COPPER AND NICKEL TOXICITY AND METAL LOADING CAPACITY OF LARVAL HAEMOLYMPH IN 
Aedes aegypti, USING A NOVEL SMALL-VOLUME LASER ABLATION INDUCTIVELY COUPLED 
PLASMA MASS SPECTROMETRY METHOD

by

Lilia Dimitrova Kotzeva

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Abstract

Much of the current literature focuses on fish as models for waterborne metal toxicology, yet few studies examine invertebrate or insect metal toxicology. The present study examined copper and nickel waterborne toxicity in *Aedes aegypti* larvae. The 24-hour LC$_{50}$ for copper in fourth instar larval *Aedes aegypti* was 2.28 mmol L$^{-1}$ (95% C. I. 1.97, 2.67) and 4.63 mmol L$^{-1}$ (95% C. I. 4.25, 5.05) in the unfed and fed treatments respectively. The 24-hour LC$_{50}$ for nickel in fourth instar larval *Aedes aegypti* was 17.2 mmol L$^{-1}$ (95% C. I. 15.58, 18.66) and 27.2 mmol L$^{-1}$ (95% C. I. 25.49, 29.11) in the unfed and fed treatments respectively. To determine the metal loading capacity of haemolymph following acute and chronic copper and nickel exposures, a novel laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) was developed as an alternative approach to analysis of small-volume samples. Matrix matching of liquid samples and standards was achieved by using oiled TLC plates. Sample chamber orientation was assessed, and the orientation used was opposite to the manufacturer’s specified orientation, resulting in better ablation characteristics. The relationship between the volume and surface area of samples spotted on the TLC plates was found to be linear. The method was validated using a certified reference material (Seronorm). It was determined that the average peak height of the chromatographs were best for analysis, after spatial profiling established the area of the most stable analyte signal. Further, the analytical method was independent of volume, avoiding the need to accurately measure small-volume samples. Using the developed method, it was found that *Aedes aegypti* larvae have an innate level of copper in their haemolymph and can concentrate copper in their haemolymph when chronically exposed to a low concentration (0.25 mmol L$^{-1}$ – 1 mmol L$^{-1}$). Conversely,
larvae did not concentrate nickel in their haemolymph, and nickel was not found in control samples of haemolymph. Taken together, the data supports copper’s role as an essential metal and implies that nickel may not be an essential metal for larval *Aedes aegypti*. 
Preface

All copyright permissions have been obtained for any figures or photographs which have been reused in this dissertation, and are in Appendix D. The statistical analysis of the 24-hour LC₅₀ toxicities for copper and nickel were calculated using a custom-made R-script written by Dr. Jason Loepkky, UBC Okanagan Department of Computer Science, Mathematics, Physics, and Statistics (Appendix B). Dr. Rob O’Brien, UBC Department of Chemistry contributed to the conceptual design of the analytical method described in Chapter 3. The conceptual design and data obtained in Chapter 3 was done in collaboration with Karli Kilkus, B.Sc. Chemistry, UBC Okanagan. This document was copy edited by Dania Sheldon, DPhil, English Language and Literature.
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List of Abbreviations

" 
" inches
°C 
°C degrees Celsius
µg 
µg microgram
µg L⁻¹ micrograms per litre
µL 
µL microlitre
µm 
µm micrometre
µmol L⁻¹ micromoles per litre
¹³C 
¹³C carbon-13
⁴³Ca calcium-43
⁴⁵Sc scandium-45
⁵⁶Fe iron-56
⁶⁰Ni nickel-60
⁶⁵Cu copper-65
ArF argon fluoride
ATPase adenosine triphosphatase
Au gold
BLM biotic ligand model
CaCO₃ calcium carbonate
Cd²⁺ cadmium
cm centimetre
CPS counts per second
CuO₄S•₅H₂O cupric sulphate pentahydrate
g gram
GAAS graphite atomic absorption spectrometry
h hours
He helium
HNO₃ nitric acid
Hz hertz
ICP inductively coupled plasma
ICP-OES inductively coupled plasma optical emission spectrometry
ISME ion-selective microelectrodes
J joules
K⁺ potassium
L litre
LA laser ablation
LC₅₀ lethal concentration for 50% of the population
LOD limit of detection
LOQ  limit of quantification
m  metres
Mg\(^{2+}\)  magnesium
min  minutes
mL  millilitre
mm  millimetre
mmol L\(^{-1}\)  millimoles per litre
MS  mass spectrometry
MT  Malpighian tubule
MΩ-cm  Mega ohm-centimeter
N  Biological replicate
n  Technical replicate
Na\(^+\)  sodium
Nd:YAG  neodymium-doped yttrium aluminum
NiSO\(_4\)•6H\(_2\)O  nickel sulphate hexahydrate
NIST  National Institute of Standards and Technology
nL  nanolitre
nm  nanometre
\(^n\)X  any element
OES  optical emission spectrometry
P  phosphorous
Pd  lead
PM  peritrophic membrane
ppb  parts per billion
ppm  parts per million
Pt  platinum
r  radius
Rb  rubidium
Re  rhenium
rpm  revolutions per minute
s  seconds
SEM  standard error of the mean
Seronorm  Seronorm™ Trace Element Serum Level II
Stdev  standard deviation
Ti  titanium
TLC  thin layer chromatography
UV  ultraviolet light
V-ATPase  vacuolar-type H\(^+\)-ATPase
W  watts
\(\pi\)  approximately 3.14
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Dedication

To my Parents

for their love and Support...
Chapter 1: General Introduction

Introduction

Insects are medically and economically relevant, perhaps none more so than the mosquito (Gallup and Sachs 2001). Mosquitoes have plagued mankind throughout the centuries, being responsible for millions of deaths annually by the spreading diseases like malaria and dengue fever, while putting billions more at risk for infection (Malavige et al. 2004). Once only endemic to tropical regions, many vector species of mosquito, like *Aedes aegypti*, have spread globally (figure 1) in response to climate change (Gian-Reto et al. 2002). To mitigate the risk of infection and reduce the economical impact of these diseases, studies have examined the pathogens carried by mosquitoes (Rico-Hesse et al. 1997; Holmes and Burch et al. 2000; Le Roch et al. 2003; Malavige et al. 2004), the effects of these infections on humans (Rigau-Pérez et al. 1998; Monath 2001; Germi et al. 2002; Dewi et al. 2004), and the effects on the mosquitoes themselves (Kokoza et al. 2000; Christensen et al. 2005; Michel and Kafatos 2005), yet few studies have focused on environment factors affecting the aquatic life stages of mosquitoes, such as metals toxicology (Rayms-Keller et al. 1998; Mireji et al. 2008; Mireji et al. 2010a,b). In order to develop better management strategies an understanding of larval mosquito physiology in an environmental context is needed.

Current trends in aquatic toxicology focus on fish as model organisms (Wood 2005), with numerous studies addressing the impact of heavy metals (transition metals) on various species of fish (Grosell et al. 2004; Pane et al. 2005; De Boeck et al. 2007; Alsop and Wood 2011).
Figure 1. World distribution of *Aedes aegypti* (yellow) and dengue fever (red) (Malavige et al. 2004). *Aedes aegypti* originate from Africa but have spread globally. (Reproduced by permission.)

Figure 2. Photograph of an adult female *Aedes aegypti* mosquito acquiring a blood meal from a human host. Photograph by James Gathany, CDC, 2006 (reproduced by permission).
Studies in fish toxicology have deduced the mechanisms of metal toxicity in fish for a number of metals, such as copper, nickel, silver, and lead (Wood et al. 1996; Pane et al. 2003a; Rogers et al. 2003; Alsop and Wood 2005). These studies have been used to set environmental guidelines and regulations to determine safe levels of heavy metals in the environment (Wood 2005). In comparison, few studies have examined heavy metals toxicology in insects, in particular mosquitoes (Mireji et al. 2008; Mireji et al. 2010a,b). Recently, there is evidence to suggest that mosquitoes are becoming increasingly tolerant to heavy metals pollution and are exploiting novel habitats in heavily polluted urban areas, elevating the risk of spreading mosquito borne diseases (Mireji et al. 2008; Mireji et al. 2010a,b).

Copper and nickel are both extensively used heavy metals that inevitably find their way into the environment in both natural and urban settings (Eisler 1997; Eisler 1998). Copper is an essential metal for all aerobic organisms, and copper regulation is highly conserved (Eisler 1997; Balamurugan et al. 2007). For example, a P-type ATPase copper transporter has been mapped in the *Drosophila* genome; larvae lacking this transporter die early in development (Southon et al. 2004). In humans, Menkes disease (an inability to take up dietary copper) and Wilson’s disease (an accumulation of copper) are both lethal conditions involving copper transporters, underlining the importance of copper regulation (Fontaine et al. 1998). However, despite copper’s essentiality, it is also highly toxic to both aquatic vertebrates and aquatic invertebrates (Eisler 1997), typically acting as an ionoregulatory disruptor (Grosell et al. 2002; Grosell and Wood 2002; Grosell et al. 2007). Similarly, in trace amounts nickel is implicated in to act as a co-factor in a number of enzymes, such as superoxide dismutase (Wuerges et al. 2004), while acting as a respiratory toxin to fish if present in excess amounts (Pane et al. 2003). However,
unlike copper, nickel’s role as an essential or non-essential metal in aquatic organisms is unknown (Pane et al. 2003; Muyssen et al. 2004), and further investigation in different taxa is needed.

Recently, some studies have examined the toxic effects of copper on Aedes aegypti (Beaty et al. 2002; Mireji et al. 2008), while to the best of the author’s knowledge, there are no studies that have examined the toxic effects of nickel on any mosquito species. Studying the toxicological effects of copper and nickel on mosquitoes and other insects presents challenges because measuring physiological parameters in insects is difficult due to their small size and small volume of physiological fluids. Some traditional methods used to measure the concentration of metals in biological tissues include ion-selective microelectrodes (ISME), graphite furnace atomic absorption spectrometry (GAAS), and inductively coupled plasma mass spectrometry (ICP-MS). However, as will be discussed later in this chapter, each of these methods presents challenges associated with quantification.

This chapter will discuss the physiology of Aedes aegypti, as well as copper and nickel toxicity in fish models in comparison to insects or other invertebrates, and the difficulty of studying metal contaminants in biological samples using the current methods available. The chapter will conclude with the outlined aims of the study.

**Introduction to Aedes aegypti**

* Aedes aegypti, more commonly known as “the yellow fever mosquito”, is an insect in the order Diptera and a member of the family Culicidae (Clements 1992, pg. xiii–xxii). Adult male *Aedes aegypti* feed exclusively on plant sugars (Scott et al. 2000), while the females of the
species require a protein-rich blood meal in order to produce eggs (Harrington et al. 2000). *Aedes aegypti* females are unique in that they prefer to feed on human blood over other types of animal blood and will forgo feeding on plant sugars if an abundant supply of human blood is available (Scott et al. 2000). The preference of the female *Aedes aegypti* mosquito to feed solely on human blood, coupled with the need to feed many times during their adult life, increases the risk of spreading disease (Harrington et al. 2000; Scott et al. 2000). Approximately two days after taking a blood meal and mating, the female mosquito will find a suitable aquatic environment in which to deposit her eggs (Briegel and DiMarco 2002). *Aedes aegypti* live in close association with humans and are prolific container breeders, laying their eggs in virtually any type of water-containing vessel (Chan et al. 1971).

Eggs laid by a female can remain viable for over a year under favourable conditions; when submerged under water, eggs are stimulated to hatch by a drop in dissolved oxygen (Clements 1992, pg. 70–72). Typically the larval stage lasts approximately 7–14 days, depending on temperature, larval density and food availablity (Appendix A). As larvae grow from 1<sup>st</sup> to 4<sup>th</sup> instar, they shed their cuticle at the beginning of each instar and will do so three times until they pupate (Appendix A). Shortly after pupation (24–48 hours), the adult mosquito emerges (Appendix A). Thus, *Aedes aegypti* undergo complete metamorphosis, exhibiting four distinct stages: egg, larva, pupa, and adult (figure 3) (Clements 1992, pg. xiii-xxii).
Figure 3. The life cycle of *Aedes aegypti*. Starting from the top and continuing clockwise, eggs (photo by Lily Kotzeva, UBC Okanagan, 2009), 4th instar larva, pupa (photographs by Ray Wilson—see Appendix D for copyright permission), and an adult female mosquito (photograph by James Gathany, CDC, 2006 (reproduced by permission).
Aedes aegypti are vectors for yellow fever, dengue fever and chikungunya (Nene et al. 2007). These diseases are arboviruses and are spread when an infected female mosquito injects virally tainted saliva into her host while taking a blood meal (Rigau-Pérez et al. 1998; Lourenço-de-Oliveira 2004; VanLandingham 2005). The mosquito acquires the virus by having previously fed from an infected host (McMeniman et al. 2009). The viruses replicate inside the mosquito and any subsequent feedings on humans result in further transmission (McMeniman et al. 2009).

Aedes aegypti body plan

To study mosquito toxicology, a general understanding of their physiology is required, and thus the following sections provide a brief overview of the anatomy and physiology of the mosquito. Mosquito larvae are covered in an impermeable cuticle (Wigglesworth 1933) and, unlike fish, do not have any respiratory tissues in direct contact with the water (Clements 1992, pg. 118). Instead, mosquito larvae breathe air through a siphon (figure 4) located on the posterior end of the insect near its anus (Wigglesworth 1937; Clements 1992, pg. 118). Many mosquito species breathe by sticking their siphon out through the surface of the water and floating upside down (figure 3, larva) (Wigglesworth 1937; Clements 1992, pg. 118). The siphon is connected to an internal network of trachea and treacheoles transporting air throughout their bodies (Clements 1992, pg. 118). In fish, the gills are the site of toxicity for a number of metals, such as copper, nickel, and silver, and have been used as the site of action in the biotic ligand model (BLM) (Playle et al. 1995; Playle 2004; Paquin et al. 2002; Wood 2005).
The BLM was developed to predict the toxicity of metals to fish, based on various water parameters, and uses the fish gill as the site of action for interaction between the toxins present in the environment and the organism (Paquin et al. 2002). However, since mosquito larvae do not have respiratory tissues in direct contact with their water environment (Wigglesworth 1937; Clements 1992, pg. 118), other tissues in direct contact with water must be the sites of interaction between the organism and toxins. The larval mosquito’s anal papillae and alimentary canal (Wigglesworth 1933), are likely the sites of interaction between the organism and a toxin. The anal papillae are implicated in ion regulation, osmoregulation, and nitrogenous waste removal (Donini and O’Donnell 2005; Clark et al. 2007; Donini et al. 2007), while the alimentary canal is responsible for digestion, osmoregulation, and nutrient uptake (Clements 1992, pg. 100–116).

Larvae reared in a dilute medium have larger anal papillae compared to those reared in a higher saline medium (Wigglesworth 1938). The anal papillae of larvae reared in dilute media are responsible for sodium, chloride, and potassium uptake (Donini and O’Donnel 2005). Although the anal papillae play an important role in ion regulation and nitrogenous waste removal (Donini and O’Donnell 2005; Clark et al. 2007; Donini et al. 2007), larvae can survive to adulthood without these appendages (Wigglesworth 1933). Thus, any toxic interactions causing damage to the anal papillae can be mitigated by other osmoregulatory organs, such as the midgut, Malpighian tubules, and hindgut (Clements 1992, pg. 124–135). However, internal damage of the alimentary canal has greater physiological consequences (Beaty et al. 2002). A closer examination of the alimentary canal is necessary to understand any toxicological consequences of metals exposure.
Figure 4. A diagram of the internal anatomy of a mosquito, from left to right: the different sections of the alimentary canal (foregut, midgut, and hindgut), the organs of the alimentary canal, and the approximate corresponding external anatomy (head, body, and siphon).
Organisation of the alimentary canal of larval *Aedes aegypti*

The alimentary canal of the mosquito is divided into three regions (figure 4): the foregut, the midgut, and the hindgut (Wigglesworth 1933). The foregut starts with the pharynx and leads into the oesophagus (Clements 1992, pg. 100–104). The pharynx and oesophagus aid in swallowing food, and extending on either side of the oesophagus are the salivary glands, used for the production of saliva (Clements 1992, pg. 100–104). Past the salivary glands, the oesophagus extends into the midgut and folds back onto itself, joining the cardia (Wigglesworth 1930). The cardia is the junction between the midgut and foregut (Wigglesworth 1930). The cardia is implicated in the immune response (Hao et al. 2003) and the formation of the peritrophic membrane (Edwards and Jacobs-Lorena 2000). The peritrophic membrane (PM) is secreted by the cardia and extends the length of the midgut, protecting the thin epithelium of the midgut from abrasive food particles, toxins, and microbes (Wigglesworth 1930; Edwards and Jacobs-Lorena 2000). The midgut contains the gastric caeca and the stomach, and is anatomically separated into the anterior and posterior midgut (Clark et al. 1999). The gastric caeca are eight blind-ended tubes with a globular-like appearance, which join the midgut directly below the cardia (Volkman and Peters 1989a). The gastric caeca are used in osmoregulation, nutrient re-absorption, and nutrient storage (Volkman and Peters 1989 b). Following the gastric caeca, the stomach extends from the anterior midgut to the posterior midgut, leading into the hindgut (Wigglesworth 1933; Bradley 1987).

The PM extends into the hindgut, which includes the Malpighian tubules (MTs), the pylorus, the intestine, the rectum, and the anal canal (Wigglesworth 1933; Bradley 1987). The whole length of the alimentary canal, apart from the midgut and MTs, is lined by cuticle
(Wigglesworth 1933; Bradley 1987). *Aedes aegypti* have five blind-ended MTs which filter the haemolymph, forming primary urine. Primary urine flows into the hindgut via the pylorus and is excreted by the anal canal (Bradley 1987).

**The midgut of larval *Aedes aegypti***

As the mosquito larva ingests food, the food bolus moves past the cardia (figure 5) and into the midgut (Clements 1992, pg. 100–104). The food bolus is surrounded by the PM in the endoperitrophic space and makes its way from the anterior midgut to the posterior midgut (Edwards and Jacobs-Lorena 2000). The anterior larval midgut is responsible for alkalisation of the gut content (Clark et al. 2000; Onken et al. 2009), while the posterior midgut absorbs products of digestion (Clements 1992, pg. 107–108). The high alkalinity (pH>10) of the midgut, is thought to aid in the breakdown of ingested tannins, which readily cause proteins and other compounds to precipitate, interfering with downstream digestion and nutrient uptake (Dadd 1975; Zhuang et al. 1999; Seron et al. 2004).

In the endoperitrophic space, the food bolus is subject to enzymatic breakdown by enzymes secreted in the midgut epithelium (Edwards and Jacobs-Lorena 2000). Enzymes such as amylase and trypsin, secreted by the gut epithelium, must move from the ectoperitrophic space into the endoperitrophic (figure 5), while any products of digestion must move in the opposite direction, transversing the PM (Terra 1990).
Figure 5. A closer look at the foregut and midgut. The diagram depicts the peritrophic membrane, outlined in red, and shows both the endo- and ectoperitrophic spaces. The peritrophic membrane is secreted by the cardia and extends the length of the midgut, creating the endoperitrophic space (blue) and the ectoperitrophic space, compartmentalising digestion (Edwards and Jacobs-Lorena 2000; reproduced by premission).
As the food bolus moves from the anterior midgut to the posterior midgut in the endoperitrophic space, fluid and secondary products of digestion move in the opposite direction in the ectoperitrophic space, where they are further broken down by localised enzymes (Terra 1990), absorbed in both the posterior midgut and the gastric caeca (Volkmann and Peters 1989; Terra 1990).

The PM is a vital midgut structure. Not only does it defend the midgut from microbial infection, toxins, and abrasive food particles, but it compartmentalises digestions (Terra 2001; Jacobs and Edwards-Lorena 2000; Beaty et al. 2002). Made up of chitin, proteins, and glycosaminoglycans, the PM’s continual secretion from the cardia creates the ecto- and endoperitrophic spaces (figure 5), allowing for concurrent movement of the food bolus and digestive fluids, as described above (Jacobs and Edwards-Lorena 2000). The PM is semi-permeable and allows small molecules to permeate (Terra 2001), resulting in the localisation of enzymes to either the endo- or ectoperitrophic space, based on their size (Jacobs and Edwards-Lorena 2000; Terra 2001). Enzyme localisation increases the efficiency of digestion by separating primary and secondary products of digestion. By separating primary and secondary products of digestion, absorption efficiency is increased and the excretion of digestion products by peristaltic movements is prevented (Clements 1992, pg. 106–108; Terra 1990; Terra 2001). Further, the localisation of enzymes allows for enzyme recycling, preventing enzyme excretion (Terra 2001).

