2002). Both wings were measured, and the mean recorded. Wing lengths were only measured for water bioassays, as they are used as a measure of adult body size (Xue and Ali 1994) and could provide an explanation for different emergence times. As wing length measurements are a lengthy procedure, these were only done when there were significant differences in emergence day.

Sediment Bioassays

140 g of sediment was added to 24 acid-washed, oven-baked, clean 600 ml beakers. Artificial water with P concentrations of 0, Control (0.17), 1.6, 3.62, and 13.1 mg/L were prepared and used as the overlying water for the sediment with 8 replicates per treatment and 12 for the Controls. The P-spiked overlying water was added to the sediment and the beakers were randomly placed onto shelves in a temperature-controlled room at 21^{o}C with 16:8hr L:D cycle for 24 hours, to allow P to become incorporated into the sediment.

After 24 hours ten 2nd instar larvae were randomly added to each beaker and aeration was provided. The larvae were fed two drops of Tetramin® food slurry twice a week and one drop when emergence was detected. Beakers were covered with fine mesh stockings and larvae provided with aeration for the duration of the test.

96-hour sediment bioassay

After 96 hours, 4 replicates were randomly removed from each treatment and 8 replicates were removed from the Control. The sediment was filtered through a 250 µm sieve and larval survival was recorded. Four larvae from each replicate were snap frozen for biomarker determination and stored at -80^{o}C until analysis and the remaining larvae were put into microcentrifuge tubes with 70% ethanol for growth determination. The overlying water from each treatment was pooled and temperature, pH, salinity, and dissolved oxygen were measured using a water quality meter (TPS Instruments, Queensland, Australia). Phosphate was measured at the beginning and end of the test by a commercial laboratory for analysis of TP and TRP (ALS Environmental, Australia).

Emergence Sediment Bioassay

Emerged adults were collected daily. The numbers of males and females were recorded and preserved in Eppendorf tubes in 70% ethanol. At the end of the experiment, sediment was filtered through a 250

µm sieve and any surviving larvae or pupae were collected. Sex and proportion of adults emerged were recorded, and mean emergence day was calculated.

The pH, temperature (ºC), dissolved oxygen (%), and conductivity (µS/cm) were measured weekly throughout the test (Table 2). A sample of sediment was sent to ALS Environmental (Australia) pre- and post-test to measure Total and Reactive Phosphorus.

2.2.5 Weight determination

Larvae collected at 96 h were placed onto pre-weighed tin trays and ethanol was allowed to evaporate, following this they were dried at 60 °C for 24 hrs and then weighed to determine dry weight. Dry weight is expressed as mg/larvae.

2.2.6 Phosphorus mass balance

To ascertain that the P addition to water would be the most significant part of added P, a mass balance calculation of the P in a 600 ml beaker sediment-water system was undertaken. This mass balance was calculated for one Control treatment with food added after 24 hours. For ease of calculation and measurement Total Phosphorus (TP) was measured. The sources of TP were the overlying water, sediment and food, and the sinks were uptake by phytoplankton and bacteria and uptake from *C. tepperi*. The phytoplankton and bacteria value was calculated based on a 41% figure from Boyd and Musig (1981).

2.2.7 Energy reserves

Protein Determination

One frozen larva per replicate was homogenised using a mixermill, (Retsch, MM 300, Retsch GmbH, Haan, Germany) in 50 µl ultra-pure water, centrifuged (Eppendorf 5804R, Eppendorf, Hamburg, Germany) at 14,000g at 4ºC for 5 minutes and the supernatant collected. Protein concentration was determined in triplicate using the DC Protein assay kit (BioRad), a modified Lowry assay (Lowry et al. 1951), using a Synergy 2 microplate reader (Biotek instruments), with bovine serum albumin as the standard.

Lipid and glycogen measurements

The lipid and glycogen analysis followed the methods of Vu et al. (2015), Van Handel (1985), and Plaistow et al. (2001), modified for use with chironomids. For each analysis, 1 frozen larva per replicate was used and analysis was done in triplicate. Briefly, for glycogen analysis larvae were homogenised in sodium sulphate solution, methanol and anthrone in sulfuric acid were used to determine glycogen content, and samples were read at 625 nm, with laboratory-grade glucose as the standard. For the lipid analysis, larvae were homogenised in a chloroform-methanol solution, sulfuric acid and vanillin reagents were used to determine lipid content and samples were read at 490 nm, with commercial vegetable oil as the standard. All analyses were carried out on a Synergy 2 microplate reader (Biotek Instruments) and each sample and standard were read in triplicate.

2.2.8 Statistical analysis

One-way analysis of variance (ANOVA) was carried out to determine whether P exposure significantly affected survival, growth (body weight and wing length), emergence and time to emergence ($\alpha = 0.05$).

If significance was observed, a Tukeys post hoc test was used to determine which concentrations were causing these differences. Proportion data (i.e. survival and chironomid emergence) were arcsine transformed prior to statistical analysis. All data were checked to ensure they conformed to the assumptions of analysis of variance by evaluating Skewness and Kurtosis values and Levene's Test for Equality of Error Variances. If data did not conform and was significant, a Dunnets T3 post-hoc test or Kruskal-Wallis test was carried out. All statistical analyses were carried out using SPSS, Version 22.

2.3 Results

2.3.1 Water Quality

Temperature, pH, conductivity and P concentration were measured in both the water-only and sediment bioassays and found to be within the acceptable limits for a viable *C. tepperi* test (Batley and Simpson 2016) (Table 1 and 2).

Table 1. Water quality data for the water-only emergence bioassay. Conductivity (µS/cm), pH, temperature (°C) and P concentrations (mg/L) were measured at the beginning and end of the experiment. Water from each treatment was pooled and measured. TP = Total Phosphorus; TRP = Total Reactive Phosphorus; PO4 = Phosphate concentration is calculated by taking TRP and multiplying by 3.006.

Water-Only Emergence Bioassay Water Quality								
	Date	**Treatments**	**TP**	**TRP**	**PO4**	**Temp.**	**pH**	**Conductivity**
Before Test	22/08/2014	0	<0.01	<0.01	N/A	21.1	6.81	206.1
		Control	0.17	0.17	0.521	21.3	6.65	186.4
		1.6	1.6	1.75	5.365	21.3	6.69	207.1
		3.62	3.62	3.25	9.964	21.3	6.72	205.3
		6.75	6.75	6.23	19.101	21.4	6.67	219.3
		13.1	13.1	13.1	40.165	21.5	6.55	240.8
		25	25	25	76.650	21.4	6.26	282
After Test	10/09/2014	0	0.85	0.26	0.797	20.8	7.48	210.1
		Control	0.99	0.45	1.380	20.7	7.24	198.8
		1.6	2.51	1.78	5.457	20.6	7.05	201.6
		3.62	4.04	2.98	9.137	20.8	7.00	203.5
		6.75	6.58	5.19	15.913	20.7	6.90	269
		13.1	11.5	9.51	29.158	20.7	6.82	228.3
		25	25.2	24.5	75.117	20.7	6.56	266

Table 2. Water quality for the sediment emergence bioassay. Water quality was measured weekly throughout the test. Values are Mean ± SD (n=4) for each treatment.

Date	Treatment	Temperature	pH	DO	Conductivity
20/07/2016	0	20.3 (± 0.2)	7.2 (± 0.0)	82.8 (± 11)	683.3 (± 151)
	Control	20.4 (± 0.3)	7.2 (± 0.0)	88.2 (± 11)	556.3 (± 14)
	1.6	20.3 (± 0.2)	7.0 (± 0.1)	94.2 (± 1.2)	559.8 (± 9.6)
	3.62	20.3 (± 0.2)	7.0 (± 0.0)	92.5 (± 1.6)	567 (± 28)
	13.1	20.2(± 0.1)	7.1 (± 0.0)	94.0 (± 0.9)	593 (± 17)
29/07/2016	0	21 (± 0.3)	7.7 (± 0.2)	91.9 (± 1.9)	703 (± 77)
	Control	21 (± 0.1)	7.8 (± 0.1)	90 (± 4.8)	736.3 (± 24)
	1.6	21 (± 0.1)	7.7 (± 0.1)	86.4 (± 3.2)	715 (± 22)
	3.62	21 (± 0.1)	7.7 (± 0.2)	85.0 (± 3.6	724.8 (± 37)
	13.1	20.1 (± 0.1)	7.5 (± 0.1)	91.7 (± 4.9)	725.3 (± 35)
01/08/2016	0	21.3 (± 0.1)	7.5 (± 0.2)	92.4 (± 0.8)	758 (± 122)
	Control	21.2 (± 0.1)	7.5 (± 0.1)	94.3 (± 2.0)	693 (± 22)
	1.6	21.3 (± 0.2)	7.5 (± 0.0)	94.2 (± 1.3)	700 (± 8.1)
	3.62	21.1 (± 0.2)	7.5 (± 0.2)	92.7 (± 1.1)	709.5 (± 81)
	13.1	21.1 (± 0.1)	7.3 (± 0.0)	94.7 (± 1.7)	707.8 (± 12)

2.3.2 Emergence Water-Only Bioassay

Percent Emergence

There was no significant effect of P concentration on C. *tepperi* percent emergence (data not shown, $F_{(6, 21)} = 2.184$, $p > 0.05$). There was greater than 90% emergence in the Control, 1.6, and 25 mg/L treatments. The treatment with the lowest mean percent emergence was the 6.75 mg/L treatment with 67.5% emerged, however the variability was much higher in the treatments with lower percent emergence. There was also no significant difference in the sex ratio of *C. tepperi* adults emerged. Most treatments had slightly more males emerge than females, although the Control was the closest to a 1:1 ratio of males to females.

Mean emergence day

Compared to the Control, chironomids exposed to all concentrations of added P emerged significantly earlier (Figure 1, $F_{(6, 21)} = 11.901$ $p < 0.05$). *Chironomus tepperi* emerged in the Control treatment at 16.83 days post hatch (dph), in contrast to the other treatments which emerged from 14.1 to 14.6 dph. Interestingly, the 0 mg/L treatment also had a mean time to emergence of 16 days, but the variability was much higher than the Control (SD: 1.182 and 00.641, respectively).

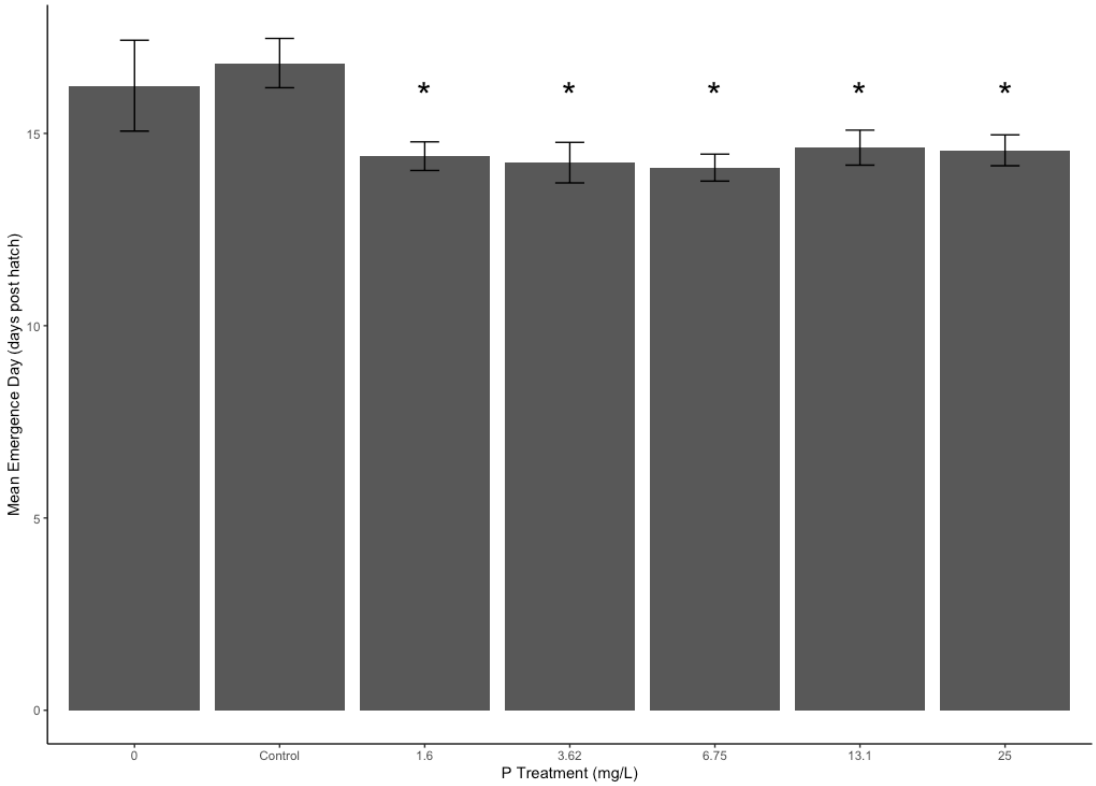

Figure 1. Mean (± SD) emergence day of *Chironomus tepperi* larvae exposed for 15 days to different concentrations of P in water. N = 4 replicates per treatment. Treatments with * are significantly different from the Control ($p < 0.05$)

Females emerged significantly earlier in the 1.6 and 3.62 mg/L treatments compared to the 0 mg/L and Control treatments (Figure 2, $F_{(6, 21)} = 9.900$, $p < 0.05$). There was no significant effect of P on male mean emergence (data not shown, $F_{(6, 21)} = 1.498$, $p > 0.05$). Males emerged between 13.59 and 16.1 dph. The Control had the latest male mean emergence day of 16.1 dph and the 13.1 mg/L treatment had the earliest at 13.59 dph.

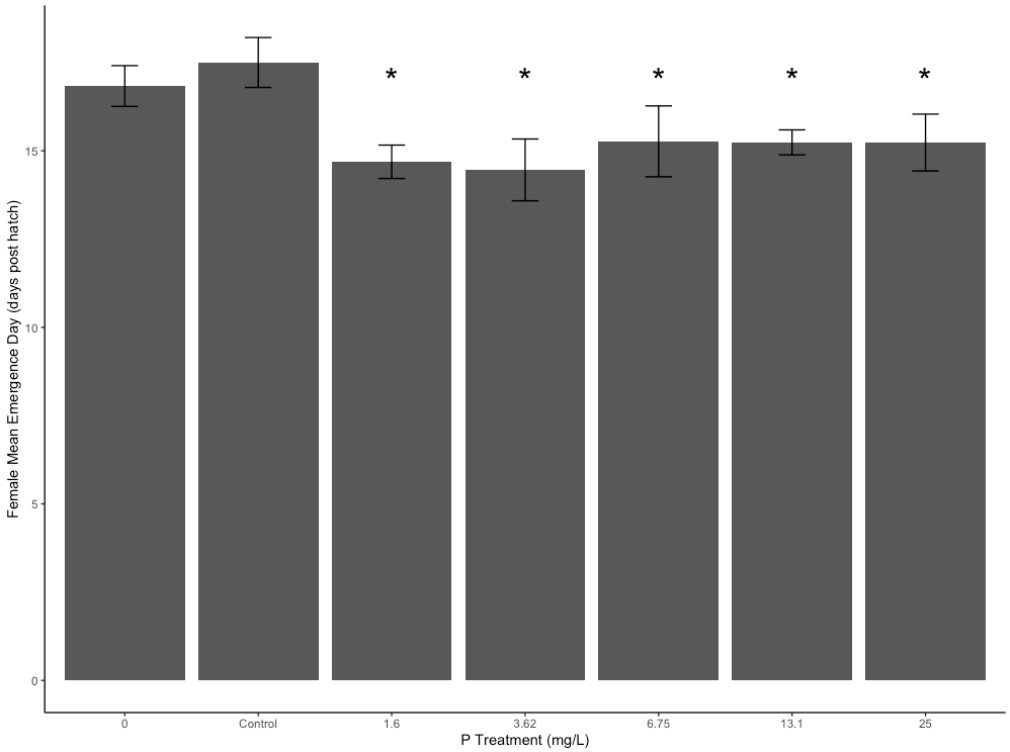

Figure 2. Mean (± SD) emergence day of female *Chironomus tepperi* larvae exposed for 20 days to different concentrations of P in water. N = 4 replicates per treatment. Treatments with * are significantly different from the Control ($p < 0.05$).

Wing lengths/Growth

Mean wing length in the Control adults was 8.06 mm and there was a trend of increasing wing length with increased P concentration (Data not shown, $F_{(6, 43)} = 4.625$, $p < 0.05$). The 1.6 mg/L treatment showed a significant increase in average wing length in comparison to the Control ($F_{(6, 43)} = 4.625$, $p < 0.05$). The 1.6, 3.62, and 13.1 mg/L treatments all showed significant increased mean wing length in comparison to the 0 mg/L treatment ($F_{(6, 43)} = 4.625$, $p < 0.05$).

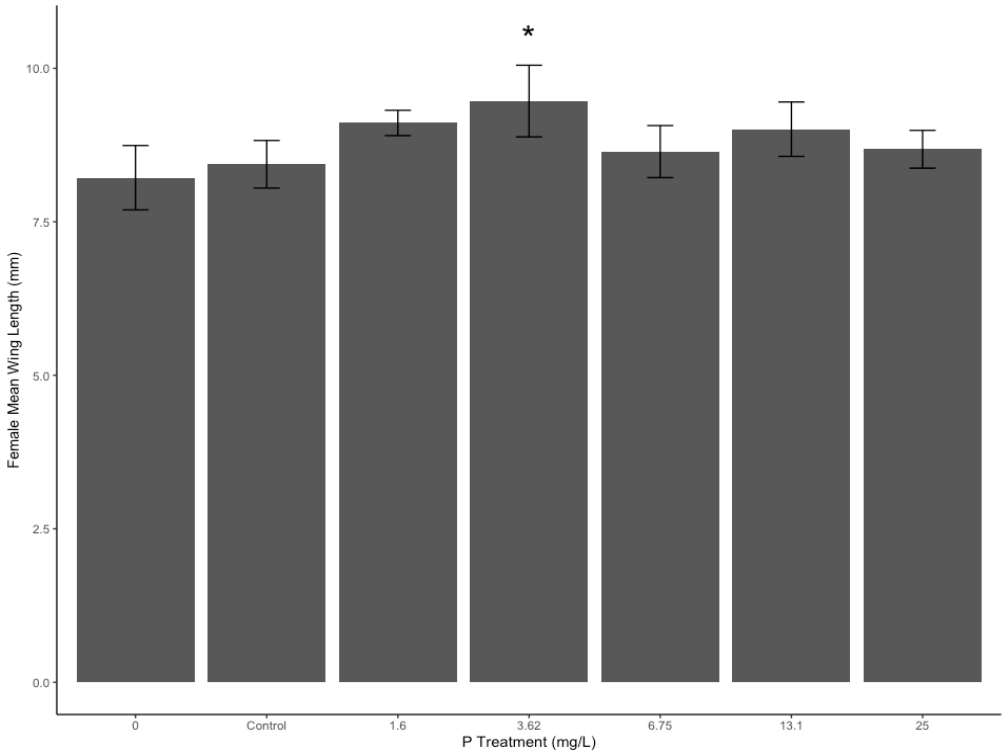

Figure 3. Mean (± SD) female wing length of adult *Chironomus tepperi* exposed for 15 days to different concentrations of P in water. N = 4 replicates per treatment. * represents treatments that are significantly different to Control ($p < 0.05$).

Male and female wing lengths were analysed separately and showed similar trends. The mean wing length of the males in the Control treatment was smaller than the mean female wing length (7.85 and 8.43 mm, respectively), this is not unexpected as male *C. tepperi* adults are naturally smaller. In the females, adults from the 3.62 mg/L treatment were significantly larger than the Control (Figure 3, $F_{(6, 31)} = 4.625$, $p < 0.05$). Male wing lengths followed similar patterns; adults from the 1.6 and 3.62 mg/L treatments were significantly larger than those from the Control and 0 mg/L treatments (Figure 4, $F_{(6, 38)} = 5.475$, $p < 0.05$).

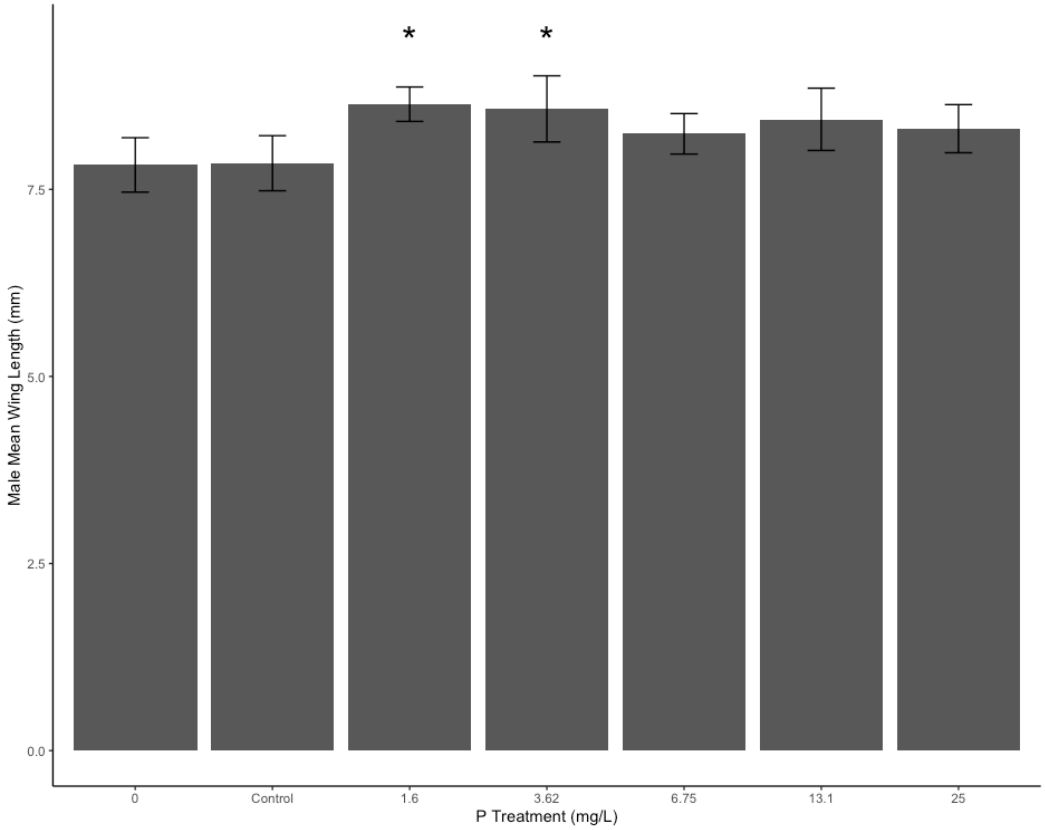

Figure 4. Mean (± SD) male wing length of adult Chironomus tepperi exposed for 15 days to different concentrations of P in water. N = 4 replicates per treatment. Treatments with * are significantly different from the Control ($p < 0.05$).

2.3.3 Phosphorus mass balance

The mass balance study showed that the sediment and food were adding P to the 600 mL beaker system in a controlled environment and that after food, sediment was the major source of P (107 mg/kg, 12.5% of total) (Table 3). To be certain of the P measurements in the sediment experiment, Total Phosphorus and Total Reactive Phosphorus were measured pre and post emergence test (Table 4)

Table 3. Phosphorus mass balance for Control sediment in 1 beaker replicate after 96 hours. All values were measured using ALS Laboratories.

Measure	**Water (mg/L)**	**Sediment (mg/kg)**	**Food (mg/kg)**	**Uptake of tepperi (mg/larvae)**	**Uptake of phytoplankton and bacteria (mg)**	**Total P present (mg)**
Total P	0.17	107	1600	-15.41	-699.9	853.1

Table 4. Sediment phosphorus measurement pre and post sediment emergence test. Total Phosphorus and Total Reactive P were measured by ALS Environmental.

	Treatment	Total Phosphorus	Total Reactive Phosphorus
Pre-test	0	149	<0.1
	Control	150	<0.1
	1.6	157	<0.1
	3.62	165	<0.1
	13.1	197	<0.1
Post test	0	96	0.2
	Control	160	0.4
	1.6	243	0.3
	3.62	166	0.2
	13.1	258	0.3

2.3.4 Sediment Emergence Bioassay

Percent Emergence

There was no significant effect of P concentration on proportion of emerged C. *tepperi* (Data not shown, Figure 1, $F_{(4, 15)} = 0.188$, $p > 0.05$). Every treatment had at least 85% emergence.

Mean Emergence

Mean emergence day was also unaffected by P concentration, however the variability of the 3.62 mg/L treatment was much higher than any of the other treatments (SD = 2.54) (Data not shown). As there was no significant difference in emergence time in the sediment bioassay, wing length measurements are not presented as we were only interested in determining the effects of earlier emergence on adult size. This is because wing length is a measure of adult body size which is a measure of development and there was no difference in development time in this study.

96-hour survival and weight

There was no significant effect of P treatment on survival or weight (Weight: data not shown, Figure 3, Survival: Kruskal-Wallis Test, $p > 0.05$. Weight: $F_{(4, 19)} = 0.941$, $p > 0.05$) after 96 hours. The mean survival was at least 97% in all treatments and was 100% in the 3.62 and 13.1 mg/L treatments. Larvae dry weight was between 0.80 and 0.98 mg per larvae. The highest larval weight was in the 13.1 mg/L treatment and the lowest was in the 0 mg/L treatment. There was a trend of increasing larval weight with an increase in P.

2.3.5 Energy Reserves

There was no significant effect of P treatment on protein (Data not shown, Figure 4, $F_{(4, 19)} = 1.039$, $p > 0.05$) or glycogen concentrations ($F_{(4, 17)} = 1.139$, $p > 0.05$). There was a slightly decreasing trend in protein concentration per mg wet weight as P increased. However, the highest protein concentration was in the 1.6 mg/L P treatment at 0.057 mg/mg wet weight. There was increasing variability in glycogen responses with increasing P treatment, which made analysis difficult. The results also tended towards a U shape, with the lowest glycogen concentrations at 1.6 and 3.62 mg/L and the highest at 0 and 13.1 mg/L.

There was a significant effect of P on lipid concentration (Figure 5, $F_{(4, 13)} = 9.246$ $p < 0.05$) when compared to the 0 mg/L treatment. The 0 mg/L treatment had the highest mean lipid concentration of 3.951 µg/mg (SD ± 0.270). The 1.6 and 3.62 mg/L treatments had significantly lower lipid concentrations compared to the 0 mg/L treatment with values of 3.076 and 3.282 µg/mg, respectively (1.6 mg/L: $F_{(4, 18)} = 9.246$, $p < 0.05$, 3.62 mg/L: $F_{(4, 18)} = 9.246$, $p < 0.05$:). No treatment was significantly different from the Control ($F_{(4, 18)} = 9.246$, $p > 0.05$).

