Organism to ecosystem responses to copper contaminated sediments in model freshwater ecosystems

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CSIRO Land and Water

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The model freshwater ecosystems in June 2013

Abstract

Most knowledge of the effects of metal contamination is derived from laboratory studies investigating molecular, biochemical, physiological or individual organism responses. The applicability of this knowledge to the effects of metals at higher levels of biological organisation (population, community and ecosystem) in the natural environment has been questioned for some time, but the challenge remains. Further, in studies that have explored the effects of metals at higher levels of biological organisation, exposure pathways to the metal in question have often been unrealistic. Copper is a classic example; the majority of studies exploring the effects of copper analysed responses at low levels of biological organisation and those that explored responses at higher level of biological organisation performed experiments with dissolved exposures in the overlying waters. However, in the natural environment sediments have acted as a sink of historical contamination and dissolved concentrations of copper are now generally present in the low µg/L range. Thus, exposure pathways of copper to biota in the natural environment are likely to be very different to those that were present in previous high level studies.

The main aim of this research was to identify relationships between the effects of copper at low and high levels of biological organisation under a realistic exposure scenario. Twenty artificial semi-field ecosystems (mesocosms) were created with environmentally relevant copper spiked sediments. At the organism level, the effects of copper on the snail *Physa acuta* and the shrimp *Paratya australiensis* were assessed via *in situ* exposures. The development of the invertebrate community within the mesocosms was monitored by traditional optical techniques, allowing an assessment of population and community effects of copper. In addition, changes over time of the eukaryote community composition were monitored by environmental DNA (eDNA) metabarcoding. Finally, the effects of copper on ecosystem function were assessed by measuring primary production and organic matter decomposition within the mesocosms.

Identifying the relationships between levels of biological organisation proved to be most useful when unexpected responses, contrary to known direct effects of copper, were observed. Increased decomposition of organic matter on the sediment surface with increasing copper concentrations was linked to an associated increase in periphyton cover; both of these responses were unexpected based on previous studies that explored the direct effects of copper on organic matter decomposition and periphyton biomass. However, the increase in periphtyon cover could be explained by a decrease in grazing pressure in the high copper treatments compared to the control. Snails, particularly *Physa acuta*, were abundant in the control and low copper treatments, but had low abundances in the high copper treatments. This was probably a direct effect of copper, as the *in situ* exposures demonstrated an inhibition of growth (i.e. fitness) of individuals of *Physa acuta* in the high copper treatments.

Beyond the main aim, this research demonstrates the importance of performing an environmentally realistic exposure scenario in manipulative studies. It provides ecologically relevant data, which will aid the further development of guideline threshold concentrations for copper. The study also contains a valuable comparison of community level analysis by traditional techniques of optical analysis and eDNA metabarcoding. The latter technique proved to be more sensitive because it incorporated a much greater proportion of the community present, from the micro- to macro- scale. Perhaps most importantly, the study demonstrates the variety of assessments that can be performed in the pursuit of understanding ecosystem health and the value of each type of assessment. It demonstrates that by using multiple lines of evidence, which assess the effects of a contaminant across levels of biological complexity, a comprehensive understanding of the interactions that drive the biotic response within an ecosystem can be gained.

Statement of Candidate

I certify that the work in this thesis entitled ‘Organism to ecosystem responses to copper contaminated sediments in model freshwater ecosystems’ has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree to any other university of institution other than Macquarie University.

I also certify that the thesis is an original piece of research and it has been written by me. Any help and assistance that I have received in my research work and the preparation of the thesis itself have been appropriately acknowledged.

In addition, I certify that all information sources and literature used are indicated in the thesis.

Stephanie Gardham (41922654)

March 2014

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I also want to thank Dr Michael Ahrens, without whom my search for a PhD would never have reached Grant and Anthony's inboxes. Thank you!

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Chapter 1. Introduction

“Unfortunately, it seems that we do not know much about the effects of metals on aquatic assemblages.”

(Mayer-Pinto et al., 2010)

In 2010, Mayer-Pinto et al. (2010) sought to determine the 'real' state of knowledge on the effects of metals in aquatic ecosystems. They surmised that most data on the effects of metals is derived from laboratory studies, but that there is little evidence that the effects observed in the laboratory occur in natural environments, where conditions are very different (Mayer-Pinto et al., 2010). The question of the environmental applicability of laboratory tests to the real world is nothing new (Chapman, 1995; Clements & Kiffney, 1994; Cairns, 1983). In John Cairns Jr's (1983) paper entitled "Are single species toxicity tests alone adequate for estimating environmental hazard?" he recognised that "the need to go beyond single species testing to evaluate hazard to the environment posed by toxic chemicals is gaining momentum" (Cairns, 1983). However, 30 years on, the challenge remains and, as a result, Mayer-Pinto et al. (2010) still identified the need for properly designed experimental field studies to truly identify cause and effect relationships between metal exposure and changes at population, community and ecosystem levels.

Copper is a classic example of the challenge in metal ecotoxicology identified by John Cairns Jr (1983) and Mayer-Pinto et al. (2010). The effects of copper on aquatic biota have been extensively studied for nearly a century. However, the overwhelming majority of studies have taken place in the laboratory and the environmental realism of those studies can be questioned. The, relatively few, studies that have assessed the effects of copper on population, community and ecosystem function end points have focused on dissolved exposures in the overlying waters. Contrary to this, in natural environments, copper is now predominantly found in the particulate phase with low concentrations of the metal dissolved in pore waters and overlying waters. Thus, true cause and effect relationships between environmentally realistic copper exposure and changes at population, community and ecosystem levels remain to be determined.

The question of biological complexity

The ultimate aim of ecotoxicologists is to preserve and restore the integrity of populations, communities and ecosystems by studying the effects of anthropogenic contaminants on ecological systems (Fleeger et al., 2003; Maltby, 1999). Contaminants manifest a response across levels of biological organisation (Figure 1.1) (Clements, 2000; Maltby, 1999), and analysis of the response at each level of biological organisation provides different information. At lower levels of biological organisation, the specific mechanisms that lead to an effect of a contaminant can be identified, but the responses measured often have questionable ecological relevance (Clements, 2000). In contrast, at higher levels of biological organisation, endpoints are generally ecologically relevant and cover a high spatiotemporal scale, but the responses are often so complex that it is hard to identify the mechanisms of action (Clements, 2000).

A mechanistic understanding of the effects of a contaminant at the molecular and biochemical level can be gained by identifying how chemicals interact at target sites in organisms (Maltby, 1999). This can potentially be linked to observed physiological (e.g. respiration) and then individual (e.g. mortality) level responses (Clements, 2000). For example, copper inhibits Na^+/K^+ ATPase activity, which is likely to lead to an increase in the metabolic costs associated with osmoregulation, and therefore could lead to physiological responses like reduced growth rates (Brooks & Mills, 2003). The responses observed at these lower levels of biological complexity can be specific to individual contaminants and therefore directly linked to exposure (Clements, 2000).

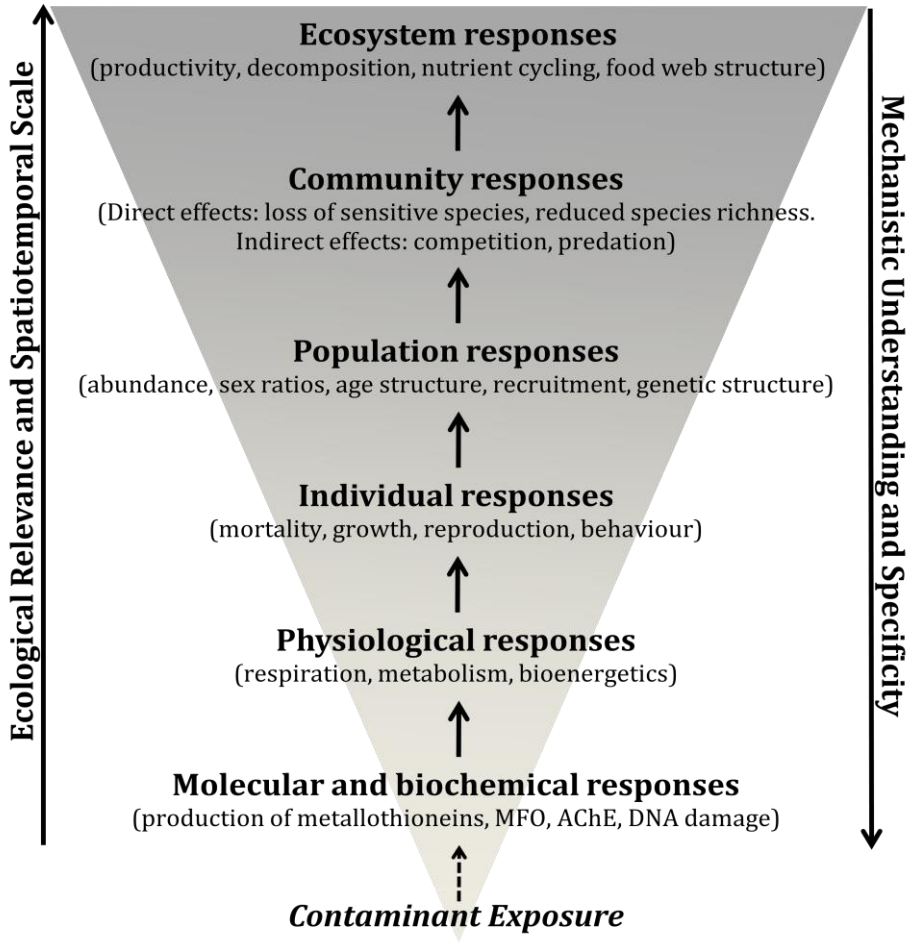

Figure 1.1: Levels of biological organisation at which effects of a contaminant can be measured and the relationships between biological complexity and ecological relevance, spatiotemporal scale, mechanistic understanding and specificity (Clements, 2000).

However, the responses observed at lower levels of biological organisation are often derived from laboratory studies and may, for several reasons, lack ecological relevance. First, laboratory tests tend to focus on a small number of test organisms, which are primarily chosen because they are easily cultured. These organisms are unlikely to be the most sensitive species present in the field, where biota often exhibit a wide range of sensitivities to contaminants (Fleeger et al., 2003; Cairns, 1983). Therefore, even though it is possible to identify a contaminant threshold concentration below which a measured response does not occur using laboratory test organisms, the contaminant may affect more sensitive organisms in the field at lower concentrations (Preston, 2002; Cairns, 1983). A simple example of this in relation to copper considers nematodes; the nematode *Caenorhabditis elegans* has been used extensively in toxicity testing, however when the effect of

copper on survival of *C. elegans* was compared to that on *Pristionchus pacificus,* the latter nematode was the most sensitive (Boyd & Williams, 2003).

Assays that consider responses at low levels of biological organisation also tend to be carried out over short exposure times. In contrast, organisms in the field are generally exposed for extended periods of time (Preston, 2002). This is important when considering the ecological relevance of tests because the direct effects of contaminants vary with the duration of exposure and longer exposures to a contaminant may reveal effects at lower concentrations that are not evident over short exposure times (Fleeger et al., 2003).

When extrapolating data obtained in the laboratory to the field, because the tests are usually performed on single, or few species, an assumption generally has to be made that individual organisms function as discrete units (Preston, 2002). However, in their natural habitat organisms are affected by intra- or inter- specific interactions with other organisms and indirect effects of a contaminant may occur (Mayer-Pinto et al., 2010; Chapman et al., 2003; Fleeger et al., 2003; Maltby, 1999). A common indirect effect of contamination is where primary production is increased because of a reduction in abundance of grazers (Mayer-Pinto et al., 2010). The effects may also change depending on season, stage of life and the community composition (Mayer-Pinto et al., 2010). Such effects simply cannot be predicted by laboratory tests using single or few species.

Responses at low levels of biological organisation are often measured under very controlled conditions. This further reduces their ecological relevance, as the physical dynamics of a field system are constantly changing and can directly affect an organisms behaviour, add additional stress and alter the bioavailability of a contaminant (Mayer-Pinto et al., 2010; Preston, 2002). Biological responses to contaminants under different abiotic conditions have been studied in laboratory assays to consider changing physical dynamics. For example, there has been much research into the influence of different sediment properties on metal speciation and therefore toxicity (e.g. Costello et al., 2011; Strom et al., 2011; Di Toro et al., 2005; Besser et al., 2003). In addition, for surface waters, the biotic

ligand model was developed to incorporate abiotic factors into the bioavailability of certain metals (e.g. Paquin et al., 2002). Some guidelines now take the biotic ligand model into account; for example, to calculate water quality criteria for copper in the USA, temperature, pH, dissolved organic carbon, calcium, magnesium, sodium, potassium, sulfate, chloride and alkalinity of the specific water body must be known (USEPA, 2007).

Ultimately, the ecological significance of the responses observed at lower levels of biological organisation is generally unknown and extrapolating from these studies to effects in the field may not be sufficient to protect a population, community or ecosystem (Clements, 2000). Increasing the biological complexity of assessments, by measuring responses at population (e.g. abundance), community (e.g. species richness) and ecosystem levels (e.g. productivity), in the field can provide information that is more ecologically relevant than that attained from measuring responses at lower levels of biological organisation.

However, the responses observed at higher levels of biological organisation are more complex (Clements, 2000). In natural habitats, indirect effects may be more common, more significant, and may confound the identification, of direct toxic effects (Fleeger et al., 2003). In addition, in the field there are often multiple stressors, making it hard to identify the contribution of one stressor to the perceived response (de Deckere et al., 2011; Chapman et al., 1998). Field studies, therefore, generally provide a description of the effects of stress, but it is hard to decipher the mechanisms and establish cause and effect relationships between contaminants and observed responses (Clements, 2000; Maltby, 1999).

There is no level of biological organisation, which should solely be used to carry out ecotoxicological investigations (Clements, 2000). At higher levels of biological organisation, the presence, or absence, of a species in an ecosystem may be observed, but without the toxicity assessments at lower levels of biological organisation, the reason for this may not be clear (Maltby, 1999). Linking across levels of biological organisation aids understanding of the ecological relevance of lower-level responses and, *vice versa*, helps to establish cause and effect

relationships between contaminants and observed responses at higher levels of biological organisation (Clements, 2000; Maltby, 1999). However, although there must be links between the lower and higher levels of biological organisation, identifying these is limited and remains a fundamental challenge in ecotoxicology (Preston, 2002). In the past, ecotoxicologists have focused on the lower levels of biological organisation, carrying out experiments in laboratories rather than the field because they are less costly and provide more rapid responses (Mayer-Pinto et al., 2010; Preston, 2002; Clements, 2000).

Mesocosm studies can provide links aross levels of biological organisation. Such studies considerably improve the degree of experimental control compared to field studies and are more environmentally applicable than laboratory tests (Costello et al., 2011; Shaw & Manning, 1996). Nevertheless, there remain limitations with these studies. For example, due to their scale, there are often low numbers of replicates, which inherently lowers the statistical power to demonstrate effects (although, with appropriate statistical design this can be avoided) (Van den Brink, 2006). Further, although more environmentally applicable, mesocosm studies often still contain artificial or simplified communities compared to true field studies. Ultimately, a weight of evidence approach that integrates multiple methods across multiple levels of biological organisation is necessary (Chapman et al., 1998).

Copper in the aquatic environment

Copper is found naturally as a metal, and occurs in at least 160 minerals and within organic compounds (Joseph, 1999; Dameron & Howe, 1998). Humans have used copper for millennia; for example, it was used to make domestic implements during the Chalcolithic period (8000 BC to 4000 BC) (Joseph, 1999). Anthropogenic releases of copper into the environment occur at every stage of its production and manipulation; as a result of extraction from copper ore and during the manufacturing, use and final disposal of products containing copper (Figure 1.2) (Lifset et al., 2012). Inputs of copper into the aquatic environment have dramatically increased within the last 100 years through urban wastewater, industrial and mine effluents, agricultural run off and atmospheric deposition (Serra

& Guasch, 2009; Eisler, 1998). As such, environmental quality criteria for copper in aquatic ecosystems are often exceeded; for example, as of January 2007, 629 rivers and streams within the USA did not meet water quality standards for copper (EPA, 2012).

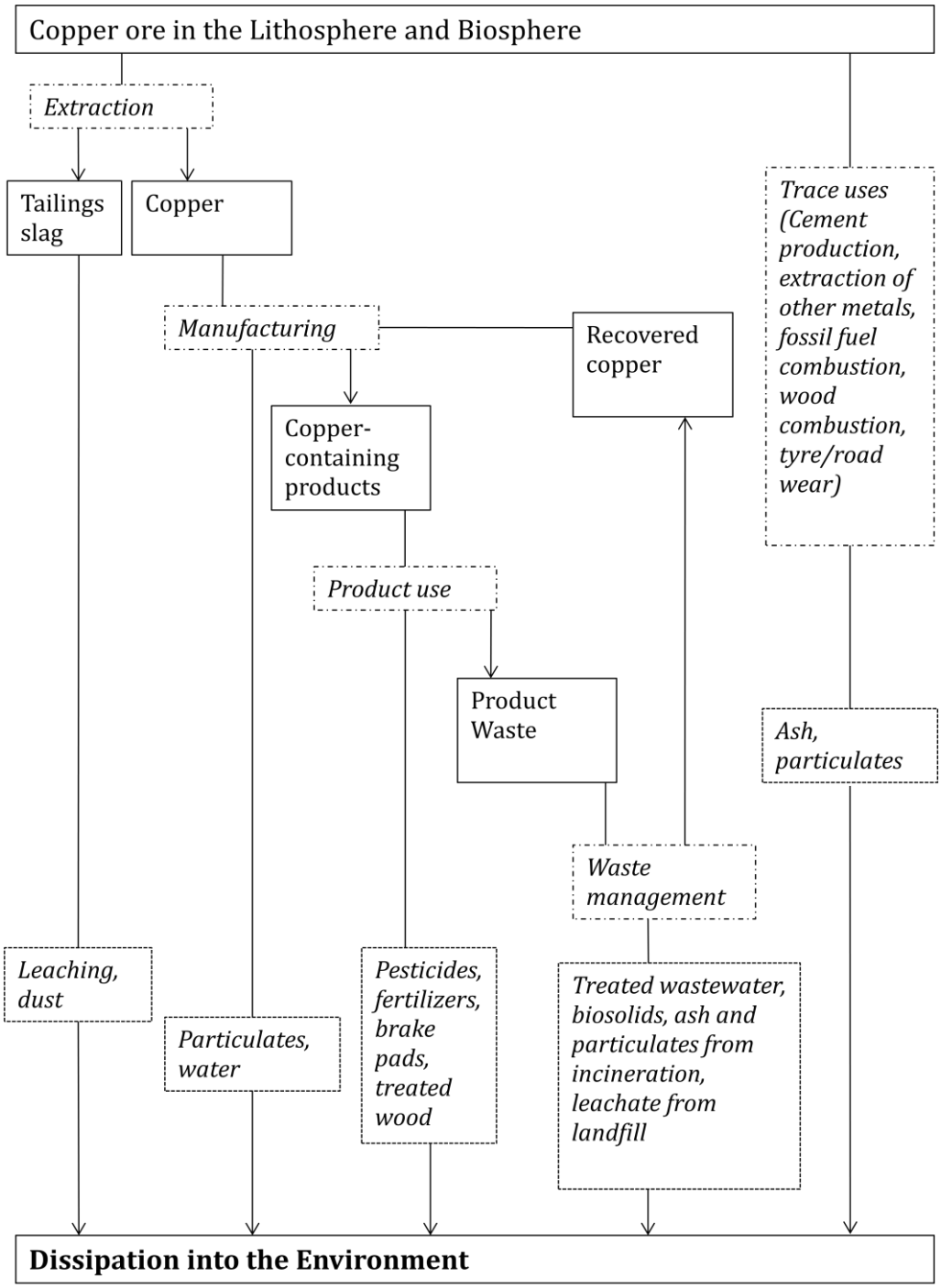

Figure 1.2: The release of copper into the environment from anthropogenic activities (adapted from Lifset et al., 2012).

When copper enters the aquatic environment it partitions between dissolved (pore water and overlying water) and particulate (sediment, suspended particulate matter and biota) phases (Figure 1.3) (Eggleton & Thomas, 2004). It is important to consider this speciation as it is a major determinant of the bioavailability of copper to biota (Chapman, 2008). The degree to which copper partitions to each phase depends on complex interactions between multiple factors. In freshwater environments, these include the attributes of the sediments, such as grain size, the presence of clay, organic matter and iron and manganese oxyhydroxides, as well as the surrounding environment, including the pH, redox potential and hardness (Eggleton & Thomas, 2004; Warren & Haack, 2001; Eisler, 1998; Elder, 1988). Small changes in any factor can change the balance between dissolved and particulate phases (Table 1.1). The rate at which changes in partitioning occur is dependent on temperature (Elder, 1988).

Table 1.1: Influence of factors important to the partitioning of copper between dissolved and particulate phases in freshwater environments (Sprang et al., 2008; Eisler, 1998; Elder, 1988).

Factor	Influence
Clay, organic matter and iron/manganese oxyhyroxides	An increase in each increases partitioning to the particulate phase as they provide binding sites for copper.
Grain size	A smaller grain size has a higher surface to volume ratio so provides more binding sites and increases partitioning to the particulate phase.
Oxidation-reduction potential	Oxidising conditions cause the precipitation of iron and manganese oxyhydroxides, increasing partitioning to the particulate phase.
pH	An increase in pH leads to the precipitation of copper, increasing partitioning to the particulate phase. In the dissolved phase, an increase in pH increases copper carbonate complexes compared to the cupric ion species.
Hardness	A decrease in calcium and magnesium ions reduces their interference with complexation and adsorption to dissolved and particulate, organic and inorganic, ligands, increasing particulate and copper carbonate complexes. A decrease in calcium and magnesium ions also reduces their competition with copper at active uptake sites in biota, leading to increased uptake of copper by biota.