Disruption of the PM has been shown to adversely affect larval development, increasing the susceptibility of larvae to microbial infection (Wang and Granados 1998) and to toxins,
allowing for non-specific binding of biotic and abiotic factors to the midgut epithelium, leaving the midgut vulnerable to physical damage from abrasive food particles (Terra 2001). PM membranes that are severely damaged or compromised prevent larvae from being able to properly digest food and obtain nutrients, leading to starvation (Terra 2001). The multiple functions of the PM outlined above make it a vital component of the larval midgut and a potential target for toxins (Beaty et al. 2002).

**Larval haemolymph**

Products of digestion that are absorbed by the midgut will enter the haemolymph and circulate within the haemocoel or body cavity (Bradely 1987). Haemolymph is analogous to blood in vertebrates; however, the haemolymph of many insects, including mosquitoes, does not contain oxygen-carrying pigments like haemoglobin, so mosquito haemolymph is clear (Wyatt 1961). Mosquitoes have an open circulatory system, and haemolymph is a combination of circulatory fluid and interstitial fluid containing water, solutes, fats, proteins, amino acids, hormones, and sugars (Wyatt 1961). Haemocytes are also found in haemolymph, but the exact number, type, and function of haemocytes is under question (Clements 1992, pg. 200–201; Castillo et al. 2006). Haemocytes have been implicated in detoxification, wound healing, and defence (Gillespie et al. 1997; Nation 2002, pg. 312–313; Castillo et al. 2006).

Haemolymph has a number of important functions as outlined by Nation 2002, pg. 301–317, some of which include: (1) lubrication and hydraulic support; (2) transport of hormones and nutrients throughout the haemocoel; (3) transport of metabolic waste products and excess water to excretory organs like the Malpighian tubules; and (4) immune response. Any
substance that enters the haemolymph can come into contact with the nervous system, musculature, heart, organs of digestion, and organs of excretion, due to the mosquito’s open circulatory system.

The larval excretory system

The excretory system in mosquitoes is made up of the MTs and the hindgut (Wigglesworth 1933; Bradley 1987). As mentioned previously, the MTs tubules filter the haemolymph (Bradley 1987; Maddrell 1981). The MTs are also responsible for maintaining haemolymph composition and volume (Beyenbach 2003). Excess water taken up by drinking or by the anal papillae can be secreted by the MTs (Wigglesworth 1938; Maddrell 1981). Some toxins and by-products of metabolism can be removed from circulation in the haemolymph by being secreted into the tubules’ lumen (Linser et al. 2009). The haemolymph filtrate passes from the tubule lumen into the hindgut (Bradley 1987). Once in the hindgut, ions and/or water are reabsorbed, while waste is excreted by the anus, removing it from the larva (Bradley 1987; Wigglesworth 1933). Metabolites which can not be further broken into less harmful metabolites, such as copper and nickel, must be sequestered and/or excreted to remove them from the haemolymph (Sohal et al. 1976; Balamurugan et al. 2007).

Introduction to copper

An estimated 16.1 million tons of copper are produced annually world-wide (US Geological Survey 2012). As a result, anthropogenic release of copper into the environment can lead to varying degrees of contamination, ranging from 1 µg L\(^{-1}\)–7 µg L\(^{-1}\) in unpolluted waters to 50 µg L\(^{-1}\)–100 µg L\(^{-1}\) in polluted waters (Eisler 1997). Moreover, copper is widely used as a
biocide in bodies of fresh water (Borkow and Gabbay 2005). Snail vectors of disease (Levine 1970) and fish parasites (Rowland et al. 2008) are routinely controlled by adding copper compounds to bodies of freshwater.

The most common copper species found in aquatic environments is Cu$^{2+}$ (Eisler 1997). The cupric ion is the most toxic species of copper; however, it readily complexes with organic matter (Flemming and Tevors 1989). Although high levels of copper are toxic, copper is also an essential trace element, with well documented deficiencies across taxa, including humans (Eisler 1997). Copper’s role as an essential trace metal warrants that it be heavily regulated within organisms and maintained within a narrow window of essentiality, as any deviation may lead to either deficiency or toxicity (Walker et al. 2006).

Copper toxicology has been well studied, since it is both essential and toxic to all aerobic organisms (Eisler 1997). The vast majority of literature concerning copper toxicity in aquatic environments focuses on fish as model organisms (Wood 2005). The effects of copper on fish are well documented for a number of species, and the physiological consequences of copper in fish have been extensively studied (Grossell et al. 2002; Grosell et al. 2004; De boak et al. 2007; Hoyle et al. 2007; Hashemi et al. 2008; Duarte et al. 2009). In comparison, relatively few studies focus on invertebrate copper toxicology, and little is known about the physiological effects of copper on mosquito larva. The majority of these studies focus on *Daphnia* as model invertebrate organisms (De toro et al. 2001; Satore et al. 2001; Schamplaere and Janssen 2002; Schamplaere and Janssen 2004). The following few sections will give a brief overview of the
underling mechanisms of toxicity in fish, followed by an overview of copper toxicity in aquatic invertebrates for comparison.

**Waterborne copper toxicity in fish**

The trout has been extensively studied as a model organism for aquatic toxicology, and the mechanisms of copper toxicity in trout are well established (Wang et al. 1998; Handy et al. 1999; Grosell et al. 2001; Grosell et al. 2002; Grosell et al. 1997; Grosell et al. 1998; McGeer et al. 2002; Wilson and Taylor 1993; Grosell and Wood 2002). Fish, like all aerobic organisms, harness the redox potential of copper, utilising it as a cofactor in cellular respiration and a wide range of enzymes, such as cytochrome oxidase and haemocyanin (Flemming and Tevors 1989). Freshwater fish exposed to excess amounts of copper succumb to circulatory failure (Grossell et al. 2002a) because copper is an ionoregulatory toxin and disrupts sodium homeostasis (Zahner et al. 2006). Freshwater environments contain very little sodium compared to the extracellular fluids of freshwater animals (Grossell et al. 2006; Grossell et al. 2007). Consequently, the perpetual loss of sodium to the environment by diffusion places freshwater animals under physiological pressure to both conserve and take up sodium from the environment (Grossell et al. 2006; Grossell et al. 2007). Thus, the inhibition of sodium uptake, coupled with the perpetual loss of sodium to the environment, results in thick, concentrated blood (Wilson and Taylor 1993). Blood is concentrated as extracellular fluid enters cells and leaves the circulating plasma, causing the force of cardiac contractions to increase (Wilson and Taylor 1993). Cardiac stress is further compounded by the release of hormones like cortisol (Dethloff et al. 1999) in response to stress. Eventually, the heart can no longer cope and cardiac arrest results (Grossell et al. 2002a).
The trout gill model

The trout gill is utilised as a model for freshwater metals toxicology (Grosell et al. 2002) and has been the focus of models aimed at predicting the toxicity of heavy metals (Playle 2004), including the biotic ligand model (Paquin et al. 2002). The gill is a delicate organ responsible for osmoregulation, respiration, acid–base balance, and nitrogenous waste removal and is sensitive to waterborne pollutants (Grosell et al. 2002).

The Na⁺/K⁺-ATPase pump located in the basolateral membrane of branchial gill cells, plays a pivotal role in cellular physiology (Grosell et al. 2002; Grosell and Wood 2002); by exchanging extracellular potassium for intracellular sodium (Skou 1957). The pump creates an electrochemical gradient favouring the influx of sodium across the apical membrane (Skou and Esmann 1992). Further, the pump is crucial in maintaining cellular membrane potentials as well as intracellular pH, volume, and calcium concentrations (Skou and Esmann 1992; Li et al. 1996).

Na⁺/K⁺-ATPase inhibition

Copper toxicity in the rainbow trout gill is due to non-competitive inhibition of the basolateral Na⁺/K⁺-ATPase pump (Li et al. 1996; Grosell et al. 2002). The Na⁺/K⁺-ATPase pump normally extrudes 3 sodium ions in exchange for two potassium ions by ATP phosphorylation and dephosphorylation (Skou and Esmann 1992; Li et al. 1996). The phosphorylation of the Na⁺/K⁺-ATPase pump requires magnesium as a co-factor (Skou 1990) and because copper interferes with the binding of magnesium to Na⁺/K⁺-ATPase (Li et al. 1996), it results in a loss of function.
There are two proposed mechanisms for apical sodium uptake: a sodium-proton exchanger and/or a V-ATPase pump coupled to a sodium channel in the apical membrane (Potts 1994; Grosell et al. 2002). In either case, both mechanisms are dependent on the electrochemical gradient generated by the Na\(^+\)/K\(^+\)-ATPase pump (Grosell et al. 2002). The electrochemical gradient favours the influx of sodium across the apical membrane (Skou 1990; Skou and Esmann 1992). Therefore, inhibition of the Na\(^+\)/K\(^+\)-ATPase pump results in reduced apical sodium uptake (Lauren and McDonald 1987) (figure 6). The reduction of sodium uptake, coupled with continual sodium loss to the environment, leads to disruption of sodium homeostasis and eventual cardiovascular collapse, as described previously (Grosell et al. 2002).

**Copper toxicity in aquatic invertebrates**

Many of the studies regarding copper toxicity in aquatic invertebrates are focused on *Daphnia magna* as model organisms. These studies primarily focus on using the BLM to predict the concentration of copper exposure that is toxic to *Daphnia* (Di toro et al. 2001; Satorel et al. 2001; Schamplaere 2004; Schamplaere and Janssen 2002). The studies focus on various water parameters, such as temperature and pH, and not the underlining physiological consequences of copper exposure. Evidence from the water flea *Daphnia magna* indicates that copper acts as an ionoreulatory toxin, similar to what has been observed in fish (Bianchini and Wood 2008). However, *Daphnia* are not insects and like fish *Daphnia* rely on a Na\(^+\)/K\(^+\)-ATPase pump to maintain cellular potentials (Bianchini and Wood 2008).
Figure 6. Schematic of the overall direction of sodium movement through a fish gill cell (A) under normal conditions, when the Na⁺/K⁺-ATPase is uninhibited, and (B) when the Na⁺/K⁺-ATPase is non-competitively inhibited by waterborne copper. Other transporters are not depicted as copper primarily affects the Na⁺/K⁺-ATPase, however movement of Na⁺ across membranes is facilitated by transporters.
Conversly, some insects, like mosquitoes, use an altogether different approach, relying on a vacuolar type V-ATPase to energize transport epithelia (Beyenbach and Helmut 2006); therefore it is likely that the mechanism of copper toxicity in mosquitoes and other insects is unlike that seen in fish and *Daphnia*. A study by Leonard et al. (2009) investigated the physiology of cadmium toxicity in 4th instar *Chironomids* larva. The authors reported on the transport of cadmium in the MT and gut, and the amount of cadmium in the haemolymph of exposed larvae. To the best of my knowledge, no such study regarding the physiology of copper toxicity in *Daphnia, Chironomids, or Aedes* exists. Studies by Mireji et al. (2010a,b) focus on molecular markers of metals exposure and the high tolerance of mosquitoes to heavy metals including (2008). No studies to date have reported baseline measurements of copper accumulation in the haemolymph of *Aedes aegypti*.

**Introduction to nickel**

Nickel is extensively used in a number of industries, primarily in the application of metal alloys for the production of stainless steel (Eisler 1998; Reck et al. 2007). Globally, some 1.8 million metric tons of nickel were produced in 2011 by mining activities alone (US Geological Survey 2012). There is concern that there may be a global nickel shortage in the coming years (Reck et al. 2007), as nickel consumption has driven annual global mining production to increase from 7,500 metric tons in 1900 (Eisler 1998) to the 2011 amount (US Geological Survey 2012), while many current nickel mines are reaching the end of their life spans. To offset future nickel shortages, ongoing exploration for natural nickel deposits is underway (US Geological Survey 2012).
Canada has one of the world’s largest nickel deposits, located in Sudbury, Ontario (Eisler 1998). Near Sudbury, nickel concentrations in fresh bodies of water have been known to exceed 180 mg L\(^{-1}\) (Kasprzak 1987) due to mining activities in the area, while unaffected bodies may contain as little as 10 µg L\(^{-1}\) (Pane et al. 2003b). In 2007, a large natural deposit of nickel sulphide was identified in the James Bay lowlands of Northwestern Ontario (US Geological survey 2012). Future mining activities in the area will lead to anthropogenic release of nickel into the surrounding environment, resulting in adverse environmental effects.

Nickel is typically a divalent ion (Ni\(^{2+}\)) in aquatic systems and is a known toxin to plants and animals at high concentrations (Eisler 1998). Unlike copper, nickel’s role as an essential trace element is not clear (Eisler 1998). Although an essential dietary trace element for a number of terrestrial vertebrates, such as goats, cattle, rats, and chickens (Neilsen and Ollerich 1974; Mertz 1993), nickel deficiency has never been demonstrated in humans (Eisler 1998). Similarly, the role of nickel as an essential trace element in aquatic organisms has not been proven (Pane et al. 2003b; Muyssen et al. 2004). Thus, the study of nickel in aquatic ecosystems warrants further investigation, particularly in Canada and other regions where nickel mining is taking place.

As previously mentioned, trout are a common freshwater model organism in toxicological studies (Wood 2005). Nickel toxicity has been studied in the rainbow trout (Pane et al. 2003a; Brix et al. 2004; Pane et al. 2004a,b), but not as extensively as copper toxicity (Grosell et al. 2002a). Further, even fewer studies focus on nickel toxicity in aquatic invertebrates (Pane et al. 2003b; Kozlova et al. 2009; Doig and Lieber 2006), and none thus far
investigate the toxic effects of nickel on mosquitoes. The following overview will examine the differences between nickel and copper toxicity in the rainbow trout, followed by what is known about nickel toxicity in invertebrates.

**Nickel toxicity in the rainbow trout**

Like copper, nickel is implicated in a number of enzymes, such as superoxide dismutase (Szilagyi et al. 2004) and glyoxalase (Thorvaldsson 2003). However, in rainbow trout (*Oncorhynchus mykiss*), the mechanism of nickel toxicity is much different than that of copper. While copper is an ionoregulatory toxin in freshwater fish (Grosell and Wood 2002; Grosell et al. 2002), the mechanism of nickel toxicity in the fish gill results from a disruption of the respiratory process rather than disruption of ion regulation. Although the gill appears to be a target of both metals, it is affected differently by each. Pane et al. (2003) measured the movement of sodium and chloride in the gill epithelia of nickel-exposed trout. The authors demonstrated that the net movement of sodium and chloride was unaffected. Brix et al. (2004) similarly concluded that nickel, unlike copper, is not an ionoregulatory toxin, as it did not disrupt Ca$^{2+}$ and Mg$^{2+}$ homeostasis.

Nickel exposure targets the delicate epithelium of the gill and alters gill morphology (Pane et al. 2004a,b). Typically, each gill filament is comprised of many secondary lamellae filaments, resembling a comb (Wilson and Laurent 2002). The secondary lamellae consist of a blood capillary system covered by a thin respiratory epithelium (Wilson and Laurent 2002). Fish exposed to nickel present lamellar curbing, fusion, and separation of the respiratory epithelium from the underlying blood capillary system (Pane et al. 2004a,b). This type of nickel-induced
damage adversely affects gas exchange at the water/cell surface, since swelling and fusion of the gill epithelium impairs oxygen diffusion (Pane et al. 2004a,b). Along with impaired oxygen uptake, carbon dioxide excretion was negatively affected, resulting in respiratory acidosis as indicated by increased circulating levels of carbon dioxide and a corresponding decrease in arterial pH (Pane et al. 2003a). Ultimately, the breakdown of gas exchange at the gill surface results in hypoxia and respiratory acidosis, leading to death (Pane et al. 2003a; Brix et al. 2004; Pane et al. 2004a).

**Nickel toxicity in aquatic invertebrates**

There is a lack of data on the effects of nickel toxicity on aquatic invertebrates. *Daphnia magna* and *Hyalella azteca* are common model organisms used to further develop the BLM with regards to nickel toxicity in invertebrates (Pane et al. 2003b; Doig and Lieber 2006; Kozlova et al. 2009). As in the case of copper invertebrate toxicity, many published studies focus on various water chemistry parameters when determining the toxicity of nickel to aquatic invertebrates (Doig and Lieber 2006; Deleebeeck et al. 2007; Kozlova et al. 2009), rather than the underlying physiological effects of nickel exposure. The only study to date to determine the mechanism of nickel toxicity in an aquatic invertebrate was done by Pane et al. (2003a). The authors measured whole-body magnesium content in *Daphnia* after exposure to nickel and determined that nickel toxicity in *Daphnia* is ionoregulatory, disrupting magnesium homeostasis. While nickel has been shown to be a respiratory toxin in fish (Pane et al. 2003a; Brix et al. 2004; Pane et al. 2004a,b), it is unlikely that nickel will be a respiratory toxin in *Aedes aegypti* and other mosquito larvae. Since mosquitoes do not have respiratory tissue in direct
contact with their aquatic environment (Clements 1992, pg. 118), nickel could potentially be an ionregulatory disruptor as in the case of *Daphnia maga* (Pane et al. 2003b).

Currently no studies describe the physiological consequences of copper or nickel exposure in aquatic insects, specifically mosquitoes. A reason for the lack of studies measuring physiological parameters in aquatic invertebrates, in particular aquatic insects, may in part be due to the current methods available. Insects are small and the haemolymph volumes that can be collected are often less than 1 µL as in mosquitoes (Beyenbach 2003), and upwards of 300 µL in some lepidopterous caterpillars (Nation 2002, pg. 318). In either case, measuring the concentration of metal contaminants is difficult using currently available methods (described later in the chapter), such as graphite atomic absorption spectrometry (GAAS), inductively coupled plasma optical emission spectrometry (ICP-OES), or inductively coupled plasma mass spectrometry (ICP-MS), without pooling several samples from multiple individuals together (Guo-Xing et al. 2006); this can be time-consuming and laborious. Leonard et al. (2009) were able to measure the amount of cadmium present in the haemolymph of *Chironomids* because a cadmium-selective microelectrode had been developed, making it possible to measure cadmium concentrations in nanolitres of fluid (Pinéros et al. 1998). Unfortunately, functioning copper and nickel-selective microelectrodes are not currently available; in fact, ion-selective electrodes are only available for a handful of elements (Ammann 1989). This study proposes a novel method for measuring the concentration of metals (copper and nickel) in small-volume samples, involving the use of laser ablation inductively coupled mass spectrometry (LA-ICP-MS). The difficulty in measuring physiological parameters in small invertebrates may be the reason for this lack of data. Various common methods used to measure metal content in biological
samples will be discussed in the following sections and will highlight the difficulties in measuring metals in small-volume samples. An alternative method will then be proposed.

**Traditional analytical techniques**

Measuring metal contaminants in the environment and biological tissues presents challenges based on the nature of the sample to be analysed. Many analytical techniques are available—for example, GAAS, ICP-OES, and laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS), to name a few. GAAS is a well-established technique and has few interferences and good sensitivity, down to ppt (Erickson 2000). However, GAAS is only suitable for single element analysis (Baffi et al. 2002; Zeini 2007), so analysis of multiple elements in a sample over a wide dynamic range is not possible. ICP-OES is suitable for multi-elemental analysis over a larger dynamic range (ppb–ppm) and is a widely used technique for the analysis of trace metals in water and food samples (Ikem et al. 2002; Buschmann et al. 2008). Commercially it is cost effective and has the ability to protocols a large number of samples (Marjanovic et al. 2000). Although more costly, ICP-MS is another multi-elemental technique that offers higher sensitivity (ppt), a wider dynamic range (ppt–ppm), higher resolution, and fewer interferences than ICP-OES (Ulrich and Ulrich 1994; Tyler tech. note). ICP-MS is a powerful method offering the sensitivity of GAAS coupled with the multi-elemental capabilities of ICP-OES (Tyler tech. note).

In brief, traditional ICP-MS or solution ICP-MS involves the introduction of a liquid sample into the ICP (Oleski 1995). Typically the sample is pumped into a nebuliser via a peristaltic pump, where it is converted into an aerosol mist (Oleski 1995; Nageswara and Kumar
Once the sample is nebulised, it is introduced to the plasma, where atomisation and ionisation take place. As the sample is atomised and ionised, an electrostatic lens focuses the ion beam on the mass separation device (Oleski 1995; Jakubowski et al. 1998). There are different types of mass separation devices, some of which include quadrupole, time-of-flight, and double-focusing magnetic sector (Glish and Vachet 2003). In this study a double-focusing magnetic sector was used.

