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Effects of Multiple Heavy Metals on Estuarine Communities

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A thesis submitted in partial fulfilment of the requirements for
the degree of Doctor of Philosophy in Marine Science,
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Frontispiece



The rolling mill and barrels that were used to produce metal-spiked sediments.

Abstract

Aquatic ecosystems are threatened by various natural and anthropogenic stressors. Their simultaneous effects can result in additive or complex non-additive effects. In the region of Auckland, New Zealand, the primary sediment contaminants of concern include stormwater-derived copper (Cu), lead (Pb) and zinc (Zn). The simultaneous effects of these metals on estuarine infauna were assessed through field and laboratory experiments. In the field, Cu, Zn and a mixture of Cu, Pb and Zn negatively affected the colonisation of infauna. In the laboratory, the survival rate of the deposit feeding bivalve *Macomona liliana* was reduced by Cu and Zn, and the combined effects of these metals were cumulative. The effects of Pb, however, were not evident.

A second field experiment explored the nature of the simultaneous effects of Cu and Zn on colonisation. Results depended on the particular response variable being examined. Additive effects were detected for the mean log abundances of the polychaetes *Prionospio* sp. and *Scoloplos cylindifer*, for species richness and for the multivariate response of the community as a whole. In contrast, antagonistic effects were detected for the mean log abundances of total infauna and the polychaete *Heteromastus* sp.

Sub-lethal impacts can result in longer-term effects on benthic communities, including through bioaccumulation of metals. A second laboratory experiment measured bioaccumulation of heavy metals in the bivalves, *M. liliana* and *Austrovenus stutchburyi*. Both species accumulated Pb and Zn, but bioaccumulation of Cu was slight in *A. stutchburyi* and not evident at all in *M. liliana*. The presence of Pb increased the bioavailability of Cu and/or Zn and, therefore, uptake of these metals by the bivalves in some cases.

These results clearly showed direct negative and cumulative effects of Cu and Zn and potential indirect effects of Pb on estuarine infauna, highlighting the importance of considering the co-occurrence of multiple metals when assessing their ecological impacts. Manipulative field experiments need to be combined with laboratory ecotoxicological studies in order to unravel the combined and interactive effects of multiple metals so that their potential impacts on estuarine communities may be accurately modelled and predicted.

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Chapter One

General Introduction

Background

An estuary is a complex and variable environment that is characterised by changes in salinity, tide, current and sediment (McLusky and Elliott 2004). Estuaries support abundant and unique communities of plants and animals (Little 2000), and they are ecologically important for their role in making land-derived carbon sources available to aquatic organisms (Wilson 2002), for providing habitats for small invertebrates such as snails, bivalves and polychaetes (Little 2000) and for providing feeding, breeding and nursery grounds for fish and birds (McLusky 1999). Estuaries also have economic importance, providing sheltered areas for port and harbour facilities, industrial processes requiring water cooling and aquaculture (Wilson 1988, McLusky 1999, Wilson 2002).

During the industrial revolution, estuaries received a large amount of waste disposal, and the present condition of estuaries in developed countries in Europe is a direct result of economic pressures over the past 200 years (McLusky 1999). Industrial activities involving highly polluting processes (e.g. candle factories, paper production and leather manufacture) and discharge of sewage effluent introduced a large amount of organic matter and nutrients into estuaries, which resulted in decreases in the number of benthic fauna, benthic biomass and diversity (Unnithan et al. 1975, Nicholls et al. 1981, Billen et al. 1999). Disposal of sewage sludge introduces not only organic components but also metals and polychlorinated biphenyls (PCBs) when accompanied with an industrial waste component (MacKay 1986). Runoff from residential and light-industrial areas transports

heavy metals, pesticides, pathogens and litter into estuaries (Laws et al. 1994).

New Zealand has a relatively small population (approximately 4.3 million), and estuaries in New Zealand are relatively unmodified by engineering works compared to many other industrialised countries (Hume 2003). While environmental problems in New Zealand aquatic environments may not be as severe as those in many overseas locations, accelerated sediment runoff due to land clearance is one of the most widespread changes brought by anthropogenic activities in New Zealand (Hume 2003). In the region of Auckland, effects of polluted stormwater runoff are of particular concern due to its relatively large population (approximately 1.3 million) and the discharge of its stormwater into sheltered harbours and estuaries (ARC 2004). In these environments, pollutants tend to become incorporated in sediments (ARC 2004). Polluted stormwater runoff contains contaminants such as heavy metals, hydrocarbons and toxic exhaust emissions. The primary sediment contaminants of concern in this region include copper (Cu), lead (Pb) and zinc (Zn) (ARC 2004).

The Auckland Regional Council developed the environmental response criteria (ERC) for sediment contaminants based on various environmental guidelines in other parts of the world and classified sediment metal concentrations into three levels: green, amber and red, in order of increasing contamination levels (ARC 2004). According to this classification, concentrations in the green zone ($Zn < 124 \mu\text{g g}^{-1}$, $Cu < 19 \mu\text{g g}^{-1}$, $Pb < 30 \mu\text{g g}^{-1}$) present a low risk to estuarine organisms, while those in the red zone ($Zn > 150 \mu\text{g g}^{-1}$, $Cu > 34 \mu\text{g g}^{-1}$, $Pb > 50 \mu\text{g g}^{-1}$) indicate that the biota at the sites are probably impacted. Although metal concentrations in estuaries in New Zealand are relatively low compared to those measured overseas (Table 1.1; Bryan and Langston 1992, Marsden et al. 2003, França et al. 2005, Kelly 2007), estuaries in the central Auckland area tend to have metal concentrations that exceed the ERC red zone values (Kelly 2007). Concentrations

of Cu, Pb and Zn in sediments also tend to be positively correlated with one another spatially across the Auckland region (Hewitt et al. 2009). Ecological responses of estuarine infauna have been detected at relatively low metal concentrations in field studies in this region (Thrush et al. 2008, Hewitt et al. 2009).

Table 1.1. List of concentrations of heavy metals in sediment recorded from estuaries in New Zealand and Europe.

Location	Cu ($\mu\text{g g}^{-1}$)	Pb ($\mu\text{g g}^{-1}$)	Zn ($\mu\text{g g}^{-1}$)	References
Orewa estuary Auckland, New Zealand	2.6 - 4.2	3.0 - 4.6	21.0 - 32.9	Kelly, 2007
Waitemata harbour Auckland, New Zealand	2.6 - 42.9	6.3 - 86.1	24.9 - 264.0	Kelly, 2007
Avon-Heathcote estuary Christchurch, New Zealand	8 - 78	-	29 - 87	Marsden et al., 2003
Tamar estuary, UK	330	235	452	Bryan & Langston, 1992
Fal estuary, UK	648	150	750	Bryan & Langston, 1992
Tagus estuary, Portugal	28 - 89	65 - 127	168 - 427	França et al. 2005

Bioavailability of heavy metals

The bioavailability of sediment-associated metals is controlled by the metal binding to and releasing from the sediment particles (Chapman and Long 1983). The most bioavailable form of metals in sediments is generally considered to be free metal ions in sediment porewater, while metals that are bound to the sediments may not be toxic to organisms (Chapman and Long 1983). Metal ions also attach to the surface of suspended particulate matter (SPM) in the water column and the particulate-bound metals can settle in sediments through flocculation (Webster-Brown 2005). In aerobic sediments, iron (Fe) and manganese (Mn) (hydr)oxide are the major metal-binding surfaces, while Fe and Mn

monosulfides, which are termed acid-volatile sulfides (AVS), determine the bioavailability of metals in anaerobic sediment (Chapman et al. 1998). Acid-volatile sulfides are unstable and when other trace metals are present, the metals displace Fe and Mn to form metal sulfides. The metals are not likely to be toxic if AVS are in excess. Particulate organic carbon also has a strong binding affinity for metals in aerobic sediments, and can also be important in anaerobic sediments when the concentration of trace metals exceed the amount of AVS binding sites available (Chapman et al. 1998).

The surface of Fe hydroxide consists of several distinct types of adsorption sites that have varying affinities for metal ions (Benjamin and Leckie 1981b). Thus, metal ions would preferentially occupy the strongest binding sites, and as those sites become limiting, metal ions would be forced to bind to sites of lower interaction energy. The preferred adsorption sites for Cu, Pb, Zn and cadmium (Cd) have been shown to be distinct from one another on the surface of Fe hydroxide, and competition for the same sites, when these metals simultaneously exist, has been shown to be minimal (Benjamin and Leckie 1981a). Competitive adsorption of heavy metals has been detected, however, on Mn oxide, for which the presence of Pb considerably decreased the adsorption of Zn and Cd (Gadde and Laitinen 1974). Different heavy metals have different binding affinities for the surfaces, and Pb generally has a stronger binding affinity than Cu, Zn or Cd (Gadde and Laitinen 1974, Guy and Chakrabarti 1976, Benjamin and Leckie 1981a).

Metal adsorption is highly pH dependent. For cationic metals (e.g. Cu, Pb, Zn, Cd), adsorption increases with increasing pH and the increase generally occurs rapidly over a narrow range of pH, known as an adsorption edge (Hatje et al. 2003, Webster-Brown 2005). A shift in an adsorption edge to more alkaline pH may occur for some cationic trace metals with increasing salinity (Millward and Moore 1982, Hatje et al. 2003). The effects of salinity on the adsorption of heavy metals are mainly due to increased metal

complexation with seawater anions (Cl^- and SO_4^{2-}) and increased competition for binding sites with seawater divalent cations (Ca^{2+} and Mg^{2+}), both resulting in desorption of heavy metals (Millward and Moore 1982, Hatje et al. 2003, Riba et al. 2004). Metal adsorption and desorption are, therefore, greatly influenced by different environmental conditions, and the behaviour of multiple heavy metals and their bioavailability, especially in estuarine conditions, can be difficult to predict.

Accumulation and toxicity of heavy metals

Heavy metals are divided into two types: essential metals such as Cu and Zn that play an essential role in metabolism and are therefore required by organisms (up to a certain amount), and non-essential metals including Pb and Cd that organisms do not require (Rainbow 2002). Aquatic organisms take up both types of heavy metals by feeding, respiration and/or absorption onto the body surface, and can store these metals in various body parts (Denton and Burdon-Jones 1981, Vigh et al. 1996). Organisms have to either detoxify or excrete excess heavy metals to avoid potential toxic effects. When the rate of metal uptake exceeds the combined rate of detoxification and excretion, concentrations of metabolically available metals increase, and toxic effects may occur (Rainbow 2002).

Different organisms employ different strategies to regulate concentrations of heavy metals (Phillips and Rainbow 1989). Regulators (e.g. for Cu and Zn in decapods) are able to maintain tissue concentrations of metals within specific ranges, over a broad range of ambient concentrations through excretion of metals, while non-regulators (e.g. for Zn in barnacles) instead accumulate and store all metals in a detoxified form without excretion (Phillips and Rainbow 1989, Rainbow 2002). Partial regulators do accumulate heavy metals, but maintain their concentrations in relatively narrow ranges through

detoxification and excretion (Phillips and Rainbow 1989). Such partial regulation of Zn has been found in mussels (Phillips 1985, Chan 1988). For non-essential metals, accumulation in detoxified forms with either some or no excretion are the predominant strategies, and concentrations of non-essential metals in organisms generally increase with increasing ambient concentrations (Rainbow 2002, Vijver et al. 2004).

Bioaccumulation of heavy metals differs among different organisms and for different metals, and is also affected by changes in temperature and salinity (Denton and Burdon-Jones 1981, Blackmore 2001). Changes in these environmental conditions are likely to modify metabolic activities and, in turn, rates of metal uptake of organisms. Changes in salinity may affect the chemical form and chemical interactions of metals (Denton and Burdon-Jones 1981). Bioaccumulation and biological effects of heavy metals are, therefore, affected by the particular forms of metals, environmental conditions and physiological mechanisms of metal uptake, storage, metabolism and detoxification. This means that generalisations about the toxicity of metals for different organisms living in different environments can rarely be made.

Individual-level response to metals

The effects of metal contaminants begin with subtle physiological alterations to individual organisms. Such physiological responses measured in individual organisms are often called biomarkers; these are defined as “any biological response to an environmental chemical at the below-individual level, measured inside an organism or in its products, indicating a departure from the normal status, that cannot be detected from the intact organism” (Van Gestel and Van Brummelen 1996). DNA base modification is an example of a biomarker, and a positive correlation between concentrations of bioaccumulated Pb and levels of DNA base modification has been found in the oyster,

Saccostrea commercialis (Avery et al. 1996). Free radical oxidation reactions are known to mediate DNA damage arising from chemical agents in environments, and this correlation may have been the result of Pb-mediated free radical formation. Lipid peroxidation has also been shown to increase in the mussel, *Mytilus galloprovincialis*, when exposed to heavy metals, including Cu and Pb, perhaps through the production of reactive oxidative species associated with oxidative damage to membrane lipids (Vlahogianni and Valavanidis 2007).

Emergence of infaunal organisms from metal-contaminated sediments reduces their exposure to the metals but increases their susceptibility to predation, and is regarded as a sublethal behavioural effect. Such changes in the behaviour of organisms can be measured by determining their EC₅₀ values, which are the concentrations of contaminant in which the behavioural changes are observed in 50% of tested individuals. Emergence and reburial behaviour have been examined using estuarine amphipods, polychaetes and bivalves (Roper and Hickey 1994, Roper et al. 1995, Bat and Raffaelli 1998). The estuarine bivalve, *Macomona liliana*, has been shown to crawl away from sediments contaminated with Cu or Zn, indicating that some organisms might be capable of actively avoiding contaminated sediments (Roper et al. 1995).

Mortality of organisms is the most obvious endpoint of a negative physiological response to metal toxicity. Numerous toxicity tests have been done to determine LC₅₀ values (concentrations of contaminants resulting in 50% mortality of tested organisms) in response to heavy metals for estuarine organisms such as amphipods (Bat and Raffaelli 1998, Gale et al. 2006), polychaetes and bivalves (King et al. 2004). Amphipod crustaceans have been used widely for sediment toxicity testing because of their short life cycle and suitability for laboratory experiments (Marsden and Rainbow 2004). The results of these toxicity tests have shown that physiological responses vary greatly among species

and for different metals.

Metal toxicities can also differentially affect organisms at various stages of development. Various developmental abnormalities (e.g. to eyes, body pigments and body morphology) have been found in hatched larvae of the estuarine crab, *Chasmagnathus granulatus*, exposed to dissolved Cu, Zn and Pb at concentrations that did not cause any apparent lethal effects on adult females (Lavolpe et al. 2004). Different metals also affected embryos of *C. granulatus* at different stages: Pb during early embryogenesis, and Cu and Zn during late embryogenesis (Lavolpe et al. 2004). The toxic effects of Zn have also been shown to cause abnormalities in the shells of larvae of the red abalone, *Haliotis rufescens*, and also to produce significantly fewer metamorphosed larvae (Hunt and Anderson 1989).

The toxic effects of heavy metals have been shown to interact with other environmental stressors (Alutoin et al. 2001, Heugens et al. 2001, Lenihan et al. 2003). For example, the hermatypic coral, *Porites lutea*, showed a decrease in primary production when exposed to either aqueous Cu or reduced salinity separately, but their primary production was not affected when they were simultaneously exposed to both an aqueous Cu and reduced salinity, indicating an antagonistic effect of these two stressors (Alutoin et al. 2001). Decreases in the level of food have been shown to result in increased metal toxicity in most cases, probably because organisms with poor food availability could not meet the extra energy costs required to cope with the toxic effects of metals (Heugens et al. 2001). Adverse effects on body length and reproduction have been detected, however, for the cladoceran, *Daphnia magna*, in the presence of elevated Cd when food was plentiful, perhaps due to an elevated metabolic rate resulting in increased metal uptake (Klüttgen and Ratte 1994).

Community-level response to metals

The presence of metal contaminants can affect faunal communities directly by changing the relative abundances of organisms, or indirectly through effects on trophic interactions. Responses of organisms to contaminants are less predictable for higher trophic levels. Faunal communities show considerable temporal and taxonomic variations in their responses, depending on environmental and biological conditions (Ward and Young 1982, Breitburg et al. 1999b). Nevertheless, high concentrations of metals in sediments are generally associated with reductions in abundance, species richness and species diversity. For example, Rygg (1985) found a strong negative correlation between benthic faunal species diversity and sediment concentrations of Cu in data from Norwegian fjords. Moderate and weak negative correlations were also detected for concentrations of Pb and Zn (Rygg 1985). Lande (1977) found that abundance and species richness of benthic fauna were low in Orkdalsfjorden in Norway where high concentrations of Cu and Zn were found in sediments, due to effluents from mining industries, compared to surrounding non-polluted areas. However, some species in Orkdalsfjorden were found in relatively high abundances (e.g. the polychaetes *Heteromastus filiformis* and *Cirratulus cirratus*), suggesting their tolerance to high metal concentrations (Lande 1977). These tolerant species that flourish in polluted areas where other less-tolerant species are unable to live can be used as indicators of pollution (Lande 1977, Hickey and Clements 1998). The displacement of sensitive species by tolerant species may result in reductions in biodiversity (Pearson and Rosenberg 1978) and may affect ecosystem processes, especially when functional traits of species that are lost are not replaced by other organisms in the system (Raffaelli et al. 2003, Covich et al. 2004).

Multiple stressors

Infaunal organisms are important components of estuarine ecosystems due to their role in the food chain, obtaining carbon energy from detritus or suspended materials in a water column and passing it to higher trophic levels (Coull 1999, Wilson 2002). Thus, examining potential effects of metal contaminants on estuarine infaunal communities can provide information that is vital in the assessment of estuarine ecosystem health. Previous investigations of the effects of heavy metals on estuarine infauna have mostly focused on elucidating the individual effects of heavy metals by examining only one metal at a time (Roper and Hickey 1994, Roper et al. 1995, Morrissey et al. 1996, Bat and Raffaelli 1998, Marsden and Wong 2001). The simultaneous and interactive effects of multiple metals, on the other hand, have not been well examined through experimental manipulations (Hagopian-Schlekat et al. 2001, Lindegarth and Underwood 2002).

Folt et al. (1999) provided a modelling framework for assessing multiple stressors, categorising the simultaneous effects of multiple stressors into three possible models: additive, multiplicative, and simple comparative effects, in which the combined effect of multiple stressors is equal to the effect of a single dominant stressor (see Chapter 4 for details). Previous investigations of the simultaneous effects of multiple anthropogenic stressors have shown that interactions among multiple stressors may generate complex non-additive effects that cannot be predicted based on the effects of individual stressors alone (Christensen et al. 2006, Crain et al. 2008). The simultaneous effects of multiple heavy metals and their interactions, therefore, need to be examined; such an investigation would be meaningful and highly relevant to real field situations, where multiple heavy metals simultaneously exist.

Methodological issues

Research in estuarine ecology was generally dominated by descriptions of the distribution and abundance of organisms until the 1960s and 1970s (Woodin 1999). Numerous previous investigations of the potential impacts of pollutants and contaminants have used such an approach of analysing the distribution and relative abundances of organisms along a pollution gradient, or by contrasting fauna living in polluted *versus* unpolluted areas (Pearson and Rosenberg 1978, Rygg 1985, Somerfield et al. 1994, Warwick 2001, Hewitt et al. 2009). These investigations generally rely on the idea that increases in concentrations of heavy metals reduce infaunal abundances, species richness and species diversity, potentially revealing correlative relationships between degrees of metal contamination and changes in these univariate measures or in the community structure as a whole in multivariate analyses. However, the presence or absence of certain organisms and documented changes in community structure can be due to fluctuations in abiotic and/or biotic factors other than the contaminants of concern (Chapman and Long 1983, Hickey and Clements 1998).

Manipulative field experiments have become more common in recent years, as they allow researchers to elucidate causal relationships between the introduction of contaminants and specific changes to faunal communities in the field (Morrisey et al. 1996, Lindegarth and Underwood 2002, Lenihan et al. 2003). Manipulative experiments assessing the effects of heavy metals generally use sediments artificially spiked with specific metals to specific target concentrations (Lenihan et al. 2003, Trannum et al. 2004). The use of metal-spiked sediments in field experiments, as well as in the laboratory, may require some caution when results are interpreted, as spiked sediments may not behave geochemically in the same way as sediments naturally contaminated over a long period of time (Simpson et al. 2004, Hutchins et al. 2009).

Various laboratory bioassays have also used aquatic organisms to examine the effects of heavy metals (Bat and Raffaelli 1998, King et al. 2004, Gale et al. 2006). Laboratory bioassays that test either lethal or sublethal effects on a single organism at a time generally cannot be used to predict indirect or community-level effects, but they can clearly identify direct effects of contaminants on target species in isolation (Taub 1997). Life table response experiments use particular organisms at different life stages in order to assess the effects of contaminants on various life history parameters and thus allow researchers to evaluate population-level effects (Levin et al. 1996). Bioassays are also a useful means to examine rigorously the effects of multiple metals, potentially unravelling interactions among different metals and explicitly identifying the effect of each metal on target species. Such detailed investigations may not be possible in the field, as large-scale manipulative field experiments can be very labour intensive and large numbers of factors and treatments may simply not be feasible for practical reasons.

Chapman and Long (1983) listed three components required in pollution studies: chemical surveys to determine contaminant concentrations, ecological surveys to measure community structure and presence or absence of certain species, and bioassays, including lethal and sublethal tests, to provide a strong link between the presence of contaminants and effects on fauna. In order to examine the simultaneous effects of multiple heavy metals and to unravel their effects in the field, manipulative field experiments should also be included in pollution studies. The use of multiple approaches is vital for researchers to understand clearly how different metals, individually and collectively, affect ecological communities in variable estuarine environments.

Aims and scope of thesis

This thesis aims to investigate the simultaneous effects of the multiple metal

contaminants, Cu, Pb and Zn, on estuarine infaunal organisms and communities in the region of Auckland, New Zealand. These three metals were specifically chosen as they are the primary sediment contaminants of concern in this region. All studies in this thesis manipulated concentrations of Cu, Pb and/or Zn by spiking sediments with these metals, allowing experimental examinations of their effects and therefore establishing clear causal relationships between the presence of these metals and observed responses of infauna. All spiking methodologies followed the method of Lu et al. (2008; see Chapter 3 for a brief summary of the method).

Previous investigations in the field (e.g. Rygg 1985, Warwick 2001, Calabretta and Oviatt 2008) have revealed correlative relationships between levels of heavy metals and infaunal responses, concluding negative effects of metals. Previous investigations in the laboratory (e.g. Bat and Raffaelli 1998, King et al. 2004) have also shown negative impacts of individual heavy metals on specific test organisms. This thesis focuses on identifying causal effects of heavy metals, individually and in combination, on infauna both in the field and the laboratory, and also investigates the contributions of each metal in the combined effects of and interactions amongst these metals.

Thesis outline

This thesis describes two manipulative field experiments (Chapters 2 and 4) and two laboratory experiments (Chapters 3 and 5) designed to examine the effects of Cu, Pb and Zn on estuarine infaunal organisms. Prior to these experiments, an infaunal and environmental survey was done at an intertidal mudflat in Orewa estuary, Auckland, in order to assess its suitability for the field experiments, to choose appropriate infaunal organisms for the laboratory experiments, and to source sediments required for metal-spiking. Appendix A discusses the rationale for these assessments and the results of this

preliminary survey.

The two chapters describing field experiments investigate the effects of heavy metals on estuarine infaunal communities by examining recolonisation of metal-spiked, defaunated sediments by estuarine infauna. Chapter 2 focuses on the effects of Cu, Pb and Zn, individually and in a mixture, using concentrations of these metals as high as would be realistic for Auckland's estuaries given predictions of potential inputs expected over the next 100 years. Chapter 4 examines the nature of the combined effects of Cu and Zn on infauna by testing hypotheses regarding the additivity of the effects on various infaunal (univariate and multivariate) variables.

The two chapters describing laboratory experiments investigate the simultaneous effects of Cu, Pb and Zn on bivalves that are commonly found in the region of Auckland. Chapter 3, focusing on lethal effects of Cu, Pb and Zn individually and in all possible combinations, examines whether the effects of treatments with multiple metals are greater than those of single-metal treatments on the survival of the bivalve, *Macomona liliana*. Chapter 5 investigates bioaccumulation of Cu, Pb and Zn in the deposit-feeding bivalve, *Macomona liliana*, and the suspension-feeding bivalve, *Austrovenus stutchburyi*, based on their different feeding behaviours and the differential sensitivities of these two bivalves to heavy metals found in Chapter 2.

Finally, the General Discussion (Chapter 6) synthesises and discusses the overall findings in a broader context. The efficacy and appropriateness of current environmental guidelines and criteria for these heavy metals are reviewed, considering the coexistence of these metals in the field and their potential interactive effects. Advantages and limitations of experimental investigations that use metal-spiked sediments are also discussed in order to provide recommendations for future assessments of the effects of heavy metals in estuarine environments.

Chapter Two

Individual and Combined Effects of Heavy Metals on Estuarine Infaunal Communities

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Introduction

Aquatic ecosystems are often exposed to multiple anthropogenic stressors simultaneously (Halpern et al. 2007). Heavy metals entering estuaries through urban runoff are of environmental concern (Laws et al. 1994, ARC 2004), and under estuarine conditions, suspended sediments transporting these pollutants coagulate and tend to rapidly settle, becoming incorporated into sediments (ARC 2004). Heavy metals have the potential to cause individual-level effects, such as DNA base modification (Avery et al. 1996), developmental abnormalities (Lavalpe et al. 2004), depression in post-exposure feeding (Moreira et al. 2006), avoidance behaviour (Roper and Hickey 1994, Bat and Raffaelli 1998) and mortality (Bat and Raffaelli 1998, Gale et al. 2006). Such individual-level effects, in turn, can affect these systems as a whole at population or community levels by changing recruitment, abundances, species richness, diversity and community composition (Lande 1977, Rygg 1985, Watzin and Roscigno 1997, Warwick 2001). Concerns regarding the cumulative or interactive effects of multiple stressors on aquatic ecosystems were raised a decade ago (Breitburg et al. 1999a), and the effects of heavy

metals have also been examined in combinations with other environmental stressors such as salinity, temperature and other toxicants (Heugens et al. 2001, Millward et al. 2004).

Estuarine infaunal organisms inhabiting soft sediments play an important role in the food chain, obtaining carbon energy from detritus or suspended organic matter in the water column and passing it to higher trophic levels (Wilson 2002). Therefore, changes in the infaunal community are likely to result in changes to the ecological functions of estuarine habitats as a whole. Hence, examining potential effects of metal contaminants on estuarine infaunal communities is vital in the assessment of anthropogenic impacts on coastal ecosystems. Field experiments have shown that increased copper concentrations in estuarine sediments negatively impact the abundances of various infaunal taxa (Morrisey et al. 1996, Lenihan et al. 2003, Trannum et al. 2004). Simultaneous effects of a mixture of multiple heavy metals have also been experimentally examined in the field or in a microcosm (Millward et al. 2004, Lu and Wu 2007). These experiments, however, were not designed to distinguish effects of individual metals from the effects of a mixture of multiple metals (but see Lindegarth and Underwood 2002).

The primary sediment contaminants of concern in the region of Auckland, New Zealand, include the heavy metals copper (Cu), lead (Pb) and zinc (Zn) (ARC 2004). Concentrations of these metals in the sediments tend to be positively correlated with one another (Hewitt et al. 2009), and Cu and Zn concentrations in estuarine sediments are predicted to increase over time across the Auckland region (Green et al. 2004a, b). In the present study, we describe a manipulative field experiment done in the Orewa estuary of the Auckland region to assess the potential effects of Cu, Pb and Zn on estuarine communities. The experiment focused on measuring the recolonisation of defaunated metal-spiked sediment by infaunal organisms over a period of 20 days.

The main purpose of the experiment was to investigate the potential effects of the three primary sediment contaminants of concern (Cu, Pb and Zn) individually and in a mixture, on Auckland's estuarine communities in the future. Thus, target metal concentrations in the experiment were set as high as would be realistic for Auckland's estuaries, given predictions of potential inputs expected over the next 100 years (Green et al. 2004a, b). It was hypothesised that sediments spiked with heavy metals (Cu, Pb or Zn) would have lower abundances of colonists and, therefore, would have different faunal assemblages compared to those without additional metals. It was also hypothesised that sediments spiked simultaneously with multiple metals would result in an even greater overall reduction in the abundances of colonists and, therefore, would differ in community structure from those spiked with either a single metal or sediments with no additional metals.

Methods

Study site and experimental design

Orewa estuary (Fig. 2.1) is located approximately 30 km north of Auckland city and is a small to medium sized estuary, with an area of 1.28 km² below mean high water spring tide level and a catchment area of 17.5 km². The estuary is almost completely flushed with each tide and contains extensive intertidal areas separated by channels at low tide. A square 50 m × 50 m study area (36° 35' 45S, 174° 40' 51E) within an expansive mudflat at low tide was set up approximately 2 km upstream of the mouth of the estuary.

A colonisation experiment was done within the study area using defaunated, metal-spiked sediments. Sediments were collected from the surface aerobic layer (< 20 mm in depth) at the mudflat, sieved through 500 µm mesh and spiked with metals to specific target concentrations in the laboratory, following the method of Lu et al. (2008). The

initial spiking concentrations for Cu and Zn were $110 \mu\text{g g}^{-1}$ and $500 \mu\text{g g}^{-1}$, respectively. These concentrations were expected to decrease over the experimental period and to reach target concentrations of approximately $90 \mu\text{g g}^{-1}$ and $400 \mu\text{g g}^{-1}$, respectively. Although these concentrations are higher than those currently found in Auckland's estuaries, they are realistic with regard to increasing levels of Cu and Zn expected with further urbanisation in the future (Green et al. 2004a, b). The target Pb concentrations were set at close to the highest levels that have been recorded in Auckland's estuaries, with $85 \mu\text{g g}^{-1}$ as the initial concentration and $70 \mu\text{g g}^{-1}$ as the ending target (Kelly 2007). There is currently some uncertainty in predictions of Pb concentrations over time as the main source of Pb in stormwater runoff is from residues of historic paint and petrol, and these contributions are expected to diminish with time (Timperley et al. 2005). Lead is, however, still present on road surfaces, and there may be some contribution from vehicle tires (Kennedy 2003). Therefore, Pb concentrations may or may not increase over time.

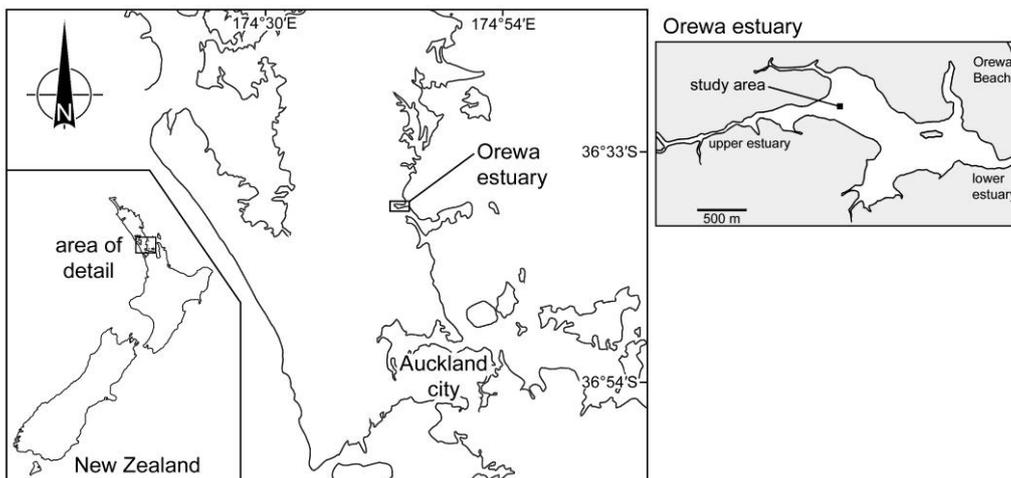


Fig. 2.1. Map of New Zealand, the Auckland region, Orewa estuary and the study area, located approximately 30 km north of the city of Auckland.

The metal-spiked sediments were poured into polyvinylchloride (PVC) moulds (300 mm in diameter, 30 mm in depth) to create sediment discs 25 to 30 mm thick and were frozen at -20 °C for a maximum of two weeks. On 5 August 2008 (day 0), these discs were taken out of the moulds and laid out within the study area by replacing existing surface sediments with the prepared discs. A small piece of PVC (20 mm × 20 mm) was placed underneath each sediment disc to determine relative changes in the thickness of the sediment discs in the course of the study. There were six different treatments in the experiment: unmanipulated (no replacement of the surface sediment), a manipulated control (surface sediments that were removed, defaunated and replaced but had no additional metals), Cu treatment (sediments spiked to a target Cu concentration of 110 to 90 $\mu\text{g g}^{-1}$), Pb treatment (spiked to 85 to 70 $\mu\text{g g}^{-1}$), Zn treatment (spiked to 500 to 400 $\mu\text{g g}^{-1}$) and mixed treatment (sediments spiked with all three metals at their respective target concentrations). The placement of sediment discs at the site was based on a randomised block design with 16 blocks (Fig. 2.2). Each block contained the six treatments placed in a randomised array of 2 × 3 sampling sites, 3 m apart from one another. Sediment discs in different blocks were at least 6 m apart.

Infaunal sampling

Sediment cores (200 mm in diameter, 50 mm in depth) from eight of the 16 blocks (n = 48) were taken inside each sediment disc on 15 August (day 10) and from the remaining eight blocks (n = 48) on 25 August (day 20). The blocks sampled at a given point in time were chosen in a way that avoided sampling any adjacent blocks on the same day (Fig. 2.2). Sediment cores were extracted and sieved through 500 μm mesh at the site. Material retained on sieves from each sample was brought back to the laboratory and preserved in 10% formalin for a minimum of 48 hours. Organisms were sorted from this

material, placed in 70% isopropyl alcohol, identified to the lowest practical taxonomic level and counted. All organisms identified and counted were assumed to be alive at the time of sampling. Although separating living from non-living organisms would have been desirable in order to quantify explicitly any potential post-colonisation mortality (e.g. see the Discussion), this was not strictly possible, as organisms were necessarily killed by fixation using formalin as part of the sampling procedure.

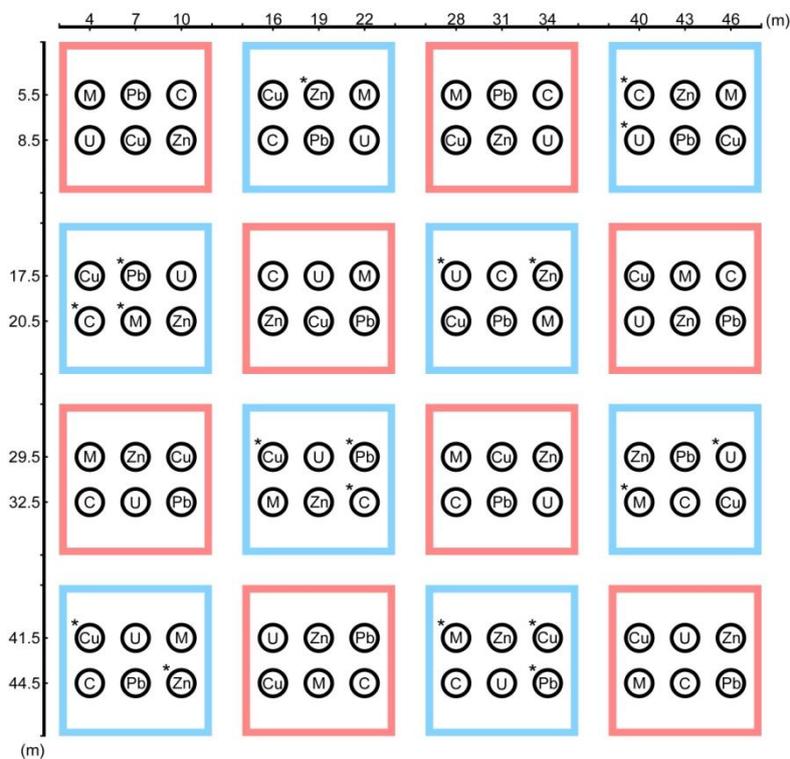


Fig. 2.2. Diagram of the field experimental design containing 16 blocks. U = unmanipulated, C = control, Cu = the copper treatment, Pb = the lead treatment, Zn = the zinc treatment, and M = the mixed metal treatment. Eight blocks (red) were sampled on day 10, and the rest (blue) on day 20. Asterisks “*” show discs assigned for the monitoring of chlorophyll *a* and porewater metal concentrations.

Environmental variables

Before the experiment began, levels of contaminants and total organic matter

(TOM) in the unmanipulated and spiked sediments were assessed. For spiked sediments, three replicate samples of sediment were taken from each treatment prior to freezing sediment discs. For the unmanipulated treatment, three sediment cores (20 mm in diameter, 20 mm in depth) were randomly taken from the study area on day 0, prior to the introduction of the sediment discs. Sediments were dried in an oven at 60 °C for 24 hours. Metal concentrations in the sediment samples were determined by flame atomic absorption spectroscopy (FAAS) following digestion of 0.5 g of the total sediment fraction in aqua regia (HCl:HNO₃ = 3:1) using high-purity HCl and HNO₃ (Chen and Ma 2001). The remaining samples were used to measure TOM by combusting the dried sediment at 500 °C for 4 hours (Byers et al. 1978). TOM (%) was calculated by the weight loss after combustion. At the time of infaunal sampling on days 10 and 20 each, sediment cores were taken adjacent to the sediment cores extracted for infaunal sampling to measure the sediment concentrations of metals and TOM associated with each disc. Metal concentrations and TOM were determined as described above.