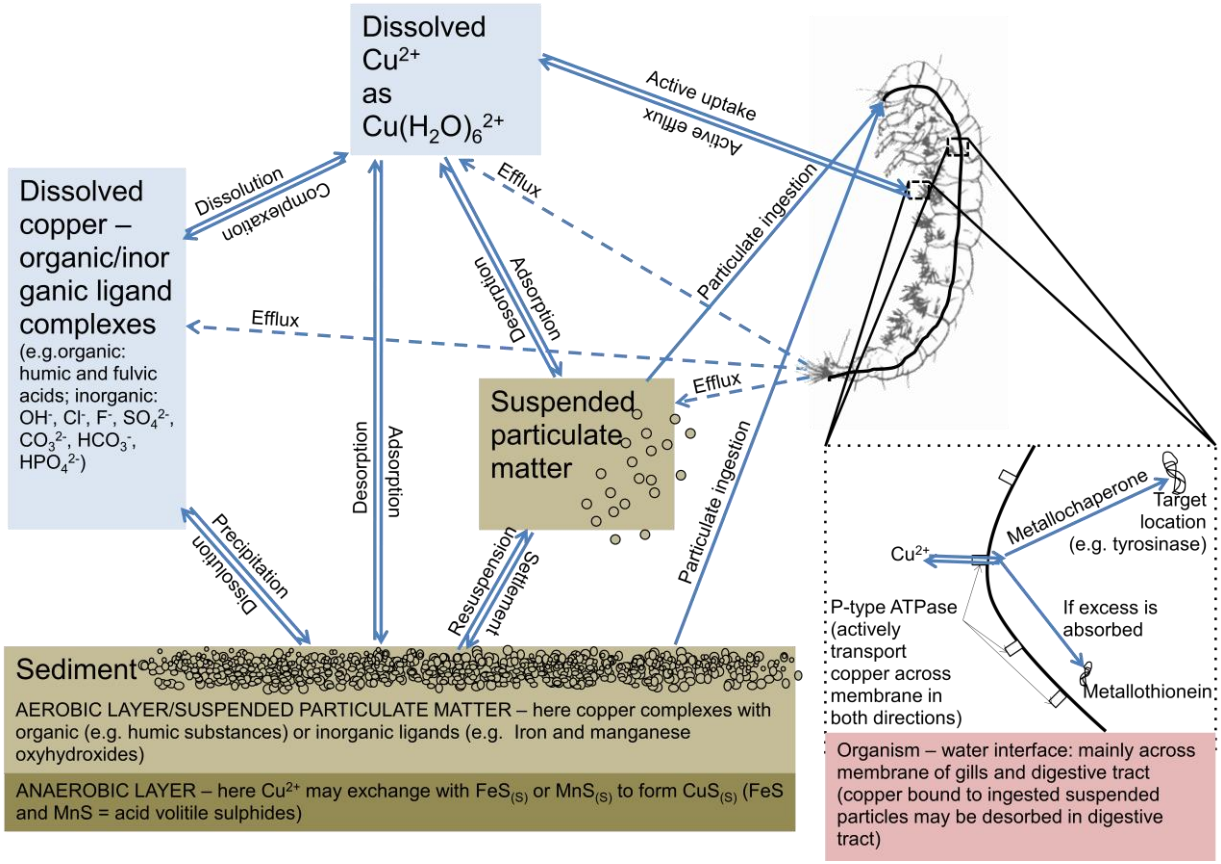

Figure 1.3: Partitioning of copper between dissolved and particulate phases in the aquatic environment (collated from information/figures of Eggleton & Thomas, 2004; Janssen et al., 2003; Chapman et al., 1998).

Historically, sediments acted as a sink for contaminated surface waters, because adsorption of copper is usually the dominant process in partitioning (de Deckere et al., 2011; Elder, 1988). Thus, in aquatic sediments worldwide, there is a large range of particulate copper concentrations due to historical contamination (Figure 1.4). However, dissolved concentrations of metals are now generally present in the low µg/L range because surface water quality has improved (due to better management of contamination sources) (de Deckere et al., 2011). Due to the low dissolved metal concentrations and because copper speciation is dynamic, sediments that have historically been contaminated with copper are now considered to pose an ecological risk as a source of contamination to overlying waters and biota (de Deckere et al., 2011; Chapman et al., 1998).

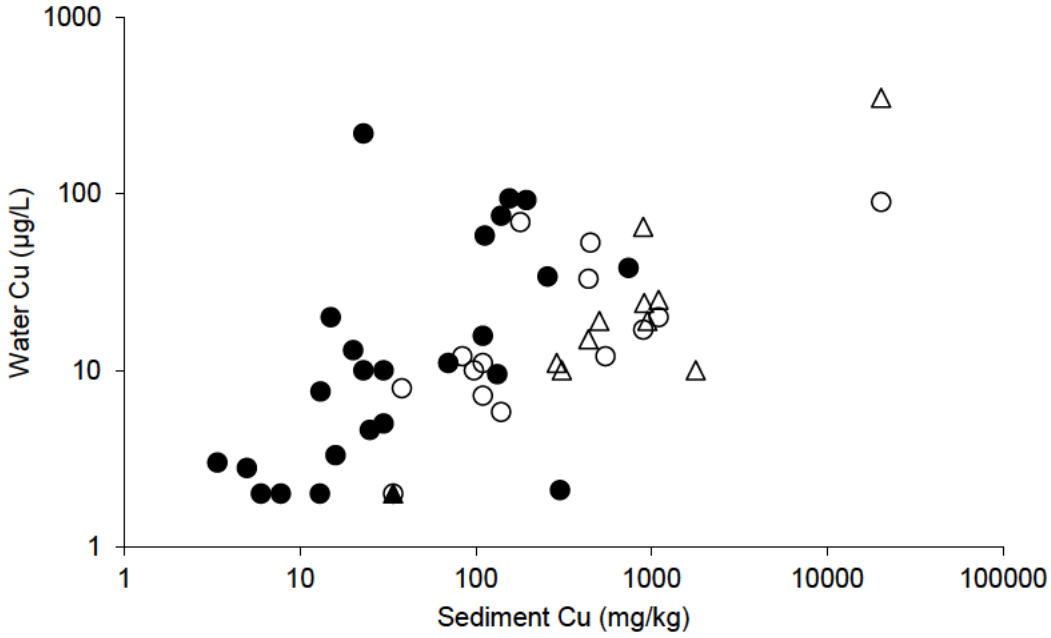

Figure 1.4: Measured concentrations of copper in sediments at field sites worldwide with their corresponding concentrations of copper in overlying waters (circles) and pore waters (triangles). Site descriptions are detailed in Table A1.1. Symbols that are not filled represent sites that have been affected by mine contamination, which often have higher dissolved metal concentrations due to acid mine drainage (Ríos et al., 2008).

Effects of copper on biota

Biota accumulate copper through respiration, skin contact or ingestion (Chapman, 2008). One mechanism of uptake is by actively absorbing cupric ions across their biological membranes through P-type ATPases, particularly within specialised organs like the gills and digestive tract (Figure 1.3) (Sprang et al., 2008). The free cupric ion is generally thought to be the most bioavailable species of metal, however there are instances where observed toxicity has not correlated well with the cupric ion, indicating other metal species may also be important (Chapman et al., 1998). Feeding behaviour can influence metal bioavailability for individual organisms; for example deposit feeders may ingest copper bound to sediment particles and in the digestive tract it could be desorbed becoming available for absorption into cells (Fukunaga & Anderson, 2011; Eggleton & Thomas, 2004).

Copper is an essential metal in eukaryotes and prokaryotes, involved in a wide range of metabolic processes (Flemming & Trevors, 1989). For example, it is an essential component of tyrosinase, an enzyme involved in melanin production, and dopamine beta-hydroxylase, which is involved in catecholamine production (Eisler, 1998). All organisms have developed a level of homeostatic control for copper because it is essential; P-type ATPases are able to pump copper in both directions across cell membranes and many organisms are able to buffer surplus copper within cells by binding the cupric ion with metallothioneins (Sprang et al., 2008; De Boeck et al., 2003).

Even though copper is an essential metal, adverse reproductive, biochemical, physiological and behavioural effects of copper on aquatic organisms have been shown at concentrations as low as 1 – 2 µg/L (Dameron & Howe, 1998). For example, a recent study found several physiological disturbances, including the inhibition of Na^+ K^+-ATPase activity and decrease in whole-body sodium content (i.e. an ionoregulatory disturbance) in juvenile freshwater mussels (*Lampsilis siliquodidea*) exposed to 2 and 12 µg/L of copper (Jorge et al., 2013). The toxicity of copper on freshwater plants and animals has been extensively studied – 21,423 records of endpoints for copper toxicity were returned from the U.S. EPA

Ecotoxicology database (USEPA, 2013); the earliest study was from 1926, in which the effects of copper on the germination of frog spawn and growth of tadpoles were assessed (Dilling & Healey, 1926).

The need for this research

Nearly all (96%) of the records held within the U.S. EPA Ecotoxicology database on freshwater copper toxicity were derived from laboratory studies; only 2% of these were for endpoints involving more than one species (USEPA, 2013). Field studies (natural and artificial), which assess the effects of copper, were identified in just 85 papers (USEPA, 2013). Most of the papers (58), described results from natural, presumably correlative, studies – there were just 27 'artificial, field' papers (likely to allow true cause and effect to be identified) (Table A1.2). A further thirteen mesocosm studies were identified through searches on google scholar using the terms 'mesocosm' 'freshwater' and 'copper' (last search 7/3/13) (Table A1.2). In total twenty artificial field studies were identified that considered the effects of copper at multiple levels of biological organisation, but just four of these assessed responses at both lower (i.e. biochemical/molecular/physiological and/or organism) and higher (i.e. population, community and ecosystem) levels (Serra & Guasch, 2009; Roussel et al., 2007ab, 2008; Real et al., 2003; Girling et al., 2000). However, links between the levels within those four studies were often tenuous, if considered at all (Table A1.3). For example, although Roussel et al. (2007a) found effects at lower levels of biological organisation in stickleback, they could not be related to the population level changes that they observed in the fish. There is a need for more studies in this area of research to allow links between effects at low and high levels of biological organisation to be explored.

Not only are there few studies which link between effects of copper at low and high levels of biological organisation, nearly all of the artificial field studies identified performed experiments with unrealistic exposure pathways (in reference to historically contaminated sites). They only considered copper dissolved in the overlying water as a source to biota and very few considered the speciation of copper once it entered the experimental system. As previously discussed, copper

is predominantly found in the particulate phase at historically contaminated sites and particulate forms of copper can be an important source of the metal to organisms, so should be considered. Only one other study was identified that considered sediments as a source of contamination. Hoang et al. (2011) aimed to characterise the effects of agriculturally contaminated soils on the Florida apple snail (*Pomacea paludosa*) and mosquito fish *(Gambusia affinis*). They assessed the effects with three soils, two that were historically contaminated and one reference soil. When the soil was characterised, copper was the notable contaminant and organochlorine, organophosphate, arsenic, cadmium and selenium were not detected. From this analysis, Hoang et al. (2011) attributed the responses they observed in the Florida apple snail and mosquito fish to copper. However, cause and effect cannot be determined from the study, because the three soils were from different places, and there may have been an unknown characteristic that was different between them causing the response observed.

Thesis aims and structure

The overall aim of this research was to identify cause and effect relationships between copper and biological responses observed at high levels of biological organisation. In order to do this, the study explores the effects of copper on aquatic ecosystems at multiple levels of biological complexity. The research was performed using a series of semi-field artificial pond ecosystems (mesocosms), in which sediments were artificially contaminated with a gradient of copper concentrations representative of historically contaminated sites. The facility was set up at Macquarie University, Sydney, NSW, Australia (33.76946 S, 151.11496 E) in 2010.

Chapters within this thesis have been written in a format for publication, with some additions that add value to the thesis. At the start of each chapter, the contributions of other individuals to the work presented are acknowledged.

Chapter 2: Preparation of environmentally relevant metal – contaminated sediments for use in outdoor mesocosms

The aim of the work presented in this chapter was to create a series of mesocosms representative of sites in the 'real world' that have been historically contaminated with copper. The most important aspect of this was the preparation of copper-spiked sediments with environmentally realistic partitioning and sediment geochemistry. The chapter incorporates a pilot study, carried out to assess the binding capacity of the sediment and develop an appropriate spiking method. It then details the *in situ* spiking procedure and partitioning of copper between sediments, pore waters and overlying waters within the mesocosms during the first 18 months. This allowed the effects of copper in the following chapters to be assessed with the knowledge that copper was the only stressor differing between mesocosms.

Chapter 3: The effects of copper on Parataya australiensis and Physa acuta in an outdoor manipulative experiment

The aim of the work presented in this chapter was to characterise responses to copper within the mesocosms at the organism level. A further aim was to provide some insight into the environmental applicability of the effects of copper on *Physa acuta* and *Paratya australiensis*, known from laboratory studies. A series of *in situ* exposures within the mesocosm facility were performed to assess the effects of copper on *Physa acuta* (growth, reproduction and survival) and *Parataya australiensis* (growth and survival). The chapter discusses the merits of *in situ* versus laboratory exposures to consider the effects of contaminants at the organism level. The effects of copper on *P. acuta* could be linked to effects at population, community and ecosystem function levels.

Chapter 4: The effects of copper on populations and communities of invertebrates in a manipulative experiment

The aim of the work presented in this chapter was to characterise the population and community level effects of copper within the mesocosms by traditional techniques of optical analysis. The development of the invertebrate communities within the benthos and water column of each mesocosm was monitored. The response of *P. acuta* at the organism level could be linked to the different populations of snails that were observed, which drove the invertebrate community response and effects at the ecosystem function level.

Chapter 5: Environmental DNA metabarcoding meets ecotoxicology: the response of the eukaryote community to copper exposure in an empirical outdoor mesocosm study

The aim of the work presented in this chapter was to characterise the effect of copper on the whole of the eukaryote community. The development of the community from micro- to macro- fauna was assessed by environmental DNA (eDNA) metabarcoding. This is a new technique of community-level analysis and is still in the early stages of development as a bioassessment tool. Therefore, a further aim was to compare eDNA metabarcoding with traditional techniques of optical analysis (Chapter 4). Although the technique can currently only provide presence/absence data, eDNA metabarcoding proved to be more sensitive than analysis of the invertebrate community because it incorporated a larger proportion of the biotic community.

Chapter 6: Exploring the direct and indirect effects of copper at the ecosystem level

The aim of the work presented in this chapter was to characterise effects of copper on the functioning of the mesocosms. A further aim was to identify the importance of direct and indirect effects in driving the biological responses measured. Organic matter decomposition and primary production were assessed through *in situ*

exposures of leaf litter and cotton strips and by quantifying chlorophyll *a* concentrations, periphyton cover and plant growth, respectively. A variety of direct and indirect effects were observed, which could be explained in part by the responses of *P. acuta* measured at the organism, population and community levels.

Chapter 7: Discussion

The discussion summarises the findings of this research and integrates the responses observed across levels of biological organisation. Within the chapter future opportunities and directions for the research are also identified.

Chapter 2. Preparation of environmentally relevant metal-contaminated sediments for use in outdoor mesocosms

The aim of the work presented in this chapter was to create a series of mesocosms representative of sites in the 'real world' that have been historically contaminated with copper. The most important aspect of this was the preparation of copper-spiked sediments with environmentally realistic partitioning and sediment geochemistry. The chapter incorporates a pilot study, carried out to assess the binding capacity of the sediment and develop an appropriate spiking method. It then details the in situ spiking procedure and partitioning of copper between sediments, pore waters and overlying waters within the mesocosms during the first 18 months. This allowed the effects of copper in the following chapters to be assessed with the knowledge that copper was the only stressor differing between mesocosms.

Authors

	Problem formulation	Experimental design/ methodology	Statistical analysis	Results interpretation	Paper preparation
Stephanie Gardham[1,2]	85	75	95	85	85
Grant C. Hose[1]	5	5	0	2.5	5
Stuart L. Simpson[2]	5	5	5	5	7.5
Chad Jaromilek[2]	0	10	0	0	0
Anthony A. Chariton[2]	5	5	0	2.5	2.5

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Abstract

Understanding the effects of sediment contaminants is pivotal to reducing their impact in aquatic environments. Outdoor mesocosms enable us to decipher the effects of these contaminants in environmentally realistic scenarios, providing a valuable link between laboratory and field experiments. However, because of their scale, mesocosm experiments are often complex to set up and manage. The creation of environmentally realistic conditions, particularly when using artificially contaminated sediment is one issue. Here, changes in geochemistry of copper-spiked sediments over 1.5 years within a large freshwater mesocosm facility are described. The majority of copper within the spiked mesocosm sediments partitioned to the particulate phase with low µg/L concentrations measured in the pore waters and overlying waters. The minimum partition coefficient following equilibration between pore waters and sediments was 1.5×10^4 L/kg, which is well within the range of observed for field-contaminated sediments (1×10^4 – 1×10^6 L/kg). Recommendations are made for the *in situ* spiking of sediments with metals in large outdoor mesocosms. These include selecting an appropriate sediment type, adjusting the pH, allowing sufficient equilibration time, and regular mixing and monitoring of metal partitioning throughout the experimental period.

Introduction

Assessment of the potential impacts of contaminants in aquatic sediments typically involves a comparison of measured concentrations against accepted sediment quality guidelines (Batley et al., 2005; ANZECC & ARMCANZ, 2000). These guidelines are mostly based on empirical effects, involving ranking of data largely derived from sediment toxicity tests using benthic organisms. Such laboratory-based tests have previously been criticised as they do not consider the influence of different sediment properties, are often based on single or few sensitive species, and are performed under very controlled conditions (Hose et al., 2003). Much research has been undertaken to improve understanding of how sediment properties influence toxicity thresholds (Costello et al., 2011; Simpson et al., 2011; Strom et al., 2011; Di Toro et al., 2005; Besser et al., 2003). However, biological interactions between species and the effects of natural environmental factors are much less well understood and often not considered (Hutchins et al., 2007; Simpson & Batley, 2007; King et al., 2006; Berry et al., 1996). Field studies can better address the contributions and influence of environmental variables and biological interactions to the perceived responses, but the results are often correlative, and do not always permit a direct link to causality (Chariton et al., 2010b; Pettigrove & Hoffmann, 2005). Experiments that utilise outdoor mesocosm systems provide a valuable link between field and laboratory tests; they considerably improve the degree of experimental control compared to field studies and are more environmentally applicable than laboratory tests (Costello et al., 2011; Shaw & Manning, 1996).

Metal contamination is a major concern for management of aquatic environments (Lourino-Cabana et al., 2009; Santoro et al., 2009; Terra et al., 2007; Hickey & Golding, 2002). Mesocosm, and microcosm, studies have been utilised to aid understanding of the effects of metals in these environments. Such studies have included the analysis of the response of natural meiofaunal communities to cadmium in microcosms (Brinke et al., 2011), the flux of nickel from sediments into overlying periphyton mats in streamside flow-through mesocosms (Burton et al.,

2009) and effects of nickel contamination on macroinvertebrate colonisation in streams (Costello et al., 2011).

Copper is frequently a metal of concern, entering aquatic environments via storm water runoff, sewage discharges, industrial effluents, mining wastes and fossil-fuel combustion (Fukunaga & Anderson, 2011; Khangarot & Das, 2010; Sodré & Grassi, 2006; Hickey & Golding, 2002). Mesocosm studies on the effects of copper have focused predominantly on its presence in the dissolved phase. For example, Serra and Guasch (2009) investigated the effects of dissolved copper on periphyton growth over a six-week period, while Roussel et al. (2007ab, 2008) considered the influence of dissolved copper on leaf litter decomposition and the community composition of macrophytes, phytoplankton and periphyton over 18 months. To our knowledge, no mesocosm studies have investigated the effects of copper contaminated sediment on biota.