When a double-focusing magnetic sector is used as the mass separation device, the ions first enter a magnetic field produced by an electromagnet, where their varying angles of motion are focused based on their momentum-to-charge ratio (Glish and Vachet 2003). The ions then pass into the electrostatic sector, where they are focused again according to their kinetic-to-charge ratio; the end result is that ions are separated based on their mass-to-charge ratio as they reach the detector (Glish and Vachet 2003). The arrangement of the magnetic and electric sectors can also be reversed, as in figure 7 (Glish and Vachet 2003).

Despite the many advantages of ICP-MS, traditional or solution ICP-MS presents challenges when it comes to analysing complex biological samples because these samples must first be digested and solubilised (Ashoka et al. 2009; Rodushkin and Huhtasaari 1999). Nebulisation of samples requires them to be completely solubilised; thus, complete digestion of samples is vital to achieving precision and accuracy (Ashoka et al. 2009).
Figure 7. The path travelled by ions as they emerge from the ion source and enter the electric sector, followed by the magnetic sector, and finally reach the detector. As the ions pass through the electric sector they are focused according to their kinetic energy-to-charge ratio; as they pass through the magnetic sector they are focused based on their varying angles of motion according to their momentum-to-charge ratio (Glish and Vachet 2003; reproduced by permission).
Sample digestion is destructive, time-consuming, and costly. In addition, the use of highly toxic acids such as hydrofluoric acid and peroxides puts the researcher at risk (Rodushkin and Huhtasaari 1999; Wang et al. 2004). A typical digestion process may involve the acidified sample being placed under heat and pressure to aid digestion, or the use of a microwave (Rodushkin and Huhtasaari 1999; Ashoka et al. 2009); this process can produce noxious gases such as hydrofluoric gas and facilitate the loss of volatile species (Wang et al. 2004). In addition, the digestion process can take hours or days (Rodushkin and Huhtasaari 1999). The sample is then reconstituted in a dilute acid matrix (Rodushkin and Huhtasaari 1999; Ashoka et al. 2009). The process of acid digestion is complex and varies from sample to sample; a cocktail of other chemicals may be needed, and thus a digestion protocol is typically formulated to fit each individual sample type (Rodushkin and Huhtasaari 1999; Wang et al. 2004; Ashoka et al. 2009). Each step in the digestion process is also a potential source of error and contamination. In the case of very small samples, one risks magnifying these errors (Todoli and Merment 2006; Hsieh et al. 2009).

**Advantages of laser ablation**

The diversity of real-world samples calls for ongoing development of sample introduction methods. In 1986 Gray demonstrated the use of laser ablation as an introduction method to ICP-MS, as an alternative to traditional liquid ICP-MS. This use of laser ablation eliminates the need for a sample to be fully digested and dissolved in solution (Jarvis and Williams 1993; Chery et al. 2003; Hsieh et al. 2009). Rather, a sample can be taken directly and ablated with a laser, which liberates sample vapour from its solid matrix for introduction into the ICP via an inert carrier gas such as helium (Jarvis and Williams 1993; Chery et al. 2003; Hsieh et al. 2009).
This is a rather attractive sample introduction method, as it eliminates the need for costly, time-consuming, and labour-intensive digestion protocols that may also put researchers at risk by exposing them to highly toxic and corrosive substances (Ashoka et al. 2009; Rodushkin and Huhtasaari 1999; Wang et al. 2004). In addition, this method reduces the chances of contaminating or losing the sample in the process; instead it is possible to directly analyse solid samples (Hsieh et al. 2009).

Certain interferences can also be reduced by using laser ablation rather than traditional liquid-based sample introduction methods. Since traditional liquid sample introduction requires the sample to be introduced as an aqueous aerosol, the presence of water may interfere with the measurement of ions like $^{56}\text{Fe}^+$ because the oxygen from the water can react with the argon torch to form argon oxides (Evans and Giglio 1993). By eliminating the presence of water, oxide interferences can be mitigated (Evans and Giglio 1993; Durrant 1998; Hsieh et al. 2009).

In addition to interferences caused by the presence of water, traditional liquid nebulisation methods may require at minimum 1 mL of solution (Todoli and Merment 2006). Where very small samples are concerned (i.e., less than milligram quantities), reconstitution of these samples in a dilute acid solution, after the digestion process, may dilute the analyte signal of interest below detection limits (Todoli and Merment 2006). Laser ablation uses far less sample, thus making it possible to measure pictogram and femtogram quantities (Russo et al. 2002).

A recent application of LA-ICP-MS, which takes advantage of its minimal sample preparation and minimal sample quantity requirements coupled with the sensitivity of ICP-MS,
was demonstrated by Hsieh et al. 2009. The authors propose LA-ICP-MS as a new method to
detect trace levels of lead in the blood of children in countries where exposure to lead is a
concern. Other conventional methods such as ICP-OES or ICP-MS require large volumes of
blood (several millilitres), which can be difficult to obtain from infants and children (Goullé et
al. 2005).

Hsieh et al. 2009 propose the use of LA-ICP-MS as a way to eliminate the need for the
addition of preservative cocktails, the need for complex digestion protocols with risk of
contamination, and the need for millilitre quantities of blood. The authors demonstrate this by
spotting 0.5 µl quantities of whole blood onto filter paper and using LA-ICP-MS to quantify the
levels of lead present. The authors report that these small volumes of blood are easy to obtain
from children by heel or finger prick, and reduce the problems associated with transport,
storage, and pre-treatment of blood samples.

Another advantage of laser ablation is its ability to provide spatial information about
analytes of interested. Bio-imaging of sections of whole organisms or various tissues (figure 8)
with laser ablation can provide analyte distribution maps (Becker et al. 2005; Becker et al. 2007;
Becker et al. 2010). Metalloproteins and their role in human pathologies are of great interest
(Ge and Sun 2009); laser ablation can not only be used to image various tissues, such as brain
tissue (Becker et al. 2005), but can also be used alongside gel electrophoresis to identify metal-
containing proteins (Becker and Jakubowski 2009). In addition, LA-ICP-MS can be used in
microarrays or immunoassays by elemental tagging of antibodies (Giesen et al. 2012).
Figure 8. An unstained photomicrograph of a 40 µm thin section of mouse heart tissue (A) is imaged by LA-ICP-MS (B) and the elemental distributions of zinc, copper, and iron are shown. A haematoxylin and eosin stained 40 µm thin section of mouse heart tissue (C) displays the anatomy of the heart (Becker et al. 2010; reproduced by permission).
Laser types

Many types of lasers have been employed in laser ablation ICP-MS, including solid-state crystal lasers such as ruby lasers, titanium-sapphire lasers, or neodymium-doped yttrium aluminum garnet (Nd:YAG) lasers (Günther and Hattendorf 2005). More recently, excimer lasers have been used because they can achieve wavelengths as low as 157nm; these are not solid-state lasers but rather rely on gas to produce UV light (Günther and Hattendorf 2005). The wavelength of UV light depends on the type of gas utilised (Bea et al. 1996). For the purpose of this study, an excimer laser with an ArF gas-operated laser was used, which can achieve a wavelength of 193 nm.

Shorter wavelengths are desirable because they are believed to reduce fractionation and improve ablation characteristics (Russo et al. 2000; Russo et al. 2002). Higher photon energies associated with shorter wavelengths are more efficient at vaporising ablated material and decreasing fractionation (Russo et al. 2000). If the laser energy is not strong enough to vaporise the sample, only a portion of that sample will be ionised, introducing bias as a result of fractionation (Jackson and Günther 2003).

Laser ablation sample chamber

The sample chamber is an important aspect of laser ablation and has an effect on the quality of data generated (Arrowsmith and Hughes 1988). Typically the sample and calibration materials are placed in an airtight chamber under atmospheric pressure on an adjustable stage, much like that of a microscope, allowing the sample to be viewed via a microscope and camera set-up, as in figure 9 (Sinclair et al. 1998; Russo et al. 2002).
Figure 9. A diagram illustrating a sample chamber. The camera allows the researcher to view the sample and ablation process. As the sample is ablated by the laser beam, a mixture of gases (argon and helium) sweeps and carries the sample vapour into the ICP-MS (Sinclair et al. 1998; reproduced by permission).
In the case of this study, ablated material was transported from the sample chamber into the ICP via tubing by a mixture of helium and argon gas.

The geometry and cell volume of the sample chamber, along with the length of tubing used, rate of gas flow, and placement of the sample chamber in relation to the ICP, are all factors that influence the quality of data (Liu and Horlick 1995; Bleiner and Günther 2001). These factors influence memory effects, transport efficiency, amount of sample entering the plasma, and wash-out time (Russo et al. 2002). In general, small cell volumes, higher carrier gas flow rates, and a reduction in transport path are optimal (Bleiner and Günther 2001; Russo et al. 2002).

**Quantification with laser ablation**

Quantification with laser ablation as a sample introduction method presents challenges (Russo et al. 2002). Currently a few strategies are employed in quantifying ablation data: solution calibration, dual introduction of aqueous standards and sample, and matrix-matched standards; however, there is no universally accepted calibration method (Russo et al. 2002).

Direct solution ablation involves ablating liquid standards to generate a calibration curve; this can be useful in the absence of a commercially available reference material (Pickhart et al. 2000). Günther et al. (1997) demonstrated the use of liquid calibration in the analysis of National Institute of Standards and Technology (NIST) glass using LA-ICP-MS; however the authors reported variable precision and accuracy. Pickhart et al. (2000) applied liquid calibration in the analysis of geological samples and reported good agreement with reference materials for most of the elements analysed. However, as previously mentioned, this
calibration method may result in matrix effects and spectral inferences associated with the formation of oxides in the presence of water (Evans and Giglio 1993; Durrant 1999; Hsieh et al. 2009).

Dual introduction of liquid standards and samples via two separate pathways was first demonstrated by Thompson et al. (1990) to ensure that the sample and standard were under similar plasma conditions. In this approach, the sample is ablated at the same time as the liquid standard is nebulised, and both are introduced into the plasma simultaneously (O’Connor et al. 2006). A desolvation device can be used to dry the liquid aerosol as it enters the plasma, and can reduce oxide interferences (Russo et al. 2002). However, because the transport efficiencies of aerosols and ablated samples differ, the ionisation and atomisation properties of the sample and the aerosol will also differ; therefore, the addition of an internal standard to both sample and aerosol is recommended (O’Connor et al. 2006).

Another way to quantify LA-ICP-MS data is through matrix-matched solid calibration materials (Bruguier et al. 2001). Matrix-matched calibration materials are not available for every type of solid. However, there are certified reference materials for some types of solid samples, such as metals, glass, cements, and ceramics (Russo et al. 2002). For example, external matrix-matched calibration has been successfully applied in quantifying Re, Au, Pd, Pt, and Rh in natural basalt glass with the use of NIST certified glasses (Sylvester and Eggins 1997).

In cases where a certified material is not commercially available, matrix-matched standards can be made by mixing various compounds into a powdered matrix and pressing the mixture into pellets (Heuzen and Morsink 1991; Borisov et al. 2001). One of the advantages of
this technique is that an internal standard can also be added to the standards and the sample of interest, provided it is powdered and pressed into a pellet (Heuzen and Morsink 1991; Borisov et al. 2001). The quality of the data will depend on how homogeneous and uniform the pellets are (Perkins et al. 1991). To make an external calibration material, the standard and the solid matrix of the sample need to be closely matched because the structure and composition of a sample affects the interaction between it and the laser beam (Holá et al. 2006), which will in turn affect the quality of the data. However, this type of matrix matching may not be possible for all sample types—for example, in the case of precious samples that would be destroyed in the process, or for small amounts of sample that could be contaminated or lost during the pellet-making process.

Despite some challenges associated with the quantification of data and the use of LA-ICP-MS, it remains an invaluable analytical tool (Russo et al. 2002). However, the uses of laser ablation in biology to not only qualitatively identify but also quantify elemental data while providing spatial and depth profiling are only now emerging (Becker and Jakubowski 2009).

**Scope of the study**

This study investigates the differences in toxicity of copper (an essential metal) and nickel (a metal of questioned essentiality in aquatic organisms) in *Aedes aegypti* larvae. The toxic accumulation of copper and nickel in the haemolymph of larval *Aedes aegypti* is investigated by a novel method of analyte identification and quantification in biological tissue utilising LA-ICP-MS. Fourth instar larvae were exposed to various concentrations of copper or nickel to establish the 24-hour LC$_{50}$ for each metal under both fed and unfed conditions. Once
the LC$_{50}$ values were established, larvae were then exposed to levels of copper or nickel at various concentrations below the LC$_{50}$ to determine the metal loading capacity of the larval haemolymph. The resulting small volume (< 1 µL) haemolymph samples were difficult to analyse. To overcome the challenges associated with small-volume samples, a novel method using LA-ICP-MS was established. In brief, the method entails spotting haemolymph samples onto an oiled thin layer chromatography (TLC) plate, along with a series of aqueous standards and Seronorm—a certified reference material (blood plasma)—thus making it possible to quantify the amount of copper and nickel present in larval haemolymph as well as spatially profile the analyte on the TLC plate.

**Aims of the study**

1) Explore the toxicological differences between copper, a known essential element, and nickel, an element of questioned essentiality in aquatic organisms, in larval *Aedes aegypti*.

2) Develop a novel LA-ICP-MS methodology for the measurement of copper and nickel in small-volume larval haemolymph samples.

The following chapters will describe the methodology employed in both analytical and biological contexts (Chapter 2), the results and conclusions generated in the development of a novel LA-ICP-MS method for small-volume analysis (Chapter3), and the resulting data collected from the biological assays undertaken, made possible by the development of this novel method.
Chapter 2: Analytical and Biological Methodology

Reagents and consumables

The following reagents were purchased from Fisher Scientific (Mississauga, Canada): alcohol denatured, cupric sulphate pentahydrate, nickel sulphate hexahydrate, nitric acid, 121-4 mineral oil light, sucrose, and bakers dried active yeast. Sylgard® and high vacuum grease were purchased from Dow Corning (Florida, USA). The following reagents were purchased from Delta (Vancouver, Canada): Ni 1000 µL g⁻¹ in 2 % HNO₃, Cu 1000 µL g⁻¹ in 2 % HNO₃, and nitric acid, environmental grade. Seronorm™ Trace Element Serum Level II was purchased from Accurate Chemical (New York, USA). Sheep blood with Alsevers solution was purchased from Cedarlane (Burlington, Canada). TetraMin® Tropical Fish Flakes were purchased from a local supplier. TLC plates were purchased from EMD Chemicals Inc. (Darmstadt, Germany).

Equipment used

The following equipment was purchased from BioQuip (Rancho Dominguez, California): mosquito jars, plastic rearing trays, mosquito rearing cages, and stockinette sleeves. The following equipment was purchased from Fisher Scientific (Mississauga, Canada): blood feeder, Isotemp 210 water bath, variable flow mini-pump, and gooseneck halogen lights. The following equipment was purchased from Motic (Richmond, Canada): dissecting microscopes and Moticam camera. The incubators were purchased from VWR (Mississauga, Canada), the thermo-freezer was purchased from Forma Scientific (Markham, Canada), and the refrigerator was purchased from Frigidaire (local supplier). The magnetic hotplate was purchased from Corning Inc. (New Jersey, USA), the scale was purchased from Denver Instrument (New Jersey,
USA), and the micro-scale was purchased from Sartorius (Massachusetts, USA). The MiliporMili-Q water filter system was purchased from Milipak Express (Massachusetts, USA). Finally, any Tupperware used was purchased from a local supplier.

Insect rearing

Adult *Aedes aegypti* were reared at 23 °C on a 12 h : 12 h light:dark cycle in 12” x 12” x 12” BioQuip rearing cages. They were fed a 10 % sugar/ultrapure water solution (w/v) ad libitum. Adult female mosquitoes were fed sheep blood warmed to 37 °C once weekly in order to stimulate egg production. Eggs were collected from the adult colonies and were stored in an incubator at 27 °C and 55 % humidity, on a 12 h : 12 h light:dark cycle. Emergent larvae were fed a 1:3 mixture of ground yeast and TetraMin® Tropical Fish Flakes. All larvae used in experiments were 4th instar. See Appendix A for further details.

Acid washing

All glassware, plastic ware, forceps, pipette tips, and Sylgard dissecting dishes used were washed in 1 % lab-grade nitric for 24 hours at room temperature prior to use, to remove any metals contamination. All acid solutions were made using ultrapure water (18.2 MΩ-cm). In addition to being soaked in acid for 24 hours, pipette tips were also soaked in acid and heated to 105 °C for 6–8 hours prior to use.

Determination of copper and nickel 24-hour LC_{50} for both fed and unfed treatments of larval *Aedes aegypti*

Preliminary experiments measuring percent mortality in a range of copper and nickel concentrations for 24 hours were useful in determining an appropriate range of concentrations
in which to best test the toxicity of waterborne copper and nickel. The range of concentrations most suitable for the determination of the 24-hour copper LC$_{50}$ was found to be between 1 mmol L$^{-1}$ and 10 mmol L$^{-1}$ for both fed and unfed treatments. The most suitable range for nickel was determined to be between 10 mmol L$^{-1}$ and 40 mmol L$^{-1}$ for both fed and unfed treatments.

To determine the LC$_{50}$ for waterborne copper and nickel, n=20 4$^{th}$ instar larvae were placed in polyethylene beakers that each contained 90 mL of ultrapure water, their rearing medium, as the control treatment, or various dilutions of cupric sulphate pentahydrate (CuO$_4$S•5H$_2$O) or nickel sulphate hexahydrate (NiSO$_4$•6H$_2$O). Copper and nickel treatment solutions were made by serial dilution from 100 mmol L$^{-1}$ stock solutions with ultrapure water (see table 1 for treatment concentrations). All experimental treatments were replicated 3–6 times for both fed and unfed treatments. The fed treatments received 0.01 g of crushed TetraMin® Tropical Fish Flakes at the start of the experiment. During the experiment, dead larvae were counted and removed every 2 hours for up to 48 hours. The endpoint for mortality was defined as lack of normal motor response when manually stimulated with a glass probe, and an inability to maintain a normal position in the water column. All experiments mentioned above were completed at room temperature.

**ICP-OES instrumentation parameters and water sample analysis**

The water treatments for a subset of the copper LC$_{50}$ experiments (table 1) were analysed to ensure that the concentration of analyte was consistent over the duration of the experiment. Water samples were taken before and after the 24-hour copper exposure, and a
Thermo Electron Corporation iCAP 6000 Series ICP Spectrometer with a low dissolved solids nebuliser and standard spray chamber was used for the analysis of the water samples. The water samples were diluted 50-fold in 1 % nitric acid prior to analysis. The operating conditions of the ICP-OES are summarised in table 2. Each water sample analysis consisted of n=3 replicates and a sample flush time of 30 seconds between samples; the system was purged with 1 % nitric acid between runs. Every 10 samples, a low and high verification standard was run and the instrument was recalibrated.

**ICP-OES detection and quantification limit**

The four wavelengths used for the analysis of copper were 219.9 nm, 224.7 nm, 324.7 nm, and 327.3 nm. All calibration curves, sample concentrations, and blanks were calculated as an average of the four wavelengths used in the analysis of copper. Blanks were defined as a solution of 1 % NHO₃. The calibration linearity coefficient was 0.9999 or better using 5 standards ranging between 0.1 ppb -1000 ppb. All of the data was collected in one day, and the following equations were used to calculate the limit of detection (LOD) and the limit of quantification (LOQ):

\[
LOD = \text{(average signal of blanks)} + 3 \text{(Stdev)} \tag{1}
\]

\[
LOQ = \text{(average signal of blanks)} + 10 \text{(Stdev)} \tag{2}
\]
Where **Stdev** is the standard deviation of the average of the blank signals, and 6 blanks were used to calculate the average of the blank signal. See table 9 in Chapter 4 for LOD and LOQ values.

**Acute and chronic copper and nickel exposure for the determination of haemolymph metal loading capacity**

During the acute copper and nickel exposure, n=20, 4\textsuperscript{th} instar larvae were placed in 100 mL polyethylene beakers with 90 mL of treatment solution (copper or nickel) at the concentrations listed in table 3, and with N=3 replicates of each treatment concentration, as previously stated. The control treatments for both chronic and acute exposures were the normal rearing media for these larvae. Similarly, nominal concentrations of CuO\textsubscript{4}S\textbullet{}5H\textsubscript{2}O and NiSO\textsubscript{4}•6H\textsubscript{2}O were made, as previously explained.