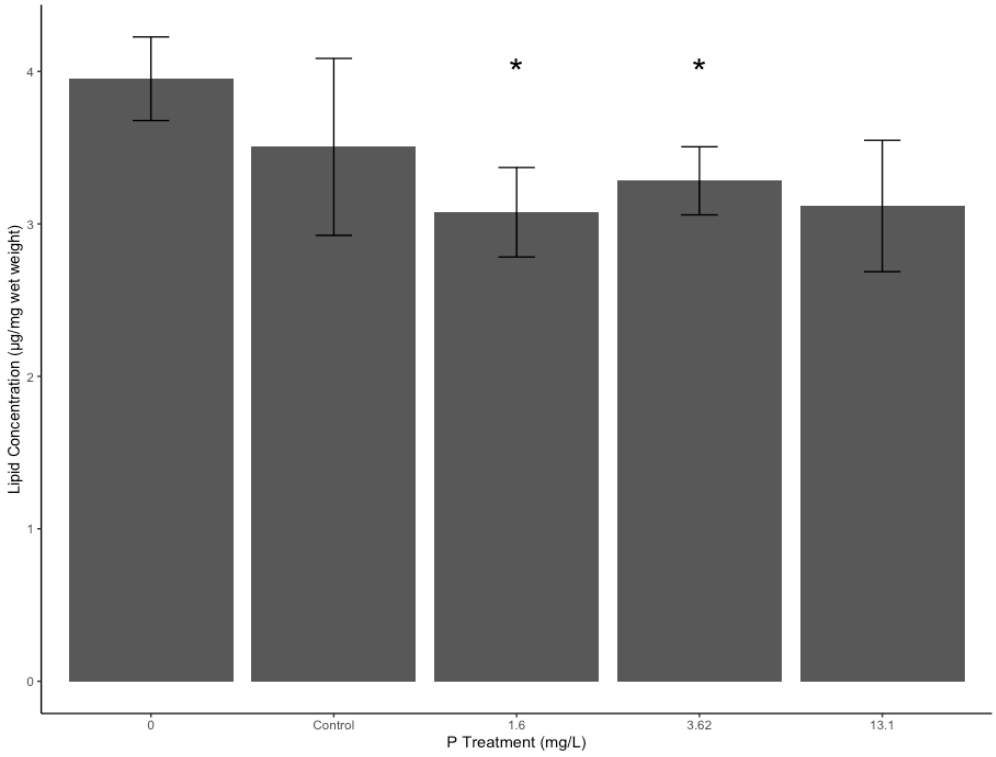

Figure 5. Mean (± SD) lipid concentration of *Chironomus tepperi* larvae exposed for 96 hours to different concentrations of P in sediment. N = 4 per treatment and N = 8 replicates for Control. * represents treatments that are significantly different from the **0 mg/L** treatment (One-way ANOVA, $p<0.05$).

2.4 Discussion

Chironomus tepperi are often found in newly inundated areas (Martin and Porter 1978; Stevens 1993) which suggests it can adapt to changes in both water quality and sources of food; for example, eutrophic conditions where P is in abundance. In these cases, food abundance and quality would be higher, which would allow *C. tepperi* to develop more quickly. This study shows that the development of *C. tepperi* is affected by an increase in P in either sediment or water and that the effects of P on the rate of development of C. *tepperi* is dependent on the type of study system.

2.4.1 Water-only vs. Sediment tests

Increasing P concentrations had a more pronounced effect on *C. tepperi* emergence in the water-only bioassay than in the sediment bioassay. It is likely that the sediment had a moderating effect on the P added to the system. Despite being filtered to 63 µm, a size that generally removes other invertebrates and larger particles, it does not remove microalgae and bacteria, two large sinks of P in freshwater systems (Haygarth and Jarvis 2002). Sediments are known to be large sinks of P in freshwater systems, particularly bio-available orthophosphates (Boström et al. 1988). This is also supported by the mass balance analysis, which showed sediment and food were the two largest contributors to the amount of TP in the sediment test system. The same amount of food was added to both water and sediment tests, so this is not likely to be the cause of the differences in TP. These factors would have had a mitigating effect on the additional P added to the overlying water. This has some interesting implications for toxicity tests. Water-only tests are likely to be more influenced by P concentration than sediment tests, where there is more P available to buffer concentrations. In particular, water-only tests must ensure that there is not a lack of P available, especially if it is a short test where the animals are not being fed.

2.4.2 Chironomid growth

Elevated P concentrations resulted in accelerated *Chironomus tepperi* development. Chironomids from high P concentrations in the water only bioassays emerged earlier. This is similar to results from a study by Ramírez and Pringle (2006) that showed an increase in the growth rate of several Chironomidae species after they were exposed to increased concentrations of P in field conditions. Wissinger et al. (2004) found that caddisfly (*Asynarchus nigriculus*) larvae emerged earlier and larger when given a diet supplemented with animal material. This is very similar to the response seen by *C. tepperi* in this study. As P is mostly incorporated into α-glycerophosphate, which is critical in cell generation (Nation 2008), *C. tepperi* may be using the higher amounts of P during pupation when there is high levels of cell generation. When developing, larvae require extra energy for ecdysis and pupation (Hamburger et al. 1996; Moon and Carefoot 1972), and *C. tepperi* would be no exception in this regard.

Woods et al. (2002) found that the total absorption of P in *Mandua sexta* (tobacco hornworm) was higher in animals that were fed higher P diets, but that the absorption rate was much slower. Phosphorus is stored in the haemolymph of insects as α-glycerophosphate (Wyatt 1961) and Woods et al. (2002) suggest that insect larvae regulate their P-intake, at least into the tissues. An imbalance of P may cause an imbalance in phosphorylation rates, calcium and magnesium regulation, energy and metabolism or affect intracellular signalling (Williams 1997). These cellular-level effects can have toxic consequences in an animal with an imbalance of P, including cell death, malnutrition, energy loss, and

death. Therefore, it is to the advantage of insects to regulate P absorption as there are potentially devastating consequences if this does not occur.

P regulation can explain the significant increase a lack of P has on *C. tepperi* lipid levels. The lack of P (in the 0 mg/L treatment) may be causing the organism to retain its energy reserves, rather than expend them. The fat body in insects is the largest store of energy reserves, which are kept in the form of triglyceride lipids (Arrese and Soulages 2010). Organic forms of P are essential to the formation of triglycerides, often from the α-glycerophosphate pathway (Chapman and Chapman 1998), which – although information is limited – has shown to increase in organisms exposed to higher concentrations of P(Woods et al. 2002). This indicates that lipid content should be higher in organisms that are exposed to more P. In the present study, lipid content was determined after the completion of the 96-h test, when larvae were in the 4^{th} instar (Stevens 1993) and approaching pupation, which is an energy-demanding process so are likely to be using any stored energy (Arrese and Soulages 2010). Our results suggest that larvae in the P-addition treatments are using more energy than the 0 mg/L treatment as the concentration of lipid is lower. This suggests that larvae in the 0 mg/L treatment are less well-developed than those in the P treatments. This is further substantiated by the observations of earlier emergence in the higher P treatments in the water-only bioassay. Lipids were used more rapidly in these treatments and so the larvae developed and emerged faster. However, despite there being a significant result in the 96-hr sediment bioassay biochemistry these results do not translate to developmental rate effects later in the life cycle in the sediment tests.

The early emergence of adults, especially females, in the water-only bioassay at all concentrations of added P may have significant impact at the population or ecosystem level. In chironomids, males generally emerge earlier than females as females are larger, more physiologically complex, and therefore take longer to mature (Martin and Porter 1978). For this reason, it is less of a concern that females are emerging earlier in the P treatments. The mean emergence day for a female C. *tepperi* in the 3.62 mg/L P treatment was 14.45 days post hatch. The mean emergence day for a male in the 3.62 mg/L P treatment was 14.73 days post hatch. As the females must mate within 24 hours of emergence, the difference in emergence between the sexes following exposure is still within the time frame for mating, and so should not pose a threat for mis-timed reproduction (Martin and Porter 1978). As there are so few studies on the developmental effects of P exposure, further investigation is needed into the impact of earlier emergence on the reproductive fitness of these adults. Wing lengths show that adults in the P treatments are significantly larger than the Control and 0 mg/L treatments, which would indicate that they are still reproductively fit, but multiple generation testing would be needed for this to be confirmed.

It is possible, however, that earlier emergence of the overall population could create ecosystem-wide problems. Briggs et al. (1985) showed that the diets of many temperate Australian waterfowl are made up of *Chironomus* species; Crome (1986) and Maher and Carpenter (1984) found a correlation between *C. tepperi* abundance and waterfowl breeding. This shows that *C. tepperi* is particularly important for water bird breeding in temperate Australia and shows that the potential effects of a decreased development time, as demonstrated by earlier emergence, could have repercussions on the wider ecosystem, and especially for waterfowl.

This study shows that the development of C. *tepperi* is accelerated by increases in P load, depending on the test system. In water bioassays, there is faster emergence with higher P concentrations.

However, this effect was not observed in the sediment systems where there are other sources of P present. In sediment tests, lipid energy reserves are in lower concentrations at higher P treatments in larval *C. tepperi,* but the time to emergence was not affected. It is possible that differences in energy reserves is mitigated by the time the organisms reach pupation when there are different energy demands and they are able to effectively regulate their P intake between the haemolymph and the fat body (Garey and Wyatt 1963).

The influence of P on the development and energy use of chironomids is a potential confounding factor in ecotoxicology tests where these metrics are used to assess the toxicity of contaminants. This effect is evident in water-only tests but not in sediment bioassays. Many ecotoxicology bioassays are conducted in water only to isolate the effects of a particular contaminant but, according to these results, the outcomes of such tests could be dependent on the P content of the water. It is possible that water-only tests with too much or too little P for the organism's ideal range may show false results.

Assessing how animals respond to changes in nutrient concentrations is especially important for understanding how they may respond to other stressors, such as pesticides. If an increase in nutrient concentrations gives the animal greater energy availability through increases in their energy stores, then they will have a greater amount of energy to draw upon to detoxify the contaminant, or to accelerate development allowing earlier emergence from the stressor (Boyle et al 2016). Having access to larger energy stores may therefore increase the organism's capacity to manage and survive such stressors. There are many examples of this, such as animals that survive the cold stress of winter through hibernation after increasing the energy reserves in the months beforehand (Humphries et al. 2003), and in particular there is evidence of the Antarctic chironomid *Belgica Antarctica* overwintering and using more energy when exposed to the stress of freezing temperatures (Sugg et al. 1983; Teets et al. 2011).

2.4.3 Conclusions

There needs to be greater consideration of the test system when assessing nutrient loads and their potential effects on toxicity. As this study highlights, there is a difference in effect between sediment and water tests, which has implications for future ecotoxicology work. The test used must depend on whether nutrients and other toxicants are being assessed in water or in sediment. Based on the results of this study we would recommend that further environmental toxicology and nutrient work examine the test system and ensure that nutrients are measured throughout the tests.

CHAPTER 3: THE EFFECTS OF PHOSPHORUS ON THE TOXICITY OF PERMETHRIN TO THE AUSTRALIAN MIDGE, *CHIRONOMUS TEPPERI*

3.1 Introduction

In traditional laboratory-based ecotoxicology experiments, a test organism is exposed to a range of concentrations of one or, less frequently, a mixture of chemicals to determine its toxicity. These values are then used by policymakers to set safety limits (for example guideline values) for acceptable concentrations of chemicals present in sediment and water that are likely to pose minimal impact on biota. Only recently has this approach considered other water and sediment quality factors that are present in the environment but not considered in laboratory assays. For instance, the toxicity of organic substances are usually described in terms of the amount of organic carbon content (e.g. ANZECC and ARMCANZ (2018)) and temperature can affect the toxicity of synthetic pyrethroids (Brown 1987; Day 1991; de Perre et al. 2014; Harwood et al. 2009; Mehler et al. 2017). The capacity of an organism to tolerate exposure to a toxicant may be also affected by indirect effects. Exposure to a toxicant may require the expenditure of energy to detoxify the substance (e.g. production of metallothionein proteins to detoxify metals) or to excrete toxic substances. It would be reasonable to assume that an organism with greater energy stores would have greater resilience to cope with exposure to a toxicant than one with low energy stores. In freshwater environments, a broad range of environmental conditions occur that may influence the toxicity of a substance. There is a vast range of nutrient concentrations in waterbodies ranging from oligotrophic to eutrophic systems and this may impact the responses of organisms to pollution stress. For instance, snails exposed to oligotrophic conditions do not grow as fast or as big as those in optimal or eutrophic conditions (Krist et al. 2014). In contrast, many mesotrophic and even eutrophic waters are highly productive, and growth rates can be rapid (Correll 1998). This raises the question whether organisms living in oligotrophic waterbodies are more susceptible to detrimental effects than those living in eutrophic systems.

In Australia, waters, soils, and sediments are naturally low in some nutrients, particularly nitrogen and phosphorus (Bartley et al. 2012; Rutherford and Gippel 2001; Young et al. 1996). This means that water and sediment quality guidelines, which are partially derived from overseas studies (Warne et al. 2014), may not be protective of local ecosystems with relatively low nutrient concentrations. The additional stress that oligotrophic or eutrophic conditions lead to on top of exposure to other chemicals, is a relatively recent topic of study (Aristi et al. 2016). Overall, it seems that the assumption that increased nutrient concentration has an antagonistic effect when combined with contaminants is correct (Jackson et al. 2015).

Phosphorus (P) is an important nutrient present in all freshwater environments. It occurs at high concentrations in many aquatic environments, especially in urban and agricultural run-off and wastewater. Fertilisers are a major source of pollution and their runoff from agricultural land can cause eutrophication on a large scale in waterways around the world (Smith 2003). In Australia, high concentrations of P occur in most human-impacted lakes, particularly in the populated south-eastern areas of the continent (Water 2015).

The effects of P on ecosystems have been studied extensively (Azevedo et al. 2015; Correll 1998; Smith 2003; Smith et al. 1999). High concentrations of P lead to excessive plant and algal growth, which can

create an anoxic environment that leads to fish kills and general environmental degradation (Anderson et al. 2005; Elser et al. 2009; Elser et al. 2001; Schindler 1974). Much of the research involving P has focused on the effects on primary producers, however there is little information on its effects on invertebrates.

The current research into the effects of P on invertebrates largely uses P enrichment in an altered diet. A study by Elser et al. (2001) found that higher concentrations of dietary phosphorus enriched the growth rate of *Daphnia dentifera* compared to controls. Meng et al. (2014) also found an increase in growth and development of *Daphnia similoides* and *Moina irrasa*, as well as increased population density when each species was fed a high P diet. Krist et al. (2014) found that a phosphorus-limited diet decreased the growth rate of *Potamopyrgus antipodarum* – a New Zealand freshwater mud snail found throughout Australia. Chapter 2 described the effects of indirect and direct exposure to P on *C. tepperi.* This showed a clear effect of P on sublethal endpoints, including decreased emergence time and increased wing length in water only exposures, as well as a decrease in some energy reserves in sediment exposures. Based on this work, I hypothesized that increasing nutrient concentrations should increase growth rates of freshwater invertebrate species. However, none of these previous studies have examined the relationship between nutrient availability and exposure to toxicants.

Synthetic pyrethroid insecticides (SPs) are widely used because of their low mammalian toxicity, as a result they are present in aquatic environments all over the world (Marshall et al. 2016; Wang et al. 2012; Weston et al. 2009a). They are insoluble and in a freshwater system will adsorb to any solid particulates, most often sediment. They are highly toxic to aquatic life, including fish and invertebrates (Maund et al. 2012). Furthermore, they also have a relatively long half-life (ranging up to 18-24 months depending on the chemical), so are persistent as sediment-bound pollutants for a relatively long time. This means benthic dwelling organisms are likely to be exposed to SPs during their life cycle. Permethrin is one of the most common synthetic pyrethroid insecticides, partly because it was one of the first of the commercially available photostable SPs (Spurlock and Lee 2008). It is utilised in a wide range of industries and products, including agriculture, as flea treatment for household pets, insect repellent textiles, and increasingly as an urban pesticide in areas of new housing developments (Marshall et al. 2016; Spurlock and Lee 2008). As it is so widely used, it often occurs in conjunction with other water quality issues (such as nutrient pollution). Therefore, the effects of exposure to different P and permethrin concentrations are poorly understood and represents a clear gap in the research in this area.

There have been several studies on the effects of permethrin on a wide range of organisms in both water and water-sediment systems. Spehar et al. (1983) worked with fathead minnows (*Pimephales promelas*) and snails (*Helisoma trivolvis*) in 30 day water exposures and found LC_{50}s of 1.4 and 0.33 μg/L, respectively. McLeesc et al. (1980) ran basic 96-hour water only toxicology tests with Atlantic salmon (*Salmo salar*), lobster (*Homarus americanus*), and sand shrimp (*Crangon septemspinosa*) and found that invertebrates, especially crustaceans, were more sensitive than fish, with LC_{50}s of 12 μg/L for the salmon and 0.73 and 0.13 μg/L for the lobster and shrimp. The relative sensitivity of invertebrates is well known, as SPs act on sodium channels, which invertebrates are more sensitive to, along with their smaller body size and lower body temperature (Davies et al. 2007). In a review of the ecotoxicology of SPs, Maund et al. (2012) collated acute toxicity data for several different SPs, including permethrin, and found that birds had an LD_{50} of 9,800 mg/kg body weight showing they are more tolerant to this pesticide compared to invertebrates. They also compared water only bioassays

with invertebrates and found that molluscs were generally the least sensitive group and crustaceans and insects the most sensitive (mollusc E(L)C_{50}: 14.9 -1,740 μg/L. crustacean E(L)C_{50}: 0.018 – 2.29 μg/L. insect E(L)C_{50}: 0.027 – 45 μg/L).

Although water only bioassays are useful to gain a general understanding of the toxicity of a pesticide, they are not as environmentally relevant in the case of SPs, which bind to the sediment (Laskowski 2002). As a result, investigation of sediment-dwelling organisms in ecotoxicology tests is a priority. Amweg et al. (2005) investigated the toxicity of several SPs, including permethrin, in sediment to the amphipod *Hyalella azteca* and found an LC_{50} of 1.58 – 8.05 μg/kg. Fleming et al. (1998) looked at the effects of permethrin on *Chironomus riparius* in both artificial and natural sediments to compare the toxicity between sediment types. They found that the toxicity was lower in natural sediments than in artificial sediments. The effects of synthetic pyrethroids are moderated by organic carbon content. Fleming et al. (1998) showed that organic carbon content can significantly alter the toxicity of permethrin to *Chironomus riparius*, with reduced toxicity following exposure to sediment with high carbon content. They also showed that the type of carbon content was important when creating artificial sediments, as the intention is usually to mimic natural sediments. However, they also discovered that their artificial sediment underestimated toxicity. In fact, the test that best predicted the toxicity in natural sediments was the water-only test. This highlights that there are other aspects of sediment chemistry that relate to pyrethroid toxicity that are often overlooked in laboratory-based toxicity tests.

Chironomus tepperi is an Australian standard toxicity species, but there is only limited data on effects of synthetic pyrethroid exposure on this species. Stevens (1993) exposed *C. tepperi* fourth instar larvae to various concentrations of different synthetic pyrethroid commercial formulations in water-only bioassays. These assays were conducted with an ethanol-sterilised paper tissue substrate and exposed to a 20 ml suspension of the insecticide in unaerated conditions for 24 hours. The permethrin LC_{50} for fourth instar *C. tepperi* under these conditions was 0.66 μg/L a much more toxic concentration than is found in sediment bioassays. More recent studies have focussed on effects of sediment-bound SPs to *C. tepperi*. Boyle et al. (2016) found that bifenthrin caused significant toxicity to *C. tepperi* larvae at 53.66 μg/g (normalised to OC), reduced adult emergence at 33.33 μg/g OC and caused early emergence at concentrations of 10 μg/g and above in laboratory toxicity tests, especially in females. Mehler et al. (2017) found that permethrin affected the survival of *C. tepperi* at 411.9 μg/g, and that growth was significantly retarded at 139.7 μg/g, which is in contrast to the high level of toxicity following the water-only exposure found by Stevens (1993). This shows a large difference in toxicity between using permethrin in water and sediments. The sediment techniques used by Mehler et al. (2017) are more environmentally relevant than water-only exposures but are still not a perfect model of the ecosystem. Additional factors need to be taken into account such as temperature, salinity, and nutrients that may also potentially affect the toxicity of synthetic pyrethroids to improve the environmental realism of laboratory-based tests.

It is also important to measure different endpoints to assess sublethal toxicity to biota. Measuring energy reserves in organisms is a relatively simple assay that provides a more functional and physiological understanding of the biological response to chemicals. Sak et al. (2006) investigated the effects of cypermethrin on lipid, glycogen, and protein content in the endoparasitic wasp *Pimpla turionellae* at several life stages (larvae, pupae, and adult males and females); exposure was through consumption of cypermethrin-exposed host organisms. The authors measured total glycogen, lipid

and protein in each life stage and found that lipid and protein content were significantly affected in the larval stage whereas glycogen content was affected in adult females. This study shows that cypermethrin affects energetic reserves, but that the effect is not consistent throughout the life cycle of the organism.
Saleem et al. (1998) exposed adult beetles, *Tribolium castaneum*, to a commercial formulation of cypermethrin and found that both glycogen and protein content decreased throughout the exposure period of the beetles, but that total lipid concentration increased. They believe this could be related to a change in energy utilisation of the adult beetles when put under the stress of an insecticide exposure.

There have been several studies investigating the impacts of water quality parameters on the toxicity of synthetic pyrethroids including permethrin. Harwood et al. (2009) showed that decreasing temperature increased the toxicity of permethrin to *Chironomus dilutus* in 10-d sediment bioassays, with nearly a 3-fold increase in toxicity in bioassays conducted at 13 ºC than at 23 ºC. This trend has also been observed with other pyrethroids in other species including *C. tepperi* (Boyle et al. 2016), tobacco budworm (*Heliothis virescens*) (Brown 1987), and German cockroaches (*Blattella germanica*) (Valles et al. 1998). It is well-established that temperature is an important factor that affects the toxicity of synthetic pyrethroids to invertebrates (Coats et al. 1989; Weston et al. 2009b).

There has been little work done on understanding how the major nutrients, phosphorus and nitrogen, affect the toxicity of pesticides to invertebrates. Alexander et al. (2013) examined how nutrient levels affect the toxicity of a mixture of three insecticides, two organophosphates and a neonicotinoid, on various endpoints of community and ecosystem health in nutrient-enriched and unenriched artificial streams. They found that the variability in responses was much higher in the enriched streams, and the effects of the pesticides were seen over a smaller range of pesticide concentrations (Alexander et al., 2013).

Many studies have investigated the interactive effects of nutrients and contaminants using artificial streams, microcosms, or field-based studies (Aristi et al. 2016; Mackintosh et al. 2016; Van Donk et al. 1995). However, there is a clear gap in our understanding of how nutrients affect contaminants in laboratory bioassays to improve the realism of these assays and there have been no attempts to quantify these effects using traditional ecotoxicology methods.

This chapter aims to: determine how P (low and high concentrations) affects the toxicity of permethrin to *Chironomus tepperi*, specifically on survival, weight, development, and impacts on energy reserves.

3.2 Methods

3.2.1 Sediment collection and preparation

Whole sediment was collected from a wetland in Bittern, Victoria, Australia—an unpolluted site approximately 90 km southeast of Melbourne (Appendix B). The top 2 cm of sediment was collected and filtered with site water through a 63 µm net into a 10 L plastic bucket that had been cleaned with Extran®, rinsed with ethanol, and then rinsed 6 times with RO water. Sediment was stored for 2 weeks at 4°C to allow it to settle. The overlying water was decanted, and the sediment allowed to settle again for 3 days after which overlying water was again poured off. This sediment was re-homogenised with a paint stirrer and then stored in clean 1L jars at 4^{o}C.

3.2.2 Chironomid Growth and Preparation

Chironomus tepperi were cultured in a modified Martin's artificial water solution (as described in Jeppe et al. (2014)), with ethanol-rinsed toilet paper as substrate. Larvae were fed tropical fish flakes (Tetramin) three times per week, with 0.25 mg added per tank. To obtain larvae of a known age, adult *C. tepperi* were collected from the CAPIM laboratory culture and placed into a Perspex tank with modified Martin's artificial water solution. After 24 hours, egg masses were then transferred to 2L beakers (three egg masses per beaker) to hatch. The 2 L beakers included ethanol-rinsed toilet paper substrate and were maintained at 20º C +/- 1 degree with a photoperiod of light:dark 16:8hr. Two drops of food (a slurry made up of crushed Tetramin, at a concentration of 10g/100 ml of artificial water) was provided every 2 days until the larvae reached 2nd instar (5 days old).

3.2.3 Preparation of spiked-sediment

The field sediment was spiked with a commercial, water soluble, formulation of permethrin (Brunnings Ant, Spider, and Cockroach Killer. Oakleigh South, Victoria, Australia) at low, medium, and high concentrations (55, 220, and 2200 µg/kg, normalised to 1% Total Organic Carbon (TOC)) that were known to affect *C. tepperi* growth and a control with no permethrin. These values were based on previous tests (CAPIM, unpublished data) and on field surveys that found high concentrations of permethrin in sediment (Marshall et al. 2016) which provided environmentally relevant concentrations for the test. Control sediment was prepared in the same way but without permethrin (i.e. the same volume of artificial water was added to the jar as for the permethrin treatments). Jars containing spiked sediment and control sediment jars were placed on a rolling machine for two hours each day for two weeks after spiking to ensure the permethrin was evenly distributed within the sediment. In between rolling jars were stored at 4^{o}C in the dark. All sediment permethrin concentrations were determined by the National Measurement Institute (North Ryde, NSW, Australia) using a method based on USEPA SW 846-8270B. Sediment samples were extracted using acetone and hexane and analysed using capillary injection following by high-performance gas chromatography coupled with determination by tandem mass spectrometry. Standard QA/QC procedures were followed and involved laboratory duplicates and matrix spikes. Matrix spike recoveries were between 86 and 96%.

3.2.4 Preparation of P solutions

Phosphorus treatments were prepared using a modified Martin's artificial water solution without KH_2PO_4. The regular recipe for artificial water uses a concentration of KH_2PO_4 that results in a concentration of 0.49 mg/L P, so this concentration of P was used for the control treatment. P as KH_2PO_4 was added to create a range of P concentrations to be used in the treatments. The concentrations used were 0, 0.49 (control), 3.81, and 14.5 mg/L. These concentrations were chosen based on previous work examining the effects of P on *C. tepperi* (Chapter 2) and of known concentrations of P present in waters and sediments in south-eastern Australia (Water 2015). The purpose of the highest concentration was to include a potentially toxic concentration of P and to determine whether high concentrations of P would affect the toxicity of permethrin. The following water quality parameters were measured: pH, temperature, conductivity, dissolved oxygen and P and found to be within the acceptable limits for a viable *C. tepperi* test (Batley and Simpson 2016).

3.2.5 Bioassays

One hundred grams of spiked (or sham-dosed with artificial water for controls) sediment was added to clean 600 ml beakers. Each treatment of P and permethrin was crossed once and there were four replicates per treatment. P treatments were prepared by spiking the overlying water of the sediment. The water and sediment in the beakers was allowed to settle for 24 hours at 21°C with 16:8hr day/night cycle and the overlying water tested for several water quality parameters (temperature, pH, salinity, dissolved oxygen). After this time, ten five-day old (2^{nd} instar) *Chironomus tepperi* larvae were randomly added to each beaker.

96-hour Bioassay

During the 96-hour exposure larvae were fed at the beginning of the test and after 2 days. After 96 hours, the sediment was filtered through a 250 µm sieve and larval survival was recorded. Four larvae from each replicate were frozen on dry ice for biomarker determination and the remaining larvae were put into microcentrifuge tubes with 70% ethanol for growth determination. The overlying water from each treatment was pooled and temperature (ºC), pH, conductivity (µs/cm), and dissolved oxygen (%) were measured.

Emergence Bioassay

Reverse osmosis water was added 3 times per week to the overlying water to counteract evaporation to ensure the salts did not become too concentrated in each beaker. Larvae were fed two drops of Tetramin food slurry 3 times a week until emergence was observed and then fed 1 drop twice a week for the remainder of the test, as fewer were larvae present. The pH, temperature, dissolved oxygen, and conductivity were measured weekly throughout the test.

Emerged adults were collected daily with an aspirator. The numbers of males and females were recorded and preserved in Eppendorf tubes in 70% ethanol. The experiment was terminated when there was no further emergence in the control treatments after 2 days and the sediment from all the remaining beakers was filtered through a 250 µm sieve to collect any surviving larvae or pupae. Sex, proportion of adults emerged, and emergence day were recorded.

3.2.6 Growth determination

The ethanol in each Eppendorf tube of larvae from the 96-hour bioassay was allowed to evaporate for 48 hrs in a fume hood and the larvae were placed onto pre-weighed tin trays. The trays were placed into an oven for 24 hrs at 60 °C and then weighed to determine larval dry weight. This weight was divided by the number of larvae in the tube and used as an average for that replicate. Dry weight is expressed as mg/larvae.