Sediments are major sinks of metal contamination (which enters aquatic environments in dissolved or particulate forms), and consequently, the majority of metals in historically contaminated sites are found within the particulate phase. The literature on the partitioning of copper in freshwater environments is quite extensive (Table A2.1). For sediments with copper concentrations >65 mg/kg (the Interim Sediment Quality Guidline low value of ANZECC and ARMCANZ (2000) guidelines), partition coefficients, which describe sediment-water partitioning relationships (K_d (L/kg = [Sediment-M; mg/kg]/[Water-M; mg/L]), typically range from 1×10^4 to 1×10^6 L/kg (between sediment and pore waters) and from 1×10^3 to 2×10^5 L/kg (between sediment and overlying waters) (Figure 2.1). These K_d values are similar to those reported in marine and estuarine sediments, where values typically range from 1×10^3 to 5×10^5 L/kg (Hutchins et al. 2008; Simpson 2005). This literature indicates that naturally contaminated environments typically contain low dissolved copper concentrations; even when particulate concentrations are very high. As metals in the dissolved phase are usually more bioavailable than those associated with particles, the position of this partitioning is

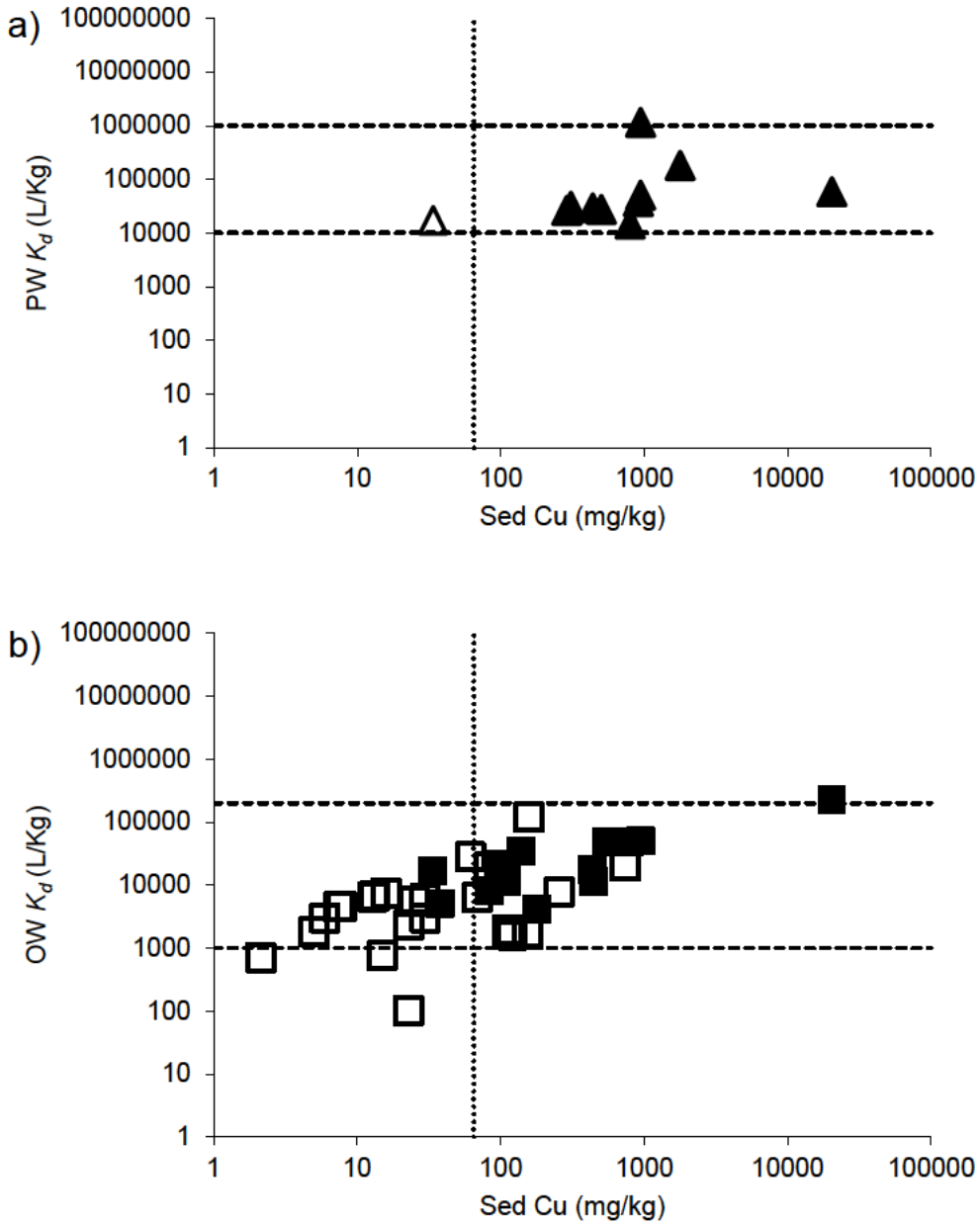

Figure 2.1: Partition coefficients (K_d) between sediments and (a) pore waters and (b) overlying waters. Filled in symbols are from mining impacted sites. Vertical line is 65 mg/kg, i.e. the low trigger value of the Australia and New Zealand Interim Sediment Quality Guidelines (ANZECC and ARMCANZ 2000). Horizontal lines depict the upper and lower range of K_d values above 65 mg/kg. Table A2.1 provides site descriptions and references.

one of the most important factors influencing the bioavailability and therefore bioaccumulation within aquatic biota (Fukunaga & Anderson, 2011; Ahlf et al., 2009; Sodré & Grassi, 2006; Kunz & Jardim, 2000; Höss et al., 1997).

In order to understand the potential effects of specific contaminants, experimental studies frequently utilise reference sediments artificially spiked with one or more contaminants, which allow a direct assessment of the contaminants of interest and ensure that there are no other toxicants that may be exerting synergistic or antagonistic effects (Hutchins et al., 2008). However, the spiked contaminant can take a long time to equilibrate to suitably represent field-contaminated sediments (which have equilibrated over long periods of time). Frequently high dissolved concentrations remain in either the pore waters, burrow waters or overlying waters due to decreases in water pH, changes in sediment redox potential, or slow reaction kinetics.

Along with abnormally high pore water contaminant concentrations, other cations that are relatively weakly bound within the particulate phase, like calcium and magnesium in freshwater sediments, may also be displaced, leading to dissolved concentrations that are much higher than typical field-contaminated sediments (Hutchins et al., 2007, 2008; Burton et al., 2006; Simpson et al., 2004; Lee et al., 2000). An increase in calcium and magnesium ions in the dissolved phase is expected to decrease the bioavailability of other metals due to competitive displacement and physiological effects on membrane permeability (Welsh et al., 2000; Bradley & Sprague, 1985; Calamari et al., 1980). The differences in sediment geochemistry between laboratory and field-contaminated sediments in the past may have altered the exposure routes of metals to the test organisms.

Simpson et al. (2004) provides key recommendations for creating realistic sediment geochemistry in artificially spiked sediments for whole sediment toxicity tests, including: (i) minimising air penetration into sediments by using deoxygenated waters for metal spike solutions and an inert gas to occupy head space during mixing; (ii) increasing the pH to greater than pH 7.5; (iii) allowing sufficient equilibration times to restore altered redox equilibria and reduce pore

water metals (it was shown that the redox equilibrium in the sediments gradually re-established over a period as long as 14 days during which the pore water concentration dramatically reduced to µg/L); and, (v) equilibrating sediments at 18 – 25°C (rather than 4°C). The recommendations were based on metal-spiked marine sediments, and some of these recommendations will be less important for copper-spiked freshwater sediments. For example, increasing the spiked sediment to a pH of greater than 7.5 makes it less applicable to many freshwater environments, for which the natural pH range is 6.5 – 8 (Hobday & Lough, 2011). More recently, for freshwater sediments, (Brumbaugh et al., 2013) have recommended the use of a 2-step method in which a portion of the sediment is spiked with a high metal concentration and pH adjusted as per Simpson et al. (2004), then the 'super-spiked' sediment is diluted with unspiked sediment to create the target sediment concentrations. While these methods are useful guidelines for spiking laboratory sediments, the *in situ* spiking in large-scale, outdoor mesocosm experiments provides additional challenges. For example, there is limited scope to minimise air penetration into the sediments, control the temperature during equilibration, and create an even dilution of super-spiked sediments.

The aim of this study was to create environmentally realistic copper partitioning and sediment geochemistry within mesocosms at a large outdoor freshwater facility. A pilot study was performed to optimise methods for large-scale spiking, which included analysis of the effect of time alone on equilibration as well as a comparison of reagents for pH adjustment. Sediments in the outdoor mesocosms were then spiked and the copper partitioning between sediments, pore waters and overlying waters was monitored for 1.5 years. Based on the pilot study, the hypothesis for the main study was that with pH adjustment and two months equilibration time, environmentally realistic partitioning could be achieved.

Methods

Mesocosm infrastructure

Twenty mesocosms were established on the Macquarie University campus (33.76946 S, 151.11496 E), in northern Sydney, NSW, Australia. The polyethylene mesocosms (Polyworld, Brisbane) had a diameter of 2.0 m, depth of 0.63 m and total volume of 1500 L. The mesocosms were sunk into the ground for insulation and were shaded by 70% shade-cloth at an average height of 2.5 m (Figure 2.2). A lip around the edge of the mesocosms limited the extent to which terrestrial insects and plants entered them. A water supply and drainage system was installed to maintain the mesocosms at a consistent depth and divert overflow water into a retention pit preventing contamination of the surrounding environment[1]. However, once established, the mesocosms remained full with natural rainwater inputs. The mesocosms were aerated with approximately 18 L/min of air using air pumps (O2 4000; Aqua One, Sydney).

Treatment design

The experimental design consisted of five artificial sediment concentrations, each with four replicates. Target concentrations of copper in the sediments were multiples of 65 mg/kg and 270 mg/kg, the low and high guideline values from (ANZECC & ARMCANZ, 2000). The nominal treatments were: (i) 0 mg/kg, Control (C); (ii) 32 mg/kg, Very Low (VL); (iii) 65 mg/kg, Low (L); (iv) 270 mg/kg, High (H); and (v) 540 mg/kg, Very High (VH). Treatments were allocated randomly to each mesocosm.

[1] A float valve to each mesocosm was connected to the mains water supply on site via a network of 25 mm polypipe and a carbon block filter cartridge, which removed chlorine from the water. An overflow pipe, which led into a network of 90 mm diameter stormwater pipe, ensured overflow water drained into a retention pit (3 x 4 x 1 m). The manning formula was used to ensure there was ample pipe diameter and a sufficient slope for appropriate drainage.

Figure 2.2 The mesocosms photographed at Macquarie University March 2013.

Sediment selection

A wetland soil mix was sourced from a local landscaping company (Wetland Smartmix #8, Benedicts Industries, Sydney). Sydney Environmental and Soil Laboratory had previously characterised the soil mix as a sandy loam with an organic matter content of 3.4% by mass. It had a low conductivity (0.04 mS/cm) and produced a pH of 6.4 and 5.2 when mixed in a ratio of 1:5 with deionised water and $CaCl_2$ respectively (methods 4A1 and 4B2 of Rayment and Higginson 1992). The soil mix contained 3.0 mg P/kg as PO_4^{3-}, 0.9 NH_4 mg/kg, 0.8 NO_3 mg/kg, 914 mg Ca/kg, 244 mg Mg/kg and 59 mg K/kg, which represent relatively low concentrations of nutrients major cations (Sydney Environmental and Soil Laboratory, 2009). It also contained relatively low concentrations of trace metals: iron 73 mg/kg, manganese 17 mg/kg, copper 1.5 mg/kg and zinc 0.5 mg/kg. Additional analyses by laser diffraction (Mastersizer 2000, Malvern) found the soil mix consisted of coarse sand (13% of particles 500-1000 µm), medium-fine sands (70% of particles 63-500 µm), and small proportions of silt (14% of particles 2 - 62.5 µm) and clay (3.0% of particles 0.01 - 2 µm). Analyses (see 'sample collection and analysis' below) of the original soil mix found 3.1% total organic carbon (TOC), <1.0 mg/kg nitrate, 3.0 mg/kg ammonia and 8.0 mg/kg of extractable phosphorus.

Pilot study

A small-scale pilot study was carried out to determine whether the copper-binding capacity of the soil/sediment was sufficient for the intended target copper concentrations under the environmental conditions that the spiked-sediments would be subjected to at the mesocosm facility. The effect of equilibration time and pH amendment on equilibration was examined by targeting H and VH treatment concentrations. For all treatments plastic buckets were lined with greenhouse film (Gro-Tuff; Geoff Miller, Melbourne) as this was to be used to line the mesocosms. Treatments (C, H and VH) were created by mixing soil (2.0 kg dry weight) with 0, 0.54 and 1.08 g of copper (in the form of copper sulfate pentahydrate; AR grade; Chem Supply, Adelaide) dissolved in 1.0 L Milli-Q water to create C, H and VH treatments, respectively. The copper-spiked sediments were covered with greenhouse film to minimise air penetration, placed outside so they would be subject to daily fluctuations in temperature and were fully mixed twice a week with a plastic mixing spoon. After one week, the pH of the H and VH treatments was increased to match that of the C treatment (pH 5.1) by addition of 50% NaOH (AR grade; Chem Supply, Adelaide). After 47 days, samples of the overlying water, sediment and pore waters were taken for copper analysis (see 'Sample collection and analysis' below). Sediment-slurry from the VH treatment was then sub-sampled (35-40 ml) into eighteen 50 mL tubes and, over a period of 11 days the pH of the each sub-sample was then increased using garden lime (32.5% Ca as $CaCO_3$, 44.8% Ca as CaO and 2.7% Mg as $MgCO_3$ corresponding to 80% equivalent $CaCO_3$; Bunnings, Melbourne), calcium carbonate powder (AR grade, Chem Supply, Adelaide) or a 50% NaOH solution. Pore water samples and pH measurements were taken daily.

Mesocosm study

The mesocosm study was split into two periods. During an initial 61-day period, conditions in the mesocosms were optimised to allow environmentally relevant partitioning of the copper. This period is hereafter referred to as the 'spiking phase'. In the subsequent period, the conditions in the mesocosms were optimised

for biotic colonisation. This second period is hereafter referred to as the 'colonisation phase'. The time at which the mesocosms were opened to biotic colonisation is referred to as t = 0 d. Times during the spiking phase are referred to with negative time, i.e. the spiking phase started at 't = -61 d'.

Spiking phase

Approximately 200 L (~ 210 kg dry weight) of sediment was placed in each mesocosm. The sediment was spiked *in situ* with the target amount of copper (in the form of copper sulfate pentahydrate; Yates bluestone, Auckland) dissolved in 100 L of tap water. Additional tap water was added to give a final sediment dry weight (kg) : water (L) ratio of 2:1 and aid mixing, which was carried out with a large plastic mixing spoon (Figure 2.3a). During the first two weeks (t = -55 d and t = -52 d) the pH of each mesocosm (including the controls) was increased to the target range of pH 6.7-7.2 (based on the pilot study) using garden lime. For the C, VL, L, H and VH treatments, 1.7 ± 0.0, 3.3 ± 0.0, 4.2 ± 0.0, 6.0 ± 0.4 and 12.3 ± 0.4 kg (mean ± S.D.) of garden lime was required, respectively. The mesocosm sediments were mixed *in situ* three times a week for five weeks and were covered with black plastic between mixing to limit air movement, rain inputs and minimise biotic colonisation (Figure 2.3b). Seven weeks after spiking, the overlying water was carefully removed from the mesocosms (t = -5 d to t = -4 d) and they were left without overlying water until the start of the colonisation phase in an attempt to ensure all mesocosms were biotically similar at t = 0 d (i.e. no aquatic organisms present).

Colonisation phase

The covers were removed 61 days after spiking and unfiltered tap water (approximately 500 L) was used to half fill the mesocosms (t = 0 d). They were then left to fill naturally with rainwater and to allow biotic colonisation.

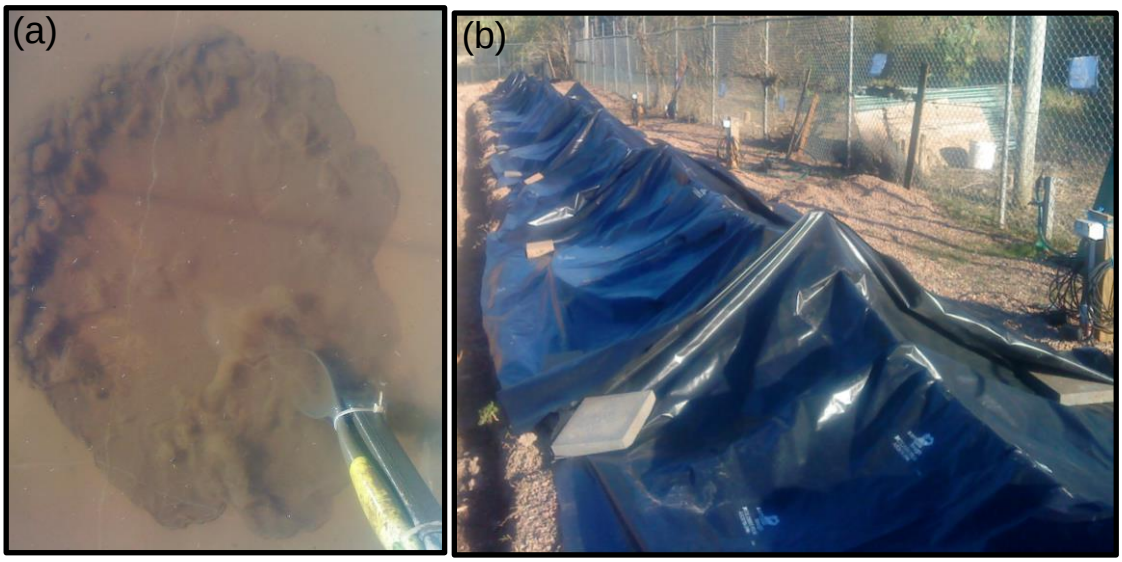

Figure 2.3: (a) Large plastic mixing spoon attached to plastic rod for physical mixing of sediments within mesocosms; and (b) black plastic sheeting covering the mesocosms during the spiking phase to limit air movement and rain inputs.

Sample collection and analysis

All plasticware used for sample collection and analyses was acid-washed overnight using 10% (v/v) HNO_3 (AR grade; Chem supply, Adelaide) and rinsed three times with Milli-Q water. The plasticware was dried in a laminar-flow cabinet prior to storage in sealed plastic bags until use.

The pH of the overlying water was measured during spiking using a hand held pH meter (HI 9125; Hanna, Woonsocket) calibrated against pH 4 and 7 buffers. After the start of the colonisation period (t = 0 d), overlying water pH (calibrated against pH 7 and 11 buffers) and redox measurements (versus the standard hydrogen electrode) were made using a hand held multimeter (HI 9828; Hanna, Woonsocket) prior to each sample collection for copper analysis.

Sediment samples (50 mL) were taken from the original batch of soil mix and from each mesocosm in March 2011 (t = 126 d) and March 2012 (t = 500 d). Nitrate (as NO_3-N) was determined following method 7C2 of Rayment and Higginson (1992). Ammonia (as NH_4-N) was determined after reaction with sodium salicylate and dichloro-isocyanurate, modified from Rayment and Higginson (1992) according to ISO 11732 (1997). Extractable phosphorus was determined by flow injection

colorimetery after Colwell extraction following Method 9B2 of Rayment and Higginson (1992). Total organic carbon (TOC) was determined by loss of ignition (LOI) using 10 g of sediment sample heated at 550°C for four hours, following the methodology of Heiri *et al*. (2001).

Surface water samples (50 mL) were taken in April 2011 (t = 161 d) and March 2012 (t = 497 d). Ammonia was measured by flow analysis (CFA and FIA) and spectrometric detection as per the International Standard ISO 11732 (1997). Nitrate was measured following method 4500-NO_3 F of Clesceri et al. (1998). Dissolved reactive phosphorus was determined following method 4500-P F of Clesceri et al. (1998).

Water and sediment samples were collected for analysis of dissolved and particulate copper, calcium and magnesium. Calcium and magnesium partitioning was monitored as the bioavailability of metals to organisms is affected by water hardness (Welsh et al., 2000; Bradley & Sprague, 1985; Calamari et al., 1980). Samples (overlying water, pore water and sediment) were collected from each mesocosm twice during spiking, then every fortnight for six weeks after the start of the colonisation period (t = 0 d), then monthly for six months and then at twelve (t = 368 d), thirteen (t = 409 d), and sixteen (t = 500 d) months.

Overlying water (5 mL) was sampled at a depth of 5 cm using a preconditioned 10 mL syringe. Each sample was filtered through a preconditioned 45 µm filter (Minisart, Sartorius, Göttingen) and acidified to 2% using 35 µL 70% (v/v) HNO_3 before storage for analysis by inductively coupled plasma - atomic emission spectrometry (ICP-AES). Duplicates were taken randomly for QA/QC analysis (Table A2.2).

A 1-L sample of the surficial sediment layer (top 1-2 cm) was obtained and from this, two 50 mL sub-samples were taken for sediment and pore water analyses. Duplicates were taken randomly and in subsequent sampling rounds previously sampled areas were avoided, although these appeared to recolonise quickly. Pore waters were extracted from sediments by centrifugation at 2000 rpm for 5 minutes

(modified from Simpson et al. 2004). The supernatant was filtered and acidified before storage for analysis by ICP-AES using the same methodology as the overlying water samples (above). For particulate analyses, the second sub-sample was dried for three days at 80°C, then ground to a fine powder, homogenised, and stored for analysis. Total particulate copper, calcium and magnesium were measured by ICP-AES following a low pressure microwave-assisted aqua regia digestion (8 mL of 3:1 concentrated HCl:HNO_3, 90 min microwave at 800 W in closed 50 mL vessels) using 0.3 g of homogenised oven-dried sediment (80°C) (Chariton et al., 2010b). To check analytical quality, a certified reference material, PACS-2 (National Research Council Canada, Ottawa, Canada) was analysed in each batch of sediment samples. All results were within acceptable ranges of QA/QC analysis (Table A2.2).

Descriptive statistics

For the pilot study, absolute pH values, K_d values and copper pore water and overlying water concentrations are reported (n = 1) unless otherwise stated. For the mesocosm study mean ± 1 standard deviation of pH, concentrations of copper, calcium and magnesium and K_d are reported (n = 4), unless otherwise stated.