In the acute exposure, larvae were exposed to the concentrations of copper and nickel listed in table 1, for 24 hours at room temperature before they were removed from their respective treatments and their haemolymph was sampled. Treatment concentrations were staggered an hour apart to ensure that larvae were sampled at 24 hours; in addition, copper and nickel exposures were done on separate days. Ten larvae were randomly selected from each treatment concentration for haemolymph collection; an excess number of larvae were exposed to ensure that at least 10 larvae would survive to 24 hours. All other live larvae were humanly euthanised by freezing at –20 °C freezer.
Table 1. Waterborne treatment concentrations of copper and nickel for the determination of both fed and unfed 24 hour LC$_{50}$ exposures

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Treatment concentrations (mmol L$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copper</td>
<td>0.25* 0.5* 1* 2.5* 5 6.25 7.5 8.75 10</td>
</tr>
<tr>
<td>Nickel</td>
<td>1 10 17.5 21.25 25 32.5 40 ---- ----</td>
</tr>
</tbody>
</table>

* Indicates treatments that were analysed by ICP-OES for total concentration of copper.

Table 2. Summary of the instrumental operating parameters of the ICP-OES

<table>
<thead>
<tr>
<th>Plasma Settings:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>RF Power</td>
<td>1150W</td>
</tr>
<tr>
<td>Pump Rate</td>
<td>45 rpm</td>
</tr>
<tr>
<td>Auxiliary Gas Flow</td>
<td>0.5 L/min</td>
</tr>
<tr>
<td>Nebulizer Gas Flow</td>
<td>0.6 L/min</td>
</tr>
<tr>
<td>Coolant Gas Flow</td>
<td>12 L/min</td>
</tr>
<tr>
<td>Additional Gas Flow</td>
<td>N/A</td>
</tr>
<tr>
<td>Purge Gas Flow</td>
<td>Normal</td>
</tr>
<tr>
<td>Radial Viewing Height</td>
<td>15.0mm</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample Pump:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Flush Pump Rate</td>
<td>100rpm</td>
</tr>
<tr>
<td>Analysis Pump Rate</td>
<td>45rpm</td>
</tr>
<tr>
<td>Pump Stabilization Time</td>
<td>5s</td>
</tr>
<tr>
<td>Pump Tubing Type</td>
<td>Tygon Orange/White</td>
</tr>
</tbody>
</table>

Table 3. Waterborne treatment concentrations of copper and nickel for both acute and chronic exposures.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Treatment concentrations (mmol L$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copper</td>
<td>0.25* 0.5 1 2.5 5</td>
</tr>
<tr>
<td>Nickel</td>
<td>1* 10 15 25 30</td>
</tr>
</tbody>
</table>

* Indicates chronic treatment concentrations
**Sylgard plates**

Petri dishes used for haemolymph collection were filled with approximately 7 mL of an optically clear silicone elastomer (Sylgard) and cured according to manufacturer specifications. After curing, the elastomer was placed under vacuum for several minutes, to remove any resulting air bubbles stuck inside the Sylgard for optical clarity, and allowed to set as specified by the manufacturer. The Sylgard provides a firm yet flexible dissecting surface and reduces static charge build-up, which can cause insect tissues to stick to the bottom of the plate making them very difficult to remove.

**Larval dissection and haemolymph collection**

After exposure to metal treatment, larvae were individually removed with a plastic transfer pipette into a weight boat. Any excess water was removed with a pipette and by blotting the larvae dry with a tissue. The larvae were then transferred to three consecutive ultrapure water baths for an approximate cumulative total of 30 seconds, to remove any surface-bound metal. After the final ultrapure water bath, all water was removed using a plastic transfer pipette and the larvae were again gently padded dry with a tissue. With the aid of a dissecting microscope, each larva was held under oil, in a Sylgard lined petri dish. A small tear in the larva’s body wall was made with a pair of forceps and the emergent haemolymph was collected under oil using a micropipettor (figure 10). Care was taken to avoid touching the haemolymph with the metal forceps to prevent possible metal contamination. Additionally, care was taken to avoid tearing the gut of the larva to prevent the contents of the gut mixing with the haemolymph.
Figure 10. Photograph of a 4\textsuperscript{th} instar \textit{Aedes aegypti} larva, submerged under mineral oil, with a tear in the body wall; haemolymph appears as a clear fluid surrounding the larva and as a clear sphere in the upper right-hand corner.

Figure 11. A photograph of a TLC plate with a printed grid. Once deposited onto a TLC plate, the samples appear as white spots; however, when allowed to dry the spots disappear. The two empty cells in the bottom row of the grid resemble the other cells in the grid prior to drying.
Since haemolymph is water-based and not soluble in oil, it is easily removed by means of a pipette. For each larva sampled, the above procedure was repeated and ≤0.2 µL of haemolymph was taken from each larva for analysis.

**Matrix matching and sample spotting using TLC plates**

Aluminum-backed TLC plates with a silica 60 F$_{254}$ gel coating were used as a means of matrix matching. TLC plates were cut into 4.4 cm x 2.5 cm pieces before a 5 cm by 7 cm grid was printed onto the plate; the 5 cm by 7 cm grid was constructed in PowerPoint for Mac and each square of the 5 cm by 7 cm grid was 3 mm x 4 mm. Using an Epson Artisan 710 printer the 5 cm by 7 cm grid was printed onto each plate using coloured ink. Prior to use, plates were soaked in mineral oil for at least 1 hour and then air-dried and stored in a covered Petri dish. Before aqueous samples were spotted, plates were once again re-submerged in mineral oil. With the aid of a dissecting microscope, the aqueous standards and samples were individually pipetted under oil and allowed to fall onto the appropriate area of the plate via gravity, thus spotting the plate. The grid on the plate was such that 0.2 µL of standard or sample could be spotted into each square of the grid without touching the borders of the grid. Once the plate had been spotted, it was left submerged in oil for at least one hour to allow the standards and samples to fully saturate the plate (figure 1). Before the plate could be ablated, it was removed from the oil and allowed to air-dry once again.

**Relationship between volume and surface area of samples spotted onto TLC plates**

To determine the relationship between the volume and surface area of a sample spotted onto an oiled TLC plate, aqueous standards containing copper, nickel, and red food dye
were spotted onto oiled TLC plates in a range of volumes, from 0.1 µL–1.0 µLs. The aqueous standards were spotted onto oiled TLC plates in triplicate, in increasing increments of 0.1 µL. Each volume was first ejected into the oil with a pipettor; under oil, aqueous solutions formed a perfect sphere, and thus it was possible to calculate their volume by measuring their diameter. A picture of each droplet was taken with a Motic microscope camera, and Motic Image Plus software was used to measure the diameter of each sphere (droplet) so that the volume of each sphere could be calculated using equation [3]. Once the diameter of each sphere was measured, the droplets were spotted onto the TLC plates, as previously described, with the aid of a dissecting microscope. The spots were then allowed to dry for 1 hour before a picture was taken, once again with the Motic microscope camera. Using the Motic Image Plus software, a circle was drawn around each spot and its surface area was calculated by the Motic software. The average surface area and volume of each spot were then plotted in Excel, and the standard error of the mean (SEM) was calculated for both volume and surface area:

\[ V = \left( \frac{4}{3} \right) \pi r^3 \]  

[3]

Where \( V \) is the volume of a sphere and \( r \) is the radius.

**Certified reference materials and standards for LA-ICP-MS**

Seronorm Trace Element Serum Level II (Seronorm) was used as the certified reference material to validate the novel LA-ICP-MS method. Seronorm is a powdered animal blood
product and needs to be reconstituted prior to use. Seronorm was reconstituted according to the manufacturer’s specifications and stored at 4 °C until use.

A stock solution of 1000 µg L⁻¹ copper and 1000 µg L⁻¹ nickel was used to make a set of calibration standards ranging from 0.05 ppm to 300 ppm (w/w), containing both analytes. All standards used were made up in 1 % environmental-grade nitric acid and re-made every two weeks. In an effort to validate and compare $^{13}$C to $^{45}$Sc as an internal standard, a set of standards were made ranging from 0.005 ppm to 50 ppm containing 10 ppm $^{45}$Sc, as well as copper and nickel, and were prepared as described above. $^{13}$C was not added to any of these standards, as it was found to be present in all ablations at a constant and consistent level. A limit of detection was determined for the developed method using either $^{13}$C or $^{45}$Sc as internal standards (table 7).

**The effect of different volumes of sample ablated on the signal detected**

To determine whether the amount of material ablated affects the signal detected, and thus the concentration quantified, two volumes of Seronorm (0.2 µL and 0.5 µL) were spotted onto a TLC plate and ablated (n=4 of each volume). The ablation line for 0.5 µL was on average 3–4 times longer than the ablation line for the 0.2 µL spot, thus liberating 3–4 times more sample. The amount of $^{65}$Cu present was quantified and compared to the acceptable range of $^{65}$Cu present in Seronorm, as specified by the manufacturer. In addition, the amounts of $^{65}$Cu present in the 0.2 µL and 0.5 µL spots were compared.
**LA-ICP-MS instrumentation and ablation parameters**

A Photon Machines 193 excimer series laser with an ArF gas was used to introduce the sample into the ICP-MS. The laser ablation parameters for the excimer series are summarised in table 4. A Thermo Scientific Element 2 ICP-MS was used for the detection of the following ionised analytes: $^{13}$C, $^{45}$Sc, $^{60}$Ni, $^{65}$Cu; the operating parameters of the ICP-MS are summarised in table 4.

**Orientation of the laser ablation sample chamber and effects on analyte signal**

The sample cell, which contains the sample chamber, has a teardrop shape and a specific orientation in regard to the end at which the carrier gas enters the cell and the end at which the gas exists the cell along with the sample (figures 12 and 14). In addition, the sample chamber has different sized openings at either end of the teardrop shape (figure 13); the difference in the size of these openings and the shape of the cell contribute to the quality of data produced (see Chapter 3). According to the manufacturer, the carrier gas should enter the sample cell at the top of the teardrop and exit at the concave bottom of the teardrop (figure 12). For all data collection, the orientation of the cell was switched so that the carrier gases entered the cell at the bottom of the teardrop, the concave end, and exited at the top of the teardrop (figure 14). This cell orientation produced better quality data (see Chapter 3, figure 18).
Table 4. Summary of the instrumental operating parameters of the LA-ICP-MS

<table>
<thead>
<tr>
<th>Laser Ablation Parameters</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength</td>
<td>193 nm</td>
</tr>
<tr>
<td>Ablation square</td>
<td>79.8 μm²</td>
</tr>
<tr>
<td>Repetition rate</td>
<td>10 Hz</td>
</tr>
<tr>
<td>Laser fluence (energy)</td>
<td>5.67 J cm⁻²</td>
</tr>
<tr>
<td>Output energy</td>
<td>100%</td>
</tr>
<tr>
<td>Sample gas</td>
<td>1.16 L/min Argon; Teed in before ICP</td>
</tr>
<tr>
<td>Chamber flush gas</td>
<td>0.60 L/min Helium</td>
</tr>
<tr>
<td>Trigger delay</td>
<td>10 seconds</td>
</tr>
<tr>
<td>Scanning Speed</td>
<td>80 μm/s</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ICP-MS Parameters</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>RF Power</td>
<td>1100 W</td>
</tr>
<tr>
<td>Auxiliary gas</td>
<td>0.93 L/min Argon</td>
</tr>
<tr>
<td>Cooling gas</td>
<td>16.23 L/min Argon</td>
</tr>
<tr>
<td>Skimmer cone</td>
<td>Nickel</td>
</tr>
<tr>
<td>Acquisition mode</td>
<td>Triple mode; low resolution</td>
</tr>
<tr>
<td>Acquisition time</td>
<td>84 seconds</td>
</tr>
</tbody>
</table>
Figure 12. A photograph of the teardrop shaped (outlined in yellow) sides of the sample chamber. As specified by the manufacturer, gas flows in the direction of the arrow from A to B.

Figure 13. The photographs show the opening at either end of the teardrop shaped sample chamber; note the opening on one end (A) is smaller than the opening on the other end (B).
Figure 14. The sample chamber has been assembled with the teardrop shaped (outlined in yellow) sides placed over the bottom of the chamber where the sample sits; the top of the sample chamber has not been replaced so that the sample and teardrop shaped sides can be viewed. Note that the orientation of the teardrop is opposite that of figure 12 and that the flow of gas is from B to A. The orientation of the sample chamber as shown in this figure is the orientation used during the study.
Analysis of LA-ICP-MS chromatographs

To create calibration curves (see appendix C) and quantify the amount of analytes in the Seronorm and samples, each ablation file was opened in Show (element software) and exported as a “cvs” file. The “cvs” file was then opened in Microsoft Excel and copied and pasted into a template in another Excel file. The template was specifically made to accommodate this data and to put it in a format suitable for analysis. The first step in analysing the data was to baseline correct each analyte signal. During each sample analysis there was a 10-second delay before the laser is fired and the sample was ablated; this 10-second delay allowed for the detection of any background analyte signal, which can then be subtracted from the sample analyte signal. The average counts per second (CPS) of each analyte was calculated for the first 10 seconds and subtracted from the CPS of that analyte for each time point; CPS is the signal for a given analyte as reported by the instrument. Equations 4 and 5, below, account for background analyte signal and subtract the background signal from the analyte of interest signal.

\[
CPS_{10} = \frac{1}{10} \left( \sum_{i=1}^{10} y_i \right) \quad \text{[4]}
\]

\[
BCS = y_k - \left[ \frac{1}{10} \left( \sum_{i=1}^{10} y_i \right) \right] \quad \text{[5]}
\]
Where is $\text{BCS}$ is the baseline corrected signal and $y_k$ is the $\text{CPS}$ at a given time point for the analyte of interest. Once baseline corrected (BSC), each analyte signal is normalised [6] to the $^{13}\text{C}$ signal, as this is the internal standard.

$$NS = \frac{y_k}{[5]}$$

$$NS = \frac{y_k}{\left[y_k - \left(\frac{1}{10}\left(\sum_{i=1}^{10} y_i\right)\right)\right]}$$

Variables are the same as in equations [4] and [5] and $\text{NS}$ is the normalized signal.

Once the signal for each analyte was normalised in the standards, samples, and Seronorm, the average peak height of the “valley” for each analyte was calculated. Where the valley region is defined as the most stable region of the analyte signal (figure 18). The average of $n=3$ analyte signals was plotted for each standard to generate a calibration curve (appendix D).

Given that the curve is linear with an $R^2$ value $\geq 0.99$, Seronorm was used to validate the calibration curve by quantifying the analyte of interest in $n=4$ Seronorm ablations using equation [7].

$$y = mx + b$$

Where $Y$ is the $\text{CPS}$, $x$ is the time point, $b$ is the $y$-intercept, and $m$ is the slope.

Given a linear calibration curve and Seronorm values that fall within the acceptable range as specified by the manufacturer, the concentration of analyte in haemolymph was
determined in the same way it was determined for Seronorm, using n=10 ablations per
exposure concentration/time.

**Imaging and spatial profiling of copper and nickel aqueous standards spotted onto a TLC plate using LA-ICP-MS**

Imaging refers to rastering an entire spot on a TLC plate to create a quasi-quantitative intensity map of analyte infiltration of the TLC plate. To image the movement of copper and nickel into a TLC plate, a 0.1 µL spot of a 50 ppm standard containing both nickel and copper was spotted onto an oiled TLC plate and rastered. To determine the CPS pertaining to each unit travelled in the x-axis, the following equation was used:

\[
Distance = [T - (\beta + z \left(\frac{L}{A}\right))]A
\]  

[8]

Where \( T \) is time corresponding to the CPS of an analyte of interest; \( \beta \) is the sum of all time delays associated with the movement of the aperture, stage, and length of sample tubing from the sample chamber to the nebulizer etc. starting from zero and increasing in increments of 5 seconds; and \( z \) is the number of lines ablated starting at 1 and increasing in increments of one, \( L \) is the length of every line ablated (2282 µm), and \( A \) is the rate of ablation (78.9 µm/s). The instrumentation parameters are as previously mentioned (table 4), with the exception of the acquisition time, which in this case was approximately 13 minutes instead of 84 seconds. Once the data was analysed, a free mapping software called Quickgrid was used to plot the relative analyte signal intensity of the 0.1 µL spot.
\(^{13}\text{C} \text{ as an internal standard compared to }^{45}\text{Sc as an internal standard and method limit of detection and quantification}\)

Ablation data was collected over the course of several weeks, and thus the average limit of detection (LOD) and limit of quantification (LOQ) were determined over the course of the study for both nickel and copper. The equations used [9, 10] are as previously described for the determination of the LOD and LOQ for waterborne copper [1, 2]. However, a pooled standard deviation is used since blanks were collected on different days for a total of n=11 blanks (see Chapter 3 for values).

\[
\text{LOD} = (\text{average signal of blanks}) + 3(\text{pooled Stdev})
\]  \hspace{1cm} [9]

\[
\text{LOQ} = (\text{average signal of blanks}) + 10(\text{pooled Stdev})
\]  \hspace{1cm} [10]

In addition, the use of two internal standards was tested: \(^{13}\text{C} \text{ and }^{45}\text{Sc}. \text{ The standards were spiked with }^{45}\text{Sc, while no additional step was required for the use of }^{13}\text{C as an internal standard, as it is present in the TLC plates. For the analysis of all the samples, blanks, and Seronorm, }^{13}\text{C was used as the internal standard. Blanks are defined as a solution of 1% NHO}_3. \text{ Chapter 3 describes the data generated using either standard, and demonstrates why }^{13}\text{C was chosen as the internal standard.}\)

**Statistical analysis of data**

In analysing the LC\(_{50}\) data, a custom-made R-script was generated by Dr. Jason Loeppky (see appendix B). The data was fitted to a probit model with a 95% C.I. The acute and chronic exposure data was analysed using Prism (California, USA). A One-Way ANOVA and the Tukey-
Kramer method were used in cases where the variances did not vary significantly. To normalize data for which the variances varied significantly, the data was log transformed and the same statistics applied. In cases where log transforming did not normalize the data and the variances varied significantly, a non-parametric test was performed, specifically a Kruskal-Wallis analysis followed by a Dunn’s Multiple Comparision test. Equivalence among variance was tested using Bartlett’s test for equal variance. Variance was concered to be significant if $P < 0.05$. To analyse the ratio of metal concentration in the haemolymph to the treatment metal concentration, a one-sample student’s $t$-test was performed to determine whether or not the ratio differed significantly from a ratio of 1. In determining whether or not the two volumes of Seronorm spotted differed in their analyte signal, a two-sample student’s $t$-test was applied. In all case differences among means were considered to be significant if $P < 0.05$.

**Methodology Discussion**

Chapter 2 describes the methodology of the biological assays used in this study, specifically the 24-hour LC$_{50}$ for nickel and copper in both fed and unfed conditions, as well as the collection and analysis of larval haemolymph after exposure to various waterborne concentrations of either copper or nickel. As described in Chapter 1, there are many challenges associated with analysing small-volume samples, therefore the development of a novel method to quantify the metal loading capacity of haemolymph was necessary. This chapter describes the application of LA-ICP-MS to quantify copper and nickel in small-volume biological samples, as well as validation of the method using a certified reference material, Seronorm. Figure 15 provides a graphical summary of the overall method used to analyse the haemolymph samples.
While this chapter has discussed the methods used in analysing the haemolymph samples, Chapter 3 will discuss the results associated with the optimization of the LA-ICP-MS method.
Figure 15. Method flow chart summary: (1) Larvae are dissected under oil after toxicity treatment, and haemolymph (large clear sphere) is collected with a pipettor. Haemolymph, standards, and Seronorm are treated in the same manner by being ejected under oil with a pipettor and (2) deposited onto a TLC plate with a printed grid. Once deposited onto a TLC plate the haemolymph, standards, and Seronorm® are (3) introduced into the ICP-MS via laser ablation and the raw data is collected.
Chapter 3: Analytical Results and Discussion

LA-ICP-MS analytical results and discussion

Small-volume samples (µL range) present challenges for traditional analytical techniques such as conventional ICP-MS and ICP-OES (Oliesk et al. 1994; Hsieh et al. 2009). As previously mentioned, these solution-based techniques require biological samples to first be digested and then reconstituted in a dilute acid matrix (Oliesk et al. 1994; Hsieh et al. 2009). When dealing with samples in the microlitre range, accurately measuring the amount of sample present is difficult and the risk of sample contamination or sample loss is magnified (Hsieh et al. 2009). In addition, reconstituting the samples in millilitre quantities, as required for some types of analysis, may suppress the analyte signal (Pozebon et al. 1999). Techniques such as GAAS allow for direct analysis of samples without the need to digest samples; however, GAAS requires much more sample volume (Resano et al. 2007) than the proposed method (table 5). Due to the small volume of the haemolymph samples required for this study, a novel LA-ICP-MS method was developed to overcome the problems associated with traditional solution-based techniques. The following chapter describes and discusses the results obtained in validating the method.