3.2.7 Energy Reserves

Protein Determination

One frozen larva per replicate was homogenised in 50 μl ultra-pure water, centrifuged at 14000g at 4^{o}C for 5 minutes and the supernatant collected. Protein concentration was determined based on the Lowry method (Lowry et al. 1951) using the DC Protein assay kit (BioRad) using Synergy 2 microplate reader (Biotek instruments), with bovine serum albumin as the standard.

Lipid and glycogen measurements

The lipid and glycogen analysis followed the methods of Vu et al. (2015), Van Handel (1985), and Plaistow et al. (2001), modified for use with chironomids. For each analysis, 1 frozen larva per replicate was used. Briefly, for glycogen analysis larvae were homogenised in 2% sodium sulphate solution, 99% methanol and a 1.415g/L anthrone in sulfuric acid solution were used to determine glycogen content at room temperature. The resulting solutions were read at 625 nm in a Synergy 2 microplate reader, with laboratory-grade glucose as the standard. For the lipid analysis, larvae were homogenised in a 1:1 chloroform-methanol solution, 98% sulfuric acid and 1.2g/L vanillin solution were used to determine lipid content at room temperature. Resulting solutions were read at 490 nm, with commercial vegetable oil as the standard. Each sample and standard were read in triplicate.

3.2.8 Statistical Analysis

Two-way analysis of variance (ANOVA) with P and permethrin as separate factors was carried out to determine whether P exposure significantly affected all endpoints ($\alpha = 0.05$). If significance was observed, a Tukeys post hoc test was used to determine which concentrations were causing the differences between treatments. Proportion data (i.e. survival and emergence) were arcsine transformed prior to statistical analysis. All data were checked to ensure they conformed to the assumptions of analysis of variance by evaluating Skewness and Kurtosis values and Levene's Test for Equality of Error Variances. If data did not conform, a Kruskal-Wallis test was carried out for each factor (permethrin and P and combined treatment). All statistical analyses were carried out using SPSS, Version 22.

3.3 Results

3.3.1 Permethrin concentrations

The measured permethrin concentrations (Table 1) were slightly higher than the intended treatments, however the magnitude of difference between permethrin treatments was similar. The highest treatment was 2200 µg/kg and the Control had <LOR µg/kg. Nominal concentrations are used for graphical results.

Table 1. Measured permethrin concentrations (µg/kg) in sediment for both the emergence and 96-hour bioassay at the beginning of testing (National Measurement Institute, North Ryde, NSW, Australia).

Treatment	Permethrin Concentration (µg/kg)
0	<LOR
55	72
220	240
2200	2100

3.3.2 Water Quality

The mean water quality for both the emergence and 96-hour bioassays is found in Table 2. The mean temperature for all treatments was 20.95°C, the mean pH was 7.31, the mean DO was 89.9%, and the mean electrical conductivity was 448 µS/cm. Electrical conductivity of the overlying water was higher than that recommended by Batley and Simpson (2016), however this was the case for all treatments including the Control and testing with *C. tepperi* and salinity work in Chapter 4 confirms that these conductivities would have had little bearing on the results.

Table *2.* Mean (± SD) water quality for the emergence and 96 hour bioassays measured once a week after the first adult had emerged. Mean is of three measurements for each treatment. *Treatment is represented as permethrin concentration (µg/kg)/total phosphorus concentration (mg/L).

Treatment*	Temperature (°C)	pH	DO (%)	Electrical Conductivity (µS/cm)
0/0	21.13 (± 0.4)	7.14 (± 0.2)	87.2 (± 2.9)	473 (± 34)
0/CTL	21.03 (± 0.4)	7.23 (± 0.3)	91.1 (± 3.0)	463 (± 53)
0/3.81	21.00 (± 0.4)	7.26 (± 0.2)	88.5 (± 1.9)	451 (± 4.0)
0/14.5	21.00 (± 0.4)	7.14 (± 0.1)	86.0 (± 5.3)	474 (± 18)
55/0	20.93 (± 0.4)	7.22 (± 0.2)	89.0 (± 2.1)	430 (± 1.4)
55/CTL	21.10 (± 0.5)	7.28 (± 0.2)	90.2 (± 1.0)	443 (± 13)
55/3.81	21.00 (± 0.4)	7.34 (± 0.2)	91.4 (± 2.5)	442 (± 20)
55/14.5	20.97 (± 0.4)	7.24 (± 0.2)	89.1 (± 1.1)	432 (± 8.0)
220/0	21.00 (± 0.4)	7.33 (± 0.2)	90.0 (± 3.0)	447 (± 12)
220/CTL	20.90 (± 0.4)	7.33 (± 0.2)	91.7 (± 2.4)	456 (± 7.4)
220/3.81	20.93 (± 0.4)	7.36 (± 0.1)	91.7 (±3.9)	439 (± 24)
220/14.5	20.97 (± 0.4)	7.34 (± 0.1)	92.2 (± 1.9)	445 (± 13)
2200/0	20.87 (± 0.4)	7.39 (± 0.2)	89.7 (± 6.2)	433 (± 21)
2200/CTL	20.97 (± 0.5)	7.44 (± 0.1)	86.7 (± 3.0)	479 (± 21)
2200/3.81	20.77 (± 0.5)	7.51 (± 0.1)	93.9 (± 3.5)	438 (± 18)
2200/14.5	20.67 (± 0.3)	7.39 (± 0.1)	89.8 (± 4.8)	423 (± 13)

3.3.3 96 Hour Sediment Test

Survival

There was a significant effect of the combined treatment on chironomid survival (Kruskal-Wallis p=0.002) due to all treatments with the highest permethrin concentration having significantly reduced survival compared to other treatments (Figure 1). The 0 permethrin mixed with 0, control (0.49 mg/L), and 3.81 mg/L P treatments had >95% survival. The highest permethrin treatment (2,200 µg/kg) had no survival above 70% irrespective of P. In the 0 mg/L P treatment (simulating oligotrophic conditions) the 220 µg/kg permethrin treatment trended towards a much higher mortality than any treatment with P addition.

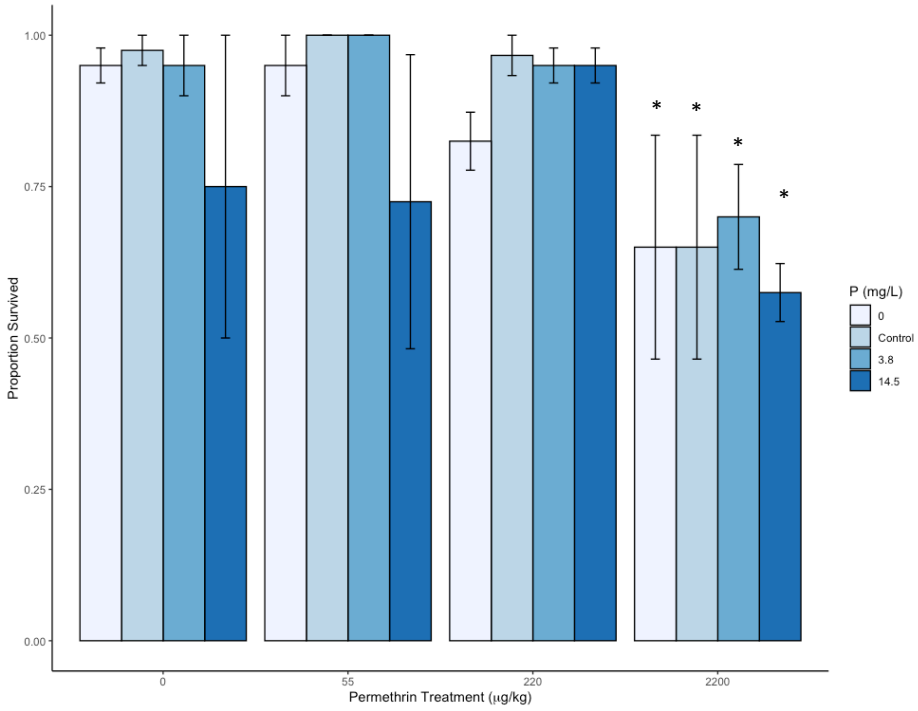

Figure 1. Mean (± SE) survival of *Chironomus tepperi* larvae exposed for 96 hours to different concentrations of P and permethrin in sediment. N = 4 replicates per treatment. Treatments with an asterisk (*) are significantly different from 0/CTL. Bars with no asterisk represent no significant difference between treatments.

Weight

There was a significant effect of treatment on larval weight after 96 hours (Figure 2

Figure , Kruskal-Wallis p=0.005). Larvae from all treatments with the highest permethrin concentration had significantly lower weights than larvae from other treatments. There was a significant decrease in larval weight with increased permethrin concentration ($p < 0.001$). In the 0, 220, and 2,200 μg/kg treatments, the 14.5 mg/L P treatment tended to have lower larval weight than all other P treatments (0.352 mg vs 0.447 mg in the control).

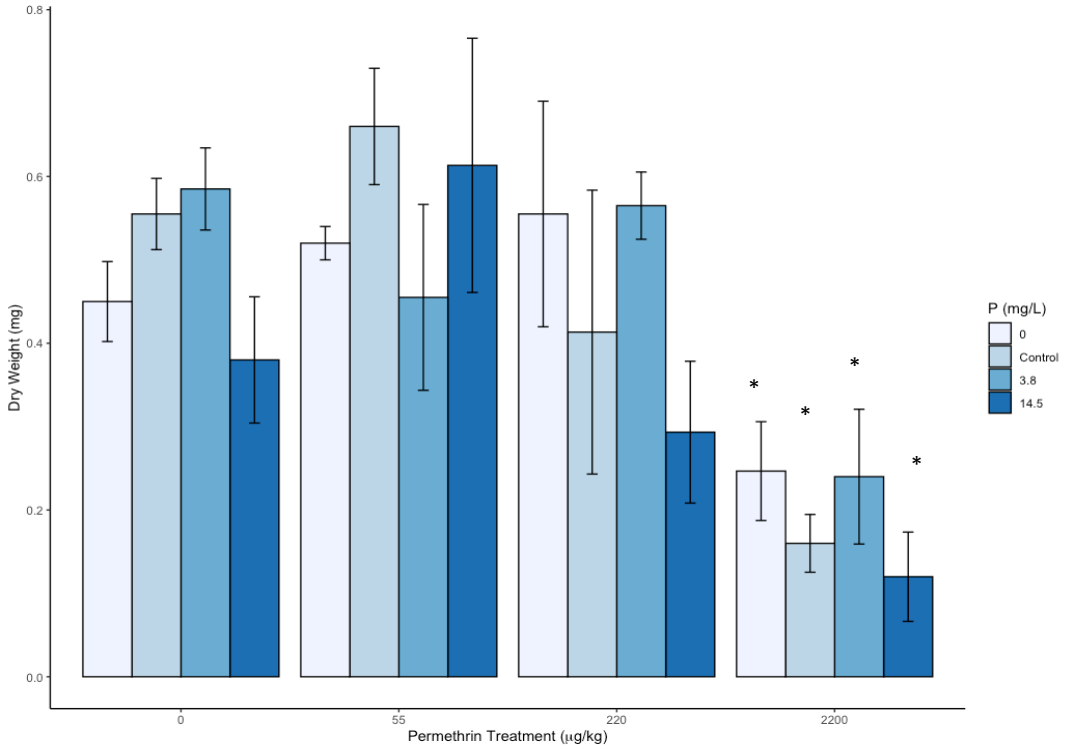

Figure 2. Mean (± SE) weight of *Chironomus tepperi* larvae exposed for 96 hours to different concentrations of P and permethrin in sediment. N = 4 replicates per treatment. Treatments with an asterisk (*) are significantly different from 0/CTL. Bars with no asterisk represent no significant difference between treatments.

3.3.4 Energy Reserves

Glycogen

There was no effect of either permethrin or P independently of each other on glycogen concentrations in larvae (Figure 3, Kruskal-Wallis $p > 0.05$). However, when P and permethrin were combined, there was a significant effect (Kruskal-Wallis $p < 0.001$). At the lowest permethrin concentration (55 μg/kg), any treatment that contained P (control, 3.81 and 14.5 mg/L) had elevated glycogen concentrations compared to the control. The 55/0 (permethrin/P) treatment had a mean glycogen concentration per wet weight of 1.218 μg/mg whereas all other treatments had mean glycogen concentrations greater than 4.6 μg/mg. The glycogen concentration is not reported for the 2200/14.5 treatment as there were insufficient larvae for analysis due to low survival rates in this treatment.

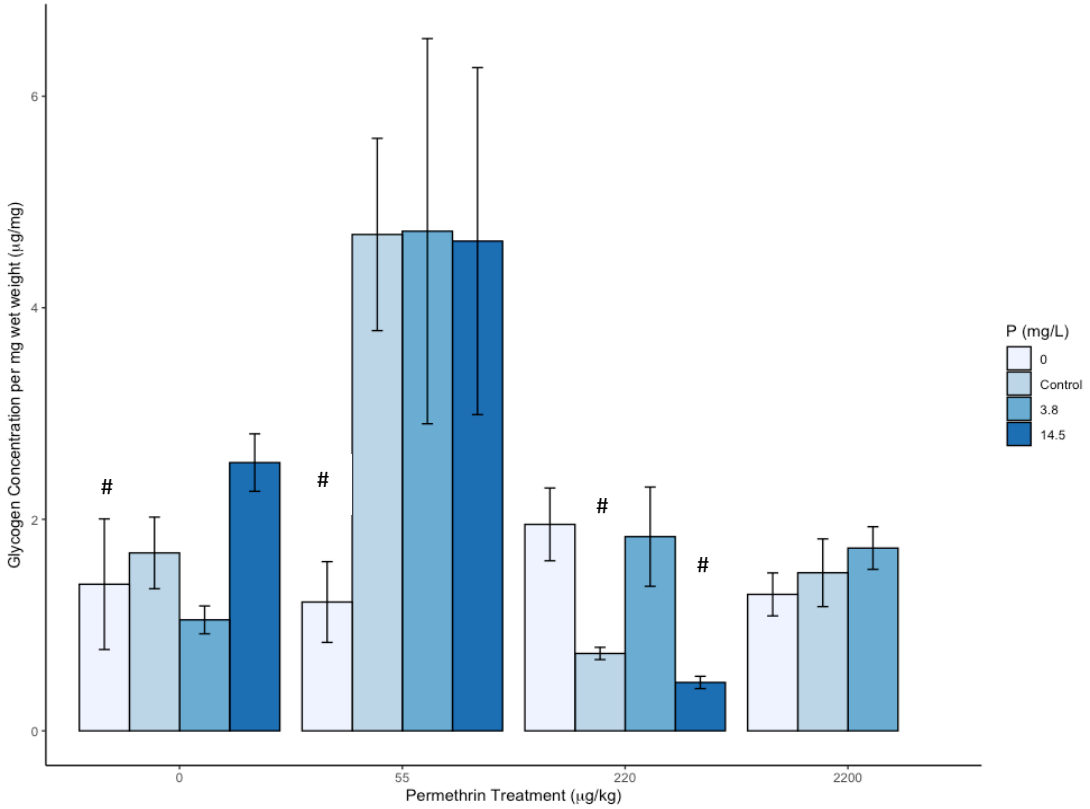

Figure 3. Mean (± SE) glycogen concentration per mg of wet weight *for Chironomus tepperi* larvae exposed to P and permethrin treatments for 96 hours. N = 4 replicates per treatment. Treatments with (#) are significantly different from the 55/CTL treatment.

Protein

When analysed singly, permethrin exposure significantly increased the protein concentration in larvae (Kruskal-Wallis p = 0.005). However, this was negated when P was added, irrespective of the concentration of P (Figure 4, Kruskal-Wallis p = 0.67). In the highest permethrin concentration, irrespective of P, the mean protein concentration was 0.376 µg/mg, in comparison to the 0 permethrin treatment with an overall mean of 0.132 µg/mg. In general, there was also a slightly increasing trend of protein concentration with P treatment, with the notable exception of the 0 mg/L treatment. The control treatment had a concentration of 0.169 µg/mg in contrast to the 14.5 mg/L treatment with a protein concentration of 0.219 µg/mg. The 0 P treatment was a clear outlier with a mean protein concentration of 0.359 µg/mg. In nearly all permethrin treatments (except 220 µg/kg) the 0 P treatment had a higher protein concentration than all other P treatments.

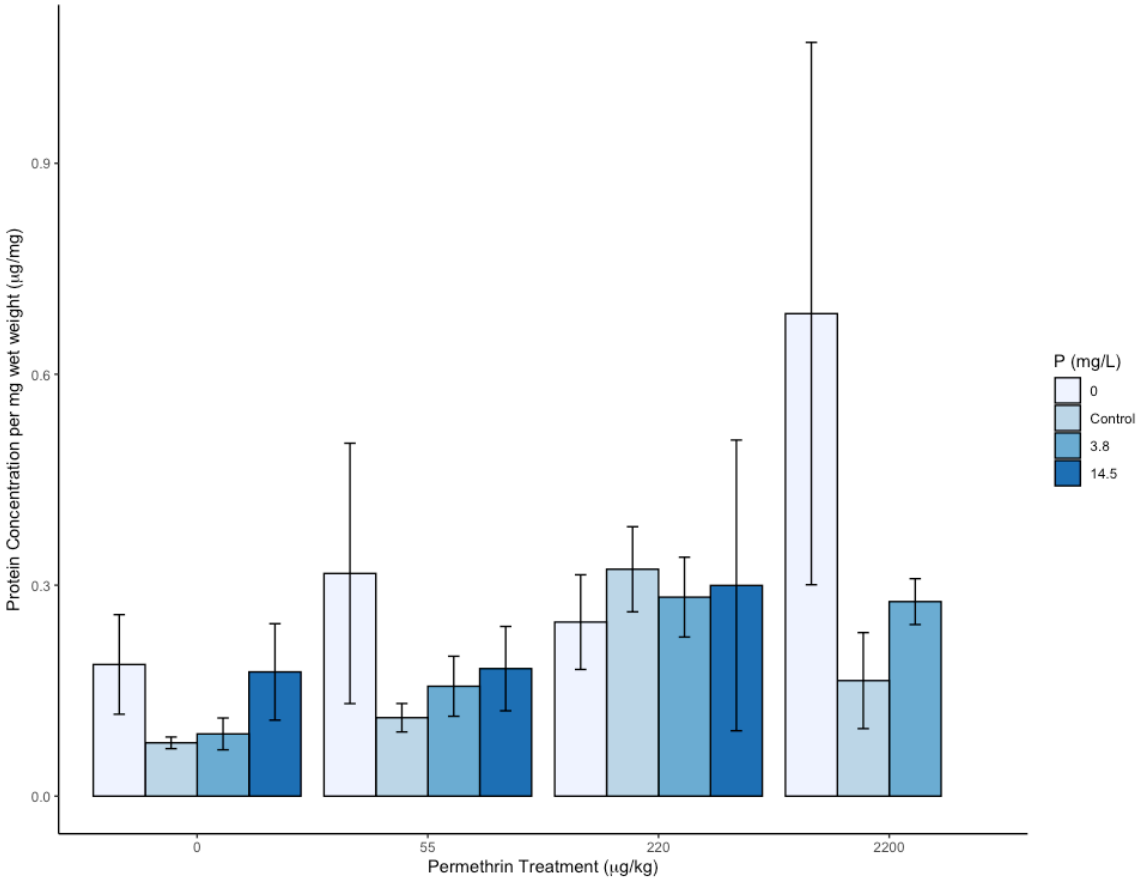

Figure 4. Mean (± SE) protein concentration per mg of wet weight for *Chironomus tepperi* larvae exposed to P and permethrin treatments for 96 hours. N = 4 replicates per treatment.

Lipid

There was a significant increasing effect of both permethrin and P exposure on the lipid concentration in the larvae (Figure 5, Kruskal-Wallis $p < 0.001$). This difference in lipid concentration was largely driven by the 14.5 mg/L P treatment which had a mean lipid concentration per wet weight of 15.3 μg/mg across all permethrin treatments, compared to all other P treatments which had mean lipid concentrations of 10.1 μg/mg or less. This effect was especially noticeable in the 0 and 2200 μg/kg permethrin treatments, where the mean lipid concentration was 379% and 239% higher than the control P treatment, respectively. There was also a slight increase in lipid concentration in all permethrin treatments compared to no permethrin.

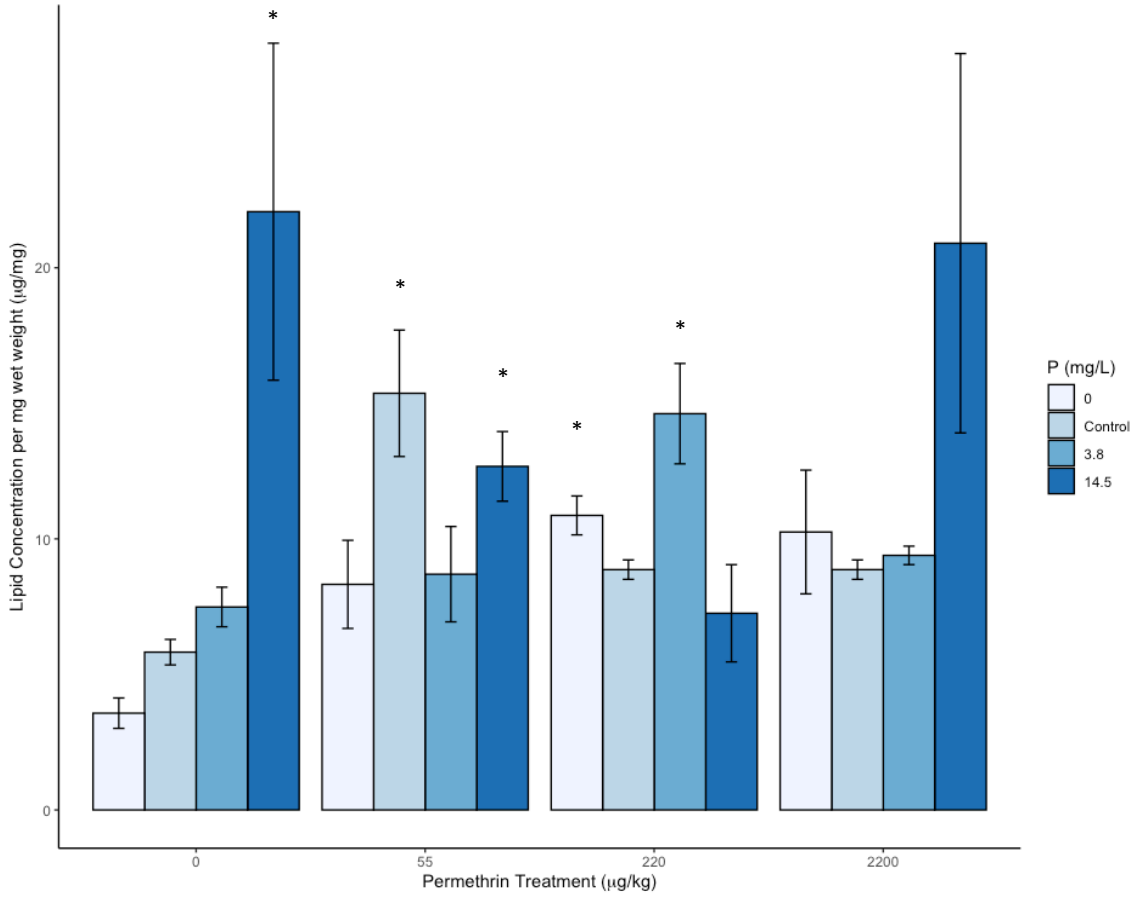

Figure 5. Mean (± SE) lipid concentration per mg of wet weight for *Chironomus tepperi* larvae exposed to P and permethrin treatments for 96 hours. N = 4 replicates per treatment. Treatments with an asterisk (*) are significantly different from the 0/0 treatment. Bars with no asterisk represent no significant difference between treatments.

3.3.5 Emergence Test

Proportion Emerged

Percent emergence was significantly reduced in the highest permethrin treatment, regardless of the P addition (Figure 6, $F_{(3, 15)} = 0.864$, $p < 0.001$). No larvae/pupae were found in the sediment at the end of the exposure period. The mean proportion emerged was 88.6% for the 0 permethrin treatment and 37.5% for the 2,200 μg/kg treatment. The addition of P did not alter the toxicity of the permethrin at any concentration.

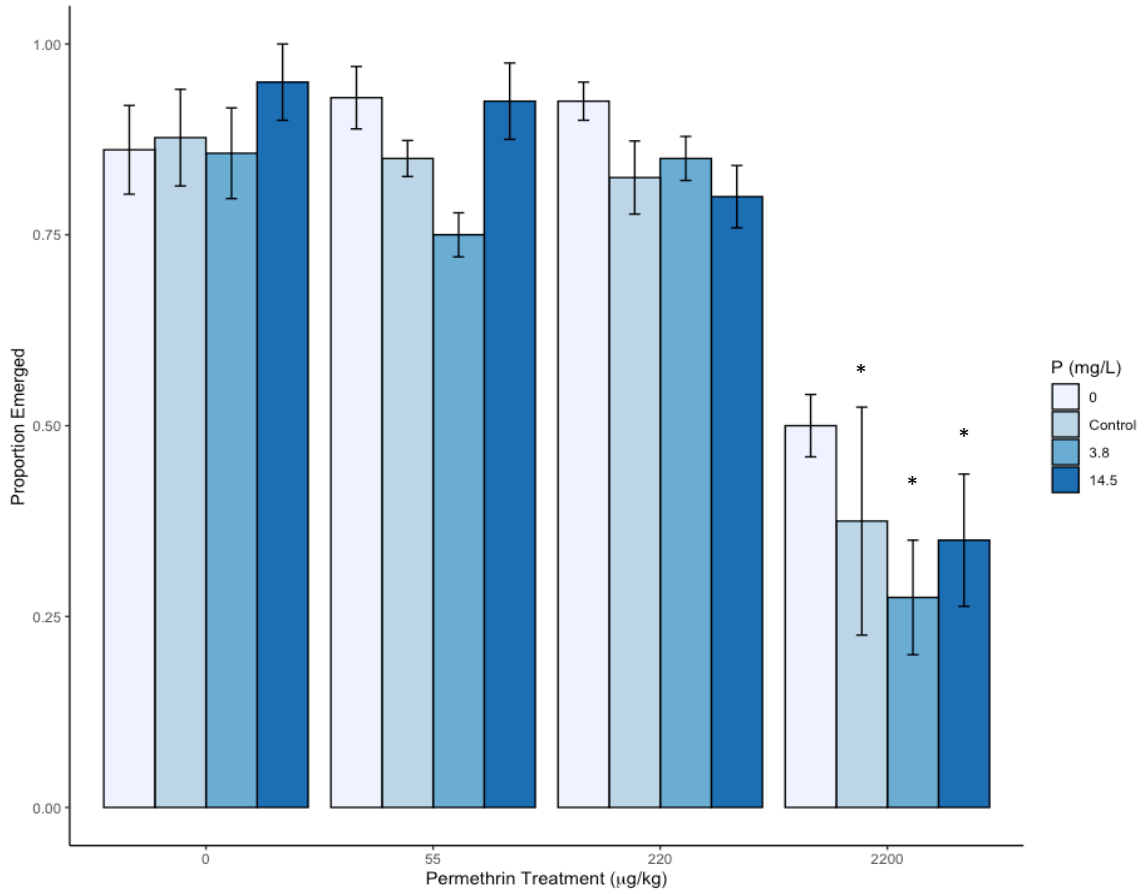

Figure 6. Mean (± SE) proportion emerged of *Chironomus tepperi* larvae exposed for 22 days to different concentrations of P and permethrin in water and sediment. N = 4 replicates per treatment. Treatments with an asterisk (*) are significantly different from 0/CTL. Bars with no asterisk represent no significant difference between treatments.