From each treatment, three discs were also assigned for temporal monitoring of chlorophyll *a* levels and concentrations of metals in the porewater. These discs were randomly chosen from the blocks scheduled for infaunal sampling on day 20. For chlorophyll *a* analysis, sediment samples (20 mm in diameter, 2 mm in depth) were taken on days 1, 5, 12 and 20 using a syringe. The amount of chlorophyll *a* was determined by the spectrophotometric method according to Parsons et al. (1984), following extraction of chlorophyll *a* in 90% acetone for 24 hours at 4 °C in the dark. To measure metal concentrations in the porewater, peepers (37 µm mesh) were inserted into the selected sediment discs to collect porewater samples from the aerobic sediment layer (Adams et al. 2003). These samples were collected over three different periods: from day 1 to 5, day 5 to 12 and day 12 to 20. Porewater samples were filtered with a 0.45 µm membrane and

acidified with high-purity HNO_3 . Porewater analysis was first attempted using graphite-furnace atomic absorption spectroscopy (GFAAS) as metal concentrations in porewater are generally relatively low. However, due to interference by components found in seawater, samples were analysed using FAAS instead. Detection limits of Cu, Pb and Zn were 0.1 mg L^{-1} , 0.5 mg L^{-1} and 0.1 mg L^{-1} , respectively. Porewater concentrations of Pb were too low to be measured by FAAS, so samples of the Pb and mixed treatments were analysed by Hill Laboratories (Hamilton, New Zealand) using inductively coupled plasma-mass spectrometry (ICP-MS). Detection limit of Pb using ICP-MS was 0.001 mg L^{-1} . The effects of chlorophyll *a* and porewater sampling on infauna were considered negligible (1) because these sampling procedures avoided the centre of the sediment discs where infaunal samples were taken, and (2) because the area disturbed by the sampling was relatively small in comparison to the size of sediment discs.

Relative changes in sediment bed height (i.e. erosion or accretion) were determined by measuring sediment thickness above the PVC pieces placed underneath sediment discs every five days. Temperature and precipitation were also monitored throughout the experiment for potential weather anomalies that could affect runoff and experimental results. A temperature recorder (HOBO[®] Temperature/Light Data Logger) was attached to a plastic peg vertically inserted at the upstream, southern corner of the study area to record sediment surface temperature every 30 minutes during the experimental period. Rainfall data in Orewa estuary were obtained from the Auckland Regional Council's Geographic Information and Mapping Service (http://maps.arc.govt.nz/website/maps/map_hydrotel.htm).

Statistical analyses

Analyses of infaunal assemblages were done using the software package PRIMER

6 (Clarke and Gorley 2006) with the PERMANOVA+ add-on (Anderson et al. 2008). The experimental design consisted of three factors: day (fixed with two levels: day 10 and day 20), treatment (fixed with six levels: unmanipulated, control, Cu, Pb, Zn and mixed) and block (random with eight levels, nested in day). Individual variables were analysed according to the full three-factor design using permutational analysis of variance (PERMANOVA; Anderson 2001, McArdle and Anderson 2001) based on Euclidean distances, with 4999 permutations of residuals under a reduced model. PERMANOVA pair-wise comparisons followed the overall partitioning when significant differences were detected among the treatments. Although each pair-wise test is exact, the PERMANOVA pair-wise comparisons do not control formally for multiple tests, so they should be interpreted with some caution in this respect. The individual variables analysed in this way were: the total abundance of all taxa (N), species richness (S), Simpson's index of species evenness ($1-\lambda$) and the abundances of several of the numerically dominant organisms. Abundance data were log transformed ($y' = \ln(y + 1)$) prior to the analyses to avoid high skewness and heterogeneity.

The structure of infaunal assemblages as a whole was examined using the modified Gower dissimilarity measure (Anderson et al. 2006a) with a logarithm base of 5. Thus, a five-fold change in abundance was weighted equally with a change in species composition (from 0 to 1). (The range of maximum abundances was between 20 and 115 per sample for most of the numerically dominant taxa in this experiment.) Non-metric multi-dimensional scaling (MDS) ordinations were done in order to visualise relationships among samples in terms of community structure. Differences in faunal assemblages among the six treatments were then examined by PERMANOVA on the basis of the full three-way experimental design using 4999 permutations of residuals under a reduced model, followed by PERMANOVA pair-wise comparisons if significant differences were

detected among the treatments.

Sediment sieving prior to spiking and freezing of sediment discs could affect the total organic content and the benthic diatom populations in the sediments, respectively. Chlorophyll *a* concentrations and TOM were therefore measured to test whether sediment discs had comparable environmental conditions. Chlorophyll *a* concentrations on day 20 were first transformed to $y' = \ln(y)$ and then analysed using univariate one-way analysis of variance (ANOVA). Variation in TOM (%) among treatments was tested separately at three times of sampling: days 0, 10 and 20. As for other univariate variables, analyses were done using PERMANOVA with 4999 permutations of raw data, based on Euclidean distances.

Results

Environmental variables

Concentrations of Cu in sediment were elevated to target levels in the Cu and mixed metal treatments, but decreased from the initial target concentration of around 110 $\mu\text{g g}^{-1}$ on day 10 to below the ending target concentration of 90 $\mu\text{g g}^{-1}$ on day 20 (Fig. 2.3a). Concentrations of Pb in the Pb and mixed metal treatments showed similar trends, being elevated to around 85 $\mu\text{g g}^{-1}$ on day 10, but decreased to average concentrations of 60 $\mu\text{g g}^{-1}$ on day 20 (Fig. 2.3b). Concentrations of Zn, however, did not reach the initial target concentration of 500 $\mu\text{g g}^{-1}$; spiking only elevated them to around 400 $\mu\text{g g}^{-1}$, and the concentrations decreased during the experiment to, on average, 290 $\mu\text{g g}^{-1}$ in the Zn treatment and 220 $\mu\text{g g}^{-1}$ in the mixed treatment (Fig. 2.3c).

Concentrations of Cu in porewater were relatively stable during the experiment: around 0.3 mg L^{-1} in the Cu treatment and between 0.15 mg L^{-1} and 0.5 mg L^{-1} in the mixed metal treatment (Fig. 2.4a). Porewater concentrations of Cu in other treatments

were mostly below 0.1 mg L^{-1} , the detection limit of FAAS. Porewater concentrations of Zn in the treatments spiked with Zn were initially high but decreased by day 12 to approximately 7 mg L^{-1} and stabilised (Fig. 2.4b). Porewater Zn concentrations in other treatments were mostly below the detection limit of 0.1 mg L^{-1} . Porewater concentrations of Pb in the Pb and mixed metal treatments were mostly below 0.001 mg L^{-1} , the detection limit of ICP-MS, except for two samples in the mixed treatment. These samples had Pb concentrations of 0.0022 mg L^{-1} (day 5 to 12) and 0.0027 mg L^{-1} (day 12 to 20).

Sediment chlorophyll *a* concentrations were mostly higher, on average, in the unmanipulated treatment compared to all the other treatments. Chlorophyll *a* concentrations on day 20 were, however, not significantly ($\alpha = 0.05$) different among treatments ($F_{5,12} = 1.36$, $P = 0.306$). There was initially no significant difference in TOM levels among treatments ($F_{5,12} = 1.02$, $P = 0.459$). TOM levels were, however, significantly different among treatments on day 10 ($F_{5,42} = 15.24$, $P = 0.0002$) and day 20 ($F_{5,42} = 5.08$, $P = 0.0022$). TOM in sediments taken from the unmanipulated treatment were significantly ($P < 0.01$) higher than those taken from all other treatments on day 10 and those taken from the Cu, Zn or mixed metal treatments on day 20. There were no significant differences in TOM levels among the control, Cu, Pb, Zn and mixed metal treatments. Monitoring of sediment disc thickness showed that the site experienced some overall erosion; the average change in bed height ranged from 0 to -5 mm (erosion) during the experiment. Changes in the thickness of sediment discs were, on average, -3.5 mm ($SD = 2.2$) for those sampled on day 10 and -0.65 mm ($SD = 4.2$) for those sampled on day 20.

Sediment surface temperature at the study area mostly fluctuated daily between $5 \text{ }^{\circ}\text{C}$ and $25 \text{ }^{\circ}\text{C}$. The maximum and minimum temperatures were $25.7 \text{ }^{\circ}\text{C}$ and $2.3 \text{ }^{\circ}\text{C}$, respectively, and these values were both recorded on day 5. The greatest amount of

rainfall recorded with a 24-hour period was 33.8 mm on day 19.

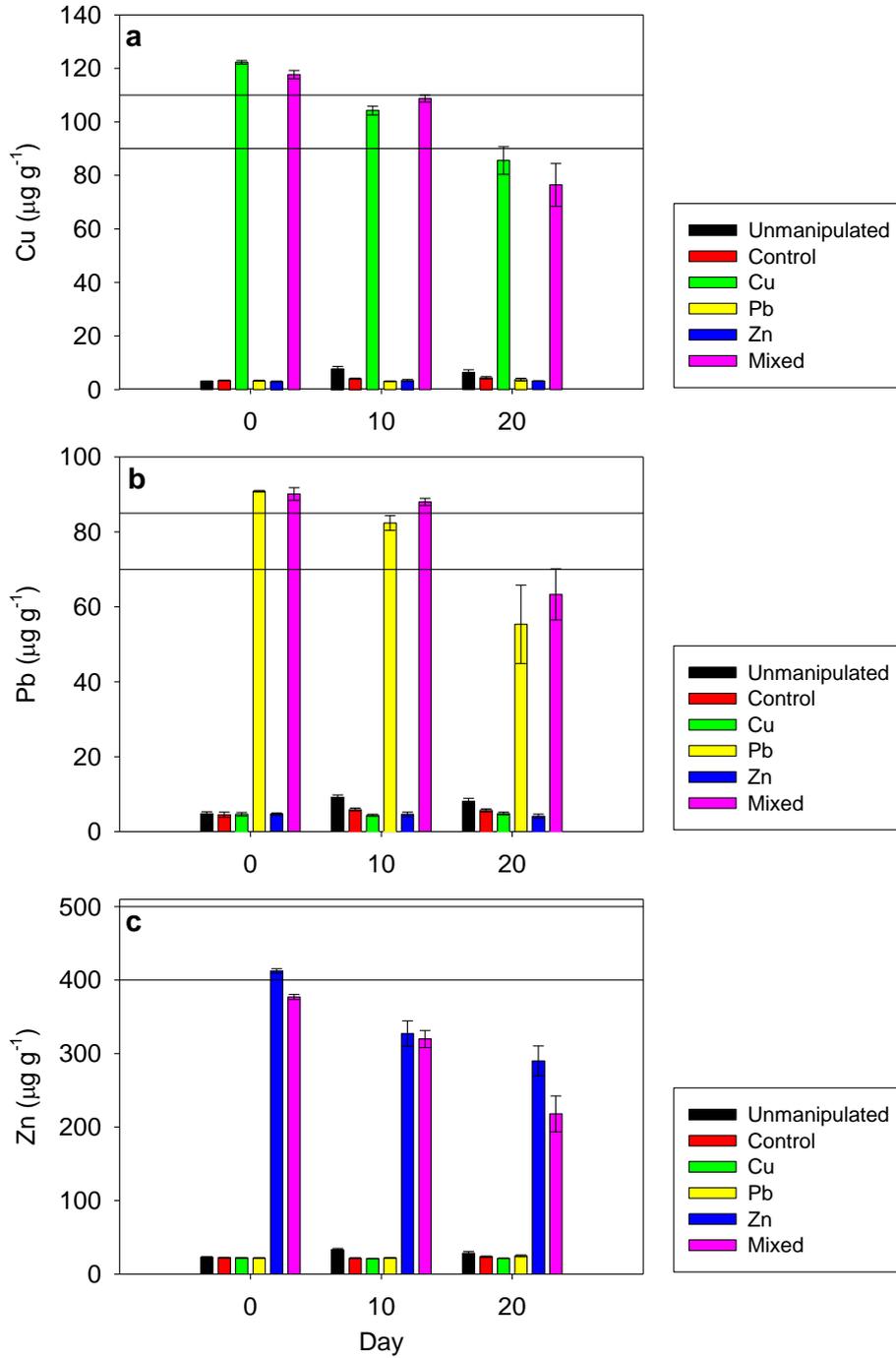


Fig. 2.3. Initial and final metal concentrations in sediments (mean \pm SE, n = 3), for (a) Cu, (b) Pb and (c) Zn. The horizontal lines indicate the initial and ending target concentrations of metals.

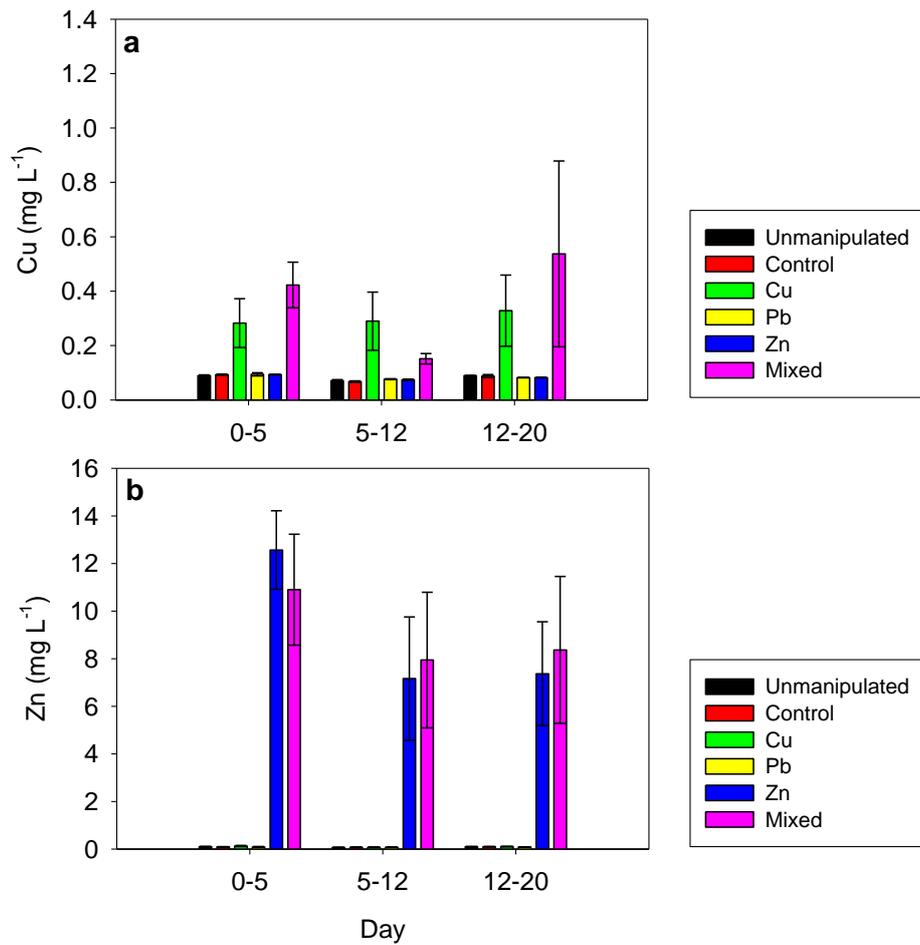


Fig. 2.4. Concentrations of metals in porewater samples (mean \pm SE, $n = 3$) over the 20-day experimental period, for (a) Cu and (b) Zn.

Infauna

In total, 31 taxa were identified from the 48 sediment cores collected on day 10 and 27 taxa from the 48 cores collected on day 20. The numerically dominant taxa in this study were, in order of decreasing abundance, *Scoloplos cylindifer*, oligochaetes, *Prionospio* sp., *Capitella* spp., *Ceratonereis* sp., *Polydorida* spp., *Paracalliope* sp., *Austrovenus stutchburyi*, *Macomona liliana*, and *Pectinaria australis*. These 10 taxa accounted for approximately 90% of the total abundance of all infaunal organisms. The mean log-transformed total abundance of infauna differed significantly among treatments

(Table 2.1). The control and Pb treatments had significantly greater abundances of organisms than the Cu, Zn or mixed metal treatments, on average, but significantly fewer than the unmanipulated treatment (Fig. 2.5a). There was also an overall increase in the total abundance of infauna from day 10 to day 20 (Table 2.1). Similarly, there were statistically significant effects of treatments on species richness (Table 2.1). The unmanipulated, control and Pb treatments had a significantly greater average number of taxa than the Cu, Zn or mixed metal treatments (Fig. 2.5b). Simpson's index of species evenness also differed significantly among treatments (Table 2.1). Evenness was, on average, greater in the unmanipulated treatment than in the Zn or mixed treatments, and was also greater in the Pb treatment than in the mixed treatment (Fig. 2.5c).

Significant treatment effects were also detected for individual taxa, with the exception of the amphipod *Paracalliope* sp. (Table 2.1). Average log abundances of oligochaetes and *A. stutchburyi* were both significantly higher in the unmanipulated treatments than in all other treatments, suggesting an effect of the surface sediment manipulation; however, no significant differences were found among the control, Cu, Pb, Zn and mixed metal treatments (Fig. 2.6b, h). The mean log abundances of *S. cylindifer*, *Prionospio* sp., *Capitella* sp., *M. liliiana* and *P. australis* were significantly higher in the control treatment than in the Cu, Zn or mixed metal treatments (Fig. 2.6). *Ceratonereis* sp. had significantly lower average abundances in the Cu and mixed metal treatments compared to the control (Fig. 2.6e), and Polydorid spp. had significantly lower average abundances in the Zn and mixed treatment compared to the control (Fig. 2.6f). None of the dominant taxa showed significant differences in their average abundances between the control and Pb treatments. *S. cylindifer*, *Capitella* spp. and *Ceratonereis* sp. also showed significant differences in their average abundances in the mixed metal treatment *versus* the Cu or Zn treatments: mean log abundances of *S. cylindifer* and *Capitella* spp. were

significantly lower in the mixed treatment compared to the Cu treatment, and mean log abundance of *Ceratonereis* sp. was significantly lower in the mixed treatment compared to the Zn treatment.

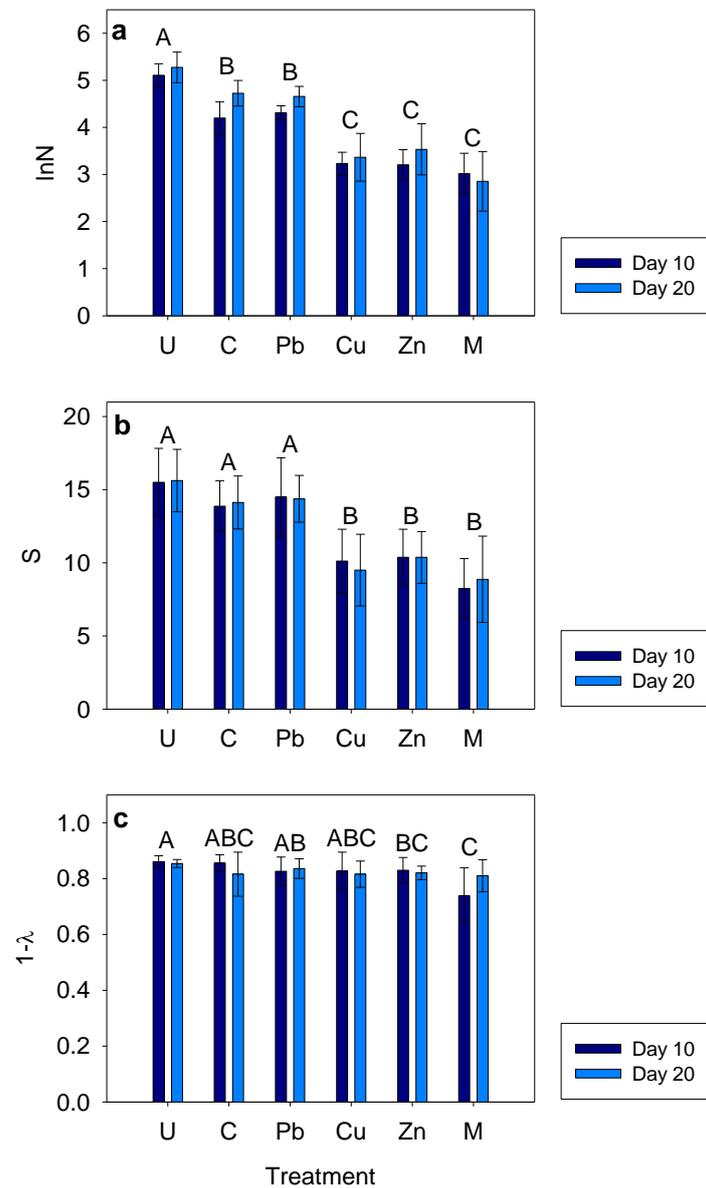


Fig. 2.5. Mean(\pm SE) on day 10 and day 20 for (a) log-transformed total infaunal abundance (lnN), (b) species richness (S) and (c) Simpson's evenness index ($1-\lambda$) in each of the treatments: U = unmanipulated, C = control, Cu = the copper treatment, Pb = the lead treatment, Zn = the zinc treatment, and M = the mixed metal treatment. Means with the same letter in each graph were not significantly different (using a significance level of $\alpha = 0.01$) based on the PERMANOVA pairwise tests.

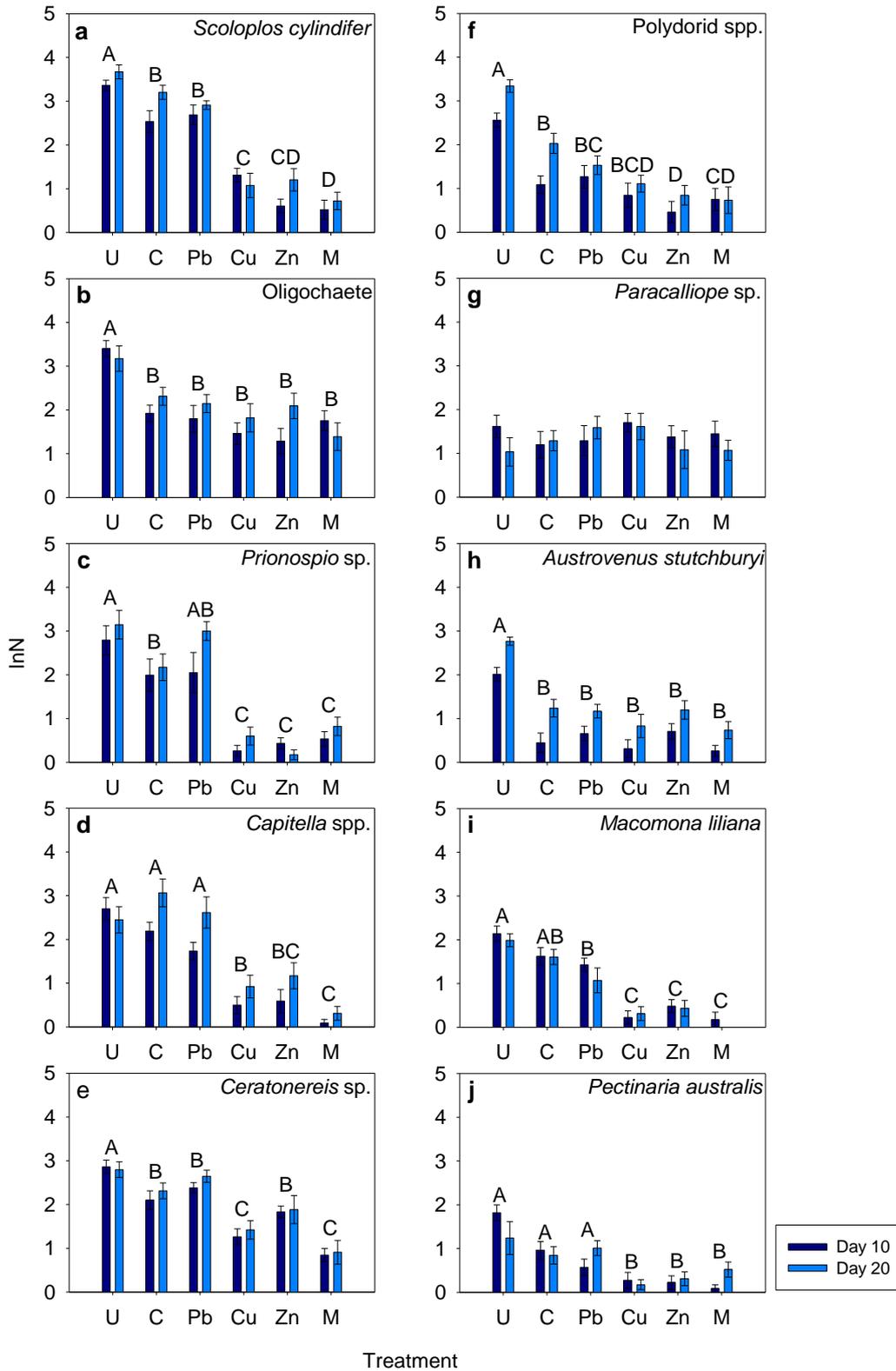


Fig. 2.6. Mean (\pm SE) log-transformed abundances of numerically dominant taxa (U = unmanipulated, C = control, Cu = the copper treatment, Pb = the lead treatment, Zn = the zinc treatment, and M = the mixed metal treatment). Means with the same letter were not significantly different (using a significance level of $\alpha = 0.01$) based on the PERMANOVA pairwise tests.

Table 2.1. Summary of results of PERMANOVA for univariate and multivariate analyses and results of pair-wise tests. Univariate analyses were for the total log abundance, species richness, Simpson’s index and numerically dominant taxa, and multivariate analysis was done based on the modified Gower dissimilarity measure (log base 5), calculated on infaunal abundances on day 10 and day 20. Treatments underlined together did not show significant ($\alpha = 0.01$) difference in pair-wise tests (U = unmanipulated, C = control, Cu = copper, Pb = lead, Zn = zinc, and M = mixed metal treatments). Results of pairwise tests for the multivariate analysis are shown in Table 2.

	Day		Treatment		Block(Day)		Day \times Treatment		Treatment
	$F_{1,14}$	<i>P</i>	$F_{5,70}$	<i>P</i>	$F_{14,70}$	<i>P</i>	$F_{5,70}$	<i>P</i>	Pair-wise test
Univariate									
Total log abundance	6.31	0.027	96.54	0.0002	1.52	0.133	1.70	0.154	<u>U</u> <u>Pb</u> <u>C</u> <u>Zn</u> <u>Cu</u> <u>M</u>
Species richness	0.01	0.967	29.15	0.0002	1.14	0.330	0.15	0.983	<u>U</u> <u>Pb</u> <u>C</u> <u>Zn</u> <u>Cu</u> <u>M</u>
Simpson’s index	0.03	0.865	3.08	0.011	1.24	0.250	1.43	0.225	<u>U</u> <u>Pb</u> <u>C</u> <u>Cu</u> <u>Zn</u> <u>M</u>
<i>Scoloplos cylindifer</i>	3.40	0.082	94.29	0.0002	2.40	0.007	1.67	0.156	<u>U</u> <u>C</u> <u>Pb</u> <u>Cu</u> <u>Zn</u> <u>M</u>
Oligochaetes	1.50	0.247	13.20	0.0002	0.13	0.125	1.52	0.194	<u>U</u> <u>C</u> <u>Pb</u> <u>Zn</u> <u>Cu</u> <u>M</u>
<i>Prionospio</i> sp.	2.54	0.130	42.72	0.0002	1.76	0.062	1.18	0.328	<u>U</u> <u>Pb</u> <u>C</u> <u>M</u> <u>Cu</u> <u>Zn</u>
<i>Capitella</i> spp.	8.39	0.010	36.29	0.0002	1.22	0.279	1.52	0.188	<u>C</u> <u>U</u> <u>Pb</u> <u>Cu</u> <u>Zn</u> <u>M</u>
<i>Ceratonereis</i> sp.	0.76	0.394	30.04	0.0002	1.44	0.159	0.20	0.960	<u>U</u> <u>Pb</u> <u>C</u> <u>Zn</u> <u>Cu</u> <u>M</u>
Polydorid spp.	9.43	0.010	28.40	0.0002	1.19	0.299	1.27	0.289	<u>U</u> <u>C</u> <u>Pb</u> <u>Cu</u> <u>M</u> <u>Zn</u>
<i>Paracalliope</i> sp.	0.33	0.578	0.96	0.451	3.91	0.0004	0.91	0.485	
<i>Austrovenus stutchburyi</i>	16.92	0.002	32.55	0.0002	2.10	0.024	0.35	0.888	<u>U</u> <u>Zn</u> <u>Pb</u> <u>C</u> <u>Cu</u> <u>M</u>
<i>Macomona liliana</i>	0.87	0.378	47.37	0.0002	1.53	0.130	0.43	0.824	<u>U</u> <u>C</u> <u>Pb</u> <u>Zn</u> <u>Cu</u> <u>M</u>
<i>Pectinaria australis</i>	0.05	0.819	14.13	0.0002	1.07	0.395	2.02	0.084	<u>U</u> <u>C</u> <u>Pb</u> <u>M</u> <u>Zn</u> <u>Cu</u>
Multivariate									
Modified Gower	2.22	0.007	13.00	0.0002	1.64	0.0002	1.20	0.098	

Table 2.2. Summary of results of PERMANOVA pair-wise tests ($\alpha = 0.01$) based on the modified Gower dissimilarity measure (log base 5), calculated on infaunal abundances on day 10 and day 20.

	Unmanipulated	Control	Cu	Pb	Zn
Control	$t_{14} = 2.74$ $P = 0.0002$				
Cu	$t_{14} = 5.37$ $P = 0.0002$	$t_{14} = 3.62$ $P = 0.0002$			
Pb	$t_{14} = 2.92$ $P = 0.0002$	$t_{14} = 0.98$ $P = 0.473$	$t_{14} = 3.20$ $P = 0.0002$		
Zn	$t_{14} = 5.06$ $P = 0.0002$	$t_{14} = 3.37$ $P = 0.0002$	$t_{14} = 1.32$ $P = 0.061$	$t_{14} = 3.32$ $P = 0.0002$	
Mixed metal	$t_{14} = 6.04$ $P = 0.0002$	$t_{14} = 4.23$ $P = 0.0002$	$t_{14} = 1.51$ $P = 0.014$	$t_{14} = 4.24$ $P = 0.0002$	$t_{14} = 1.89$ $P = 0.0012$

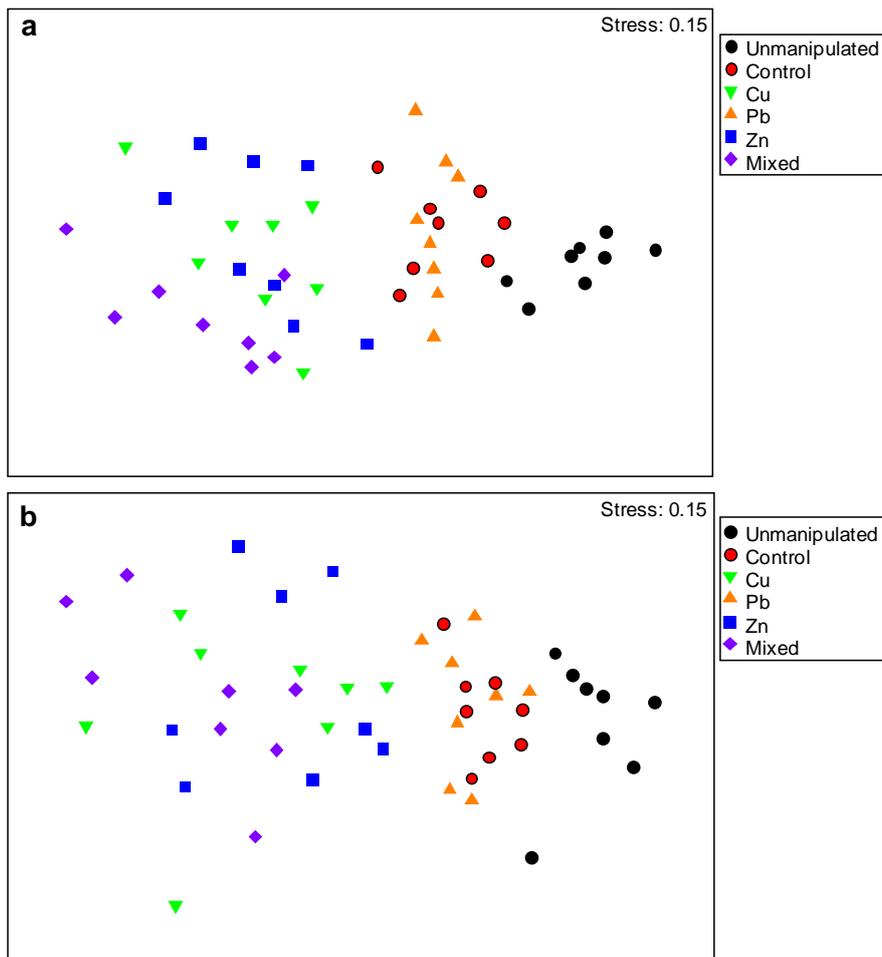


Fig. 2.7. MDS plots based on the modified Gower dissimilarity measure (log base 5) calculated from infaunal abundances for 31 taxa (a) on day 10 and (b) on day 20.

Overall increases in abundance from day 10 to day 20 were found in *Capitella* spp., Polydorid spp. and *A. stutchburyi* (Table 2.1). Significant variation among blocks was also detected for *S. cylindifer*, *Paracalliope* sp. and *A. stutchburyi* (Table 2.1), indicating high spatial variability in their distributions at scales of tens of meters.

There were significant ($\alpha = 0.05$) differences in the structure of infaunal assemblages between day 10 and day 20, among blocks and among treatments (Table 2.1). Assemblages in the unmanipulated treatment differed from all other treatments (Table 2.2). Assemblages in the control and Pb treatments did not differ significantly from one another, but were significantly different from those in the Cu, Zn or mixed metal treatments (Table 2.2). Importantly, there was also a significant difference between assemblages in the mixed metal treatments and assemblages from any of the treatments spiked with only a single metal alone, whether it was Cu, Zn or Pb. These results were supported by patterns seen visually in the MDS plots (Fig. 2.7): assemblages from the mixed metal treatment (with all three metals, on the left of the diagram) were most dissimilar from those in the unmanipulated treatment (on the right). In between these, assemblages from the Cu and Zn treatments were interspersed with one another, placed close to the mixed treatment. Assemblages from the control and Pb treatments were also well interspersed with one another, placed in-between the unmanipulated treatment and the Cu and Zn treatments. The overall pattern in the MDS was a clear gradient in effect from unmanipulated treatments on the right, to assemblages experiencing sediment manipulation or those spiked with Pb, followed by assemblages in sediments spiked by either Cu or Zn alone, and finally assemblages which experienced elevated levels of all three heavy metals (the mixed metal treatment) on the far left. Although the pattern of difference between the mixed treatment and either the Cu or Zn treatments is somewhat less clear for day 20 than for day 10 in the reduced-space (2-dimensional) MDS ordination (Fig. 2.7), the

PERMANOVA test results, which used the full resemblance matrix of information, demonstrated clear and consistent differences between the mixed treatment and either the Cu or Zn treatments (Tables 2.1 and 2.2).

Discussion

Total infaunal abundance and species richness were significantly reduced by the Cu, Zn and mixed treatments. Several of the numerically dominant organisms reflected this pattern. Direct negative effects of Cu and Zn on polychaetes, crustaceans and bivalves have also been found in laboratory bioassays (Bat and Raffaelli 1998, King et al. 2004). For example, Bat and Raffaelli (1998) showed that sediment Cu and Zn concentrations of $100 \mu\text{g g}^{-1}$ each caused 100% mortality of the amphipod *Corophium volutator*, and $90 \mu\text{g g}^{-1}$ of Cu and $100 \mu\text{g g}^{-1}$ of Zn caused 100% mortality of the polychaete *Arenicola marina*. Some previous manipulative field experiments have also shown that Cu in sediments can significantly reduce abundances of various taxa (Morrisey et al. 1996, Lenihan et al. 2003, Trannum et al. 2004).

The bioavailability of sediment-associated metals is controlled by the metal binding strength to the sediment, and metals bound to sediments may not be toxic to organisms (Chapman and Long 1983). Lead generally has high affinity for suspended particulate matter (Webster-Brown 2005), and binds strongly to iron and manganese oxyhydroxides, which are the dominant metal-binding phases in aerobic sediments (Gadde and Laitinen 1974, Benjamin and Leckie 1981b). The strong binding of Pb to the sediments was also evident from the extremely low Pb concentrations in porewater (mostly below 0.001 mg L^{-1}) measured in this experiment. Concentrations of Cu and Zn in porewater in spiked-sediments were, on the contrary, elevated to levels comparable to those reported from estuaries in the UK that had been affected by mining activities (Bryan

et al. 1987, Bryan and Langston 1992). Borgmann and Norwood (1999) examined bioavailability of Pb to the amphipod *Hyalella azteca* and showed that dissolved Pb, rather than Pb associated with sediments, almost entirely explained the bioavailability and toxicity of Pb. The lack of any apparent effects of Pb on recolonisation of estuarine infauna, therefore, may be explained by low bioavailability of this metal to organisms.

The experimental target concentration of Pb ($85 \mu\text{g g}^{-1}$) might be considered as relatively low by international standards (Long et al. 1995, ANZECC 2000). However, this concentration falls within the range of the sediment quality guidelines of Long et al. (1995), which list sediment Pb concentrations of $46.7 \mu\text{g g}^{-1}$ and $218 \mu\text{g g}^{-1}$ as the effects range-low (ERL) and the effects range-median (ERM), respectively. Similarly, the target Cu concentration of $110 \mu\text{g g}^{-1}$ in the present experiment was also higher than the ERL ($34 \mu\text{g g}^{-1}$) but lower than the ERM ($270 \mu\text{g g}^{-1}$) for Cu (Long et al. 1995). Josefson et al. (2008) suggested a threshold Pb concentration of approximately $200 \mu\text{g g}^{-1}$ for deterioration of faunal communities to occur in the field. However, the sediments in that study were also contaminated with Zn, so the contribution of Pb to the observed effects could not be separated and identified unambiguously. The target and ending concentrations of Pb in the present experiment were comparable to Pb concentrations in some of the most highly contaminated estuaries of the Auckland region (Kelly 2007). Our results suggest that Pb alone will be unlikely to cause important changes in estuarine faunal communities in this region if current Pb levels are maintained. Increases in concentrations of Pb, however, may cause acute adverse effects on estuarine fauna as indicated by other studies. Potential long-term or sublethal effects of Pb and other metals also remain to be examined.

Different organisms had different sensitivities to heavy metals. Three taxa that apparently were not affected by Cu, Pb or Zn in this experiment were the amphipod

Paracalliope sp., the bivalve *Austrovenus stutchburyi* and oligochaetes. The insensitivity of *Paracalliope* sp. to heavy metals contradicts findings from other studies reporting reduced abundances of amphipods in sediments contaminated with heavy metals in the field (Morrisey et al. 1996, Warwick 2001, Lenihan et al. 2003). Laboratory bioassays have also shown sensitivity of amphipods to Cu and Zn (Bat and Raffaelli 1998, Marsden and Wong 2001). These laboratory bioassays, however, suggested that the LC₅₀ values (concentrations responsible for 50% mortality) could vary greatly between species and for different metals. It is therefore possible that *Paracalliope* sp. simply has higher tolerance to heavy metals. Another possible explanation is that this amphipod does not experience full metal toxicity as the species is patchily distributed in the field and highly mobile.