Matrix matching with TLC plates

TLC plates were chosen as the substrate onto which to spot samples as a way of matrix matching. As mentioned previously, matrix matching is essential when performing quantitative analysis with laser ablation, as it reduces fractionation and matrix effects (Russo et al. 2002).
Table 5. Sample volume, risk contamination, and sample prep comparison between current method* and other analytical techniques.

<table>
<thead>
<tr>
<th>Sample volume</th>
<th>Contamination Risk</th>
<th>Sample prep</th>
<th>Single element</th>
<th>Multi-element</th>
</tr>
</thead>
<tbody>
<tr>
<td>&gt;5 nL</td>
<td>low</td>
<td>none</td>
<td>ISMEØ</td>
<td>GFAASa</td>
</tr>
<tr>
<td></td>
<td>low</td>
<td>minimal</td>
<td>LA-ICP-MS*</td>
<td>ICP-MSb</td>
</tr>
<tr>
<td></td>
<td></td>
<td>direct analysis</td>
<td></td>
<td>ICP-OESc</td>
</tr>
<tr>
<td>50-100 μL</td>
<td>low</td>
<td>minimal</td>
<td>≥0.20 μL</td>
<td>200-1000 μL</td>
</tr>
<tr>
<td>200-1000 μL</td>
<td>high</td>
<td>laborious</td>
<td>2 mL</td>
<td>high</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Wet digestion</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ISME: ion-selective microelectrodes; GAAS: graphite furnace atomic absorption spectrometry; LA-ICP-MS: laser ablation inductively coupled plasma mass spectrometry; ICP-OES inductively coupled optical emission spectrometry; Ø indicates ISME are not available for copper and nickel (Ammann 1989); *current method; a Resano et al. 2007; b Zhang et al. 1997, Hsieh et al. 2009; c Suleiman et al. 2007.
TLC plates were chosen as the matrix because they are absorbent, have a consistent and known make-up, and are readily available in a variety of compositions.

The TLC plates used in this study have silica pressed and bound onto an aluminum backing. The absorbent surface is flaky and easily chips off the aluminum backing. As the laser ablates the surface of a dry TLC plate, small particles from the plate are liberated and scattered randomly on the surface of the rest of the plate (figure 16A). This liberated material contaminates nearby samples and creates an uneven ablation surface, translating into a noisier analyte signal (figure 17A).

To visualise the effect of ablating a dry TLC plate, spots of black ink were printed onto the surface of a plate prior to ablation; as can be seen in figure 16 (A, the path of ablation is black and the surrounding area is scattered with black specks. These specks are small pieces of debris from the ablated black ink spot. As the ink spot is ablated, the liberated debris can be seen to fill the path of ablation and scatter across the surface of the plate. In contrast, when a TLC plate is first soaked in oil prior to use, the path of ablation is clear and the amount of debris present is minimal.

When comparing the signal obtained from a dry and an oiled plate (figure 17), the dry plate produces a much noisier signal than the oiled plate, particularly in the “valley” region of the chromatograph, as indicated by the red arrows. The valley region is of most importance as this is the region from which the average peak height of the analyte signal is taken and used to calculate its concentration. The increase signal noise observed in figure 17A is likely due to random debris from previously ablated samples contaminating the path of ablation.
Figure 16. The photographs depict a dry TLC plate (A) and an oiled TLC plate (B); each plate has had black ink printed on it with a printer. The yellow arrows run parallel to the path of ablation.

Figure 17. Representative chromatographs produced by LA-ICP-MS of a 50 ppm standard spotted onto a dry TLC plate (A) and an oiled TLC plate (B). The signals are normalised to $^{13}$C as the internal reference material. The red arrows indicate the “valley” region from which the analyte signal is taken. The valley region in chromatograph A is much noisier than the valley region in chromatograph B.
The oil essentially acts as a binder, much like the addition of binders to pressed pellets, and improves laser ablation properties (Borisov et al. 2001).

Further, oiling the plates prior to use served as an additional way of matrix matching, as haemolymph is often collected under oil (Donini and O’Donnell 2005; Leonard et al. 2009). When haemolymph samples are collected by pipettor, there is always an unknown amount of oil associated with the sample coating the pipette tip. Since it is not possible to separate the sample from the oil without further processing the sample, at the risk of sample loss or contamination, ablating dry plates with an unknown amount of oil associated with each sample adds another layer of complexity. Along with the issue of contamination and an uneven ablation surface caused by the liberation of particles when ablating dry plates, the addition of an unknown amount of oil creates an inconsistent matrix, thus making it difficult to achieve reliable quantitative data. By oiling the plates prior to sample addition, the issues associated with nearby sample contamination, signal noise, and matrix matching have been overcome.

Orientation of the laser ablation sample chamber and effects on analyte signal

Typically, a sample chamber with a small volume is desirable (Bleiner and Günther 2001; Russo et al. 2002). A sample chamber with a smaller volume will reduce the time it takes the analyte signal to return to baseline once ablation is stopped, making the analysis more efficient and reducing memory effects (Russo et al. 2002). The laser ablation chamber of the instrument used in this study is teardrop shaped (Chapter 2, figures 12–14). As previously explained in Chapter 2, carrier gas enters the cell at the top of the teardrop and exits at the bottom or concave end (figure 12), as specified by the manufacturer. The manufacturer-specified cell
orientation produces an analyte chromatograph, as seen in figure 18A. This cell orientation arrangement was found to be less than optimal, since the analyte signal does not return to baseline during the course of the analysis (figure 18A). This may be as a result of the smaller opening (figure 12) through which gas enters the chamber in the manufacturer-specified orientation (figure 13); the opening may not be large enough to allow a sufficient supply of carrier gas to fill and flush the sample chamber rapidly over the given time course. In addition, the geometry of the sides of the sample chamber likely plays a role in the quality of data generated (Liu and Horlick 1995; Bleiner and Günther 2001), and the chosen orientation geometry (figure 14) may be more favourable than the manufacturer-specified orientation geometry (figure 12). However, with just one sample chamber type it is not possible to test the full extent to which either the geometry or the gas openings on either end of the chamber may affect the quality of the data.

When the sample chamber orientation is reversed, as in figure 14 (Chapter 2), an analyte chromatograph, as seen in figure 18B, is produced. The chromatograph in figure 18B is desirable because the analyte signal returns to baseline quickly after ablation has stopped (approximately 4–5 seconds). This is important as the next ablation cannot begin until the signal has reached baseline. If the ablation is started prematurely, memory effects will generate an artificially high analyte signal that could be compounded as the analysis progresses. Thus, the sample chamber orientation as seen in figure 14 (Chapter 2) was used throughout the study because it provided greater transport efficiency of sample vapours, allowing the analyte signal to return to baseline during the allotted acquisition time (figure 18B).
Figure 18. A representative chromatograph of a $^{13}$C signal (bold black line) obtained from ablating an oiled TLC plate; lighter black lines represent other analytes. The first panel (A) depicts the analyte signal obtained with the sample chamber in the orientation as specified by the manufacturer (Chapter 2, figure 12). The second panel (B) depicts the analyte signal obtained with the sample chamber in the reverse orientation. The dashed red line indicates when ablation was stopped, while the dashed blue line indicates when the analyte signal returned to baseline in panel B, as outlined by the dashed yellow lines.
**Relationship between volume and surface area of samples spotted onto TLC plates**

A range of 0.1 µL – 1.0 µL samples were spotted onto a TLC plate and their surface areas were measured (figure 19). It was found that in this range the relationship between volume and surface area, on the TLC plates used, is linear. As the volume of sample increased, so too does its surface area (figure 19). Thus, analysis of two samples of the same concentration but of a different volume can be done independently of volume; a sample with a larger volume will not occupy the same surface area as a sample with a smaller volume (figure 19). If the above relationship was not linear and a sample of a larger volume occupied the same surface area as a sample with a smaller volume, the analyte present would be concentrated, generating an artificially high signal.

**Volume and concentration relationship of samples spotted onto TLC plates**

To confirm that volume does not affect analyte signal, Seronorm samples were spotted onto a TLC plate in two different volumes (0.2 µL and 0.5 µL) (figure 21). As figure 20 indicates, there is no statistical difference between the concentrations measured in the 0.2 µL spot and in the 0.5 µL spot, even though the length of the ablation line used to ablate the 0.5 µL spot is roughly 2.5–3 times larger. Therefore, roughly 2.5–3 times as much material is ablated in the 500 µL spot than in the 0.2 µL spot. Figure 20 also shows that the concentration of copper measured in the Seronorm falls within the manufacturer-specified concentrations, as indicated by the dashed lines. The conversion of copper analyte signal to copper concentration was done using a calibration curve, which was generated as previously described in Chapter 2.
Figure 19. Aqueous standards containing copper, nickel, and food dye were spotted onto a TLC plate, under oil in triplicate, in a range of 0.1 µL–1 µL. The average volume of each droplet (sphere) was measured under oil before contact with the TLC plate and is represented by the left y-axis. The right y-axis represents the volume equivalent in µL, as it was ejected into the oil with a pipettor. The x-axis represents the average surface area of each droplet once it had been spotted onto the TLC plate and allowed to absorb. The vertical and horizontal error bars represent SEM (n=3). The goodness of fit, $R^2 = 0.986$. Where error bars are not visible, they are present but hidden by the plot symbol.
Figure 20. The concentration of copper as measured by LA-ICP-MS, in two volumes (0.2 µL and 0.5 µL) of Seronorm. Each volume of Seronorm (n=4) was spotted onto a TLC plate under oil. A two-sample t-test (*P<0.05) indicates that the two ablated volumes do not statistically differ in their concentration of copper. Dashed lines (-----) indicate the acceptable range of copper in Seronorm, as specified by the manufacturer. Data is presented as means and the error bars represent ± SEM. The LOQ for copper for this method is 0.0691 ppm ± 0.000 Stdev (table 7).
Two important conclusions can be drawn from figure 20. First, ablating more material (larger volume) does not generate an artificially higher analyte signal, because as seen in figure 19, the relationship between surface area and volume is linear, meaning that the sample infiltrates the TLC plate at a constant rate within the range specified (0.1 µL–1.0 µL); and second, the calibration method developed was validated using Seronorm as a certified reference material. Since it has been shown that analyte signal is independent of volume (surface area) spotted onto the TLC plate, determining the exact volume of haemolymph sample is not necessary.

**Imaging and spatial profiling of copper and nickel aqueous standards spotted onto a TLC plate using LA-ICP-MS**

As seen in figures 21 and 22, the signal intensity of copper and nickel, respectively, is highest and most variable along the perimeter of the spot. However, in the centre the signal intensity for both copper and nickel stabilises, forming a relatively stable intensity signal in the centre of the spot. This stable region is larger for the copper signal than for the nickel signal. In contrast, the area of highest signal intensity and variability, around the perimeter, is larger for the nickel signal than for the copper signal. This represents one of the attractive qualities of laser ablation, its ability to spatially profile analytes (Becker et al. 2005; Becker et al. 2007; Becker et al. 2010), allowing the researcher to find the location of particular elements, rather than just the overall amount. As previously mentioned, this is of particular interest in biological tissues (Becker et al. 2005; Becker et al. 2007; Becker et al. 2010).

Panel B of both figures 21 and 22 illustrates a typical chromatograph of an aqueous standard solution. Rather than rastering the entire area of the spot, the chromatograph was
generated by ablating a single line across the centre of a spot from one end to the other. In short, a chromatograph is like one slice of the analyte signal intensity map, a 2-dimensional image of signal intensity. What can easily be visualised from the chromatograph is that the analytes are spatially separated in the TLC plate; the copper signal (green) consistently remains within the nickel signal (red). The signal for both copper and nickel is collected at the same time during analysis, and with the chromatograph it is much easier to graph the signal for both copper and nickel on one graph. This demonstrates that the particular type of TLC plate used interacts differently with copper than it does with nickel, allowing nickel to spread out further on the plate than copper, indicating a potential to combine chromatography and laser ablation in identifying metal-containing compounds.

It is important to note that the average peak height of the valley region is taken and not an integration of the entire chromatograph or an integration of the valley region. Although it would be possible to integrate under the curve and use the value generated for the subsequent concentration calculations, this would add complexity to the experiments in terms of sample ablation and sample collection. By taking the average peak height of the valley region and not integrating under the curve, the length of the ablation line does not need to be taken into account. Integrating under the curve would require that the integrations were consistently done in the same time frame or for the entire chromatograph. To ensure that the same region of each chromatograph fell within that time frame, each sample or standard would have to be ablated with the same length of ablation line.
Figure 21. The spatial distribution of copper in an aqueous standard as imaged by LA-ICP-MS (A). 0.1 µL of a 50 ppm copper and nickel standard solution was spotted onto an oiled TLC plate and the area of the spot, including some of the surrounding area, was rastered. Quickgrid software was used to create a quasi-quantitative intensity map of the copper signal, with red representing the highest signal intensity and blue representing the lowest signal intensity. (B) A typical chromatograph of copper (green) and nickel (red) analyte signal after ablating a single line across the diameter of a 0.2 µL 50 ppm spot of copper and nickel standard solution spotted onto an oiled TLC plate.
Figure 22. The spatial distribution of nickel in an aqueous standard as imaged by LA-ICP--MS (A). 0.1µL of a 50 ppm copper and nickel standard solution was spotted onto an oiled TLC plate and the area of the spot, including some of the surrounding area, was rastered. Quickgrid software was used to create a quasi-quantitative intensity map of nickel signal, with red representing the highest signal intensity and blue representing the lowest signal intensity. (B) A typical chromatograph of copper (green) and nickel (red) analyte signal after ablating a single line across the diameter of a 0.2 µL 50 ppm spot of copper and nickel standard solution spotted onto an oiled TLC plate.
Ablating each sample with the same length of ablation line would slow the ablation process, as the user would have to ensure that each line of ablation was the same length and also in the same region of the spot for each sample or standard ablated. Since the standards are not visible once the TLC plate is dry (figure 11), this would be very difficult to achieve. Further, an ablation line of the same length for all of the samples and standards run would require that all of the samples or standards occupy the same surface area and thus the same volume (figure 19). As previously mentioned, there is great difficulty in measuring the exact volume of the haemolymph samples collected, as there is always an unknown amount of oil associated with each sample; in addition, the natural variability in haemolymph volume between larvae would make collecting the exact same amount of haemolymph from each larva difficult in the presence of mineral oil. Since the analyte signal is independent of volume (figure 20), taking the average peak height of the valley region negates having to determine the volume of the sample. This is a major advantage of this technique, since with the use of traditional methods, like ICP-OES or ICP-MS, knowing the exact volume or mass of a sample is paramount in determining the concentration of analytes (Becker and Jakubowski 2009).

\textbf{\textsuperscript{13}C as an internal standard compared to \textsuperscript{45}Sc as an internal standard and the method for limit of detection and quantification for each standard}

An additional benefit of oiling the TLC plates is that the oil was used as an internal standard. Upon initial test ablations, it was found that the oil contained a high but consistent signal of \textsuperscript{13}C (figure 18), several times higher than the analytes of interest. Since \textsuperscript{13}C is naturally present in the mineral oil used to oil the TLC plates, it was evaluated as an internal standard and compared to the addition of \textsuperscript{45}Sc as an internal standard.
Initially calibration curves with a wide range (0.005 ppm – 50 ppm) were generated for both copper and nickel, using either $^{13}\text{C}$ or $^{45}\text{Sc}$ (figures 23 A, C and 24 A, C) as an internal standard to visualise the detection limit. At a glance, both the copper and the nickel calibration curves exhibit good linearity when either $^{13}\text{C}$ or $^{45}\text{Sc}$ is used as the internal standard (figures 23 A, C and 24 A, C). In the case of the copper calibration curve, a goodness of fit of 0.9999 (figure 23 A, C) is observed when either $^{13}\text{C}$ or $^{45}\text{Sc}$ is used as the internal standard. Similarly, a goodness of fit of 0.9994 (figure 24A) and 0.9975 (figure 24C) is observed when $^{13}\text{C}$ and $^{45}\text{Sc}$, respectively, are used as the internal standard in generating a calibration curve for nickel. However, focusing on the lower end of the calibration range for both copper and nickel (figures 23B and 24D) dramatically changes the goodness of fit of the curve, depending on which element is used as the internal standard, $^{13}\text{C}$ or $^{45}\text{Sc}$. The goodness of fit for the copper calibration curve in the range of 0.005 ppm – 0.50 ppm is 0.9453 (figure 23B) when $^{13}\text{C}$ is used as the internal standard, compared to a goodness of fit of 0.4020 (figure 23D) when $^{45}\text{Sc}$ is used as the internal standard. Similarly, the goodness of fit for the nickel calibration curve in the range of 0.05 ppm – 1 ppm is 0.9978 (figure 24B) when $^{13}\text{C}$ is used as the internal standard compared to a goodness of fit of 0.1783 (figure 24D) when $^{45}\text{Sc}$ is used as the internal standard. The goodness of fit values for both copper and nickel dramatically improve when $^{13}\text{C}$ is used as the internal standard (figures 23AB and 24A,B), particularly in the lower end of the calibration curve (0.05 ppm – 1 ppm), suggesting that $^{13}\text{C}$, which is already present in the matrix, is a better internal standard for this methodology.
Figure 23. Calibration curves generated for $^{63}$Cu, with $^{13}$C as the internal standard, by spotting aqueous standards onto a TLC plate, ranging from 0.005 ppm –50 ppm of n=3 (A) and 0.005 ppm –0.50 ppm of n=3 (B). The signal of $^{63}$Cu was normalised to the signal of $^{13}$C, already present in the TLC plate, to generate the curve. Similarly, calibration curves were generated for $^{63}$Cu, with $^{45}$Sc as the internal standard, by spotting aqueous standards onto a TLC plate, ranging from 0.005 ppm –50 ppm of n=3 (C) and 0.005 ppm –0.50 ppm of n=3 (D). Aqueous standards were spiked with $^{45}$Sc prior to spotting on the TLC plate, and the signal of $^{63}$Cu was normalised to the signal of $^{45}$Sc to generate the curve. In all cases, data presented as means ±1 stdv. Where error bars are not visible, they are present but hidden by the plot symbol.
Figure 24. Calibration curves generated for $^{60}$Ni, with $^{13}$C as the internal standard, by spotting aqueous standards onto a TLC plate, ranging from 0.005 ppm – 50 ppm of n=3 (A) and 0.05 ppm – 1 ppm of n=3 (B). The signal of $^{60}$Ni was normalised to the signal of $^{13}$C, already present in the TLC plate, to generate the curve. Similarly, calibration curves were generated for $^{60}$Ni, with $^{45}$Sc as the internal standard, by spotting aqueous standards onto a TLC plate, ranging from 0.005 ppm – 50 ppm of n=3 (C) and 0.05 ppm – 1 ppm of n=3 (D). Aqueous standards were spiked with $^{45}$Sc prior to spotting on the TLC plate, and the signal of $^{60}$Ni was normalised to the signal of $^{45}$Sc to generate the curve. In all cases, data presented as means ±1 stedv. Where error bars are not visible, they are present but hidden by the plot symbol.
In addition to the dramatic change in the observed goodness of fit values for both copper and nickel, depending on whether $^{13}$C or $^{45}$Sc is used as the internal standard, there is also a difference in the analyte concentration of the blanks. Table 6 represents the concentrations of analytes in blank ablations, based on the calibration curves in figures 23 and 24. When $^{45}$Sc is used as the internal standard, for normalisation, the concentrations generated using the above calibration curves are invalid, ranging in the negative. This is not surprising as the calibration curves generated when $^{45}$Sc is the internal standard exhibit poor goodness of fits for both copper and nickel (figures 23 and 24). Based on the observed goodness of fits in figures 23 and 24, and the negative concentration values observed for both analytes in blank ablations, $^{45}$Sc is not suitable as an internal standard for this method. Conversely, when $^{13}$C is used as the internal standard the concentrations generated using the above calibration curves are acceptable (table 6) and were used in determining the LOQ for this method (table 7). The observed difference between $^{13}$C and $^{45}$Sc as internal standards could be the result of how homogeneous and consistent their distribution is throughout the TLC plate. Craig et al. (2000) observed a similar response when testing different calibration strategies in naturally occurring biological samples that were primarily composed of CaCO$_3$. In two of the calibration strategies used by the authors, a comparison was made between the use of indium as an internal standard versus $^{43}$Ca as an internal standard. Indium was added to the binding material used to make pressed pellets of samples, while $^{43}$Ca was already present in the samples as the predominant matrix. The authors found that indium showed large variations in signal response ($>$50%) among different ablations, while $^{43}$Ca improved both precision and long-term reproducibility.
Table 6. Concentrations (ppm) of analyte present in blank ablations when $^{13}$C or $^{45}$Sc is used as the internal standard for the developed LA-ICP-MS.