Mean Emergence Day

There was no significant effect of P or permethrin, or the interaction between the two on the mean day of emergence (Figure 7, $p > 0.05$). All treatments' mean emergence day was between 15 and 17 days after hatching. The 0 μg/kg treatment had a mean day of emergence of 17.062 days post hatch compared to the permethrin treatments, which were all within the 16 days post hatch range. There was also a slight trend of earlier emergence in the P treatments as the mean day of emergence in the 0 mg/L treatment was 17 days as opposed to 16 in all other P treatments.

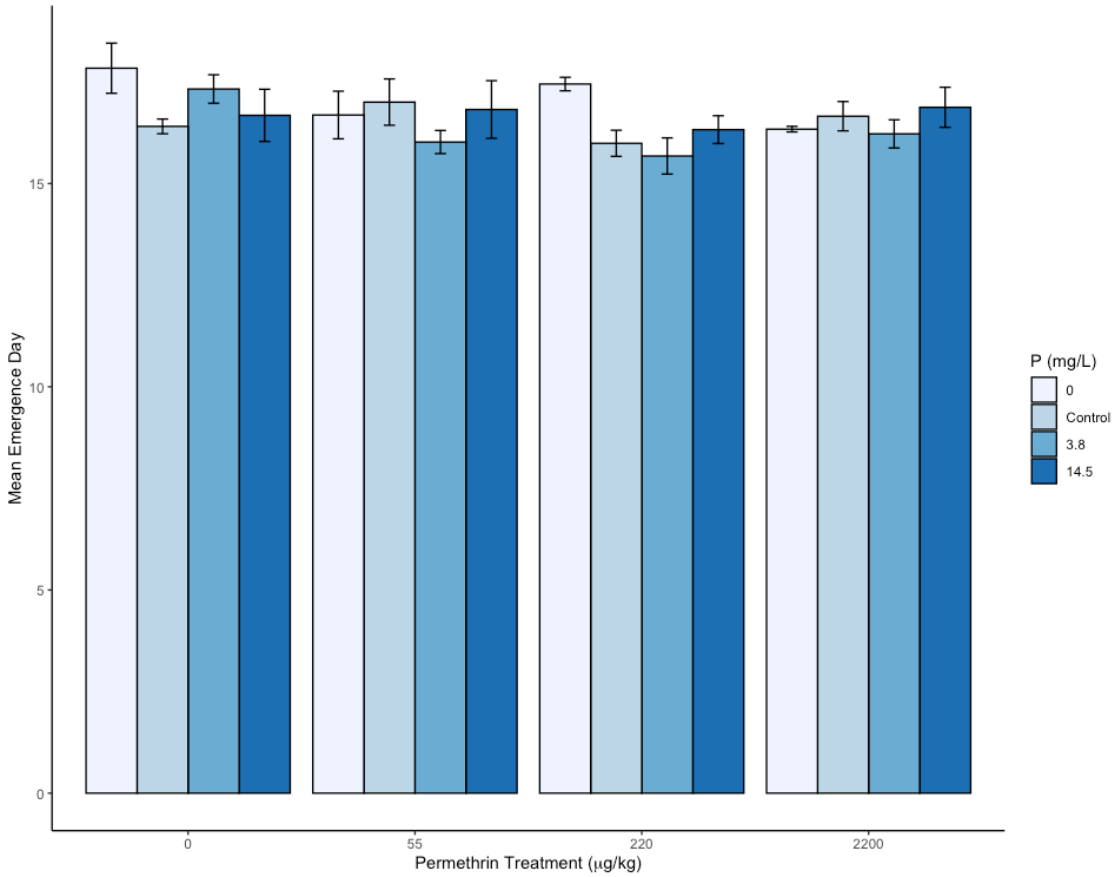

Figure 7. Mean (± SE) emergence day of *Chironomus tepperi* larvae exposed for 22 days to different concentrations of P and permethrin in water and sediment. N = 4 replicates per treatment.

Female Mean Emergence Day

Mean emergence day of females occurred between 16 and 19 days post hatch (Figure 8). There was a significant decrease in female emergence day when exposed to permethrin (Kruskal-Wallis p = 0.027), however this effect was negated when P was also present (Kruskal-Wallis p = 0.077). In the 2200 μg/kg permethrin treatment the average day of emergence was 16.7 days, which was two days earlier than the 0 μg/kg treatment mean of 18.5 days post hatch. There was a small trend of earlier emergence in the permethrin treatments, however this trend was negated by the opposing trend in the P treatments. In the P treatments there was a small increase in the mean female day of emergence in the 14.5 mg/L treatment (18.4 days) compared to the control (17.3 days). These opposing trends cancelled each other out to result in no overall effect on the female mean day of emergence.

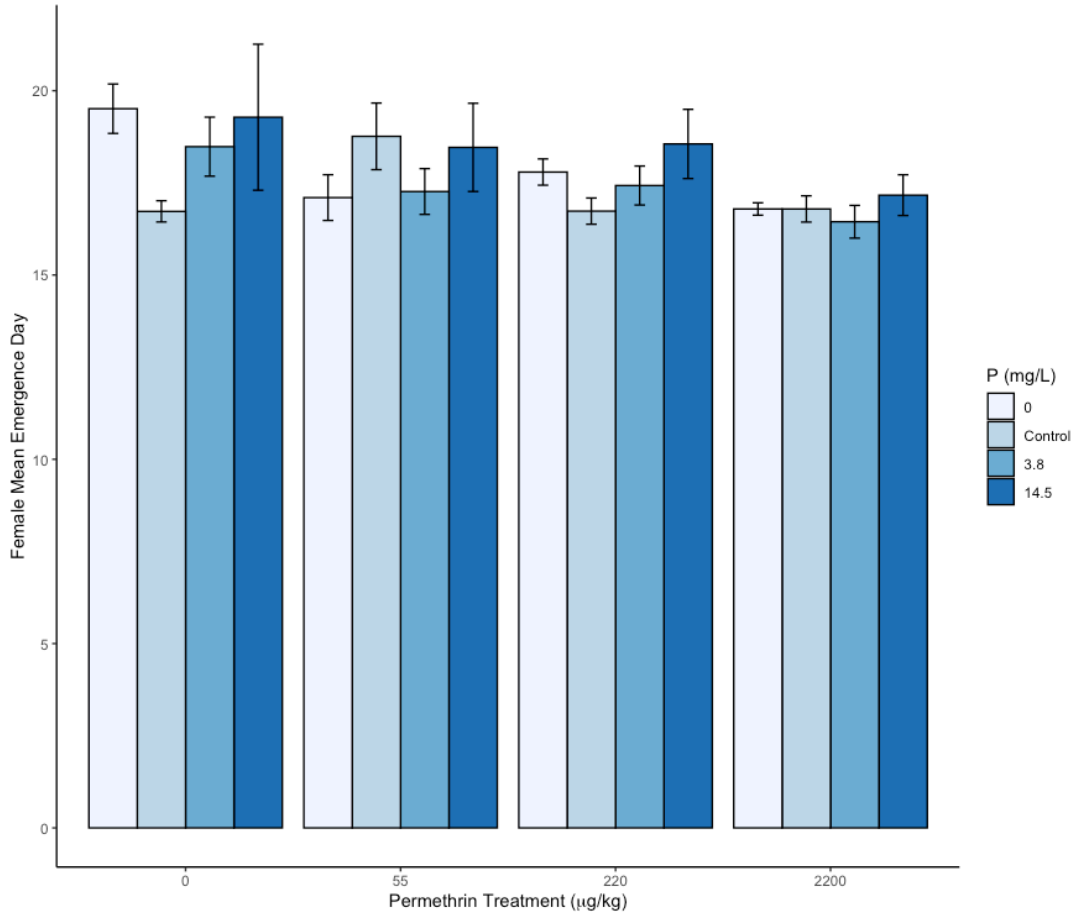

Figure 8. Mean (± SE) female emergence day of *Chironomus tepperi* larvae exposed for 22 days to different concentrations of P and permethrin in water and sediment. N = 4 replicates per treatment.

Male Mean Emergence Day

There was a significant increase in male emergence day when P was present (Kruskal-Wallis p = 0.024), but this effect was negated when permethrin was present (Figure 9, Kruskal-Wallis p = 0.154). All males had a mean emergence day between 15 and 17 days post hatch. However, this significant increase was a weak trend and was negated by the fact that there was almost no difference in male mean emergence day in the permethrin treatments.

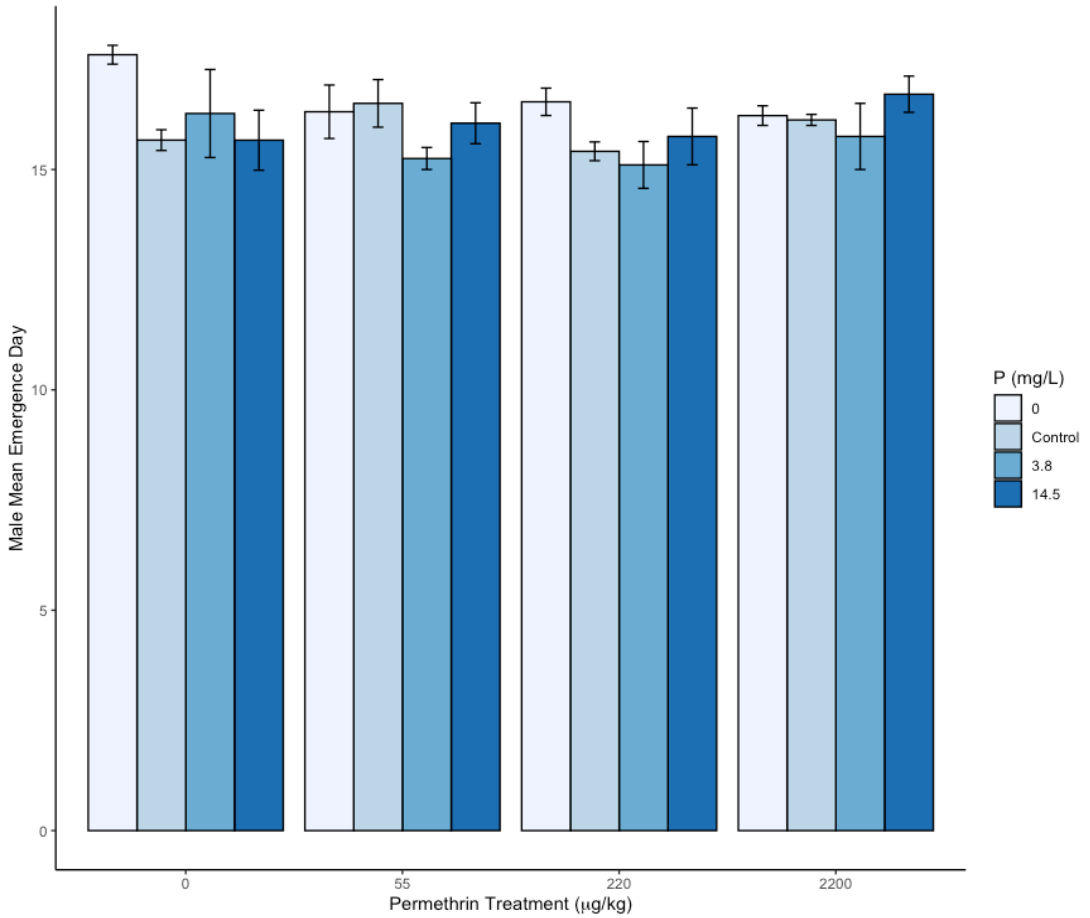

Figure 9. Mean (± SE) male emergence day of *Chironomus tepperi* larvae exposed for 22 days to different concentrations of P and permethrin in water and sediment. N = 4 replicates per treatment.

3.4 Discussion

The aim of this study was to determine whether P had any effect on the toxicity of permethrin on *C. tepperi* survival, weight, development, and energy reserves. We found that the 96-hour time point was a more sensitive time to observe effects of exposure to permethrin and P compared to the longer exposure period of up to 20 days for the emergence endpoints. This is in contrast to other studies, which have found that emergence day and percent emergence is generally a more sensitive endpoint (Hale et al. 2014; Langer-Jaesrich et al. 2010; Stoughton et al. 2008). This is a surprising result, as it would be assumed that any effects in acute toxicity tests would translate to developmental effects in chronic tests. However, as this study shows, that is not always the case when aspects of water and sediment quality are included in the test. This is a known pattern and similar mistranslation of effects in chironomids have been found by Pascoe et al. (1989) who found that significant reductions in survival and growth after *Chironomus riparius* were exposed to cadmium showed a reduction in percent adult emergence, but no significant alteration of emergence day**.** Their explanation for acute effects not translating to chronic ones was that the study may not have used high enough concentrations of cadmium to elicit a response. While this hypothesis may apply to the present study, it is more likely that P was mediating the toxicity of permethrin over the period of the chronic test, which did not happen over the acute testing period. Although the emergence findings are interesting, the more statistically significant results for this study occurred in the 96-hour exposure, which will be the emphasis of this discussion.

3.4.1 Acute Effects

There was a clear effect of high permethrin treatment that reduced survival of *C. tepperi* regardless of P concentration or exposure duration. This concurs with Mehler et al. (2017) who found similar concentrations of permethrin reduced *C. tepperi* survival. In the present study, there was also a decrease in larval dry weight with increased permethrin concentrations. These effects were exacerbated at the highest (14.5 mg/L) and lowest (0 mg/L) P treatments. This decrease in larval weight was an indication that permethrin has a sublethal effect on *C. tepperi*, which can translate into lethal effects later in development. Similar results were found by Maul et al. (2008) who also showed that increasing exposure to permethrin resulted in decreased larval dry mass and growth rate in *Chironomus tentans* (now *C. dilutus*) after a 10 day exposure.

This research analysed protein, lipid, and glycogen content in larvae exposed for 96 hours to determine if exposure results in changes in their energy storage and usage. The effects of P and permethrin on the energy reserves depended largely on P treatment. The 0 mg/L P treatment (simulated oligotrophic conditions) produced a different response in glycogen content compared to other treatments. This interesting response has implications for acute ecotoxicology tests. There was a clear hormesis response across permethrin treatments; which was reflected in all P treatments except 0 mg/L. Hormesis is a common response found in ecotoxicology where a low dose of a toxicant can induce a beneficial effect (Chapman 2001). In this study, the 55 μg/kg permethrin treatment had increased glycogen concentrations compared to all other treatments. This is not an effect that has been previously observed as a response to either synthetic pyrethroids or nutrients. Choi et al. (2001) found that glycogen concentration in *C. riparius* larvae decreased in hypoxic environments and in response to an organochloride pesticide exposure. Sak et al. (2006) found a decrease in glycogen concentration across life-cycle stages in parasitic wasps following exposure to the synthetic pyrethroid

cypermethrin. It is possible energy was being stored in the form of glycogen because the low permethrin treatment was exerting a low level of stress to stimulate storing the extra glycogen for future energy-requiring needs, but insufficient stress to justify the expenditure of that energy. This is consistent with the allocation of energy in insects, as glycogen is a relatively short term store of energy that is released quickly – often used for flight in adult insects – as opposed to lipids, which are mostly stored long-term in the fat body (Thompson 2003). When an insect is exposed to a sublethal concentration of pesticide, it is possible that the short-term energy stores increase to help the animal handle the low level of exposure. This may not happen at the higher permethrin concentrations because the glycogen stores may have already been depleted as a result of coping with the higher level of stress from either detoxification or the additional movement that sometimes results from high stress environments (Azevedo-Pereira et al. 2011b). This also explains why the 0 mg/L P treatment is generating a similar response across all permethrin concentrations, as the lack of P increases the stress for the larvae and their response is more akin to the stressed animals in the higher permethrin concentrations. The effects of a lack of P on glycogen concentration in the larvae is expected. P is essential to glycogenesis – the synthesis of glycogen from glucose molecules – and it follows that a lack of P would result in a lower concentration of glycogen, as illustrated in this study, as there would be insufficient P for glycogenesis to occur. A study by Sakamoto and Yone (1978) showed diets containing different levels of P fed to Red Sea Bream (*Chrysophrys major*) resulted in differing glycogen concentrations and that diets which contained low P decreased glycogen concentrations in the fish. However, there are very few studies investigating the effects of P on glycogen storage in aquatic organisms, and none could be found on invertebrates at the time of writing.

There was a nearly opposite effect of P concentration when lipids were analysed. The concentration of lipids was slightly higher in the 14.5 mg/L treatment and was more variable, than in all other treatments, especially the 0 and 2200 µg/kg permethrin concentrations. This may be because the larvae that were exposed to higher concentrations of P were storing their excess nutrients in the form of fat energy. Most of the lipids in insects are stored in the fat body, which uses P to create triglyceride lipids for long term storage (Gilby 1965). It seems, from these results, that the larvae exposed to higher concentrations of P are using that excess P to put into long term storage as lipids. Sakamoto and Yone (1978) also found that that a decrease in P led to an increase in lipids in Red Sea Bream, so it is possible that this trend is found across taxonomic groups.

There have been some investigations into how nutrient deficient diets decrease insect glycogen and lipid stores (Hahn 2005; Thompson 1982), but none that investigate the effects of environmental nutrient exposure on these energy reserves. This study better approximates a real-world exposure scenario as the *C. tepperi* larvae will likely take up P through the food located in the sediment.

3.4.2 Chronic Effects

The effects of permethrin and P on *C. tepperi* in the chronic test were varied. The was a significant decrease in the proportion of emerged adults as permethrin increased, regardless of P concentration. This followed a similar, but exaggerated, pattern as the survival and weight endpoints in the acute tests. It is not a surprise that the reduction in survival and weight following the short-term, acute exposure would translate to reduced emergence in the chronic test. Other studies on *Chironomus* species and various pesticides have also found that acute or larval endpoints (especially weight) are an indication of adult emergence (Cavallaro et al. 2017).

The reduction in the proportion of emerged adults did not affect the mean day of emergence. Despite there being a slight trend towards earlier emergence with higher permethrin concentrations, there was no real trend in emergence with P concentration, which negated the very slight trend in the permethrin treatment. This general effect was replicated across both male and female emergence.
Early emergence of *C. tepperi* in response to a synthetic pyrethroid insecticide has been seen in previous studies. Boyle et al. (2016) exposed *C. tepperi* to bifenthrin and found that concentrations of 10 μg/g OC and higher induced an early emergence response, particularly for males. The authors also observed a similar early emergence response in *C. oppositus* in a field-based microcosm experiment. This response to SPs seems to be unique to *C. tepperi* and *C. oppositus* among standard *Chironomus* species. Goedkoop et al. (2010) exposed *C. riparius*, a Northern Hemisphere species, to cypermethrin and found that they responded with delayed development and increased adult size. It should be noted that *C. riparius* has a slightly longer life cycle than *C. tepperi* and that the larvae in those experiments were exposed from 1st instar, just after hatching.

The explanation for early emergence given by Boyle et al. (2016) was that bifenthrin may have been disrupting the development hormone pathway for ecdysis. Synthetic pyrethroids are known to cause endocrine disruption in vertebrates (Moore and Waring 2001; Singh and Singh 2008), and it is possible that this is also a response in some invertebrates. It may also be that Australian invertebrate species have a slight difference in either hormone levels or hormone pathways that makes them particularly susceptible to SPs. There is also a possible ecological explanation. Unlike *C. riparius, C. tepperi* tend to inhabit temporary pools. This accelerated development may have developed as a response to signs of stress (i.e. a pool or puddle drying). Earlier emergence may have developed as a response to the time pressure of a drying puddle or pool where accelerated development is needed. Currently, there are no studies on the effects of drying or drought on *C. tepperi* development, however a thorough investigation from Cantrell and McLachlan (1982) into the response of dipterans to drying rain pools found that other stressors (such as the presence of frog larvae) can cause larvae to emerge earlier. In the case of flies that inhabit temporary pools emerging earlier when other stressors are present can be a fitness advantage as it reduces the chance of desiccation.

Even though the response to permethrin in the chronic tests was early emergence, there was no significant effect of treatment when P was introduced. As previously mentioned, it is likely that P is negating the response seen in the permethrin treatments. This was especially noticeable in the female's mean emergence day where there was a significant decrease in emergence day in permethrin treatments and a trending increase in P treatments. As the significant decrease in emergence day associated with permethrin was a very weak trend, it is possible that the additional P added to the system was enough to strengthen the ability of the larvae to deal with the pesticide stressor.

3.4.3 Conclusions

The effects of P on the toxicity of permethrin to *C. tepperi* are difficult to unravel. In both acute and chronic tests, increased concentrations of permethrin increased mortality, reduced emergence and reduced dry weight at high concentrations irrespective of the concentration of P. However, at lower concentrations of permethrin the toxicity of the pesticide was mitigated by the presence of higher concentrations of P, particularly in measures of energy reserves. These sublethal indicators showed that a lack of P can affect the overall energy reserves in *C. tepperi* which could cause a change in energy expenditure and storage which may explain the observed reduced growth. In emergence measures,

the opposing trends of later emergence in the P treatments and earlier emergence in the permethrin treatments are further evidence of an antagonistic relationship between these two stressors.

The results of this study show that traditional ecotoxicology tests need to take nutrient and water quality parameters into account when interpreting laboratory toxicity studies. This is especially true in countries outside of Europe and North America where the science is still being standardised. It is possible that local conditions are concealing potential toxicity that would be found in more traditional test species and that it is essential to monitor water quality parameters (including nutrients) in a management context. This study also highlights that a more thorough understanding of the test medium in ecotoxicology bioassays is required, and that researchers should be analysing for nutrient concentrations in "clean" sediment before testing or amendment. Based on the results of this study nutrient (and particularly P) concentrations need to be considered in ecotoxicology bioassays as well as in the management of Australian waters in order to alleviate any concerns about toxicity masking by differing water quality parameters.

CHAPTER 4: THE EFFECTS OF SALINITY ON THE TOXICITY OF IMIDACLOPRID TO THE GROWTH AND DEVELOPMENT OF THE AUSTRALIAN MIDGE *CHIRONOMUS TEPPERI*.

4.1 Introduction

In many regions of the world there is predicted to be less rainfall due to climate change. In Australia, for example, most of Southern Australia is predicted to have a 15% decrease in average rainfall by 2055 (CSIRO and BOM 2015). One of the effects of decreased average rainfall is an increase in the salinity of fresh waters and there is evidence from freshwater environments worldwide that salinity is becoming an increasing issue (Herbert et al. 2015; Kaushal et al. 2005; Williams 1999). This is particularly a problem in Australia, where many freshwaters are already saline, but with increased evaporation rates, land clearing, and irrigation this has become an even bigger problem (Hart et al. 1991; Williams 1999).

Consequently, the effects of increasing salinity on freshwater species has been investigated extensively on nearly every continent (Cheng and Chen 2000; Kefford et al. 2012; Richmond and Woodin 1996; Sarma et al. 2006; Waterkeyn et al. 2008). In particular, there has been much work done in Australia in the last decade or so (Hassell et al. 2006; Kefford et al. 2003; Nielsen et al. 2003) that has led to an increased understanding of the effects of salinization on both individual species (Hassell et al. 2006; Kefford et al. 2007) as well as communities and ecosystems (Schäfer et al. 2012; Szöcs et al. 2012). Overall, invertebrate species seem to be less tolerant and more stressed as salinity increases. However, as shown by (Kefford et al. 2012) there can be variation in salinity tolerance across geographic regions. In that study, species from relatively wet regions in France (Lorraine and Brittany) were found to be more sensitive than those species from relatively arid regions (Australia and South Africa). This highlights the need for research on locally endemic species especially in areas, such as Australia, where salinisation is an increasing issue.

Imidacloprid is a neonicotinoid pesticide that has received much attention over the last two decades due to its toxicity to invertebrates (Aizen and Harder 2009; CSIRO and BOM 2015; Decourtye et al. 2004; Winfree et al. 2009). It has been implicated in the reduction of honey bee populations in the northern hemisphere and it's use has recently been banned in the European Union (Iwasa et al. 2004; Stokstad 2018; Suchail et al. 2009). There is increasing evidence that neonicotinoids, particularly imidacloprid, have an undesirable effect on freshwater and benthic insect survival, development, and population (Colombo et al. 2013; LeBlanc et al. 2012; Morrissey et al. 2015). However, as described in Morrissey et al. (2015), there are still gaps in our understanding of the impacts of neonicotinoids on aquatic organisms, especially in regards to chronic exposure, despite the fact that their review shows that freshwater communities are more sensitive when exposed for longer periods. Research into the effects of imidacloprid is particularly important for the Australian context as neonicotinoid insecticides make up approximately 1/5 of total insecticide sales (APVMA 2013). The detrimental effects of imidacloprid have been found in Australia in acute exposures in local aquatic invertebrates. In a study by Stevens et al. (2002) a simple exposure experiment was carried out with the non-biting midge *Chironomus tepperi* that found that after 24 hours of exposure to a commercial formulation of imidacloprid the LC50 was 1.60 μg/L. Similar work by Gagliardi (2017) on *C. tepperi* found a sublethal concentration of a commercial imidacloprid formulation to be approximately 1.30 μg/L. Stevens et al.

(2005) reported 24 hr LC50 values of other neonicotinoid insecticides (thiacloprid, acetamiprid, and clothianidin) of between 1.06 and 2.83 μg/L. However, none of these studies sought to investigate various chronic test endpoints or the effects of imidacloprid on larval energy resources. This study will extend our understanding of the effects of imidacloprid on Australian ecotoxicology species.

There are limited studies on the combined effects of imidacloprid and salinity on aquatic organisms. In particular, Song and Brown (1998); Song and Brown (2006); Song et al. (1997) have shown that salinity can increase pesticide-induced mortality in several insect species. These authors generally found that increased salinity also increased the toxicity of imidacloprid. When the two test species (*Aedes taeniorhynchus* and *Artemia* sp.) were exposed to hyperosmotic conditions as well as a range of imidacloprid concentrations, increased salinity increased the mortality of both species, particularly in *A. taeniorhynchus* which had a 50% higher mortality at the highest concentration of imidacloprid when in a hyperosmotic environment. Although there are some studies showing the effects of imidacloprid exposure on freshwater insects, there are few studies conducted on Australian species and even fewer investigating chronic exposure. Similarly, few studies have investigated the effects of salinity on the life history of Australian *Chironomus* species. Kefford et al. (2004) and (Kefford et al. 2007) investigated the effects of increasing salinity on the survival of Australian *Chironomus* eggs and larvae. They concluded that *Chironomus* salinity tolerance is relatively consistent throughout the larval lifespan. However, these studies only examined salinity tolerance with either 24 or 72 h exposures depending on the life stage. Hassell et al. (2006) investigated the chronic effects of salinity on an Australian field collected *Chironomus* species and found that survival/emergence was highest at a low salinity and significantly less at both the highest (15 mS/cm) and lowest (0.15 mS/cm) concentrations. They also found that emergence time increased in the higher salinities but not in the lowest salinities and that overall there was a decrease in growth rate with increasing salinity. Some of the data from the present study was incorporated into Hale et al. (2019) which formed one of the first studies into the salinity tolerance of *C. tepperi.* In that research salinity concentrations of 6,000 μS/cm were found to reduce survival and concentrations above 4,000 μS/cm were found to reduce emergence of *C. tepperi*.

There currently are no default water quality guideline values for imidacloprid concentrations in Australia (ANZECC and ARMCANZ 2018). This, combined with the relative lack of data on its effects on Australian species, means that any water quality regulation of imidacloprid will be from overseas sources. This raises the question of whether guideline values or regulation would be protective of waterbodies with extreme salinities (low or high) as can be found throughout Australia. For example, in relatively pristine alpine areas in Victoria the average stream salinity is around 120 μS/cm in comparison to a relatively impacted site with an average salinity of approximately 3500 μS/cm (DELWP 2018). It is possible that imidacloprid will be more toxic to invertebrates in relatively pristine waters and for this reason it was essential that this study include a very low or 0 salinity treatment.