A. stutchburyi are filter-feeding, venerid bivalves commonly found in estuaries in New Zealand, and it is perhaps surprising that no deleterious effects of metals on their colonisation were detected in this experiment. Potential effects of certain environmental stressors on this species (such as organic chemicals and sedimentation) have been examined (Hickey et al. 1995, Norkko et al. 2006), but the potential direct effects of heavy metals have not been previously tested in field-based manipulative studies. Thrush et al. (2008) demonstrated a negative correlation between abundances of *A. stutchburyi* and Cu concentrations in Auckland's estuaries and harbours. De Luca-Abbott (2001) showed that sediments contaminated with Cu, Pb, Zn and polycyclic aromatic hydrocarbons had sublethal effects on *A. stutchburyi* in the field. These studies, however, did not manipulate concentrations of heavy metals directly, so direct causality cannot be inferred. Nevertheless, potential sublethal or long-term effects should be considered and investigated for this species, based on this prior work.

In contrast, the deposit-feeding tellinid bivalve *Macomona liliana* did show sensitivity to Cu and Zn in this experiment. The different responses to the Cu, Zn and

mixed metal treatments for *M. liliana* versus *A. stutchburyi* may have been due to differences in their feeding behaviour. Bioaccumulation of sediment-associated organic contaminants has been found to be higher in *M. liliana* than in *A. stutchburyi*, probably because the filter-feeding *A. stutchburyi* is unlikely to directly ingest highly contaminated surface sediments, unlike the deposit-feeding *M. liliana* (Wilcock et al. 1993, Hickey et al. 1995). Juvenile *M. liliana* can also actively disperse using bedload and water column transport (Lundquist et al. 2004), and will crawl away from sediments with Cu concentrations of $10 \mu\text{g g}^{-1}$ or Zn concentrations of $40 \mu\text{g g}^{-1}$ (Roper et al. 1995). Such behaviour indicates the potential ability of this species to actively avoid metal contaminants.

Annelid worms have been shown to be relatively tolerant to heavy metals in the laboratory and in the field. The polychaetes *Australonereis ehlersi* and *Nephtys australiensis* did not show lethal effects with sediment-bound Cu at $1300 \mu\text{g g}^{-1}$ or Zn over $3000 \mu\text{g g}^{-1}$ in laboratory bioassays (King et al. 2004). Polychaetes have also been found in high abundances in sediments contaminated with heavy metals in the field (Lande 1977, Rygg 1985). The relative tolerance of annelids to heavy metals described in other studies is only supported by the insensitivity of oligochaetes to Cu, Pb and Zn in the present experiment; the numerically dominant polychaetes studied here were, in contrast, all sensitive to the Cu and Zn treatments. Rygg (1985) found that the metal-tolerant species in Norwegian fjords were mostly carnivorous polychaetes, suggesting that feeding behaviour might play a role in determining the sensitivity of organisms to metals. Carnivorous polychaetes might be less affected by sediment metals than deposit-feeders because they would not ingest sediment-associated metals directly.

Capitellids have been found in high abundances in areas contaminated with heavy metals (Lande 1977) and identified as highly tolerant to Cu (Rygg 1985). Such findings

from previous studies contradict the apparent sensitivity of *Capitella* spp. found in the present experiment. Capitellids (especially *Capitella* species) are known as *r*-selected, highly opportunistic polychaetes that quickly colonise polluted habitats (Borja et al. 2000), but their increased abundances have been found mostly in association with organic enrichment (Tsutsumi 1987, Blanchard and Feder 2003, Lee et al. 2006), even with elevated sediment Cu concentrations of $500 \mu\text{g g}^{-1}$ (Lenihan et al. 2003). Thus, the sensitivity of capitellids to heavy metals may be affected by the amount of organics in sediments, and potential interactions between heavy metal contaminants and organics in their effects on these organisms need to be examined.

Multivariate analyses revealed that faunal assemblages in the mixed metal treatment were different from those in either the Cu or Zn treatments, suggesting possible cumulative (e.g. additive) effects of multiple metals. Such differences were not clearly observed, however, in any univariate analysis of individual taxa. For example, the significantly lower average log abundance of *Scoloplos cylindifer* in the mixed *versus* the Cu treatments may have been caused by combined effects of Cu and Zn or because the mixed treatment was simply dominated by the effect of Zn alone (see Fig. 2.7a). While combined effects of metals may not be clear at the population (individual species) level, in the present study differences in the responses of individual taxa to different metals were reflected at the community-level with the help of multivariate analyses, which distinguished mixed metal treatments from the others. For example, the polychaete *Ceratonereis* sp. was insensitive to the Zn treatment but negatively responded to the Cu and mixed treatments. Polydorid spp., on the other hand, was insensitive to the Cu treatment but negatively responded to the Zn and mixed treatments. This differential sensitivity led to community-level effects caused by the simultaneous presence of several different heavy metals.

Because of the fairly short experimental period of 20 days, recolonisation in this experiment is more likely to be explained by the migration of organisms to sediment discs, rather than by recruitment. Similar experiments examining recolonisation of contaminated sediments have used much longer experimental periods (up to 14 months), potentially allowing organisms to colonise sediments through recruitment, as well as migration (Trannum et al. 2004, Lu and Wu 2007). A pilot study in Orewa showed, however, that the sediments spiked with heavy metals were subject to continuous decreases in metal concentrations, so deploying a longer experimental period would not have been feasible here. For this reason, inferences are restricted to short-term effects of heavy metals on colonisation only. The reduced abundances of infauna in metal-spiked treatments might be explained either by post-migration mortality or avoidance behaviour. For example, the bivalve *Macomona liliana* has been shown to be capable of avoiding sediment contaminated with Cu (Roper and Hickey 1994, Roper et al. 1995). In contrast, the shells of dead *Macomona* were frequently observed on the surface of sediment discs spiked with Cu and Zn during this experiment, suggesting that migration was followed by mortality. Empty *Macomona* shells were also found, however, on sediment surfaces outside sediment discs, so these shells might simply have been transported to the site from surrounding areas by water movement. This experiment could not distinguish the potential underlying processes driving observed effects (e.g. recruitment, migration followed by mortality or avoidance behaviour). Such mechanisms would need to be examined by subsequent studies to elucidate how contaminants in sediments might ultimately affect population dynamics for individual species across larger spatial scales.

The potential contribution of Pb to the effects of mixed treatments also remains somewhat unclear: While the Pb treatment alone did not have any detectable effects on the recolonisation of estuarine fauna, the presence of Pb might have affected the

bioavailability of Cu and Zn in the mixed treatment (Gadde and Laitinen 1974, Benjamin and Leckie 1981a). Multiple stressors can interact in additive, multiplicative or comparative (the effect of stressors in combination equals the effect of a single, dominant stressor) fashions, and antagonisms or synergisms may also occur (Folt et al. 1999). Crain et al. (2008) have shown that interactions among three stressors tended to be synergistic, suggesting that synergistic interactions of multiple stressors might commonly occur in nature. Although strong indication of synergism was not found in the present experiment, it is important to further investigate the potential simultaneous effects of these three metals as concentrations of Cu, Pb and Zn do tend to correlate with one another in Auckland's estuaries.

Conclusions

This manipulative field experiment showed that increased amounts of Cu and Zn in estuarine intertidal sediments significantly reduced the colonisation of infaunal organisms. Although the current levels of Pb in the sediments of Auckland's estuaries are unlikely to cause adverse effects on estuarine fauna, its potential contribution to simultaneous effects in the presence of Cu and Zn, as well as sublethal and chronic effects, still needs to be investigated. Nevertheless, the fact that both Cu and Zn generally have negative effects and the fact that different taxa have differential sensitivities to these two metals demonstrate that their combined effects in contaminated sediments will have important impacts on estuarine community structure as a whole.

Chapter Three

Acute Toxicity of Copper, Lead and Zinc to the Deposit-Feeding Bivalve

Macomona liliana

Introduction

Heavy metals are anthropogenically introduced into estuaries through polluted stormwater runoff and tend to become incorporated in sediments (McLusky 1999, ARC 2004). These metals are then taken up and accumulated by various estuarine organisms (Bryan and Langston 1992). The effects of certain heavy metals on estuarine organisms such as bivalves, polychaetes and crustaceans have been fairly well examined through numerous laboratory experiments (e.g. Roper and Hickey 1994, Bat and Raffaelli 1998, Lavalpe et al. 2004, Gale et al. 2006). These experiments have examined only one metal at a time, but clearly identified lethal and sublethal effects. The effects of individual heavy metals have also been studied in combination with other potential environmental stressors (e.g. salinity and temperature), as aquatic ecosystems are generally exposed to multiple anthropogenic stressors (Denton and Burdon-Jones 1981, Alutoin et al. 2001, Heugens et al. 2001). The simultaneous effects of multiple metals, however, have not generally been well examined (Hagopian-Schlekat et al. 2001).

The toxicity of heavy metals is affected by bioavailability and the detoxification and excretion rates of organisms (Rainbow 2002). The bioavailability of sediment-associated metals is, in turn, controlled by metals being bound to and subsequently released from the sediments (Chapman et al. 1998). The most bioavailable phase of metals in sediments is, in general, free metal ions in sediment porewaters, while metals

that are bound to the sediments may not be toxic to organisms (Chapman and Long 1983). When multiple heavy metals exist simultaneously in an environment, the bioavailabilities of these metals are difficult to predict because adsorptive bonding strength between metals and sediments differ for different metals and types of sediment (Guy and Chakrabarti 1976, Benjamin and Leckie 1981b). Bioavailability is also affected by the presence or absence of other metals (Gadde and Laitinen 1974, Benjamin and Leckie 1981a).

Various environmental guidelines have been proposed and implemented to regulate the amounts of metal contaminants in aquatic environments (Long et al. 1995, ANZECC 2000, ARC 2004). Such guideline values are generally derived based on data from laboratory bioassays and field studies (Long et al. 1995). Field studies, in which sediments are often contaminated with multiple contaminants, cannot establish causal links between a specific contaminant and responses of organisms (Borgmann 2003). When environmental guidelines are largely based on laboratory experiments that tend to examine only one metal at a time, guideline values may not be relevant for real field situations where multiple metals simultaneously exist. As different heavy metals have been shown to affect different physiological processes of organisms (Grosell et al. 2002, Muysen et al. 2006), the simultaneous effects of multiple metals may be additive (Folt et al. 1999). However, the combined effects of multiple anthropogenic stressors have also been shown to result in complex non-additive effects (Christensen et al. 2006, Crain et al. 2008). Laboratory experiments that rigorously examine the effects of multiple metals and their interactions are needed. Such experiments can provide useful information to assess the appropriateness of environmental guidelines.

Bivalves have been used for decades to monitor or assess anthropogenic impacts on estuarine organisms, including heavy metals (Roper et al. 1995, King et al. 2004) chemical contaminants (Phillips 1985, Burgess and McKinney 1999) or sedimentation

(Cummings and Thrush 2004, Norkko et al. 2006). Bivalves, especially the common mussel, *Mytilus edulis*, have been used to monitor levels of heavy metals in their surrounding environment, due to their ability to accumulate metals and their relatively wide distribution in temperate waters (i.e. 'Mussel Watch'; Goldberg 1975, Goldberg et al. 1978). The use of bivalves in bioassays to assess the toxicity of contaminated sediments has been recommended; the desirable characteristics of test species include ease of collection, high tolerance to laboratory and experimental conditions, sensitivity to toxicants and ecological relevance to the environments of concern (EPA/USACE 1991). In New Zealand, the deposit-feeding bivalve, *Macomona liliana*, may be suitable for an assessment of the effects of heavy metals, due to their widespread distribution and common occurrence in estuaries. They are an important prey item for large predators including the eagle ray, *Myliobatis tenuicaudatus* (Thrush et al. 1991) and, therefore, potentially play an important role in estuarine food chains. *M. liliana* have been shown to be sensitive to heavy metals in the field (Fukunaga et al. 2010; Chapter 2 herein) and in the laboratory (Roper and Hickey 1994, Roper et al. 1995). Previous laboratory experiments involving this species to examine the effects of various environmental stressors (Roper and Hickey 1994, Hickey et al. 1995, Cummings and Thrush 2004) also suggest their robustness to laboratory conditions.

In the region of Auckland, New Zealand, effects of polluted stormwater runoff are of particular concern, due to Auckland's relatively large population and the discharge of its stormwater into sheltered harbours and estuaries (ARC 2004). The primary sediment contaminants in runoff include copper (Cu), lead (Pb) and zinc (Zn). Concentrations of these metals in estuarine sediments tend to spatially correlate with one another across the region (Hewitt et al. 2009). Here, I describe two series of laboratory bioassays designed to examine the acute toxicity of Cu, Pb and Zn, individually and in combination, on the

deposit-feeding bivalve *M. liliانا* at concentrations relevant to Auckland's estuaries. The bioassays used sediments spiked with Cu, Pb and/or Zn and examined mortality of *M. liliانا* after 10 days. Although sediment-associated metals are generally not the most bioavailable form of metals, deposit-feeders ingest sediments as a food source, and therefore, they may take up considerable amounts of heavy metals through contaminated sediments (Chapman et al. 2002). It was hypothesised that increases in metal concentrations would result in increased mortality rates for this species. It was also hypothesised that sediments spiked with multiple metals would cause greater mortality of *M. liliانا* than those spiked with single metals alone.

Methods

Sediment spiking and general procedure

The study was done at the Leigh Marine Laboratory, University of Auckland. Prior to each bioassay, the bivalves, *Macomona liliانا*, were collected from Puhoi estuary (36° 31' 53S, 174° 42' 16E), located approximately 40 km north of the city of Auckland, by sieving sediments through 800 µm mesh. They were kept in buckets with running seawater in the laboratory for a maximum of four days. Bivalves that were collected were 12 – 20 mm in size. Sediments were collected from the surface aerobic layer at an intertidal mudflat in Orewa estuary (36° 35' 45S, 174° 40' 51E), approximately 30 km north of the city of Auckland, brought back to the laboratory and sieved through 500 µm mesh to remove macrofauna. Sediments were homogenised and small amounts of sediment samples were taken to determine the water content (%) by the weight loss after drying at 60 °C for 24 hours. The sediments were then spiked with metals following the spiking method of Lu et al. (2008). Briefly, sediments and seawater were poured into small containers or large barrels, depending on the amount of sediment being spiked. The

solid nitrate salts ($\text{Cu}(\text{NO}_3)_2 \cdot 3\text{H}_2\text{O}$, $\text{Pb}(\text{NO}_3)_2$ and/or $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$) were dissolved in deionised water and then added to the mixture of sediments and seawater. The ratios of liquid to solid in the mixture were approximately 5 to 5.5. The containers/barrels were placed on rotating wheels, and the contents were continuously mixed for 36 hours, during which time adsorption of heavy metals onto the sediments would occur. No adjustments to pH or temperature were made during the spiking procedure.

Metal-spiked sediments were rinsed and placed in plastic containers (150 mm × 150 mm × 85 mm). Each container had 450 mL of sediment to a thickness of 20 mm, and was equipped with an independent input and output of seawater. Tidal cycles were mimicked by alternating six-hour inflow and six-hour outflow of seawater through individual containers. The average flow of seawater through the system was 0.4 cm³ per second, so the entire content of water in each 2 L container was replaced approximately every 80 minutes. Containers were placed outside at the laboratory in a randomised array and exposed to natural weather conditions. For each bioassay, containers were first established for 24 hours (day 0) to condition the sediments, and then 10 individuals of *M. liliana* were added to each container the next day (day 1). Bioassays were run for 10 days, with only the input and output of filtered seawater (200 µm), as described above, during this period. The number of live individuals in each container was recorded after 10 days (day 10).

Experiment 1

A series of seven bioassays was done from August to November 2008. Each bioassay investigated different combinations of metals: Cu, Pb, Zn, Cu + Pb, Pb + Zn, Cu + Zn, and Cu + Pb + Zn (Table 3.1). The target concentrations of these metals for each combination were chosen to preserve the regional average ratio of their concentrations,

Cu:Pb:Zn = 10.7:16.8:72.5 by weight. This ratio was calculated based on the heavy metal concentrations measured from each of 84 sheltered intertidal soft-sediment sites across the broader Auckland region (Anderson et al. 2006b). Each bioassay had six different levels of metal concentration and a control, with four replicates each (Table 3.1).

Table 3.1. List of starting dates and target concentrations of metals ($\mu\text{g g}^{-1}$) for each bioassay in Experiment 1 from August to November 2008.

Bioassay	Day 0	Metal concentrations ($\mu\text{g g}^{-1}$)						
		Metals	Level 1	Level 2	Level 3	Level 4	Level 5	Level 6
Cu	Sep. 6	Cu	15.0	25.0	35.0	45.0	75.0	100.0
Pb	Aug. 20	Pb	30.0	55.0	80.0	160.0	320.0	640.0
Zn	Sep. 20	Zn	75.0	140.0	200.0	300.0	400.0	500.0
Cu + Pb	Nov. 15	Cu	25.0	45.0	60.0	80.0	100.0	120.0
		Pb	39.3	70.7	94.2	125.6	157.0	188.4
Pb + Zn	Nov. 1	Pb	39.3	70.7	94.2	125.6	157.0	188.4
		Zn	169.4	304.9	406.5	542.1	677.6	813.1
Cu + Zn	Oct. 18	Cu	25.0	45.0	60.0	80.0	100.0	120.0
		Zn	169.4	304.9	406.5	542.1	677.6	813.1
Cu + Pb + Zn	Oct. 4	Cu	25.0	45.0	60.0	80.0	100.0	120.0
		Pb	39.3	70.7	94.2	125.6	157.0	188.4
		Zn	169.4	304.9	406.5	542.1	677.6	813.1

A small amount of sediment was collected from each container on day 1, prior to the addition of animals, to measure the concentrations of metals and total organic matter (TOM) in sediments. The sediment samples were dried in an oven at 60 °C for 24 hours. Concentrations of metals were determined by flame atomic absorption spectroscopy (FAAS) following digestion of dry sediment in hot aqua regia (HCl:HNO₃=3:1) using high-purity HCl and HNO₃ (Chen and Ma 2001) and dilution ($\times 30$) of digested sediment with deionised water. The amount of TOM was determined by combusting dried

sediments at 500 °C for 4 hours (Byers et al. 1978). TOM (%) was calculated by the weight loss after combustion.

Porewater samples were collected in each container over the 10-day experimental period by burying a peeper (mesh size 37 µm) filled with deionised water in the sediments (Adams et al. 2003). The deionised water equalised with porewater over the experimental period. Porewater samples were filtered through a 0.45 µm membrane and acidified with high-purity HNO₃. Porewater analysis was first attempted using graphite-furnace atomic absorption spectroscopy (GFAAS) as concentrations of metals in porewater are generally relatively low. However, due to interference by components found in seawater, samples were analysed using FAAS instead. Detection limits of Cu, Pb and Zn were 0.1 mg L⁻¹, 0.5 mg L⁻¹ and 0.1 mg L⁻¹, respectively.

Experiment 2

Another series of bioassays was done from June to July 2009. These bioassays supplemented Experiment 1, in which some of the bioassays suffered low survival of *M. liliانا* in the control treatment (see results for details) and were also designed to elucidate the potential effects of Pb in combination with Cu and/or Zn. The bioassays examined Cu, Zn and a mixture of Cu and Zn (Cu + Zn) at three different concentrations (low, mid and high) and either with or without Pb (Table 3.2). Concentrations of these metals for each combination again preserved the regional average ratio described above. Each bioassay also included a control treatment with no addition of metals. There were four replicates for each treatment. Sediments with different concentrations of metals were produced in a different manner from those for Experiment 1, in which each level was separately spiked with metals in small containers. For Experiment 2, different concentrations of metals were achieved by spiking sediments to the highest target level and then mixing the spiked

sediments with control sediments, which also went through the spiking procedure but without added metals.

Table 3.2. List of starting dates and target concentrations of metals ($\mu\text{g g}^{-1}$) for each bioassay in Experiment 2 from June to July 2009.

Bioassay	Starting date (day 0)	Metal	Metal concentrations ($\mu\text{g g}^{-1}$)					
			Without Pb			With Pb		
			Low	Mid	High	Low	Mid	High
Cu	Jul. 10	Cu	24.0	48.0	72.0	24.0	48.0	72.0
		Pb				37.7	75.4	113.0
Zn	Jun. 26	Zn	165.0	330.0	495.0	165.0	330.0	495.0
		Pb				38.2	76.5	114.7
Cu + Zn	Jun. 12	Cu	17.0	34.0	51.0	17.0	34.0	51.0
		Zn	115.2	230.4	345.6	115.2	230.4	345.6
		Pb				26.7	53.4	80.1

To determine concentrations of metals and TOM in sediments, a small amount of sediment was collected from each container on day 1, prior to the addition of animals, and day 10 when the number of live bivalves was counted. Porewater was also collected from each container over each experimental period. The sediment and porewater samples were processed and analysed as described above, with one exception; for Pb concentrations, porewater samples taken from sediment spiked with Pb were analysed by Hill Laboratories (Hamilton, New Zealand) using inductively coupled plasma-mass spectrometry (ICP-MS). Four samples were also randomly chosen from those collected in the control treatment and were analysed by ICP-MS in order to determine a reference background concentration for Pb in these bioassays. The detection limit of Pb using ICP-MS was 0.001 mg L^{-1} .

The mortality rate of *M. liliانا* was modelled using binomial logistic regression with logit link function in the statistical software package R (version 2.10). This was done separately for concentrations of Cu and Zn in sediments, treating different combinations of

metals as an additional factor in the analysis. Thus, the analysis with Cu concentrations had one treatment factor with four levels (Cu, Cu + Pb, Cu + Zn, and Cu + Pb + Zn). The analysis with concentrations of Zn also had one treatment factor with four levels (Zn, Cu + Zn, Pb + Zn, and Cu + Pb + Zn). Note that concentrations of other metals (i.e. Pb or Zn for Cu analysis, Cu or Pb for Zn analysis) varied in a fashion that is proportional to the occurrence of the metal being analysed based on the regional average ratio. From these models, concentrations of Cu and Zn resulting in 50% mortality of the bivalves (LC_{50}) were estimated individually and also in the presence of other metals.

Results

Experiment 1

Sediment collected from Orewa estuary for metal-spiking had a water content ranging from 37.4% to 46.1% by weight, and a TOM content of 3 – 6% (Table 3.3). Sediment spiking mostly elevated concentrations of Cu, Pb and Zn in sediments to their target levels (Fig. 3.1). At higher target levels, however, concentrations of Cu and Zn undershot target levels by up to 20 – 30%, while concentrations of Pb overshot target levels by approximately 25%. Although the spiking could not precisely achieve higher target levels of Cu and Zn, it successfully created gradients of metal concentration, with the highest concentrations of Cu and Zn substantially exceeding concentrations in the control sediments (at least 45 and 20 times higher for Cu and Zn, respectively).

Average concentrations of Cu and Zn in porewater increased with increasing concentrations of these metals in sediments (Fig. 3.2). The examination of Zn between sediments and porewater showed two distinguishable groupings: the Zn and Cu + Pb + Zn bioassays and the Pb + Zn and Cu + Zn bioassays (Fig. 3.2b). This is possibly due to a dilution effect resulting from the higher water content of sediments used for the Zn and Cu

+ Pb + Zn bioassays (above 45%) in comparison to those for the Pb + Zn and Cu + Zn bioassays (below 40%, Table 3.3). Such a difference was not clearly observed with Cu (Fig. 3.2a). Concentrations of Cu in porewater were, however, generally low, being under or close to the detection limit for FAAS of 0.1 mg L^{-1} , and therefore it was extremely difficult to obtain reliable measurements. Similarly, concentrations of Pb in porewater were below the detection limit of FAAS and could not be determined.

Table 3.3. Water content (WC; %) in sediments collected for metal-spiking in Experiment 1, measured prior to spiking, and TOM \pm SE (%) in sediments on day 1.

Bioassay	WC (%)	TOM \pm SE (%)
Cu	45.3	5.86 ± 0.12
Pb	43.3	5.84 ± 0.15
Zn	45.5	4.53 ± 0.07
Cu + Pb	40.6	3.69 ± 0.10
Pb + Zn	37.4	3.26 ± 0.07
Cu + Zn	39.2	4.03 ± 0.07
Cu + Pb + Zn	46.1	5.36 ± 0.11

No mortality of *Macomona liliana* was observed in the bioassay with Pb, even at the highest target level of $640 \text{ } \mu\text{g g}^{-1}$ of Pb in sediments. Survival rates of *M. liliana* in all other bioassays decreased with increasing levels of metals in sediments (Fig. 3.3).

Nominal concentrations of Cu and Zn that individually caused 50% mortality were approximately $90 \text{ } \mu\text{g g}^{-1}$ and $450 \text{ } \mu\text{g g}^{-1}$, respectively. Adding Pb to Cu or Zn reduced survival rates, but adding Pb to a mixture of Cu and Zn increased overall survival. The lowest overall survival was observed in the Cu + Zn bioassay; 50% mortality of *M. liliana* was caused at nominal concentrations of approximately $30 \text{ } \mu\text{g g}^{-1}$ of Cu and $200 \text{ } \mu\text{g g}^{-1}$ of Zn. The Cu + Zn and Pb + Zn bioassays had relatively lower average survival rate (< 85%) in the control treatments (Fig. 3.3), suggesting that some unknown factors affected

these two bioassays, causing lower overall survival.

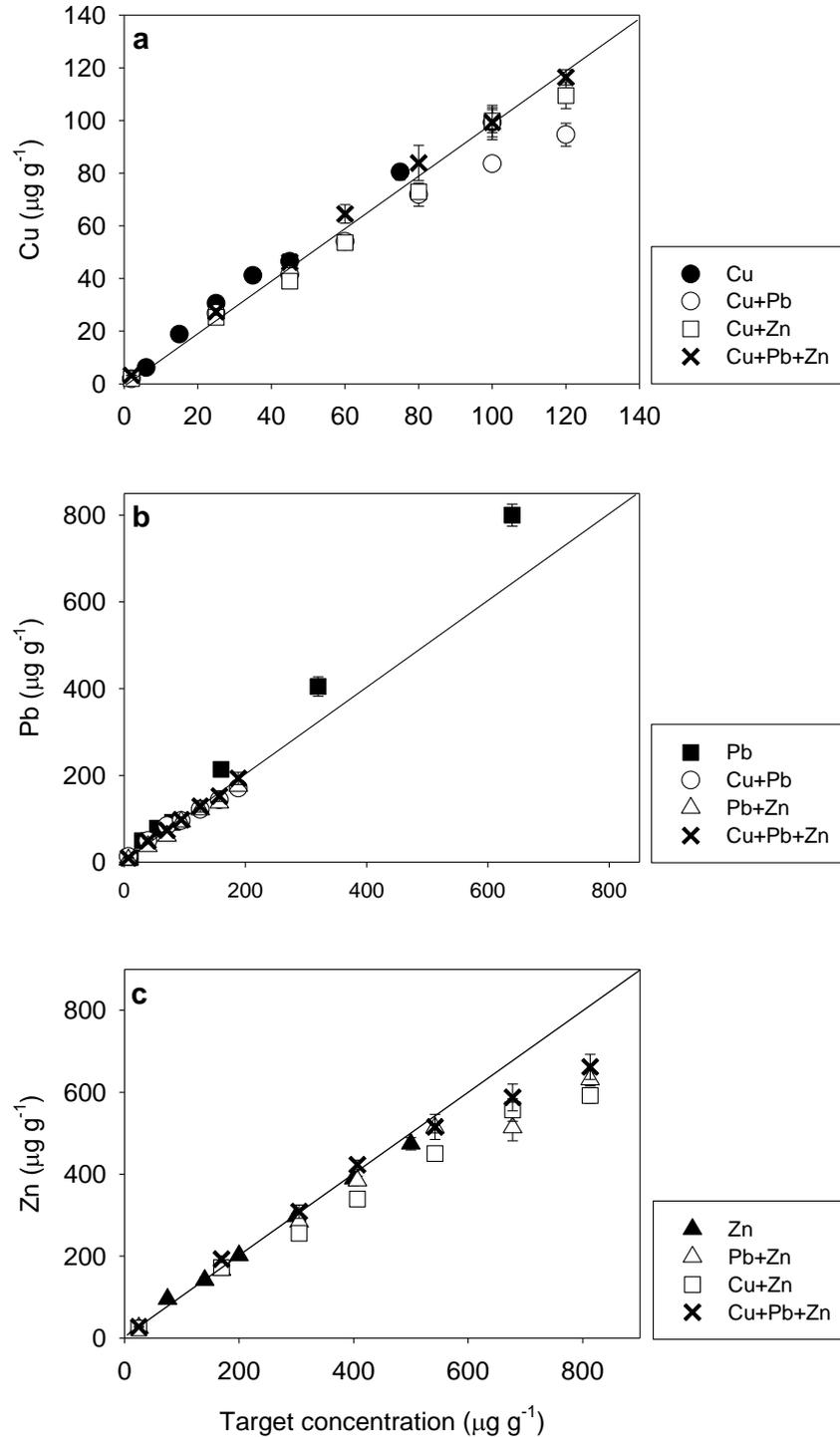


Fig. 3.1. Initial concentrations of metals in sediments (mean \pm SE) in Experiment 1, for (a) Cu, (b) Pb and (c) Zn. The solid line indicates a one-to-one relationship (i.e. where the measured concentration is equal to the target concentration).

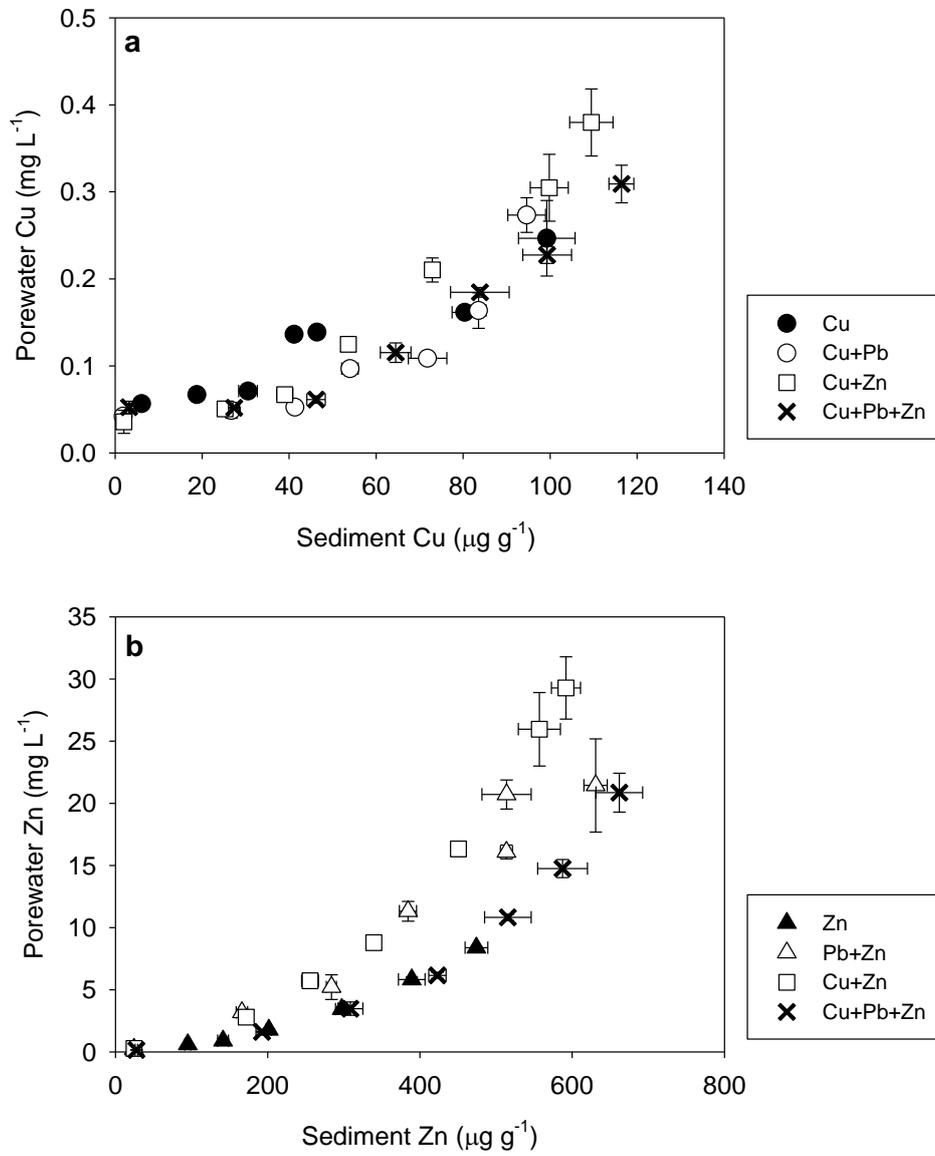


Fig. 3.2. Relationship between concentrations of metals in porewater (mean \pm SE) and those in sediments (mean \pm SE) in Experiment 1, for (a) Cu and (b) Zn.

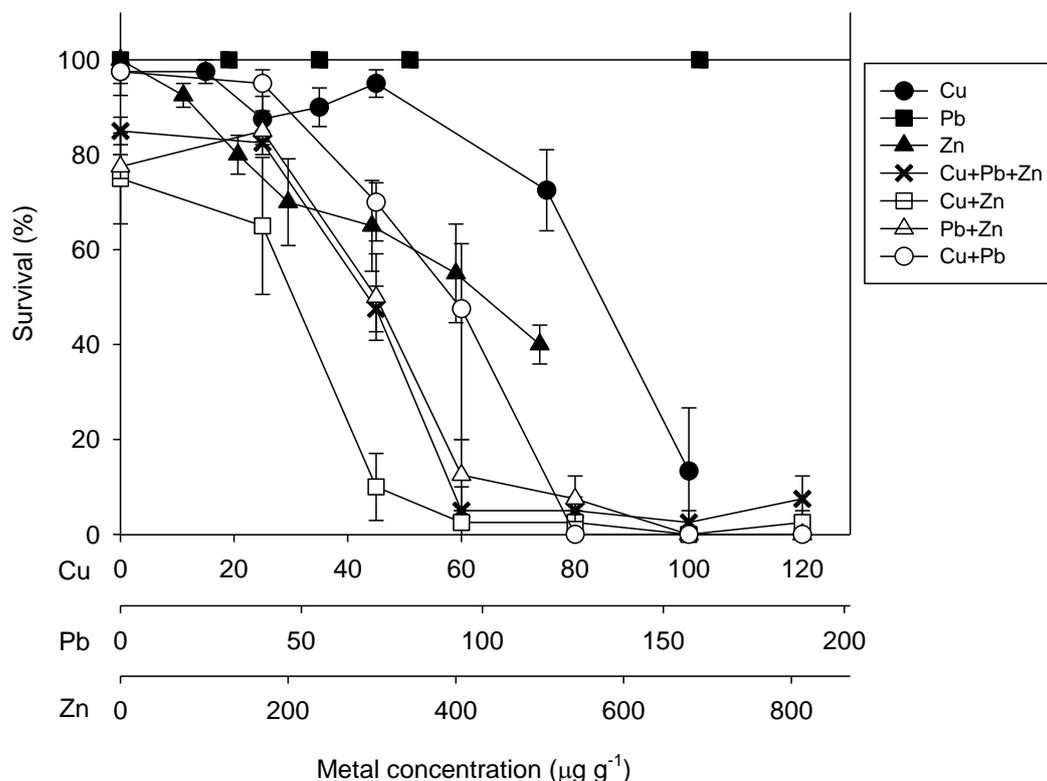


Fig. 3.3. Survival rates (%) of *M. liliana* plotted against nominal (target) concentrations of metals in sediments in the seven bioassays in Experiment 1. Those for the two highest target levels in the Pb bioassay (320 and 640 $\mu\text{g g}^{-1}$) all had 100% survival (not shown). The control treatments for each bioassay had nominal metal concentrations of 0 $\mu\text{g g}^{-1}$.

When survival rates of *M. liliana* were examined directly with porewater concentrations of Cu or Zn, the Cu + Zn and Cu + Pb + Zn bioassays showed similar patterns of survival, having the lowest overall survival of *M. liliana* (Fig. 3.4). The Zn and Pb + Zn bioassays also showed a somewhat similar pattern of survival with respect to concentrations of Zn (Fig. 3.4b). With respect to concentrations of Cu, the Cu bioassays showed considerably higher survival rates than any other bioassays with multiple metals (Fig. 3.4a).