<table>
<thead>
<tr>
<th></th>
<th>$^{63}$Cu</th>
<th>$^{60}$Ni</th>
<th>$^{63}$Cu</th>
<th>$^{60}$Ni</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{13}$C</td>
<td>0.054</td>
<td>1.854</td>
<td>-0.382</td>
<td>-3.780</td>
</tr>
<tr>
<td></td>
<td>0.046</td>
<td>1.757</td>
<td>-0.382</td>
<td>-3.781</td>
</tr>
<tr>
<td></td>
<td>0.052</td>
<td>1.632</td>
<td>-0.382</td>
<td>-3.783</td>
</tr>
</tbody>
</table>

Concentrations based on calibration curves from figures 23 and 24.

Table 7. Limit of detection (LOD) and limit of quantification (LOQ), in ppm and mmol L$^{-1}$, for the developed LA-ICP-MS method, using $^{13}$C as the internal standard.

<table>
<thead>
<tr>
<th></th>
<th>$^{63}$Cu</th>
<th>ppm</th>
<th>mmol L$^{-1}$</th>
<th>$^{60}$Ni</th>
<th>ppm</th>
<th>mmol L$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOD</td>
<td>0.068</td>
<td>0.001</td>
<td>1.580</td>
<td>0.027</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LOQ</td>
<td>0.069</td>
<td>0.001</td>
<td>1.613</td>
<td>0.028</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stdev</td>
<td>±0.00012</td>
<td>±1.574x10$^{-6}$</td>
<td>±0.0047</td>
<td>±7.971x10$^{-5}$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Similarly, when $^{45}$Sc is used as an internal standard, in this study, there are large variations in signal response, which correspond to poor goodness of fit values (figures 23 and 24), resulting in negative blank analyte concentrations (table 6). Both precision and long-term reproducibility improve when $^{13}$C is used as the internal standard; because the TLC plates are oiled prior to the sample being spotted on them, the oil becomes an additional way of matrix matching, since it becomes incorporated into the TLC plate matrix. In contrast, standards were spiked with $^{45}$Sc under oil prior to spotting onto the TLC plate. The use of an internal standard is vital for reliable quantitative data, as an internal standard can correct for variable signals and loss of instrument sensitivity over time (Craig et al. 2000; Russo et al. 2002).

**Measurements of copper and nickel in haemolymph**

The LA-ICP-MS method described and validated in the preceding sections was used to measure 4$^{th}$ instar larval *Aedes aegypti* haemolymph copper and nickel concentrations prior to and after 24-hour exposure to waterborne copper or nickel. Fourth instar *Aedes aegypti* larvae were exposed to either control conditions, 1 mmol L$^{-1}$ copper, or 15mmol L$^{-1}$ nickel for 24 hours, and the concentration of copper and nickel was measured in the larval haemolymph. The exposure concentrations were chosen based on 24-hour LC$_{50}$ experiments done prior to the analytical method development (Chapter 4), and represent values near or at the 24-hour LC$_{50}$ of nickel and copper. Larval haemolymph in the control group and the copper-treated group contained no detectable nickel (table 7). While larva in the 15 mmol L$^{-1}$ nickel treatment contained 1.18 mmol L$^{-1}$ ± 0.28 SEM of nickel in their haemolymph, which was statistically significant from the amount of nickel present in the control group and the 1 mmol L$^{-1}$copper group. In contrast, all three treatment groups contained copper above the LOQ (table 7): 0.018
mmol L\(^{-1}\) ± 0.0014 SEM, 0.51 mmol L\(^{-1}\) ± 0.091 SEM, and 0.027 mmol L\(^{-1}\) ± 0.0020 SEM for the control, 1 mmol L\(^{-1}\) copper, and 15 mmol L\(^{-1}\) nickel treatment groups, respectively, with the 1 mmol L\(^{-1}\) copper treatment group being statistically different than the other two treatment groups. From figure 25 it is important to note that the control treatment group contains copper but not nickel. This is likely due to the fact that copper is an essential element (Walker et al. 2006); nickel essentiality, however, has only been established for terrestrial vertebrates, and its role in aquatic organisms has yet to be determined (Muysen et al. 2004). It follows that the nickel treatment group contains copper as well as nickel. Since copper is an essential trace element for all aerobic life forms (Eisler 1997), the amount of copper present in the control group and the nickel treatment group is not statistically different, indicating the observed values are likely representative of innate copper levels present in *Aedes aegypti* larvae. In contrast, nickel is only found in the haemolymph after external exposure. Another important conclusion to draw from figure 25 is that the developed analytical method can measure both nickel and copper simultaneously in small-volume insect haemolymph, making the investigation of nickel and copper toxicity in larval *Aedes aegypti*, as well as in other insects, possible without the need to pool samples. It follows that other analytes of interest could also be measured, and that more than two analytes at a time can be measured in small-volume samples with precision and reproducibility, depending on the type of detector system used. These values chosen are believed to be physiologically relevant concentrations for the larvae tested, since they are near the respective LC\(_{50}\) values of copper and nickel and were initially chosen to determine whether these metals are able to penetrate the cuticle of the larva.
Figure 25. Concentration of copper and nickel in the haemolymph of 4th instar Aedes aegypti after a 24-hour exposure to a control (Ctrl) treatment, a 1 mmol L⁻¹ Cu²⁺ (Copper) treatment, and a 15 mmol L⁻¹ Ni²⁺ (Nickel) treatment. A Kruskal-Wallis test (P<0.05) was used to test for statistical significance and a Dunn’s multiple comparison test (P<0.05) * was used to compare significance across all treatments; ND = not detectable. Data plotted as means ± 1 SEM (n=9 for control, n=10 for all other treatments). The LOQ for copper for this method is 0.001 mmol L⁻¹ ± 1.574x10⁻⁶ Stdev; the LOQ for nickel for this method is 0.028 ± mmol L⁻¹ ± 7.971x10⁻⁵ Stdev (table 7).
Analytical Results Discussion

As previously stated, quantifying analytes in small volumes is a challenge, and before biological assays addressing the accumulation and physiological consequences of copper and nickel accumulation in the haemolymph of larval *Aedes aegypti* could be undertaken, a method to quantitatively identify the presence of copper and nickel in small volumes of haemolymph was needed.

The novel LA-ICP-MS method developed for the analysis of small-volume haemolymph samples, and the results generated in developing that method, were discussed in this chapter. Matrix matching of samples and standards was achieved by spotting onto oiled TLC plates. The oil acted as a binder and produced better quality data than non-oiled plates (figures 16 and 17); further, the oil served as an additional way of matrix matching, since all haemolymph samples were collected under oil, and an unknown amount of oil was associated with each sample. The oil further lent itself as an internal standard, as it was found to be present in oiled TLC plates at a constant level (figures 18, 23, and 24).

The optimal sample chamber orientation was found to be opposite to that specified by the manufacturer (figure 12). The chosen sample chamber orientation (figure 14) was found to produce better quality data by allowing the analyte signal to return to baseline shortly after ablation of the sample was stopped, thus allowing the next sample to be ablated without generating an artificially high analyte signal by preventing signal carry over and memory effects.

The relationship between volume of samples and surface area was found to be linear (figure 19) in the TLC plate, ruling out the possibility of artificially concentrating the analyte
signal by spotting two differing volumes that occupied the same surface area, and thus liberating more or less analyte. In addition, different volumes of ablated Seronorm (figure 20) generated the same manufacturer-specified concentration for copper, thus validating the accuracy of this method. Spatially profiling aqueous standards on the TLC plate by imaging them using LA-ICP-MS showed that the most stable region of analyte signal was in the centre of the spot, and this therefore was the region of each chromatograph used for analysis. To summerize, the linear relationship between volume and surface area, the constant concentration generated by ablating different volumes, and the identification of the region of analyte stability allowed for sample analysis independent of volume. This method provides advantageous for small-volume sample analysis, which can be difficult to measure or separate from their surrounding medium, as was the case with larval haemolymph. Once the method was developed, it was applied to the measurement of copper and nickel in the haemolymph of larval *Aedes aegypti* (figure 25). A more in dept assessment of haemolymph metal loading capacity will be discussed in Chapter 4, using the described method.
Chapter 4: Biological Results and Discussion

Biological results and discussion

Anthropogenic release of heavy metals into the environment is of growing concern for many aquatic organisms (Hare 1992). Insects, however, exhibit high tolerance to heavy metals, including copper, nickel, cadmium, and lead, with LC_{50} values typically orders of magnitude higher than values found in unpolluted environments (Buchwalter et al. 2007). Such high tolerance to heavy metals implies that insects have strong coping mechanisms. A strong ability to excrete (Timmermans and Walker 1989) or sequester (Gills and Wood 2008) heavy metals is likely responsible for their observed high tolerance. A combination of both excretion and sequestration was observed by Leonard et al. (2009) when exposing Chironomid larvae to Cd^{2+}, indicating some species of insects may rely on both strategies to overcome exposure to heavy metal pollutants.

It is unclear which strategies Aedes aegypti larvae employ to cope with exposure to heavy metals. However, like other insects (Leonard et al. 2009), they exhibit a high tolerance to heavy metals (Mirjie et al. 2008). This chapter discusses the effects of both acute and chronic exposure to copper and nickel on 4\textsuperscript{th} instar Aedes aegypti, and provides baseline measurements of copper and nickel in the haemolymph of exposed and control larvae. The measurement of analytes in haemolymph was only made possible by the development of the method described in Chapter 3.
**Determination of copper and nickel 24-hour LC$_{50}$ for both fed and unfed treatments of larval *Aedes aegypti***

Copper and nickel share similar atomic properties, such as a 2+ charge, atomic radii of 128 pm and 124 pm, respectively, and similar atomic weights of 63.55 and 58.70, respectively (Petrucci et al. 2002). However, they have different mechanisms of toxicity. Copper has been shown to be an ionoregulatory toxin in aquatic vertebrates (Li et al. 1996; Grosell et al. 2002) and some aquatic invertebrates (Grosell et al. 2002), while nickel has been shown to be a respiratory irritant in aquatic vertebrates and a suggested ionoregulatory disruptor in some aquatic invertebrates (Pane et al. 2003a,b).

The data in figures 26 and 27 suggests that copper and nickel may have different mechanisms of toxicity in *Aedes aegypti*, based on the dramatic difference in mortality between copper exposures (2.28 mmol L$^{-1}$ (95% C. I. 1.97, 2.67) unfed, 4.63 mmol L$^{-1}$ (95% C. I. 4.25, 5.05) fed) and nickel exposures (17.2 mmol L$^{-1}$ (95% C. I. 15.58, 18.66) fed, 27.2 mmol L$^{-1}$ (95% C. I. 25.49, 29.11) unfed). Despite their atomic similarities, different detoxification strategies may be employed by larval *Aedes aegypti* in order to increase their tolerance to such high levels of copper and nickel. Although an increase in waterborne copper or nickel exposure concentrations resulted in an increased probability of mortality (figures 26 and 27), the LC$_{50}$ values for copper and nickel exposure are different. Further, there is a difference between fed and unfed treatments. The LC$_{50}$ for the unfed copper treatment is 2.28 mmol L$^{-1}$ (95% C. I. 1.97, 2.67) and for the fed treatment is 4.63 mmol L$^{-1}$ (95% C. I. 4.25, 5.05), as determined by probit analysis (figure 26). The LC$_{50}$ for the unfed nickel treatment is 27.2 mmol L$^{-1}$ (95% C. I. 25.49, 29.11), an order of magnitude higher than that of the unfed copper treatment (figure 27).
Figure 26. The relationship between the waterborne copper concentration and the probability of mortality in 4th instar Aedes aegypti in fed (black) and unfed (red) conditions. The 24-hour LC₅₀ data were fit using a custom probit model designed in R-script (see Chapter 2). At each exposure concentration, biological replicates for both fed (+) and unfed (o) conditions are denoted individually. The 95 % C.I. for both fed (4.25 mmol L⁻¹, 5.05 mmol L⁻¹) and unfed (1.97 mmol L⁻¹, 2.67 mmol L⁻¹) conditions are indicated by the corresponding dashed lines. Data represents N=3–6 biological replicates of n=20 larvae per replicate.
Figure 27. The relationship between the waterborne nickel concentration and the probability of mortality in 4th instar *Aedes aegypti* in fed (black) and unfed (red) conditions. The 24-hour LC$_{50}$ data were fit using a custom probit model designed in R-script (see Chapter 2). At each exposure concentration, biological replicates for both fed (+) and unfed (o) conditions are denoted individually. The 95 % C.I. for both fed (15.58 mmol L$^{-1}$, 18.66 mmol L$^{-1}$) and unfed (25.49 mmol L$^{-1}$, 29.11 mmol L$^{-1}$) conditions are indicated by the corresponding dashed lines. Data represents N=3 biological replicates of n=20 larvae per replicate.
The LC₅₀ for the fed nickel treatment is 17.2 mmol L⁻¹ (95% C.I. 15.58, 18.66), 74 % higher than that of the fed copper treatment (figure 27). These results indicate that in 4th instar *Aedes aegypti* copper is more toxic than nickel. These results support trends in current literature, with copper being generally more toxic than nickel to other aquatic organisms (table 8). The unfed 24-hour LC₅₀ values (2.28 mmol L⁻¹ (95% C.I. 1.97, 2.67)) for copper in 4th instar *Aedes aegypti* (figure 26) are higher than values in the current literature (table 8). This can in part be explained by the fact that 3rd instar larva were used by Rayms-Keller et al. (1998), in determining the unfed 24-hour LC₅₀ value for copper, instead of 4th instar larva. Larva in the 3rd instar are smaller than 4th instar larva and are thus likely more sensitive to elevated levels of copper. Aside from LC₅₀ values for nickel in other aquatic organisms, to the best of the author’s knowledge, there are currently no published LC₅₀ values for waterborne nickel exposure in *Aedes aegypti* or other mosquito species.

The role of copper as an essential metal (Eisler 1997) may explain why *Aedes aegypti* appear to be more sensitive to copper than nickel. Copper’s role as an essential metal necessitates an efficient means of copper uptake from the environment; copper transporters have been identified in a number of taxa, including humans (Puig et al. 2002) and *Drosophila* (Norgate et al. 2006; Balamurugan et al. 2007). Copper taken from the environment can then be incorporated into enzymes, including cytochrome oxidase (Eisler 1997), and thus must be tightly regulated within an organism (Eisler 1997; Balamurugan et al. 2007). However, the existence of these transporters creates copper-specific routes of entry into an organism, and copper in excess disrupts normal enzyme function (Eisler 1997; Balamurugan et al. 2007).
Table 8. Comparison of copper and nickel LC$_{50}$ values across different taxa

<table>
<thead>
<tr>
<th>Organism</th>
<th>Duration (h)</th>
<th>Copper LC$_{50}$ (mmol L$^{-1}$)</th>
<th>Nickel LC$_{50}$ (mmol L$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fish</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rainbow trout, <em>Oncorhynchus mykiss</em>$^1$</td>
<td>48</td>
<td>1.18E-02</td>
<td>5.45E-01</td>
</tr>
<tr>
<td>Zebrafish, <em>Danio rerio</em>$^2$</td>
<td>96</td>
<td>1.84E-04</td>
<td>1.01E-02</td>
</tr>
<tr>
<td>Common carp, <em>Cyprinus carpio</em>$^3$</td>
<td>96</td>
<td>4.72E-03</td>
<td>2.62E-02</td>
</tr>
<tr>
<td><strong>Amphibians</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sunda toad, <em>Duttaphrynus melanostictus</em>$^4$</td>
<td>96</td>
<td>4.72E-04</td>
<td>6.82E-03</td>
</tr>
<tr>
<td><strong>Crustations</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Copepod, <em>Amphiascus tenuiremis</em>$^5$</td>
<td>96</td>
<td>1.96E-03</td>
<td>1.17E-02</td>
</tr>
<tr>
<td>Crayfish, <em>Cherax destructor</em> $^5$</td>
<td>96</td>
<td>7.77E-03</td>
<td>5.57E+00</td>
</tr>
<tr>
<td><strong>Insects</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Midge fly, <em>Chironomus riparius</em> $^7$</td>
<td>24</td>
<td>3.29E-02</td>
<td>4.26E-01</td>
</tr>
<tr>
<td>Mosquito, <em>Anopheles gambiae</em> $^8$</td>
<td>24</td>
<td>1.95E-04</td>
<td>------</td>
</tr>
<tr>
<td>Mosquito, <em>Aedes aegypti</em> $^8$</td>
<td>24</td>
<td>5.19E-01</td>
<td>------</td>
</tr>
<tr>
<td>Mosquito, <em>Aedes aegypti</em> $^{10}$</td>
<td>24</td>
<td>2.28</td>
<td>17.2</td>
</tr>
</tbody>
</table>

Notes: $^1$ Browne and Dalton 1970; $^2$ Alsop and Wood 2011; $^3$ Alam and Maughan 1992; $^4$ Shuhaimi-Othman et al. 2011; $^5$ Hagopian-Schlekat et al. 2001; $^6$ Khan and Nugegoda 2007; $^7$ Bechard et al. 2008; $^8$ Mireji et al. 2010a; $^9$ Rayms-Keller et al. 1998; $^{10}$ present study, values shown are from unfed exposures. ------ indicates no published values.
Turchot and Boitel (1992) found that copper disrupted the affinity of haemocyanin, a copper-containing respiratory pigment analogous to haemoglobin, for O$_2$ in the gills of shore crabs. *Aedes aegypti* larvae may be more permeable to copper, since there are copper specific transporters, than nickel because of its essentiality and thus more sensitive to copper. Figure 25 in Chapter 3 agrees with copper’s role as an essential metal, as copper was found in the haemolymph of larvae reared in ultrapure water, while nickel was not. Nickel’s role as an essential or non-essential element in *Aedes aegypti* has yet to be determined.

As indicated by the 95% C.I. in figures 26 and 27, there is a statistical difference in the LC$_{50}$ values between fed and unfed treatments, regardless of whether larvae were exposed to copper or nickel. In the case of the copper exposures, the larvae in the unfed treatments experienced a higher probability of mortality at lower concentrations than those in the fed treatments (figure 26). The opposite was true of the nickel treatments, where larvae in the fed treatment experienced a higher probability of mortality at lower concentrations than those in the fed treatments (figure 27).

In the case of copper, the unfed treatment was more toxic than the fed treatment. Copper is known to bind to organic matter in nature (Eisler 1997; Santos et al. 2008); hence, a possible explanation may be that the food binds Cu$^{2+}$ in the external media, thus reducing the concentration of Cu$^{2+}$ to which the larvae are exposed. To test this theory, ICP-OES was used to test the concentration of Cu$^{2+}$ (table 1) of the fed and unfed treatments, at the beginning and end of the experiment (table 9). It was found that the concentration of copper stayed the same within the unfed treatment from the beginning of the experiment to the end (table 9).
Table 9. The concentration of total waterborne copper (mmol L⁻¹) in both unfed and fed treatments, at the start of the exposure (0 h) and at the end of the exposure (24 h), as analysed by ICP-OES. The values represent an average of n=3 technical replicates ± 1 Stdev.

<table>
<thead>
<tr>
<th></th>
<th>Unfed</th>
<th></th>
<th>Fed</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0h</td>
<td>24h</td>
<td>0h</td>
<td>24h</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.243 ± 0.006</td>
<td>0.240 ± 0.000</td>
<td>0.250 ± 0.000</td>
<td>0.220 ± 0.010*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.490 ± 0.000</td>
<td>0.493 ± 0.006</td>
<td>0.490 ± 0.000</td>
<td>0.463 ± 0.006*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.990 ± 0.000</td>
<td>0.987 ± 0.006</td>
<td>0.980 ± 0.010</td>
<td>0.957 ± 0.012</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.503 ± 0.012</td>
<td>2.513 ± 0.006</td>
<td>2.487 ± 0.006</td>
<td>2.413 ± 0.038</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Indicates statistical significance (P<0.05) by two-tailed Student’s t-test.
In the fed treatment, the external concentration of waterborne copper decreased after 24h in the 0.25 mmol L\(^{-1}\) and 0.5 mmol L\(^{-1}\) treatments. The decrease in waterborne copper is slight but statistically significant, dropping from 0.25 mmol L\(^{-1}\) \(\pm\) 0.00 Stdev to 0.22 mmol L\(^{-1}\) \(\pm\) 0.01 Stdev and from 0.49 mmol L\(^{-1}\) \(\pm\) 0.00 Stdev to 0.46 mmol L\(^{-1}\) \(\pm\) 0.01 Stdev; in both cases a difference of 0.03 mmol L\(^{-1}\) is observed (table 9). Although a drop of 0.03 mmol L\(^{-1}\) in the concentration of waterborne copper over the course of the 24-hour exposure in the fed treatment may not seem significant, food concentrated in the gut of fed larvae is likely to bind ingested copper, thus reducing the bioavailability of copper (Santos et al. 2008; Heilaan et al. 2011) and its damaging effects on the PM (Rayms-Keller et al. 1998). This is likely the reason larvae in the fed treatments experienced a lower rate of mortality (figure 26). The PM, described earlier, is a site of metals toxicity in some aquatic arthropods (Beaty et al. 2002). Disruption of the PM exposes the delicate epithelium of the midgut to physical damage from food, infections, and potential toxins. The eventual damage to the midgut lining due to the absence of a PM leads to the disruption of homeostasis and death. Ramsy-Keller et al. (1998) found that *Aedes aegypti* larvae failed to produce a PM when exposed to copper and cadmium. The authors found that this effect was metal- and dose-dependent, and larvae exposed to mercury did not exhibit a disturbance in the PM. Studies on copper toxicity in *D. magna* demonstrate that *D. magna* given more food were more tolerant of copper than those given less food (Koivisto et al. 1992). The observed difference between fed and unfed copper treatments may be explained by the adverse effects of copper on the PM. Thus, the fed treatment may be less toxic because: (1) food acts as a physical barrier between ingested
copper and the PM and (2) concentrated food in the gut binds copper, reducing its bioavailability.