Chironomus tepperi is an Australian freshwater midge (Diptera) which is often used in standard aquatic toxicity tests (Batley and Simpson 2016; Martin and Porter 1978; Stevens et al. 2002) . However, there have been no studies investigating the effects of chronic exposure to imidacloprid on *C. tepperi* and there has also been little investigation into the combined effects of salinity and imidacloprid on individuals, and more specifically their life history and energy use. There is a need to conduct research into this organism's whole life history and energy resources in order to provide better environmental monitoring data and make better water management decisions.

This chapter aims to determine the effects of salinity on the acute and chronic toxicity of imidacloprid to *Chironomus tepperi*, specifically on development, survival, weight and energy reserves.

4.2 Methods

4.2.1 Chironomid Culture and Preparation

Chironomus tepperi were cultured in a modified Martin's artificial water solution (as described in Jeppe et al. (2014)), with ethanol-rinsed toilet paper as substrate. Larvae were fed tropical fish flakes (Tetramin) three times per week, with 0.25 mg per tank. To obtain larvae of a known age, adult *C. tepperi* were collected from the CAPIM laboratory culture and placed into a Perspex tank with artificial water (Jeppe et al. 2014). After 24 hours, egg masses were collected with a pipette and placed into 2L beakers (three egg masses per beaker) to hatch. The 2L beakers included ethanol-rinsed toilet paper substrate and were maintained at 20º C +/- 1 degree with a photoperiod of light:dark 16:8hr. Two drops of food (a slurry made up of crushed Tetramin, tropical fish food flakes, at a concentration of 10g/100 ml of artificial water) was provided every 2 days until the larvae had reached 2nd instar (5 days old).

4.2.2 Salinity and Imidacloprid spiking

Both salinity and pesticide treatments were prepared using artificial water (as described above) to create a range of conductivities and imidacloprid concentrations, with the exception of the 0 µS/cm salinity treatment which used Reverse Osmosis (RO) water. The conductivity solutions were prepared with NaCl (Univar, analytical reagent) added to the salts already present in the artificial water and stirred for 1 min until fully dissolved. The conductivity of the solution was measured with a water quality meter (TPS, Australia). Imidacloprid solutions were prepared with the commercial formulation Confidor (Bayer, 50g/kg active ingredient). Imidacloprid solutions were sent to a commercial laboratory (Advanced Analytical Australia, North Ryde, NSW) for determination of imidacloprid concentration using liquid chromatography–tandem mass spectrometry. QA/QC procedures for the analysis included a laboratory control spike or matrix spike in every 20 samples and a duplicate sample for every 10 samples analysed. Prior to analysis an internal standard (Atrazine-d5) was added to the samples and standards. Matrix spike recoveries were between 89 and 101%.

The conductivities of water used in this experiment were 0, 150-220 (salinity of artificial water), 4000 and 8000 µS/cm. The concentrations of imidacloprid used were 0 (control), 0.28, 0.47 and 1.30 µg/L. Throughout this manuscript, the control treatment refers to a 150-220 µS/cm and 0 µg/L mixture of salinity and imidacloprid in order to most closely mimic both the natural environment and laboratory conditions. These values were determined based on results of previous literature (Gagliardi 2017) and the salinity of water in south-eastern Australia (DELWP 2018).

4.2.3 Bioassays

For both the 96 hour and emergence bioassays 400ml of prepared treatment water was added to clean 600ml beakers with 4 replicates per treatment. Ten five-day old (2nd instar) *Chironomus tepperi* larvae were randomly added to each beaker. The beakers were then put into a temperature-controlled room at 21°C with 16:8hr day/night cycle. The overlying water was tested for the following water quality parameters: temperature, pH, salinity, and dissolved oxygen.

96 hour Bioassay

After 96 hours, the water was filtered through a 250 µm sieve and larval survival was recorded. Four larvae from each replicate were frozen on dry ice for biomarker determination (and stored at -80°C until further analysis) and the remaining larvae were put into microcentrifuge tubes with 70% ethanol for growth determination. The overlying water from each treatment was pooled and temperature, pH, and salinity were measured. Samples of the pooled treatment water were sent to a commercial laboratory to determine imidacloprid concentration (Advanced Analytical Australia, North Ryde, NSW).

Emergence Bioassay

Reverse osmosis water was added 3 times a week to counteract evaporation and to maintain appropriate salinity for each treatment. Larvae were fed two drops of Tetramin food slurry 3 times a week until emergence was observed and then fed 1 drop two times a week for the remainder of the test, as there were fewer larvae present. The pH, temperature (ºC), and salinity (µs/cm) were measured weekly throughout the test.

Emerged adults were collected daily with an aspirator. The numbers of males and females were recorded and preserved in Eppendorf tubes in 70% ethanol. The experiment was terminated when there was no further emergence in the Control treatments after 2 days. The remaining water was filtered through a 250 µm sieve to collect any surviving larvae or pupae. Sex, proportion of adults emerged, and emergence day were recorded. The overlying water from each treatment was pooled and temperature, pH, salinity, and dissolved oxygen were measured. Samples of the pooled treatment water were sent to a commercial laboratory to determine imidacloprid concentration (Symbio Laboratories, Brooklyn, VIC).

4.2.4 Growth determination

The ethanol in each Eppendorf tube of larvae from the 96 hour bioassay was allowed to evaporate for 48 hrs in a fume hood and the larvae were placed onto pre-weighed tin trays. The trays were placed into an oven for 24 hrs at 60 °C and then weighed to determine larval dry weight. This weight was divided by the number of larvae in the tube and used as an average for that replicate. Dry weight is expressed as mg/larvae.

4.2.5 Energy Reserves

Protein Determination

One frozen larva per replicate was homogenised in 50µl ultra-pure water, centrifuged at 14000g at 4°C for 5 minutes and the supernatant collected. Protein concentration was determined using the Lowry method (Lowry et al. 1951) using the DC Protein assay kit (BioRad) using Synergy 2 microplate reader (Biotek instruments), with bovine serum albumin as the standard.

Lipid and glycogen measurements

The lipid and glycogen analysis followed the methods of Vu et al. (2015), Van Handel (1985), and Plaistow et al. (2001), modified for use with chironomids. For each analysis, 1 frozen larva per replicate was used. Briefly, for glycogen analysis larvae were homogenised in 2% sodium sulphate solution, 99% methanol and a 1.415g/L anthrone in sulfuric acid solution were used to determine glycogen content at room temperature, and samples were read at 625 nm in a Synergy 2 microplate reader, with laboratory-grade glucose as the standard. For the lipid analysis, larvae were homogenised in a 1:1 chloroform-methanol solution, 98% sulfuric acid and 1.2g/L vanillin solution were used to determine

lipid content at room temperature. Samples were read at 490 nm, with commercial vegetable oil as the standard. Each sample and standard were read in triplicate.

4.2.6 Statistical Analysis

Two-way analysis of variance (ANOVA) was carried out to determine whether salinity and imidacloprid exposure significantly affected all endpoints ($\alpha = 0.05$). If significance was observed, a Tukeys post hoc test and a simple effects analysis were used to determine which concentrations were causing the differences between treatments. Proportion data (i.e. survival and adult emergence) were arcsine transformed prior to statistical analysis. All data were checked to ensure they conformed to the assumptions of normality and analysis of variance by evaluating Skewness and Kurtosis values and Levene's Test for Equality of Error Variances. If data did not conform and was significant, a Kruskal-Wallis test was carried out for each factor (salinity, imidacloprid and combined treatment). All statistical analyses were carried out using SPSS, Version 23 (IBM Analytics, Chicago, Illinois, USA).

4.3 Results

4.3.1 96 hour experiment

Survival

There was a significant effect of salinity ($F_{(4,67)}$ = 3.04, p = 0.036), imidacloprid ($F_{(3,67)}$ = 12.713, p = 0.000) and their interaction ($F_{(9,67)}$ = 3.922, p = 0.001 – Figure 1) on *C. tepperi* larval survival. There was decreased survival in the highest imidacloprid treatment (1.3 µg/L) regardless of salinity, with a mean survival of 65%, in contrast to greater than 84% for all other imidacloprid treatments. In comparison, the lowest salinity treatment (0 µS/cm) had reduced survival compared to the control (73.7% in contrast to >83.4%). However, the interaction of both treatments exacerbated the effect on survival, which is especially noticeable in the 0.28/0 (imidacloprid/salinity) treatment, where the survival was lowest of all treatments at 52.5%. Overall, the interaction of salinity and imidacloprid was generally synergistic and especially exacerbated by a lack of salts in the 0 µS/cm treatment.

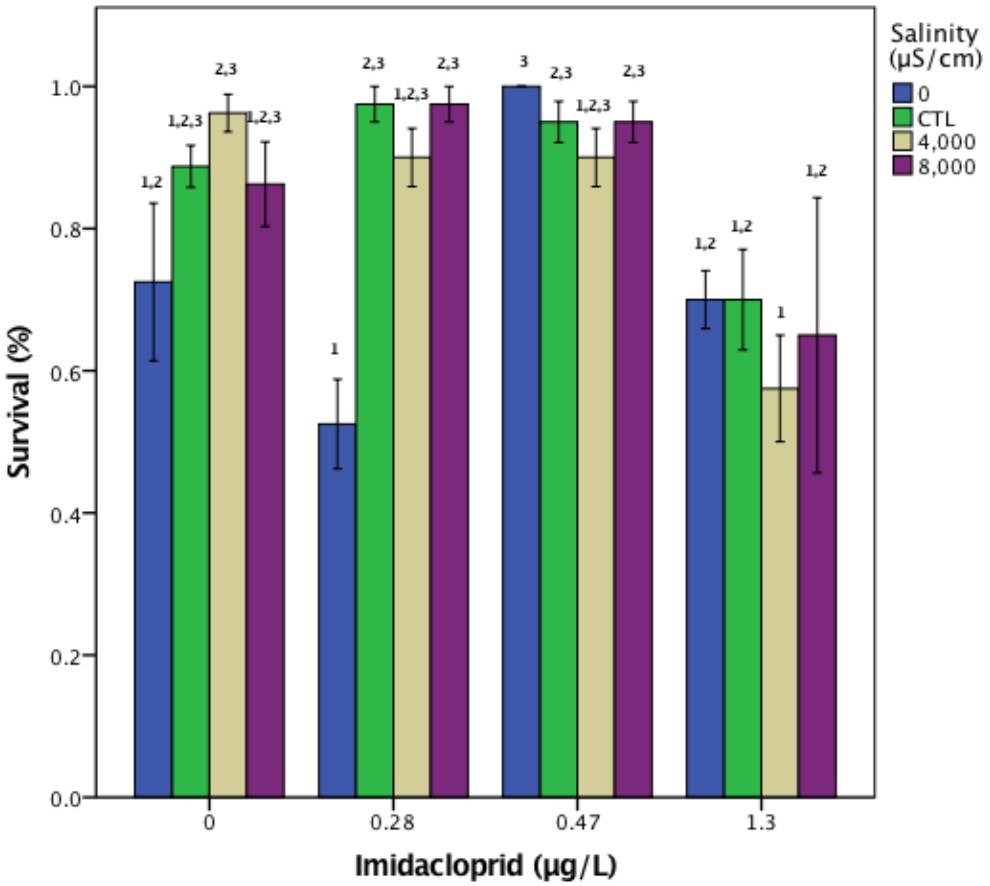

Figure 1. Mean (± SE) proportion survived of *Chironomus tepperi* larvae exposed for 96 hours to different concentrations of imidacloprid and salinity in water. N = 8 replicates per 0/CTL control and n = 4 replicates per treatment. Treatments that do not share a number are significantly different from each other.

Weight

There was a significant effect of imidacloprid, salinity, and the interaction between them on the weight of *C. tepperi* (Figure 2, Kruskal – Wallis $p = 0.000$, $p = 0.011$, and $p = 0.000$ respectively). The 1.30 µg/L imidacloprid treatment had the lowest overall mean weight at 0.137 mg (SEM ± 0.032) in contrast to all other imidacloprid treatments with mean weights above 0.236 mg. In the salinity treatments there was a significant difference between the 8000 µS/cm treatment at 0.167 mg and the Control at 0.321 mg. Imidacloprid had a more significant effect than salinity on the weight of larvae, however there was an overall decrease in weight with increasing salinity, indicating that there was a synergistic effect between the two treatments.

The effects of high and low salinity can be seen in the 0.28 µg/L imidacloprid treatment where the highest and lowest salinity treatments had low weight, but the 4000 and Control treatment were nearly identical.

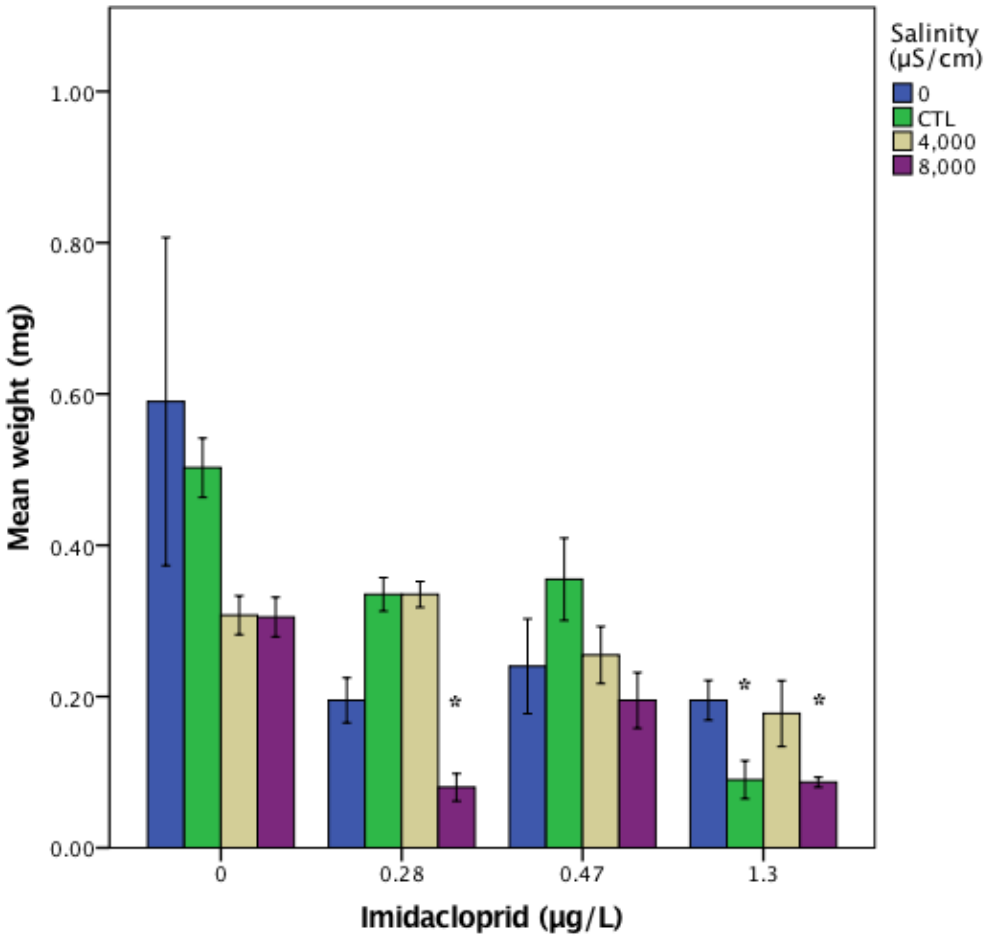

Figure 2. Mean (± SE) dry weight of *Chironomus tepperi* larvae exposed for 96 hours to different concentrations of imidacloprid and salinity in water. N = 8 replicates per control (CTL µS/cm and 0 µg/L) and n = 4 replicates per treatment. Treatments with * are significantly different from CTL/0 ($p \leq 0.05$).

4.3.2 Energy Reserves

Protein

There was no significant difference of treatment on the protein concentration in *C. tepperi* larvae (Figure 3, Kruskal-Wallis p = 0.054), although it was trending towards an increase in protein concentration with treatment. However, there was a significant effect of imidacloprid on protein treatment (Kruskal-Wallis p = 0.013), but significance was eliminated by the interaction with salinity, which was not significant on its own (Kruskal-Wallis p = 0.413). The 1.30 µg/L imidacloprid treatment had a higher overall protein concentration than all other imidacloprid treatments (0.438 µg/mg compared to 0.347 to 0.370 µg/mg). This difference seemed to drive any trend in protein concentration as all other imidacloprid treatments had similar values (between 0.358 and 0.400 µg/mg). Interestingly there was again some separation of the 0/0.28 treatment, which had the lowest protein concentration of all treatments (0.206 µg/mg).

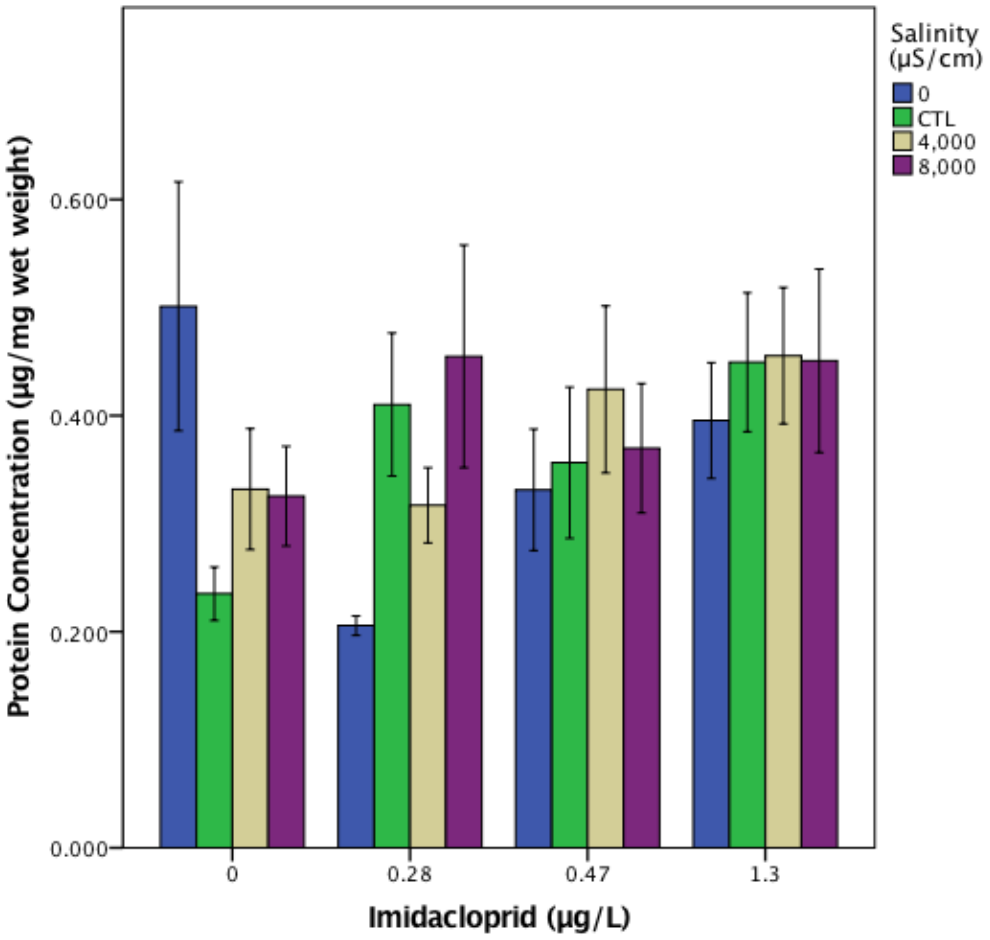

Figure 3. Mean (± SE) protein concentration of *Chironomus tepperi* larvae exposed for 96 hours to different concentrations of imidacloprid and salinity in water. N = 8 replicates per control and n = 4 replicates per treatment. No treatments were significantly different from each other.

Glycogen

There was a significant effect of treatment on glycogen concentration (Kruskal-Wallis p = 0.000). This was largely driven by the imidacloprid treatment (Figure 4, Kruskal-Wallis p = 0.000). Averaging the imidacloprid treatments, glycogen concentration decreased from 3.763 µg/mg in the 0.47 µg/L treatment to 1.841 µg/mg in the 1.30 µg/L treatment regardless of salinity; a greater than two-fold decrease. Interestingly, despite there being no significant effect of salinity on its own there was an interactive effect of salinity and imidacloprid at each concentration of imidacloprid (Kruskal-Wallis p = 0.000). Compared to the 0 µg/L imidacloprid treatment any treatment that had some imidacloprid present had an increase in glycogen at the 8000 µS/cm treatment. The highest salinity treatments had the greatest effect on glycogen concentration once mixed with imidacloprid further illustrating that imidacloprid concentration was driving the response.

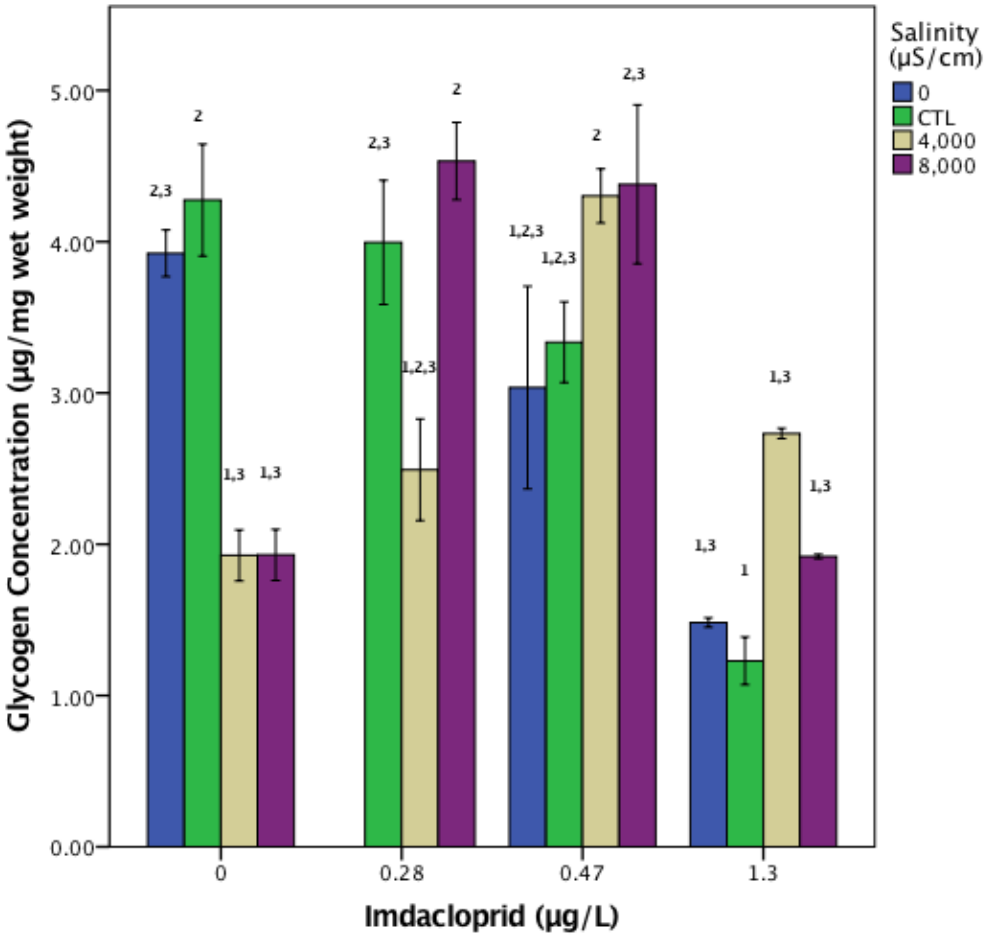

Figure 4. Mean (± SE) glycogen concentration of *Chironomus tepperi* larvae exposed for 96 hours to different concentrations of imidacloprid and salinity in water N = 8 replicates per control and n = 4 replicates per treatment. Treatments that do not share a number are significantly different from each other (p ≤ 0.05).

Lipid

There was a significant effect of treatment on the lipid concentration of *C. tepperi* larvae after 96 hours (Figure 5, Kruskal – Wallis p = 0.000). Overall, the Control salinity treatment had the highest mean lipid concentration with an average lipid concentration of 7.197 µg/mg compared to the other salinity treatments, with 6.347 µg/mg or less. In general, the 0 µS/cm salinity treatment had a lower lipid concentration across imidacloprid treatments. There seemed to be a much stronger interactive effect in lipid concentration than there were in the other energy reserves. The 0.47 imidacloprid treatment had the largest effect on lipid concentration, with the overall highest lipid concentration across salinity treatments. However, there were interesting results in the 0.28 µg/L imidacloprid concentration. The 0 salinity and 0.28 imidacloprid treatment had the lowest lipid concentration at 2.373 µg/mg, followed by the 4000/0.28, 8000/0.28 and the Control/0.28. This indicates that again the 0/0.28 treatment had a much more dramatic effect on lipid response in the larvae.

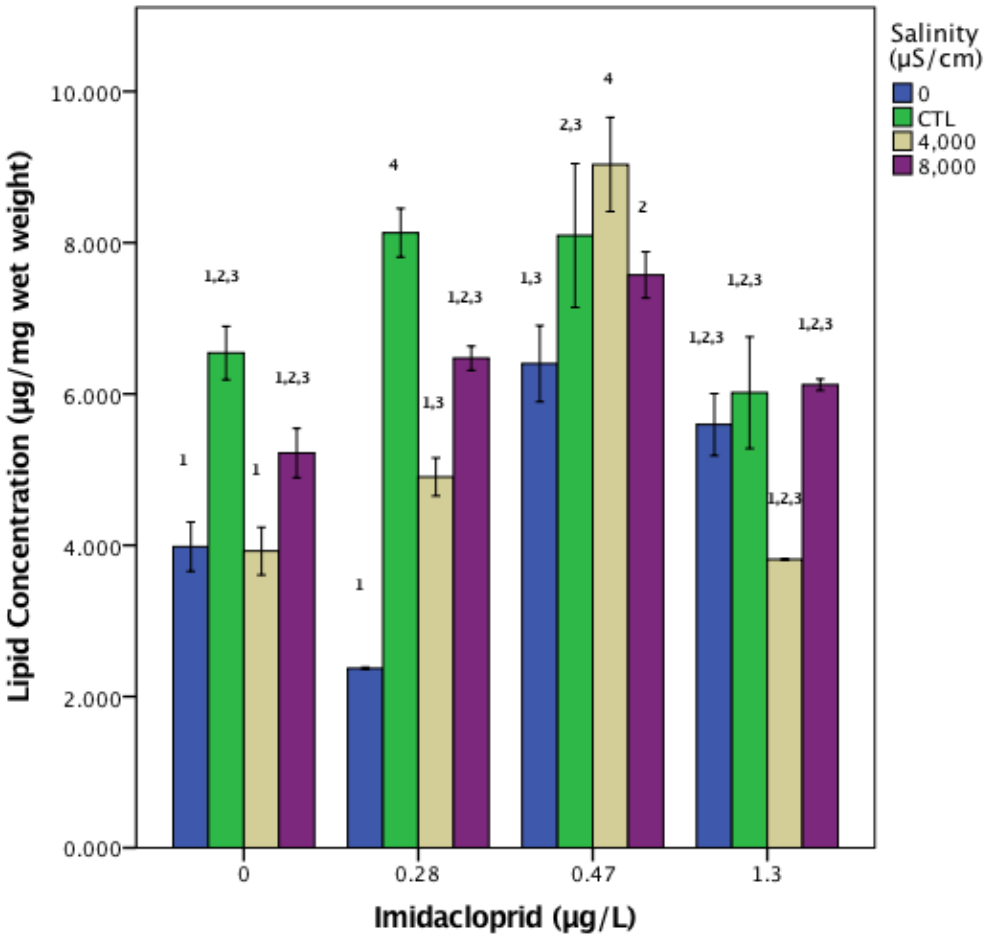

Figure 5. Mean (± SE) lipid concentration of *Chironomus tepperi* larvae exposed for 96 hours to different concentrations of imidacloprid and salinity in water. N = 8 replicates per control and n = 4 replicates per treatment. Treatments that do not share a number are significantly different from each other (p ≤ 0.05).