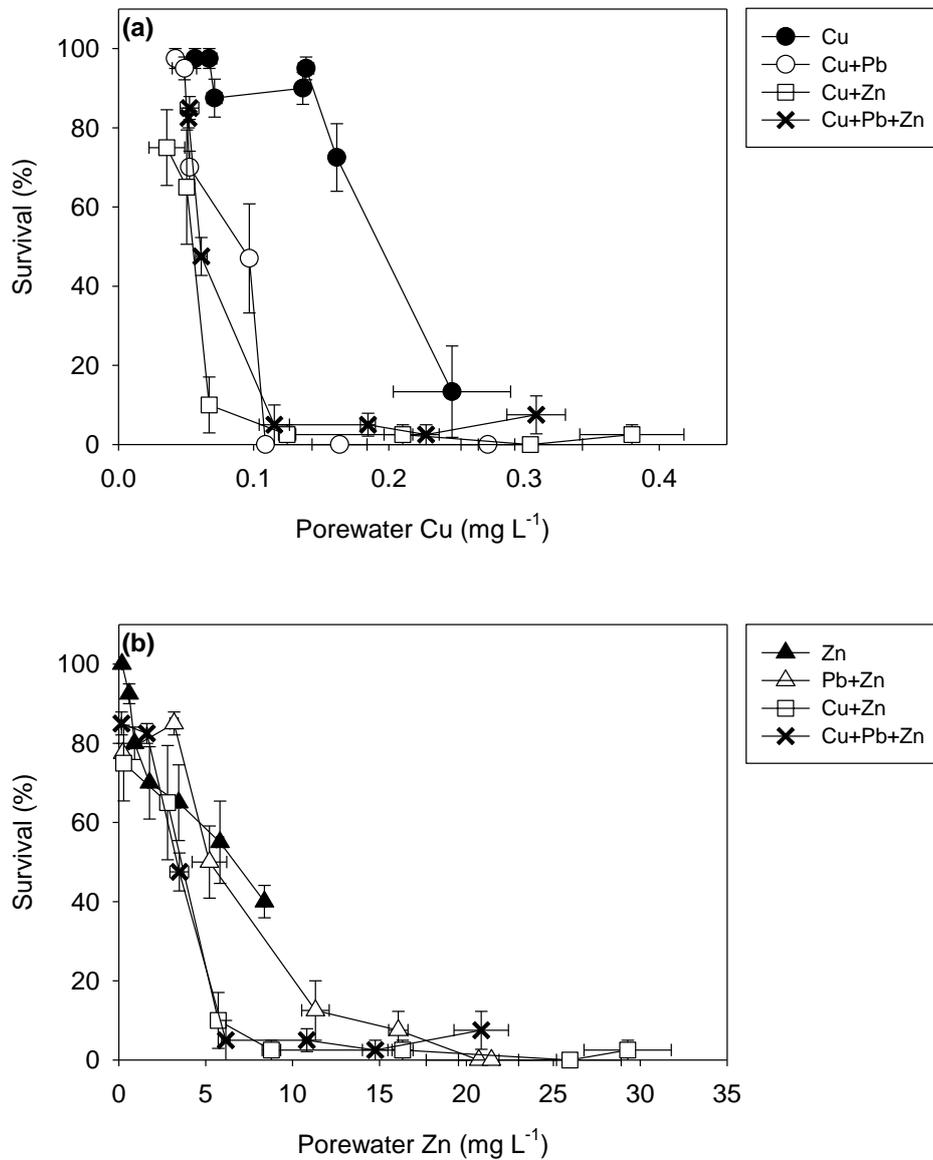


Fig. 3.4. Survival rates (%) of *M. liliana* plotted with concentrations of (a) Cu and (b) Zn in porewater in Experiment 1.

Experiment 2

Sediment collected from Orewa estuary for metal-spiking had a water content ranging from 31.1% to 33.7%, and a TOM content of 1.5 – 2.5% (Table 3.4). Sediment spiking mostly elevated concentrations of Cu, Pb and Zn in sediments to their target levels

(Fig. 3.5), although concentrations of Zn were generally somewhat lower than target concentrations. Concentrations of Pb in sediments without added Pb were below the detection limit of $0.5 \mu\text{g g}^{-1}$ for this element. Considering the $30\times$ dilution after the acid digestion in sample preparation, background concentrations of Pb in sediments were determined to be less than $15 \mu\text{g g}^{-1}$.

Table 3.4. Water content (WC; %) in sediments collected for metal-spiking in Experiment 2, measured prior to spiking, and TOM \pm SE (%) in sediments on day 1 and day 10.

Bioassay	WC (%)	TOM \pm SE (%)	
		Day 1	Day 10
Cu	33.7	1.94 ± 0.04	1.65 ± 0.03
Zn	31.1	2.42 ± 0.06	2.29 ± 0.04
Cu + Zn	31.4	2.39 ± 0.05	2.36 ± 0.06

Average concentrations of Cu, Pb and Zn in porewater were mostly positively correlated with concentrations of these metals in sediments (Fig. 3.6). The highest average concentrations of these metals in porewater were observed, however, at the mid target levels in some cases: i.e. for Cu and Pb in the Cu bioassay (Fig. 3.6a, b) and for Cu and Zn in the Cu + Zn bioassay without Pb (Fig. 3.6a, c). Average concentrations of Cu or Zn in porewater at each target level in each bioassay differed between samples without added Pb and those with added Pb, but these effects were not consistent in their direction or magnitude. In the Zn bioassay, however, concentrations of Pb in porewater mostly stayed under or around the detection limit of 0.001 mg L^{-1} even at the highest target level (Fig. 3.6b), suggesting the possibility that Pb competed with Cu for binding sites on sediments but not with Zn.

No mortality of *M. liliانا* was observed in the control treatment, nor at the low target level in any of the bioassays (Fig. 3.7). Mortality rates, however, significantly

increased with increases in concentrations of either Cu or Zn ($p < 0.0005$, Table 3.5). The LC_{50} values for Cu and Zn were $81.0 \mu\text{g g}^{-1}$ and $474.8 \mu\text{g g}^{-1}$, respectively. The combined effects of Cu and Zn on mortality of *M. liliانا* were significantly greater than the effects of either metal alone, with LC_{50} values being decreased to $68.0 \mu\text{g g}^{-1}$ for Cu when combined with Zn ($p = 0.040$) and $348.1 \mu\text{g g}^{-1}$ for Zn when combined with Cu ($p < 0.0005$). Adding Pb to the mixture of Cu and Zn had apparently no significant effects on average survival rates of *M. liliانا*, with negligible differences in LC_{50} values between the Cu + Zn and the Cu + Pb + Zn bioassays (Table 3.5). There was no statistically significant difference in mortality of *M. liliانا* when Pb was added to Cu ($p = 0.656$), while adding Pb to Zn resulted in a marginal p-value ($p = 0.072$), with a reduction in the LC_{50} value to $443.9 \mu\text{g g}^{-1}$ for Zn.

Table 3.5. Summary of results of binomial logistic regression analysis of mortality rates of *M. liliانا* with logit link function using concentrations of Cu or Zn in sediments and calculated LC_{50} values ($\mu\text{g g}^{-1}$) of Cu and Zn.

Predictor	Coeff	SE Coeff	p	Odds Ratio	95% CI		LC_{50}
					Lower	Upper	
Sediment Cu							
Constant	-6.9396	0.9877	< 0.0005				
Cu	0.0857	0.0158	< 0.0005	1.09	1.06	1.12	81.0
Metal							
Cu+Pb	-0.2204	0.4945	0.656	0.80	0.30	2.11	83.5
Cu+Zn	1.1133	0.5409	0.040	3.04	1.05	8.79	68.0
Cu+Pb+Zn	0.9570	0.5457	0.080	2.60	0.89	7.59	69.8
Sediment Zn							
Constant	-14.2922	2.7096	< 0.0005				
Zn	0.0301	0.0063	< 0.0005	1.03	1.02	1.04	474.8
Metal							
Pb+Zn	0.9313	0.5179	0.072	2.54	0.92	7.00	443.9
Cu+Zn	3.8142	0.9645	< 0.0005	45.34	6.85	300.23	348.1
Cu+Pb+Zn	3.7552	0.9739	< 0.0005	42.74	6.34	288.31	350.1

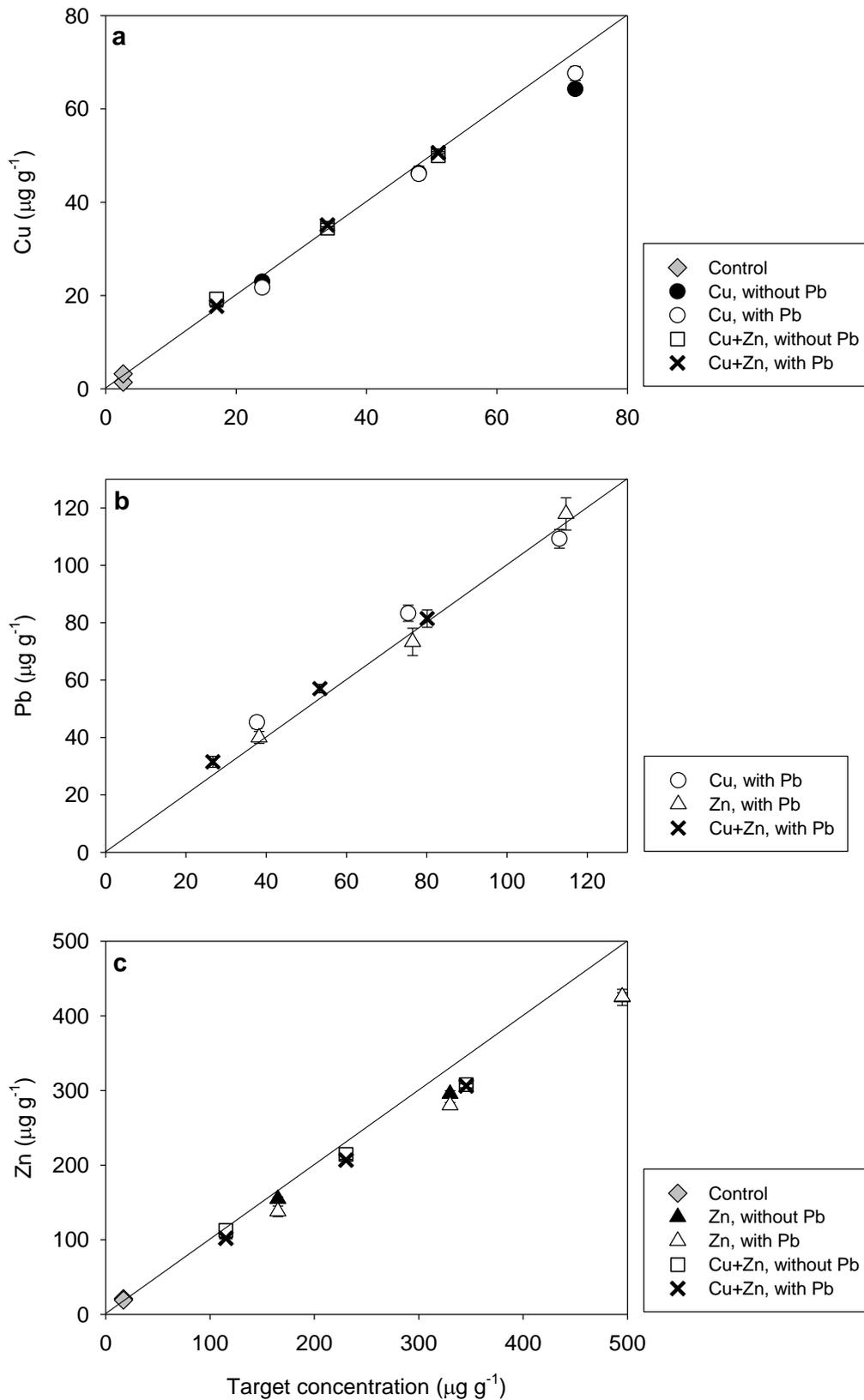


Fig. 3.5. Average metal concentrations in sediments (mean \pm SE) in Experiment 2, for (a) Cu, (b) Pb and (c) Zn. The solid line indicates a one-to-one relationship (i.e. where the measured concentration is equal to the target concentration).

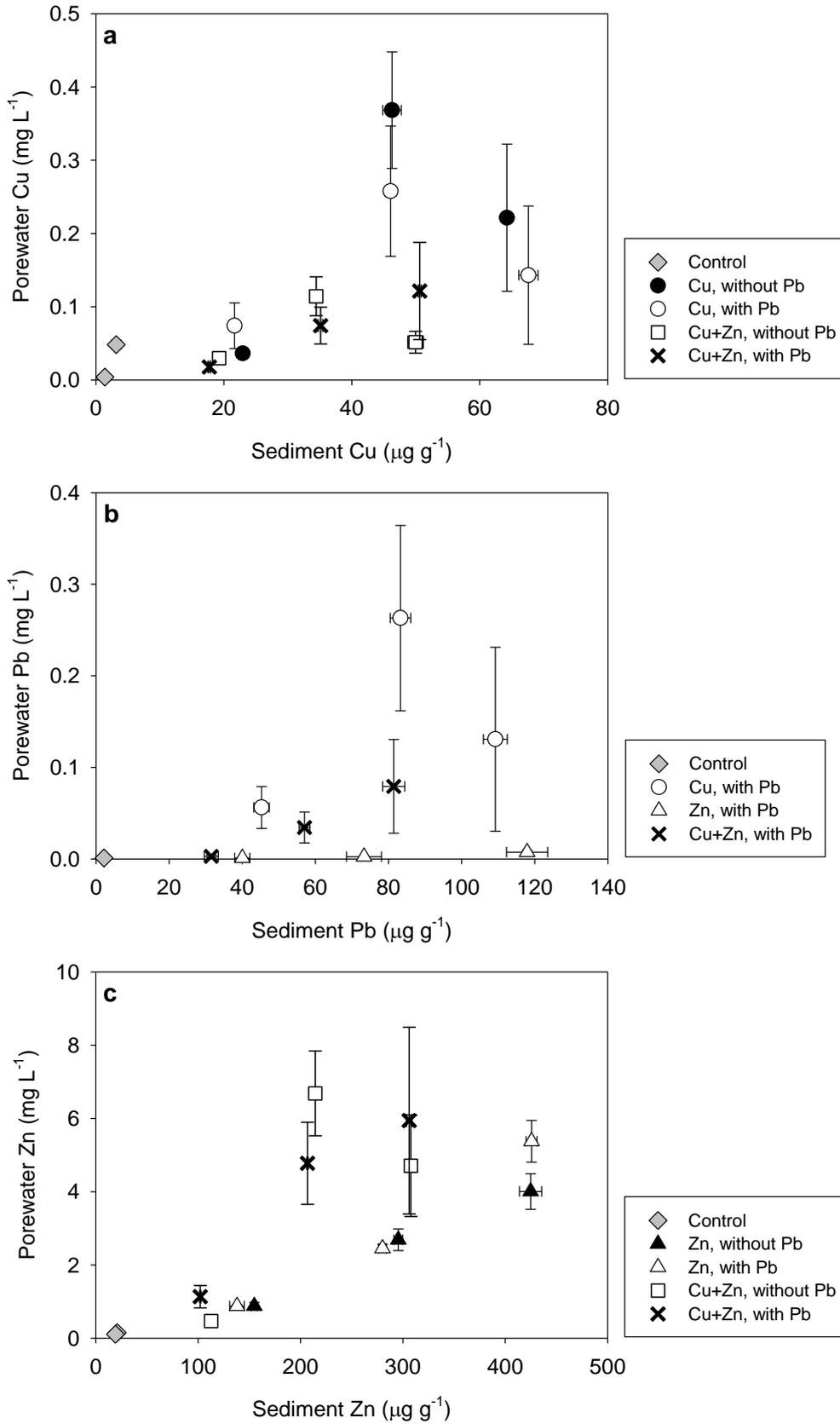


Fig. 3.6. Metal concentrations in porewater (mean \pm SE) with respect to those in sediments (mean \pm SE) in Experiment 2, for (a) Cu, (b) Pb and (c) Zn.

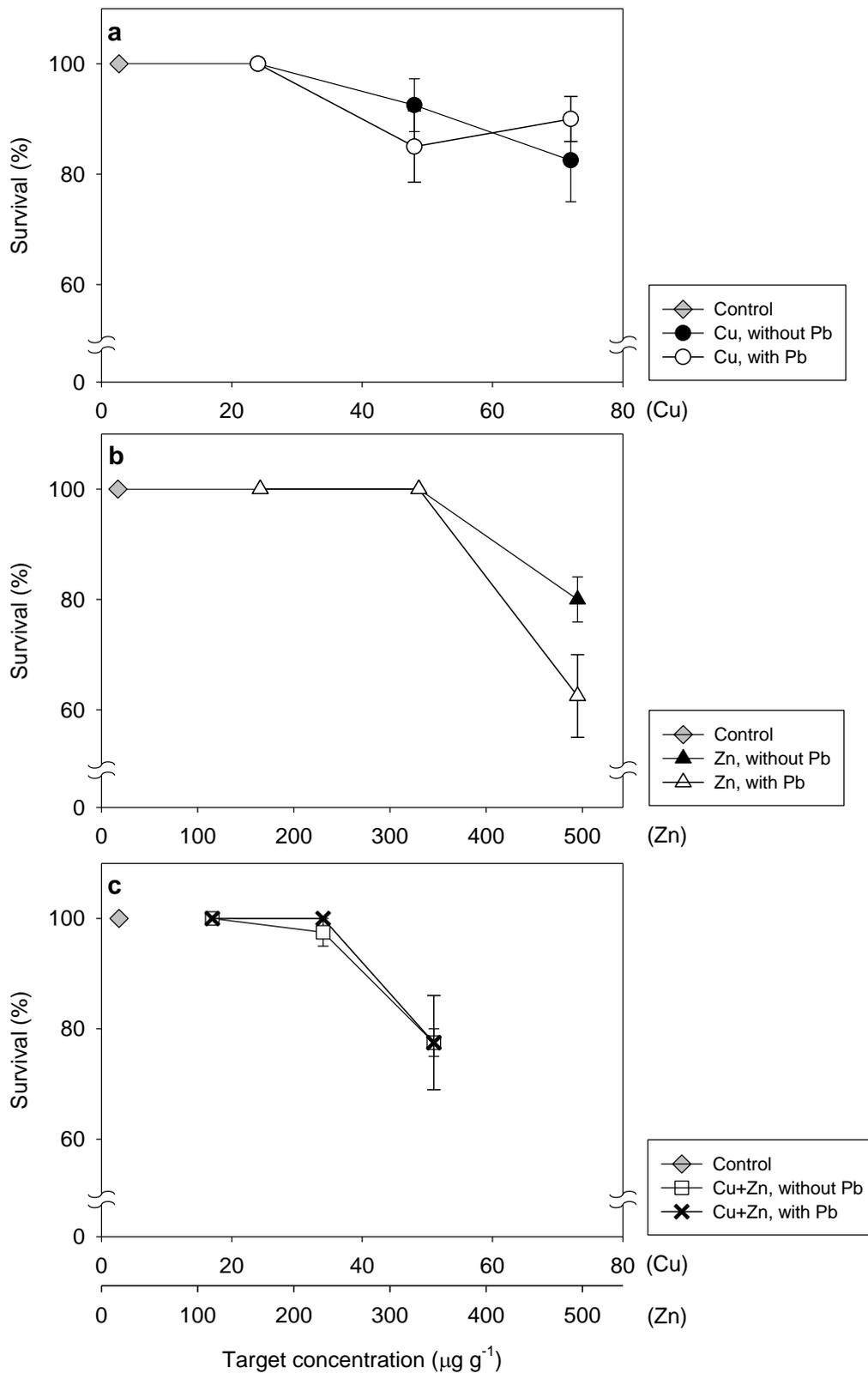


Fig. 3.7. Survival rate (%) of *M. liliiana* plotted with nominal (target) concentrations of metals in sediments in the three bioassays in Experiment 2: the bioassay with (a) Cu, (b) Zn and (c) Cu and Zn, with and without Pb.

Discussion

Previous laboratory investigations of the acute effects of heavy metals on estuarine organisms commonly involved 10-day exposure of target organisms to sediments contaminated with individual metals of interest, followed by determination of LC₅₀ values as one of the standard point estimates of metal toxicity (Roper and Hickey 1994, Bat and Raffaelli 1998, Marsden and Wong 2001, King et al. 2004). LC₅₀ values could then be used to compare the toxicities of individual metals to a particular organism and/or the sensitivities of different organisms to a particular metal (Bat and Raffaelli 1998). The present study, in contrast, focused on examining the simultaneous effects of multiple metals and over a range of concentrations that were specifically relevant to Auckland's estuaries. Folt et al. (1999) categorised the simultaneous effects of multiple stressors into three possible models: additive, multiplicative and simple comparative effects, in which the simultaneous effect of multiple stressors is equal to the effect of a single dominant stressor. The term "cumulative effect" will be used here to describe the situation of simultaneous effects of multiple metals being greater than the effects of a single dominant metal, as the present study was not designed to classify effects explicitly as either additive or multiplicative (but see Chapter 4). Cumulative effects, as described here, therefore may include (but are not limited to) additive and multiplicative effects.

Mortality of *Macomona liliana* increased as concentrations of either Cu or Zn increased. Separate individual effects of Cu and Zn on *M. liliana* have been demonstrated previously in the laboratory (Roper et al. 1995) and in the field (Fukunaga et al. 2010; Chapter 2 herein). The LC₅₀ value for juvenile *M. liliana* has been reported to be 17 µg g⁻¹ for Cu, and complete mortality has been observed at 75 µg g⁻¹ of Cu (Roper and Hickey 1994). Juvenile *M. liliana* have been shown to crawl or drift away from sediments spiked with Zn at a concentration of 40 µg g⁻¹ and to slow burial behaviour at 80 µg g⁻¹ (Roper et

al. 1995). The adverse effects of Cu and Zn have also been found in the estuarine amphipod *Corophium volutator* and the polychaete *Arenicola marina*, with LC₅₀ values of 37 µg g⁻¹ for Cu and 32 µg g⁻¹ for Zn for *C. volutator* and of 20 µg g⁻¹ for Cu and 50 µg g⁻¹ for Zn for *A. marina* (Bat and Raffaelli 1998). Results of the present experiments supported these previous findings, clearly showing the toxicity of Cu and Zn to *M. liliانا*, although adult individuals of *M. liliانا*, showing the LC₅₀ value of 81.0 µg g⁻¹ for Cu and 474.8 µg g⁻¹ for Zn, were apparently less sensitive to these metals than juvenile *M. liliانا* or other organisms in previous studies. The combined effects of Cu and Zn on mortality of *M. liliانا* were clearly cumulative. As the ranges of concentrations of Cu and Zn examined in this study were similar to those currently observed in estuaries in the Auckland Region (Kelly 2007, Stewart et al. 2009), heavy metal contamination in sediments may well be one of the important factors limiting the distribution of this bivalve in Auckland's estuaries.

There was no individual effect of Pb detected on the survival of *M. liliانا*. The combined effects of Pb with either Cu and/or Zn were apparently comparative in most cases. The potential cumulative effects of Pb were observed, however, in the presence of Cu in Experiment 1 and in the presence of Zn in Experiment 2. The variability in the effects of Pb may be due to differences in experimental conditions among different bioassays and/or the complex nature of the toxicity of heavy metals. The uptake of different heavy metals by a particular organism can be differentially affected by environmental conditions such as salinity and temperature (Phillips 1976, Denton and Burdon-Jones 1981). The simultaneous presence of multiple heavy metals may also affect the uptake of any individual metals (Phillips 1977). Once metals are taken up, the assimilation efficiency of heavy metals differ for different metals and can be correlated with assimilation of food (Janssen et al. 1991). For example, the assimilation efficiency of

the mussel *Mytilus edulis* has been shown to be affected by abundances of phytoplankton; a higher abundance of phytoplankton resulted in an increase in assimilation of Zn but a decrease in assimilation of cadmium (Wang et al. 1996). It would have been ideal to run all of the bioassays simultaneously using sediments collected all at once. This would have reduced variability in experimental conditions, but logistically it was not possible, due to the large amount of sediment required for these experiments, limitations on the number of treatments (combinations of metals) that could be spiked at any one time, and limits on the space available for the seawater system and the number of containers it could hold.

Mortality of *M. liliانا* in Experiment 2 was overall lower than that in Experiment 1. The only difference in the methods used for these two experiments was the way sediments were spiked: they were spiked separately to specific target levels in Experiment 1, while they were spiked to the high target level and then mixed with control sediments to achieve lower levels in Experiment 2. However, this alone cannot explain differences in results, because the spiked sediments for the high target level were not mixed with control sediments in either experiment, and therefore should have been comparable. Another difference in these two experiments was that the water content and TOM of the sediment in Experiment 1 were higher than for sediment in Experiment 2. TOM has a high binding affinity for some trace metals (Chapman et al. 1998), and the higher water contents should have resulted in dilution of metals in porewater. Therefore, these factors should have rather reduced bioavailabilities of metals in Experiment 1, not enhanced toxicity of the sediments.

These two experiments were done at slightly different times of year: Experiment 1 in late winter – spring and Experiment 2 in early – mid autumn. As containers for the experiments were placed outside the laboratory, water temperature and salinity (due to differences in rainfall) might have varied between these two experiments. Toxicities of

metals are affected by variations in salinity and temperature (Heugens et al. 2001). It is, therefore, possible that the difference in overall mortality between Experiment 1 and Experiment 2 originated from slightly different water conditions in the two experiments. Although the difference in mortality between these two experiments does not change the primary conclusion about the effects of these heavy metals, it calls for a caution in interpretation, as biological impacts of heavy metals can vary considerably depending on a host of environmental and sediment geochemical factors.

The overall difference in mortality of *M. liliana* between the two experiments also somewhat argues against the usefulness of LC₅₀ values as point estimates of the toxicity of individual heavy metals. The use of bioassays for an assessment of marine pollution, in combination with other means of investigation (i.e. chemical and ecological surveys), has been strongly advocated (Chapman and Long 1983). Laboratory bioassays clearly identify the effects of bioavailable contaminants on the species tested, under the conditions of the test (Chapman and Long 1983). In the case of heavy metals, however, results of bioassays measured as LC₅₀ values can differ considerably under different environmental conditions. For example, the LC₅₀ values of dissolved mercury for the hard clam *Meretrix lusoria* have been shown to be 341 and 140 µg L⁻¹ at salinities of 20 and 30, respectively (Chin and Chen 1993). In contrast, the LC₅₀ values of a mixture of heavy metals (Cu, cadmium, Pb and Zn) for the estuarine clam *Ruditapes philippinarum* have been shown to decrease (i.e. to have increased toxicity) with decreasing pH or salinity (Riba et al. 2004).

Defining LC₅₀ values of particular heavy metals in the presence of other metals is also difficult, as the nature of their combined effects may not necessarily be additive; they can be multiplicative or comparative, and synergism and antagonism may also occur (Folt et al. 1999). Thus, it is not clear how much each metal in a mixture contributes to the

observed toxicity. Nevertheless, the LC_{50} values for *M. liliانا* determined here clearly demonstrated increased toxicity as multiple metals were added to sediments, especially in the case of Cu and Zn. The LC_{50} values were then compared with existing sediment guidelines (Fig. 3.8): the Auckland Regional Council's environmental response criteria (ERC red zone value; ARC 2004) and the Australian/New Zealand interim sediment quality guideline (ISQG-Low and ISQG-High; ANZECC 2000). These guideline values were based on the North American sediment quality guidelines of Long et al. (1995), that were derived by compiling and arranging chemical and biological effects data from numerous field and laboratory studies in ascending order of concentration and choosing the lower 10th percentile of the effects data as the effects range-low (ERL, below which adverse effects are expected to rarely occur; equivalent to ISQG-Low) and the 50th percentile of the effects data as the effects range-median (ERM, above which adverse effects are predicted to frequently occur; equivalent to ISQG-High). The data used to determine the guidelines included LC_{50} values determined in laboratory bioassays using sediments spiked with a single metal. The ISQG-High apparently predicts the Zn toxicity to *M. liliانا* well when Cu does not co-occur (Fig. 3.8b). In the presence of both Cu and Zn, however, the ISQG-High underestimates the toxicity of the sediment to *M. liliانا*, if only the concentration of Zn is considered. The ISQG-High for Cu greatly underestimates the toxicity of Cu to *M. liliانا* (Fig. 3.8a). This is even more dramatic when Cu is accompanied by Zn, with 50% mortality of *M. liliانا* estimated to occur at the ISQG-Low value. Thus, these comparisons emphasize the importance of considering the co-occurrence of multiple metals when deriving environmental guidelines.

A discrepancy between environmental guideline values and results of the current study was detected for the effects of Pb. The highest target concentration of Pb tested in the bioassay as a single metal treatment was $640 \mu\text{g g}^{-1}$, which is more than five times the

highest concentration recorded from soft-sediment intertidal areas in the Auckland region (Hewitt et al. 2009) and well above the ERC red zone value of $50 \mu\text{g g}^{-1}$ (ARC 2004) or the ISQG-High value of $220 \mu\text{g g}^{-1}$ (ANZECC 2000). Environmental guideline values could overestimate the toxicity of heavy metals when data from field chemical and biological surveys are used to derive the values, as sediments in the field are often contaminated by more than one contaminant (Borgmann and Norwood 1999). Such surveys may reveal correlative relationships between levels of a mixture of contaminants and biological responses, but the correlative relationships do not necessarily mean that particular measured levels of individual contaminants present in the mixture are responsible for the observed effects (Borgmann 2003).

Another possible explanation for the discrepancy detected for the lack of any consistent effects of Pb is the extremely low bioavailability as a consequence of its strong binding to the surface of sediment particles (Borgmann and Norwood 1999). Strong binding of Pb to sediments was evident in the current study from the generally low concentrations of Pb measured in porewater (generally below detection limits). In Experiment 2, the highest average concentration of Pb in porewater was observed in the mid target level of the Cu bioassay (+Pb), in which mortality of *M. liliانا* was, on average, higher than that in the concomitant Cu treatment without Pb. These results highlight the sensitivity of *M. liliانا* to heavy metals in porewater. Additional water-only bioassays would have been useful in order to separate metal-uptake from sediments *versus* from solution, determining their relative contributions to bioavailability for *M. liliانا*, and therefore potentially providing a deeper understanding of simultaneous effects of multiple metals. This was, however, not possible as *M. liliانا* could not be kept alive in the laboratory without sediment media for a long time period (personal observation).

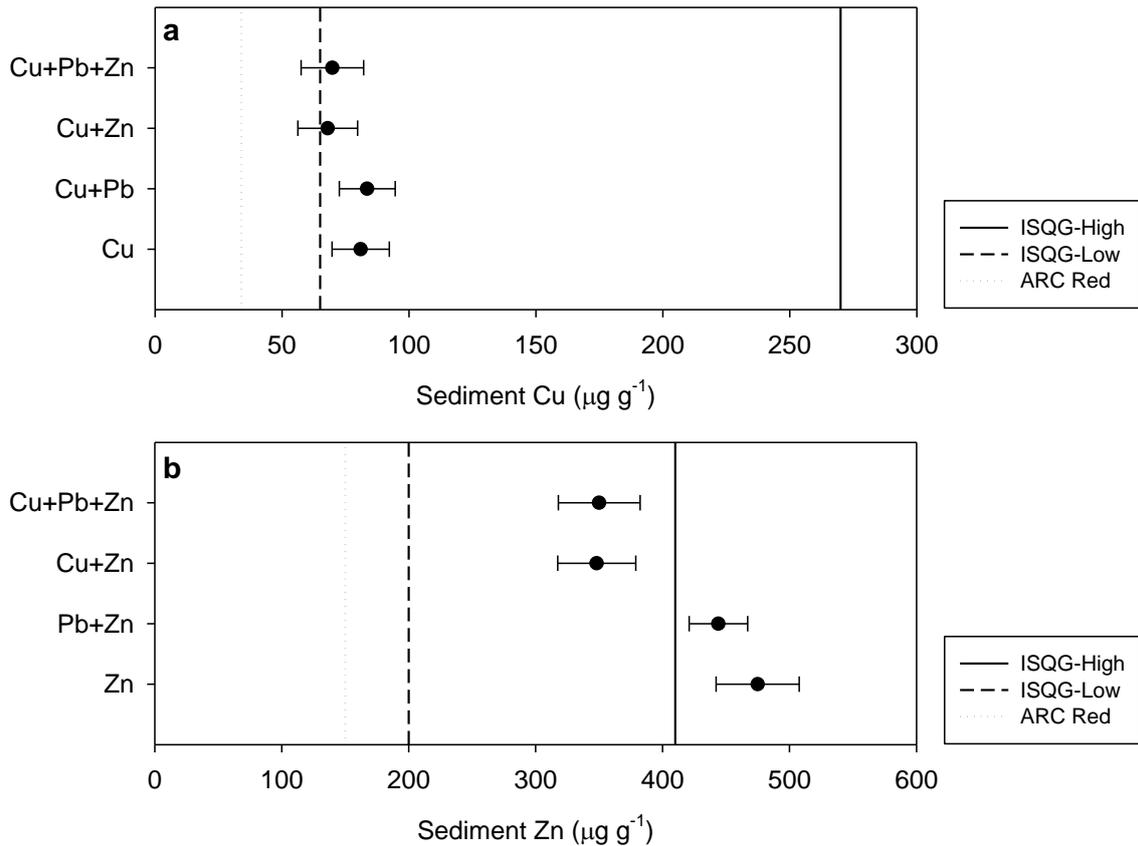


Fig. 3.8. LC₅₀ values and 95% confidence intervals for *M. liliiana* for (a) Cu and (b) Zn in the presence/absence of other metals. Vertical lines indicate the Australian and New Zealand guideline values (ISQG-High and ISQG-Low) and the Auckland Regional Council’s environmental response criteria (ARC Red). For Cu, ISQG-High = 270 $\mu\text{g g}^{-1}$, ISQG-Low = 65 $\mu\text{g g}^{-1}$ and ARC Red = 34 $\mu\text{g g}^{-1}$. For Zn, ISQG-High = 410 $\mu\text{g g}^{-1}$, ISQG-Low = 200 $\mu\text{g g}^{-1}$ and ARC Red = 150 $\mu\text{g g}^{-1}$.

Conclusions

Laboratory bioassays provide useful tools to examine closely the simultaneous effects of multiple metals on a specific organism in a controlled environment. The present study examined the effects of Cu, Pb and Zn at ranges of concentrations that were relevant to estuaries in the Auckland region. The current levels of Pb in the sediments of Auckland’s estuaries are unlikely to cause mortality of the estuarine bivalve *Macomona liliiana*, and may or may not exacerbate the effects of Cu and Zn. In contrast, elevated

concentrations of Cu and Zn significantly increased the mortality rates of *M. liliانا*. The combined effects of Cu and Zn were also clearly cumulative. The fact that Cu and Zn have negative effects that can be cumulative and which can vary, depending on other environmental factors, highlights the need for great care in the derivation of appropriate environmental guidelines for monitoring. Laboratory bioassays testing a single metal at a time may not be useful when considering the simultaneous and interactive effects of multiple heavy metals. On the flip side, collecting field data alone may not necessarily provide insights regarding the causal effects of a particular metal or the underlying interactive biological and geochemical mechanisms of the effects of multiple metals on individual species. Laboratory bioassays testing multiple heavy metals, such as the present study, can reveal interactions among multiple heavy metals in their effects. This information, when combined with field data, is crucial information to derive appropriate environmental guidelines for these species.

Chapter Four

Assessing the Nature of the Combined Effects of Copper and Zinc on Estuarine Infaunal Communities

Atsuko Fukunaga, Marti J. Anderson and Jenny G. Webster-Brown

This study is currently in review in *Environmental Pollution*.

Introduction

Aquatic ecosystems are threatened by various natural and anthropogenic stressors (Halpern et al. 2007). Interactions of multiple stressors can be complex, and their simultaneous effects are often difficult to predict based on the effects of individual stressors alone (Heugens et al. 2001, Christensen et al. 2006, Crain et al. 2008). A modelling framework for assessing multiple stressors was provided by Folt et al. (1999), who categorised the simultaneous effects of multiple stressors into three possible models: additive, multiplicative, and simple comparative effects, in which the combined effects of multiple stressors is equal to the effects of a single dominant stressor (Fig. 4.1). Folt et al. (1999), furthermore, considered that deviations could be observed, being either greater than (synergistic) or less than (antagonistic) predicted under any one of these models. For the simple comparative effects model, the terms “increased stress” and “decreased stress” were used to describe such deviations (see Fig. 4.1).

Estuarine benthic environments are often considered a ‘sink’ for metal pollutants (McLusky 1999). In soft-sediment systems, the bioavailability of heavy metals is

controlled by the strength of metal binding to the sediment. The most bioavailable form of a metal in sediment is, in general, the portion present as free metal ion in the sediment porewater; metals that are bound to the sediments are unlikely to be toxic to organisms unless ingested (Chapman and Long 1983). In aerobic sediments, iron and manganese hydroxides and particulate organic carbon are the dominant binding surfaces for metals (Olsen et al. 1982, Chapman et al. 1998). Adsorption of a given heavy metal onto these surfaces depends on the metal species present and is affected by the presence of other metals and environmental factors, such as salinity and pH (Gadde and Laitinen 1974, Guy and Chakrabarti 1976, Benjamin and Leckie 1981b, Millward and Moore 1982, Turner et al. 2004). Thus, the behaviour of heavy metals and their bioavailability in estuaries, especially when multiple metals are present, is highly complex.

In the region of Auckland, New Zealand, the primary sediment contaminants of concern include copper (Cu) and zinc (Zn) (ARC 2004). Concentrations of these metals in Auckland's estuaries and harbours are relatively low compared to those measured in similar environments in other industrialised countries (Kelly 2007). Ecological responses of estuarine infauna have been detected, however, at such relatively low levels (Thrush et al. 2008, Hewitt et al. 2009). Concentrations of these metals in the sediments also tend to correlate with one another spatially across the region (Hewitt et al. 2009). Although examining the simultaneous effects of multiple metals is important, due to such correlations, most studies have focused only on the effects of individual heavy metals in isolation (Roper and Hickey 1994, Morrissey et al. 1996, Bat and Raffaelli 1998) or in combination with other potential environmental stressors, such as temperature, salinity or total organic carbon (Denton and Burdon-Jones 1981, Alutoin et al. 2001, Lenihan et al. 2003), but rarely in combination with other metals (Hagopian-Schlekat et al. 2001).

Heavy metals have the potential to cause lethal and sublethal effects on estuarine

organisms (Bat and Raffaelli 1998). Although physiological mechanisms of metal toxicity have not been well studied for estuarine and/or marine organisms, it has been shown that Cu and Zn affect different physiological processes in freshwater fish and crustaceans: Cu induces disturbance of sodium balance, while Zn affects calcium uptake (Grosell et al. 2002, Muysen et al. 2006). Extending inferences from freshwater to estuarine conditions is complex (Bianchini et al. 2004, Pinho et al. 2007), but the freshwater examples do suggest that Cu and Zn may affect different physiological processes in estuarine organisms as well. If stressors affect different physiological processes, their simultaneous effects on individual organisms are predicted to be additive: i.e., equal to the sum of the effects caused by the individual stressors (Folt et al. 1999).