The results of the fed and unfed nickel treatments are perplexing because the fed treatment is more toxic than the unfed treatment (figure 27). Typically, decreasing the amount of food or not providing food increases toxicity in aquatic organisms (Heugens et al. 2001). A potential explanation may be that by ingesting food, larvae increase their intake of waterborne nickel; however, unlike freshwater fish (Pérrott et al. 1992), mosquito larvae have high drinking rates in the presence or absence of food—approximately 309 nL larva⁻¹ per hour for 4th instar Aedes aegypti larvae (Clements 1992). Further, it is believed that larvae take in almost no water when eating (Clements 1992). This implies that larvae are taking in similar amounts of waterborne nickel by drinking, whether they are in a fed or unfed treatment. The lack of nickel toxicity studies on other invertebrates (table 8) makes it difficult to speculate on a possible mechanism of toxicity without further investigation.

The difference between the copper treatments and the nickel treatments indicates that toxicity is both dose-dependent and metal-dependent, and that the mechanism of toxicity between the two metals is likely different. Nickel is known to competitively interact with Mg²⁺ in cells, and the addition of Mg²⁺ decreases its toxic effects (Eisler 1998). A study by Pane et al. (2003b) on D. magna found that nickel is an ionoregulatory toxin adversely affecting Mg²⁺ homeostasis and disrupting the function of DNA polymerase, which contains Mg²⁺ (Costa 1991). It is not known whether nickel disrupts Mg²⁺ homeostasis in Aedes aegypti or whether the PM is
the site of nickel toxicity. Histological studies on various midgut sections following nickel
exposure could highlight tissues of interest for further study.

**Acute copper exposure and determination of haemolymph copper loading capacity**

When unfed 4th instar larvae were exposed to increasing concentrations of copper (table
1), the haemolymph copper concentrations measured using LA-ICP-MS showed a significantly
increased copper concentration relative to the non-exposed control (figure 28). In the three
lowest concentrations of waterborne copper exposures (0.25 mmol L⁻¹, 0.5 mmol L⁻¹, 1 mmol L⁻¹),
which were lower than the LC₅₀ value of the unfed copper treatment (2.28 mmol L⁻¹ (95% C.
I. 1.97, 2.67) copper—see figure 27), the amount of copper in the haemolymph was significantly
different from that in the non-exposed controls (0 mmol L⁻¹ copper). However, the
haemolymph concentrations measured at these three lowest exposure concentrations were
not statistically different from each other, as determined by a Kruskal-Wallis test followed by a
Dunn’s multiple comparison test (figure 28). At the external waterborne copper concentration
of 5 mmol L⁻¹, which was nearly twice the LC₅₀ value of the unfed copper treatment (2.28 mmol
L⁻¹ (95% C. I. 1.97, 2.67) copper—see figure 26), the difference in haemolymph copper
concentration was statistically significant when compared to the haemolymph copper
concentrations determined at the four lowest waterborne copper exposure concentrations.

Figure 28 shows that copper enters the haemolymph from the external medium, likely
from some point in the alimentary canal or the anal papillae, since the cuticle is impermeable to
water (Gibbs 1998). Whether entry is via a copper-specific transporter, a generic metal
transporter, or competition with another divalent cation is unknown.
Figure 28. Concentration of accumulated Cu²⁺ in the haemolymph of 4th instar Aedes aegypti larvae after an acute 24-hour waterborne exposure to the treatments indicated above. Bars that do not share the same letter were significantly different according to a Kruskal-Wallis test followed by a Dunn’s multiple comparison test (*P<0.05). Data plotted as means ± 1 SEM (n=9–10). The LOQ for copper for this method is 0.001 mmol L⁻¹ ± 1.574x10⁻⁶ Stdev.
However, it is possible that copper enters the haemolymph via a copper-specific transporter, similar to the copper transporter Ctr1B found in *Drosophila* (Zhou et al. 2003), another dipteran insect.

Once inside the haemocoel, copper is likely bound to haemolymph proteins to protect against the conversion of oxygen to superoxide, which is highly toxic (Eisler 1998). Due to the production of reactive oxygen species by copper (Eisler 1998), exogenous copper in the haemolymph of shore crabs is almost entirely protein bound (Rtal et al. 1996). Sequestering copper by protein binding removes it from circulation and is an effective detoxification strategy employed by many animals (Rtal et al. 1996; Eisler 1998). The induction of metallothionein genes in *Drosophila* has been associated with exposure to high concentrations of copper and cadmium (Balamurugan et al. 2007). Recently, metallothionein and alpha-tubulin expression was reported in lab-reared copper- and cadmium-tolerant *Anopheles gambiae* (Mireji et al. 2010a). Thus, it is probable that *Aedes aegypti* produce metal-binding proteins like metallothionein and alpha-tubulin in response to copper exposure as a means of sequestering copper from freely circulating in the haemolymph.

When exposed to levels of copper in the range of 0.25 mmol L\(^{-1}\)–1 mmol L\(^{-1}\) (figure 28), *Aedes aegypti* larvae are able to maintain the levels of copper in their haemolymph at a consistent level, not differing statistically over this range as the external concentration of waterborne copper increases; this implies an ability to regulate copper. Expression of metallothioneins is an indication of metal-related stress and is a detoxification mechanism (Balamurugan et al. 2007); however, it is unlikely that this is the only mechanism of
detoxification employed. To keep the level of copper consistent over a range of increasing external copper concentrations, two possibilities exist: (1) copper is prevented from entering the haemolymph by being sequestered and accumulated in the cells of the midgut (Balamurugan et al. 2007) or (2) copper is exported via a copper exporter, similar to the one found in *Drosophila* (Norgate et al. 2006; Balamurugan et al. 2007).

Similar to *Drosophila*, it is likely that *Aedes aegypti* balance copper import, copper sequestration, and copper export to maintain homeostasis and avoid toxicity (Balamurugan et al. 2007). Examining the ratio of copper haemolymph concentration to that of the external copper concentration (figure 29) provides further evidence to suggest that *Aedes aegypti* larvae are able to regulate haemolymph copper concentrations. A ratio of haemolymph copper concentration <1 suggests defence against copper, a ratio = 1 implies equilibrium, and a ratio >1 implies copper is being concentrated and taken up from the environment. Defence against copper can be achieved by a number of detoxification strategies, such as export out of the haemolymph or body via copper-specific exporters (Norgate et al. 2006; Balamurugan et al. 2007), sequestration in other tissues by metal-binding proteins (Balamurugan et al. 2007; Mireji et al. 2010a), or reduced permeability of copper by downregulation of copper importers (Balamurugan et al. 2007).

In the lowest exposure treatments (0.25 mmol L\(^{-1}\) and 0.5 mmol L\(^{-1}\)), the ratio of copper haemolymph concentration to that of the external waterborne copper concentration (figure 29) is >1; although the ratios are not statistically different from 1, they support the hypothesis that copper is being actively taken up.
Figure 29. Acute 24-hour copper exposure, ratio of haemolymph copper concentration to waterborne (environmental) copper concentration ([Cu$^{2+}$]$_{hae}$/[Cu$^{2+}$]$_{Env}$) in 4$^{th}$ instar Aedes aegypti larvae. Horizontal line at ratio=1.0, passive diffusion;* indicates significantly different from a ratio of 1, as determined by one sample t-test (*P<0.05). Data plotted as means ± 1 SEM (n=9–10).
Drosophila fed a diet with low levels of copper increased their expression of Ctr1B, a copper-specific importer, in the posterior midgut (Balamurugan et al. 2007). Since larvae were not fed during these exposures, it is likely that they would exhibit high levels of expression for copper-specific importers to avoid copper deficiency. However, as the waterborne copper concentration increases to and past 1 mmol L\(^{-1}\), the ratio drops below 1, indicating an attempt to defend against copper by excluding it from the haemolymph.

In Drosophila, copper importers accumulate copper in the cells of the midgut for distribution throughout the body and in times of copper paucity (Balamurugan et al. 2007), while copper exporters send copper out of the midgut cells to avoid copper toxicity (Norgate et al. 2006; Balamurugan et al. 2007) and excess amounts of copper from entering the haemolymph. It is likely that copper enters the haemolymph of Aedes aegypti larvae via the midgut, as found in Drosophila (Norgate et al. 2006; Balamurugan et al. 2007), by drinking of the exposure water. During the acute waterborne copper exposure in the range of 0.25–1 mmol L\(^{-1}\) (figure 28), the concentration of copper in the haemolymph is consistent, likely because the majority of copper is both accumulated and exported out of the midgut cells in order to reduce copper toxicity. Expression of mucin in the midgut of Aedes aegypti larvae is associated with exposure to sublethal levels of copper and is likely one of a number of metal-binding proteins expressed by larvae to defend against copper (Beaty et al. 2002).

Beyond an external copper exposure of 1 mmol L\(^{-1}\), the amount of copper in the haemolymph jumps to roughly double the amount of haemolymph copper observed in the range of 0.25 mmol L\(^{-1}\)–1 mmol L\(^{-1}\) (figure 28). The sudden jump in haemolymph copper is
observed near the LC$_{50}$ (2.28 mmol L$^{-1}$ (95% C. I. 1.97, 2.67)), suggesting that past 1 mmol L$^{-1}$ waterborne copper exposure, the larval detoxification strategies are overcome and homeostasis is disrupted, leading to both an increase in haemolymph copper levels (figure 28) and a higher probability of mortality (figure 26).

**Chronic copper exposure and determination of haemolymph copper loading capacity**

As in the acute exposure (figure 28), haemolymph copper concentrations increased following exposure to waterborne copper. However, when unfed larvae were chronically exposed to 0.25 mmol L$^{-1}$ of copper over the course of 72 hours (figure 30), the accumulation of copper in the haemolymph measured resembles a bell-shaped curve, rather than a step-wise increase. Initially there is little difference between the amount of copper in the control and the 12-hour exposure. At 31 hours, the copper haemolymph levels peak, reaching 0.60 mmol L$^{-1}$ ± SEM 0.13, and by 48 hours, the copper haemolymph levels have dropped back down to figures that are not statistically different from those of the control and 12-hour exposure, as evaluated by a Kruskal-Wallis test followed by a Dunn’s multiple comparison test (figure 30).

In examining the ratio of copper haemolymph concentration to that of the external medium for the chronically exposed larvae (figure 31), it was found that the haemolymph to external medium copper ratio was <1 for 0 h, 12 h, 48 h, and 72 h, as indicated by a Student’s t-test. However, in the 24 h, 31 h, and 36 h treatments, the haemolymph to external medium copper ratio was above 1, and at 31 h the ratio was statistically higher than 1 (figure 31).
Figure 30. Concentration of Cu\textsuperscript{2+} in the haemolymph of unfed 4\textsuperscript{th} instar Aedes aegypti larvae after a low-dose chronic exposure to 0.25 mmol L\textsuperscript{-1} of Cu\textsuperscript{2+} over the course of 72 hours. Bars that do not share the same letter were significantly different according to a Kruskal-Wallis test followed by a Dunn’s multiple comparison test (*P<0.05). Data plotted as means ± 1 SEM (n=6–10). The LOQ for copper for this method is 0.001 mmol L\textsuperscript{-1} ± 1.574x10\textsuperscript{-6} Stdev.
Figure 31. Chronic waterborne copper exposure of 0.25 mmol L$^{-1}$; ratio of copper concentration to waterborne (environmental) copper concentration ([$Cu^{2+}]_{hae.}/[Cu^{2+}]_{Env.}$) in unfed 4th instar *Aedes aegypti* larvae. Horizontal line at ratio=1.0, passive diffusion; * indicates significantly different from a ratio of 1, as determined by a one sample t-test (*P<0.05). Data plotted as means ± 1 SEM (n=6–10).
A ratio above 1 indicates that the concentration of copper in the haemolymph is higher than the concentration of external waterborne copper. This means that larvae are concentrating copper in their haemolymph, against the concentration gradient, at 31 h. This is only possible by means of active transport, and further supports the argument that the initial accumulation of haemolymph copper may be due to the upregulation of copper importers like Ctr1B, found in Drosophila, during times of copper scarcity (Balamurugan et al. 2007).

The observed decrease in haemolymph copper concentration after 36 hours implies that mosquitoes have a copper-specific exporter, like the one found in Drosophila—DmATP7—which is upregulated in response to high levels of copper (Norgate et al. 2006; Balamurugan et al. 2007). A copper exporter must be present to move copper out of the haemolymph and across cell membranes, since any copper bound by proteins in the haemolymph would be measurable by the method described in Chapter 3. Another possible detoxification strategy, aside from a copper-specific exporter, may be to incorporate excess copper from the haemolymph into the developing cuticle. Copper is essential for cuticle sclerotisation (Terwilliger 1999), and thus it is possible that excess copper is incorporated into the developing cuticle as a means of excretion once the cuticle is shed during moulting. Future studies could measure the amount of copper present in the cuticle of control larvae versus the cuticle of larvae reared in copper-contaminated water, to determine whether excess copper is incorporated into the cuticle as a means of detoxification. The method described in Chapter 3 could be used to measure the amount of copper present in the cuticle.
Figure 32. Concentration of accumulated Ni$^{2+}$ in the haemolymph of 4$^{th}$ instar Aedes aegypti larvae after an acute 24-hour waterborne exposure to the treatments indicated above. One-way ANOVA (P<0.05) indicates means are statistically significant, and bars that do not share the same letter were significantly different, as indicated by the Tukey-Kramer method (*P<0.05). Data plotted as means ± 1 SEM (n=8–10). The LOQ for nickel for this method is 0.028 ± mmol L$^{-1}$ ± 7.971x10$^{-5}$ Stdev (table 7).
**Acute nickel exposure and determination of haemolymph nickel loading capacity**

During the acute unfed nickel exposures, larvae were exposed to the same concentrations of nickel as in the LC50 exposures (table 3). In unfed treatments the haemolymph nickel concentration increased as the external medium concentration increased (figure 32). Unlike in the acute copper treatments (figure 28), much more nickel was required in the external medium to see an increase in haemolymph nickel levels, coinciding with the much higher nickel LC50 values. For example, an external concentration of 10 mmol L\(^{-1}\) Ni\(^{2+}\) is required to increase the haemolymph nickel concentration to roughly 0.55 mmol L\(^{-1}\), while in the copper exposure only 0.25 mmol L\(^{-1}\) –1mmol L\(^{-1}\) of waterborne copper is required to increase the haemolymph copper concentration to roughly 0.5 mmol L\(^{-1}\).

Initially there is no statistical difference between the control and the 1 mmol L\(^{-1}\) and 10 mmol L\(^{-1}\) exposures. However, as the external medium concentration approaches that of the LC50 (27.2 mmol L\(^{-1}\) (95% C. I. 25.49, 29.11)) there is a sharp increase in the nickel haemolymph levels, as previously observed in the acute copper exposures (figure 28), implying that the organism’s ability to defend against nickel or copper is deteriorating. In the control exposure, as observed in figures 33 and 26 of Chapter 3, the amount of nickel present in the haemolymph was below the LOQ (table 7) for nickel using the method described in Chapter 3. This leads to three possible conclusions: (1) there may be trace levels of nickel, in the haemolymph or in other tissues, that are below the LOQ of the method used, (2) nickel is not an essential element for mosquito larvae and is therefore not present in their tissues in significant amounts and (3) a combination of the above two (1 and 2) conclusions.
High external concentrations of nickel are needed to elicit an internal response (figure 32), suggesting that there is no specific route of entry for nickel—i.e., a nickel-specific transporter. As mentioned previously, nickel’s role as an essential element has yet to be established in aquatic organisms (Pane et al. 2003b). This potential lack of essentiality would explain nickel’s absence from the haemolymph, further suggesting that there is no nickel-specific uptake transporter. The larva’s best defence against nickel toxicity may be that it is less permeable to nickel than to copper, and that its tissues are an effective physical barrier to sublethal waterborne nickel exposure (Gibbs 1998).

As larvae are exposed to increasing concentrations of waterborne nickel (figure 32), nickel appears above the LOQ. A study in Aedes albopictus cell lines, albopictus C6/36, found that cadmium accumulation in the cells was due mediated transport via a Ca\(^{2+}\) transporter and required no energy; it was further speculated that Cd\(^{2+}\) may also have entered the cells via an unidentified ionophore (Braekman et al. 1998). Thus, although a nickel-specific route of entry may not exist, it is still possible for nickel to enter the haemolymph in a similar manner via protein carriers when larvae are exposed to nickel. Once high levels of nickel are encountered internally, it is likely that the production of metallothionein and glutathione, another metal-binding protein, is induced to bind nickel and prevent it from freely circulating (Schlenk and Rice 1998; Canesi et al. 1999; Egli et al. 2006). As in the case of copper exposure, it is likely that cystine-rich metal-binding proteins play a key role in detoxification of nickel and other heavy metals (Schlenk and Rice 1998; Canesi et al. 1999).
Figure 3. Acute 24-hour nickel exposure; ratio of haemolymph nickel concentration to waterborne (environmental) nickel concentration ([Ni^{2+}]_{hae.}/[Ni^{2+}]_{Env.}) in 4^{th} instar Aedes aegypti larvae. Horizontal line at ratio=1.0, passive diffusion; * indicates significantly different from a ratio of 1, as determined by one sample t-test (*P<0.05). Data plotted as means ± 1 SEM (n=6–10).
Comparing the ratio of acute nickel haemolymph concentration to external nickel concentration (figure 33) with the ratios observed in the acute copper exposures (figure 29) provides further evidence in support of the argument that nickel is not essential. In the acute copper exposures (figures 28 and 29), the ratio of copper haemolymph concentration to external copper concentration increased in low sublethal exposures. In contrast, the ratios of the acute nickel haemolymph concentration to the external nickel concentration are well below a ratio of 1. This suggests that larvae do not have a specific transport capacity for nickel and thus prevent nickel from entering the haemolymph rather than concentrating nickel in their haemolymph, at least in sublethal exposures. The ratio of nickel haemolymph concentration to the external medium concentration remains very low throughout the acute exposure treatments from 1 mmol L\(^{-1}\) to 30 mmol L\(^{-1}\), implying a strong ability to passively defend against nickel.

**Chronic nickel exposure and determination of haemolymph nickel loading capacity**

When larvae were exposed to 1 mmol L\(^{-1}\) of nickel chronically, unfed, and over the course of 72 hours (figure 34), the accumulation of nickel in larval haemolymph was highly variable but not statistically different from that of the control as indicated by One-Way ANOVA, except for at 36 hours, and did not exceed 0.05 mmol L\(^{-1}\).
Figure 34. Concentration of accumulated Ni$^{2+}$ in the haemolymph of 4$^{th}$ instar Aedes aegypti larvae after a low-dose chronic waterborne exposure to 1 mmol L$^{-1}$ of Ni$^{2+}$ over the course of 72 hours. One-Way ANOVA indicates means are statistically significant (P<0.05). Bars that do not share the same letter were significantly different from the control, according to the Tukey-Kramer method (*P<0.05). The blue line represents the LOQ for nickel for this method (0.028 ± mmol L$^{-1}$ ± 7.971x10$^{-5}$ Stdev; see table 7). Data plotted as means ± 1 SEM (n=8–10).
In addition, the level of nickel in the haemolymph of control larvae is below the LOQ (table 7), and the error bars of the 36 hour exposure dip below the LOQ (table 7), putting into question whether any of the measured levels of nickel in the haemolymph during the chronic exposure are actual values measured or noise, and suggesting that larvae do not contain any nickel in their haemolymph and do not accumulate nickel during chronic exposure.