4.3.3 Emergence Experiment

Proportion Emerged

There was a significant effect of treatment on the percent emerged (Kruskal – Wallis p = 0.000) and, as can be seen in Figure 6, this was driven equally by salinity and imidacloprid (Kruskal – Wallis p = 0.003 and 0.000, respectively). There was no emergence in the highest imidacloprid and salinity treatments. In these treatments, larvae died before pupation but after the 96-hour timepoint, during approximately the 4th instar. *C. tepperi* had reduced emergence at both 0 µS/cm salinity and at the higher salinity treatments across all imidacloprid treatments. There was also a dramatic decrease in proportion emerged at the highest concentration of imidacloprid averaged across all salinity treatments (0. 038 compared to the 0.853 in the CTL/0 treatment).

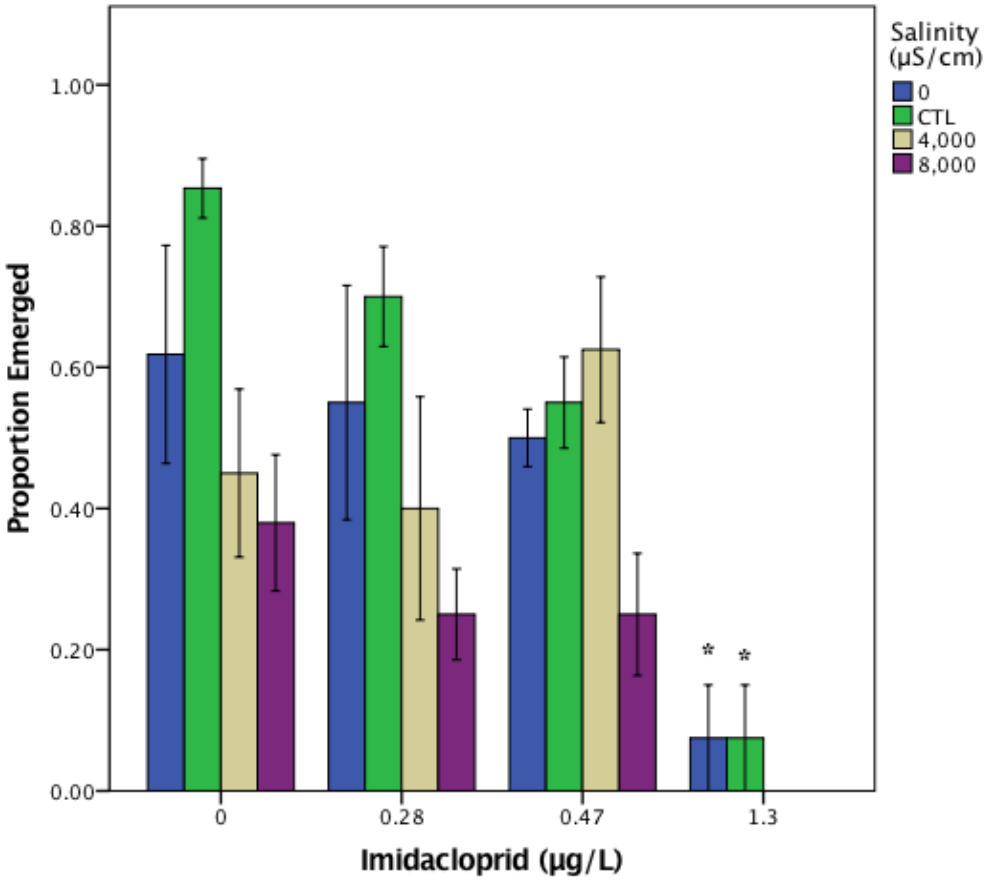

Figure 6. Mean (± SE) proportion emerged of *Chironomus tepperi* larvae exposed for 21 days to different concentrations of salinity and imidacloprid in water. N = 8 replicates per control and n = 4 replicates per treatment. Treatments with * are significantly different from CTL/0 ($p \leq 0.05$). Treatments that do not have a bar did not have any adults emerge.

Sex ratio

There was no significant effect of imidacloprid, salinity, or the interaction between them on the sex of adults emerged (Data not shown, Kruskal -Wallis, $p > 0.05$).

Mean Emergence Day

There was no significant effect of the interaction on mean emergence day. Salinity significantly increased (Figure 7, $F_{(3,39)} = 5.240$, $p = 0.004$) and imidacloprid significantly decreased emergence day ($F_{(3,39)} = 3.396$, $p = 0.027$), but the interaction between the two factors was not significant ($F_{(7,39)} = 1.140$, $p = 0.359$). This is because the two treatments had opposite effects. Salinity caused *C. tepperi* to emerge later compared to the control (15.8 days in the control treatment in contrast to 17.1 days in the 8000 µS/cm treatment). Whereas, the opposite trend was found in the imidacloprid concentrations (16.8 days in the 0 and 15.3 days in the 1.30 µg/L treatments).

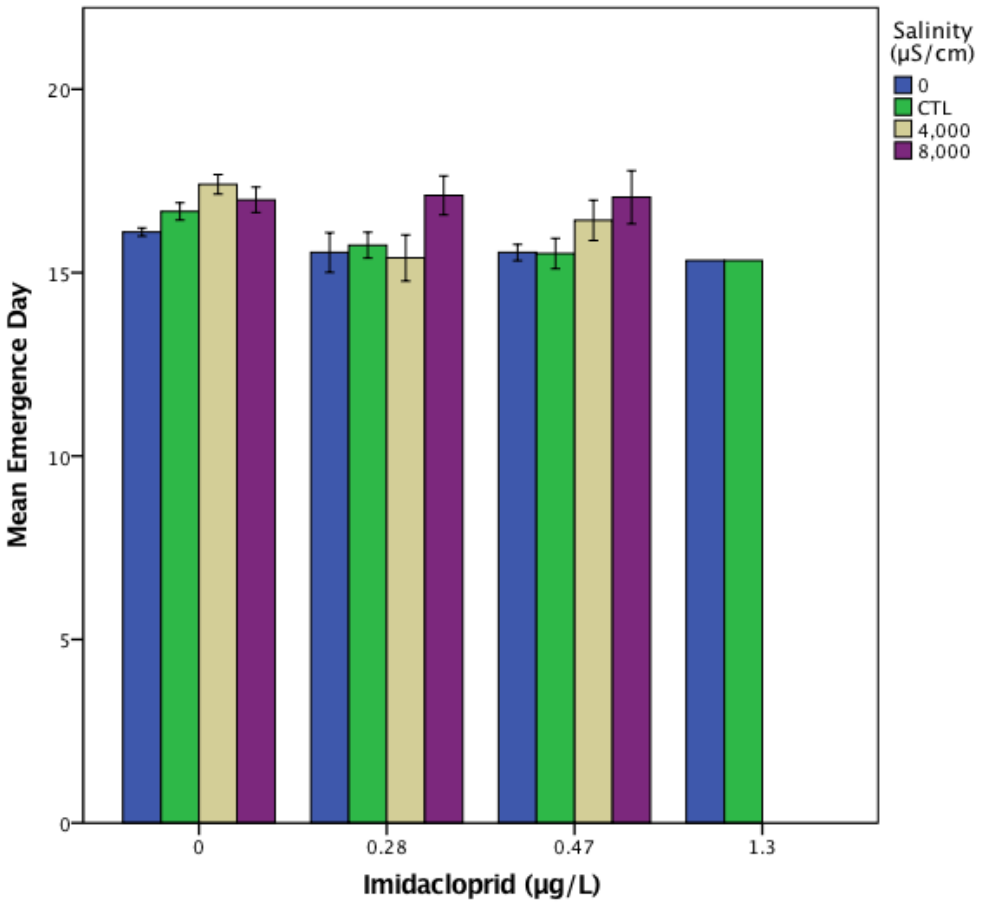

Figure 7. Mean (± SE) Mean emergence day of *Chironomus tepperi* larvae exposed for 21 days to different concentrations of salinity and imidacloprid in water. N = 8 replicates per control and n = 4 replicates per treatment. Treatments that do not have a bar did not have any adults emerge. No error bars indicate a very small number of individuals emerged at the same time. No treatments were significantly different from each other.

Male Mean Emergence Day

There was no significant effect of the interaction of salinity and imidacloprid on male mean emergence day. Salinity was found to have a significant effect (Figure 8, $F_{(3,12)} = 3.005$ $p = 0.041$) but imidacloprid was not significant ($F_{(3,12)} = 2.112$ $p = 0.116$). The interaction between the two factors was also not significant ($F_{(6,12)} = 1.239$ $p = 0.310$). There was a slight increase in male mean emergence day as salinity increased, from 15.4 days in the 0 µS/cm treatment to 16.7 days in 8000 µS/cm. However, the interaction effect of the salinity and imidacloprid treatments did not alter male emergence day.

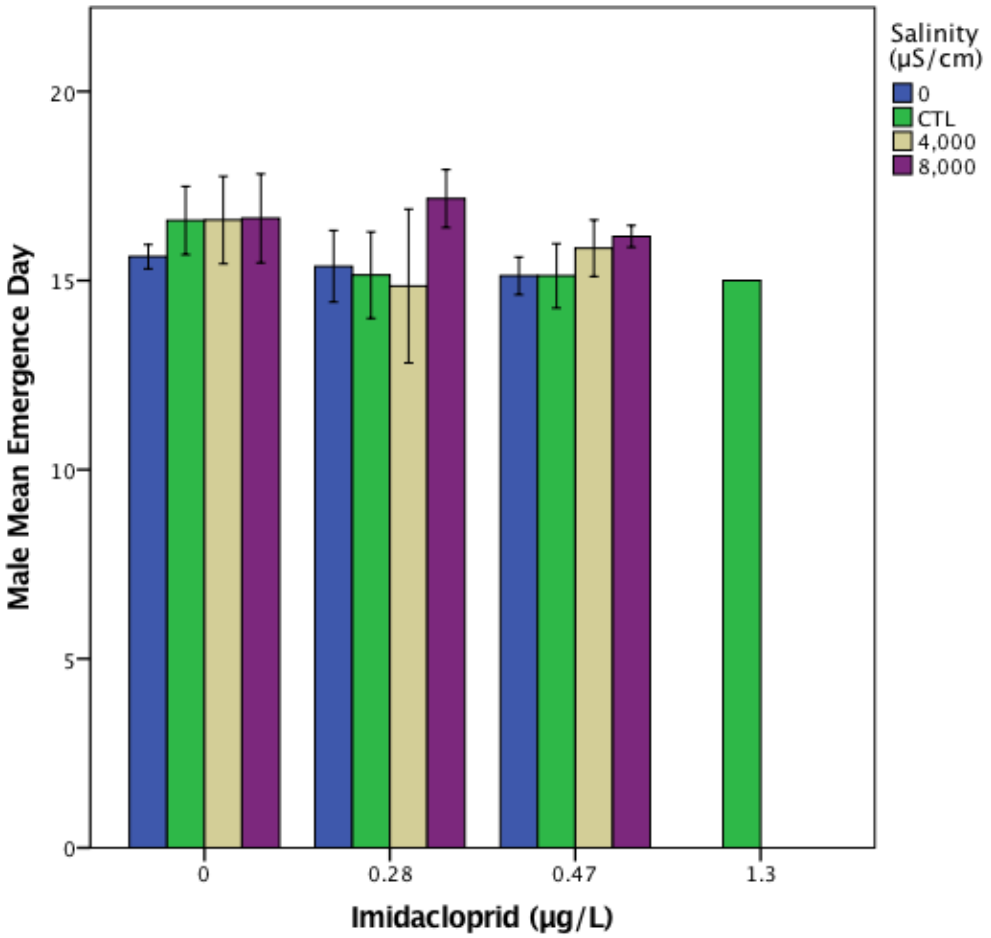

Figure 8. Mean (± SD) Mean male emergence day of *Chironomus tepperi* larvae exposed for 21 days to different concentrations of salinity and imidacloprid in water. N = 8 replicates per control and n = 4 replicates per treatment. Treatments that do not have a bar did not have any males emerge. No treatments were significantly different from each other.

Female Mean Emergence Day

There was no overall significant effect of treatment on female mean emergence day. Imidacloprid significantly decreased female emergence day (Figure 9, Kruskal- Wallis p =0.007) but salinity did not have a significant effect (Kruskal – Wallis p = 0.091). The interaction between the two factors was not significant (Kruskal – Wallis p =0.097). The effects of salinity were not significant, but they did have a similar trend to both male and overall mean emergence day in that adults emerged later in the higher salinity treatments compared to the control and 0 (from 16.2 to 17.4 days); and again, similar to the overall mean emergence day, there was a decrease in emergence day with increasing imidacloprid concentration. The 0 µg/L imidacloprid concentration had a female mean emergence day of 17.2 days post hatch compared to 15.4 days in the 1.30 µg/L treatment.

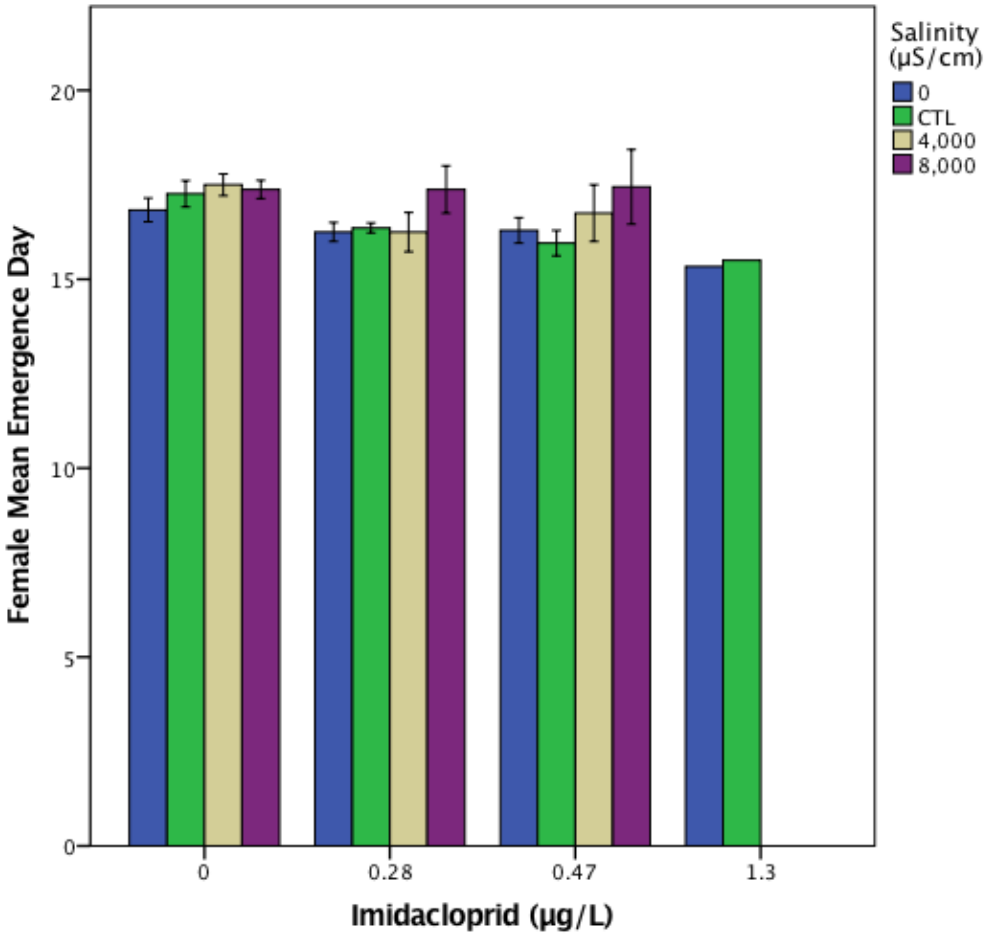

Figure 9. Mean (± SE) female emergence day of *Chironomus tepperi* larvae exposed for 21 days to different concentrations of salinity and imidacloprid in water. N = 8 replicates per control and n = 4 replicates per treatment. Treatments that do not have a bar did not have any females emerge. No treatments were significantly different from each other.

4.4 Discussion

This study shows that the effects of salinity on the toxicity of imidacloprid are dependent on the concentration of both stressors. There seems to be a synergistic effect of imidacloprid at very low and high salinities. Areas that generally have low or high salinity will be more susceptible to imidacloprid toxicity. This is especially important in a changing climate as freshwaters – especially in arid areas – all over the world increase in salinity (Williams 2001).

4.4.1 Effects on survival and emergence

The toxic effects of imidacloprid on the survival and development of *C. tepperi* were not unexpected. Previous experiments with *Chironomus* species and imidacloprid or similar neonicotinoid pesticides have found similar levels of toxicity (Cavallaro et al. 2017; Gagliardi 2017). The pesticide affects the larvae's ability to move and obtain food when foraging due to effects on the nervous system (Azevedo-Pereira et al. 2011b). In this study, imidacloprid had a negative effect on survival as concentration increased. Furthermore, these effects were reflected in emergence endpoints following the chronic exposure. The effects on the development of *C. tepperi* from larvae into adults can be partially explained by the effects of imidacloprid on larval weight after 96 hours. There was a significant decrease in larval weight in the high imidacloprid treatment after 96 hours which was likely caused by their inability to obtain food or move which then decreased the number of larvae that emerged as adults. Other studies have shown that slightly higher concentrations of imidacloprid (2.15 µg/L) can decrease survival and growth of other *Chironomus* species over similar time periods (Azevedo-Pereira et al. 2011a; Stoughton et al. 2008).

There was also earlier emergence with increased imidacloprid concentration, despite there not being a significant effect when interacting with salinity. This finding is in contrast to previous studies that exposed other species of *Chironomus* to neonicotinoid insecticides which showed either no effect on emergence or a slightly delayed development rate (Cavallaro et al. 2017; Langer-Jaesrich et al. 2010; Stoughton et al. 2008). However, a study by Cavallaro et al. (2018) on the community response to imidacloprid did show a decreased emergence day in damselfly and chironomid species. As far as we are aware, there have been no previous studies on the chronic effects of neonicotinoids on *C. tepperi*, but the early emergence response has been seen with synthetic pyrethroid pesticides (Boyle et al. 2016). As hypothesised by Boyle et al. (2016), *C. tepperi* may be emerging earlier because pupation is being brought on earlier by the pesticide. As mentioned in that study, one of the possible mechanisms for early emergence is an alteration in the ecdysteroid that brings on pupation (Weiner et al. 2014). Rewitz et al. (2006) showed that the expression of cytochrome P450 enzymes can affect insect moulting and development. They reported that P450s mediate the final hydroxylation in the synthesis of ecdysone (one of the ecdysteroids). This is relevant to imidacloprid toxicity, as imidacloprid has been found to alter the expression of cytochrome P450 (Karunker et al. 2009), which could then affect the ability of insects to moult and potentially decrease their time to pupation and emergence. There have been no specific studies linking altered cytochrome P450 expression with insect emergence, however work from Van De Wouw et al. (2006) showed that the insect growth regulator insecticide Cyromazine caused earlier emergence in *Drosophila melanogaster*. However, after exposing *D. melanogaster* to the metamorphosis hormone 20-hydroxyecdysone (20E) they found that the lethality of Cyromazine was significantly reduced. As in this study, they also hypothesised that the pesticide

could inhibit cytochrome P450 expression and alter ecdysteriod concentrations and therefore effect insect emergence and development.

With increasing salinity, there was a decrease in larval survival and weight over the 96-hour exposure. This was not unexpected, as organisms need to use energy to maintain ideal fluid and salt concentrations. These results show that both a lack of salts and a significant increase in them can affect how well *C. tepperi* survives and grows. Based on previous research from Hassell et al. (2006) and Hale et al. (2014) the salinity tolerance of Australian *Chironomus* species is fairly well known and so decreased survival at higher salinity concentrations was expected. Similar to Hassell et al. (2006), *Chironomus* survival displayed an inverted U shape, with the lowest salinity concentration and the highest both affecting survival. This is an indication that the larvae are using most of their energy for osmoregulation, rather than for growth. This is especially obvious in larval weight as the highest salinity treatments had the lowest weight, and this effect was exaggerated when larvae were exposed to imidacloprid.

For the same reason salinity affected survival and weight it also reduced the number of larvae that emerged into adults. That is, extra energy was expended on osmoregulation, rather than normal growth and development. This is likely why adults emerged later in higher salinity treatments. Hassell et al. (2006) also found that a change in the ideal salinity concentration for a *Chironomus* species delayed emergence. These results were not exactly replicated here, possibly because the 0 μS/cm was lower than any of the lowest salinity treatments used by Hassell et al.

The interaction between salinity and imidacloprid had an overall negative and synergistic effect on the survival and weight of the larvae. There was a dramatic effect of both imidacloprid and salinity on survival at 96 hours which was replicated to an even greater extent in the emergence experiment. Imidacloprid and salinity seem to act antagonistically on larval development, rather than synergistically, which contrasts with their combined effects on survival. As discussed previously, imidacloprid and salinity have opposite effects on emergence day, and it seems that this antagonistic relationship is the cause of the lack of a significant effect. However, the mechanism for such an effect is unclear. If imidacloprid is acting on the cytrochrome P450 detoxification pathway that is altering ecdysis steroids, then it is possible there is insufficient stress from imidacloprid to overcome the increased energy that osmoregulation requires. The mode of action of each stressor is different and as a result the larvae that survive to pupation and emergence are likely to have adjusted to the salinity to overcome any additional stress from imidacloprid exposure. There seems to be a fine balance, as evidenced by the lack of emergence in the higher imidacloprid/salinity treatments: larvae either survive and emerge within a normal time period, or they die.

4.4.2 Effects on Energy reserves

The effects of imidacloprid on the energy reserves did not follow the same dose-response curve as survival. At the middle concentrations of imidacloprid there was generally an increase in energetic reserves. This is contrary to other research (Nyman et al. 2013; Radwan and Mohamed 2013) which mostly showed a decrease in lipid and/or glycogen concentrations when invertebrates were exposed to imidacloprid. As in this study, Radwan and Mohamed (2013) showed an increase in protein concentration after exposure to imidacloprid, which is likely a build-up of heat shock proteins, which are a common response to stress in invertebrates (Sanders 1993).

However, there have been instances of glycogen concentrations in invertebrates increasing in response to lower imidacloprid concentrations. Sawczyn et al. (2012) exposed *Gromphadorhina portentosa* (Madagascar hissing cockroaches) to imidacloprid through the diet and found that there was an increase in glycogen concentration in the insect fat body compared to the controls. They attributed this to an increase in the concentration of trehalose, a disaccharide sugar. The larvae were storing energy to deal with the stress of the sub-lethal concentrations of imidacloprid. It is possible that the addition of higher salinity at these imidacloprid concentrations is affecting the ability of the larvae to use the energy they have stored in response to the stressor. The increased energy required for adapting to sup-optimal salinity conditions has been seen in copepods (Lee et al. 2017). This is possible as generally the 0 μS/cm salinity treatment had the opposite response pattern than all the other treatments; energetic reserves decreased compared to the control as imidacloprid concentration increased.

There was a significant interactive effect of imidacloprid and salinity on energy storage particularly in the middle range of imidacloprid concentrations. In particular, the 0 μS/cm treatment had the lowest lipid and protein concentrations of all salinity treatments. This is possibly because the larvae had expended most of their energy trying to osmoregulate from any possible source, that they were unable to store energy in the same way as at the higher salinity concentrations. The opposite effect can be seen in the 0.47/4000 treatment which had very high lipid and glycogen concentrations.

The effects of salinity on energy storage in invertebrates has been previously studied (Verslycke and Janssen 2002) and generally there seems to be an energy cost on individuals exposed to concentrations outside their adapted range (Sokolova et al. 2012). In copepods, Lee et al. (2017) showed that increasing salinity decreases lipid reserves and increases stress proteins. This is in contrast to the results in this study, which generally showed an increase in lipid concentration in response to a hypersaline environment, which is in line with other studies (Goolish and Burton 1989).

4.4.3 Conclusions

An increase in mortality, decrease in proportion emerged, and an overall increase in energy reserves was the response of *C. tepperi* to the increasing stress of imidacloprid and salinity. The initial aim of this study was to investigate how a change in salinity would alter the toxicity of imidacloprid. Overall, increased salinity amplified the toxicity response, and in some cases a decrease in salinity also increased the toxicity of imidacloprid. Understanding that increasing salinity can increase the toxicity of pesticides is important for water management as it indicates there needs to be some nuance in the water quality guidelines based on the salinity of the water. The concentrations of salinity present in this study have been found in Australia and with increasing salinisation are likely to be found in more fresh waters in the future. This makes this research increasingly more important for both ecotoxicity testing and regulation.

CHAPTER 5: GENERAL DISCUSSION

This thesis outlines how nutrients and salinity can alter the toxicity of frequently used pesticides. For the first time, the effects of P on the standard Australian toxicity species *Chironomus tepperi* were explored and showed that the acute effects of P are significant enough to warrant further monitoring in laboratory toxicity tests, particularly when assessing sublethal indicators of stress such as energy reserves (protein, glycogen, lipids). In water-only tests, P significantly decreased mean emergence day, largely driven by a reduced emergence time in females. In sediment tests the influence of P on emergence was not as clear, however there was a significant decrease in lipid concentrations in larvae after 96 hours. This indicated that there was the potential for phosphorus to mask the response of *C. tepperi* to some pesticides.

Chapter 3 confirmed that nutrients can mask a response to pesticides. This was particularly true of the more sensitive endpoints such as mean emergence day. The survival, weight, and emergence of *C. tepperi* were reduced at the highest tested concentrations of the pesticide permethrin. However, at the lower concentrations of permethrin, the toxicity of the pesticide was mediated by a potential positive effect from the P. In the more sensitive emergence endpoints, P exposure caused later emergence of adults and permethrin caused earlier emergence. This could pose a problem for toxicity tests, as increased P could mask potential indicators of permethrin contamination in *C. tepperi*.

In Chapter 4, the interaction between the insecticide imidacloprid pesticide and salinity was explored. Nutrients are generally assumed to have an overall positive effect on invertebrates (up to a certain concentration), whereas salinity is assumed to have a negative effect. This investigation found that highly saline water, when mixed with environmentally relevant high concentrations of imidacloprid, created a highly toxic environment for *Chironomus tepperi* larvae. Low salinity affected *C. tepperi* survival, growth, and additional energy reserves. However, in the emergence experiment, there was an antagonistic relationship between salinity and imidacloprid, which saw opposing emergence day trends cancelled each other out in the overall treatment analysis.

This research answers some of the questions posed by the overarching question of the thesis which is how water quality parameters can affect the toxicity of pesticides to aquatic invertebrates. Considering the threat global warming poses to our planet, there has been plenty of research done into the effects of temperature and pH on the toxicity of pesticides. For instance, DeLorenzo et al. (2009) showed that increasing temperature increased the toxicity of a synthetic pyrethroid to glass shrimp (*Palaemonetes pugio*). This is supported by work from Harwood et al. (2009) who exposed *Chironomus dilutus* to four different insecticides and found that a 10 degree decrease in temperature increased the toxicity of pyrethroids and DDT, but decreased the toxicity of chlorpyrifos. And Tsui and Chu (2003) showed that an increase of pH increased the toxicity of the glyphosate pesticide Roundup® to the cladoceran *Ceriodaphnia dubia*. The effects of temperature and pH are an important consideration, particularly when dealing with pyrethroids, and the tests done in this thesis were done a constant temperature for that reason. The results discussed are relevant at the temperatures reported, but as shown by Harwood et al. (2009) the toxicity can increase at lower temperatures.

There has been less work done on the less obvious effects of climate change such as salinisation and eutrophication. In terms of nutrients, there has been considerable research on the effects of carbon

on organic pesticides (Day 1991; Fleming et al. 1998; Mehler et al. 2017). However, this thesis has filled the gap in nutrient research, particularly in Australia. The nitrate experiments (Appendix A), showed that even very low concentrations of nutrients can affect how an invertebrate survives and develops.