Pearson correlations were performed between pH and overlying water copper concentrations, and pH and pore water copper concentrations in the mesocosms using Minitab 16.2.2; the significance level (α) was 0.05. Data passed normality assumptions (Anderson-Darling) prior to analysis. The sediment – water partition coefficients for copper (K_d, L/kg) were calculated between sediments and both overlying water and pore water copper concentrations ([Sediment, mg/kg]/[water, mg/L]).

Hardness-modified water quality guideline values for copper were calculated using the hardness (calculated from measured concentrations of calcium and magnesium) of the pore waters and overlying waters after t = 74 d (i.e. once the mesocosms had reached pseudo-equilibration and there were no further dramatic

changes in partitioning). Details of these water quality guideline calculations are provided in (ANZECC & ARMCANZ, 2000).

Results and Discussion

Pilot study

The pH of the slurry in the non-spiked control treatment over the first 47-days was 5.1 ± 0.10 (n = 13). As expected, the addition of copper resulted in a comparatively lower pH in the H (pH 4.8) and VH (pH 4.5) treatments. This is due to the hydrolysis of the added copper and displaced cations, such as iron (Hutchins et al., 2007; Burton et al., 2006; Simpson et al., 2004; Simpson & Batley, 2003; Lee et al., 2000). The addition of sodium hydroxide solution was found to be suitable for increasing the pH of the H and VH treatments to match the pH of the control treatment (5.2 ± 0.05 and 5.0 ± 0.06, respectively (n = 11)), for the initial equilibration period of 47 days. After the initial equilibration period, the dissolved copper concentrations were 300 and 13000 µg/L in the pore water and 40 and 960 µg/L in the overlying water for the H and VH treatments, respectively, with corresponding K_d values of 1100 L/kg and 40 L/kg for pore waters and 7800 and 520 L/kg for overlying waters. The dissolved copper concentrations are far greater than could be expected to be observed for most field copper contaminated sediments, and pH 5 was not considered suitable for the mesocosm study as it is more acidic than most freshwater environments. More frequently, freshwaters vary from pH 6.5 – 8 (Hobday & Lough, 2011).

Increasing the pH to 7 in sediments from the VH treatment resulted in considerably lower pore water copper concentrations (Figure 2.4), consistent with previous studies (Hutchins et al., 2007; Burton et al., 2006; Simpson et al., 2004; Lee et al., 2000). The use of garden lime and calcium carbonate generally resulted in lower pore water copper concentrations than sodium hydroxide at the same pH. When the pH was further increased with sodium hydroxide the pore water copper concentrations increased again, and were greater than those observed at pH 5 (Figure 2.4). Similar observations have been made previously and were attributed

to the formation of colloidal copper-hydroxide phases (Simpson et al., 2004; Hassan et al., 1996). For this reason, we considered the use of sodium hydroxide unsuitable as a neutralising agent.

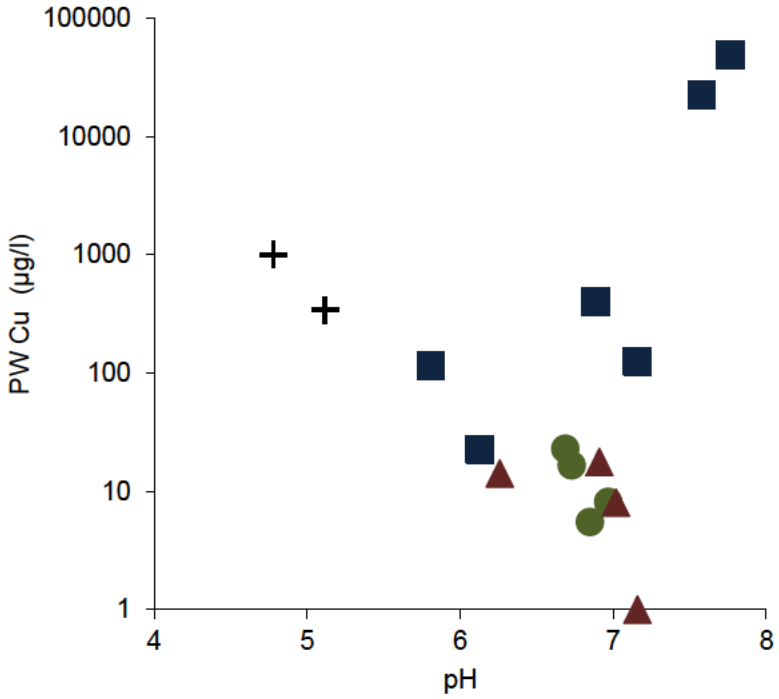

Figure 2.4: Pore water copper concentrations after 11 days of gradually increasing the pH using different bases. Key: sodium hydroxide (■); garden lime (▲); calcium carbonate (●); control (i.e. where no base was added) (+); (n = 1).

Pore water copper concentrations less than 10 µg/L were achieved with both garden lime and calcium carbonate near pH 7, which is within the pH range of most freshwater environments (pH 6.5 – 8) (Hobday & Lough, 2011). The K_d values (sediment – pore water) for the sediments adjusted with garden lime to pH 6.7 – 7.2 were between 2.2×10^4 L/kg and 9.1×10^4 L/kg. These are comparable to those commonly observed for freshwater sediments in the field (1×10^4 and 1×10^6 L/kg) (Figure 2.1, Table A2.1). The pilot study confirmed that a pH of 6.7 – 7.2 should be a suitable target for the mesocosm study. Garden lime was considered to be the most suitable as a neutralising agent. The amount of garden lime required to bring about an increase in the natural soil pH from pH 5.2

to 7 was approximately 5 g per 100 g sediment and this was used as a basis to calculate approximate amounts of garden lime to add to the mesocosms.

Mesocosm study

Overlying water pH and redox potential

At the start of the spiking phase (t = -55 d) the overlying water pH was highest in the C treatment (5.61 ± 0.04) and lowest in the VH treatment (4.51 ± 0.09) due to the hydrolysis of added copper and displaced metals (Figure 2.5). This was expected based on the results of the pilot study and past studies. After the addition of garden lime to all treatments (t = -55 d and t = -52 d), the pH increased and was within the target range. However, the pH continued to increase before reaching a plateau during the colonisation phase (Figure 2.5). The maximum pH recorded was 8.8 ± 0.1 (Control; t = 310 d), which exceeds the upper limit of the range noted by Hobday and Lough (2011) for most freshwater environments (pH 6.5 – 8). This higher than expected pH was probably due to further dissolution of lime over time, which was not accounted for when increasing the pH during the pilot study or spiking phase. It has implications for the environmental realism of the mesocosm sediments, because the higher pH could affect the copper bioavailability (Atkinson et al., 2007). Notwithstanding, a review of pH levels within freshwater environments where copper partitioning has been assessed shows that a pH of 8.8 is still applicable (Table A2.3).

Although the pH remained high, there were fluctuations in pH in all treatments – particularly in the control, which ranged from pH 6.8 ± 0.2 (t = 247 d) to 8.8 ± 0.1 (t = 310 d). Owing to less lime being added, the control had a lower hardness and lower capacity to buffer changes in pH compared to the copper-spiked mesocosm treatments. Despite the fluctuations in pH in all treatments, there was no significant correlation between pH and copper concentrations in either the overlying waters (r = 0.146, p 0.458) or pore waters (r = 0.081, p 0.682), indicating that the fluctuations in pH did not affect copper partitioning (Figure A2.1). Notably, extremely high concentrations of pore water copper did not occur when the pH exceeded 7.5 (as was observed in the pilot study with the sodium hydroxide

treatment). We believe this was because the garden lime did not result in the formation of large quantities of colloidal copper forms. This is possibly due to the higher ionic strength of the waters due to greater dissolved cation concentrations resulting in more rapid precipitation kinetics.

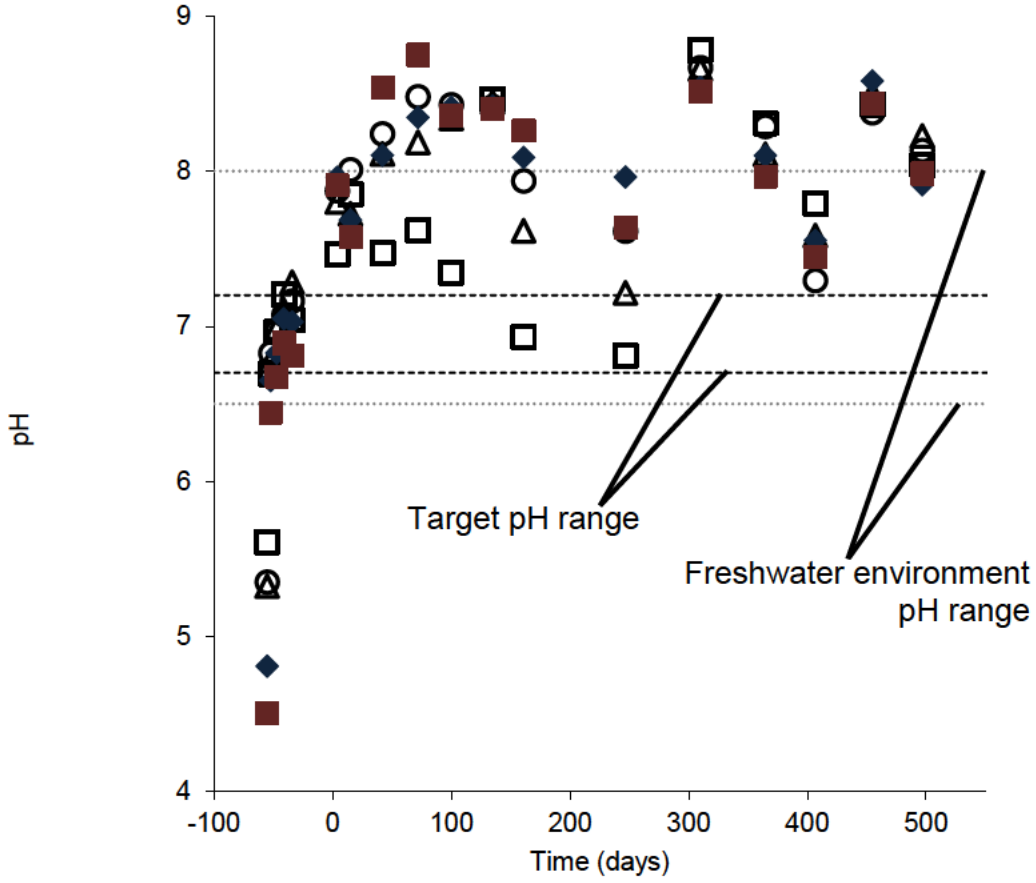

Figure 2.5: Change in overlying water pH over time. Key: Control (□); very low (△); low (○); high (◆); very high (■). Target pH range was defined by the pilot study (n=4).

When measured during the colonisation phase, the redox potential of the overlying water was similar across treatments indicating that the sediments had become equilibrated. However, the redox potential did fluctuate over time between 250 and 480 mV (Figure 2.6). This may be due to the low concentrations of redox-active species in the overlying waters and the influence of rain inputs on the chemistry of the overlying water.

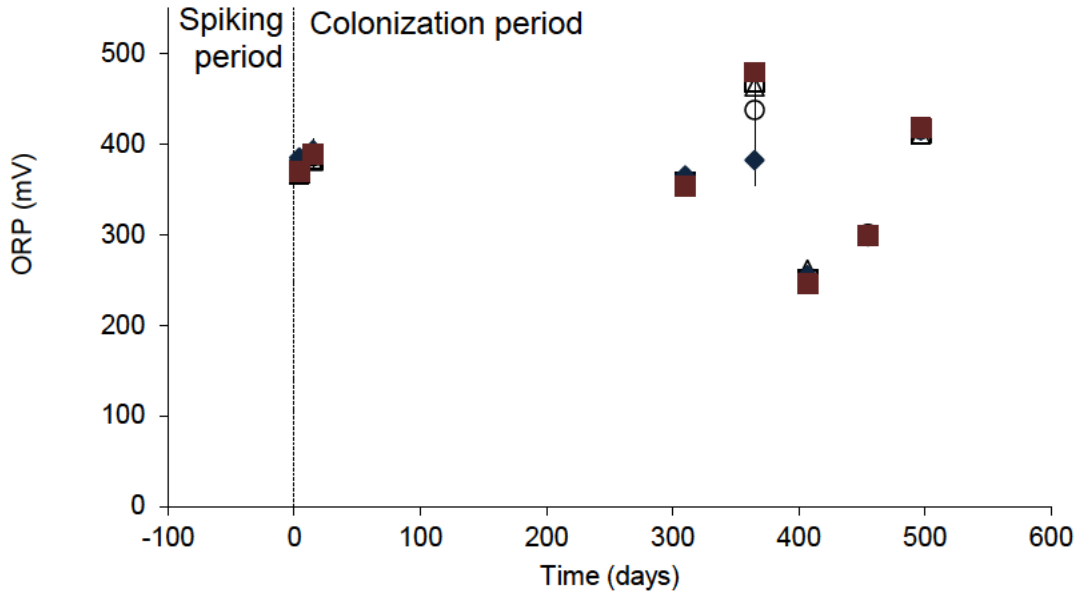

Figure 2.6: Change in overlying water redox potential (ORP) over time. Key: Control (□); very low (△); low (○); high (◆); very high (■); (n=4).

Total organic carbon, nutrients and ammonia

The concentrations of TOC, ammonia, nitrate and phosphorus remained low during the colonisation period (Table A2.4). In the surface waters, ammonia, nitrate, nitrite and phosphate were all near or below detection limits (<0.005 mg/L) in all samples.

Equilibration of copper

The total particulate copper, concentrations were reasonably consistent over time once they had stabilised (Figure 2.7a), indicating that the surficial sediment layer was suitably homogeneous following mixing. Particulate copper concentrations were 5 ± 2, 71 ± 12, 99 ± 19, 406 ± 110 and 711 ± 120 mg/kg (mean after day 74, n = 10 measurements) for the C, VL, L, H and VH treatments, respectively. For the H and VH treatments these were considerably higher than target concentrations of 270 and 540 mg/kg, which is most likely to have occurred due to inadequate mixing between surface and deeper sediments. This inadequate mixing probably also explains the larger variance within these treatments. While there is some possibility that there may have been a gradual flux of copper to the surface

sediments when disturbed (e.g. during sampling or bioturbation), we believe it is unlikely to have contributed significantly to the differences in copper with depth during the timeframe of this experiment. Nevertheless, the particulate copper concentrations displayed an excellent range and are comparable to those occurring at contaminated sites reported in the literature (Table A2.1).

Although the concentrations of particulate and pore water copper reached a plateau in all treatments (Figure 2.7a,b), the particulate copper in the H and VH treatments, and pore water copper in the VH treatment, only stabilised eighteen weeks after the sediments were initially spiked (t = 74 d); i.e. 10 weeks into the colonisation phase. Corresponding to this, in the spiked sediments, the partition coefficients (K_d) for copper between pore waters and sediments increased during the start of the colonisation phase, indicating the copper was increasingly becoming bound to the sediments (Figure 2.8a,b). The recommendation by Simpson et al. (2004) of >15 days equilibration time for copper-spiked sediments was for very fine-grained marine sediments with relatively large quantities of silt and organic matter, known to increase copper binding (Strom et al., 2011; Besser et al., 2003). The equilibration of the freshwater sediments in this study appears considerably slower than this, and based on these results a minimum of 135 days equilibration time would be recommended, though this is clearly dependent on several factors. It is not clear whether the use of deoxygenated waters would assist in shortening equilibration times (Simpson et al., 2004), however, for the large scale mesocosm experiment this was impractical. Despite the time taken to equilibrate, the K_d values between pore water and sediments generally remained above 1×10^4 L/kg thirteen weeks after spiking (after t = 32 d) (Figure 2.8a,b), following which, the K_d values were similar in magnitude for the four copper-spiked treatments and the minimum K_d value was 1.5×10^4 L/kg (L; t = 102 d). These partition coefficients are comparable to those previously observed in the field ($1 \times 10^4 – 1 \times 10^6$ L/kg) (Figure 2.1).

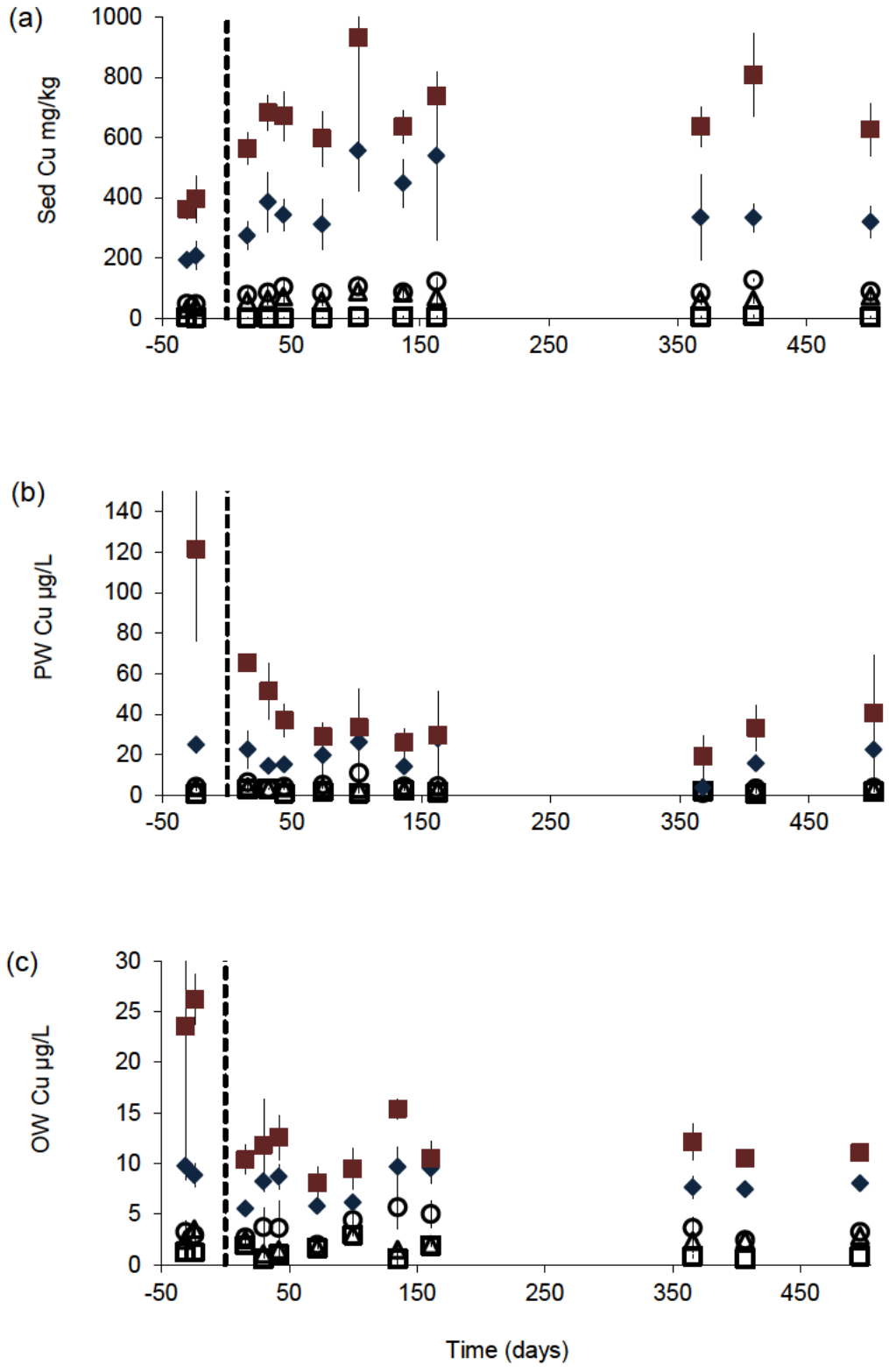

Figure 2.7 Change in (a) particulate, (b) pore water and (c) overlying water concentrations of copper over time. Key: Control (□); very low (△); low (○); high (◆); very high (■); the dotted line represents when mesocosms were opened to biotic colonisation. Target particulate concentrations were 0 mg Cu/kg (C), 32 mg Cu/kg (VL), 65 mg Cu/kg (L), 270 mg Cu/kg (H) and 540 mg Cu/kg (VH). Values are mean ± standard error (n = 4).

In contrast to the pore water copper concentrations, the mean overlying water copper concentrations appeared relatively stable from the start of the colonisation phase (t = 0 d), and the concentrations increased in the expected order C<VL<L<H<VH (Figure 2.7c). In the C and VL treatments the concentrations of copper in overlying water were near or below detection limits (typically <3 µg/L) for the duration of the colonisation period. The partition coefficient values (K_d) between overlying waters and sediments did not appear to change (Figure 2.8c,d). The overlying water K_d values were above 1×10^3 L/kg (excluding control) throughout this phase, comparable to those previously observed in the field ($1 \times 10^3 – 2 \times 10^5$ L/kg) (Figure 2.1).