Responses of organisms to multiple heavy metals in the field are difficult to predict, especially at population and community levels, due to the complex chemistry of heavy metals affecting their bioavailabilities, differences in the sensitivities of organisms to different heavy metals (Bat and Raffaelli 1998, King et al. 2004) and inter-specific interactions between organisms. A recent manipulative field experiment clearly showed negative effects of Cu and Zn on recolonisation of defaunated sediments by estuarine infauna, but the nature of their simultaneous effects was far from clear (Fukunaga et al. 2010; Chapter 2 herein). Here, we describe a manipulative field experiment to assess the nature of the simultaneous effects of Cu and Zn on estuarine infauna. The experiment examined recolonisation of defaunated metal-spiked sediments by infaunal organisms over two weeks. We focused on examining the additivity of the effects, as would be expected under the working hypothesis of fundamental differences in the physiological mechanisms of toxicity for Cu *versus* Zn. More specifically, we used analysis of variance (ANOVA), which models the effects of individual factors on a response variable in an additive fashion, and therefore, a significant interaction term in a two-way ANOVA model

indicates directly the presence of non-additivity (Billick and Case 1994).

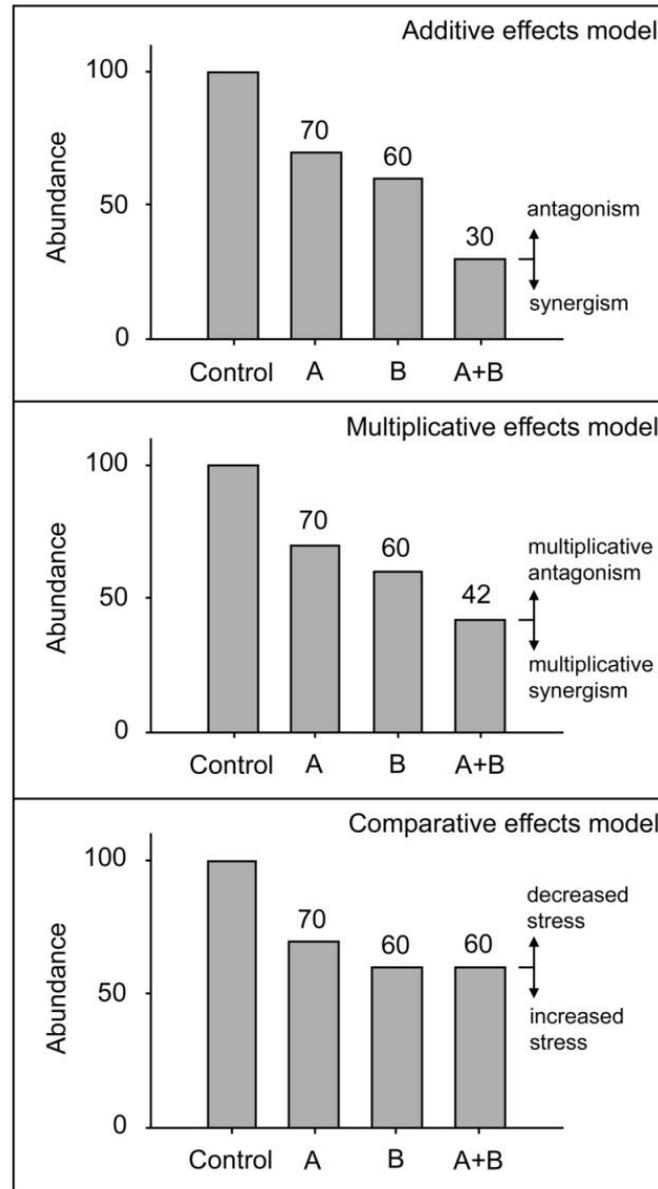


Fig. 4.1. Schematic diagram showing hypothetical models of combined effects of stressor A and stressor B according to the additive, multiplicative, and simple comparative effects models described by Folt et al. (1999). Individual effects of stressor A and stressor B reduce abundances of organisms to 70 (N_A) and 60 (N_B), respectively (100 in the control, N_{Cont}). The combined effects of stressors A and B reduce abundances (N_{A+B}) to 30 under the additive effects model ($N_{Cont} + N_{A+B} = N_A + N_B$), 42 under the multiplicative effects model ($N_{Cont} \times N_{A+B} = N_A \times N_B$), and 60 under the comparative effects model ($N_{A+B} = N_B$, as $N_B < N_A$). Synergisms and antagonisms are then superimposed on each of these particular models as deviations from expectations, *sensu* Folt et al. (1999).

Concentrations of Cu and Zn examined in this experiment were chosen to target the null hypothesis of additivity. Unravelling effects of multiple stressors experimentally is often difficult, as additive models may predict impossible (negative) population size (Wootton 1994). For example, if Cu and Zn individually reduce abundances of a certain organism by 50% and 60%, respectively, the additive effect is predicted to be a reduction of 110%, but clearly a reduction to anything beyond 100% (complete mortality) is impossible to measure accurately and distinguish from other potential models. Using concentrations that individually should cause low to moderate decreases in abundance should reduce the risk of uninterpretable results.

A further constraint on the choice of metal concentrations was imposed in the present study, to ensure that results would be relevant to estuarine communities across the Auckland region as a whole; the Cu-to-Zn ratio in one of the mixed treatments was chosen to be consistent with the empirical measurements of these ratios from a large number of intertidal sites across the entire Auckland region. Given these concentrations, it was hypothesised that increased levels of Cu or Zn in sediments would reduce abundances of individual taxa, total abundances of all taxa and species richness in an additive fashion. It was also hypothesised that the combined effects of Cu and Zn on the multivariate community structure as a whole (when measured on the basis of an appropriate dissimilarity measure, see Methods) would be additive.

Methods

Experimental design

The colonisation experiment was done at an intertidal mudflat in Orewa estuary (36° 35' 45S, 174° 40' 51E), located approximately 30 km north of the city of Auckland, using defaunated, metal-spiked sediments (Fig. 4.2). Sediment Cu and Zn concentrations

and total organic matter (TOM) at the site were measured prior to the experiment, and were $2.94 \mu\text{g g}^{-1}$ (± 0.10) for Cu, $22.80 \mu\text{g g}^{-1}$ (± 0.37) for Zn and 2.21% (± 0.06) by weight for TOM. Sediments were collected from the surface aerobic layer (< 20 mm in depth) at the mudflat, sieved through $500 \mu\text{m}$ mesh and spiked with metals to specifically chosen target concentrations in the laboratory following the methods of Lu et al. (2008). The target spiking concentrations for Cu and Zn were chosen based on laboratory bioassays using the estuarine bivalve, *Macomona liliana* (A. Fukunaga, unpublished results). *M. liliana* is one of the numerically dominant organisms at the study site and was found to be sensitive to Cu and Zn (Fukunaga et al. 2010). Target concentrations for Cu of $60 \mu\text{g g}^{-1}$ and for Zn of $325 \mu\text{g g}^{-1}$ were chosen as these concentrations were expected each to reduce abundances of *M. liliana* by approximately 40%. A second appropriate target concentration of Zn in sediments for this experiment was chosen in order to match the current estimated field-based regional average ratio of Cu to Zn of 1:6.75 (by weight) in Auckland's estuaries (Anderson et al. 2006b). Thus, for a target sediment concentration for Cu of $60 \mu\text{g g}^{-1}$, the concomitant target sediment concentration of Zn in the field based on this ratio would be $405 \mu\text{g g}^{-1}$. Based on previous research at the site (Fukunaga et al. 2010), concentrations of metals in spiked sediments were expected to decrease over the experimental period, and this was also taken into consideration when choosing the target levels.

The metal-spiked sediments were poured into moulds (300 mm in diameter, 30 mm in depth) to create sediment discs approximately 25 – 30 mm thick and were frozen at $-20 \text{ }^{\circ}\text{C}$ for a maximum of two weeks. The discs were then brought back to the estuary and laid out by replacing the surface sediment on 10 March 2009 (day 0). There were six treatments used to assess the null hypothesis of additive effects of heavy metals on estuarine fauna: a control (replacement sediment discs had no additional metals), Zn 1

($325 \mu\text{g g}^{-1}$), Zn 2 ($405 \mu\text{g g}^{-1}$), Cu ($60 \mu\text{g g}^{-1}$), Cu & Zn 1 (combining Cu and Zn at the target concentrations of “Cu” and “Zn 1”) and Cu & Zn 2 (combining Cu and Zn at the target concentrations of “Cu” and “Zn 2”). These treatments can be considered in a crossed experimental design with two factors: (1) Cu (fixed with two levels: “low” with no addition of Cu and “mid” with the target concentration of $60 \mu\text{g g}^{-1}$) and (2) Zn (fixed with three levels: “low” with no addition of Zn, “mid” and “high” with the target concentrations of $325 \mu\text{g g}^{-1}$ and $405 \mu\text{g g}^{-1}$, respectively). In addition, in order to account for the effects of manipulating surface sediments, an unmanipulated treatment (where there was no replacement of surface sediments) was included. The placement of sediment discs in the field was based on a randomised block design with eight blocks (Fig. 4.3). Each block contained the seven treatments placed in a randomised array, 3 m apart from one another. The eight blocks covered an area of $50 \text{ m} \times 25 \text{ m}$, and sediment discs in different blocks were at least 6 m apart.

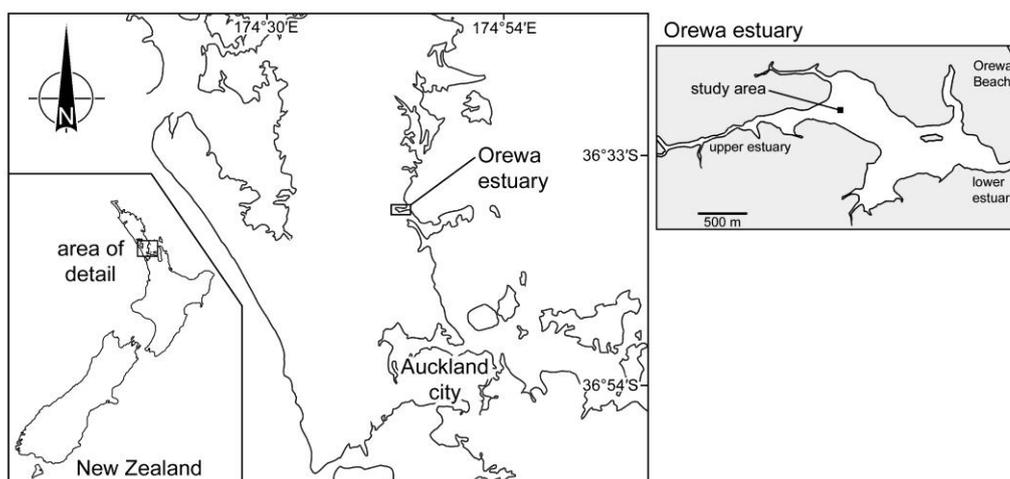


Fig. 4.2. Map of New Zealand, the Auckland region, Orewa estuary and the study area, located approximately 30 km north of the city of Auckland.

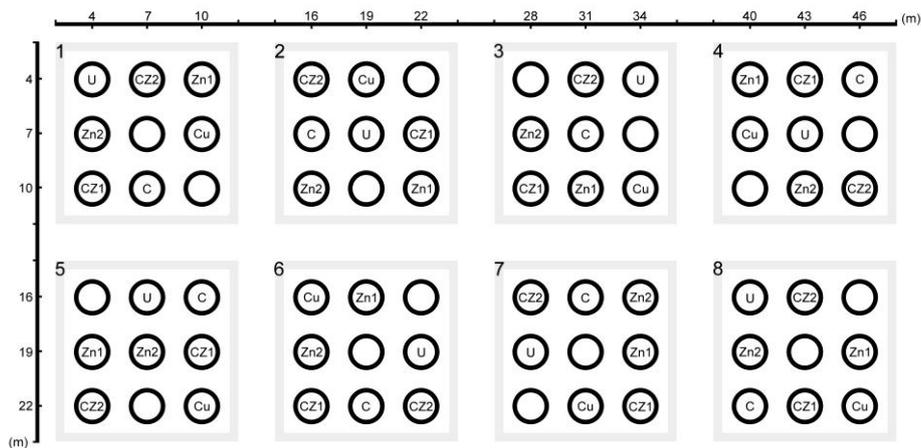


Fig. 4.3. Schematic diagram of the field experimental design containing eight blocks. U = unmanipulated, C = control, Cu = the Cu treatment, Zn1 = the Zn 1 treatment, Zn2 = the Zn 2 treatment, CZ1 = the Cu & Zn 1 treatment and CZ2 = the Cu & Zn 2 treatment. Two empty cells in each block were used to measure relative sediment bed height changes over the experimental period.

Infaunal sampling

Sediment cores (200 mm in diameter, 50 mm in depth) were taken from all blocks on 24 March 24 2009 (day 14). The sediment sampled in each core was extracted and sieved through 500 μm mesh at the site. Material retained on sieves from each sample was brought back to the laboratory and preserved in 10% formalin for a minimum of 48 hours. Organisms were sorted from this material, placed in 70% isopropyl alcohol, identified to the lowest practical taxonomic level and counted.

Environmental variables and metals

Before the experiment began, levels of contaminants and total organic matter (TOM) in the unmanipulated and spiked sediments were measured. For spiked sediments, three replicate samples of sediment were taken from each treatment prior to freezing sediment discs. For the unmanipulated treatment, three sediment cores (20 mm in

diameter, 20 mm in depth) were taken randomly from the study area on day 0, prior to sediment manipulation. Sediments were dried in an oven at 60 °C for 24 hours. Metal concentrations in the sediment samples were determined by flame atomic absorption spectroscopy (FAAS) following digestion of 0.5 g of the dried total sediment fraction in hot aqua regia (HCl:HNO₃ = 3:1) using high-purity HCl and HNO₃ (Chen and Ma 2001). Detection limits of Cu and Zn were both 0.1 µg g⁻¹. The rest of the samples were used to measure TOM by combusting the dried sediment at 500 °C for 4 hours (Byers et al. 1978). TOM (%) was calculated by the weight loss after combustion. At the time of infaunal sampling on day 14, sediment cores (20 mm in diameter, 20 mm in depth) were taken adjacent to infaunal cores to measure the sediment concentrations of metals and TOM associated with each disc. Concentrations of Cu and Zn and TOM were determined as described above.

Concentrations of Cu and Zn in porewater were also measured during the course of the experiment. A peeper (37 µm mesh) was inserted into each sediment disc to collect porewater samples from the aerobic sediment layer (Adams et al. 2003). Three blocks were randomly chosen for temporal monitoring of porewater metals, and porewater samples were collected from these blocks on days 3, 6 and 10 during the experiment. Peepers in blocks not assigned for temporal monitoring were emptied at these times. To obtain the final porewater metal concentrations in each disc, porewater samples were collected from all peepers between day 10 and 14. Samples were filtered through a 0.45 µm membrane and acidified with high-purity HNO₃. Concentrations of heavy metals in porewater are usually low, and so are commonly measured using graphite-furnace atomic absorption spectroscopy, which is complicated by chemical interferences when the matrix is seawater. However, because of the relatively high concentrations of Cu and Zn present in porewaters of this study, the samples were able to be analysed using FAAS instead. Detection

limits of Cu and Zn were 0.1 mg L^{-1} .

Relative sediment bed height changes were measured over the experimental period by burying two small PVC pieces ($20 \text{ mm} \times 20 \text{ mm}$) in the sediments inside each block (Fig. 4.3) and measuring sediment thickness above the PVC pieces on days 0, 3, 6, 10 and 14. Temperature and precipitation were also monitored throughout the experiment for potential weather anomalies that could affect runoff and experimental results. A temperature recorder (HOBO[®] Temperature/Light Data Logger) was attached to a plastic peg and vertically inserted at the downstream, northern corner of the study area to record sediment surface temperature every 30 minutes during the experimental period. Precipitation data in Orewa estuary was obtained from the Auckland Regional Council's Geographic Information and Mapping service (http://maps.arc.govt.nz/website/maps/map_hydrotel.htm).

Statistical analyses

Statistical analyses were done using the software package PRIMER 6 (Clarke and Gorley 2006) with the PERMANOVA+ add-on (Anderson et al. 2008). Univariate analyses separately examined total abundance of all taxa (N), species richness (S), Simpson's index (λ) and the individual numerically abundant organisms based on Euclidean distances. Abundance data were log transformed ($y' = \ln(y + 1)$) prior to the analyses to avoid high skewness and heterogeneity. Multivariate analyses examining the structure of infaunal assemblages as a whole in response to treatments were done on the basis of the modified Gower dissimilarity measure (Anderson et al. 2006a) with a logarithm base of 5. Thus, a five-fold change in abundance was weighted equally with a change in species occurrence (from 0 to 1). (The range of maximum abundances of numerically dominant taxa was between 10 and 75 per sample in this experiment.) Non-

metric multi-dimensional scaling (MDS) ordination was used in order to visualise relationships among samples in terms of community structure on the basis of this measure. In addition, to visualise the relationships among the treatments and the relative sizes of effects, distances among the treatment centroids (calculated on the basis of the modified Gower measure) were visualised using principal coordinates analysis (PCO, Gower 1966). We specifically chose to use PCO, rather than non-metric MDS, to visualise the relative positions of treatment centroids, because we wanted to visualise the actual sizes of the effects in the dissimilarity space to assess additivity. It was therefore appropriate to use PCO, which attempts to project and preserve actual dissimilarities in a reduced number of dimensions (Legendre and Legendre 1998) and not just their relative ranks, as in non-metric MDS.

Formal tests to compare treatments were done using permutational analysis of variance (PERMANOVA; Anderson 2001, McArdle and Anderson 2001) for either univariate or multivariate data with 4999 permutations of residuals under a reduced model. First, an analysis was done using a design with two factors: treatment (fixed with seven levels: unmanipulated, control, Cu, Zn 1, Zn 2, Cu & Zn 1 and Cu & Zn 2) and block (random with eight levels). If ‘treatment’ was significant, then specific *a priori* planned contrasts were done, as follows: (i) the unmanipulated *versus* the control treatments (to test for the effects of manipulating sediments and to monitor recolonisation); and (ii) the control *versus* all metal-spiked treatments (to test the general null hypothesis of no effects of heavy metals).

Variables that showed a significant contrast of the control *versus* the metal-spiked treatments were then examined further to test for potential interactions between Cu and Zn in their effects (i.e. non-additivity). This was done using a three-way PERMANOVA, excluding the unmanipulated treatment. The three factors for this analysis were: Cu (fixed

with two levels, low and mid), Zn (fixed with three levels, low, mid and high) and block (random with eight levels). All analyses were done using 4999 permutations of residuals under a reduced model, and relevant PERMANOVA pair-wise comparisons followed when significant effects were detected.

Potential differences among treatments in either the sediment or porewater concentrations of Cu or Zn were examined by simple one-way ANOVAs with the treatment factor alone (having seven levels). As for the other univariate variables, analyses were done using PERMANOVA with 4999 permutations of raw data, based on Euclidean distances, and relevant PERMANOVA pair-wise comparisons followed when significant effects were detected.

Results

Environmental variables and metals

Concentrations of Cu in sediments were elevated to the target level in the Cu and Cu & Zn 1 treatments, although they did not quite reach the anticipated target levels in the Cu & Zn 2 treatment (Fig. 4.4a). There was, however, no statistically significant difference in concentrations of Cu in the Cu, Cu & Zn 1 and Cu & Zn 2 treatments at the end of the experiment, which had decreased to approximately $30 \mu\text{g g}^{-1}$ in all three treatments. Concentrations of Cu in the porewater were also initially high in the Cu, Cu & Zn 1 and Cu & Zn 2 treatments, but quickly decreased by day 6 (Fig. 4.5a). Concentrations of Cu in porewater in these treatments did not differ from one another at the end of the experiment, but were significantly higher than those in the unmanipulated, control, Zn 1 or Zn 2 treatments.

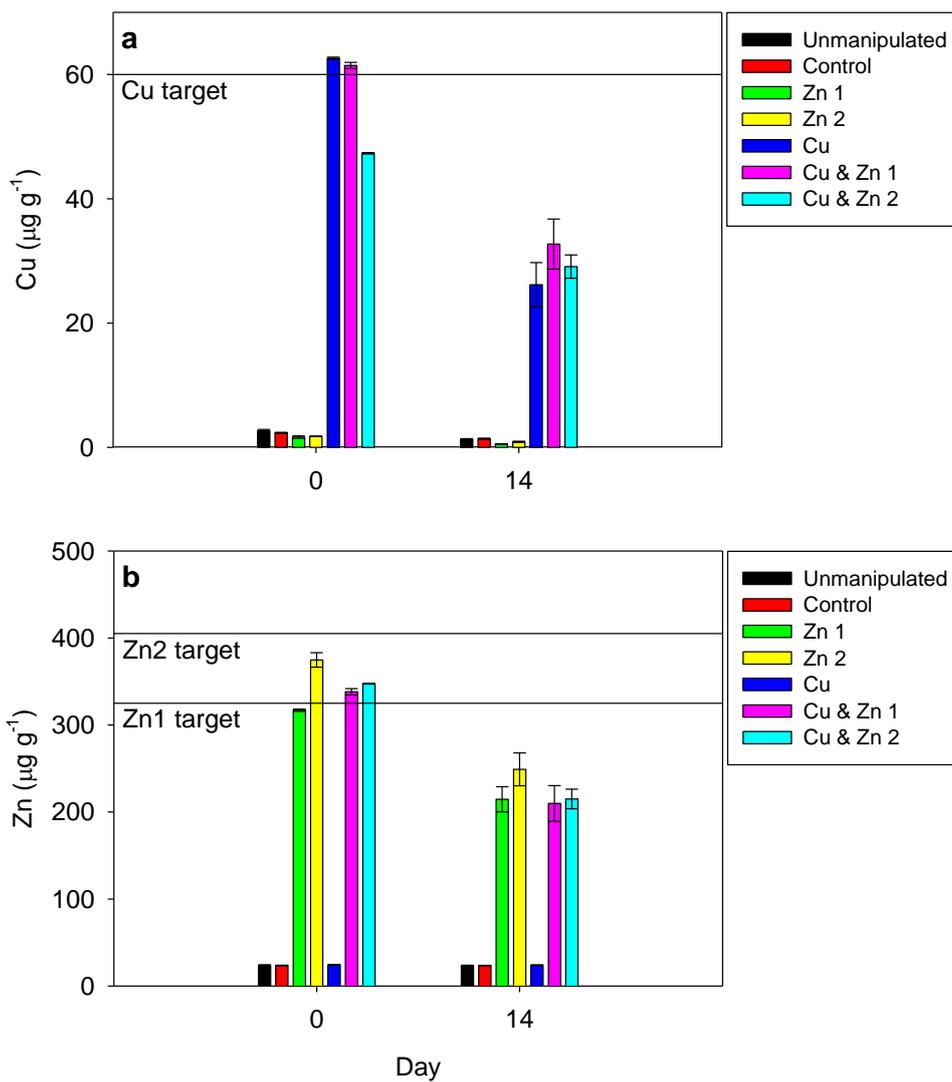


Fig. 4.4. Mean (\pm SE) of the initial and ending concentrations of sediment metals, for (a) Cu and (b) Zn.

Concentrations of Zn in sediments were elevated to the target levels in the Zn 1 and Cu & Zn 1 treatments but did not quite reach the anticipated targets in the Zn 2 and Cu & Zn 2 treatments (Fig. 4.4b). The average concentrations of Zn in these treatments had decreased to approximately $200 - 250 \mu\text{g g}^{-1}$ by the end of the experiment, when they were higher in Zn 2 and Cu & Zn 2 than in Zn 1 and Cu & Zn 1 treatments, but these differences were not statistically significant. Concentrations of Zn in the porewater were initially, on average, higher in the Zn 2 and Cu & Zn 2 than in the Zn 1 and Cu & Zn 1 treatments, but quickly decreased by day 6 (Fig. 4.5b). There were no statistically

significant differences in porewater Zn concentrations among the Zn 1, Zn 2, Cu & Zn 1 and Cu & Zn 2 treatments at the end of the experiment, but concentrations of Zn in these treatments were significantly higher than those in the unmanipulated, control and Cu treatments.

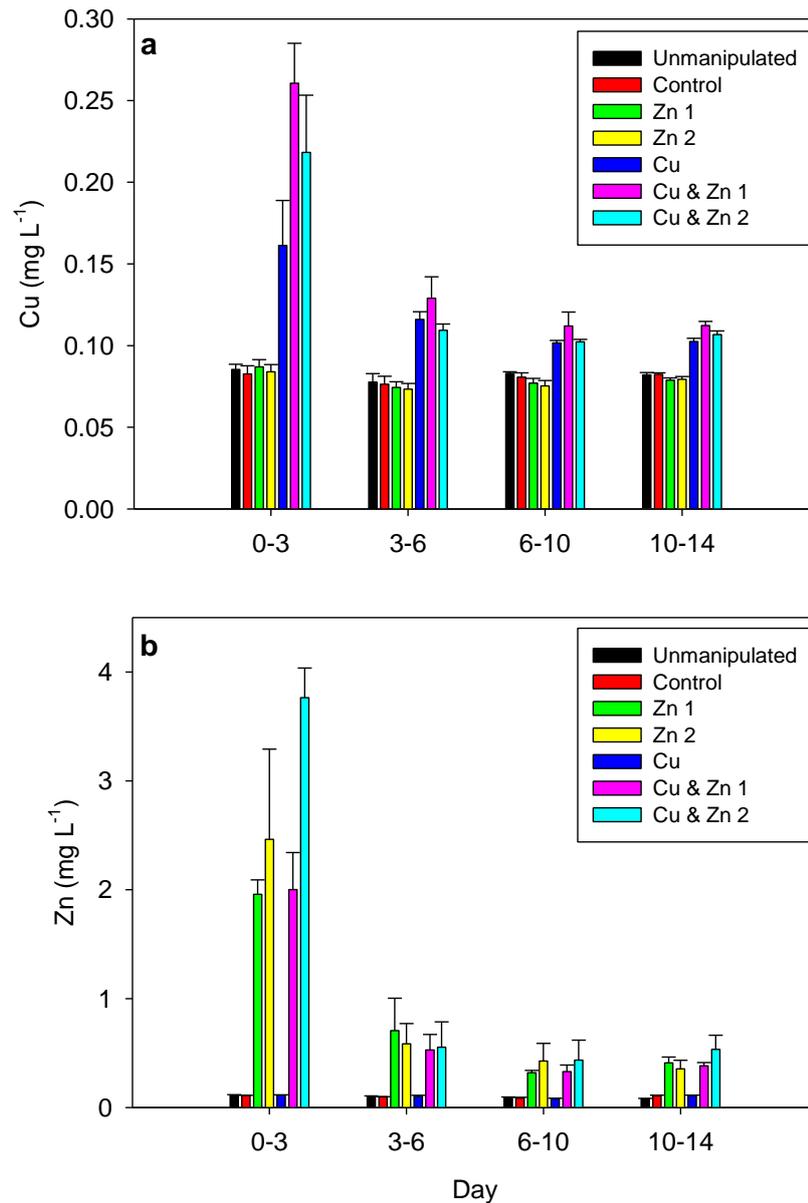


Fig. 4.5. Mean (\pm SE) of the concentrations of metals in porewater samples over the 14-day experimental period, for (a) Cu and (b) Zn.

The environmental variables measured during the experiment did not show any atypical anomalies. TOM levels in sediments were, on average, 2.22% (SD = 0.28) at the beginning and 2.14% (SD = 0.25) at the end of the experiment. Monitoring of relative bed height changes showed that the site experienced some accretion, and the average bed height changes in all blocks were between 0 and +6 mm during the experiment. Sediment surface temperature at the study area generally fluctuated daily between 15 °C and 35 °C. The smallest amount of rainfall recorded (over a 24-hour period) was 0.5 mm, and the heaviest rainfall recorded was 4.0 mm.

Infauna

In total, 26 taxa were identified from the 56 sediment cores. The most numerically dominant organisms in this study were, in order of decreasing abundance, *Prionospio* sp., oligochaetes, *Scoloplos cylindifer*, *Ceratonereis* sp., *Capitella* spp., Polydorid spp., *Austrovenus stutchburyi* and *Heteromastus* sp. These eight taxa accounted for approximately 80% of the total abundance of all infaunal organisms. Significant treatment effects were detected for the mean total log abundance of infauna, species richness and for all of the numerically dominant taxa (Table 4.1). There was no significant difference, however, in the mean value of Simpson's index among treatments (Table 4.1).

Significant effects of sediment manipulation were detected for the mean total log abundance of infauna, species richness and all of the numerically dominant taxa with the exception of *Ceratonereis* sp. (Table 4.1). The control treatment had significantly fewer abundances and taxa of infauna than the unmanipulated treatment (Figs. 4.6 and 4.7).

There were statistically significant effects of metal spiking on the mean total log abundance of infauna, the mean species richness and the mean log abundances of *Prionospio* sp., *S. cylindifer*, *Capitella* spp., Polydorid spp. and *Heteromastus* sp. (Table

4.1). Significant variation among blocks was also detected for oligochaetes and *Heteromastus* sp., indicating high spatial variability in their distributions (Table 4.1).

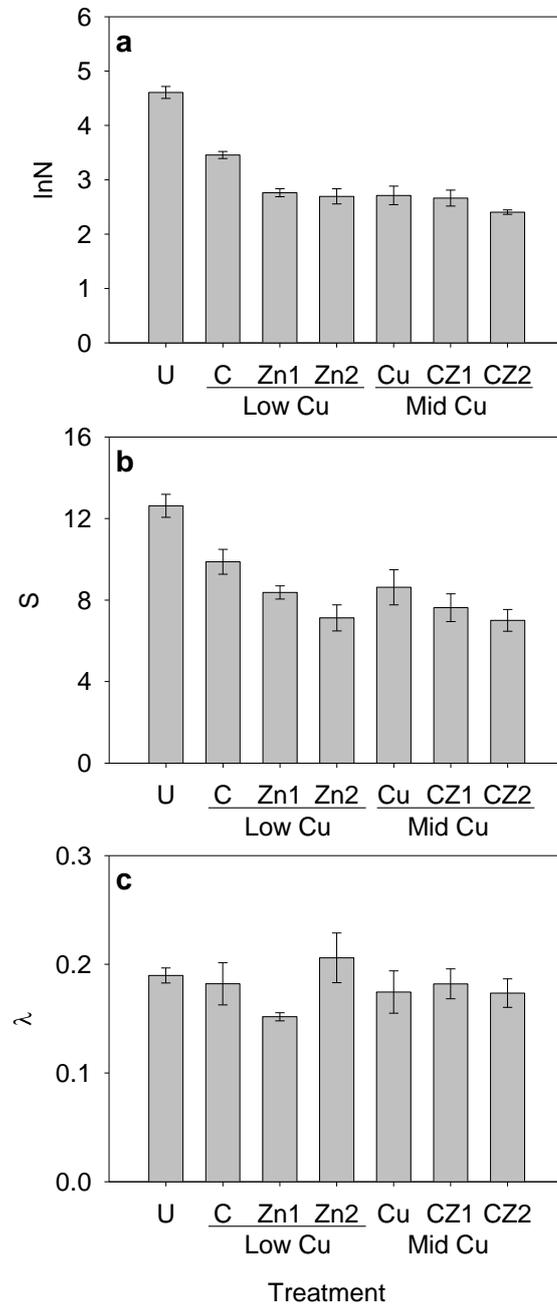


Fig. 4.6. Averages (\pm SE) for (a) log-transformed total infaunal abundance (lnN), (b) species richness (S) and (c) Simpson's index (λ) in each treatment (U = unmanipulated, C = control, Cu = the Cu treatment, Zn1 = the Zn 1 treatment, Zn2 = the Zn 2 treatment, CZ1 = the Cu & Zn 1 treatment and CZ2 = the Cu & Zn 2 treatment).

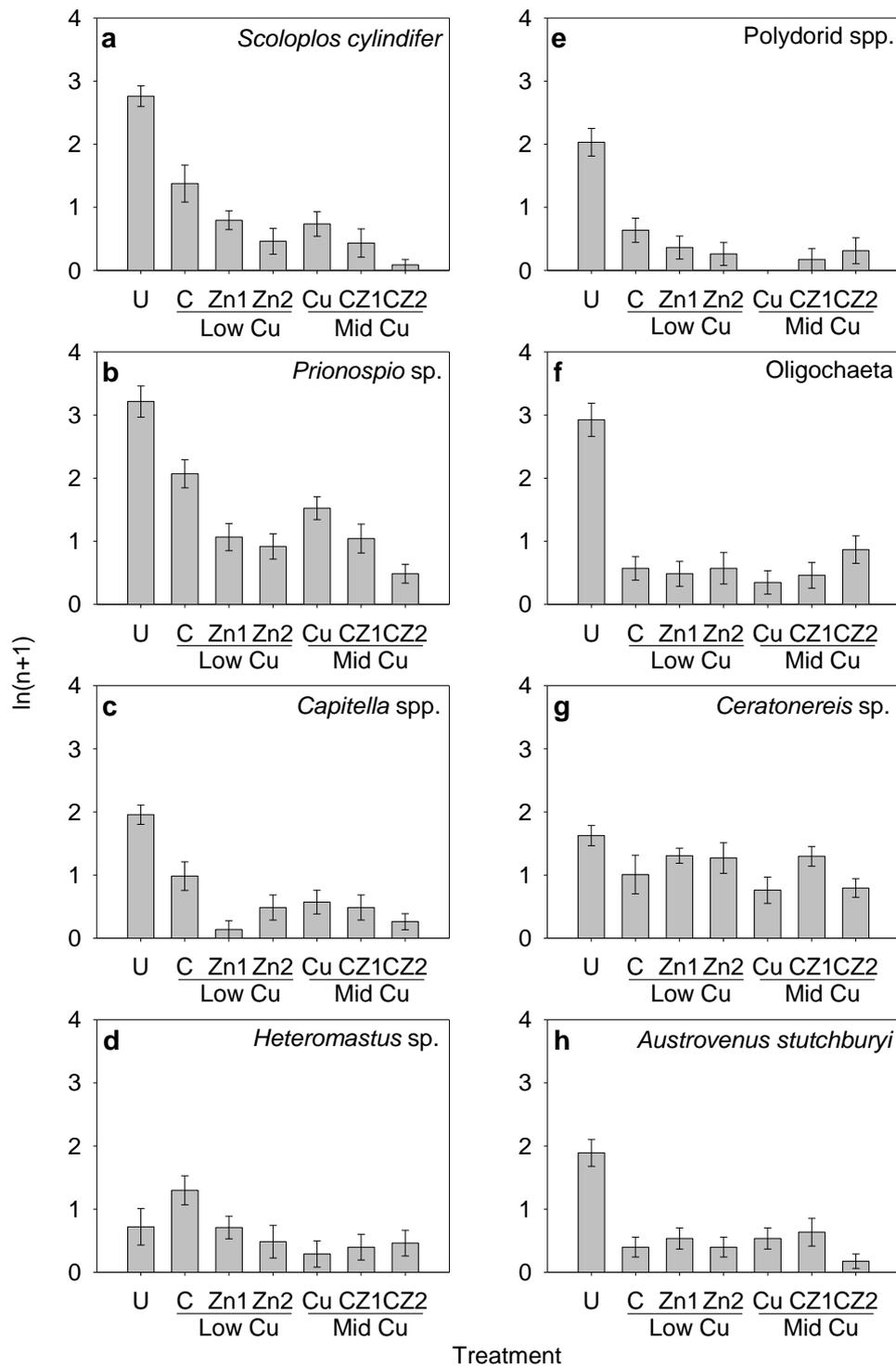


Fig. 4.7. Averages (\pm SE) of log-transformed abundances of each of several numerically dominant taxa (as indicated) in each treatment (U = unmanipulated, C = control, Cu = the Cu treatment, Zn1 = the Zn 1 treatment, Zn2 = the Zn 2 treatment, CZ1 = the Cu & Zn 1 treatment and CZ2 = the Cu & Zn 2 treatment).

Table 4.1. Summary of results of two-way PERMANOVA for univariate and multivariate data and *a priori* planned contrasts of the unmanipulated treatment *versus* the control and the control *versus* all metal-spiked treatments. Univariate analyses were for the total log abundance, species richness, Simpson's index and numerically dominant taxa, and multivariate analysis was done based on the modified Gower dissimilarity (log base = 5) measure, calculated on infaunal abundances.

	Block		Treatment		Contrast			
					unmanipulated vs. control		control vs. spiked treatments	
	$F_{7,42}$	P	$F_{6,42}$	P	$F_{1,7}$	P	$F_{1,39}$	P
Univariate								
Total abundance	0.75	0.640	41.71	0.0002	56.12	0.0002	36.23	0.0002
Species richness	0.86	0.548	11.76	0.0002	10.29	0.0174	15.92	0.0004
Simpson's index	1.35	0.247	1.19	0.330				
<i>Prionospio</i> sp.	1.32	0.260	20.11	0.0002	42.72	0.0008	18.46	0.0004
Oligochaeta	5.95	0.0006	30.14	0.0002	113.95	0.0006	0.023	0.883
<i>Scoloplos cylindifer</i>	2.17	0.055	23.95	0.0002	70.25	0.0006	15.61	0.0006
<i>Ceratonereis</i> sp.	0.84	0.574	2.36	0.045	2.63	0.145	0.116	0.745
<i>Capitella</i> spp.	0.43	0.870	10.90	0.0002	11.46	0.0142	8.16	0.0072
Polydorid spp.	1.97	0.087	16.77	0.0002	25.31	0.0024	16.77	0.0216
<i>Austrovenus stutchburyi</i>	1.87	0.098	11.83	0.0002	30.49	0.0016	0.122	0.725
<i>Heteromastus</i> sp.	7.80	0.0002	4.33	0.0016	7.44	0.0318	22.18	0.0004
Multivariate								
Modified Gower	1.97	0.0002	5.19	0.0002	7.88	0.0026	4.56	0.0002

Table 4.2. Summary of results of three-way PERMANOVA for univariate and multivariate analyses and pair-wise tests, examining interactions between Cu and Zn. Univariate analyses were for the total log abundance, species richness, and numerically dominant taxa, and multivariate analysis was done based on the modified Gower dissimilarity measure (log base = 5), calculated on infaunal abundances. Pair-wise tests were for the three different levels of Zn: low (L), mid (M) and high (H). Levels underlined together did not show significant differences ($\alpha = 0.05$). When the Cu \times Zn term was significant, pair-wise tests comparing Zn levels was done for each level of Cu; low (L) and mid (M).