This is in contrast to the accumulation of copper in the haemolymph of chronically exposed larvae (figure 30), which exhibited a clear bell-shaped pattern, exceeding the concentration of the external medium and dropping back down below the concentration of the external medium (figure 30). This type of pattern is indicative of the upregulation and downregulation of copper-specific transporters (Balamurugan et al. 2007), underlining copper’s role as an essential metal. Unlike the chronic copper exposure, the accumulation of nickel in larval haemolymph was kept low (at control levels) throughout the 72 hour exposure, with a high degree of variability (figure 34), once again suggesting that larvae defend against nickel entering the haemolymph.

Examining the ratio of nickel haemolymph concentration to external nickel concentration (figure 35) reveals that the ratios of the chronically exposed larvae resemble those of the acutely exposed larvae (figure 32). The ratio of nickel haemolymph concentration to external nickel concentration does not approach 1 or surpass it, as seen in the copper chronically exposed larvae treatments (figure 31). Leonard et al. 2009 found that during sublethal levels of Cd$^{2+}$ exposure, the concentration of Cd$^{2+}$ in the external medium was the same as the concentration of Cd$^{2+}$ in the haemolymph of *Chironomus* larvae.
**Figure 35.** Chronic nickel exposure of 1 mmol L$^{-1}$ ratio of haemolymph [Ni$^{2+}$]/environmental [Ni$^{2+}$] in 4$^{th}$ instar *Aedes aegypti* larvae. Horizontal line at ratio=1.0, passive diffusion;* indicates significantly different from a ratio of 1, as determined by one sample t-test (*P<0.05). Data plotted as means ± 1 SEM (n=8–10).
The authors suggested that because the external and internal concentrations of Cd\(^{2+}\) were the same (a ratio of 1), the gut is not a complete physical barrier to Cd\(^{2+}\) at sublethal concentrations. Since the external and internal concentrations of Cd\(^{2+}\) were the same, passive diffusion of Cd\(^{2+}\) into the haemolymph was implied, mostly like through Ca\(^{2+}\) channels (Braekman et al. 1998). However, in the case of sublethal nickel exposure, the external concentration of nickel is much higher than the internal haemolymph concentration of nickel. This represents a ratio less than 1 (figure 14) and suggests that the larval tissues of *Aedes aegypti* may be an effective barrier to sublethal nickel exposure, unlike what was observed by Leonard et al. (2009) in the case of sublethal Cd\(^{2+}\) exposure in *Chironomus* larvae. Since it appears that nickel is not present in the haemolymph of control *Aedes aegypti* larvae and it is not actively taken up from the medium in the way that copper was during chronic sublethal exposure (figure 30), it is likely that nickel is not an essential metal for *Aedes aegypti* larvae.

**Biological Results Discussion**

This chapter explored and compared the toxicity of waterborne copper, an essential metal, and nickel, a metal of questioned essentiality, in 4\(^{th}\) instar larval *Aedes aegypti*. The 24-hour LC\(_{50}\) of each metal was determined in both fed and unfed conditions, with toxicity being metal and dose dependent. The 24-hour LC\(_{50}\) of copper in the unfed treatment was 2.28 mmol L\(^{-1}\) (95% C. I. 1.97, 2.67) and in the fed treatment was 4.63 mmol L\(^{-1}\) (95% C. I. 4.25, 5.05) (figure 26). The 24-hour LC\(_{50}\) of nickel in the unfed treatment was 17.2 mmol L\(^{-1}\) (95% C. I. 15.58, 18.66) and in the fed treatment was 27.2 mmol L\(^{-1}\) (95% C. I. 25.49, 29.11) (figure 27). Copper was found to be more toxic than nickel in either fed or unfed treatments. Interestingly, the fed
copper treatment was less toxic than the unfed treatment, while the reverse proved to be true of nickel, with the fed treatment being more toxic than the unfed treatment.

Following the determination of the 24-hour LC₅₀ values for both metals, a physiologically relevant range of toxicities was established for both metals, and the haemolymph metal loading capacity for both copper and nickel was established in acute and chronic exposures, using the method previously described in Chapter 3. Following the acute copper exposure there was an initial increase in haemolymph copper concentration, plateauing over a range of 0.25 mmol L⁻¹ –1 mmol L⁻¹ (figure 28). During the chronic copper exposure, copper was concentrated in the haemolymph at 36 hours and therefore actively taken up from the environment at an exposure of 0.25 mmol L⁻¹ (figure 30). Taken together along with the innate levels of copper present in control haemolymph (figures 25, 32, and 33), the data agrees with copper’s role as an essential metal (Eisler 1997).

Higher external waterborne concentrations of nickel (10 mmol L⁻¹) were needed to elicit an increase in haemolymph nickel concentration above that of the control, during an acute 24-hour exposure (figure 32). Past 15 mmol L⁻¹, a dramatic increase in nickel haemolymph concentration was seen, corresponding to the LC₅₀. Unlike for the copper exposures, nickel was not concentrated in the haemolymph. During the 1 mmol L⁻¹ chronic exposure there appeared to be no pattern of nickel accumulation in the haemolymph (figure 33). The nickel exposures imply that nickel is not an essential metal to larval Aedes aegypti, since unlike copper, it is not present in control haemolymph samples, and at no point do the larvae actively take up nickel from the external medium.
Chapter 5: General Conclusion

Insects exhibit high tolerances to heavy metals, including copper, nickel, cadmium, and lead, with LC$_{50}$ values typically orders of magnitude higher than values found in unpolluted environments (Brix et al. 2005; Buchwalter et al. 2007). Understanding the underlying mechanisms of mosquito tolerance to heavy metals requires the measurement of physiological parameters and is vital in developing new management strategies, as mosquito vectors of disease are becoming increasingly tolerant of metal pollutants (Mireji et al. 2008; Mireji et al. 2010a,b). However, the measurement of metals and other ions in physiological fluids, like haemolymph, of mosquitoes and other insects presents challenges due to the small volume of these samples (Beyenbach 2003).

The development of the small-volume LA-ICP-MS method described in Chapter 3 allows multi-elemental analysis of small-volume insect physiological fluids. This method takes into account the relationship between volume and surface area, the relationship between volume and concentration measured of a certified reference material (Seronorm), and identifies the region of analyte stability; taken together, the method provides sample analysis of small volume samples independent of volume. The ability to analyse samples independently of volume is an attractive feature of this method, since measuring small volumes accurately and consistently can be challenging and is a prerequisite of more established techniques such as fluid ICP-OES and ICP-MS.

In applying the methodology in Chapter 3, the toxicities and metal loading capacity of copper and nickel were compared in the haemolymph of larval Aedes aegypti. The innate levels
of copper present in control haemolymph (figures 25, 32, and 33) demonstrate copper’s role as an essential metal (Eisler 1997). In contrast, larvae did not appear to have innate, or detectable, levels of nickel present in their haemolymph, and nickel was only found in larval haemolymph following >10 mmol L\(^{-1}\) waterborne exposure. This suggests that although nickel is essential for a number of vertebrates, such as goats (Neilsen and Ollerich 1974; Mertz 1993), it may not be an essential element for larval *Aedes aegypti*.

LA-ICP-MS has proven to be a versatile methodology and is a promising new tool in biological investigations. Future areas of research may develop into imaging whole sections of larval mosquito organs to determine the localisation of metals at various stages of toxicity. Organs of interest to elucidate the pattern of metal distribution may include the MT, midgut, gastric caeca, anal papillae, and cuticles of both adult and larval stages of mosquitoes. Analysing the haemolymph of adult mosquitoes that have been previously exposed to metals as larvae, in comparison to the haemolymph of larvae exposed to the same concentrations, may indicate whether mosquitoes are able to shed copper or nickel burdens upon emerging as adults, by shedding their cuticle as a potential detoxification strategy. A further application of laser ablation may be used to uncover migration patterns of insects by ablating their cuticles and identifying the presence of trace elements, much like the method used in determining the migration patterns of birds, as demonstrated by Kaimal et al. (2009); the authors indentified 14 trace elements in the tail feathers of mallard ducks, and used this information to identify the birds’ origin and make inferences about the birds’ migration patterns. Similar studies have been done on the otoliths of fish to learn more about fish migration and ecology (Brophy et al. 2004).
Aside from applications in entomology and insect physiology, LA-ICP-MS has many biomedical applications. Another potential application of LA-ICP-MS is the detection of metal-containing pharmaceuticals (Lewen 2011), which would allow for pharmokinetic studies to be carried out using μL–nL of blood rather than mL. This could potentially allow pharmokinetic studies to be conducted on mice, rather than rabbits or dogs, reducing the cost of studies and increasing efficiency due to minimal sample preparation time.
Works Cited


Grosell, M., Blanchard, J., Brix, K. V. and Gerdes, R. (2007). Physiology is pivotal for interactions between salinity and acute copper toxicity to fish and invertebrates. Aquatic Toxicology 84, 162-172.


Nation, J. L. (2002). Insect Physiology and Biochemistry. *pg. 301-308, 312-318 CRC Press*


**Rogers, J. T., Richards, J. G. and Wood, C. M.** (2003). Ionoregulatory disruption as the acute toxic mechanism for lead in the rainbow trout (*Oncorhynchus mykiss*). *Aquatic Toxicology* 64, 215-234.


Appendices

Appendix A: Mosquito Rearing

Hatching

Strips of filter paper 6x1 inch wide with eggs (figure A1) are submerged under ultrapure water in a glass tray (figure A2). The tray is filled ¾ full and placed in an incubator at 27°C, 55% relative humidity, and on a 12h:12h light/dark cycle. Once the eggs are submerged, a pinch of yeast is added to the glass tray. The larvae should hatch within an hour; they are translucent and very difficult to see at this stage due to their small size. They prefer to hatch in water containing very little oxygen; yeast creates a low oxygen environment and also acts as a food source. Once larvae are hatched, a food mixture of crushed fish flakes and yeast is added to the tray; the mixture is composed of 1 part yeast to 3 parts TetraMin® Tropical Fish Flakes and is crushed using a mortar and pestle. Only a small amount of the food mixture is needed, as overfeeding will kill the larvae (see figure 2A: a light dusting of food is seen on the surface). Larvae are left in the hatching trays for a period of 2–3 days or until they are 2nd instar. At this point the larvae are clearly visible. The larvae have reached 2nd instar when they shed their cuticle; the cuticles are visible on the surface of the water as tiny black specks. During this 2–3 day period the water is not changed but the tray is topped up daily as water evaporates. During this time more food mixture may be added daily, but in very small amounts so as not to dirty the water.

![Figure A1](image1.png)

**Figure A1.** *Aedes aegypti* eggs on strips of filter paper in a plastic tray (A). Photograph of *Aedes aegypti* eggs under a microscope (B).
Rearing larvae
Once larvae are large enough (2\textsuperscript{nd} instar), they are transferred from the hatching tray to mosquito jars filled with ultrapure water. A small amount of the food mixture is added and the jars are placed in the incubator. The water is changed daily and the larvae are fed afterwards. Larvae will grow visibly every day until they reach 4\textsuperscript{th} instar and begin to pupate; this will first happen 6–7 days after hatching. Not all larvae grow at the same rate, therefore pupation will continue to occur after 7 days up to 14 days. Once larvae start to pupate, the pupae must be removed from the mosquito jars and placed into emerging jars daily (figure 4A).
Figure A4. An emerging jar (A) placed inside a mosquito cage (B). Adult mosquitoes emerge directly into the cage.

Figure A5. A solution of 10% sucrose (w/v) with a cotton ball protruding from the centre of a sealed Petri dish; the dish is sealed with parafilm to prevent evaporation. Adult mosquitoes will drink this solution from the cotton ball.

Rearing adult mosquitoes

Once the adults emerge into the cage, a few sugar feeder dishes should be provided (figure A6). The dishes should be checked daily to make sure that the dish has solution in it and that the cotton ball is not dried out. If the dishes are not maintained regularly, most of the colony’s males will die off, as they have few fat reserves; this will lead to poor egg yields. The dishes should be replaced every two days. A stock solution of sugar water is made weekly and autoclaved before use. Both male and female mosquitoes will feed off the sugar; however, females prefer to feed on blood and require a blood meal to produce eggs.
Blood feeding

The colonies are blood fed once weekly. The photos below depict how our mosquitoes are blood fed.

Figure A6. Blood feeding procedure; approximately 10 mL of blood is poured into the mosquito feeder and sealed with parafilm (A). The inverted feeder is left on the cage for approximately 3 hours to ensure that the blood reaches the right temperature (37°C) and that most of the females have fed. Water is circulated through the outside of the beaker via a peri-pump and heated with a water bath (B).
Figure A7. Happily fed female *Aedes aegypti*; female mosquitoes swell with blood after feeding.

**Storing eggs**

Once the colony has been fed it will be two days before the females start laying eggs. The females will lay eggs daily for up to four days. The egg-laying dish should be removed daily from the cage and the eggs should be collected and stored (figure A7). To collect the eggs, the strips of filter paper are removed, which should now have eggs directly stuck to them, and placed in a plastic tray. Once the eggs are in plastic trays they should be placed in a large Tupperware container inside the incubator (figure 8A). The Tupperware container should have a small amount of ultrapure water in it to increase the humidity inside the container. The water level in the Tupperware container must not wet any of the eggs in the trays. The lid of the container should be placed on the container but not sealed. All eggs collected are dated and cataloged when they are stored.

Figure A8. An egg-laying dish with strips of filter paper cut into 2-inch-wide strips. The strips are placed around the circumference of the jar and the jar is filled with 1 inch of ultrapure water.
Figure A9. Plastic trays with strips of filter paper are stored in a Tupperware container inside an incubator. The strips of filter paper are covered in mosquito eggs.
#Function to Find the Lethal Concentration

#Assumed that the Concentration is Log

#Function Returns the LC50 on Original Scale

lethal.conc<-function(obj,p)
{
   cf=c(1,2)
   eta <- family(obj)$linkfun(p)
   x.p <- (eta - coef(obj)[[1]])/coef(obj)[[2]]
   pd <- -cbind(1, x.p)/coef(obj)[[2]]
   SE <- sqrt(((pd %*% vcov(obj)[cf, cf]) * pd) %*% c(1, 1))
   CI<-x.p+c(-1,1)*2*SE
   list(Conc=x.p,ConcSE=SE,ConcCI=CI)}

#Read the Data to be used for Fitting

#Data is a comma delimited text file

d<-read.table('datamod.csv',header=T,sep=',')

#If data contains time variable.

d$subset(d,Time==24)

#Create the Data for the Average

dm<-rowsum(d,d$conc)

dm$conc=unique(d$conc)

#USE the same procedure below to fit the average

#fit to the averages

lconc<-log(dm$conc)

#Create the Responses for fitting, Dead and Alive

resp<-cbind(dm$ndead, dm$nalive)
#Fit the model using a Probit
pro.mod<-glm(resp~lconc,family=binomial(link=probit))

#Find the LC50
LC50<-lethal.conc(pro.mod,p=0.5)

#Transform LC50 back to Conc.Scale
Conc50<-exp(LC50$Conc)
Conc50
Conc50CI<-exp(LC50$ConcCI)
Conc50CI

#Create the Log-Concentrations
lconc<-log(d$conc)

#Create the Responses for fitting, Dead and Alive
resp<-cbind(d$ndead, d$nalive)

#Fit the model using a Probit
pro.mod<-glm(resp~lconc,family=binomial(link=probit))

#Find the LC50
LC50<-lethal.conc(pro.mod,p=0.5)

#Transform LC50 back to Conc.Scale
Conc50<-exp(LC50$Conc)
Conc50
Conc50CI<-exp(LC50$ConcCI)
Conc50CI

#Plot the Data on the Original Scale with Confidence Intervals
lp=seq((floor(10*min(lconc))-1)/10, (ceiling(10*max(lconc))+1)/10,0.01)

#Create the Observed Probabilities
obsprob <- d$ndead/(d$ndead+d$nalive)

lcp <- predict(pro.mod, data.frame(lconc=lp), se=TRUE, type="response")

# Plot the Fit on the Probability scale
plot(lp, lcp$fit, type='l', ylab="Probability", xlab="log concentration", main="fitted model with probit")

# Add the confidence limits
lines(lp, lcp$fit + 2*lcp$se.fit, type='l', lty=2)
lines(lp, lcp$fit - 2*lcp$se.fit, type='l', lty=2)

# Add points with the observations
points(lconc, obsprob)

mprob <- dm$ndead/(dm$ndead+dm$nalive)

points(log(dm$conc), mprob, pch=4)

# Fit the model using a Logit
log.mod <- glm(resp ~ lconc, family=binomial(link=logit))

# Find the LC50
LC50 <- lethal.conc(log.mod, p=0.5)

# Transform LC50 back to Conc. Scale
Conc50 <- exp(LC50$Conc)

Conc50CI <- exp(LC50$ConcCI)

lclp <- predict(log.mod, data.frame(lconc=lp), se=TRUE, type="response")

plot(lp, lclp$fit, type='l', ylab="Probability", xlab="log concentration", main="fitted model with logit")

lines(lp, lclp$fit + 2*lclp$se.fit, type='l', lty=2)
lines(lp, lclp$fit - 2*lclp$se.fit, type='l', lty=2)

points(lconc, obsprob)
# Compare Probit and Logit

plot(lp,lcp$fit,type='l',ylab="Probability",xlab="log concentration",main="Compare Fitted Models")

lines(lp,lclp$fit,lty=2)
Appendix C: LA-ICP-MS Calibration Curves

Seronorm volume vs. volume – July 29, 2011, quantified copper in Seronorm ablations are the calibration curves in figures 24 and 25

Acute copper and nickel exposure-Sept 14, 2010

**Figure C1.** LA-ICP-MS calibration curves for an acute copper exposure; the instrument is calibrated for both copper and nickel. Calibration curves are used to determine the amount of copper in the haemolymph of 4th instar *Aedes aegypti* after an acute 24 h waterborne copper exposure.
Figure C2. LA-ICP-MS calibration curves for an acute copper exposure; the instrument is calibrated for both copper and nickel. Calibration curves are used to determine the amount of copper in the haemolymph of 4th instar *Aedes aegypti* after an acute 24 h waterborne copper exposure.
Chronic copper exposure-Jan 11, 2011

**Figure C3.** LA-ICP-MS calibration curves for a chronic copper exposure; the instrument is calibrated for both copper and nickel. Calibration curves are used to determine the amount of copper in the haemolymph of 4th instar *Aedes aegypti* following chronic exposure to waterborne copper at 0 h, 12 h, 24 h, 31 h, 36 h, 48 h, and 72 h.

For copper:
- Equation: $y = 0.0029x - 0.0019$
- $R^2 = 0.9993$

For nickel:
- Equation: $y = 0.001x - 0.0017$
- $R^2 = 0.9915$
**Figure C4.** LA-ICP-MS calibration curves for an acute nickel exposure; the instrument is calibrated for both copper and nickel. Calibration curves are used to determine the amount of nickel in the haemolymph of 4th instar *Aedes aegypti* after an acute 24 h waterborne nickel exposure.
Chronic nickel exposure- Feb 3, 2011

Figure C5. LA-ICP-MS calibration curves for a chronic nickel exposure; the instrument is calibrated for both copper and nickel. Calibration curves are used to determine the amount of copper in the haemolymph of 4th instar Aedes aegypti following chronic exposure to waterborne nickel at 0 h, 12 h, 24 h, 31h, 36h, 48 h, and 72 h.
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Figure 8. Becker et al. 2010

**Title:** Bioimaging of Metals and Biomolecules in Mouse Heart by Laser Ablation Inductively Coupled Plasma Mass Spectrometry and Secondary Ion Mass Spectrometry

**Author:** J. Sabine Becker, Uwe Breuer, Hui-Fang Haigh, Tobias Osterholt, Usarat Kumtabtim, Bei Wu, Andreas Matusch, Joseph A. Caruso, and Zhenyu Qin

**Publication:** Analytical Chemistry

**Publisher:** American Chemical Society

**Date:** Nov 1, 2010

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Figure 1. Malavige et al. 2004; Figure 5. Jacobs Edwards; Figure 7. Glish and Vachet 2003; Figure 11. Sinclair et al. 1998.
Figure 2. Photographs by James Gathany, CDC, 2006

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On 1 April 2012 00:15, LILY Kotzev <kotzev@hotmail.com> wrote:

Hello,

My name is [redacted]. I am in the process of writing my thesis on Aedes aegypti. I would like to ask permission to use a photograph taken by you on your website. The photographs depict fourth instar aedes larva and pupa at the surface of the water on a greenish blue background I would like to use the photo in my thesis as a visual representation of the aedes aegypti life stages. If given permission to include the photo I will cite you as the photographer and thank you in the acknowledgements.

Cheers