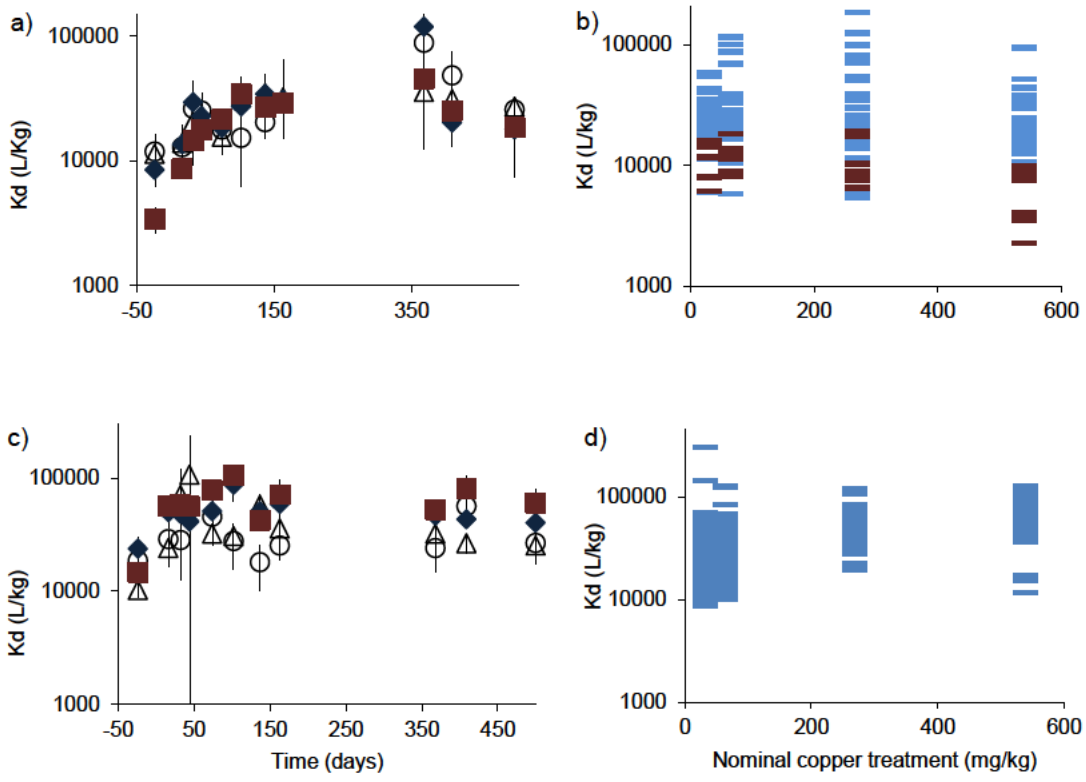

Figure 2.8: K_d values between particulate and pore water (a,b) and particulate and overlying water (c,d) copper concentrations over time (a,c) and by nominal treatment (b,d). Figures (b) and (d) display the K_d values across all time points, showing clearly that after t = 32, they remained above the minimum targets of 1 x 10^4 and 1 x 10^3 respectively. Key (a,c): Very low (△); low (○); high (◆); very high (■); (n = 4). Key (b,d): before t = 32 d (red) and from t = 32 d onwards (blue) (n = 1).

Equilibration of calcium and magnesium

Similar to copper, the total particulate calcium and magnesium concentrations were reasonably consistent over time once they had stabilised (Figure 2.9a,b). Although consistent within treatments, total particulate calcium concentrations followed the pattern C<VL<L<H<VH throughout the spiking and colonisation periods (Figure 2.9a). This pattern was observed because the higher concentrations of copper required greater amounts of garden lime to neutralise the acidity associated with the added copper and displaced metals. A large component of the particulate calcium was probably present as $CaCO_3$ and CaO, given that they were the main forms in the garden lime. Consequently, it is likely that considerable amounts of the copper adsorbed to or precipitated as hydroxy-carbonate particulate phases. These forms of the copper may have been more bioavailable than those in field-contaminated sediments, where a larger portion of the copper might be expected to be bound by sulphide (AVS), particulate organic matter, or iron/managense oxyhydroxide phases (Simpson & Batley, 2007; Chapman et al., 1998). While this may be a non-ideal artifact of using garden lime, in this instance it was considered the appropriate base to use in comparison to sodium hydroxide, which led to elevated aqueous copper concentrations in the pilot study.

Calcium and magnesium pore water concentrations declined throughout the experiments, but the decline was more rapid initially. The garden lime contained some magnesium (2.7% Mg as $MgCO_3$) and it is likely that this, along with the high calcium concentrations in the pore waters displacing magnesium from sediment phases, led to the initial high concentrations of magnesium in the pore waters. The pore water concentrations of calcium and magnesium also remained higher in the VH and H treatments compared to the C, VL and L treatments for the first six months (Figure 2.9c,d), which may have reduced the bioavailability of copper in the VH and H treatments during that period (Welsh et al., 2000; Bradley & Sprague, 1985; Calamari et al., 1980).

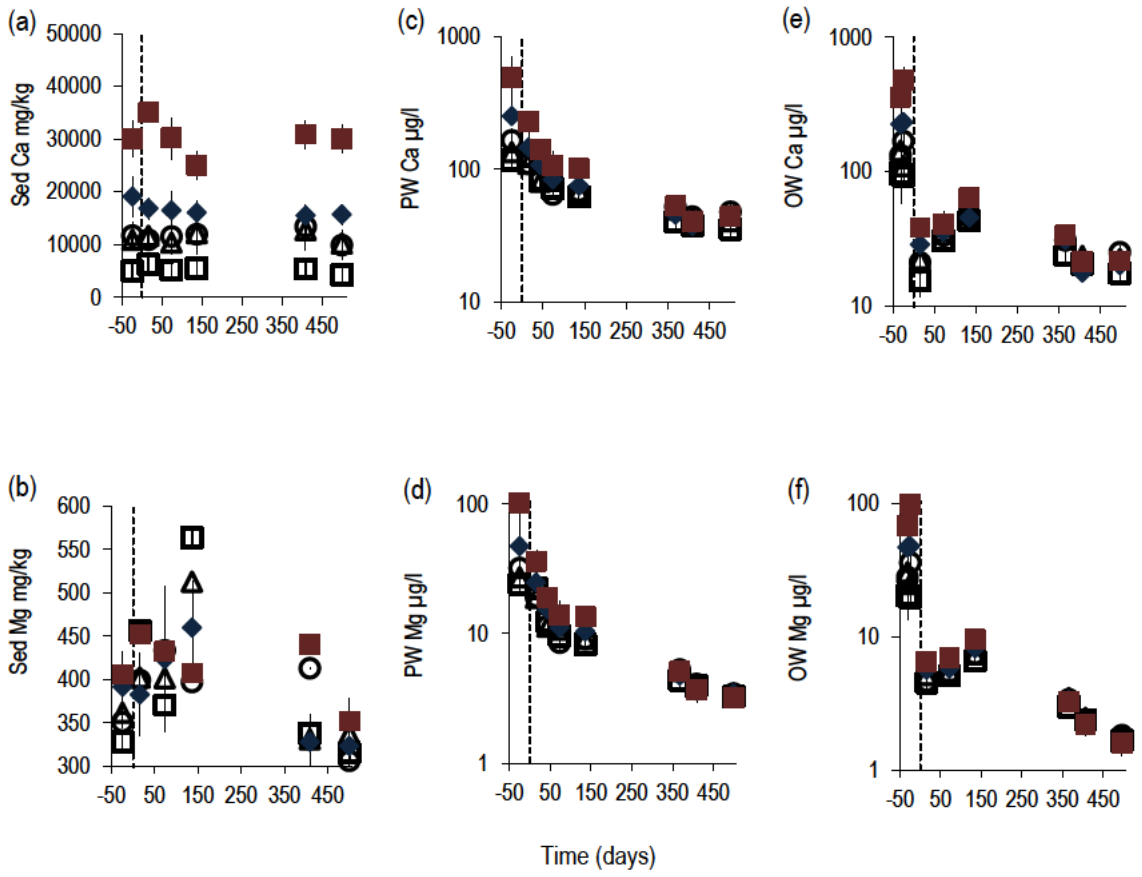

Figure 2.9: Change in particulate (a,b), pore water (c,d) and overlying water (e,f) concentrations of calcium (a,c,e) and magnesium (b,d,f) over time. Key: Control (□); very low (△); low (○); high (◆); very high (■). Values are mean ± standard error (n = 4).

The initial rapid decline in pore water calcium and magnesium concentrations demonstrates the importance of allowing the spiked sediments enough time to equilibrate. Simpson et al. (2004) spiked marine sediment in the laboratory under anoxic conditions and adjusted the pH after metal additions using 1 M NaOH. They reported >15 days equilibration times for copper-spiked sediments, but the sediments were very silty marine sediments, where calcium and magnesium concentrations in seawater are typically much higher (e.g. 0.4 and 1.3 g/L). While for marine sediments, the additional calcium and magnesium in the pore water and overlying waters may be less important, for freshwater sediments and waters the implications are much greater. Equilibration periods of >100 days appears more suitable for freshwater sediments with lime amendments (Figure 2.9).

The potential changes in copper bioavailability that may be occurring as the sediments equilibrate following copper spiking emphasises the importance of

monitoring the partitioning of both the metal of interest and other potentially weakly bound metals following spiking if data are to be used for assessing ecotoxicological effects. Monitoring allows the appropriate time to start exposure experiments to be determined and may indicate whether exposure pathways for biota have significantly changed during the experimental period. When only short equilibration times are available, the experiment should only commence after the rate in decline of pore water concentrations has slowed and pseudo-equilibration has been reached (Hutchins et al., 2009a), which in this study was after t = 74 d in the VH treatment.

In the overlying waters, there were high concentrations of both magnesium and calcium during the spiking period but they decreased and remained stable after the start of the colonisation period (t = 0 d) (Figure 2.9e,f). Magnesium concentrations in the overlying waters showed the same decrease initially and then continued to decrease at a slow rate throughout the colonisation period.

Influence of equilibrating water composition on biological effects

For a number of metals, including copper, toxicity is known to be influenced by hardness (De Schamphelaere & Janssen, 2004; Di Toro et al., 2001; Santore et al., 2001). Within the ANZECC and ARMCANZ (2000) water quality guidelines, algorithms are used for adjusting the guideline values, based on toxicity data spanning water hardness from 25 – 400 mg $CaCO_3$/L. The pore waters in the mesocosms were classified as hard waters (very hard for treatment VH) with between 147 and 204 mg/L as $CaCO_3$. Hardness-modified trigger values for the protection of 80% of species in the H (10.9 µg/L) and VH (12.8 µg/L) treatments were exceeded, while in the L treatment the hardness-modified trigger value for the protection of 99% of species of 4.2 µg/L was exceeded (Table A2.5). This indicates that the benthic biota could be affected by dissolved copper, particularly in the H and VH treatments, and indeed, effects on benthic biota were observed (Chapter 3-Chapter 6).

The overlying waters were all considered moderately hard, with between 83 and 110 mg/L as $CaCO_3$. The hardness-modified trigger values were still exceeded in the water column, even to protect 80% of species in the H (6.5 µg/L) and VH (7.5 µg/L) treatments (Table A2.5). In the L treatment, the trigger value to protect 95% of species (3.7 µg/L) was exceeded. However, analyses that considered the biota in the water column, generally did not reveal an effect of copper (Chapter 4 and Chapter 6).

Conclusions

The phenomenon of high pore water metal concentrations in the field is rare because inputs have occurred gradually and hydrolysis reactions have had an extensive equilibration time (Hutchins et al., 2007; Simpson et al., 2004; Bat & Raffaelli, 1998), omitting localised areas with high pore water metal concentrations due to recent spills, for example. It is imperative to ensure spiking procedures minimise the aqueous concentrations of the spiked metal, and other metals associated with the sediment, and encourage the partitioning of the metal to the particulate phase to create environmentally realistic K_d values. When spiking sediments for use in large-scale outdoor mesocosms, sediment properties, method of pH amendment, regime of physical mixing, and equilibration time, should all be considered. The sediment and pore water properties strongly affect equilibration times, and it appears that equilibration times for freshwater spiked-sediments may be longer than marine sediments (Hutchins et al., 2007, 2009b). The optimum choice of base for neutralising introduced acidity is dependent on the magnitude of pH change required, which depends on soil type, target concentration and equilibration time. A pilot study is recommended to identify an appropriate base to use and the magnitude of pH adjustment required. Once spiked, it is essential to monitor the metal partitioning within sediments to increase understanding of any further results obtained.

In the mesocosm sediments of this study, relatively low pore water metal concentrations were achieved, producing K_d values similar to those found in historically field-contaminated sediments. Consequently, although the final pH was

above the normal pH range of most freshwater environments, subsequent studies of biotic colonisation at the mesocosm facility could be conducted in the knowledge that the partitioning of copper in the sediments reflected, as close as possible, that which is expected in field situations.

Chapter 3. The effects of copper on *Parataya australiensis* and *Physa acuta* in an outdoor manipulative experiment

The aim of the work presented in this chapter was to characterise responses to copper within the mesocosms at the organism level. A further aim was to provide some insight into the environmental applicability of the effects of copper on Physa acuta and Paratya australiensis, known from laboratory studies. A series of in situ exposures within the mesocosm facility were performed to assess the effects of copper on Physa acuta (growth, reproduction and survival) and Parataya australiensis (growth and survival). The chapter discusses the merits of in situ versus laboratory exposures to consider the effects of contaminants at the organism level. The effects of copper on P. acuta could be linked to effects at population, community and ecosystem function levels.

Authors

	Problem formulation	Experimental design/ methodology	Statistical analysis	Results interpretation	Paper preparation
Stephanie Gardham[1,2]	90	85	90	85	92.5
Anthony A. Chariton[2]	5	5	5	2.5	2.5
Grant C. Hose[1]	5	10	5	12.5	5

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Abstract

Responses to contamination at low levels of biological organisation are generally measured in controlled laboratory studies. *In situ* exposures provide an alternative means of assessment, which incorporates changing environmental conditions at field sites. Here, the effects of copper on both *Paratya australiensis* and *Physa acuta* were assessed by performing *in situ* exposures within a manipulative mesocosm study representative of historically contaminated sites. Although at environmentally relevant concentrations in the mesocosms, copper was not lethal to either species, but effects on growth in both species were observed in the highest copper treatment. This indicated that the chronic exposure of both species to copper could ultimately influence their populations within historically contaminated sites. The study emphasises the importance of site-specific chronic assessments to truly understand the effects of contaminants on aquatic biota in the field.

Introduction

The effect of a contaminant can be measured across levels of biological organisation, from its mechanism of action at the molecular and biochemical scale to its impact on the ability of communities to perform vital functions at the ecosystem scale (Clements, 2000). Responses at lower levels of biological organisation (molecular, biochemical, physiological and organism) are generally measured in controlled laboratory studies, which lack environmental realism (Cairns, 1983). *In situ* exposure studies offer an alternative to laboratory studies, allowing an assessment of the effects of a contaminant at lower levels of biological organisation under environmentally relevant conditions.

The responses of biota to contaminants measured during *in situ* studies often differ in comparison to laboratory toxicity assays. For example, Burton et al. (2005) investigated the effects of river sediments contaminated with PCBs by exposing a variety of invertebrates via *in situ* and standard laboratory tests; they observed greater toxicity via the *in situ* exposures compared to the laboratory toxicity assays. In contrast, the natural environment can be protective of contamination; Mann et al. (2010), found no effect on fecundity in an amphipod, *Melita plumulosa*, when organisms were exposed to contaminated sediments *in situ*, while sediments from the same sites were shown to cause toxicity in the laboratory. In some cases, the effects of a contaminant in laboratory and *in situ* enclosures are similar; for example Hose et al. (2003) showed toxicity of endosulfan to *Atalophlebia* spp. to be similar in laboratory and caged benthic mesocosm studies.

There is no consistent pattern for extrapolation from laboratory to field. However a meta-analysis that compared the toxicity of sediments tested in the laboratory to those tested *in situ* showed that the toxicity of sediments in laboratory tests is generally less than their toxicity *in situ* (Hose et al., 2006). Differences may occur because the environmental conditions during exposures are very different (Mann et al., 2010). Further, intra- and inter- specific interactions with other biota are likely to be altered; for example sediment homogenisation removes predators, which may contribute to lower mortality in laboratory tests compared to *in situ*

studies (Hose et al., 2006). Because of their ecological relevance, *in situ* bioassays are increasingly being utilised to assess the applicability of laboratory toxicity estimates to field sites (Moreira et al., 2006).

Copper is an essential metal, but negatively affects most aquatic biota when homeostatic control mechanisms are overcome. In this study, the effects of copper on *Paratya australiensis* (Kemp) (Decapoda: Atyidae) and *Physa acuta* (Draparnaud) (Physidae) are explored by assessing the susceptibility of the species via *in situ* exposures in established outdoor pond mesocosms. *P. australiensis* is the most common Atyid shrimp in southeastern Australia (Williams, 1977), and is sometimes used in standard toxicity studies. However, the effects of copper on *P. australiensis* have only been explored in the laboratory: Skidmore & Firth (1983) considered the effect of temperature on copper toxicity to the shrimp, and Daly et al. (1990abc; 1992) assessed the influence of various ligands (inorganic, characterised organic and natural dissolved organic matter) on copper toxicity. *P. acuta* is abundant worldwide (Ohbayashi-Hodoki et al., 2004) and has been studied extensively outside of Australia. However, the sensitivity of *P. acuta* to copper has only been considered in four studies: three were performed in the laboratory (Arthur & Leonard, 1970; Wurtz, 1962), and one was performed in the field with unrealistically high dissolved copper concentrations (≥85 mg/L) (Massoud & Mansoorin, 1986). By performing *in situ* exposure studies using *P. australiensis* and *P. acuta* the aim of this study was to provide some insight into the environmental applicability of the outcomes reported from the laboratory studies. The effects of copper during chronic exposures on mortality, fecundity and growth were assessed.

Methods

Study design

Twenty pond mesocosms were established on the Macquarie University campus (33.76946 S, 151.11496 E), in northern Sydney, NSW, Australia. Full details of the infrastructure are included in Chapter 2. Briefly, each mesocosm had a volume of 1500 L, was sunk into the ground, shaded by 70% shade-cloth, aerated and inputs from rainwater were sufficient to maintain water depth.

Sediments were spiked with copper prior to opening up the mesocosms to biotic colonisation on the 1st November 2010 (t = 0 d). The experimental design consisted of a control (C) and four nominal sediment copper concentrations: very low (VL), low (L), high (H) and very high (VH). The copper partitioning between sediments, pore waters and overlying waters were periodically analysed (Chapter 2), and the *in situ* exposures performed in this study were carried out once the mesocosms were established and pseudo-equilibration of copper had been achieved.

The concentrations of copper in the sediments, pore waters and overlying waters at or near the time of each exposure experiment generally followed the pattern C < VL < L < H < VH, but differences among treatments were not always significant (Table 3.1). For all exposure experiments, concentrations of copper in the sediments could be clustered into four distinct groups: “C”, “VL/L”, “H” and “VH”. Pore water copper concentrations in the first *P. australiensis* exposure were similar in C/VL/L/H treatments, which were all lower than the VH treatment. In the second *P. australiensis* exposure and the first *P. acuta* exposure the pore water copper concentrations could be grouped as for the sediment copper concentrations (“C”, “VL/L”, “H” and “VH”). For the second *P. acuta* exposure, pore water copper concentrations could be grouped into two: “C/VL/L” and “H/VH”. In the overlying waters, copper concentrations in the first *P. australiensis* exposure were different among all treatments. In the second *P. australiensis* exposure/first *P. acuta* exposure, the overlying water copper concentrations in the “C/VL/L”

treatments were similar and the “H” and “VH” treatments differed from each other and the C/VL/L treatments. In the second *P. acuta* exposure, three groups were apparent: “C”, “VL/L” and “H/VH” in the overlying water copper concentrations.

Table 3.1: Sediment, pore water and overlying water copper concentrations at or near the time of each exposure experiment (mean ± S.E.M (n = 4)).

Treatment	Sediment copper (mg/kg)	Pore water copper (µg/L)	Overlying water copper (µg/L)
***Paratya australiensis* Exposure 1 (Date of copper analysis: 2/11/11)**			
Control	5.3 ± 1.0	2.0 ± 1.1	0.9 ± 0.1
Very low	63 ± 7.1	3.5 ± 2.0	2.3 ± 0.5
Low	82 ± 10	0.9 ± 0.1	3.6 ± 0.5
High	350 ± 61	3.5 ± 1.0	7.6 ± 0.5
Very high	640 ± 32	19 ± 5.3	12 ± 1
ANOVA	$F_{4,15}$ = 190, p < 0.001	$F_{4,15}$ = 9, p = 0.001	$F_{4,15}$ = 64, p < 0.001
	C < (VL = L) < H < VH	(C = VL = L = H) < VH	C < VL < L < H < VH
***P. australiensis* Exposure 2 and *P. acuta* Exposure 1 (Date of copper analysis 12/3/12)**			
Control	5.4 ± 0.7	1.6 ± 0.1	0.8 ± 0.1
Very low	70 ± 11	2.6 ± 0.3	2.8 ± 0.3
Low	88 ± 7.7	3.6 ± 0.3	3.3 ± 0.3
High	320 ± 26	24 ± 8.1	8.0 ± 0.2
Very high	340 ± 39	37 ± 6.8	11 ± 1.1
ANOVA	$F_{4,15}$ = 270, p < 0.001	$F_{4,15}$ = 67, p < 0.001	$F_{4,15}$ = 140, p < 0.001
	C < (VL = L) < H < VH	C < (VL = L) < H < VH	(C = VL = L) < H < VH
***P. acuta* Exposure 2 (Date of copper analysis 7/11/12)**			
Control	5.4 ± 1.6	10 ± 4.2	1.2 ± 0.31
Very low	54 ± 5.8	12 ± 3.2	3.4 ± 0.21
Low	88 ± 10	5.7 ± 0.7	5.4 ± 1.0
High	270 ± 35	21 ± 1.9	20 ± 10
Very high	630 ± 39	30 ± 3.7	22 ± 2.7
ANOVA	$F_{4,15}$ = 140, p < 0.001	$F_{4,15}$ = 8, p = 0.001	$F_{4,15}$ = 26, p < 0.001
	C < (VL = L) < H < VH	(C = VL = L) < (H = VH)	C < (VL = L) < (H = VH)

Parataya australiensis exposures

The species has been recorded in both lotic and lentic freshwater habitats (Walsh, 1993). Males and females live for two years and the females breed in their second summer. Juveniles grow rapidly between December – March reaching >12 mm long, but growth is minimal in winter months (April – August) (Williams, 1977). Male shrimp grow up 32 mm long, while females grow up to 35 mm long (Williams, 1977). The shrimp feed by both scraping biofilm off the substratum (browsing) and filter feeding (Gemmell, 1978).