	Block		Cu		Zn		Block \times Cu		Block \times Zn		Cu \times Zn		Pair-wise test (Zn)
	$F_{7,14}$	<i>P</i>	$F_{1,7}$	<i>P</i>	$F_{2,14}$	<i>P</i>	$F_{7,14}$	<i>P</i>	$F_{14,14}$	<i>P</i>	$F_{2,14}$	<i>P</i>	
Univariate													
Total abundance	0.72	0.661	9.48	0.023	11.96	0.001	1.81	0.161	1.01	0.503	4.43	0.034*	Cu-L: <u>L</u> <u>M</u> <u>H</u> Cu-M: <u>L</u> <u>M</u> <u>H</u>
Species richness	1.17	0.366	5.15	0.055	6.59	0.010	0.98	0.485	0.91	0.555	0.89	0.429	<u>L</u> <u>M</u> <u>H</u>
<i>Prionospio</i> sp.	1.24	0.342	4.18	0.082	12.19	0.002	1.28	0.315	1.65	0.180	1.22	0.336	<u>L</u> <u>M</u> <u>H</u>
<i>Scoloplos cylindifer</i>	1.04	0.455	13.52	0.009	17.21	0.0004	0.38	0.900	0.29	0.985	0.20	0.820	<u>L</u> <u>M</u> <u>H</u>
<i>Capitella</i> spp.	0.40	0.890	0.36	0.560	4.65	0.031	0.87	0.562	0.62	0.800	1.75	0.214	<u>L</u> <u>M</u> <u>H</u>
Polydorid spp.	2.20	0.097	2.81	0.137	0.06	0.939	1.56	0.227	0.97	0.512	2.67	0.105	
<i>Heteromastus</i> sp.	7.62	0.001	12.41	0.010	1.64	0.228	1.25	0.343	1.74	0.160	6.64	0.009*	Cu-L: <u>L</u> <u>M</u> <u>H</u> Cu-M: <u>H</u> <u>M</u> <u>L</u>
Multivariate													
Modified Gower	1.72	0.004	2.64	0.026	3.62	0.0002	0.88	0.753	0.89	0.770	1.18	0.272	<u>L</u> <u>M</u> <u>H</u>

* Pair-wise comparisons for each Zn level showed significant differences between the two Cu levels (low and mid) at the low level of Zn.

The mean log abundance of *S. cylindifer* was reduced by both Cu and Zn, and a non-significant Cu \times Zn term indicated that the combined effects were additive (Table 4.2, Fig. 4.7a). Species richness and the mean log abundance of *Prionospio* sp. were significantly reduced by Zn, and Cu also had statistically significant marginal effects on these variables (Table 4.2, Figs. 4.6b and 4.7b). The Cu \times Zn term was not significant, suggesting that Cu and Zn affected these variables in an additive fashion. Significant interaction effects between Cu and Zn were detected for the mean log abundances of total infauna and *Heteromastus* sp; with no addition of Zn to sediments, an addition of Cu reduced these variables. Similarly, with no addition of Cu to sediments, Zn had negative effects on these variables. When sediments had elevated levels of Cu or Zn, however, adding the other metal did not further affect these variables (Table 4.2, Figs. 4.6a and 4.7h). The mean log abundance of *Capitella* spp. was significantly reduced by Zn, but the Cu and Cu \times Zn terms were both non-significant (Table 4.2), indicating their relative insensitivity to Cu.

There were significant differences in the structure of infaunal assemblages among treatments ($F_{6,42} = 5.19$, $P = 0.0002$) and among blocks ($F_{7,42} = 1.97$, $P = 0.0002$). Assemblages in the unmanipulated treatment differed from those in the control treatment, which in turn were also significantly different from those in the sediments spiked with heavy metals (Table 4.1). Although the value of stress was fairly high (0.22), patterns in the responses of assemblages to treatments were apparent in the MDS plot (Fig. 4.8). The separation of the assemblages in unmanipulated sediments from all others was clearly shown visually in the MDS plot (Fig. 4.8). In addition, the control treatment (in the middle of the diagram) was very dissimilar from the Cu & Zn 2 treatment (on the left). The Cu, Zn 1, Zn 2, Cu & Zn 1 treatments were interspersed in between these, indicating a gradient in the effects of varying levels of metal-spiking on the recolonisation of

assemblages. More specifically, the Zn 1 treatment was located in between the control and the Cu & Zn 2 treatments, while the Zn 2 and Cu & Zn 1 treatments were more spread out, with some samples overlapping the Cu & Zn 2 treatment. The PCO plot of treatment centroids clarified this gradient and showed a clear pattern, with assemblages in the following ordered treatments having increasing dissimilarity from the control: Cu, Zn 1, Cu & Zn 1, Zn 2 and Cu & Zn 2 (Fig. 4.9). The structure of infaunal assemblages was significantly affected by both Cu and Zn (Table 4.2). The Cu \times Zn interaction term was not significant, suggesting the combined effects of Cu and Zn on community structure and composition were additive.

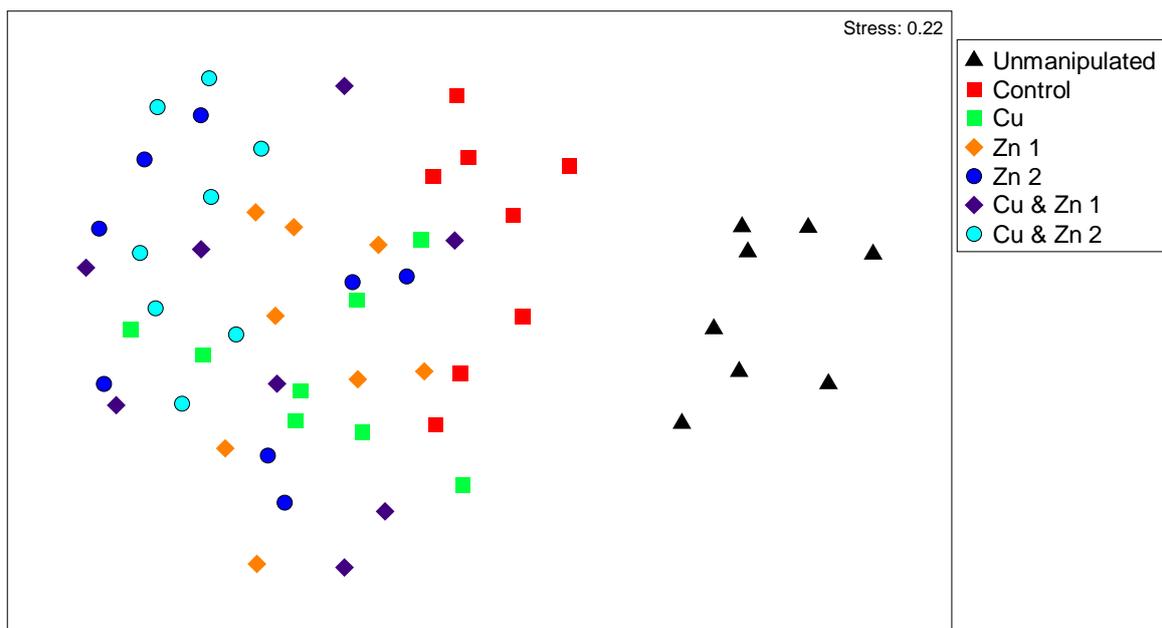


Fig. 4.8. Non-metric MDS plot based on the modified Gower dissimilarity measure (log base = 5) calculated from the abundances of 26 infaunal taxa.

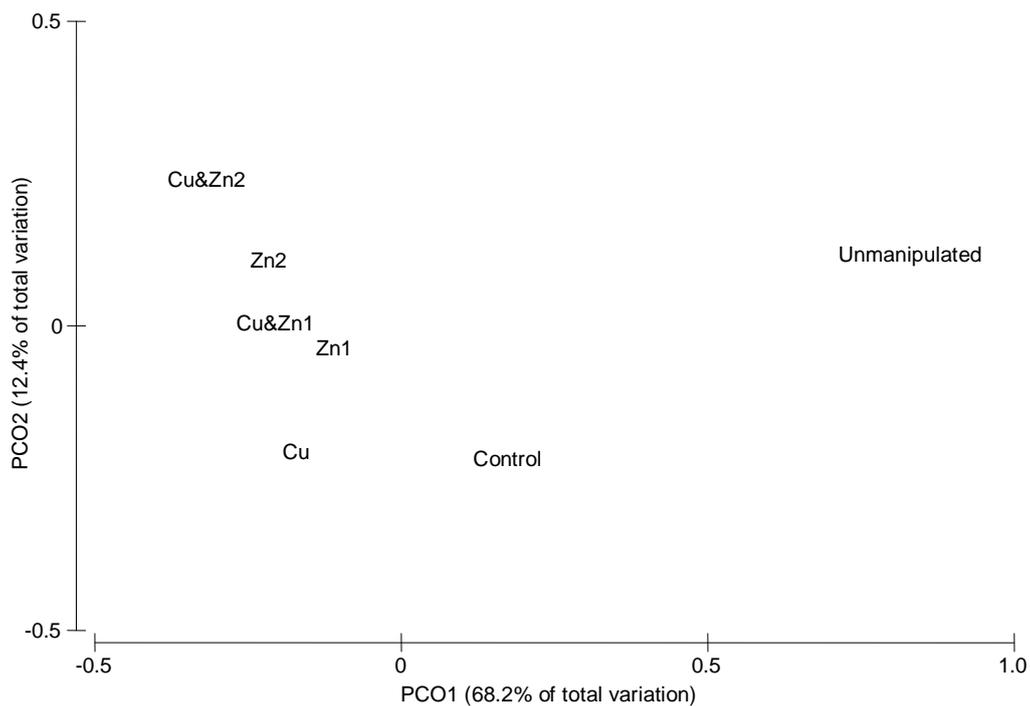


Fig. 4.9. PCO of distances among treatment centroids based on the modified Gower dissimilarity measure (log base = 5) calculated from the abundances of 26 infaunal taxa.

Discussion

Effectiveness of treatments

Spiking sediments with heavy metals elevated the concentrations of Cu and Zn in relevant treatment discs. The sediment concentrations of metals in the Cu & Zn 2 treatment, however, were not as high as anticipated for the target levels. The adsorption capacities of Cu and Zn for Orewa sediments were reported as $2000 \mu\text{g g}^{-1}$ and $910 \mu\text{g g}^{-1}$, respectively (Lu et al. 2008), and therefore the target concentrations should have been achieved by the spiking. These adsorption capacities for individual metals were not measured, however, in the presence of other metals. It is possible that the high concentrations of Cu and Zn resulted in competitive effects between these metals binding

to adsorption surfaces in the sediment and thereby reduced their adsorption. Examining adsorption capacities of heavy metals in the presence of other metals is recommended for future experiments to ensure that target concentrations are achieved using the given method for chemically spiking sediments.

The current experiment examined two levels of Cu (low and mid) and three levels of Zn (low, mid and high). The metal spiking successfully increased levels of Cu, creating a comparable mid-level of Cu in the Cu, Cu & Zn 1 and Cu & Zn 2 treatments. The three levels of Zn, on the other hand, were not well achieved by the spiking, as the mid and high levels of Zn did not differ statistically from each other. Thus, the biological differences detected between treatments having Zn 1 *versus* Zn 2 are likely due to higher concentrations of Zn in the porewater of the Zn 2 (and Cu & Zn 2) treatments in the early stages of the experiment.

Abundances of infauna and species richness were affected by the manipulation of surface sediment. The relatively large effects of sediment manipulation detected after the 14-day experimental period was somewhat surprising. In a similar experiment previously done in Orewa estuary (Fukunaga et al. 2010), there were no significant differences in species richness between the unmanipulated and control treatments after 10 days and in the total infaunal abundance after 20 days. It is possible that the timings of sediment manipulation affected the speed of infaunal recolonisation, as these two experiments were done in different seasons: the current experiment in late summer and the previous experiment in winter. Although the difference between the unmanipulated and control treatments do not affect the assessment of the effects of heavy metals (i.e. comparisons between the control and metal-spiked treatments), it should be taken into consideration when planning a future experiment.

Effects of Cu and Zn on infauna

The total infaunal abundance, species richness and abundances of some of the numerically dominant organisms were reduced by the spiking of sediments with Cu and/or Zn. Three of the numerically dominant taxa (Oligochaeta, the nereid polychaete *Ceratonereis* sp. and the bivalve *Austrovenus stutchburyi*), however, did not show any negative effects of Cu and Zn. The relative insensitivity of oligochaetes and *A. stutchburyi* to these concentrations of metals is consistent with results of a previous manipulative field experiment in Orewa estuary (Fukunaga et al. 2010). Relative tolerance of *Ceratonereis* sp. to heavy metals was also consistent with monitoring programs of Auckland's estuaries that reported increased abundances of nereid polychaetes along a pollution gradient of heavy metals (Anderson et al. 2006b). In the Fal estuary in England, the nereid polychaete *Nereis diversicolor* has also been found to be highly tolerant to Cu and Zn and to be able to maintain its distribution in an area where concentrations of metals were orders of magnitude higher than background levels (Bryan et al. 1987).

The capitellid polychaetes *Capitella* spp. were not affected by Cu, suggesting that they can tolerate higher levels of Cu compared to many other organisms that are negatively affected by this metal. Capitellids have been found to be abundant in areas with high concentrations of heavy metals in other systems as well, suggesting high tolerance (Lande 1977, Rygg 1985). However, some studies have demonstrated negative effects of heavy metals on capitellid abundances (Morrisey et al. 1996, Warwick 2001). Such inconsistencies in the responses of capitellids as a taxon suggest that their responses to heavy metals are species-specific. *Capitella (capitata)* has long been considered to be an indicator of pollution around the world (Reish 1971, Wade et al. 1972, Rosenberg 1976, Tsutsumi 1987), but this cosmopolitan species has been shown actually to comprise a

complex of sibling species that possess very different life histories and reproductive modes, showing distinct temporal adaptations to disturbed environments (Grassle and Grassle 1976). Nevertheless, the responses of *Capitella* spp. in the current experiment do indicate their relative tolerance to Cu in the Orewa estuarine system.

Contrary to multivariate analyses and species richness, Simpson's index did not show any significant differences among the treatments. This result contrasts sharply with the idea that the evenness of species abundances in ecological communities is reduced by long-term environmental pollution through decreases in the abundances of sensitive organisms and relatively large increases in the abundances of more tolerant, opportunistic organisms (Pearson and Rosenberg 1978). In the current experiment, abundances of numerically dominant taxa were either relatively unaffected or were reduced by the treatments, and none apparently increased in abundances. As the experiment had a relatively short experimental period of 14 days, long-term effects of these heavy metals could not be inferred. Considering individual size classes and life stages to distinguish the potential underlying processes driving observed effects (i.e. recruitment or migration) may shed light on the potential chronic effects of heavy metals. In order to consider recruitment of organisms as part of the recolonisation process, a much longer experimental period may also be required. Such experiments can result in a different conclusion regarding the effects of heavy metals on species evenness. Nevertheless, the results obtained here provide a caution against the use of evenness measures alone to detect acute toxicities or short-term effects of pollutants on ecological communities.

Additivity of the combined effects of Cu and Zn were detected for the mean log abundances of the polychaetes *Prionospio* sp. and *Scoloplos cylindifer*. Additivity on the log scale indicates that the effects were, in effect, multiplicative on their raw abundances. Wootton (1994) suggested that multiplicative statistical models were usually more

appropriate, compared to additive models, to apply to experimental data on abundances, biomass and survivorship when testing for interactions using ANOVA. This is because population growth observed at relatively low density is mostly multiplicative rather than additive (linear) over time, and also because combining probabilities of survivorship resulting from different treatments is usually done multiplicatively (Wootton 1994). Although abundance data were log-transformed in the present study to avoid high skewness and heterogeneity, Billick and Case (1994) cautioned against haphazardly applying such data transformation as it would change underlying dynamic models: log-transforming data assumes that treatments cause mortality to a constant fraction of the target species, while the use of untransformed data assumes that they cause mortality to a constant number of target species, regardless of the size of the population.

The community structure of colonists, measured by the modified Gower measure, and species richness, on the other hand, were affected by Cu and Zn in a directly additive fashion, and therefore the effects of Cu and Zn on these variables were consistent regardless of the presence or absence of the other metal. Analyses of additive effects on communities using multivariate dissimilarity-based methods will be strongly affected by the choice of transformation as well as the choice of resemblance measure used in the analysis. In the present study, the modified Gower measure was chosen as an appropriate measure *a priori*, in order to explicitly specify the relative contribution of a 0-to-1 change (i.e. a change in the identities of species from one sample to the next) *versus* a change in abundance towards the calculation of the measure. More specifically, a 0-to-1 change was given an equal weight to a five-fold change in abundance in the calculation of the dissimilarity, based on the ranges of abundances observed in the dataset. The log transformation (with an appropriate exception for the treatment of zeros) is intrinsic to the modified Gower measure (see Anderson et al. 2006a for details). It was considered here

that an assessment of additivity in effects should treat with abundances of organisms on a log-scale, and our choice of dissimilarity measure was a direct reflection of this philosophy. Analyses based on some other resemblance measure may or may not have resulted in similar conclusions regarding the additivity of effects on community structure.

Interactions among multiple stressors have been shown to generate complex non-additive effects that cannot be predicted based on single-stressor studies (Christensen et al. 2006). Non-additive effects of Cu and Zn detected for the mean log abundances of total infauna and *Heteromastus* sp. in the current experiment were apparently antagonistic. The antagonism on the total infaunal abundance is likely due to differential sensitivity of different taxa to Cu and Zn. Toxicants, including heavy metals, reduce abundances of sensitive organisms but favour more tolerant ones, shifting communities towards increased community tolerance (Blanck 2002). Although not statistically significant, the taxa identified as insensitive to Cu and Zn in the current experiment did have higher average abundances in the Cu & Zn treatments than in single metal treatments in some cases, perhaps compensating for some decreases in sensitive taxa in the Cu & Zn treatments.

Antagonistic effects of multiple stressors may occur when one stressor affects organisms to such an extent that the second stressor cannot have any further effects (i.e. as in a comparative effect *sensu* Folt et al. 1999). Antagonisms have also been found for toxins paired with nutrients, potentially because the positive effect of nutrients may compensate for the negative effect of toxins (Crain et al. 2008). Some organisms may develop co-tolerance to heavy metals, in which their tolerance to one metal improves their tolerance to another metal (Blanck 2002). It is difficult to determine the exact cause of the antagonism detected for *Heteromastus* sp. in the present experiment, as physiological mechanisms of metal toxicities have not been very well studied in these organisms. In other studies, the capitellid polychaete *Heteromastus filiformis* has been reported to be

tolerant to heavy metals (Lande 1977, Rygg 1985), but the biological mechanisms underlying this are unknown.

There was no strong indication of synergistic effects of Cu and Zn on any variables examined in the present experiment. Synergistic effects of multiple stressors have been found, however, in other studies. A combination of diesel fuel and a mixture of heavy metals (Cu, Cd, Hg, Cr, and Pb) has been shown to reduce abundances of copepods synergistically (Millward et al. 2004). Hagopian-Schlekat et al. (2001) have also shown that the combined effects of heavy metals (Cu, Pb, Ni and Zn) on the estuarine copepod *Amphiascus tenuiremis* were greater than the effect predicted from simple additivity of the individual metal toxicities. Synergistic effects of multiple stressors have been shown to occur more frequently with three-stressor interactions compared to two stressors, suggesting that synergisms could be common in natural environments where more than two anthropogenic stressors exist simultaneously (Crain et al. 2008). Sediment contaminants of concern in the Auckland region include not only Cu and Zn, but also lead and polynuclear aromatic hydrocarbons (ARC 2004). It is, therefore, important to further investigate the effects of Cu and Zn in combination with these contaminants for potential synergistic effects.

Some of the taxa examined in the current experiment responded differently to the two different spiking levels of Zn, even though these levels were no longer significantly different in either the porewater or sediments by the end of the experiment. Porewater concentrations of Zn were different in these two treatments early on, however, and the fact that organisms, too, reflected these differences in their colonisation patterns may indicate the importance of the metal concentrations in porewater in these systems. As noted earlier, porewater generally contains the most bioavailable form of metals (i.e. dissolved metals, particularly free metal ions), but ingestion of sediment-associated metals has also been

suggested to be an important metal-exposure pathway for deposit-feeding infauna (Chapman et al. 2002). The present experiment could not separate the effects of metals in porewater and in sediments, and so determining the most important exposure pathway of metals for infauna was outside the scope of this experiment. Laboratory-spiked sediments can, however, overestimate the sensitivity of organisms to metals, because metals in laboratory-spiked sediments tend to be less strongly associated with the sediments compared to sediments in the field which have become gradually more contaminated over a long period of time (Simpson et al. 2004). Results of metal-spiked experiments, therefore, need to be interpreted with some caution in this respect.

The Cu-to-Zn ratio most relevant to Auckland's estuaries could not be achieved at the beginning of the experiment, as metal-spiking did not achieve the high target-level for Zn. Nevertheless, at the end of the experiment, the ratios of average concentrations of Cu and Zn in the sediments of mixed-metal treatments were comparable to those recorded in some of the most highly contaminated estuaries in Auckland (Kelly 2007). The general additivity of the combined effects of Cu and Zn on estuarine infauna observed in this study, therefore, suggests that the effects of these heavy metals on estuarine communities in the Auckland region may be greater than the effects predicted from individual metals alone. The additivity of metal effects shown here also provides useful information in order to build appropriate models to predict the potential effects of heavy metals in these systems that might be expected with changes in metal concentrations anticipated for the future. This type of modelling can then be used in the development of environmental guidelines and policy.

The crossed experimental design used here shed light on the nature of the simultaneous effects of multiple metals and allowed effects to be classified as additive, synergistic or antagonistic. This approach provides an alternative, somewhat simpler set

of models to those described by Folt et al. (1999), and links specific biological models to particular statistical results in such a study design (Fig. 4.10). Significant main effects and a non-significant interaction term indicate the additivity of a response variable to the two factors (in our case, metals) being examined. In contrast, a significant interaction term indicates either a synergism or an antagonism. The difference between these two is then distinguishable by reference to the directions of the effects relative to predictions under additivity. Specifically, if in the presence of a second metal, the effects exceed what is expected from the sum of individual metals acting alone, then combined effects are synergistic. Whereas, if the effects are reduced in such cases, then the combined effects are antagonistic (i.e. one metal mollifies the effects of another, Fig. 4.10). This classification is relatively simple and particularly practical when physiological mechanisms of stressors are not well understood (i.e. when the simultaneous effects of multiple stressors cannot be classified *a priori* as either additive, multiplicative or comparative). Heavy metals are one of many anthropogenic stressors that are threatening aquatic ecosystems (Halpern et al. 2007), and contamination by a single metal is a rare occurrence. Understanding the nature of simultaneous effects of multiple metals, as well as in combination with other environmental stressors, should provide useful information to protect coastal ecosystems.

Conclusions

This manipulative field experiment examined the nature of the combined effects of Cu and Zn on estuarine infauna, with a specific test for additivity. Additivity of the combined effects was detected for the community structure based on the modified Gower dissimilarity measure, as well as for several individual variables, including species richness and log abundances of the polychaetes *Prionospio* sp. and *Scoloplos cylindifer*.

Non-additive and antagonistic effects of these metals were detected for the mean log abundances of total infauna and for the polychaete *Heteromastus* sp. No synergistic effects were detected, but different taxa had differential sensitivities to Cu and Zn, and the nature of the combined effects also varied among taxa. It is therefore important to understand the nature of the combined effects of multiple heavy metals when modelling their effects on estuarine communities under future scenarios, in which different metals are expected to have variable concentrations in the field.

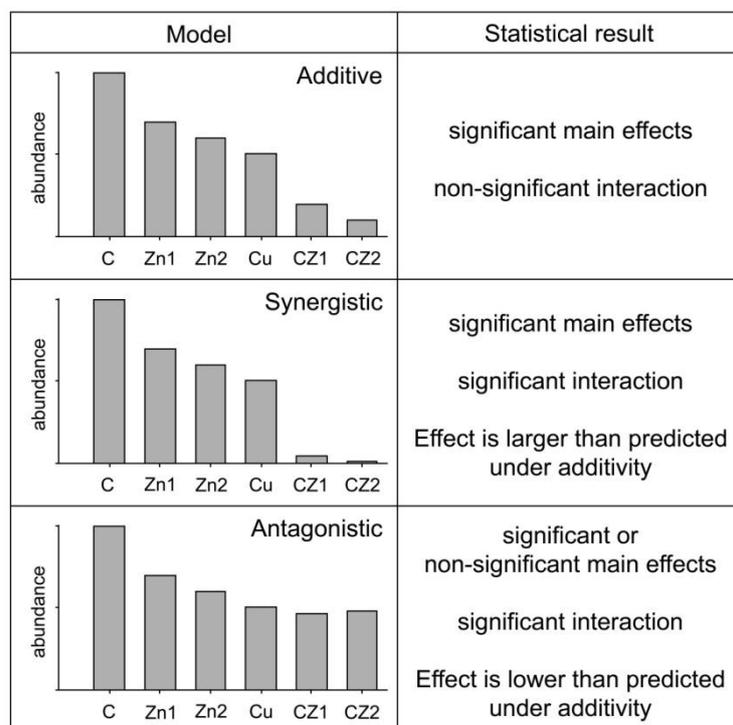


Fig. 4.10. Schematic diagram showing hypothetical results of combined effects of Cu and Zn according to the simplified models of additive, synergistic, and antagonistic effects. The nature of the combined effects can be categorised based on statistical results of ANOVA tests and the directions of deviations from expectations under the additive model as shown by the outcomes of subsequent relevant pair-wise comparisons in the case of non-additivity.

Chapter Five

Bioaccumulation of Copper, Lead and Zinc by the Bivalves

Macomona liliana and *Austrovenus stutchburyi*

Introduction

In aquatic environments, heavy metals exist as dissolved or particulate phases. Aquatic organisms take up these metals from surrounding environments and through feeding (Chapman et al. 1998). These metals can then accumulate in their body tissues and cause lethal or sublethal effects (Bryan and Langston 1992, Bat and Raffaelli 1998). The uptake of dissolved heavy metals may occur by diffusion through permeable surfaces of organisms, including the gut (Depledge and Rainbow 1990), or by absorption across respiratory surfaces (Wang 2001). The absorption efficiency of dissolved heavy metals is an important factor when assessing the toxicity of heavy metals to particular organisms in aquatic environments (Wang et al. 1996). The uptake of heavy metals as particulates may occur through feeding and is affected by the assimilation efficiency of metals and ingestion rates of organisms (Wang et al. 1996).

The bioavailability of heavy metals is defined as the amount of metals that is actually taken up by organisms and therefore has the potential to cause an effect (Plette et al. 1999). The bioavailability of sediment-associated metals is influenced by the metal binding to and subsequently being released from sediment particles. Free metal ions in sediment porewater are generally considered the most bioavailable form of metals, while metals that are bound to sediments may not be toxic to organisms (Chapman and Long 1983). The bioavailability of heavy metals cannot necessarily be determined by simply

measuring their concentrations in porewater and/or sediment (Borgmann and Norwood 1999). When multiple heavy metals are present simultaneously, their bioavailabilities are even more difficult to predict, as different heavy metals have different binding strengths to sediments, and they may interact with one another competing for sediment binding sites (Gadde and Laitinen 1974, Benjamin and Leckie 1981a).

Feeding behaviour can also affect the types of metals that are taken up by organisms. Porewater, containing dissolved metals and metals associated with suspended particulate matter, may be an important exposure pathway for filter-feeders, but ingestion of sediments is likely to be a dominant route of exposure to metals for deposit-feeders (King et al. 2004). Bioturbators and tube-dwelling organisms that actively pump overlying water through their tubes or burrows are likely exposed to metals in overlying water (Chapman et al. 2002). Once organisms take up heavy metals, they have to excrete and/or detoxify these metals to avoid potential toxic effects (Rainbow 2002). Heavy metals that are in excess of the metabolic requirement and storage capacity can be potentially toxic (Depledge and Rainbow 1990). Organisms can, on the other hand, accumulate a large amount of heavy metals without experiencing any toxic effects when stored in detoxified forms (Rainbow 2002).

Bioaccumulation of heavy metals is therefore complex, being influenced by biological processes, sediment chemistry, multiple routes of exposure and different strategies of organisms to regulate heavy metals (Luoma and Rainbow 2005). Nevertheless, concentrations of heavy metals in body tissues of organisms have been used as a measure of the supply of bioavailable metals to the organisms (Rainbow 1995) and have been suggested as a better predictor of toxicity than metal concentrations in the exposure medium (Borgmann and Norwood 1999).

Effects of polluted stormwater runoff are of particular environmental concern in

the region of Auckland, New Zealand. The primary contaminants in the runoff include copper (Cu), lead (Pb) and zinc (Zn) (ARC 2004). The effects of Cu, Pb and Zn on estuarine infauna were recently examined through a manipulative field experiment (Fukunaga et al. 2010; Chapter 2 herein) and laboratory bioassays (Chapter 3). These studies clearly demonstrated that Cu and Zn, individually and in combination, reduce colonisation and survival of estuarine infauna. However, the effects of Pb, either alone or in combination with Cu and/or Zn, were not evident. This raised two questions: (i) is Pb bioavailable to infauna? and (ii) does the presence of Pb affect the bioavailability of Cu and/or Zn? Lead generally has strong binding affinity to benthic sediments (Gadde and Laitinen 1974, Benjamin and Leckie 1981a) and suspended particulate matter in water (Webster-Brown 2005). The strong binding affinity of Pb to sediments may affect the degree of binding by other metals and therefore influence the relative partitioning of Cu and Zn between sediment and porewater.

A manipulative field experiment (Fukunaga et al. 2010; Chapter 2 herein) also revealed differential sensitivities of the two numerically dominant bivalves to these heavy metals. The deposit-feeding bivalve *Macomona liliana* was sensitive to Cu and/or Zn, while the suspension-feeding bivalve *Austrovenus stutchburyi* was not. These bivalves are commonly found throughout estuaries in New Zealand and are important components of these ecosystems. They have previously been used in laboratory bioassays to assess environmental impacts, such as sedimentation and the presence of contaminants (Hickey et al. 1995, Cummings and Thrush 2004, Norkko and Thrush 2006). Higher accumulation of organic contaminants by *M. liliana* and their higher sensitivity, in comparison to *A. stutchburyi*, have also previously been shown in the field (Hickey et al. 1995). The difference in their feeding behaviour (i.e. deposit-feeding *versus* filter-feeding) may explain their differential sensitivity to contaminants; deposit feeders are more likely to

ingest highly contaminated sediments than filter feeders (Wilcock et al. 1993). Examining the bioaccumulation of heavy metals in the tissues of these two different bivalves should provide important information regarding the bioavailabilities of heavy metals and their differential sensitivity.

A series of bioassays was done to examine the bioaccumulation of Cu, Pb and Zn in the tissues of the bivalves *M. liliana* and *A. stutchburyi*, using sediments spiked with Cu, Pb and/or Zn. The bioassays were designed to elucidate the bioaccumulation of Cu and Zn, individually and in a mixture, at different concentrations, either with or without the presence of Pb. The bioaccumulation of Pb was also of interest. It was hypothesised that the accumulation of Cu or Zn by the deposit-feeding bivalve *M. liliana* would increase as concentrations of metals in sediment increase, while the accumulation by the suspension-feeding bivalve *A. stutchburyi* would be relatively unchanged. It was also hypothesised that the accumulation of Pb would not be affected by concentrations of Pb in sediment due to its strong binding affinity, but that the presence of Pb would increase the bioaccumulation of Cu and Zn.

Methods

Sediment and animal collection

A series of laboratory bioassays was done at the Leigh Marine Laboratory in May 2009, using the bivalves *Macomona liliana* and *Austrovenus stutchburyi*. The bivalves *M. liliana* and *A. stutchburyi* were simultaneously collected from Puhoi estuary (36° 31' 53S, 174° 42' 16E), located approximately 40 km north of the city of Auckland, two days prior to each bioassay by sieving sediments through 800 µm mesh. The bivalves were brought back to the laboratory and kept in buckets with running seawater. Bioaccumulation of heavy metals in some animals has been shown to be size-dependent (Wang and Fisher

1997, Garcês and Costa 2009). In order to minimise the potential size effects within each species, bivalves of similar size were collected: 20 – 30 mm in size for *M. liliانا* and 20 – 25 mm in size for *A. stutchburyi*. Sediments for the bioassays were collected from the surface aerobic layer at an intertidal mudflat in Orewa estuary (36° 35' 45S, 174° 40' 51E), approximately 30 km north of the city of Auckland. Sediments brought back to the laboratory were sieved through 500 µm mesh to remove macrofauna and were then homogenised. Small samples of the collected sediment were taken to determine the water content (%) by weight loss after drying at 60 °C for 24 hours. Puhoi estuary and Orewa estuary have relatively low existing concentrations of Cu, Pb and Zn in their sediments, and are considered to be areas uncontaminated by these metals (Anderson et al. 2006b).

Experimental design

There were three bioassays designed to measure bioaccumulation of metals into bivalve tissues: (i) Cu, (ii) Zn and (iii) a mixture of Cu and Zn (Cu + Zn). In each bioassay, there were two concentration levels of the metals (i.e. low and high), and these were each examined either with or without the addition of Pb (Table 5.1). Each bioassay also had a control treatment with no additional metals. Concentrations of these heavy metals for each combination preserved the regional average ratio of Cu:Pb:Zn = 10.7:16.8:72.5 by weight. This ratio was calculated based on the heavy metal concentrations in 84 sheltered intertidal soft-sediment sites across the Auckland region (Anderson et al. 2006b). The high target concentrations were set, based on previous laboratory bioassays using *M. liliانا* (Chapter 3), at levels that would not result in more than 40% mortality of the test organisms, in order to obtain enough individuals for the subsequent depuration process and tissue analysis of metals. The ranges of metal concentrations used in the bioassays were similar to those currently observed in estuaries

of this region (Kelly 2007). Sediments collected from Orewa estuary were spiked with metals to the high target concentrations following the spiking method of Lu et al. (2008). Sediments having low target concentrations were produced by mixing the spiked sediment and the control sediment, which also went through the spiking process but without additional metals.

Table 5.1. List of starting dates and nominal target concentrations of metals for each bioassay.

Bioassay	Starting date (day 0)	Metal	-Pb		+Pb	
			Low	High	Low	High
Cu	May 1	Cu	40.0	60.0	40.0	60.0
		Pb			62.8	94.2
Zn	May 15	Zn	200.0	300.0	200.0	300.0
		Pb			46.3	69.5
Cu + Zn	May 29	Cu	19.6	29.5	19.6	29.5
		Zn	133.0	200.0	133.0	200.0
		Pb			30.8	46.3

The bioassays were done in plastic containers (150 mm × 150 mm × 85 mm) with 450 mL of sediment to a thickness of 20 mm. Each treatment had six replicates, three for *M. liliانا* and the other three for *A. stutchburyi*. Containers were placed outside at the laboratory in a randomised array and exposed to natural weather conditions during each bioassay. Each container was equipped with an independent input and output of seawater. Tidal cycles were mimicked by alternating six-hour inflow and six-hour outflow of seawater through individual containers. The average flow of seawater through the system was 0.4 cm³ per second, so the entire content of water in each 2 L container was replaced approximately every 80 minutes. Containers were first established for 24 hours (day 0) to condition the sediments, and 10 individuals of *M. liliانا* or *A. stutchburyi* were added to each container the next day (day 1). Experiments were run for 10 days, with the addition of only filtered seawater (200 µm) through this period. The number of live individuals in

each container was recorded after 10 days (day 10). Surviving bivalves were depurated in clean seawater for 24 hours to clear sediments in their guts and frozen at -20 °C until further analysis.

Metal concentrations in bivalves

Five individuals of *M. liliana* or *A. stutchburyi* were randomly chosen from each container. The shells were removed and the soft tissues were rinsed in deionised water. Tissues were dried at 60 °C for a minimum of 24 hours, weighed and completely dissolved in high-purity HNO₃ at 80 °C (Bat and Raffaelli 1998). The samples were then diluted with deionised water and filtered through Whatman™ filter paper. Concentrations of metals in the samples were determined using graphite-furnace atomic absorption spectroscopy (GFAAS) for Cu and flame atomic absorption spectroscopy (FAAS) for Zn. Concentrations of Pb were determined by Hill Laboratories in Hamilton, New Zealand, using inductively coupled plasma–mass spectrometry (ICP-MS). The number of tissue samples for Pb analysis was reduced to three per replicate container due to the cost of analysis. Detection limits were 0.001 µg g⁻¹ for Cu and Pb using GFAAS and ICP-MS, respectively, and 0.1 µg g⁻¹ for Zn using FAAS.

Environmental variables

Concentrations of metals in sediment were measured to determine whether the metal-spiking process elevated metal concentrations to the target levels. Small amounts of sediment were collected from each container on day 1, prior to the addition of animals, and on day 10 when live individuals were counted. The sediment samples were dried in an oven at 60 °C for 24 hours. Metal concentrations in the sediment samples were determined by FAAS after digesting approximately 0.5 g of dry sediment in hot aqua regia

(HCl:HNO₃=3:1) using high-purity HCl and HNO₃ (Chen and Ma 2001) and diluting the sediment with deionised water to approximately 15 g. Detection limits of Cu, Pb and Zn were 0.1 µg g⁻¹, 0.5 µg g⁻¹ and 0.1 µg g⁻¹, respectively. The amount of total organic matter (TOM) in each container was also determined by combusting dried sediments at 500 °C for 4 hours (Byers et al. 1978). TOM (%) was calculated by the weight loss after combustion.

Porewater samples were collected in each container over the 10-day experimental period using peepers with mesh size of 37 µm (Adams et al. 2003). Samples were filtered with a 0.45 µm membrane, and acidified with high-purity HNO₃. Porewater analysis was first attempted using GFAAS as metal concentrations in porewater are generally relatively low. However, due to interference by components found in seawater, samples were analysed for Cu and Zn using FAAS instead. Detection limits of these metals were 0.1 mg L⁻¹. Porewater samples taken from sediments with additional Pb were sent to Hill Laboratories for Pb analysis using ICP-MS. In order to determine a reference background concentration of Pb in these bioassays, three each were randomly chosen from porewater samples collected in the control treatments with *M. liliana* or *A. stutchburyi* and were analysed using ICP-MS.