The phosphorus only experiments in Chapter 2 showed that not only is the test system important for measuring the effects of nutrients (i.e. water vs sediment) but also that nutrients need to be measured per se. Measurement of nutrient concentrations is not usually recommended in standard guidelines (ASTM 2010; Batley and Simpson 2016; OECD ; OECD 2004b; OECD 2010; USEPA 2002) and if it is, it is usually only recommended in order to determine the overall quality of the water or sediment being used for the test. As the investigation in Chapter 2 shows, the addition of nutrients can change sublethal responses (e.g. lipid concentration) as well as more sensitive endpoints (e.g. mean emergence day) and there is a need to consider nutrients (other than carbon) in pesticide toxicity testing. Much of the research into mixtures of toxicants has only considered those with similar modes of action (Deneer 2000). There is an assumption in mixture modelling that two toxicants are more likely to be synergistic if they act on similar modes of action (De Zwart and Posthuma 2005; Deneer 2000; Lydy et al. 2004). This assumption does not necessarily hold when examining multiple stressors. Although two (or more) stressors may not have similar modes of action, they still have the potential for a synergistic response.

The need for P monitoring in toxicity testing was emphasised by the work in Chapter 3 where the presence of P masked some of the effects of the pesticide permethrin, particularly in emergence. In the proportion emerged results there was even a trend of increased emergence in the low permethrin/high P mixtures. In the sublethal energy reserves the masking of potential toxicity was even more apparent as the treatments with 0 P showed significant changes in energy expenditure and storage compared to any treatments with P. These investigations further show that nutrients and water quality need to be considered in the standard guidelines for laboratory toxicity testing of sediment and water.

The work until this point had largely been examining an antagonistic relationship between the nutrient water quality parameter and the pesticide, but there was space to also examine a potentially synergistic relationship. Salinisation is an increasingly concerning issue worldwide and particularly in Australia (Matthaei and Piggott 2019). Invertebrates need some salts in their environment to maintain fluid and electrolyte balance as well as for generating nerve impulses, but most insects have an ideal osmotic range (Kefford et al. 2012) and that exposure to salinity outside this range may have detrimental effects on their fitness. Salinity concentrations that are too high or two low can affect survival and development (Lee et al. 2017). This, combined with the commonly used pesticide imidacloprid, was seen as a good combination to investigate as the detrimental effects of salinity and imidacloprid alone on Australian chironomid species was already known (Gagliardi 2017; Hassell et al. 2006; Stevens et al. 2002) and they are both water soluble so the potential confounding factor of sediment could be eliminated. In contrast to the antagonistic relationship between permethrin and P, there was a generally synergistic relationship between salinity and imidacloprid. This was especially apparent in survival and emergence responses, as the higher concentrations of imidacloprid and salinity caused lower *C. tepperi* larvae survival and adult emergence. This synergistic effect had not been previously shown in *C. tepperi.* The energy reserves showed that the way energy is stored and used in this particular stressor combination is more related to salinity concentration than imidacloprid.

In nearly all energy reserves, the treatment with no conductivity (0 µS/cm) trended towards lower energy reserves. This response at lower salinity concentrations is in line with results from Hassell et al. (2006) who also showed that a field collected chironomid species had a U shaped dose-response curve with lowest and highest salinity concentrations having a detrimental effect. Salinity is generally already considered when conducting toxicity testing, but not always in a management context. Often the environmental guidelines are not specific to the waterway and the generalised water quality parameters may not give a full reflection as to the actual health of an ecosystem.

5.1 Energy reserves as a measure of toxicity

The work in this thesis is the first time energy reserves of protein, glycogen, and lipid concentrations have been used with *C. tepperi* . Previous work from Vu et al. (2015) used similar methods in an amphipod species to great success, and the same process was adapted to *C. tepperi* with some alterations. Their success was mixed. A single larva generally provided only enough tissue for one of the three tests, so it was not possible to use tissue from a single individual for all energy reserves. It would be a better measure of stress if it were possible to obtain lipid, glycogen, and protein from each individual as then the concentrations of each could be adjusted against each other. Glycogen, particularly, is stored and used quickly in comparison to lipids and protein (Hamburger et al. 1996) and so the data would be more interesting if it were able to clearly see where energy is being stored and used in a single larva. Additionally, the methods outlined in this thesis are time consuming and use many different toxic or highly acidic chemicals. There are more modern methods for determining these energy reserve concentrations, however refining these methods for this species was beyond the scope of this thesis.

Despite these drawbacks, measurement of energy reserves provides a more complete understanding of the interactions between multiple stressors. This is especially important when trying to better understand the way model ecotoxicology species respond in the environment outside the laboratory. It also gives researchers the opportunity to carry out relatively quick investigations early in a chronic toxicology assay that could provide insight into later responses. Additionally, it gives us an understanding of how energy is being used in *C. tepperi* when responding to stressful conditions.

5.2 Are energy reserves a good indicator of multiple stressor response?

Based on the results from this thesis, energy reserves should not be used as an indicator of multiple stressor response, for two reasons. The first is that it should be recognised that the response of one type of organism may not be the same across all types. Organisms store and use energy in different ways (Arrese and Soulages 2010). In chironomids, glycogen has been found to be stored and used depending on the life stage of the organism where it is used more when undergoing stress or during ecdysis and metamorphosis (Hamburger et al. 1996). In that study, the authors found that glycogen storage and use was particularly responsive to oxygen stress, where glycogen stores are severely reduced when larvae are exposed to a hypoxic environment. A decrease in glycogen concentrations when under stress was also seen in the present experiments in both the P/permethrin and the salinity/imidacloprid mixtures (Chapters 3 and 4). The knowledge of how an organism responds to stress is essential for energy reserve measurements to be of any use as a biomarker of multiple stressors.

The second reason is that there needs to be enough biological material to effectively analyse protein, glycogen, and lipid effectively. One of the largest issues with these measurements in this thesis,

particularly with glycogen, was that each biomarker came from one individual larva. Problems arose when individuals were smaller as a result of the experiments as this meant that there was higher variability in the measurements and made the analysis less certain. This could be solved by increasing replication or using a composite sample, however the later may pose some issues.

5.3 The importance of endemic species

Australia is the driest inhabited continent on earth and as a result of this the stressors that aquatic life are exposed to are unique compared to more temperate areas of the world. The two abiotic stressors outlined in this thesis (nutrients and salinity) have been identified as some of the most relevant to the Australian environment (Matthaei and Piggott 2019).

It's important to develop toxicity tests for endemic species in cases where a globally cosmopolitan species is not relevant. As mentioned in Chapter 3, *Chironomus tepperi* have a unique emergence response to stressors that are not found in other *Chironomus* species. When under pesticide-related stress *C. tepperi* tend to emerge earlier, rather than later which is the response seen in other temperate *Chironomus* species (Goedkoop et al. 2010). This highlights the importance of obtaining data on native species.

5.4 Avenues for further study

More species need to be tested in order to come to any sort of conclusions about the way water quality parameters and pesticides interact with each other. As we've seen in the salinity literature, molluscs tend to be less sensitive to high concentrations of salinity, but crustaceans are very sensitive (Hart et al. 1991). Some testing on other Australian species would be encouraged as these water quality issues are ones that affect Australia particularly and the increase in data would be welcomed by environmental regulators.

Further testing with different pesticide and water quality combinations should be investigated. Other classes of pesticides are likely to have different responses when mixed with nutrients and Personal Care Products (PCPs), pharmaceuticals, and other emerging contaminants could also have different reactions in mixtures with nutrients or salinity. It would be particularly interesting to investigate Synthetic Pyrethroids (SPs) in mixtures with salinity as both work on sodium channels. This was not done in this thesis for time and logistical reasons but would be an interesting mix as previous studies in this area show (Tu et al. 2012; Wang et al. 2013). The work from Tu et al. (2012) in particular has implications for energy reserves as they measured a variety of stress biomarkers in shrimp (*Penaeus monodon*) exposed to salinity and the SP deltamethrin and found that the mixture greatly increased some of the stress biomarkers, particularly Acetylcholinesterase (AChE). The work from (Wang et al. 2013) also found that altering salinity can increase toxicity. In this study a decrease in salinity increased the toxicity of beta-cypermethrin to the tropical shrimp *Litopenaeus vannamei* further showing that a change in the optimal salinity range for an organism can alter the toxicity of a pesticide. There are currently no studies looking at the effects of salinity of the toxicity of SPs to Australian freshwater species, so this would be a good avenue for further research.

It would also be useful to researchers and regulators to extend the energy reserves work found in this thesis to many different species. The methods outlined in this paper have more modern analogues that could be easily adapted to other species and may be useful as a relatively rapid biomarker of stress (De Coen and Janssen 1997). It would be especially useful to have refined methods for Australian

species, and particularly adults so that effects of sex could be differentiated both in *Chironomus* and other organisms as males and females have different energy requirements throughout their life cycles.

5.5 Conclusions

The work in this thesis is the beginnings of investigations into how water quality affects the toxicity of pesticides. This thesis was the first time that the effects of phosphorus on *Chironomus tepperi* have been described and had contributed to the overall understanding of that species and its tolerance to water quality issues. The effects of phosphorus on the toxicity of permethrin in that species also showed an antagonistic relationship between phosphorous and a pesticide, further illustrating the assumption that nutrients can mitigate the toxicity of pesticides up to a certain point. Lastly, the synergistic effects of salinity on imidacloprid toxicity showed that other aspects of water quality can increase the toxicity of pesticides. These results mean that researchers need to be aware of the water quality (including nutrients) in their environmental toxicology testing and regulators can better take care of the environment by also including how water quality affects the pollutants they regulate.

This thesis also highlights a fundamental question in ecotoxicology: What is the purpose of ecotoxicity testing? If the purpose is to create pristine environments in the laboratory where every aspect can be controlled, then there is no real need to mimic environmental conditions and therefore less of a need to consider endemic species and the conditions in which they live. There is a place for this kind of standardised testing as it will produce relevant results quickly and allows for every aspect of an experiment to be controlled and manipulated. However, there is also a need to bring ecology into the lab by mimicking natural conditions with native species. A need to think about the conditions a researcher is trying to reproduce and whether the test species being used is appropriate for that environment or the toxicant that is being tested. Is the species likely to encounter this contaminant under these conditions in the field? Although they often take more time, these are the tests that are most useful for regulators and environmental risk assessments. Ecotoxicology needs both types of testing, but this thesis emphasises that we should at least be considering natural conditions in our ecotoxicology work.

APPENDIX A: PROBLEMS WITH USING NITRATE IN CHRONIC TOXICITY TESTING WITH *CHIRONOMUS TEPPERI*

Introduction

Nitrogen pollution is recognised as a problem throughout the world (Camargo and Alonso 2006). In Australia, where much of the land is dedicated to agriculture, nitrogen has been found to cause concern in waterways throughout the country, particularly in marine areas (Bartley et al. 2012). A review of nutrient concentrations in Australia by Matthaei and Piggott (2019) showed that nutrient concentrations, and particularly nitrogen are an issue for freshwater ecosystems around the country.

Nitrogen takes four main forms that are relevant when referring to nitrogen pollution: ammonium ion (NH_4+), ammonia (NH_3), nitrite (NO_2-), and nitrate (NO_3-). The effects of ammonia have been widely studied on a variety of aquatic organisms, including macroinvertebrates and fish (Alonso and Camargo 2009; Arthur et al. 1987; Hickey and Vickers 1994). It is well established that ammonia is the most toxic form of nitrogen to invertebrates, followed by nitrite(Alonso and Camargo 2003; Alonso and Camargo 2004; Camargo and Alonso 2006; Camargo et al. 2005; Meade and Watts 1995). For example, in the aquatic snail *Potamopyrgus antipodarum* the 96 hr LC_{50} for Ammonia is 2.02 mg/L compared to 535 and 1042 mg/L for nitrite and nitrate, respectively (Alonso and Camargo 2003). However, ammonia and nitrite are relatively quickly converted to nitrate by bacteria and algae, which means that the exposure of organisms to both these toxic forms is usually short. The most common form of nitrogen pollution is nitrate, which persists in the environment for much longer than ammonia and nitrite (Camargo and Alonso 2006). The guidelines put forth by the US Environmental Protection Agency (USEPA) and the World Health Organisation (WHO) regulate the amount of nitrate allowed in drinking water to 10 mg NO3-N /L in order to avoid possible damaging effects to human health (Bogárdi et al. 2013); however, concentrations of nitrate up to 190 mg/L have been found in surface waters in the Melbourne, Australia area (Water 2015). Interestingly, many countries do not regulate the amount of nitrate in waters for environmental protection purposes (Weisenburger 1991).

The effects of nitrate are less studied than ammonia, likely because it is assumed to be relatively non-toxic. There are, however, some studies on the effects of nitrate on freshwater crayfish and macroinvertebrates, including caddisflies, daphnia, and aquatic snails. Camargo et al. (2005) found that early instars of European caddisfly larvae were more sensitive to nitrate than later instars and that freshwater invertebrates were much more sensitive than marine. Based on these studies the authors recommended a maximum level of 2 mg NO3-N/L in order to protect freshwater species. Alonso and Camargo (2003) found that the freshwater snail *Potamopyrgus antipodarum* had an acute toxicity value of 195 mg/L of nitrate. This shows that there is quite a large difference in the amount of nitrate different species can tolerate and deserves further research. However, there have been no nitrate toxicity studies on Australian species. This represents a major limitation in our understanding of the effects of nitrate on local species especially given the concentrations detected in water in Melbourne; furthermore, overseas guideline values are not always relevant to Australian species, so it is important to know how local species respond. The work described below is an attempt to fill the gap in the research.

Aims

To determine the effects of nitrate on the survival, weight, emergence, and mean emergence day of the Australian midge *Chironomus tepperi*.

Methods

Chironomid Culture and Preparation

Chironomus tepperi were cultured in a modified Martin's artificial water solution (as described in Jeppe et al. (2014)), with ethanol-rinsed toilet paper as substrate. Larvae were fed tropical fish flakes (Tetramin) three times per week, with 0.25 mg added per tank. Adult *C. tepperi* were collected from the CAPIM laboratory culture and placed into a Perspex tank with artificial water containing modified Martin's artificial water solution.

After 24 hours, egg masses were collected with a pipette and placed into a beaker with 100 ml of artificial water. Egg masses were then transferred to 2L beakers (three egg masses per beaker) to hatch. The 2L beakers included ethanol-rinsed toilet paper substrate and were maintained at 20º C +/- 1 degree with a photoperiod of light: dark 16:8hr. Two drops of food (a slurry made up of crushed Tetramin, tropical fish food flakes, at a concentration of 10g/100 ml of artificial water) was provided every 2 days until the larvae had reached 2nd instar (5 days old).

Preparation of nitrate solutions

Nitrate treatments were prepared using a Martin's artificial water solution. Nitrate as $NaNO_3$ was added this water to create a range of NO_3 concentrations to be used in the treatments. The following water quality parameters were measured: pH, temperature, conductivity and nitrate and found to be within the acceptable limits for a viable *C. tepperi* test (Batley and Simpson 2016). The concentrations of nitrate were Control (0), 0.0025, 0.025, 0.25, 2.5, 5 and 10 mg/L). These concentrations were chosen based on an examination of the literature, of known concentrations of nitrate present in waters and sediments in south-eastern Australia (Water 2015) and on pilot testing with a range of nitrate concentrations (0, 1.25, 12.5, 125, 250, 500, 1000 mg/L). The purpose of the highest concentrations was to include a potentially toxic concentration of nitrate to *C. tepperi*. Spiked water was sent to a commercial laboratory for analysis of Ammonia, Nitrate, and Nitrite as N as well as Total Kjeldahl Nitrogen (TKN) and Total Nitrogen as N (ALS Environmental, Australia).

Bioassay

In each 600 mL glass beaker 400 mL of treatment (nitrate-spiked) or Control water was added with eight replicates per treatment. The water level in each beaker was marked so the water level could be maintained throughout the exposure period. One sheet of 2-ply toilet paper rinsed in ethanol was added to each beaker as substrate for the larvae. Ten 2nd instar *C. tepperi* larvae were randomly added to each beaker with two drops of Tetramin food preparation. The treatments were maintained in a temperature-controlled room at 21 ± 1 ºC. Aeration was provided to each beaker. The water level was topped up three times a week with deionized water. This was to ensure the salts in the artificial water did not become too concentrated from evaporation of water. Fine mesh stockings were placed over the beakers to contain emerged adults. Water quality measures of pH, temperature, conductivity and Dissolved Oxygen (DO) were measured every second day throughout the experiment.

After 96 hours, 4 beakers were randomly selected and removed from each treatment in order to determine larval survival. The water and tissue substrate were filtered through a 250 μm sieve to collect and record surviving larvae. The water was pooled by treatment and water quality parameters

of pH, temperature, and conductivity were measured, this water was then sent to a commercial laboratory for analysis of Ammonia, Nitrate, and Nitrite as N as well as Total Kjeldahl Nitrogen (TKN) and Total Nitrogen as N (ALS Environmental, Australia).
Emerged adults were collected with an aspirator daily and the number of males and females were recorded. Proportion emerged and mean day of emergence were calculated.
When there was no further emergence in the Control treatments after 2 days the experiment was terminated, and the remaining water was filtered through a 250 μm sieve to collect any surviving larvae or pupae. The water was pooled by treatment and water quality parameters of pH, temperature, and conductivity were measured, this water was then sent to a commercial laboratory for analysis of Ammonia, Nitrate, and Nitrite as N as well as Total Kjeldahl Nitrogen (TKN) and Total Nitrogen as N (ALS Environmental, Australia).

Larvae addition experiment

The analytical results showed that the concentration of nitrate had reduced in the solutions significantly (Table 1). This was thought to be a result of adding the larvae to the beakers. A 96-hour experiment was set up with test conditions as above, however with six replicates per treatment. Larvae were added to three replicates and the remaining three beakers were left only with aeration and substrate. One drop of Tetramin food slurry was added to all beakers (including those without larvae) at the beginning and two days into the test. After 96 hours the water from each treatment was pooled and sent to ALS Environmental for analysis of the above parameters.

Nitrate Time course test

After it was discovered that the nitrate was not stable in solution long enough for an emergence test (up to 20 days), a time course test was conducted to determine the rate of nitrate conversion. Five 600ml beakers of artificial water were set up for each time point and each treatment of Control, 0.25, 2.5, and 10 mg/L spiked according to the above methods. Ten *C. tepperi* larvae (2^{nd} instar) were added to each replicate and beakers were kept in a temperature-controlled room according to previous test conditions. At each time point one replicate was removed from a randomly selected treatment and a sample of the water sent to a commercial laboratory for analysis. Water was sampled at spiking (0), 1, 6, 18, 24, 48, and 96 hours. Replicates from all treatments were sacrificed at spiking (0), 1, and 96 hours and were randomly selected for the intervening time points.

Results

Water Quality

Table 1. Nitrate as NO_3 concentrations in water at spiking, 96 hours, and post emergence test (20 days). Values are Nitrate as N multiplied by 4.428 in order to obtain nitrate as NO3 value (mg/L).

Treatment	Nitrate at spike (mg/L)	Nitrate at 96 h (mg/L)	Nitrate post-test (mg/L)
Control	<0.01	0.088536	0.044268
0.0025	<0.01	0.044268	0.132804
0.025	0.088536	0.088536	0.088536
0.25	0.309876	0.044268	0.088536
2.5	2.611812	0.044268	0.088536
5	5.002284	3.674244	0.088536
10	10.18164	8.986404	0.265608

Table 2. Ammonia as N, Total Kjeldahl Nitrogen (TKN), and Total Nitrogen (TN) as N concentrations in mg/L measured at spiking, 96 hours, and post emergence test (20 days).

Treatment	Ammonia (Spike)	Ammonia (96h)	Ammonia (post emerge)	TKN (spike)	TKN (96h)	TKN (post emerge)	TN as N (spike)	TN as N (96h)	TN as N (post emerge)
Control	0.04	0.11	0.04	<0.1	0.8	6.7	<0.1	0.8	6.7
0.0025	0.04	0.06	<0.01	<0.1	0.6	8.1	<0.1	0.6	8.1
0.025	0.04	0.07	0.12	<0.1	0.7	9.1	<0.1	0.7	9.1
0.25	0.04	0.07	0.08	<0.1	0.9	8.9	<0.1	0.9	8.9
2.5	0.04	0.09	0.06	<0.1	1	9.1	0.6	1	9.1
5	0.04	0.07	0.04	0.2	0.8	9.4	1.3	0.8	9.4
10	0.04	0.15	0.03	0.7	1.6	15.1	3	1.6	15.2

96 Hour Test

Survival

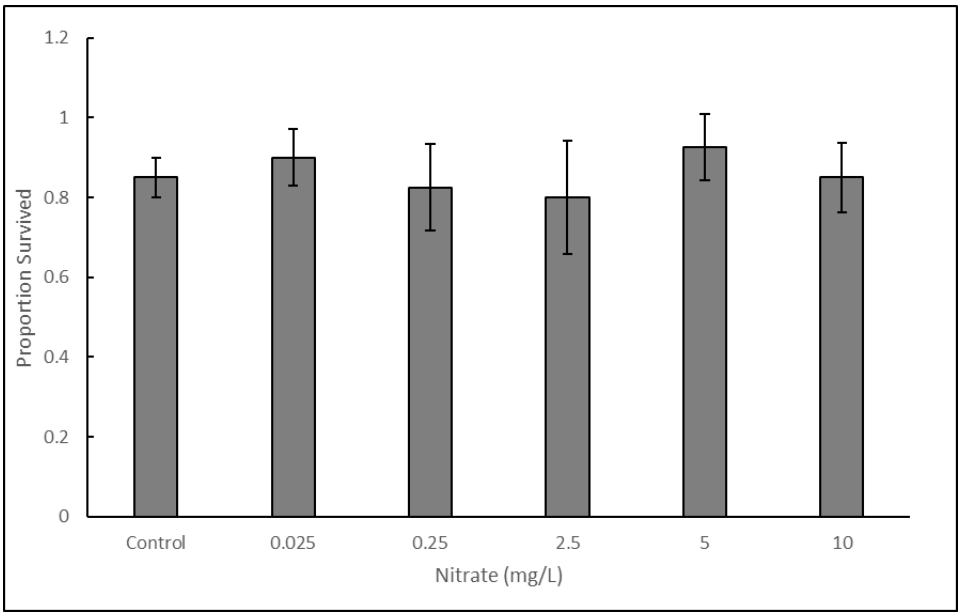

Figure 1. Mean (±SD) survival of *Chironomus tepperi* larvae exposed for 96 hours to different concentrations of nitrate. N = 4 replicates per treatment.

Emergence Test

Total Emergence

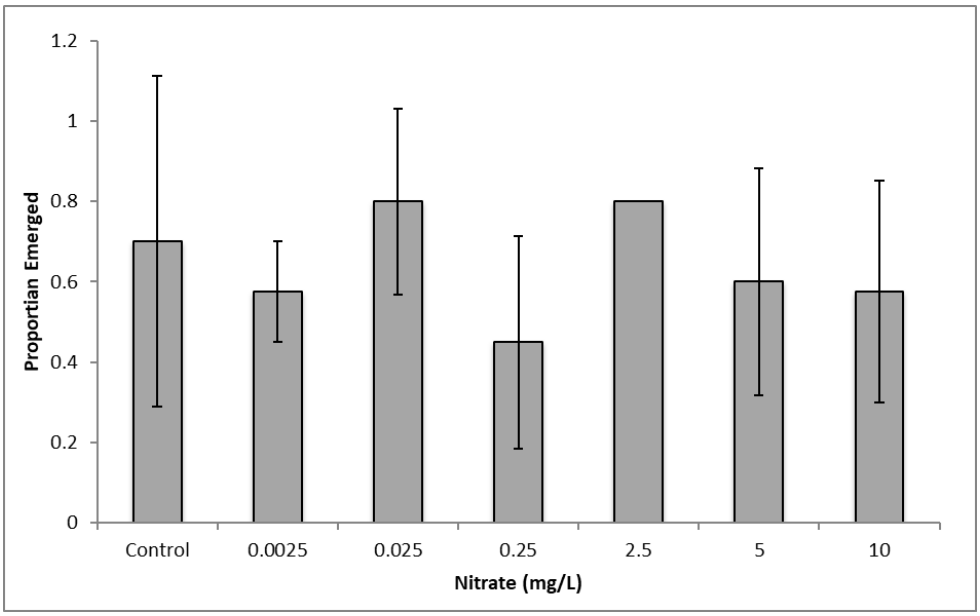

Figure 2. Mean (±SD) proportion emerged of *Chironomus tepperi* adults exposed for 20 days to different concentrations of nitrate. N = 4 replicates per treatment.

Mean Day of Emergence

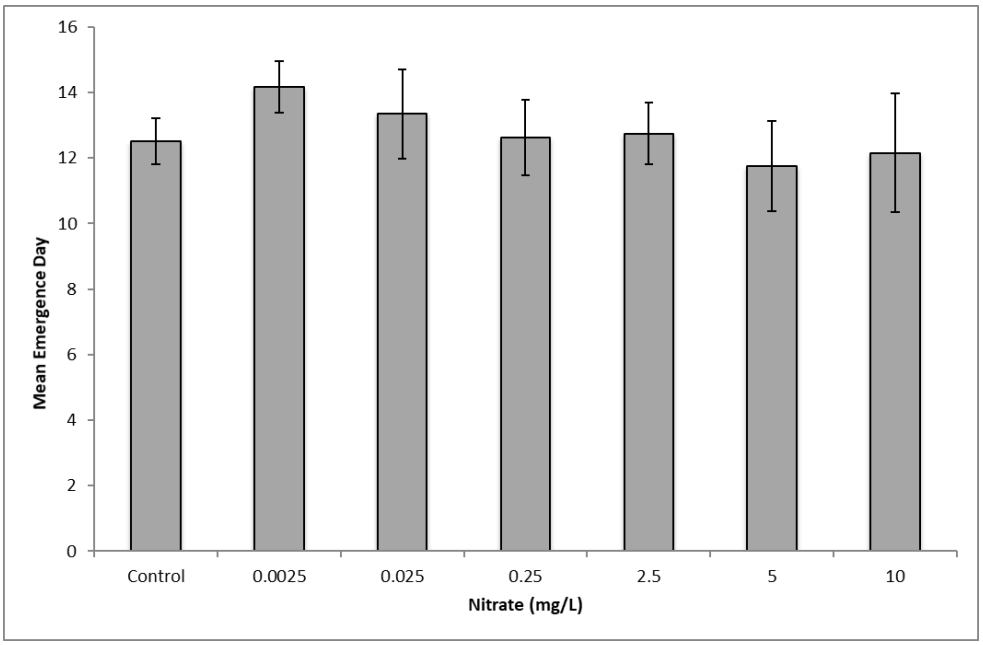

Figure 3. Mean (±SD) emergence day of *Chironomus tepperi* adults exposed for 20 days to different concentrations of nitrate. N = 4 replicates per treatment.

Larvae addition experiment

Table 3. A comparison of pooled nitrate as NO3 concentrations measured at spiking and 96 hours after the addition of larvae in half the replicates. Both sets of replicates were under test conditions.

Treatment (mg/L)	Nitrate as NO3 (Spike) (mg/L)	Nitrate as NO3 (96 h) (mg/L)
Control	<0.01	0.088536
0.0025	<0.01	0.044268
0.025	0.088536	0.088536
0.25	0.309876	0.044268
2.5	2.611812	0.044268
5	5.002284	3.674244
10	10.18164	8.986404
Control (larvae)	<0.01	0.22134
0.0025 (larvae)	<0.01	0.088536
0.025 (larvae)	0.088536	0.044268
0.25 (larvae)	0.309876	<0.01
2.5 (larvae)	2.611812	0.044268
5 (larvae)	5.002284	0.088536
10 (larvae)	10.18164	0.044268

Timepoint test

Table 4. Time Point results for 96-hour test of nitrate concentrations under standard testing conditions. Values are Nitrate as N multiplied by 4.428 in order to obtain nitrate as NO3 value (mg/L). Due to budget contraints, not all treatments were tested at every timepoint (NM is Not Measured).