Exposure 1: 4th July 2011 – 1st August 2011

Adult *P. australiensis* (15 – 20 mm length) were sourced from Aquablue seafoods (Pindimar, Australia), housed in an aquarium containing aged tap water at 23ºC and subjected to a 16:8-h light:dark cycle for one week. At the mesocosm facility, ten individuals were randomly allocated to each of twenty plastic cages 19(L) × 9(W) × 12(H) cm; the base (171 cm^2) of the cages was secured by 0.5 mm mesh and 1.2 cm diameter holes in the sides (covered in thin 0.5 mm mesh) allowed water to pass through the cages. One cage was randomly allocated to each mesocosm and pressed into the sediment surface to allow exposure to the sediments, pore waters and overlying waters. Survival was checked daily for four days, and then after 11, 21 and 28 days of exposure by removing each cage from each mesocosm and observing movement. Any dead individuals were removed and each cage was returned to its respective mesocosm in a different location.

Exposure 2: 1st February 2012 – 29th February 2012

Juvenile *P. australiensis* specimens ~10 mm length (with scope for growth) were sourced from Aquablue seafoods. To acclimate the shrimp to light and temperature conditions, they were placed in aerated tanks (30 L) within a pond adjacent to (and of the same dimensions as) the test mesocosms for two weeks prior to exposure. One specimen of *P. australiensis* of known wet weight was placed into each of 80 'small glass tube' cages (10 cm long, 3 cm diameter, with 0.5 mm mesh at either end; Figure 3.1) and four cages were pressed into the

sediment of each mesocosm randomly, allowing exposure to the sediments, pore waters and overlying waters. The survival of each specimen was recorded every 2 – 3 days until 19 days of exposure and then checked after 23 and 28 days of exposure by removing each cage from each mesocosm and observing movement. Any dead individuals were removed and then each cage was returned to its respective mesocosm in a different location. The wet weight of individuals that were still alive after 28 days of exposure was recorded.

Figure 3.1: Exposure cages for shrimp (exposure 2) and snails (both exposures). Cages were placed with open end pushed into the sediment surface to ensure sediment exposure.

Physa acuta exposures

The gastropod is a simultaneous hermaphrodite and although able to self fertilise, it prefers to outcross (Facon et al., 2007). Individuals reach sexual maturity before they are fully grown (Ohbayashi-Hodoki et al., 2004) and breed readily in captivity if kept at room temperature but in the field do not breed over winter (Duncan, 1959).

Laboratory culture

Mature *P. acuta* specimens were collected from an uncontaminated established pond at Macquarie University. Laboratory stocks were cultured from these specimens using a method adapted from Noland (1946), in a 20 L tank containing carbon filtered and aged Sydney tap water. The tank was aerated and covered to reduce evaporation. In the laboratory culture, *P. acuta* were fed iceberg lettuce (microwaved, stored frozen and washed with deionised water prior to feeding) and fish food (Algae Grazers, HBH) every 2 – 3 days. Water changes were performed every 1 – 2 weeks. Prior to each exposure experiment, *P. acuta* from the laboratory culture were transferred to aerated 10 L tanks held in a pond adjacent to the test ponds and kept for two weeks for acclimation to light and temperature conditions.

Exposure 1: 1st February 2012 – 4th March 2012

Three 'mature' *P. acuta* (i.e. largest by eye from the laboratory stock) were randomly allocated to each of twenty 'large glass tube' cages (20 cm long, 3 cm diameter, with 0.5 mm mesh at either end; Figure 3.1). Five smaller (by eye), juvenile *P. acuta* were randomly allocated to each of another twenty glass tube cages of the same dimensions. Two cages (one cage containing mature and one containing juvenile snails) were randomly placed into each mesocosm with an end pushed into the sediment, allowing exposure to the sediments, pore waters and overlying waters. Each cage was removed from the mesocosm every 2 – 3 days until 19 days then at 23 and 32 days of exposure and survival and the number of

egg masses produced were recorded. Each cage was then returned to the mesocosm in a different location.

Exposure 2: 9th October 2012 – 7th November 2012

Four 'mature' *P. acuta* (6.6 ± 0.2 mm) were randomly allocated to each of twenty 'large glass tube' cages (see above). One 'juvenile' (with scope for growth) *P. acuta* (3.6 ± 0.1 mm) was randomly allocated to each of eighty 'small glass tube' cages (see above). The length of each *P. acuta* shell was measured using digital calipers prior to allocation. One cage containing mature *P. acuta* and four cages containing juvenile *P. acuta* were placed into each mesocosm, with an end pushed into the sediment, allowing exposure to the sediments, pore waters and overlying waters. After 7, 10, 13, 16, 18, 22 and 29 days of exposure, the cages were removed from the mesocosms and the mature and juvenile *P. acuta* were checked for survival and, in the mature *P. acuta* cages, egg mass production, egg mass size (by maximum length and width) and the number of eggs per egg mass were recorded. Each cage was returned to the mesocosm in a different location. *P. acuta* were removed from their cages after 29 days of exposure and the length of the shells were recorded in order to calculate growth.

Data analysis

The difference in sediment, pore water and overlying water copper concentrations described above were analysed using one-way ANOVAs (between subjects factor: treatment). Data were all $\log_{10}(x)$ transformed, apart from sediment and pore water data from the second *P. acuta* exposure, which was square root transformed, to meet assumptions of normality (tested by Shapiro Wilk's test) and homogeneity of variance (tested by Levene's test).

To explore the effects of treatment on survival over time of *P. australiensis* and *P. acuta* a repeated measures (RM) ANOVA (between-subjects factor: treatment, within-subjects factor: time) was performed on the data set from each exposure. Data did not require transformation to meet the assumptions of normality (assessed by Shapiro Wilk's test) and homogeneity of variance (assessed by

Levene's test); if the assumption of sphericity (assessed by Mauchly's test) was not met during the RM ANOVA analyses, the Greenhouse-Geisser correction was used.

The mean growth of surviving *P. australiensis* and *P. acuta* individuals at the end of each species second exposure was calculated from wet weight and length data, respectively:

$$\frac{x_f - x_i}{x_i}$$

- where: x_f is the final weight (*P. australiensis*) or length (*P. acuta)* and x_i is the initial weight or length.

A one-way ANOVA followed by LSD posthoc analysis was performed on each data set to consider the effect of treatment. Growth data were arcsine (*P. australiensis*) and square root (*P. acuta*) transformed to meet assumptions of normality (tested by Shaprio Wilk's test) and homogeneity of variance (tested by Levene's test).

Egg mass production, egg mass size, number of eggs per egg mass and egg density (calculated by total no. eggs/total egg mass size) data from the second *P. acuta* experiment were normalised to the size of the snails (based on original length) as a relationship between body size and these factors has been shown in several studies (e.g. Norton 2006). Egg mass production from both exposures of *P. acuta* was analysed using a RM ANOVA (as described above for survival). Egg mass size, the number of eggs per egg mass and the egg density data from the second exposure were analysed using a one-way ANOVA.

Results

P. australiensis exposure 1

P. australiensis survival was >90% across all the mesocosms throughout the exposure and, as a result, neither treatment ($F_{4,15} = 0.75$, $p = 0.57$), or time ($F_{1.8,27} = 2$, $p = 0.16$) affected survival, nor was there a significant interaction between treatment and time ($F_{7,27} = 0.75$, $p = 0.64$) (Table A3.1).

P. australiensis exposure 2

There was no difference in survival of *P. australiensis* among treatments during the second exposure experiment ($F_{4,15} = 2$, $p = 0.15$) (Figure 3.2a). However, survival did decrease over time ($F_{3,45} = 23$, $p < 0.001$); there was no significant interaction between treatment and time ($F_{12,45} = 1.3$, $p = 0.28$) (Table A3.1). *P. australiensis* individuals decreased in weight in the VL, H and VH treatments and gained weight in the C and L treatments (Figure 3.2b). The decrease in weight was most notable in the VH treatment and a clear biological effect is evident; the overall effect of treatment was near significant ($F_{4,19} = 2.7$, $p = 0.068$) and further analysis showed the difference in weight change was significant between the VH and both the C ($p = 0.014$) and L ($p = 0.019$) treatments (Table A3.1).

P. acuta exposure 1

Survival of *P. acuta* decreased over time ($F_{9,135} = 2.8$, $p = 0.004$), by the end of the exposure period it was still >91% in the C/VL/L/H treatments and appeared lower (66 ± 22%) in the VH treatment (Figure 3.3). However, there was large variability and survival was not affected by treatment ($F_{4,15} = 1.9$, $p = 0.17$). There was no interaction between time and treatment ($F_{36,135} = 1.2$, $p = 0.23$) (Table A3.2).

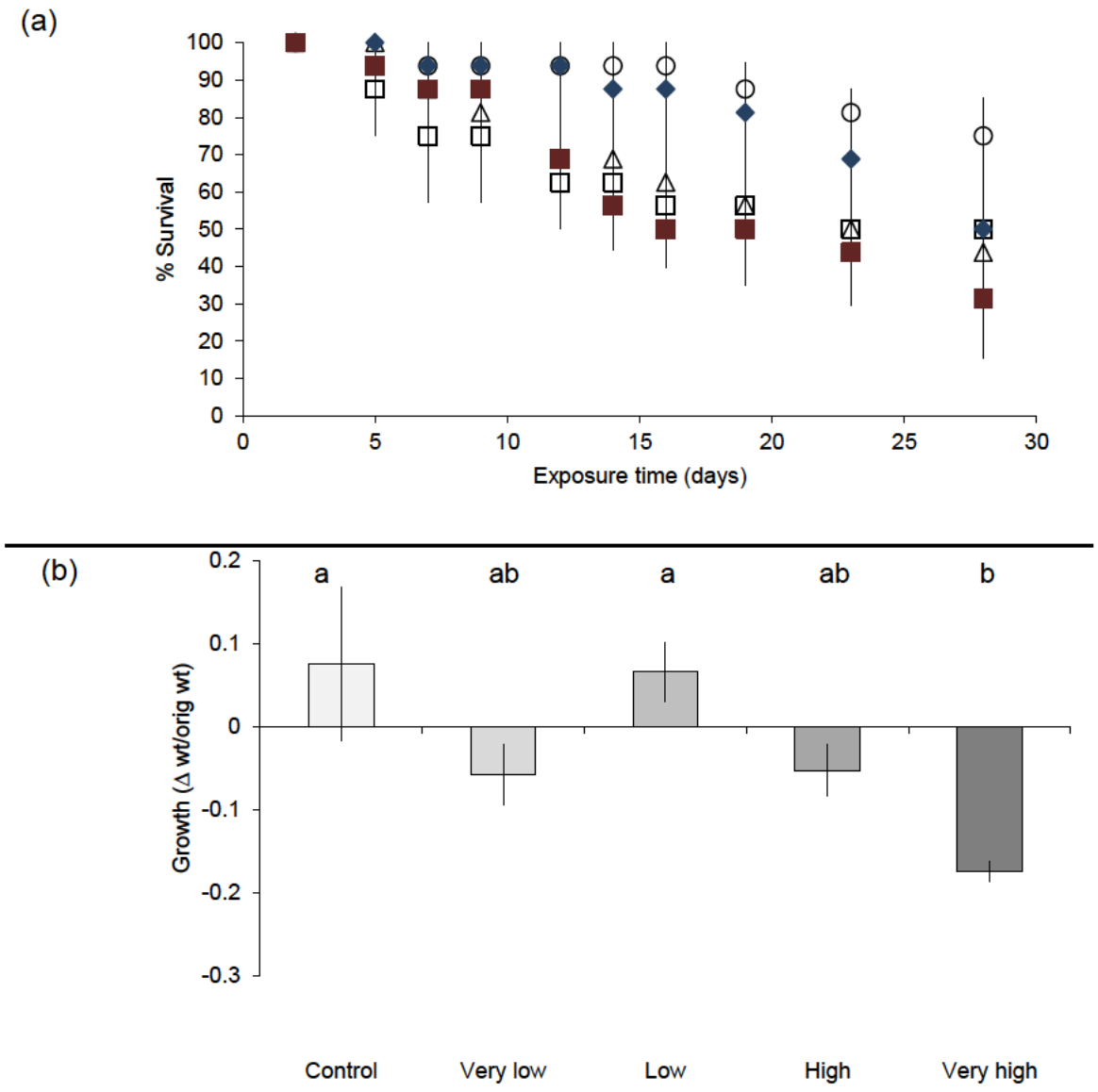

Figure 3.2: (a) Survival and (b) growth of *P. australiensis* during the second shrimp exposure. (mean ± S.E.M (n = 4)). Key: In (a) control (□); very low (△); low (○); high (◆); very high (■). In (b), treatments that do not share a letter are significantly different.

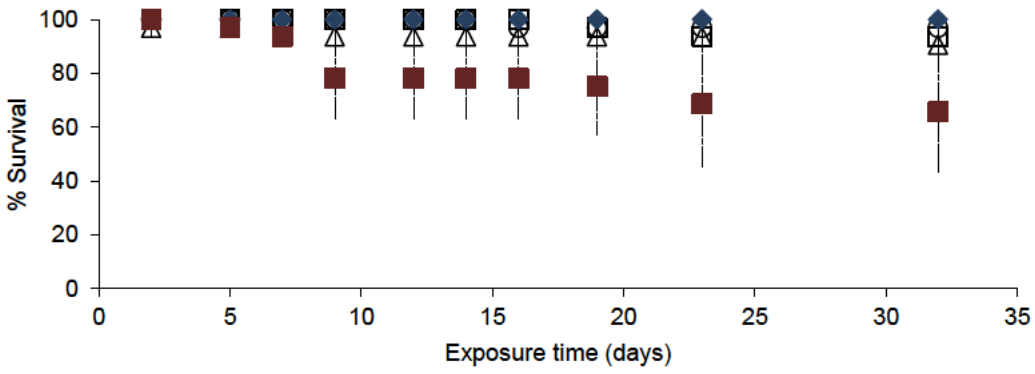

Figure 3.3: Survival of *P. acuta* during the first exposure. Key: control (□); very low (△); low (○); high (◆); very high (■).

P. acuta exposure 2

P. acuta survival was >90% across treatments throughout the second exposure experiment, but decreased over time ($F_{6,90}$ = 3.6, p = 0.003): at 7 days of exposure survival was higher than at 16 days and beyond. There was no effect of treatment ($F_{4,15}$ = 0.73, p = 0.69); although, survival did appear lower in the H/VH treatments than the C/VL/L treatments (Figure 3.4a), variability was high. There was no interaction between treatment and time ($F_{24,90}$ = 0.36, p = 1).

In total, 129 egg masses were produced during the exposure period across treatments. The cumulative number of egg masses increased over time ($F_{1.7,25}$ = 23, p < 0.001) (Figure 3.4b). Even though the number of egg masses produced by *P. acuta* in the VH treatment appeared lower than other treatments, there was no effect of treatment on egg mass production ($F_{4,15}$ = 1, p = 0.42). There was no interaction between time and treatment ($F_{6.7,25}$ = 0.73, p = 0.64). The egg masses produced by *P. acuta* varied in size from 1 – 88 mm^2, and contained between 2 – 44 eggs with an egg density of between 0.2 – 2.6 eggs/mm^2. However, there was no significant difference in egg mass size ($F_{4,15}$ = 0.11, p = 0.98), the number of eggs per egg mass ($F_{4,15}$ = 0.29, p = 0.88) or the egg density ($F_{4,15}$ = 1, p = 0.42) among treatments (Table A3.2). There was a significant difference in growth of the juvenile *P. acuta* among treatments ($F_{4,15}$ = 5.5, p = 0.006) (Figure 3.4c); juvenile snails in the VH treatment grew less than those in all other treatments (p < 0.01) (Table A3.2).

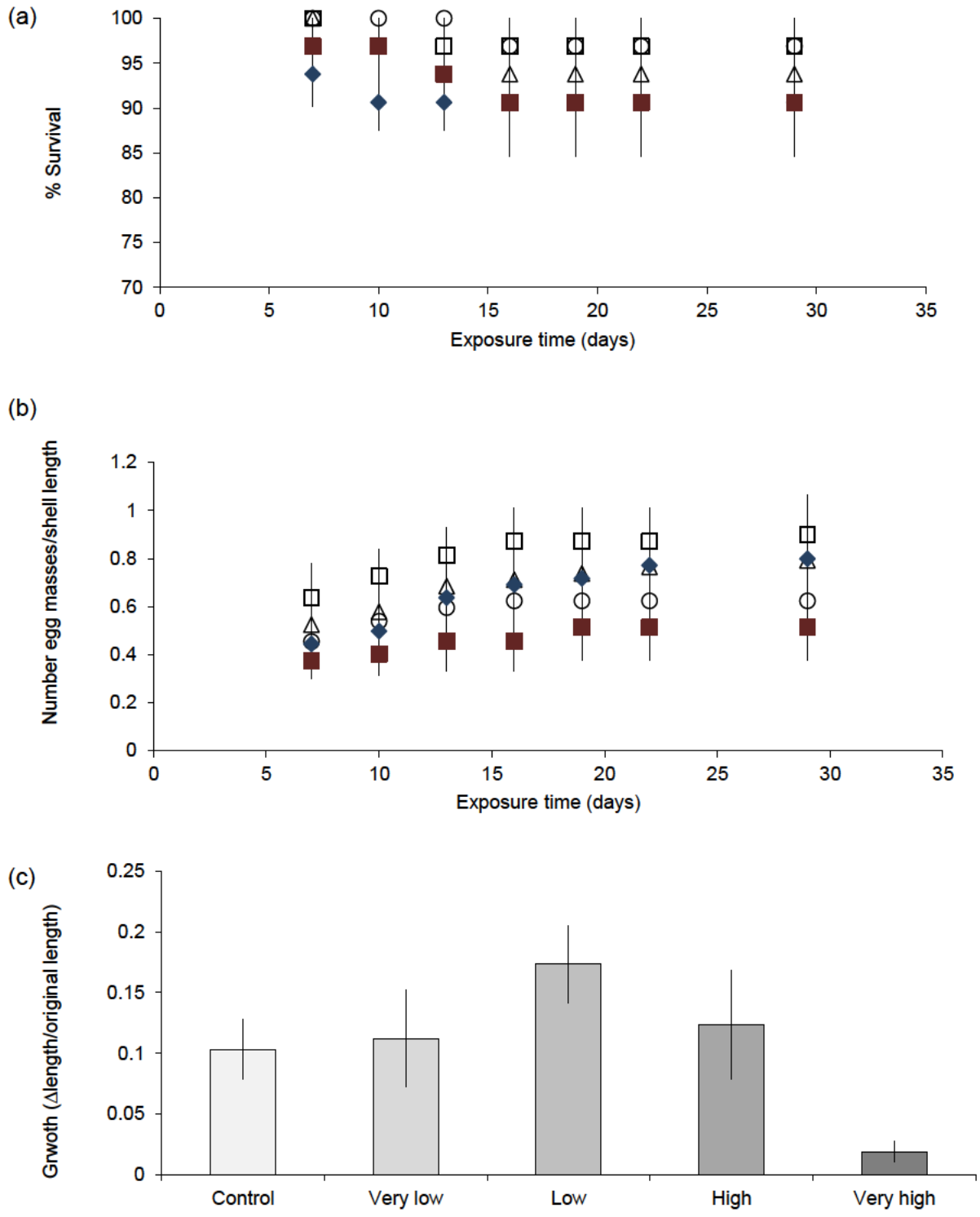

Figure 3.4: (a) Survival, (b) egg mass production and (c) growth of *P. acuta* during the second exposure. Key: in (a) and (b): control (□); very low (△); low (○); high (◆); very high (■).

Discussion

Although survival was not affected, the copper contaminated mesocosm environment had a significant effect on the growth and development of *P. acuta* and *P. australiensis*. This study demonstrates that chronic effects of copper at historically contaminated sites may influence the populations of *P. acuta* and *P. australiensis,* even though direct mortality in mature specimens of either species is unlikely. The research confirms the need for site-specific chronic assessment of the effects of contamination to verify laboratory studies.