Statistical analyses

Concentrations of heavy metals in bivalves were separately analysed for *M. liliana* and *A. stutchburyi* and for each bioassay. The experimental design consisted of two factors: treatment (fixed with five levels; control, low - Pb, high - Pb, low + Pb and high + Pb) and container (random with three levels, nested in treatment). Concentrations of Cu and Zn were analysed according to the full two-factor design using permutational analysis of variance (PERMANOVA; Anderson 2001, McArdle and Anderson 2001) based on

Euclidean distances, with 4999 permutations of residuals under a reduced model. When significant differences were detected among treatments, specific *a priori* planned contrasts were done using the Monte Carlo asymptotic approximation to the permutation tests (Anderson and Robinson 2003) as follows: (i) the control treatment *versus* the low - Pb and high - Pb treatments to test for the bioaccumulation of Cu or Zn; (ii) the low - Pb treatment *versus* the high - Pb treatment to test for the different levels of bioaccumulation; (iii) the low - Pb treatment *versus* the low + Pb treatment; and (iv) the high - Pb treatment *versus* the high + Pb treatment to test for the effects of Pb on accumulation of either Cu or Zn.

Similarly, concentrations of Pb were also analysed according to the full two-factor design using PERMANOVA based on Euclidean distance with 4999 permutations. If ‘treatment’ was significant, specific *a priori* planned contrasts were done using the Monte Carlo tests as follows: (v) - Pb treatments *versus* the + Pb treatments to test for the bioaccumulation of Pb; and (vi) the low + Pb treatment *versus* the high + Pb treatment to test for different levels of Pb accumulation. These contrasts tested for the effects of Pb in sediments on tissue concentrations of Pb in the bivalves in the presence of Cu and/or Zn. Although the solo effects of Pb cannot be inferred in this manner, one of the purposes of the experiment was to examine if Pb was bioavailable to these bivalves. Given the co-occurrence of these metals in the field, these tests are adequate for this purpose.

Results

Environmental variables

Sediment collected from Orewa estuary for metal-spiking had a water content ranging from 31.1% to 36.8%, and a TOM content of 2.1 – 2.7% (Table 5.2). Sediment spiking mostly elevated concentrations of Cu, Pb and Zn in sediments to their target levels

(Fig. 5.1), although concentrations of Zn did not quite reach target levels at higher concentrations. Concentrations of Pb in samples taken from sediments where no Pb had been added were under the detection limit of $0.5 \mu\text{g g}^{-1}$ for this element. Considering the $30\times$ dilution after the acid digestion process in the sample preparation, background concentrations of Pb in sediments were determined to be less than $15 \mu\text{g g}^{-1}$.

Average concentrations of Cu, Pb and Zn in porewater were mostly positively correlated with concentrations of these metals in sediments (Fig. 5.2). Concentrations of Cu in porewater were generally too low for accurate determination by FAAS, being under the detection limit of 0.1 mg L^{-1} (Fig. 5.2a). Average concentrations of Cu and Zn in porewater were, generally, higher in the + Pb treatments than in the - Pb treatments (Fig. 5.2a, c).

Table 5.2. Water contents (WC; %) in sediments collected for metal-spiking, measured prior to spiking, and TOM \pm SE (%) in sediments on day 1 and day 10.

Bioassay	WC (%)	TOM \pm SE (%)	
		Day 1	Day 10
Cu	33.1	2.48 ± 0.06	2.24 ± 0.04
Zn	33.3	2.71 ± 0.05	2.11 ± 0.05
Cu + Zn	36.8	2.14 ± 0.05	1.89 ± 0.06

Metal concentrations in bivalves

No mortality of *Austrovenus stutchburyi* was observed in any treatments in the three bioassays (Table 5.3). There was also no mortality of *Macomona liliana* in any of the control treatments in the three bioassays. Mortality of *M. liliana* increased with increases in concentrations of metals in sediments (Table 5.3). In the Cu bioassay and the Cu + Zn bioassay at the high target level, survival rates of *M. liliana* were, on average,

lower in the + Pb treatment than in the - Pb treatment. There was no difference in average percent survival between the + Pb and - Pb treatments in the Zn bioassay.

Table 5.3. Mean percent survival (\pm SE) of *M. liliana* and *A. stutchburyi* after the 10-day experimental period.

Bioassay	Bivalve	Control	-Pb		+Pb	
			Low	High	Low	High
Cu	<i>M. liliana</i>	100	96.7 (\pm 3.3)	86.7 (\pm 8.8)	93.3 (\pm 3.3)	76.7 (\pm 3.3)
	<i>A. stutchburyi</i>	100	100	100	100	100
Zn	<i>M. liliana</i>	100	100	96.7 (\pm 3.3)	100	96.7 (\pm 3.3)
	<i>A. stutchburyi</i>	100	100	100	100	100
Cu + Zn	<i>M. liliana</i>	100	100	93.3 (\pm 3.3)	100	90.0 (\pm 0.0)
	<i>A. stutchburyi</i>	100	100	100	100	100

Concentrations of heavy metals in the tissues of bivalves were generally much higher in *M. liliana* than in *A. stutchburyi* (Figs. 5.3 – 5.5). More specifically, concentrations of Cu in *M. liliana* ranged between $8 \mu\text{g g}^{-1}$ and $180 \mu\text{g g}^{-1}$, with the exception of a few samples in the Cu bioassay, where tissue concentrations of Cu reached as high as $485 \mu\text{g g}^{-1}$, while those in *A. stutchburyi* were between just $1 \mu\text{g g}^{-1}$ and $18 \mu\text{g g}^{-1}$. Concentrations of Pb in *M. liliana* ranged between $0.8 \mu\text{g g}^{-1}$ and $34 \mu\text{g g}^{-1}$, while those in *A. stutchburyi* were between $0.1 \mu\text{g g}^{-1}$ and $2.7 \mu\text{g g}^{-1}$ with the exception of one sample in the Zn bioassay that had $4.8 \mu\text{g g}^{-1}$. The difference in concentrations between the two bivalves was not as large for Zn, although still apparent. Concentrations of Zn in *M. liliana* were between $20 \mu\text{g g}^{-1}$ and $290 \mu\text{g g}^{-1}$, while those in *A. stutchburyi* were between $50 \mu\text{g g}^{-1}$ and $170 \mu\text{g g}^{-1}$. Average concentrations of Zn in the control treatments were higher in *A. stutchburyi* than in *M. liliana*, indicating that *A. stutchburyi* would likely have higher tissue concentrations of Zn than *M. liliana* in uncontaminated sediments.

Concentrations of Cu in the tissues of *M. liliana* did not differ significantly among

the treatments in either the Cu or Cu + Zn bioassays (Table 5.4a). Thus, *M. liliانا* does not appear to accumulate Cu in its tissues in response to increasing Cu concentrations in sediments; this was observed regardless of the presence/absence of Pb. Concentrations of Cu in *M. liliانا* were relatively variable within each treatment, ranging between about 12 $\mu\text{g g}^{-1}$ and 178 $\mu\text{g g}^{-1}$. Concentrations of Cu in *A. stutchburyi* significantly increased with increasing concentrations of Cu in sediments in the presence of Zn (i.e. in the Cu + Zn bioassay), but not in the Cu bioassay (Table 5.4a).

In contrast, concentrations of Zn in *M. liliانا* significantly increased with increasing concentrations of Zn in sediments when no Pb was added (Table 5.4b). The low + Pb treatment also had significantly higher levels of Zn than the low - Pb treatment, showing that more Zn was taken up when Pb was added. In the Cu + Zn bioassay, concentrations of Zn in *M. liliانا* were significantly higher in the Zn-spiked treatments than in the control treatment, but there was no significant difference in uptake between treatments having low *versus* high levels of Zn, when no Pb was added (Table 5.4b). The presence of Pb at the high target level, however, resulted in significantly higher concentrations of Zn in the animal's tissues (Table 5.4b). Concentrations of Zn in *A. stutchburyi* in the Zn and Cu + Zn bioassays were significantly higher in the Zn-spiked treatments than in the control, but there was no significant difference between the two levels of concentration (low *versus* high, Table 5.4b). In the Cu + Zn bioassay, significantly higher levels of Zn were taken up by *A. stutchburyi* when Pb was added (Table 5.4b).

Concentrations of Pb in *M. liliانا* and *A. stutchburyi* were significantly elevated in the + Pb treatments in all bioassays (Table 5.4c). Concentrations of Pb in *A. stutchburyi* were also significantly higher in the high + Pb treatment than in the low + Pb treatment in the Cu bioassay (Table 5.4c).

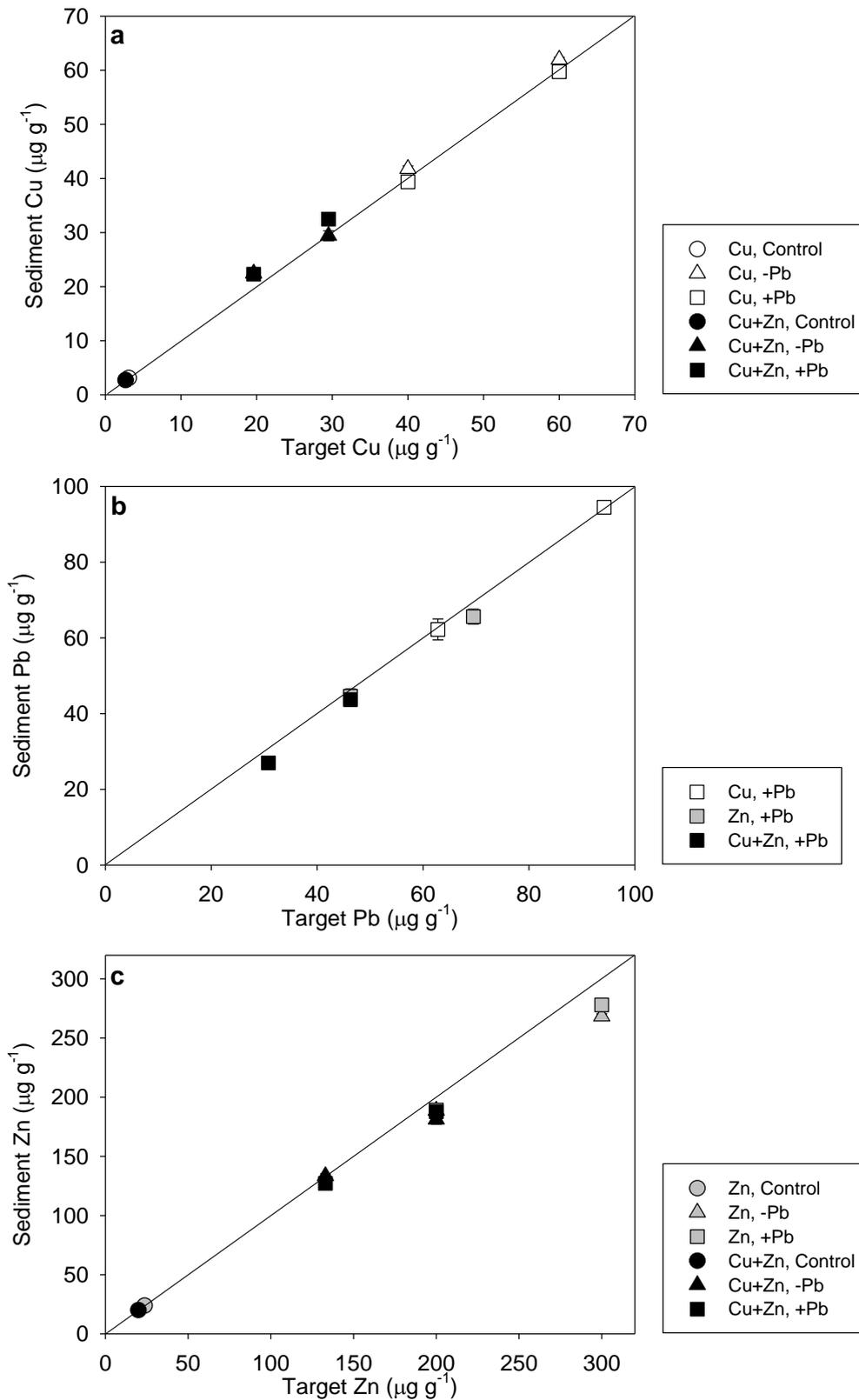


Fig. 5.1. Average concentrations ($n = 6$) of metals in sediment (mean \pm SE) at different target concentrations, for (a) Cu, (b) Pb and (c) Zn. The solid line indicates a one-to-one relationship, where the measured concentrations would be precisely equal to the target concentrations.

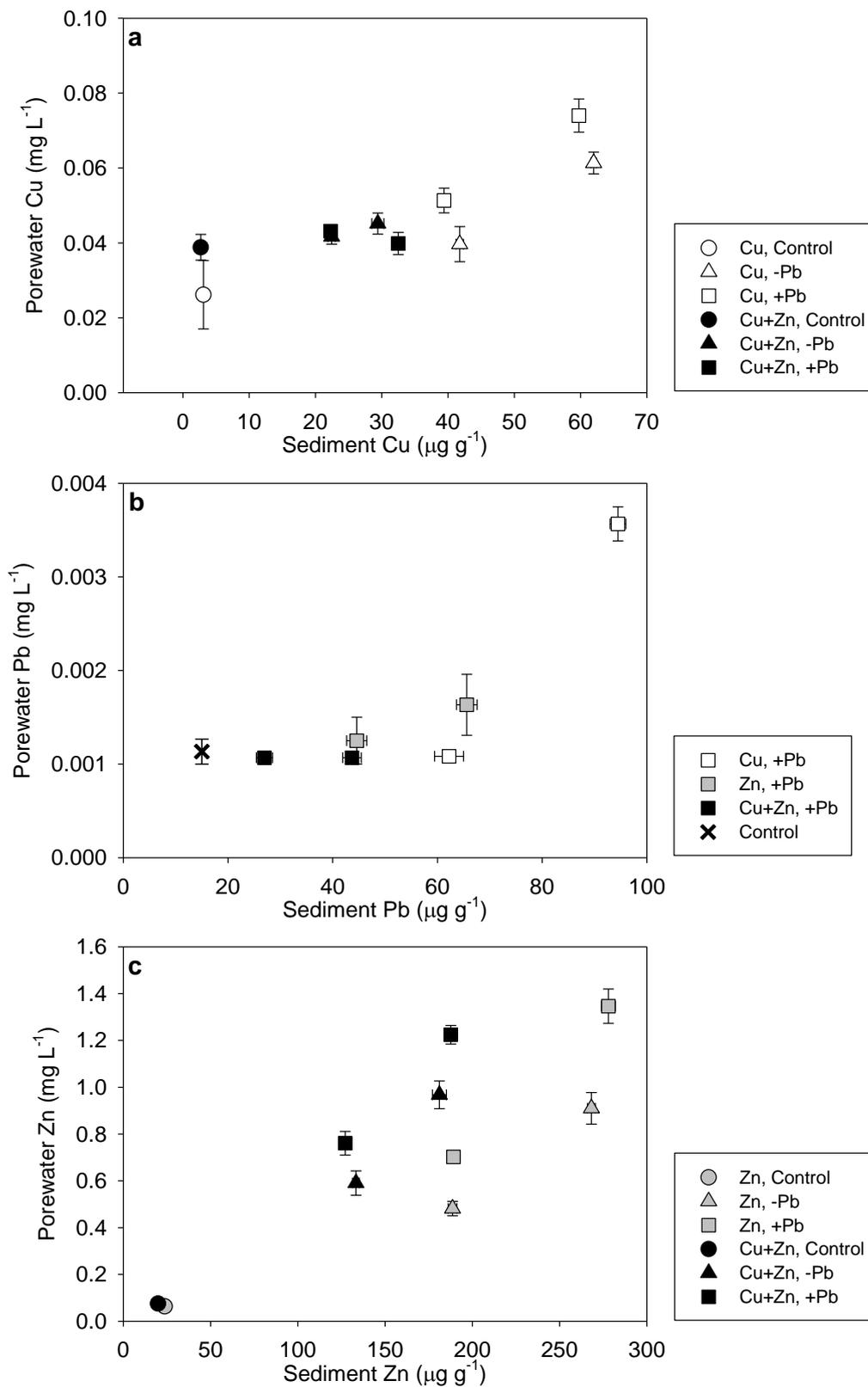


Fig. 5.2. Average concentrations ($n = 6$) of metals in porewater (mean \pm SE), at different concentrations of metals in sediments for (a) Cu, (b) Pb and (c) Zn.

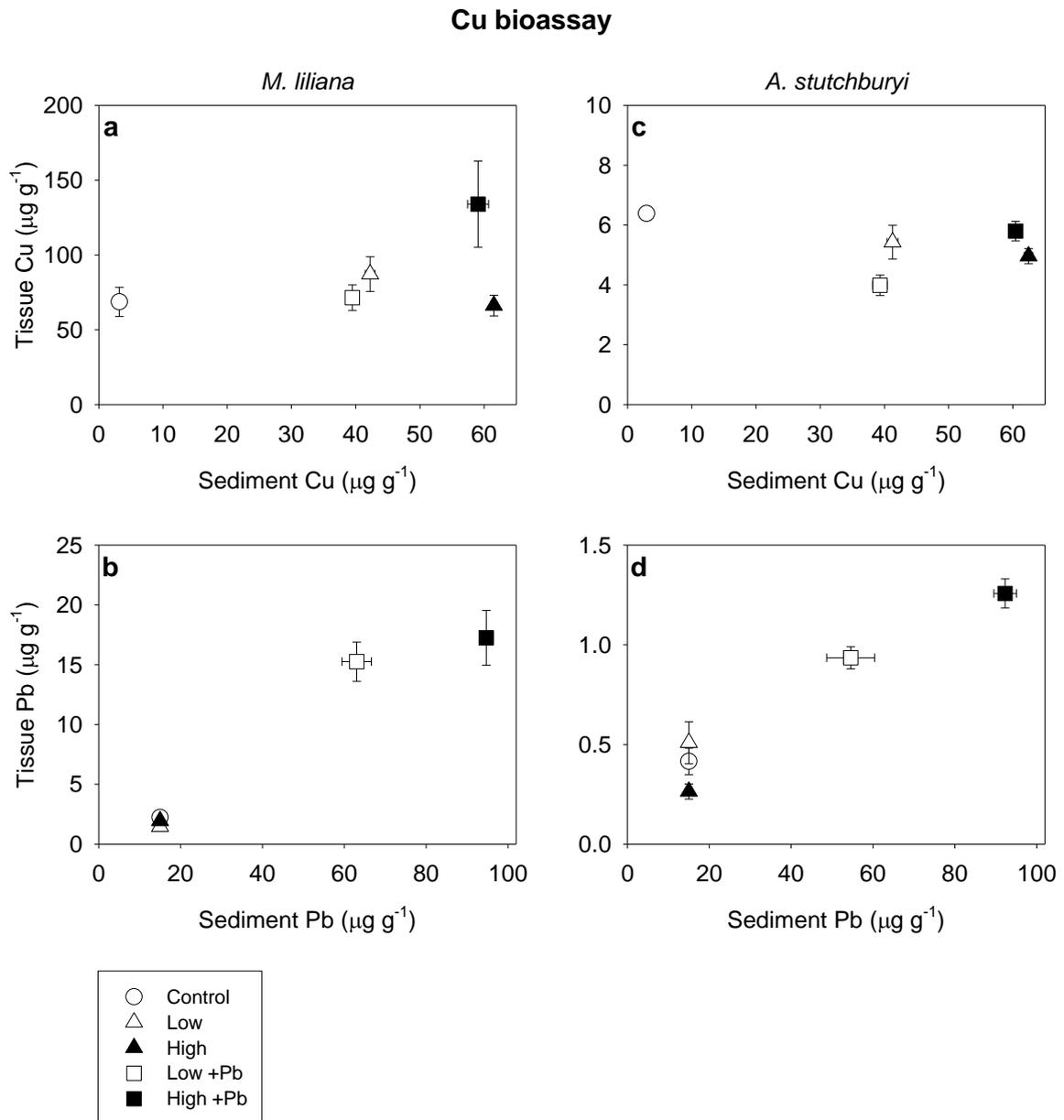


Fig. 5.3. Average concentrations of metals in the Cu bioassay in *M. liliana* for (a) Cu and (b) Pb, and in *A. stutchburyi* for (c) Cu and (d) Pb. Data from three different containers for each treatment were pooled.

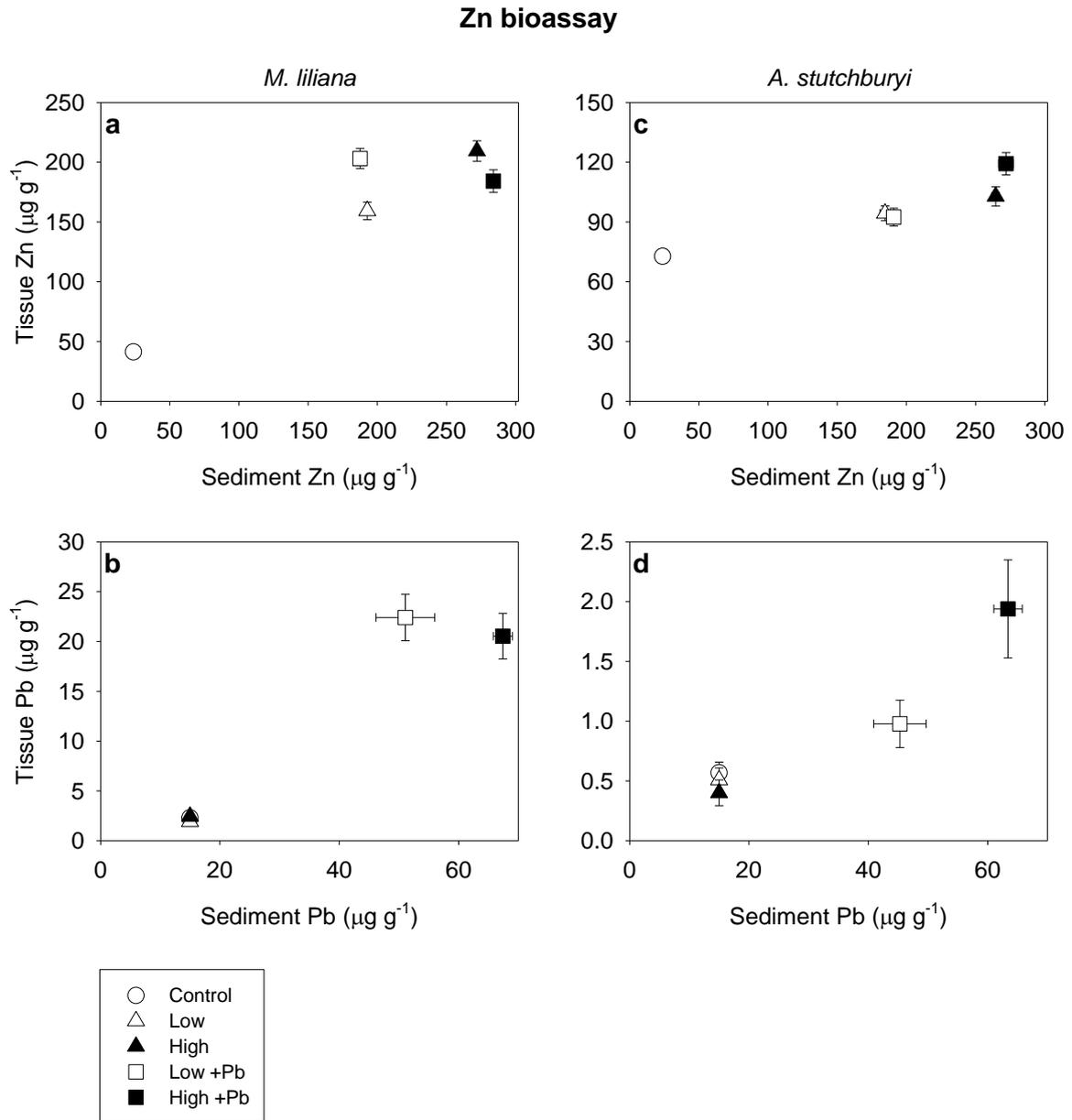


Fig. 5.4. Average concentrations of metals in the Zn bioassay in *M. liliana* for (a) Zn and (b) Pb, and in *A. stutchburyi* for (c) Zn and (d) Pb. Data from three different containers for each treatment were pooled.

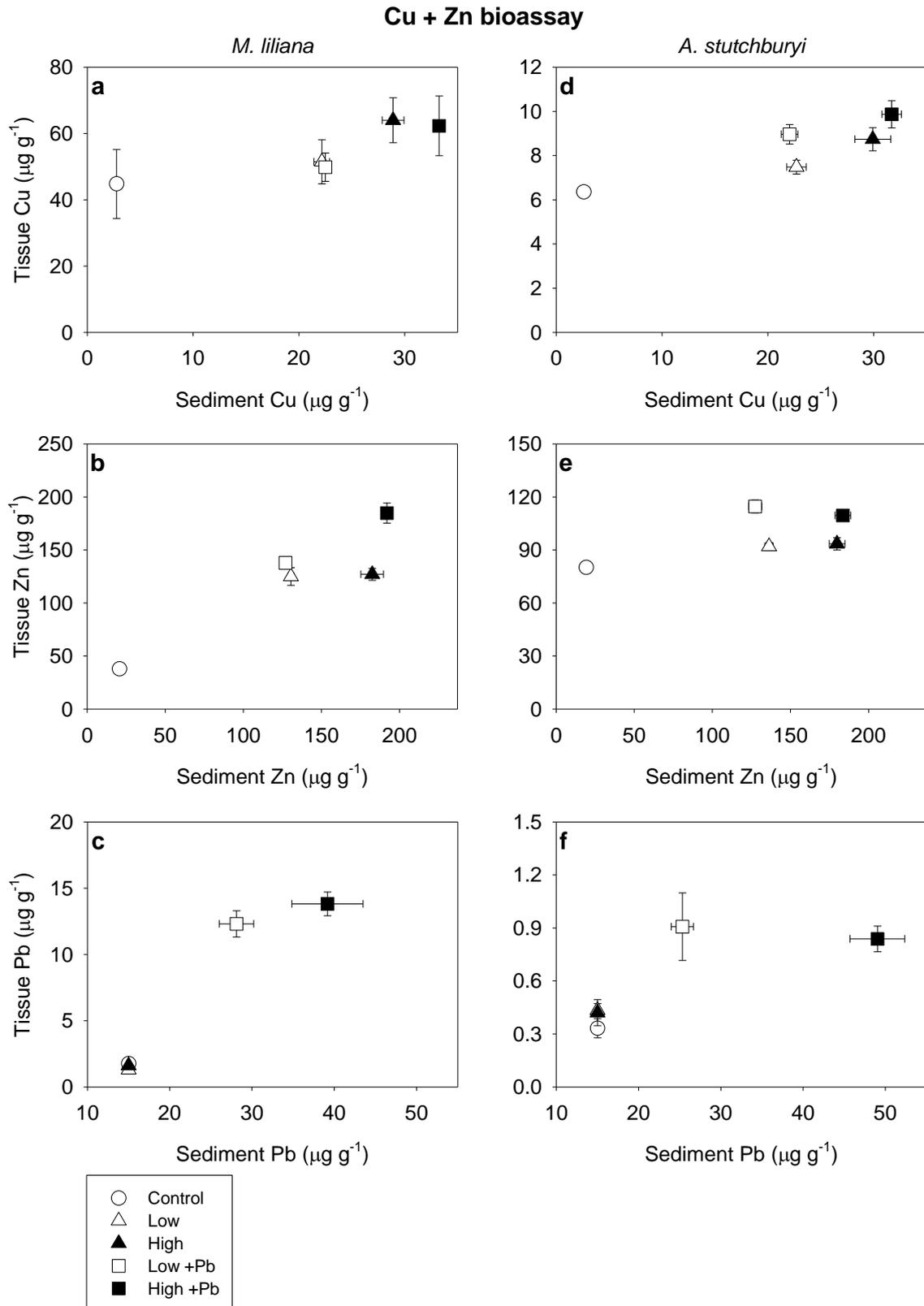


Fig. 5.5. Average concentrations of metals in the Cu + Zn bioassay in *M. liliana* for (a) Cu, (b) Zn and (c) Pb, and in *A. stutchburyi* for (d) Cu (e) Zn and (f) Pb. Data from three different containers for each treatment were pooled.

Table 5.4. Summary of results of PERMANOVA for (a) Cu, (b) Pb and (c) Zn for *M. liliana* and *A. stutchburyi*, and results of *a priori* contrasts. For Cu and Zn, statistically significant contrasts are indicated as (i) the control treatment vs. the low and high - Pb treatments, (ii) the low - Pb treatment vs. the high - Pb treatment, (iii) the low - Pb treatment vs. the low + Pb treatment and (iv) the high - Pb treatment vs. the high + Pb treatment. For Pb, significant contrasts are indicated by: (v) - Pb treatments vs. the + Pb treatments and (vi) the low + Pb treatment vs. the high + Pb treatment.

(a) Cu	Treatment		Container(Treat)		Contrast (Treatment)
	$F_{4,10}$	P	$F_{10,60}$	P	
<i>M. liliana</i>					
Cu bioassay	2.20	0.091	1.70	0.079	
Cu + Zn bioassay	0.90	0.495	1.35	0.217	
<i>A. stutchburyi</i>					
Cu bioassay	2.01	0.170	4.78	0.0002	
Cu + Zn bioassay	12.19	0.0016	0.76	0.686	(i), (ii)

(b) Zn	Treatment		Container(Treat)		Contrast (Treatment)
	$F_{4,10}$	P	$F_{10,60}$	P	
<i>M. liliana</i>					
Zn bioassay	98.44	0.0002	0.79	0.632	(i), (ii), (iii)
Cu + Zn bioassay	54.14	0.0002	1.22	0.312	(i), (iv)
<i>A. stutchburyi</i>					
Zn bioassay	7.29	0.0026	2.30	0.0216	(i)
Cu + Zn bioassay	39.42	0.0002	0.49	0.890	(i), (iii), (iv)

(c) Pb	Treatment		Container(Treat)		Contrast (Treatment)
	$F_{4,10}$	P	$F_{10,30}$	P	
<i>M. liliana</i>					
Cu bioassay	20.64	0.0008	2.65	0.0082	(v)
Zn bioassay	67.59	0.0008	0.72	0.714	(v)
Cu + Zn bioassay	55.94	0.0012	2.81	0.0120	(v)
<i>A. stutchburyi</i>					
Cu bioassay	16.60	0.0004	2.99	0.0110	(v), (vi)
Zn bioassay	6.49	0.0046	1.44	0.185	(v)
Cu + Zn bioassay	4.59	0.0308	1.78	0.090	(v)

Discussion

The bivalves *Macomona liliana* and *Austrovenus stutchburyi* accumulated Pb and Zn in their body tissues when they were exposed to elevated levels of these metals in sediments, while bioaccumulation of Cu occurred only in the presence of Zn for *Austrovenus* and was not evident for *M. liliana*. Copper is one of the metals used for metabolic purposes (an essential metal) and, therefore, many organisms have systems to regulate this metal, comprising specific mechanisms for uptake, sequestration, detoxification and excretion (Phillips and Rainbow 1989). High concentrations of this metal in some individuals in the control treatment, as well as high variation among individuals, suggest that *M. liliana* take up and store Cu in a detoxified form. When organisms store heavy metals in a detoxified form, there is no theoretical limit to the amount that can be stored and no known threshold in concentration at which toxic effects might occur (Rainbow 2002).

Metallothionins (MT) are the most important metal-binding ligand to detoxify heavy metals and are found in most living organisms (Viarengo and Nott 1993). MT are proteins with low molecular weight, mainly located in the cytosol, and rapidly produced when organisms are exposed to heavy metals (Vijver et al. 2004). MT binds and detoxifies both essential and non-essential metals, but also plays an important role in homeostasis of essential metals, providing a reservoir (Viarengo and Nott 1993, Wang and Shi 2001). In some organisms, heavy metals may also be sequestered in particular subcellular fractions, tissues and/or organs. For example, the bivalve *Macoma balthica* has been shown to store heavy metals in metal-rich granules, in which metals are precipitated into insoluble concretions (Wallace et al. 2003). The mussel, *Mytilus edulis*, has been shown to accumulate heavy metals in lysosomes in the kidney, which would be eventually excreted in the urine (George et al. 1982). The terrestrial isopod *Porcellio*

scaber has also been shown to accumulate heavy metals in lysosomes in the hepatopancreas (Dallinger and Prosi 1988). A large amount of metal sequestered in a particular tissue can mask smaller fluctuations in the concentrations of metals in their specific tissues or critical organs, so can be overlooked when measuring concentrations for the body as a whole (Depledge and Rainbow 1990).

Organisms suffer toxic effects of heavy metals when the rate of metal uptake exceeds the rates of detoxification and excretion, and therefore concentrations of metabolically available metals around biologically important metal-binding molecules rise above tolerance levels (Chan 1988, Rainbow 2002). The mortality of *M. liliانا* in Cu-spiked sediments in the present experiment may be explained by increases in concentrations of metabolically available Cu that were masked by the total concentrations of this metal in the bivalve as a whole. However, bivalves can quickly retract their body into their shells when exposed to relatively low concentrations of Cu (Wisely and Blick 1967). This behaviour would reduce their exposure to Cu while their shells remain closed, but it might also interfere with their feeding behaviour and result in starvation (Rashid et al. 2009).

Bioaccumulation of Cu by *A. stutchburyi* was found only when they were exposed to elevated levels of Cu in the presence of elevated levels of Zn, but not when exposed to either Cu alone or Cu in the presence of Pb. The uptake of Cu by the common mussel *Mytilus edulis* has been shown to be affected by the presence and variation in concentration of other metals (Phillips 1976). In that study, however, the addition of Zn caused a reduction rather than an increase in the body concentrations of Cu in *M. edulis*. It is therefore unclear whether Zn would facilitate or inhibit the uptake of Cu by bivalves in general. Further studies on the physiological mechanisms of Cu and Zn uptake by *A. stutchburyi* are required to clarify potential interactions between these two metals during

uptake.

Zinc is also an essential metal and is regulated in many aquatic organisms (Phillips and Rainbow 1989). The hard clam *Meretrix meretrix* has been shown to accumulate but also to depurate Zn at relatively similar rates (Rashid et al. 2009). Concentrations of Zn in the mussel *Perna viridis* have also been shown to increase when they were exposed to higher levels of dissolved Zn, but reached a plateau at a body concentration of around 250 $\mu\text{g g}^{-1}$ (Chan 1988). In the field, *P. viridis* maintain body concentrations of Zn within a relatively narrow range regardless of the levels of Zn in their surrounding environment, so they are considered to be a partial regulator of Zn (Phillips 1985). A similar pattern of Zn accumulation was detected for *A. stutchburyi* in the current experiment. The amount of Zn in the tissues of *A. stutchburyi* was, overall, maintained within a relatively narrow range, indicating that *A. stutchburyi* may be partially regulating body concentrations of Zn.

Although concentrations of the essential metals Cu and Zn have been reported not necessarily to reflect metal loads in their surrounding environments in the bivalves *Limnoperna fortunei* and *Corbicula fluminea* (Villar et al. 1999), and in the terrestrial collembolan *Orchesella cincta* (Van Straalen et al. 1987), this did not seem to be the case for concentrations of Zn in *M. liliانا*. Concentrations of Zn in *M. liliانا* were fairly similar to those in the surrounding sediments. Concentrations of Zn in sediment at the low target level in the Cu + Zn bioassays and those at the high target level in the Zn bioassays were similar, as they had the same target concentration of 200 $\mu\text{g g}^{-1}$. At this target concentration, the concentrations of Zn in *M. liliانا* were, on average, higher in the absence of Cu (i.e. in the Zn bioassay; Fig. 5.4a) than in the presence of Cu (i.e. in the Cu + Zn bioassay; Fig 5.5b), but mortality rates were higher in the presence of Cu (Table 5.3). Although direct comparison between these two bioassays may require some caution, as they were done separately, this nevertheless highlights the importance of interactions

among multiple metals when assessing their lethal effects. Linking the bioaccumulation of heavy metals in a particular organism and their toxicity is complex (Luoma and Rainbow 2005). A higher level of bioaccumulation of one metal does not necessarily mean a higher mortality rate of the organism, especially when multiple metals are simultaneously present.

Lead is a non-essential metal, and therefore, organisms do not have essential metabolic requirements for this metal. Aquatic organisms generally detoxify non-essential metals to avoid toxic effects (Rainbow 2002). Detoxified metals, then, may or may not be excreted (Rainbow 2002). For example, the mussel *Trirhomya hirsuta* has been shown to accumulate Pb when exposed to elevated levels of Pb, but not to excrete these accumulated metals even after being transported to clean seawater (Klumpp and Burdon-Jones 1982). In the present experiment, Pb was taken up and accumulated by the bivalves *M. liliana* and *A. stutchburyi*. Concentrations of Pb in these bivalves were, however, considerably lower compared to Cu or Zn, supporting the previous finding of no apparent acute toxicity of Pb to estuarine infauna in the field (Fukunaga et al. 2010).

The low concentrations of Pb in *M. liliana* and *A. stutchburyi* confirm the low bioavailability of Pb to these bivalves, potentially due to its strong binding affinity to sediments and suspended particulate matter (Gadde and Laitinen 1974, Benjamin and Leckie 1981a, Webster-Brown 2005). Such strong binding affinity may result in low assimilation and/or absorption rates of this metal by organisms. For example, assimilation of Pb by the collembolan *O. cincta* through feeding has been reported to be only 0.4% (Van Straalen et al. 1987). Concentrations of heavy metals examined in the present study were comparable to those currently observed in estuaries in the Auckland region (Kelly 2007). It is therefore unlikely that short-term exposure to current levels of Pb in Auckland's estuaries results in bioaccumulation of this metal in the bivalves to a level

which exceeds their tolerance. However, the combined effects of Pb and other metals, as well as the potential chronic effects of Pb exposure still need to be considered.

Concentrations of Cu and/or Zn in the bivalves in the + Pb treatments either were not significantly different from or were significantly higher than those in the - Pb treatments, suggesting that the presence of Pb can influence the bioavailability and/or uptake of other metals.