Time	Treatment			
	Control	**0.25**	**2.5**	**10**
Spike (0)	<0.01	0.309876	2.567544	10.66859
1hr	0.088536	0.354144	2.479008	10.00457
6hr	NM	0.531216	2.301936	NM
18hr	NM	0.265608	NM	9.827496
24hr	0.044268	NM	NM	8.720796
48hr	0.044268	NM	0.66402	NM
96hr	0.044268	0.044268	0.088536	0.088536

Results and Discussion

The reported water quality for the 96 hour and emergence test showed that there was a clear difference in nitrate concentration at the start of the experiment, i.e. after spiking, and at the end of the emergence test (Table 1). As a result, the survival and emergence data were not statistically analysed until the cause of the instability of the nitrate concentrations could be determined or mitigated. Because of this, I will not discuss the 96h survival, proportion emerged, or mean emergence day data in detail. Graphical representations of these results are found in Figures 1, 2 and 3.

As the intention of the study was to have consistent nitrate exposure throughout, the reduction in nitrate concentrations over the course of the experiment posed a problem. It was initially suspected that bacteria associated with the larvae were converting the nitrate into ammonia through the ammonification process (Stein and Klotz 2016). However, this process is usually associated with organic nitrogen, which was not the form of nitrate used for spiking. Furthermore, there was not a significant increase in ammonia throughout the experiment (Table 2). It was also possible that the nitrate was being converted into N_2 gas through the denitrification process. This is a common process in biological systems but is usually undertaken under anaerobic conditions (Stein and Klotz 2016Stein and Klotz 2016). As the beakers were aerated it seemed unlikely that this was the process via which the nitrate was being converted. However, an experiment over 96 hours was done comparing a test system with and without larvae. As can be seen in Table 3, there was a difference between the treatments with and without larvae after the 96-hour exposure period. Those treatments that did not contain larvae had relatively stable nitrate concentrations, whereas those that contained larvae had nitrate concentrations between <0.01 and 0.22134 mg/L which was between a 99% reduction or a >3000% increase in nitrate depending on the starting concentration. Interestingly, there was still a general change in nitrate concentrations in the beakers that did not have larvae added, although the difference was not as large. These results indicate that the larvae were influencing the nitrate concentrations, but that the nitrate was unstable even under test conditions without larvae.

As a result of the larvae addition experiment the possibility that a shorter exposure period could be done with nitrate and *C. tepperi* larvae was explored through an analysis of the nitrate concentration over several timepoints. As seen in Table 4, the nitrate concentration in the Control treatment changed 1 hr after spiking, however for the other treatments there was not a large reduction in nitrate

concentrations until 24 -48 hours after spiking. From these results it seems that the ideal time range for a nitrate test with *C. tepperi* larvae where there is no total replacement of the spiked nitrate water is 24 hours.

Ultimately, it was not logistically feasible to change the nitrate treated water every 24 hours in the static test system as outlined here. As the results of a 24-hour exposure would not have yielded productive sublethal endpoints and would not be comparable to the other acute and chronic experiments with *C. tepperi* described in this thesis, the nitrate experiments were abandoned. These data are presented here as a report on why nitrate water exposures do not work over a chronic test period in a static system with *C. tepperi*. In future, experiments looking at the effects of nitrate on freshwater invertebrates should be carried out with a 100% replacement of test solutions on a daily basis to ensure nitrate concentrations are stable throughout the exposure period.

APPENDIX B: BITTERN SEDIMENT CHEMICAL ANALYSIS

Analyte	Units	LOR	Measured
Moisture Content (dried @ 103°C)	%	1	2.3
Aluminium	mg/kg	50	18000
Antimony	mg/kg	5	<5
Arsenic	mg/kg	5	<5
Barium	mg/kg	10	60
Beryllium	mg/kg	1	<1
Boron	mg/kg	50	<50
Cadmium	mg/kg	1	<1
Chromium	mg/kg	2	27
Cobalt	mg/kg	2	4
Copper	mg/kg	5	54
Iron	mg/kg	50	24800
Lead	mg/kg	5	15
Manganese	mg/kg	5	144
Nickel	mg/kg	2	8
Selenium	mg/kg	5	<5
Silver	mg/kg	2	<2
Vanadium	mg/kg	5	49
Zinc	mg/kg	5	21
Mercury	mg/kg	0.1	<0.1
Total Phosphorus (TP)	mg/kg		107
Total Reactive Phosphorus (TRP)	mg/kg		0.1
Total Organic Carbon	%	0.02	1.2
Polynuclear Aromatic Hydrocarbons			
Acenaphthene	mg/kg	0.05	<0.05
Acenaphthylene	mg/kg	0.05	<0.05
Anthracene	mg/kg	0.05	<0.05
Benz(a)anthracene	mg/kg	0.05	<0.05
Benzo(a)pyrene	mg/kg	0.05	<0.05
Benzo(a)pyrene TEQ (half LOR)	mg/kg	0.05	0.06
Benzo(a)pyrene TEQ (LOR)	mg/kg	0.05	0.12
Benzo(a)pyrene TEQ (zero)	mg/kg	0.05	<0.05
Benzo(b+j)fluoranthene	mg/kg	0.05	<0.05
Benzo(g.h.i)perylene	mg/kg	0.05	<0.05
Benzo(k)fluoranthene	mg/kg	0.05	<0.05
Chrysene	mg/kg	0.05	<0.05
Dibenz(a.h)anthracene	mg/kg	0.05	<0.05
Fluoranthene	mg/kg	0.05	<0.05
Fluorene	mg/kg	0.05	<0.05
Indeno(1.2.3.cd)pyrene	mg/kg	0.05	<0.05
Naphthalene	mg/kg	0.05	<0.05
Phenanthrene	mg/kg	0.05	<0.05
Pyrene	mg/kg	0.05	<0.05

Sum of polycyclic aromatic hydrocarbons	mg/kg	0.05	<0.05
Phenolic Compound Surrogates			
2.4.6-Tribromophenol	mg/kg	surrogate	3.54
2-Chlorophenol-D4	mg/kg	surrogate	3.03
Phenol-d6	mg/kg	surrogate	3.32
PAH Surrogates			
2-Fluorobiphenyl	mg/kg	surrogate	2.81
4-Terphenyl-d14	mg/kg	surrogate	2.68
Anthracene-d10	mg/kg	surrogate	3.43
Total Petroleum Hydrocarbons			
C10 - C14 Fraction	mg/kg	50	<50
C10 - C36 Fraction (sum)	mg/kg	50	<50
C15 - C28 Fraction	mg/kg	100	<100
C29 - C36 Fraction	mg/kg	100	<100
Total Recoverable Hydrocarbons - NEPM 2013 Fractions			
>C10 - C16 Fraction	mg/kg	50	<50
>C10 - C40 Fraction (sum)	mg/kg	50	<50
>C16 - C34 Fraction	mg/kg	100	<100
>C34 - C40 Fraction	mg/kg	100	<100

APPENDIX C: ANOVA, KRUSKAL-WALLIS, AND TUKEY TABLES

Chapter 2:

1.1.1 Water-Only

Water-only Proportion Emerged

Tests of Between-Subjects Effects

Dependent Variable: total

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	26.000[a]	6	4.333	2.116	.094
Intercept	2023.000	1	2023.000	987.977	.000
Treatment	26.000	6	4.333	2.116	.094
Error	43.000	21	2.048		
Total	2092.000	28			
Corrected Total	69.000	27			

a. R Squared = .377 (Adjusted R Squared = .199)

Water-only Mean Emergence Day

Tests of Between-Subjects Effects

Dependent Variable: Meanemergenceday

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	12.608[a]	6	2.101	5.866	.001
Intercept	626.074	1	626.074	1747.810	.000
Treatment	12.608	6	2.101	5.866	.001
Error	7.522	21	.358		
Total	646.204	28			
Corrected Total	20.130	27			

a. R Squared = .626 (Adjusted R Squared = .520)

Meanemergenceday

Tukey HSD[a,b]

Treatment	N	Subset		
		1	2	3
10 mg/L	4	3.99097222225		
20 mg/L	4	4.11111111100	4.11111111100	
5 mg/L	4	4.40833333325	4.40833333325	
80 mg/L	4	4.56111111100	4.56111111100	
40 mg/L	4	4.62996031775	4.62996031775	
0.5 mg/L	4		5.38214285725	5.38214285725
Control	4			6.01666666675
Sig.		.736	.083	.742

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .358.
a. Uses Harmonic Mean Sample Size = 4.000.
b. Alpha =

Water-only Female Mean Emergence Day

Tests of Between-Subjects Effects

Dependent Variable: FemaleMeanemergenceday

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	30.576[a]	6	5.096	9.900	.000
Intercept	878.827	1	878.827	1707.315	.000
Treatment	30.576	6	5.096	9.900	.000
Error	10.810	21	.515		
Total	920.213	28			
Corrected Total	41.386	27			

a. R Squared = .739 (Adjusted R Squared = .664)

FemaleMeanemergenceday

Tukey HSD[a,b]

Treatment	N	Subset		
		1	2	3
10 mg/L	4	4.45833333350		
5 mg/L	4	4.68750000000		
80 mg/L	4	5.23333333325	5.23333333325	
40 mg/L	4	5.23750000000	5.23750000000	
20 mg/L	4	5.26666666675	5.26666666675	
Control	4		6.83333333350	6.83333333350
0.5 mg/L	4			7.50000000000
Sig.		.688	.061	.838

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .515.
a. Uses Harmonic Mean Sample Size = 4.000.
b. Alpha =

Tests of Between-Subjects Effects

Dependent Variable: MeanFemaleWinglength

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	5.024[a]	6	.837	4.861	.001
Intercept	2754.241	1	2754.241	15986.130	.000
Treatment	5.024	6	.837	4.861	.001
Error	5.341	31	.172		
Total	2915.367	38			
Corrected Total	10.365	37			

a. R Squared = .485 (Adjusted R Squared = .385)

MeanFemaleWinglength

	Treatment	N	Subset 1	Subset 2	Subset 3
Tukey B[a,b,c]	0	6	8.2183		
	Control	6	8.4367	8.4367	
	20	5	8.6440	8.6440	
	80	6	8.6817	8.6817	
	40	7		9.0086	9.0086
	5	5		9.1100	9.1100
	10	3			9.4667

Means for groups in homogeneous subsets are displayed.
Based on observed means.
The error term is Mean Square(Error) = .172.
a. Uses Harmonic Mean Sample Size = 5.087.
b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.
c. Alpha = .05.

Male Wing Lengths

Tests of Between-Subjects Effects

Dependent Variable: MalemeanWinglength

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	4.220[a]	6	.703	5.475	.000
Intercept	2989.308	1	2989.308	23271.522	.000
Treatment	4.220	6	.703	5.475	.000
Error	4.881	38	.128		
Total	3091.074	45			
Corrected Total	9.101	44			

a. R Squared = .464 (Adjusted R Squared = .379)

MalemeanWinglength

	Treatment	N	Subset 1	Subset 2	Subset 3
Tukey HSD[a,b,c]	0	6	7.82666666667		
	Control	7	7.84928571429		
	20	6	8.24236111117	8.24236111117	
	80	6	8.31101190483	8.31101190483	
	40	5	8.43600000000	8.43600000000	
	10	9		8.57500000000	
	5	6		8.63819444450	
	Sig.		.064	.461	

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = .128.

a. Uses Harmonic Mean Sample Size = 6.246.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. Alpha = .05.

Tests of Between-Subjects Effects

Dependent Variable: Lipid_Conc_per_mg

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	3.056[a]	4	.764	9.246	.001
Intercept	190.029	1	190.029	2299.359	.000
Treatment	3.056	4	.764	9.246	.001
Error	1.074	13	.083		
Total	211.241	18			
Corrected Total	4.131	17			

a. R Squared = .740 (Adjusted R Squared = .660)

Lipid_Conc_per_mg

	Treatment	N	Subset 1	Subset 2	Subset 3
Tukey HSD[a,b,c]	5	3	2.6604171060		
	40	3	3.2987927410	3.2987927410	
	10	3		3.3824676800	3.3824676800
	Control	6		3.4691928082	3.4691928082
	0	3			4.0723479767
	Sig.		.082	.936	.055

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = .083.

a. Uses Harmonic Mean Sample Size = 3.333.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. Alpha = .05.

1.1.2 96 Hour Tests

Survival

Tests of Between-Subjects Effects

Dependent Variable: propemergeArcsin

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	6.652[a]	15	.443	2.992	.002
Intercept	87.046	1	87.046	587.239	.000
Permethrin	5.273	3	1.758	11.857	.000
PO4	.577	3	.192	1.298	.286
Permethrin * PO4	.830	9	.092	.622	.772
Error	6.967	47	.148		
Total	100.521	63			
Corrected Total	13.619	62			

a. R Squared = .488 (Adjusted R Squared = .325)

propemergeArcsin

			Subset	
	Permethrin	N	1	2
Tukey HSD[a,b,c]	2200	16	.6871	
	220	15		1.2644
	0	16		1.3478
	55	16		1.4042
	Sig.		1.000	.739
Tukey B[a,b,c]	2200	16	.6871	
	220	15		1.2644
	0	16		1.3478
	55	16		1.4042

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = .148.

a. Uses Harmonic Mean Sample Size = 15.738.

b. The group sizes are unequal. The harmonic mean of the group sizes is used. Type I error levels are not guaranteed.

c. Alpha = 0.05.

Weight

Levene's Test of Equality of Error Variances[a]

Dependent Variable: Weight

F	df1	df2	Sig.
2.120	15	41	.029

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design: Intercept + Permethrin + PO4 + Permethrin * PO4

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Weight is the same across categories of Permethrin.	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Weight is the same across categories of PO4.	Independent-Samples Kruskal-Wallis Test	.173	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Weight is the same across categories of Perm_PO4.	Independent-Samples Kruskal-Wallis Test	.005	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Total N	57
Test Statistic	32.950
Degrees of Freedom	15
Asymptotic Sig. (2-sided test)	.005

Glycogen

Levene's Test of Equality of Error Variances[a]

Dependent Variable: Glycogen_per_mg

F	df1	df2	Sig.
15.317	14	141	.000

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design: Intercept + Permethrin + PO4 + Permethrin * PO4

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Glycogen_per_mg is the same across categories of Permethrin.	Independent-Samples Kruskal-Wallis Test	.085	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Glycogen_per_mg is the same across categories of PO4.	Independent-Samples Kruskal-Wallis Test	.214	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Glycogen_per_mg is the same across categories of Perm_PO4.	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Total N	156
Test Statistic	41.820
Degrees of Freedom	14
Asymptotic Sig. (2-sided test)	.000

Protein

Levene's Test of Equality of Error Variances[a]

Dependent Variable: Concentration_per_mg

F	df1	df2	Sig.
4.420	14	39	.000

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design: Intercept + Permethrin + PO4 + Permethrin * PO4

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Concentration_per_mg is the same across categories of Permethrin.	Independent-Samples Kruskal-Wallis Test	.005	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Concentration_per_mg is the same across categories of PO4.	Independent-Samples Kruskal-Wallis Test	.299	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Concentration_per_mg is the same across categories of Perm_PO4.	Independent-Samples Kruskal-Wallis Test	.067	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Levene's Test of Equality of Error Variances[a]

Dependent Variable: Concentration_per_mg

F	df1	df2	Sig.
23.932	15	140	.000

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design: Intercept + Permethrin + Phosphate + Permethrin * Phosphate

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Concentration_per_mg is the same across categories of Permethrin.	Independent-Samples Kruskal-Wallis Test	.013	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Concentration_per_mg is the same across categories of PO4.	Independent-Samples Kruskal-Wallis Test	.039	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Concentration_per_mg is the same across categories of Perm_PO4.	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Total N	156
Test Statistic	55.767
Degrees of Freedom	15
Asymptotic Sig. (2-sided test)	.000

1.1.3 Emergence Tests

Proportion Emerged

Tests of Between-Subjects Effects

Dependent Variable: arcsinesqrt

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	2.903[a]	15	.194	8.756	.000
Intercept	55.901	1	55.901	2528.776	.000
Permethrin	2.553	3	.851	38.500	.000
PO4	.178	3	.059	2.687	.057
Permethrin * PO4	.172	9	.019	.864	.563
Error	1.061	48	.022		
Total	59.866	64			
Corrected Total	3.965	63			

a. R Squared = .732 (Adjusted R Squared = .649)

3. Permethrin * PO4

Dependent Variable: arcsinesqrt

Permethrin	PO4	Mean	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
0	0	1.048	.074	.899	1.198
	10	1.044	.074	.895	1.194
	40	1.181	.074	1.031	1.330
	CTL	1.065	.074	.916	1.215
220	0	1.107	.074	.958	1.256
	10	1.011	.074	.861	1.160
	40	.966	.074	.817	1.116
	CTL	.990	.074	.841	1.139
2200	0	.723	.074	.573	.872
	10	.457	.074	.307	.606
	40	.589	.074	.439	.738
	CTL	.596	.074	.446	.745
55	0	1.112	.074	.963	1.261
	10	.923	.074	.773	1.072
	40	1.132	.074	.982	1.281
	CTL	1.011	.074	.861	1.160

Mean Emergence Day

Tests of Between-Subjects Effects

Dependent Variable: Meanemergenceday

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	19.552[a]	15	1.303	1.678	.089
Intercept	17368.288	1	17368.288	22356.753	.000
Permethrin	4.300	3	1.433	1.845	.152
PO4	4.918	3	1.639	2.110	.112
Permethrin * PO4	10.356	9	1.151	1.481	.183
Error	36.513	47	.777		
Total	17523.213	63			
Corrected Total	56.064	62			

a. R Squared = .349 (Adjusted R Squared = .141)

3. Permethrin * PO4

Dependent Variable: Meanemergenceday

Permethrin	PO4	Mean	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
0	0	17.837	.441	16.951	18.724
	10	17.326	.441	16.439	18.212
	40	16.677	.441	15.791	17.564
	CTL	16.406	.441	15.519	17.292
220	0	17.447	.441	16.561	18.334
	10	15.677	.441	14.791	16.564
	40	16.326	.441	15.439	17.212
	CTL	15.990	.441	15.103	16.876
2200	0	16.337	.441	15.451	17.224
	10	16.222	.509	15.198	17.246
	40	16.875	.441	15.988	17.762
	CTL	16.656	.441	15.770	17.543
55	0	16.686	.441	15.800	17.573
	10	16.021	.441	15.134	16.907
	40	16.822	.441	15.935	17.708
	CTL	17.003	.441	16.117	17.890

Female Mean Emergence Day

Levene's Test of Equality of Error Variances[a]

Dependent Variable: femalemeanemergenceday

F	df1	df2	Sig.
2.799	15	47	.004

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design: Intercept + Permethrin + PO4 + Permethrin * PO4

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of femalemeanemergenceday is the same across categories of Permethrin.	Independent-Samples Kruskal-Wallis Test	.027	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of femalemeanemergenceday is the same across categories of PO4.	Independent-Samples Kruskal-Wallis Test	.605	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of femalemeanemergenceday is the same across categories of Perm_PO4.	Independent-Samples Kruskal-Wallis Test	.077	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Male Mean Emergence Day

Levene's Test of Equality of Error Variances[a]

Dependent Variable: malemeanemergenceday

F	df1	df2	Sig.
2.381	15	43	.013

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design: Intercept + Permethrin + PO4 + Permethrin * PO4

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of malemeanemergenceday is the same across categories of Permethrin.	Independent-Samples Kruskal-Wallis Test	.400	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of malemeanemergenceday is the same across categories of PO4.	Independent-Samples Kruskal-Wallis Test	.024	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of malemeanemergenceday is the same across categories of Perm_PO4.	Independent-Samples Kruskal-Wallis Test	.154	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

1.1.4 96 Hour Tests

Survival

Tests of Between-Subjects Effects

Dependent Variable: Arcsin_survival

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	6.736[a]	15	.449	5.560	.000
Intercept	86.870	1	86.870	1075.554	.000
Conductivity	.737	3	.246	3.040	.036
Imidacloprid	3.080	3	1.027	12.713	.000
Conductivity * Imidacloprid	2.851	9	.317	3.922	.001
Error	4.846	60	.081		
Total	108.953	76			
Corrected Total	11.582	75			

a. R Squared = .582 (Adjusted R Squared = .477)

3. Conductivity * Imidacloprid

Dependent Variable: Arcsin_trans_survival

Conductivity	Imidacloprid	Mean	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
0	0	.846	.142	.562	1.131
	0.28	.559	.142	.274	.843
	0.47	1.571	.142	1.287	1.855
	1.3	.780	.142	.496	1.065
4000	0	1.434	.100	1.233	1.635
	0.28	1.184	.142	.900	1.469
	0.47	1.184	.142	.900	1.469
	1.3	.621	.142	.337	.906
8000	0	1.166	.100	.965	1.367
	0.28	1.458	.142	1.174	1.742
	0.47	1.345	.142	1.061	1.630
	1.3	.843	.142	.559	1.128
CTL	0	1.160	.100	.959	1.361
	0.28	1.458	.142	1.174	1.742
	0.47	1.345	.142	1.061	1.630
	1.3	.788	.142	.504	1.073

Weight

Levene's Test of Equality of Error Variances[a]

Dependent Variable: Weight_in_mg

F	df1	df2	Sig.
10.619	15	59	.000

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design: Intercept + Conductivity + Imidacloprid + Conductivity * Imidacloprid

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Weight_in_mg is the same across categories of Conductivity.	Independent-Samples Kruskal-Wallis Test	.011	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Weight_in_mg is the same across categories of Imidacloprid.	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Weight_in_mg is the same across categories of Cond_Imid.	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Total N	75
Test Statistic	51.127
Degrees of Freedom	15
Asymptotic Sig. (2-sided test)	.000

Protein

Levene's Test of Equality of Error Variances[a]

Dependent Variable: Concentration_per_mg

F	df1	df2	Sig.
3.917	15	164	.000

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design: Intercept + Conductivity + Imidacloprid + Conductivity * Imidacloprid

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Concentration_per_mg is the same across categories of Conductivity.	Independent-Samples Kruskal-Wallis Test	.413	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Concentration_per_mg is the same across categories of Imidacloprid.	Independent-Samples Kruskal-Wallis Test	.013	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Concentration_per_mg is the same across categories of Cond_Imid.	Independent-Samples Kruskal-Wallis Test	.054	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Levene's Test of Equality of Error Variances[a]

Dependent Variable: Concentration_per_mg

F	df1	df2	Sig.
6.425	14	144	.000

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design: Intercept + Conductivity + Imdacloprid + Conductivity * Imdacloprid

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Concentration_per_mg is the same across categories of Conductivity.	Independent-Samples Kruskal-Wallis Test	.204	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Concentration_per_mg is the same across categories of Imdacloprid.	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Concentration_per_mg is the same across categories of Cond_Imid.	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Total N	159
Test Statistic	80.351
Degrees of Freedom	14
Asymptotic Sig. (2-sided test)	.000

Lipid

Levene's Test of Equality of Error Variances[a]

Dependent Variable: Concentration_per_mg

F	df1	df2	Sig.
6.946	15	155	.000

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design: Intercept + Conductivity + Imidacloprid + Conductivity * Imidacloprid

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Concentration_per_mg is the same across categories of Conductivity.	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Concentration_per_mg is the same across categories of Imidacloprid.	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Concentration_per_mg is the same across categories of Cond_Imid.	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Total N	171
Test Statistic	86.020
Degrees of Freedom	15
Asymptotic Sig. (2-sided test)	.000

1.1.5 Emergence Tests

Proportion Emerged

Levene's Test of Equality of Error Variances[a]

Dependent Variable: Arcsine_transf

F	df1	df2	Sig.
2.425	15	52	.009

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design: Intercept + Conductivity + Imidacloprid + Conductivity * Imidacloprid

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Arcsine_transf is the same across categories of Conductivity.	Independent-Samples Kruskal-Wallis Test	.003	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Arcsine_transf is the same across categories of Imidacloprid.	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Arcsine_transf is the same across categories of Cond_Imid.	Independent-Samples Kruskal-Wallis Test	.000	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Total N	68
Test Statistic	49.872
Degrees of Freedom	15
Asymptotic Sig. (2-sided test)	.000

Mean Emergence Day

Tests of Between-Subjects Effects

Dependent Variable: Mean_emergence_day

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	25.576[a]	13	1.967	2.736	.008
Intercept	7286.641	1	7286.641	10132.418	.000
Conductivity	11.305	3	3.768	5.240	.004
Imidacloprid	7.327	3	2.442	3.396	.027
Conductivity * Imidacloprid	5.739	7	.820	1.140	.359
Error	28.047	39	.719		
Total	14084.261	53			
Corrected Total	53.623	52			

a. R Squared = .477 (Adjusted R Squared = .303)

3. Conductivity * Imidacloprid

Dependent Variable: Mean_emergence_day

Conductivity	Imidacloprid	Mean	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
0	0	16.105	.424	15.248	16.963
	0.28	15.550	.424	14.692	16.408
	0.47	15.550	.424	14.692	16.408
	1.3	15.333	.848	13.618	17.049
4000	0	17.408	.424	16.551	18.266
	0.28	15.400	.424	14.542	16.258
	0.47	16.425	.424	15.567	17.283
	1.3	.[a]	.	.	.
8000	0	16.982	.424	16.124	17.840
	0.28	17.104	.424	16.247	17.962
	0.47	17.056	.490	16.065	18.046
	1.3	.[a]	.	.	.
CTL	0	16.667	.300	16.060	17.273
	0.28	15.746	.424	14.889	16.604
	0.47	15.521	.424	14.664	16.379
	1.3	15.333	.848	13.618	17.049

a. This level combination of factors is not observed, thus the corresponding population marginal mean is not estimable.

Male Mean Emergence Day

Tests of Between-Subjects Effects

Dependent Variable: Male_Mean_Emergence_Day

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	25.390[a]	12	2.116	2.094	.043
Intercept	5749.521	1	5749.521	5690.099	.000
Conductivity	9.262	3	3.087	3.055	.041
Imidacloprid	6.403	3	2.134	2.112	.116
Conductivity * Imidacloprid	7.512	6	1.252	1.239	.310
Error	36.376	36	1.010		
Total	12347.377	49			
Corrected Total	61.766	48			

a. R Squared = .411 (Adjusted R Squared = .215)

3. Conductivity * Imidacloprid

Dependent Variable: Male_Mean_Emergence_Day

Conductivity	Imidacloprid	Mean	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
0	0	15.633	.503	14.614	16.653
	0.28	15.375	.503	14.356	16.394
	0.47	15.125	.503	14.106	16.144
	1.3	.[a]	.	.	.
4000	0	16.600	.580	15.423	17.777
	0.28	14.854	.503	13.835	15.873
	0.47	15.854	.503	14.835	16.873
	1.3	.[a]	.	.	.
8000	0	16.644	.580	15.467	17.821
	0.28	17.167	.580	15.990	18.344
	0.47	16.167	.580	14.990	17.344
	1.3	.[a]	.	.	.
CTL	0	16.588	.355	15.867	17.308
	0.28	15.146	.503	14.127	16.165
	0.47	15.125	.503	14.106	16.144
	1.3	15.000	1.005	12.961	17.039

a. This level combination of factors is not observed, thus the corresponding population marginal mean is not estimable.

Female Mean Emergence Day

Levene's Test of Equality of Error Variances[a]

Dependent Variable: Female_Mean_Emergence_day

F	df1	df2	Sig.
2.519	13	37	.014

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Design: Intercept + Conductivity + Imidacloprid + Conductivity * Imidacloprid

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Female_Mean_Emergence_day is the same across categories of Conductivity.	Independent-Samples Kruskal-Wallis Test	.091	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Female_Mean_Emergence_day is the same across categories of Imidacloprid.	Independent-Samples Kruskal-Wallis Test	.007	Reject the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of Female_Mean_Emergence_day is the same across categories of Cond_Imid.	Independent-Samples Kruskal-Wallis Test	.097	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

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