In this study, there was no effect of copper on mortality of *P. australiensis*, indicating that the bioavailable fraction of copper in the mesocosms to the shrimp was too low to elicit a response. *P. australiensis* would have been exposed to copper through the overlying waters, sediments and porewaters as biota are exposed to copper through respiration, skin contact and ingestion (Chapman, 2008). However, it is likely that the overlying water was the main route of exposure; previous studies have shown that, apart from burrowing benthic invertebrates, those living on the surface of the sediment accumulate cadmium almost exclusively from the overlying water (Hare et al., 2001; Warren et al., 1998). The maximum concentrations of copper in the overlying waters were 12 ± 1 and 11 ± 1.1 µg/L (during each respective exposure). These concentrations are lower than overlying water concentrations previously shown to cause mortality; the acute toxicity of copper at 15ºC and 24ºC was assessed by Skidmore & Firth (1983), who determined 96-h LC50 values of 110 and 120 µg/L respectively and Daly et al. (1990abc, 1992), who determined multiple 96-h LC50 values between 34 µg/L (exposure to the free cupric ion) and 317 µg/L (exposure to copper in the presence of natural organic matter). No other studies have considered the chronic effect of copper on *P. australiensis* and the pore water and sediment exposure pathways, to allow comparison of the sediment and pore water concentrations of copper. Nevertheless, because the copper partitioning in this study is comparable to historically contaminated sites (Chapter 2), this study indicates that at such sites, chronic copper exposure is unlikely to be lethal to *P. australiensis,* because the concentrations are too low.

Although copper did not affect the survival of *P. australiensis* in this study, it did affect growth. Effects of copper on growth have been observed in many crustaceans, including *Farfantepenaeus paulensis* (Decapoda: Penaeidae) *Artemia fraciscana* (Anostraca: Artemiddae) and *Penaeus indicus* (Decapoda: Penaeidae) (Paila & Yallapragada, 2010; Santos et al., 2000; Spicer, 1995). Lower growth rates were linked to a decrease in food consumption (*F. paulensis*), oxygen consumption (*F. paulensis* and *P indicus*) and metabolic rates (*P. indicus*) (Paila & Yallapragada, 2010; Santos et al., 2000). Further research could confirm if those physiological effects of copper could also be linked with a decrease in growth of *P. australiensis.* Ultimately, an effect on growth indicates a decreased fitness of individuals, which could lead to population level effects. Therefore, because copper partitioning in this study is representative of historically contaminated sites, at such sites, the shrimp population may still be affected due to the chronic effects of copper on the fitness of individuals.

An effect of treatment on survival in *P. acuta* was expected because the overlying water copper concentrations during each respective exposure was 11±1.1 and 22±2.7 µg/L and Arthur & Leonard (1970) observed mortality during chronic six week laboratory exposure studies at copper concentrations above 14.8 µg/L. However, Arthur & Leonard (1970) used a lake water as their diluent, which was soft. The waters in this study were hard (Chapter 2) and, as an increase in total hardness is known to decrease bioavailability of copper (Welsh et al., 2000), it is likely that the environmental conditions in the mesocosms reduced the bioavailability of copper to such an extent that it no longer caused mortality. This emphasises the need for site-specific assessment as differences in the environmental conditions, like hardness, between sites strongly influence the bioavailability of copper, which in turn affects the survival of test organisms. However, as this study is generally applicable to historically contaminated sites, similar to *P. australiensis*, it suggests that concentrations of copper at such sites are unlikely to be lethal to mature specimens of *P. acuta*.

Although no effect on mortality was observed, within the mesocosms there were healthy snail populations in the control and low copper treatments while in the

higher copper treatments snails were scarce, if present at all (Chapter 4). As such, given that the *in situ* exposures revealed no effect of copper on survival, an effect on reproduction was expected, which would explain the population level response. However, no effect on reproduction was observed. This was even more surprising given that during their six-week exposure experiment, Arthur & Leonard (1970) only observed successful reproduction at ≤8 µg/L. This is much lower than the overlying water concentration in the highest treatment (22±2.7 µg/L), although, as discussed previously, the bioavailability of copper in the waters in this study was probably lower than that in Arthur & Leonard (1970), due to different environmental conditions i.e. hardness.

The lack of a significant reproductive response to copper observed during the *in situ* exposures may be explained by the end points measured. The effect of copper on the ability of snails to reproduce was assessed on snails that were cultured in optimal conditions, and only exposed to the mesocosms when mature. The viability of the embryos laid, their survival to maturity or the fitness of those individuals in the F1 generation was not recorded. Therefore, from this study, it can only be concluded that mature snails chronically exposed to copper were still able to produce egg masses of equal size, and containing equal numbers of eggs, to those in control mesocosms. It is possible that during the early development of the embryos, mortality occurred in the higher copper treatments, resulting in the population response observed (Chapter 4). This seems plausible as the early life stages of aquatic organisms are usually more susceptible to contaminants (Khangarot & Das, 2010). Alternatively, if they survived to maturity individuals in the F1 generation may not have been able to reproduce. Such latent effects of metals have been demonstrated in other gastropod species: early exposure to cadmium was shown to affect time to reproduction, egg mass density, hatching success and size in *Physa pomilia* offspring (Kimberly, 2012). This warrants further research to obtain a greater understanding of the population level response to copper that was observed in the mesocosms.

Evidence that copper did lead to some decrease in fitness of *P. acuta* was demonstrated by measuring growth rates, which were lower in the VH treatment.

Inhibition of growth of *P. acuta* due to copper exposure was also observed by Arthur & Leonard (1970) who found no growth occurred at and above 14.8 µg/L copper during their six-week study (although note that their water was soft, as discussed above). There was also no effect of copper on *Lymnaea stagnalis* survival when exposed via the diet, but growth inhibition did occur (Ng et al., 2011). Similar to *P. australiensis*, the effect of copper on growth of *P. acuta* indicates population level effects at historically contaminated sites may still occur due to chronic exposure, even if there is no change in the mortality of mature individuals.

Interestingly, a significant effect on growth of *P. acuta* was only observed in the VH treatment, even though copper concentrations in the overlying waters and pore waters of the H and VH treatments were not significantly different (overlying water concentrations were 20 ± 10 and 22 ± 2.7 µg/L and pore water concentrations 21 ± 1.9 and 30 ± 3.7 µg/L for each respective treatment). However, particulate copper was significantly different between treatments (270 ± 35 and 630 ± 39 mg/kg in the H and VH treatments respectively). This indicates that the primary source of copper leading to the effect on growth of *P. acuta* may have been in the particulate phase. The copper may have affected the snails through direct exposure, i.e. direct ingestion of sediment, or biofilm with high concentrations of copper. Biofilms are known to readily accumulate metals, with the rate of uptake being dependent on their biomass (Burton & Johnston, 2010), and may therefore may be important in controlling metal partitioning between dissolved and particulate phases (Nimick et al., 2003). However, in contradiction, non-burrowing benthic invertebrates have previously been shown to be primarily exposed to cadmium in the overlying water (Hare et al., 2001; Warren et al., 1998). Alternatively, an indirect effect may have occurred whereby particulate copper could have affected the quality of the biofilm, changing the energy resources available to the snails; for example, copper has been shown to induce changes in biofilms causing them to lose the capacity to metabolise certain organic substrates (Barranguet et al., 2003). This demonstrates the importance of environmentally realistic exposure scenarios.

This study has highlighted that when assessing the effects of contaminants at a particular site, it is impossible to predict them from laboratory studies alone. It has emphasised the importance of chronic studies, which enable a clearer understanding of the sub-lethal effects of contaminants that may ultimately affect populations, communities and ecosystems. However, even these studies may not extrapolate well to field sites, where multiple stressors are likely to be present. Ultimately, this study indicates that at historically contaminated sites, where dissolved concentrations of copper are generally low, acute exposures using *P. acuta* and *P. australiensis* may indicate that they are not being affected by copper, when in fact chronic effects could be leading to changes at the population level in both species.

Chapter 4. The effects of copper on populations and communities of invertebrates in a manipulative experiment

The aim of the work presented in this chapter was to characterise the population and community level effects of copper within the mesocosms by traditional techniques of optical analysis. The development of the invertebrate communities within the benthos and water column of each mesocosm was monitored. The response of P. acuta at the organism level could be linked to the different populations of snails that were observed, which drove the invertebrate community response and effects at the ecosystem function level.

Authors

	Problem formulation	Experimental design/ methodology	Statistical analysis	Results interpretation	Paper preparation
Stephanie Gardham[1,2]	85	85	85	90	90
Grant C. Hose[1]	7.5	7.5	10	5	7.5
Anthony A. Chariton[2]	7.5	7.5	5	5	2.5

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Abstract

Historical contamination has left a legacy of high copper concentrations in the sediments of freshwater ecosystems worldwide. Previous mesocosm studies have focused on dissolved copper exposures in the overlying waters which, due to altered exposure pathways, may not accurately predict the effects of copper exposure on invertebrate communities at historically contaminated sites. This study assessed the effects of copper on the establishment of invertebrate communities within a large outdoor lentic pond mesocosm facility containing environmentally relevant copper spiked sediments. Copper caused a pronounced effect on the benthic community richness, abundance and structure in mesocosms contaminated with high concentrations of copper, but did not affect the invertebrate community present in the mesocosms contaminated with low concentrations of copper. Further, there were no effects of copper on the invertebrate communities within the water column, even in the highest copper treatment. This study demonstrates the importance of using environmentally realistic exposure scenarios. It also emphasises that site specific assessments on the health of aquatic ecosystems are paramount to understand the effects of contaminants at those sites because of the influence of interspecific interactions and interspecies variation in driving the biotic response.

Introduction

Within the last 100 years, a sharp increase in the discharges of metals, including copper, into aquatic ecosystems has occurred (Eisler, 1998). However, with better management of contamination sources, concentrations of dissolved metals, like copper, are now generally low in surface waters worldwide (de Deckere et al., 2011). In contrast, sediments have been, and remain, a sink for metals and, as the metals cannot be degraded, the legacy of historical contamination remains in the sediments of aquatic ecosystems (Feiler et al., 2013; Cardwell et al., 2002; Maier et al., 2000). Concentrations of copper in sediments are now often orders of magnitude greater than those in the overlying waters (Harrahy & Clements, 1997). A review of copper concentrations measured in sediments at field sites worldwide found that they varied from 3.4 mg/kg (Frakes et al., 2008) to 744 mg/kg (Mal et al., 2002) (N.B. this range does not include mine-impacted sites, where exposure pathways are often very different to other historically contaminated sites (Ríos et al., 2008)) (Chapter 2).

In the field, complex and unknown stressors may exert synergistic or antagonistic effects on biota making it difficult to elucidate the impact of a specific toxicant (Hutchins et al., 2008). Mesocosms and microcosms avoid many such issues by permitting some control over the experimental conditions and allowing direct tests of causality under realistic environmental conditions. Consequently, they provide a useful tool to assess direct and indirect effects of a pollutant on diverse biological communities (Brinke et al., 2011; Relyea & Hoverman, 2006). From the community-level perspective, the general trend from past studies has been that an increase in copper concentrations leads to shifts in the composition of invertebrate communities and a decline in biomass and total abundance (e.g. Girling et al., 2000; Shaw & Manning, 1996; Havens, 1994a; Winner et al., 1990; Hedtke, 1984).

The majority of benthic organisms inhabit surface (rather than deep) sediments, and uptake of metals by those organisms is strongly influenced by the speciation of the metal in the sediment (Campana et al., 2012). Organisms can take up metals, like copper, through exposure to the dissolved phase (e.g. facilitated

diffusion through permeable surfaces) or the particulate phase (e.g. active uptake by feeding) (Fukunaga & Anderson, 2011; Simpson & Batley, 2007). The dominant exposure route for an individual organism depends on its feeding behaviour; for example, benthic filter-feeders are most likely to accumulate metals from the pore waters while deposit feeders are more likely to be exposed through ingestion of sediment particles (Fukunaga & Anderson, 2011). Ultimately, for benthic invertebrates, sediment and pore water exposures probably represent the most important exposure pathways, while exposure to dissolved metals in surface waters is negligible (MacDonald et al., 2011).

In manipulative studies, it is important to establish environmentally relevant partitioning of copper between dissolved and particulate phases in order to create exposure pathways for organisms that are similar to those present in field-contaminated sites. However, the handful of lentic mesocosm studies that have considered the effects of copper on invertebrate communities have not demonstrated copper partitioning representative of historically contaminated sites (Shaw & Manning, 1996; Havens, 1994ab; Winner et al., 1990; Moore & Winner, 1989; Hedtke, 1984). These studies exposed invertebrate communities to copper dissolved in the overlying waters at concentrations between 2 – 420 µg/L. In the context of historically contaminated sites, overlying water copper concentrations at the mid-upper end of this range are unrealistically high. Concentrations at the lower end of the range represent those likely to occur in overlying waters present at historically contaminated sites, so may be useful for determining effects on invertebrates present in the water column. However, sediment and pore water concentrations are likely to have been unrealistically low (although they were generally not measured). For example, Shaw & Manning (1996) exposed their mesocosm systems to overlying water copper concentrations between 20 – 260 µg/L and recorded sediment concentrations of between 4.7 – 9.1 mg/kg at the end of their study. Hedtke (1984) recorded a maximum sediment copper concentration of 146 mg/kg at the end of their study, but this coincided with an unrealistically high (420 µg/L) concentration of copper in the overlying water.

Here, the effects of copper on the establishment of invertebrate communities within a series of copper contaminated lentic mesocosms is described. The mesocosms were manipulated to produce realistic field copper partitioning, with the majority of copper present in the particulate phase of the sediment and low concentrations in the dissolved phase (Chapter 2). The hypothesis was that, with increasing copper concentrations, the structure of invertebrate communities in the water column and benthos would be altered and a decline in abundance, richness and diversity of invertebrates would be observed relative to the controls.

Methods

Study design

Twenty pond mesocosms were established on the Macquarie University campus (33.76946 S, 151.11496 E), in northern Sydney, NSW, Australia. Mesocosms are described in detail in Chapter 2, and briefly below. Each mesocosm had a volume of 1500 L, was sunk into the ground, shaded by 70% shade-cloth, aerated and inputs from rainwater were sufficient to maintain water depth. Sediments were spiked *in situ* with copper and allowed two months to equilibrate and create environmentally relevant sediment contaminated with copper (Chapter 2). The mesocosms were opened to allow biotic colonisation on the 1st November 2010 (t = 0 d). The mesocosms were allowed to colonise naturally and some biota colonised the mesocosms following the introduction of a specimen of *Vallisneria spiralis* to each mesocosm after the first six months.

The experimental design consisted of a control (C) and four sediment copper concentrations (very low, VL; low, L; high, H; and very high, VH), each with four replicates. Mean concentrations of copper in the sediments, pore waters and overlying waters following t = 74 d (once the sediments appeared to have reached pseudo-equilibration; see Chapter 2) are included in Table 4.1. The invertebrate communities and water quality parameters (see below) were monitored every month in the first six months (first spring/summer season) and then at twelve, thirteen and sixteen months (second spring/summer season). The final sample was taken on 12th March 2012 (t = 497 d).

Water quality

Copper concentrations in the sediments, pore waters and overlying waters were measured as per Chapter 2. Conductivity, pH, turbidity, dissolved oxygen and oxidation-reduction potential (redox potential) (versus the standard hydrogen electrode) of the overlying water were recorded using a multimeter (HI 9828; Hanna, Woonsocket, USA).

Table 4.1: Mean ± S.E.M. (n = 7) copper concentrations in the sediment, pore waters and overlying waters by treatment.

Treatment	Sediment copper (mg/kg)	Pore water copper (µg/L)	Overlying water copper (µg/L)
Control	4.6 ± 0.7	1.5 ± 0.2	1.3 ± 0.3
Very low	71 ± 4.6	2.8 ± 0.2	2.2 ± 0.2
Low	99 ± 7.1	4.6 ± 1.2	3.8 ± 0.5
High	410 ± 41	18 ± 3.1	7.8 ± 0.6
Very high	711 ± 46	30 ± 2.5	11 ± 0.9

Invertebrate community sampling

For the benthos, three cores (6 cm diameter) were collected from the surficial layer of the sediment (top 1 – 2 cm) and were mixed together. Then, 100 mL of that pooled sediment was retained and preserved in 100% ethanol for later processing. Samples were processed by a flotation procedure adapted from that described by Anderson (1959). In this procedure, each sample was drained of ethanol through a 106 µm mesh sieve then placed in a container and flooded with saturated sugar (glucose) solution. The sample was stirred to assist organisms to float to the surface and then the sugar solution was decanted through the 106 µm mesh sieve to collect the floating organisms. Each sample was processed twice and soaked in water for five minutes between processing to remove any residue of the sugar solution. The animals collected from both extractions were pooled and identified.

Water column invertebrates were collected using a plastic tube (5 cm diameter) that was inserted vertically from the water surface to the sediment at a random location within the mesocosm. A stopper was placed at the bottom of the tube, then the tube was removed from the mesocosm and the water collected was retained. This process was repeated four times and the samples pooled. The pooled sample was mixed then a sub-sample (~1 L) was filtered through a 106 µm sieve and animals retained were preserved in 70% ethanol.

At sixteen months, standardised sweep net samples were also collected using a kick net (0.12 m^2 opening) with 500 µm mesh. The nets were swept across the surface of the sediment and macrophytes in one half of each mesocosm. This process was repeated three times. Large macroinvertebrates (i.e., those obvious to the eye without magnification) were live picked by two people until all large animals were removed.

Data analysis

Water quality data (including copper concentrations) were analysed to identify key differences in water quality among treatments over time. A repeated measures (RM) ANOVA was performed on the data of each parameter (between-subjects factor: treatment, within-subjects factor: time) followed by least significant difference (LSD) pair-wise multiple comparison tests to assess the differences between individual treatments and time points using PASW Statistics 18.0.3. Where a significant interaction between treatment and time was found, a one-way ANOVA followed by LSD pair-wise multiple comparison was performed on the data from within each time point to identify where a significant difference among treatments occurred, and a separate RM ANOVA was performed for the data of each treatment to identify changes in the parameter within each treatment over time. Data were transformed to meet assumptions of homogeneity of variance and normality as described in Table A4.1. Where transformed data failed Levene's test, the significance level (α) was 0.01, for all other comparisons α was 0.05 (Underwood, 1997). If the assumption of sphericity (assessed by Mauchly's test) was not met during the RM ANOVA analyses, the Greenhouse-Geisser correction was used.

The effects of treatment and time on the invertebrate assemblages identified in the benthic, water column and sweep net sample data were analysed separately. For each data set, firstly, community indices (total abundance, taxonomic richness and diversity [H']) were each analysed using a RM ANOVA with post hoc tests (as described for the water quality data). Distanced-based linear modeling (DistLM) (McArdle & Anderson, 2001) was then performed to identify the significance of

measured copper concentrations in driving community compositional change and produce distance-based RDA (dbRDA) plots. The invertebrate data sets were interrogated against copper concentrations in the sediment (all three invertebrate data sets), pore waters (benthic and sweep net data sets) and overlying waters (water column and sweep net data sets). The analysis was based on Bray-Curtis similarities of appropriately transformed biological data and normalised environmental data (Table A4.1).

The benthic and water column biota data sets were analysed using principal response curves (PRC) (Van den Brink & Ter Braak, 1999) with Canoco version 4.5; recommended software settings were used on appropriately transformed data (Table A4.1). PRC is a multivariate method of analysis, which shows differences in community composition between treatments and the control at each time point. The species weights enable consideration of the effect at the species level, the higher the weight (positive or negative), the more similar the response to that identified in the figure; taxa with negative weights show the opposite pattern to the PRC. The significance of the PRC was tested using Monte Carlo permutation tests (using α 0.05). To further interrogate the pattern observed in the PRC, the variation in community composition between treatments at each time point was analysed using Permutational Multivariate Analysis of Variance (PERMANOVA) (Anderson, 2001) followed by post hoc pair-wise comparisons. The analysis was based on Bray-Curtis similarities of transformed data (Table A4.1) and performed in Primer 6+ (PrimerE Ltd, Plymouth, UK).

To understand the responses of individual taxa to copper treatment, a RM ANOVA was performed (as described for the water quality data) on the appropriately transformed (Table A4.1) abundance data of sensitive taxa identified by the PRC within each invertebrate data set. Where present, even if not identified in the PRC analysis, the response of *Physa acuta* (Gastropoda: Physidae) to copper treatment was characterised because field observations indicated that the gastropod was abundant in the C/VL/L treatments and rare in the H/VH treatment. For the sweep net data set, a one-way ANOVA (as there was only one time point)

with LSD post hoc analysis was performed on the transformed (Table A4.1) abundance data of *P. acuta*.

Results

Water quality and copper concentrations

There was an interaction between treatment and time on pH ($F_{16,61}$ = 4.6, p = <0.001), which varied between 6.8 ± 0.1 (C, t = 247 d) and 8.8 ± 0.1 (C, t = 310 d) (Figure 4.1a; Table A4.2). Within each sample point there was generally (60% of the time) no effect of treatment (Figure 4.1a). Where a significant effect of treatment was found, the pH of the C treatment was lower than the other treatments; this generally occurred in the first spring/summer season. The maximum difference in pH between treatments at an individual time point was 1.3 units (t = 161 d). Although there were significant fluctuations in pH (p < 0.001) (Table A4.2), there was no consistent directional trend over time (Figure 4.1a).

The redox potential varied between 250 ± 4.0 mV (VH, t = 407 d) and 480 ± 5.9 mV (VH, t = 365 d) (Figure 4.1b); although there was no consistent directional trend in redox potential over time, it did fluctuate significantly ($F_{2.4,37}$ = 293, p = <0.001) (Table A4.2). The redox potential was not affected by treatment ($F_{4,15}$ = 1.1, p = 0.39) and there was no interaction between treatment and time ($F_{9.8,37}$ = 2.5, p = 0.021; α 0.01) (Table A4.2).