The potential chronic effects of Pb and other metals can be difficult to predict based on results of short-term bioassays, as some organisms may develop tolerance to heavy metals (Van Straalen et al. 1987, Posthuma et al. 1992, Viarengo and Nott 1993). In contrast, the toxicity of heavy metals can be time-dependent; Crommentuijn et al. (1994) examined bioaccumulation of Cd in six different species of terrestrial arthropods and found that LC₅₀ values (concentrations of Cd that resulted in 50% mortality) decreased over time, depending on the ability of each species to excrete Cd. This indicates that concentrations of heavy metals that are not high enough to cause acute toxicity to particular organisms can still have serious chronic effects to the organisms. Life table response experiments that examine the effects of metal contaminants on various life history parameters may provide insight regarding the chronic population level effects of heavy metals on test organisms (Levin et al. 1996). A negative correlation between the distribution of *A. stutchburyi* and concentrations of Cu has been shown in the field (Thrush et al. 2008), potentially indicating the importance of assessing chronic effects of heavy metals.

The bioavailability of heavy metals is not absolute, being affected by the feeding behaviour of organisms. Thus, patterns of metal accumulation in organisms also vary qualitatively and quantitatively from species to species (Phillips and Rainbow 1988). The higher concentrations of heavy metals found in *M. liliana* than in *A. stutchburyi* in the

present study suggest that the bioavailability of heavy metals is generally higher for *M. liliana*, perhaps as a consequence of the difference in their feeding behaviour. However, the relatively high concentrations of Zn in *A. stutchburyi* in the control treatment in all bioassays indicate that the difference in their feeding behaviour alone cannot explain the different levels of bioaccumulation in these bivalves. In another study, the isopods *Oniscus asellus* and *Porcellio scaber* were shown to have considerably different levels of metals, despite their similarities of diet and structure of the digestive system, due to differences in the rates of uptake and loss of metals by these two species (Hames and Hopkin 1991a). Although dietary uptake of heavy metals is an important factor (Luoma and Rainbow 2005), excretion efficiency has been suggested to have a greater influence on the bioaccumulation of metals than the efficiency of food assimilation (Janssen et al. 1991). Relatively high concentrations of Zn in *A. stutchburyi* may reflect the metabolic requirement of Zn for this species. Such a high equilibrium concentration may be caused by low excretion (Janssen et al. 1991). Although the generally higher levels of metal accumulation in *M. liliana* may potentially explain the higher sensitivity of this bivalve to heavy metals, bioaccumulation of heavy metals does not always reflect the bioavailability or toxicity of the metals.

Conclusions

This study examined the bioaccumulation of Cu and/or Zn in the presence/absence of Pb, as well as bioaccumulation of Pb, by the bivalves *Macomona liliana* and *Austrovenus stutchburyi*. *M. liliana* and *A. stutchburyi* both accumulated Pb and Zn, although *A. stutchburyi* might partially regulate body concentrations of Zn. *A. stutchburyi* also accumulated Cu to a small extent in the presence of Zn, suggesting an interaction between these two metals during uptake. There was some evidence that the presence of

Pb could affect bioaccumulation of Cu and Zn. Bioaccumulation of heavy metals was generally much lower in *A. stutchburyi* than in *M. liliانا*, potentially explaining the higher tolerance of *A. stutchburyi* to heavy metals. Previous investigations have indicated no acute toxicity of Pb to either *M. liliانا* or *A. stutchburyi*, as well as the relative tolerance of *A. stutchburyi* to heavy metals. However, the fact that these bivalves do accumulate the metals, when exposed to concentrations currently observed in Auckland's estuaries, highlights the need for further investigations of potential chronic toxicity of these heavy metals.

Chapter Six

General Discussion

Salient results

The core essential results and findings presented in this thesis, obtained from field and laboratory experiments are as follows:

- Elevated levels of Cu, Zn and a mixture of Cu, Pb and Zn reduced the colonisation of various infaunal taxa (Chapter 2).
- A relative tolerance to Cu, Pb and Zn was found for oligochaetes, the bivalve *Austrovenus stutchburyi* and the amphipod *Paracalliope* sp. (Chapter 2).
- The survival rate of the deposit feeding bivalve *Macomona liliana* was reduced by Cu and Zn, and the combined effects of these metals were cumulative (Chapter 3).
- No strong effects of Pb (either alone or in combination with Cu and/or Zn) on either colonisation of infauna or survival of *M. liliana* were detected (Chapters 2 and 3).
- The nature of the simultaneous effects of Cu and Zn on the colonisation of infauna differed for different response variables being examined. Effects were additive for the mean log abundances of the polychaetes *Prionospio* sp. and *Scoloplos cylindifer*, for species richness and for the multivariate response of the infaunal community composition as a whole, while those for the mean log abundances of total infauna and the polychaete *Heteromastus* sp. were non-additive and antagonistic (Chapter 4).
- The filter feeding bivalve *A. stutchburyi* accumulated, overall, much lower amounts of heavy metal than the deposit feeding bivalve *M. liliana*, which may

partially explain their relative insensitivity to heavy metals (Chapter 5).

- *A. stutchburyi* and *M. liliana* both accumulated Pb and Zn, but bioaccumulation of Cu occurred in *A. stutchburyi* only in the presence of Zn and was not evident in *M. liliana* (Chapter 5).
- The presence of Pb could increase the bioavailability of Cu and/or Zn and, therefore, uptake of these metals by the bivalves (Chapter 5).

The effects of heavy metals on estuarine communities

The adverse effects of copper (Cu) and zinc (Zn) on estuarine infauna were found consistently throughout this study. Given the ranges of sediment concentrations of these heavy metals examined in each experiment, which were comparable to the current levels of concentrations in Auckland's estuaries (Kelly 2007, Stewart et al. 2009), these metals are likely to play an important role in limiting the present distributions of infauna. This is further supported by previous field investigations in the Auckland region, in which correlative relationships between concentrations of these metals and the structure of estuarine infaunal communities were found (Thrush et al. 2008, Hewitt et al. 2009). The effects of Cu and Zn on estuarine communities intensified when they were considered in tandem; additivity of Cu and Zn in their effects was detected for a number of individual taxa (Chapter 4). As concentrations of Cu and Zn in estuarine sediments are predicted to increase over time across the Auckland region (Green et al. 2004a, b), these heavy metals need to be treated as serious environmental threats to estuarine communities.

Acute toxicity of lead (Pb) to estuarine infauna, on the other hand, was not evident. There was no individual effect of Pb detected on the colonisation of infauna (Chapter 2) or on the mortality of the estuarine bivalve, *Macomona liliana* (Chapter 3). As the main source of Pb in stormwater runoff in the Auckland region is from residues of historic paint

and petrol (Timperley et al. 2005), the lack of Pb toxicity at current measured sediment concentrations indicates that Pb can potentially be given lower priority for management compared to Cu or Zn. Changes in the structure of Auckland's estuarine infaunal communities along a gradient of Cu, Pb and Zn concentrations (Hewitt et al. 2009) is also more likely to be due to the effects of Cu and Zn, rather than that of Pb. There are, however, still concerns regarding the presence of Pb in estuarine sediments due to the indirect effect of Pb through its interactions with Cu and/or Zn; the presence of Pb may increase the bioavailability of Cu and/or Zn, and therefore uptake of these metals by organisms, potentially exacerbating their effects (Chapters 3 and 5). Bioaccumulation of Pb in the bivalves, *M. liliانا* and *Austrovenus stutchburyi*, also highlights the need for further investigations of the potential effects of chronic exposure to heavy metals. For example, Stewart (2005) reported a disrupted reproductive cycle and a potential decrease in size at reproductive maturity for *A. stutchburyi* in Tamaki estuary, Auckland, which is contaminated by heavy metals and polycyclic aromatic hydrocarbons. This suggests that heavy metals can also cause important negative sublethal population-level effects on these bivalves (Stewart 2005).

Previous investigations of the effects of pollutants on infaunal communities around the world have shown negative correlative relationships between levels of pollutants and the abundance, biomass and diversity of benthic fauna (Unnithan et al. 1975, Nicholls et al. 1981, Billen et al. 1999). Similar correlative relationships in the concentrations of heavy metals and the structure of infaunal assemblages have also been found throughout the world, including Europe (Lande 1977, Rygg 1985), North America (Balthis et al. 2002), Australia (Stark 1998b) and New Zealand (Hewitt et al. 2009). In the region of Auckland, New Zealand, 5% of intertidal infaunal taxa were predicted to be affected by a mixture of Cu, Pb and Zn at sediment concentrations of 6.5 – 9.3 $\mu\text{g g}^{-1}$ for Cu, 18.8 – 19.4 $\mu\text{g g}^{-1}$ for

Pb and $114 - 118 \mu\text{g g}^{-1}$ for Zn (Hewitt et al. 2009). A general response of macrobenthic communities to environmental pollution is thought to be a reduction of species diversity, through decreases in the abundances of sensitive organisms and relatively large increases in the abundances of more tolerant, opportunistic organisms (Pearson and Rosenberg 1978). The effects of Cu and/or Zn found here through manipulative field experiments in Orewa estuary (Chapters 2 and 4) are generally consistent with this idea, clearly causing decreases in the abundances of sensitive taxa and species richness. Relatively tolerant species (i.e. oligochaetes, the bivalve *A. stutchburyi* and the amphipod *Paracalliope* sp.) were also found in the present study, although these species did not exhibit large increases in their abundances. Given the relatively short experimental periods used in the present study, the acute toxicities of heavy metals may not cause a dramatic reduction of species diversity, as reported in association with long-term organic pollution (e.g. Tsutsumi 1987, Lee et al. 2006), since no organisms apparently benefit from high metal concentrations. The effects of chronic exposure to heavy metals can cause, however, a shift in faunal assemblages from sensitive organisms to more tolerant ones (Blanck 2002, Gerhardt et al. 2004). Such tolerant organisms may or may not show large increases in abundances through recruitment over a long period of time.

Both lethal effects and the bioaccumulation of heavy metals in the soft tissue of infauna found here have important implications for higher trophic levels, as infaunal organisms provide an important link in estuarine food webs. Bioaccumulation of heavy metals depends on the physiological strategies used by specific organisms to regulate the metals (Rainbow 2002). For example, the isopods *Oniscus asellus* and *Porcellio scaber*, with similar diets, have been shown to accumulate considerably different levels of metals, due to differences in the rates of uptake and loss of metals by these two species (Hames and Hopkin 1991a, b). The assimilation efficiencies of cadmium (Cd) by four different

species of terrestrial arthropods have been found to be related to trophic levels (more specifically, predator species have higher assimilation efficiencies), but concentrations of Cd in these arthropods were mainly influenced by their excretion efficiencies rather than their trophic levels (Janssen et al. 1991). However, several metals, including methylmercury, Zn and selenium (Se), are possibly biomagnified during their transfer through marine food webs (Wang 2002). Luoma et al. (1992) showed that, in San Francisco bay, concentrations of Se in the water were much lower than the level that would cause toxicity to organisms in water-only bioassays, but such concentrations could potentially cause toxicity to organisms at higher trophic levels (e.g. fish and birds) through transfer of this metal through the food chain.

The subcellular distribution of heavy metals in prey species also potentially affects the bioavailability of the metals to their predators. For example, increases in concentrations and durations of Cd exposure have been shown to result in increases in the amount and proportion of Cd in cytosol in the oligochaete *Limnodrilus hoffmeisteri*, perhaps due to induction of metallothioneine (MT) in cytosol, which binds and detoxifies heavy metals (Wallace and Lopez 1996). In that study, a strong positive relationship was found between the amount of Cd in cytosol of the oligochaetes and Cd transferred to their predator, the grass shrimp *Palaemonetes pugio*, indicating increases in MT-associated metals in prey could increase the bioavailability of the metals to their predators (Wallace and Lopez 1996). In a subsequent study, Wallace et al. (1998) also showed that the oligochaete *L. hoffmeisteri* could develop Cd resistance after long-term exposure to high levels of Cd by producing insoluble metal-rich granules, as well as MT, for detoxification, while non-resistant oligochaetes only produced MT. The production of metal-rich granules affected trophic transfer of Cd to the grass shrimp *P. pugio*, as metals in these granules were much less bioavailable to *P. pugio* than soluble MT-associated metals

(Wallace et al. 1998). Trophic transfer of heavy metals is affected, therefore, by various physiological factors of prey and predator species. These issues all serve to complicate the development of useful models of the real effects of metals on ecosystems as a whole, either for scientific understanding or applied environmental management.

Limitations of experimental investigations in estuarine environments

Manipulating concentrations of heavy metals in sediments provided a useful means to investigate clearly causal relationships between concentrations of specific metals and responses of infauna throughout this study. Laboratory-spiked sediments can overestimate, however, the sensitivity of organisms to heavy metals, because metals in laboratory-spiked sediments are less strongly bound to the surfaces of sediment particles and so metals leach into porewater readily, rendering them more bioavailable to organisms compared to sediments contaminated in the field over longer periods of time (Simpson et al. 2004). Thus, the results of all of the experiments in this study which used spiked sediments need to be interpreted with some caution in this respect. One alternative methodology would be to use sediments that had already acquired excess pollutants *in situ* (e.g. Riba et al. 2004, Lu and Wu 2007). Riba et al. (2004) examined the effects of sediments contaminated with multiple heavy metals by long-term, continuous mining activities and those contaminated with an isolated, accidental spill of mining wastes. In that study, the sediments contaminated with an accidental spill were found to be much more toxic to the estuarine clam, *Ruditapes philippinarum*, than the sediments contaminated by historical mining activities, pointing towards the importance of considering time-scales over which contamination of sediments has occurred when assessing the toxicity of heavy metals.

The use of sediments contaminated *in situ* may be more appropriate for assessing the effects of heavy metals in terms of geochemistry; sediments contaminated *in situ* could

be used, for example, in the place of dissolved metal solution in the spiking process, yielding “naturally-spiked” sediments. However, a recent study by Little (2009) showed that there was little difference in the development of redox profiles, as indicated by porewater concentrations of manganese (Mn) and iron (Fe), between laboratory- and naturally-contaminated sediments, and therefore unlikely to be significant differences in the amount of Cu, Zn and Pb released from Fe- and Mn-oxides in these sediments. Moreover, sediments contaminated *in situ* are often contaminated by multiple contaminants, potentially including some that are not of interest. Such sediments would only be useful to assess the overall effects of those particular sediments, but could not be used to assess the individual effects of specific contaminants and their interactions. The use of laboratory-spiked sediments is therefore necessary to tease out the effects of specific metal contaminants. As different metal-spiking methodologies become available, with some potentially mimicking sediments contaminated *in situ* better than others (Hutchins et al. 2009), inferences regarding causal effects of contaminants as they occur in the field can become even more directed and rigorous.

The field investigations examining the effects of heavy metals (Chapters 2 and 4) were limited to examine only short-term recolonisation, as elevated metal concentrations could not be maintained over a long period of time in the field, primarily due to the movement of sediments (erosion and accretion) at the intertidal study site. Similar previous studies in subtidal environments have used much longer experimental periods (up to 14 months) and placed contaminated sediments in containers, instead of replacing the surface sediments at study sites (Lenihan et al. 2003, Trannum et al. 2004, Lu and Wu 2007). Such long-term studies would be useful to perform in estuarine environments, as they would allow researchers to examine the effects of heavy metals on the recruitment of infauna and/or the chronic effects of heavy metals over longer time scales (see Chapter 2).

However, due to strong currents generated by tidal activities in estuarine intertidal environments, implementation of the methodologies described in subtidal studies could be quite difficult. Another potential methodology to investigate the potential effects of heavy metals in intertidal habitats is to bury plaster mixed with metals in sediments (e.g. Morrissey et al. 1996, Stark 1998a). This method, however, can initially cause a large increase in metal concentration in porewater (Stark 1998a) and can also be subject to the differences in binding strength between sediments and heavy metals in naturally *versus* artificially contaminated sediments. A suitable methodology to study the long-term effects of heavy metals on estuarine intertidal communities in the field needs to be developed.

Linking ecotoxicological studies and environmental guidelines

Developing environmental criteria and guideline values for heavy metals involves a process of relating concentrations of heavy metals in porewater and/or sediments to the toxic effects on various organisms in laboratory and field investigations (Long et al. 1995). Generalisations regarding the toxicity of heavy metals are, however, problematic to articulate, as not all heavy metals in porewater and/or sediment are bioavailable to organisms, depending on a variety of biotic and abiotic factors (Plette et al. 1999). The use of concentrations of contaminants found in organisms, instead of concentrations in ambient sediments or porewater, has been advocated for the ecotoxicological assessment of various pollutants (Escher and Hermens 2004). This can be achieved by directly comparing concentrations of pollutants in organisms with some threshold values at which the toxic effects occur; researchers can thus avoid the complexity associated with predicting the bioavailability of the pollutants to the test organisms. Such a use of contaminant concentrations in organisms has been successful in the assessment of the

effects of a wide range of nonpolar organic chemicals, but this does not necessarily work for heavy metals (Vijver et al. 2004). Bioaccumulation of Cu by the bivalve *Macomona liliana* clearly demonstrated this difficulty, as concentrations of Cu in the bivalves were highly variable and did not seem to reflect either the toxicity or the ambient concentrations of Cu (Chapter 5). This is because the bioaccumulation of heavy metals by aquatic organisms is also influenced by different strategies employed by organisms to regulate heavy metals, through excretion and/or accumulation in a detoxified form (Rainbow 2002).

One approach to link bioaccumulation of heavy metals and their toxic effects is the use of the biotic ligand model (BLM). The concept of BLM is based on the assumption that the toxicity of heavy metals to organisms is proportional to the concentration of metals bound to the physiologically active binding sites at the site of action: a biotic ligand (Di Toro et al. 2001). For fish, the biotic ligand is assumed to be the sodium or calcium channel proteins in the gill surface, and BLMs have been developed successfully to predict the toxicity of Cu in varying water conditions such as pH, water hardness and organic matter (Di Toro et al. 2001, Santore et al. 2001). BLMs have also been developed for the cladoceran *Daphnia magna* (De Schampelaere and Janssen 2002) and the alga *Pseudokirchneriella subcapitata* (Heijerick et al. 2002), paving the way for the development of BLMs in other organisms. The potential use of BLMs to predict the effects of a mixture of metals has also been considered (Di Toro et al. 2001). The need for further research has been emphasised, however, to make BLMs a more successful tool for ecological risk assessment. For example, the effects of chronic and dietary exposure need to be integrated (Szebedinszky et al. 2001, Niyogi and Wood 2003). BLMs were originally developed for fish, in which the biotic ligand was assumed to be in direct contact with the external environment, but such an assumption may not be valid for all organisms (Vijver et al. 2004). BLMs also conventionally assume that the binding

characteristics of biotic ligands are fixed regardless of changes in environmental condition, but this may not be valid in some cases (Heijerick et al. 2002, Niyogi and Wood 2003). Further developments in ecotoxicological studies, including BLMs, should help increase scientists' understanding of the effects of heavy metals on test organisms. For example, radiotracers and cellular fractionation may be useful tools to determine the uptake, assimilation and elimination rates and subcellular distribution of heavy metals (Reinfelder and Fisher 1991).

The difficulty of ecological risk assessment lies, however, in extrapolating from laboratory observations on a relatively small number of individuals of a few test species to groups of many individuals and species (Calow and Forbes 2003). In the present study, the effects of Cu and Zn on the bivalve *M. liliانا* were consistent, reducing the abundance of this bivalve in the field (Chapter 2) and decreasing its survivorship in the laboratory (Chapter 3). The mortality rates observed in the laboratory did not necessarily correspond in size to decreases in the abundance of *M. liliانا* measured in the field. More specifically, concentrations of Zn in the field experiment were approximately 300 – 400 $\mu\text{g g}^{-1}$, which resulted in a more than 50% reduction of the abundance of *M. liliانا* in the Zn treatment compared to that in the control treatment (Fig. 2.6i), while the same level of Zn did not cause more than 20% mortality in the laboratory (Fig. 3.7b). Extrapolating from the results of bioassays with *Macomona* to other species would obviously be problematic, as different species responded differently to Cu, Zn and a mixture of Cu and Zn in the field (Chapter 2). This becomes further complicated when the co-existence of multiple heavy metals are considered, as interacting effects of multiple metals can differentially affect different species (Chapter 4).

Laboratory ecotoxicological studies on specific organisms, therefore, have the disadvantage of not allowing extrapolation of results to other species or to real field

settings, although their advantage is to identify clearly the direct biological effects of heavy metals on the chosen test organisms. As discussed earlier, the use of metal-spiked sediments in laboratory studies can overestimate the toxicity of heavy metals, as metals tend to be less strongly associated with sediment in laboratory spiked sediments than in naturally contaminated sediments (Simpson et al. 2004). Laboratory experimental conditions may also not closely mimic natural environments. For example, the terrestrial isopod *Oniscus asellus* has been shown to accumulate considerably higher levels of Cu in the laboratory than in the field, when they were fed the same leaf material, possibly because the isopods held under the controlled laboratory condition with an abundant food supply became hyperphagic and consumed much more food (Hopkin 1990).

Further investigations are also required to study the effects of heavy metals on organisms through long-term exposure in the field, as toxicity may increase or decrease through time (Neely 1984, Crommentuijn et al. 1994). Some organisms may also develop resistance. For example, pre-exposure to sublethal concentrations of heavy metals can stimulate synthesis of MT, making organisms more resistant to higher concentrations of the metals which would otherwise be lethal (Viarengo and Nott 1993). The terrestrial collembolan *Orchesella cincta* has been shown to increase excretion efficiency for Pb and Cd after long-term exposure through adaptation (Van Straalen et al. 1987, Posthuma et al. 1992). Heritable tolerance to heavy metals has also been found in the isopod *Porcellio scaber* (Donker et al. 1996), the oligochaete *L. hoffmeisteri* (Wallace et al. 1998) and the polychaete *Nereis diversicolor* (Grant et al. 1989, Hately et al. 1989) in populations inhabiting areas highly contaminated with metals.

The use of data from field investigations in developing environmental guideline values, which examine only existing correlative relationships between levels of contaminants and infaunal distributions, has been criticised for the lack of clear cause-

effect relationships (Borgmann 2003). Correlative field data does provide, however, ecologically and environmentally relevant information regarding chronic effects of heavy metals on ecological communities in the field. This is especially important where multiple contaminants simultaneously exist and potentially interact with one another or with other environmental conditions. Leung et al. (2005) proposed to use field data of benthic communities and contaminant levels in the sediments to construct field-based sensitivity distributions of species, from which sediment quality guidelines could be derived. Thrush et al. (2008) also used multiple regression models to analyse the effects of Cu, Pb, Zn, sediment particle size and organic content on the distributions of individual macrofauna found in intertidal habitats in the Auckland region. Useful models predicting the effects of Cu, Pb and Zn in this region have also been developed based on multivariate analyses of infaunal data along an overall gradient in these heavy metals (Hewitt et al. 2005, 2009).

Results of these field investigations, which inherently incorporate the broader-scale effects of multiple contaminants, can be further compared with results of laboratory and smaller-scale manipulative field experiments, providing useful information to build appropriate models for the assessment of ecological risk. For example, a negative correlation between the distribution of *M. liliانا* and concentrations of Cu has been shown in the field (Thrush et al. 2008). The laboratory and field experiments in the current study (Chapters 2 and 3) further indicated the adverse effects of Zn on the distribution of *M. liliانا* and the cumulative effects of these metals. While adult individuals of *A. stutchburyi* were found to be relatively tolerant to acute toxicity of heavy metals (Chapters 2 and 5), a negative correlation between the distribution of *A. stutchburyi* and concentrations of Cu has been found in the field (Thrush et al. 2008). This could be due to bioaccumulation and eventual toxicity of heavy metals after long-term exposure (see Chapter 5), heavy metals affecting the bivalves at different stages of development, or

interactions of metal effects with other unmeasured contaminants or environmental variables. Bioaccumulation of heavy metals may not always reflect the toxicity of the metals, but such investigations along with other ecotoxicological studies that investigate the potential chronic or sublethal effects could be used to test whether the correlative relationship is caused by Cu or by some other correlated variables.

The additive or interactive effects of Cu and Zn on various infaunal taxa (Chapter 4) and general insensitivity of organisms to Pb (Chapter 2) help to unravel the combined effects of these three metals that have previously been detected in multivariate analyses of field data (e.g. Hewitt et al. 2009). Chapman and Mann (1999) suggested that environmental guideline values should be used only as an initial screening tool to identify contaminants of potential concern. Such a use of environmental guideline values, followed by site-specific assessments using appropriate models based on field and laboratory investigations, should provide more meaningful, ecologically and environmentally relevant predictions of ecological impacts of contaminants on specific ecosystems. More specifically, clear causal relationships between potential metal contaminants and biological effects can be established using field and laboratory experiments, as described in this thesis. This process is also potentially useful for determining the priority of each contaminant for management purposes. Interactive effects of these contaminants then need to be examined in detail using ecologically relevant organisms, at various stages of development, through ecotoxicological studies both in the field and in the laboratory. These studies, in turn, provide important information to revise or modify the existing environmental guidelines. While examining single metals at a time is an important first step towards establishing causal relationships, given the co-occurrence of multiple heavy metals in the field and their interactive effects as described here, the true ecological risk of heavy metals cannot be fully modelled and

predicted unless their simultaneous effects are considered.

Appendix A

Assessing the Suitability of the Orewa Estuary as a Site for Sediment Collection and Manipulative Field Experiments

Introduction

Polluted stormwater runoff introduces contaminants into estuarine environments (ARC 2004). In the region of Auckland, New Zealand, copper (Cu), lead (Pb) and zinc (Zn) are the primary sediment contaminants of concern (ARC 2004). In order to assess the effects of these heavy metals on benthic macrofauna, field recolonisation experiments (Chapters 2 and 4) and laboratory bioassays (Chapters 3 and 5) were proposed. These experiments require a suitable estuarine site to source sediments for metal-spiking to artificially create sediments contaminated with heavy metals. In the field experiments, the metal-spiked sediments are then brought back to the site to examine recolonisation of infauna. It is desirable for the site to have an infaunal community dominated by polychaetes, bivalves and crustaceans, as these organisms have been shown to rapidly recolonise sediments (Thrush et al. 1991), and therefore, assessment of recolonisation is possible within a relatively short experimental period.

Another desirable characteristic of the site is to have sediments fine enough on texture to bind metals, as metals are generally associated with fine grain-size particles (Martinčić et al. 1990, Singh et al. 1999). Fine-grained sediments are also considered the most ecologically relevant component of sediments (Luoma and Davis 1983). In addition, the process of “spiking” sediments with metals requires sediments to be sieved using 500 µm mesh to remove macrofauna (Lu et al. 2008). Thus, a site having sediment grain size

< 500 μm is suitable as the sieving is unlikely to change the sediment texture. The metal-spiking process binds metals to sediments, and binding strength between metals and sediments is affected by environmental conditions such as total organic matter (TOM) and sediment texture (Chapman et al. 1998). Low variability in environmental variables should reduce confounding effects of these variables on experimental results.

The intertidal mid-estuary mudflat in Orewa estuary in Auckland was proposed as a site for field experiments and to collect sediments for metal-spiking. Preliminary biological and environmental surveys of benthic intertidal habitats were done at this site prior to the proposed field and laboratory experiments. The surveys aimed to evaluate suitability of the site and to provide baseline information about the macrofaunal community and environmental variables (chlorophyll *a*, TOM and sediment texture) for the field experiments. The biological survey also identified infaunal organisms available for laboratory bioassays. It is desirable to use common organisms for laboratory experiments as (i) they are easy to collect, and (ii) they potentially make up a large and even indicative fraction of the community.

Methods

Study site

Orewa estuary is located approximately 30 km north of the city of Auckland and is mid to small in size, with an area of 1.28 km² below mean high water spring tide level and a land catchment area of 17.5 km². The estuary has been shown to have low sediment metal concentrations, with 2.7 $\mu\text{g g}^{-1}$ for Cu, 2.2 $\mu\text{g g}^{-1}$ for Pb and 17.1 $\mu\text{g g}^{-1}$ for Zn (Lu et al. 2008). It contains a diverse array of organisms including mangroves, shellfish, birds, fish and benthic invertebrates. Orewa estuary is almost completely flushed with each tide and contains extensive intertidal areas separated by channels at low tide. A square, 50 m \times

50 m, study area (36° 35' 45S, 174° 40' 51E), was set up by marking four corners with pegs. The area is located approximately 2 km upstream of the mouth of the river and characterised by an expansive mudflat at low tide. Ten randomly chosen sampling locations inside the study area were sampled in April, 2007. Four separate cores of sediments were taken at each sampling location in order to survey infauna, chlorophyll *a*, TOM and sediment texture.

Infaunal survey

A core of sediment (130 mm in diameter, 150 mm in depth) was extracted at each sampling location, and material collected on 500 µm mesh was retained. Samples were preserved in 10% formalin for a minimum of 48 hours. All macro-organisms were then removed and placed in 70% isopropyl alcohol, sorted and identified to the lowest practical taxonomic level. Mean abundances and standard deviations (SD) of these taxa were calculated, and the numerically dominant taxa were determined. The coefficient of variation (SD/mean) and frequency of occurrence were also calculated for each taxon.

Chlorophyll a

A sediment core (20 mm in diameter and in depth) was collected at each sampling location, stored on ice away from direct sunlight and brought back to the laboratory. The amount of chlorophyll *a* was determined following the spectrophotometric method (Parsons et al. 1984). Briefly, chlorophyll *a* was extracted from the samples by adding 20 mL of 90% acetone to sediments for 24 hours at 4 °C in the dark. The acetone solution was extracted, and extinction at 665 nm and 750 nm (E_{665_0} and E_{750_0} , respectively) were measured. Two drops of 10% HCl were then added to the extract and absorptions were remeasured at 665 nm and 750 nm (E_{665_a} and E_{750_a} , respectively). The path-length of the

cuvette used was 1 cm. Chlorophyll *a* concentrations in the extract were calculated by the following equation (Parsons et al. 1984):

$$\text{Chlorophyll } a \text{ } (\mu\text{g/ml}) = 26.7[(E665_0 - E750_0) - (E665_a - E750_a)]$$

Sediments in the samples were dried in an oven at 60 °C for 48 hours to obtain dry sediment weights, and sediment chlorophyll *a* concentrations were calculated in $\mu\text{g g}^{-1}$.

TOM

A core of sediment (36 mm in diameter, 20 mm in depth) was collected at each sampling location to measure TOM. TOM was determined by loss of weight on ignition of sediment (Byers et al. 1978). Samples were oven dried at 60 °C for 48 hours, then combusted at 500 °C for 4 hours. TOM (%) was calculated by the weight loss after combustion.

Sediment texture

Another sample of sediment (36 mm in diameter, 20 mm in depth) was extracted in the field at each sampling location for granulometry. Samples were pre-treated to remove organic matter and disaggregate before wet-sieving (Lewis and McConchie 1994): The sediment samples were oven dried at 60 °C for 48 hours and treated with 9% hydrogen peroxide (H_2O_2) until frothing ceased in order to dissolve organics. They were then oven-dried at 60 °C for 48 hours, weighed to obtain total sediment weights and then dispersed with Calgon (5 g/L) for at least 4 hours. Sediments were wet-sieved using a series of nested sieves (500 μm , 250 μm , 125 μm and 63 μm) by gently pouring water through the sieves. All fractions were then separately oven-dried at 60 °C for a minimum of 24 hours and weighed. The four grain size fractions were: coarse sand (> 500 μm), medium sand (250 μm to 500 μm), fine sand (125 μm to 250 μm) and very fine sand (63 μm to 125 μm).

The silt and clay fraction (grain size < 63 μm) was estimated by subtracting the sum of the weights of the four other grain size fractions from the total sediment weight.

Results

An average of 235 individuals (SD = 233.2) were found in each of the 10 sediment samples. The most abundant organisms found at the site were copepods and nematodes, with mean abundances of 80.7 (SD = 169.4) and 69.9 (SD = 63.3) individuals, respectively. Many of these organisms were smaller than 500 μm diameter (i.e. meiofauna) and were probably retained as they were attached to organic debris (i.e. dead plant material) in the sediment samples. Abundances were highly variable among samples for these two groups, ranging between 3 and 557 individuals per sample for copepods and 14 and 208 individuals for nematodes. The high variability in their abundances was also evident from their high coefficients of variation (2.10 for copepods and 0.91 for nematodes).

Polychaetes and oligochaetes were also abundant, and the dominant polychaete taxa were *Ceratonereis* sp., Capitellidae spp., *Prionospio* sp., *Scoloplos cylindifer* and Polydorid spp. (Table A.1). These taxa mostly had relatively low coefficients of variation, suggesting that they are more consistently abundant at the site than copepods or nematodes. The amphipods *Paracalliope* sp. and *Paracorophium* sp. were also found in most of the samples. Other organisms found at low densities included the bivalve *Macomona liliana* and the mud crab *Helice crassa*. These organisms are possibly more ecologically important than their numbers would suggest, due to their relatively large biomass and bioturbation activities, respectively.

Environmental variables showed low variability among samples. The mean chlorophyll *a* content was 4.09 $\mu\text{g/g}$ dry sediment (SD = 1.29), and the mean TOM was

3.38% (SD = 0.60). The granulometry showed that sediments in the study area were mostly fine sands, silt and clay (Table A.2). The mean percentage of silt and clay was 50.8% (SD = 4.72), suggesting that the sand-to-silt ratio at the study area was approximately one. When only sand fractions (grain size $\geq 63 \mu\text{m}$) were considered, 99.5% of the sands were fine or very fine sands.

Table A.1. List of macrofaunal organisms found at the Orewa study site and their mean abundances, standard deviations, coefficients of variation, frequency of occurrence and percent abundances. Frequency of occurrence is shown as the number of samples in which each taxon was present.

	Mean abundance	SD	Coeff. of variation	Freq.	Percent abundance
<i>Ceratonereis</i> sp.	21.6	11.45	0.58	10	8.39
Oligochaeta	19.7	11.03	0.51	10	9.20
Capitellidae spp.	8.9	5.90	0.66	10	3.79
<i>Prionospio</i> sp.	8.1	5.67	0.70	9	3.45
<i>Scoloplos cylindifer</i>	6.8	4.69	0.69	10	2.90
Polydorid spp.	5.9	4.47	1.66	10	1.15
<i>Paracalliope</i> sp.	4.0	4.18	0.71	9	2.51
<i>Paracorophium</i> sp.	3.7	3.62	0.98	8	1.58
Kinorhyncha	2.7	3.56	0.89	7	1.70
Nemertea	0.8	1.26	2.11	5	0.26
<i>Notomastus</i> sp.	0.6	0.92	1.15	3	0.34
<i>Macomona liliana</i>	0.4	0.70	1.75	3	0.17
<i>Helice crassa</i>	0.2	0.42	2.11	2	0.09
<i>Glycera</i> sp.	0.1	0.32	3.16	1	0.04
Sabellidae sp.	0.1	0.32	3.16	1	0.04
<i>Scolecopides</i> sp.	0.1	0.32	3.16	1	0.04
Decapoda (shrimp)	0.1	0.32	3.16	1	0.04
<i>Halicarcinus</i> sp.	0.1	0.32	3.16	1	0.04
Isopoda	0.1	0.32	3.16	1	0.04
Ostracoda	0.1	0.32	3.16	1	0.04

Table A.2. Sediment texture, TOM and chlorophyll *a* content for the Orewa sediment.

	Mean	SD	Coefficients of variation
Granulometry (%)			
Coarse sand (> 500 µm)	0.15	0.077	0.496
Medium sand (250 µm to 500 µm)	0.23	0.047	0.201
Fine sand (125 µm to 250 µm)	14.15	7.808	0.552
Very fine sand (63 µm to 125 µm)	34.71	6.642	0.191
Silt and clay (< 63 µm)	50.75	4.719	0.093
TOM (%)	3.38	0.601	0.178
Chlorophyll <i>a</i> (µg/g)	4.09	1.289	0.315

Discussion

The infauna at the Orewa study area was numerically dominated by copepods and nematodes. Their abundances were, however, highly spatially variable. In addition, many of these organisms were probably retained on 500 µm mesh as they were attached to organic debris in the sediment samples. Modification of the infaunal separation method, using a nested series of sieves, should improve the separation of organisms from debris, and this is likely to reduce their counts. Nematodes and copepods would not be suitable for laboratory bioassays because (i) their abundances were highly variable, (ii) individuals larger than 500 µm represent only a small fraction of their populations, and (iii) their small body sizes make collection and handling of these organisms difficult.

Polychaetes, amphipods and bivalves are commonly used in bioassays to examine the effects of metals (Rainbow et al. 1993, Roper and Hickey 1994, Bat and Raffaelli 1998, King et al. 2004, Gale et al. 2006, Allen et al. 2007). Polychaetes were consistently present in the sediment samples, and *Ceratonereis* sp. (Nereididae) were especially abundant. Nereids have been found throughout estuaries in the Auckland region, and they were present in areas relatively high in metal pollution (Anderson et al. 2006b). The amphipods *Paracalliope* sp. and *Paracorophium* sp. were consistently present in the

sediment samples in relatively low densities compared to polychaetes. Bivalves were not common in the study area; *Macomona liliانا* was the only species found in this survey.

Organisms in different taxonomic categories often have different feeding modes (e.g. deposit feeding and filter feeding), and the types of metal phases that play an important role in metal uptake could differ among different taxonomic groups (King et al. 2004). Therefore, amphipods could be included in bioassays as their response to sediment metals may differ to that of polychaetes. *M. liliانا*, also, could be included because this species is common in Auckland's estuaries and is an important prey item for large predators including the eagle ray, *Myliobatis tenuicaudatus* (Thrush et al. 1991). *M. liliانا* are expected to be relatively sensitive to metal pollution (Roper and Hickey 1994, Roper et al. 1995), and have a larger body size in comparison to *Ceratonereis* sp. or amphipods, making collection and handling relatively easy. *Ceratonereis* sp., amphipods (*Paracalliope* sp. or *Paracorophium* sp.), and/or *M. liliانا* would be suitable for laboratory bioassays.

The Orewa estuary site was found to be a suitable site to collect sediments for metal spiking and to do field experiments. The sediments in the study area mostly consisted of fine sands, silt and clay, and therefore the site has enough fine sediment for experiments involving “spiking” of metals. There was low spatial variability in environmental variables (as measured by SD values for chlorophyll *a*, TOM and sediment texture) in the study area. The dominant organisms at the site are suitable for short-term recolonisation experiments (i.e. rapid recolonisers; Thrush et al., 1991). Moreover, organisms found at the site are fairly common in estuaries and harbours in New Zealand (Morton and Miller 1968) and, therefore, may be representative of estuarine infaunal communities of New Zealand.

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