There was no directional trend in conductivity over time but it fluctuated significantly ($F_{1.8,27}$ = 110, p < 0.001); it varied between 100 ± 24 µS/cm (VL, t = 15 d) and 250 ± 14 µS/cm (C, t = 100 d), apart from at t = 135 d where a higher conductivity was recorded across all treatments (390 ± 11 µS/cm, n = 20) (Figure 4.1c). There was no significant effect of treatment on conductivity ($F_{4,15}$ = 0.60, p = 0.67), nor was there an interaction between treatment and time ($F_{7.3,27}$ = 1, p = 0.44) (Table A4.2).

At the start of the colonisation period, dissolved oxygen concentrations were low (e.g. 28 ± 0.4%, t = 4 d (n = 20)). However, after t = 31 d, the overlying waters in the mesocosms were generally well oxygenated (Figure 4.1d). This change in dissolved oxygen over time was significant ($F_{3.7,55}$ = 190, p < 0.001). Treatment did

not affect dissolved oxygen concentrations ($F_{4,15} = 0.59$, $p = 0.68$) and there was no interaction between treatment and time ($F_{15,55} = 0.75$, $p = 0.72$) (Table A4.2).

Turbidity changed over time ($F_{4.2,64} = 60$, $p < 0.001$); it was highest when the mesocosms were first opened up to colonisation, but gradually decreased and was between 0.6 ± 0.1 and 5.5 ± 2.3 NTU from t = 135 d onwards (Figure 4.1e; Table A4.2). There was no effect of treatment on turbidity ($F_{4,15} = 0.13$, $p = 0.97$), nor was there a significant interaction between treatment and time ($F_{17,64} = 1.2$, $p = 0.28$) (Table A4.2).

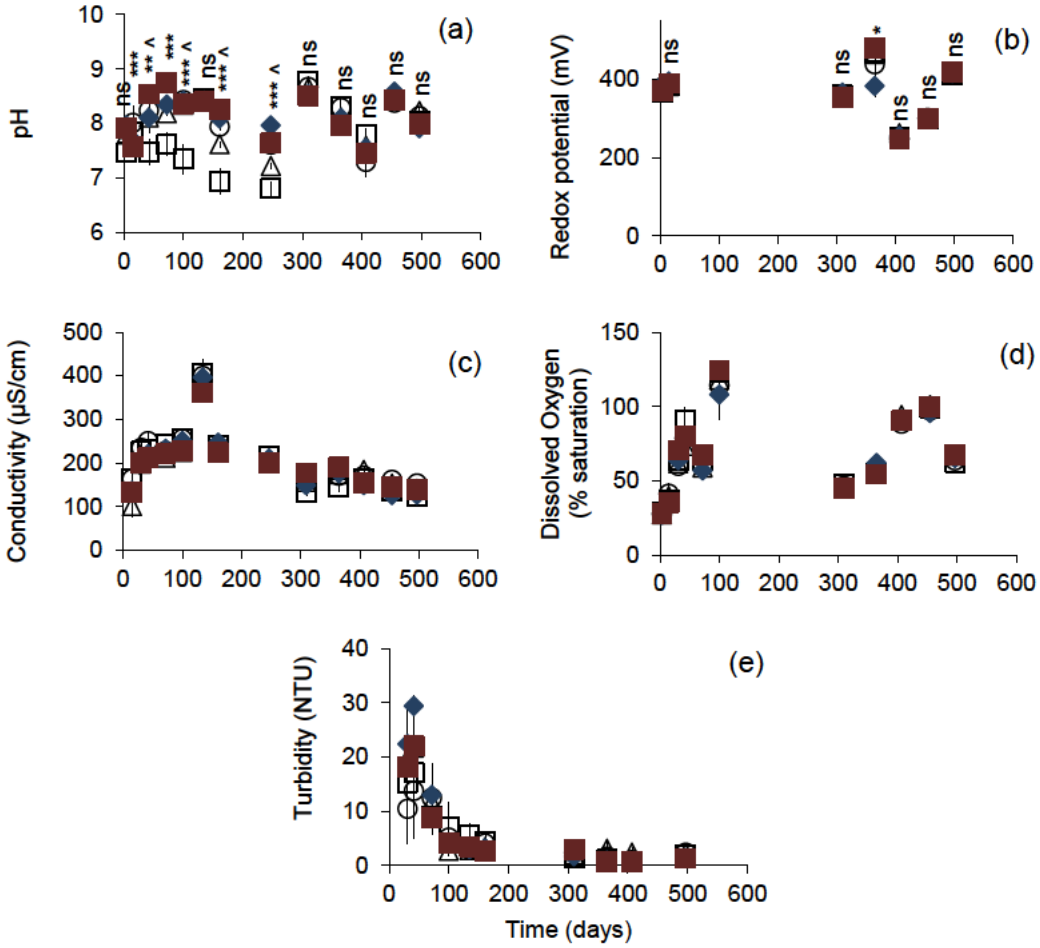


Figure 4.1: Change in (a) pH, (b) redox potential, (c) conductivity (d) dissolved oxygen and (e) turbidity over time. Key: C (□), VL (△), L (○), H (◆) and VH (■). Values are mean ± S.E.M., (n = 4). The main results of the one-way ANOVAs performed at each time point on the pH and conductivity data (due to a significant interaction between time and treatment for those data) are displayed above each respective time point: ns = not significant, * = p<0.05, ** = p<0.01, *** = p<0.001, ^ = homogeneity of variance $p < 0.05$ (therefore α reduced to 0.01).

Concentrations of copper in the sediments, pore waters and overlying waters generally increased from the C < VL < L < H < VH treatments (Figure 4.2). There was a significant interaction between treatment and time on copper concentrations in the sediments ($F_{17,64} = 2.6$, $p = 0.003$), pore waters ($F_{14,53} = 3.2$, $p < 0.001$) and overlying waters ($F_{13,48} = 4.2$, $p < 0.001$) (Table A4.2).

In general, there was a significant difference between the copper concentrations in the sediments among treatments; however, the VL and L treatments were only significantly different ($p < 0.05$) on four occasions (Figure 4.2a). In addition, at t = 161 d, there was no significant difference between sediment copper concentrations in the H and VH treatments ($p = 0.12$). Within the C, L, H and VH treatments, copper concentrations changed significantly over time ($p < 0.05$), however, in general, post hoc analysis showed no difference between individual time points.

Pore water copper concentrations were similar among C, VL and L treatments and they were usually significantly higher in the H and VH treatments (Figure 4.2b) (Table A4.2). Within the C, L, H and VH treatments, pore water copper concentrations changed significantly over time ($p < 0.05$); initially they decreased (up to t = 42 d), but then they plateaued (Figure 4.2b).

In the overlying waters, the concentrations of copper in the C, VL and L treatments were quite similar, although they were often significantly lower in the C than the VL treatment (Figure 4.2c, Table A4.2). The H and VH treatments were also generally significantly different from each other (70% of the time $p < 0.01$). Within each treatment, overlying water copper concentrations changed significantly over time but there was no underlying directional trend (Figure 4.2c).

In summary, the primary abiotic difference between the treatments was copper. Statistically, treatments could be grouped into “C”, “VL/L”, “H” and “VH” by sediment copper concentrations, “C/VL/L” and “H/VH” by pore water copper concentrations and “C/VL/L”, “H” and “VH” by overlying water copper

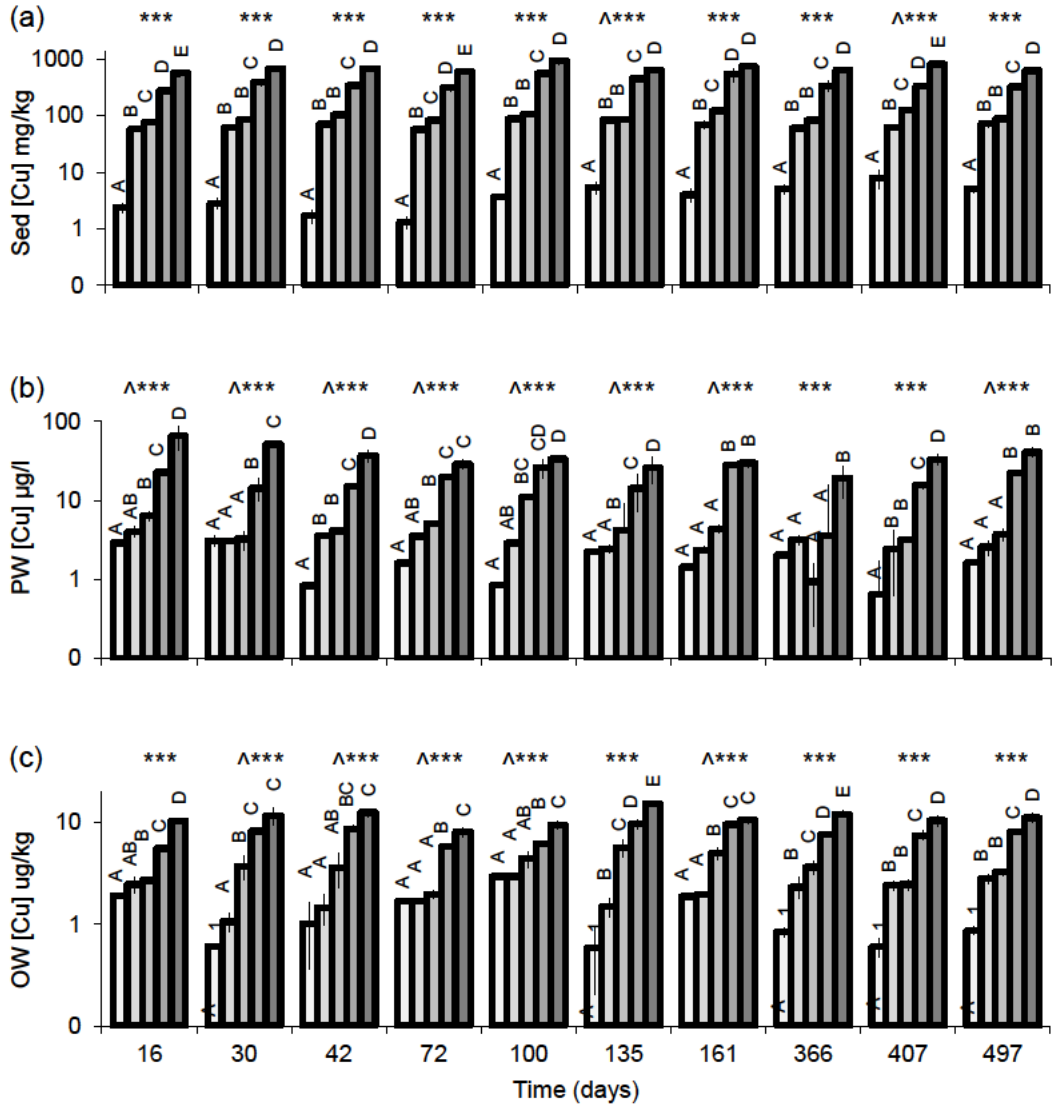


Figure 4.2: Change in (a) Sediment, (b) pore water, and (c) overlying water copper concentrations over time. Key: C (□), VL (□), L (□), G (□) and VH (■). The main results of the one-way ANOVAs performed at each time point on the data (due to a significant interaction between time and treatment for those data) are displayed above each respective time point: *** = $p < 0.001$, ^ = homogeneity of variance $p < 0.05$. Letters above each bar indicate the outcomes of LSD post hoc analyses, where treatments that do not share a letter within a time point are significantly different.

concentrations. Conductivity, dissolved oxygen and turbidity were stable among treatments. While the overlying water pH and conductivity was significantly affected by treatment at some time points, this was rare and did not reflect pronounced differences in the environment; for example, the overlying water was still neutral/slightly alkaline where the pH was significantly different among treatments.

The aquatic community

In total, 35 taxa were recorded across all datasets (Table A4.3). Meiofauna comprised 88% of the total abundance, and were dominated by three Ostracoda taxa (collectively 36%) and Nematoda (26%). The most abundant macrofauna were Diptera (10%), which were dominated by the Chironomidae subfamilies Chironominae and Tanypodinae. Other relatively abundant macroinvertebrates were Libellulidae (Odonata), *P. acuta* (Gastropoda: Physidae) and *Mesovelia* sp. (Hemiptera: Veliidae).

Benthic community response

The total abundance of benthic invertebrates increased up to t = 161 d, as the benthic communities within the mesocosms became established ($F_{7,105} = 58$, $p < 0.001$). After t = 161 d the total abundance was quite stable among treatments but on occasion a spike in total abundance was recorded in the VL, L or H treatments (Figure 4.3a). There were differences in the total abundance of benthic invertebrates among treatments ($F_{4,15} = 6.9$, $p = 0.002$); in the VH treatment the benthic invertebrate abundance was significantly lower compared to all other treatments ($p < 0.01$; Table A4.4). There was no significant interaction between time and treatment on total abundance of benthic invertebrates ($F_{28,105} = 1.6$, $p = 0.058$; α 0.01).

There was a significant interaction between treatment and time on taxonomic richness of the benthic invertebrates ($F_{28,105} = 1.6$, $p = 0.047$). Within each treatment, taxonomic richness increased as the benthic communities in the mesocosms became established (Figure 4.3b; $p < 0.001$, Table A4.4). During the establishment of the benthic communities, the taxonomic richness in the H/VH treatments was consistently lower than the taxonomic richness in the C/VL/L treatments and, corresponding to this, a significant effect of treatment was found within each time point from t = 74 d to t = 368 d ($p < 0.05$) (Figure 4.3b; Table A4.4). Once the benthic communities had become established, taxonomic richness was reasonably constant over time and there appeared to be little

difference in taxonomic richness among treatments (Figure 4.3b); at t = 409 d and t = 500 d, no significant effect of treatment on taxonomic richness was found ($p > 0.05$; Table A4.4).

There was a significant interaction between treatment and time on benthic diversity ($F_{28,105} = 1.9$, $p = 0.012$). Within each treatment, benthic diversity changed over time ($p < 0.05$); it increased up to t = 137 d (during the community's establishment) and then leveled out (Figure 4.3c). Within each time point there was generally no effect of treatment on benthic diversity; diversity was only significantly different among treatments at t = 100 d ($F_{4,15} = 3.3$, $p = 0.04$).

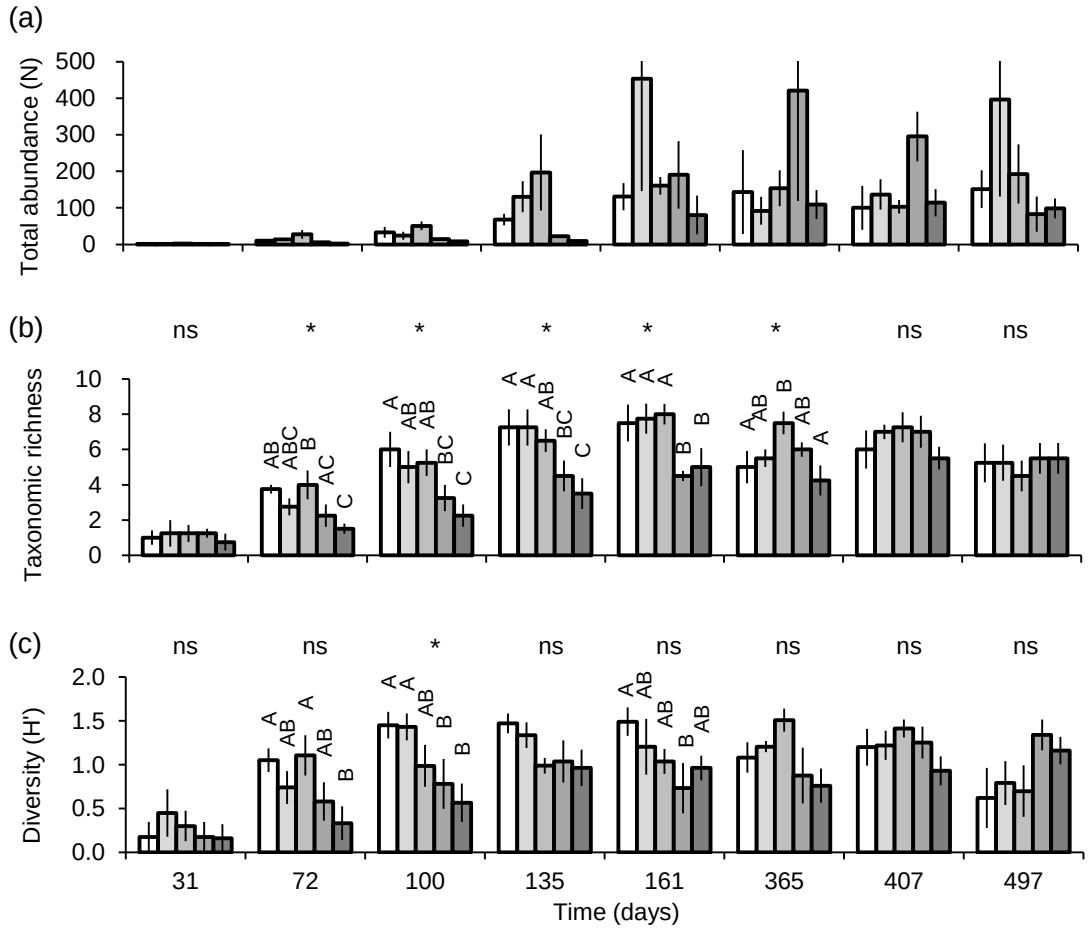
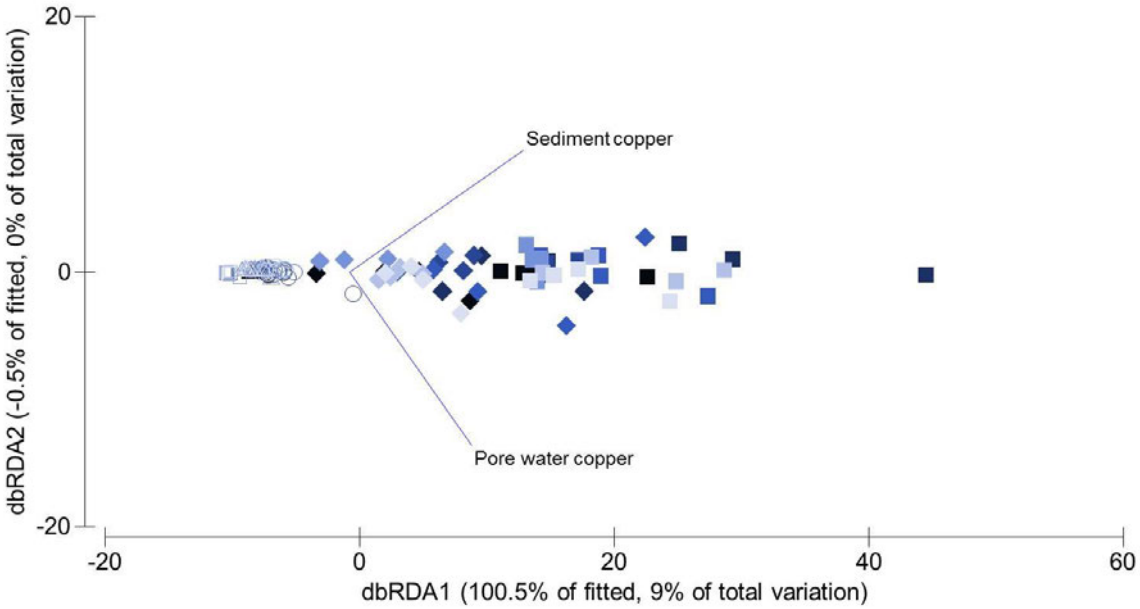


Figure 4.3: Benthic community (a) total abundance, (b) taxonomic richness and (c) diversity over time. Key: C (□), VL (□), L (□), H (□) and VH (□). The main results of the one-way ANOVAs performed at each time point on the taxa richness and diversity data (due to a significant interaction between time and treatment for those data) are displayed above each respective time point: ns = not significant, * = $p < 0.05$. Letters above each bar indicate the outcomes of LSD post hoc analyses, where treatments that do not share a letter within a time point are significantly different.

When the mesocosms were first opened up for biotic colonisation, the benthic communities were similar across all treatments (Pseudo-$F_{4,15}$ = 0.33, *p*(perm) = 0.94). Changes in benthic community composition were significantly correlated with both sediment ($p < 0.001$) and pore water ($p < 0.001$) copper concentrations (Figure 4.4a; Table A4.5). Over the first spring/summer season (up to t = 161 d), as the benthic communities became established, the H/VH treatments became more different from the C/VL/L treatments (evidenced by the H/VH treatment lines moving further from C/VL/L treatments in Figure 4.4b). At the start of the second spring/summer period (t = 368 d) the apparent difference between H/VH and C/VL/L treatments was still present, but gradually all the treatments appeared to become more similar, and at t = 500 d there appeared to be little difference among treatments in Figure 4.4b. Treatment accounted for 25% of the total variance in the benthic community across all time points. The 1st axis of the PRC for the benthic community was significant ($p = 0.002$) and accounted for 44% of the variance explained by the treatment regime (Figure 4.4b). The second axis accounted for 18% of the variance explained by the treatment regime and was not significant ($p = 0.13$); thus only the 1st axis is presented and discussed.

There was a significant interaction between time and treatment on the benthic community assemblages (Pseudo-$F_{28,120}$ = 1.4, $p = 0.002$). Within each time point, there was no significant difference between the benthic communities in the C/VL/L treatments. The apparent difference between C/VL/L treatments and the VH treatment was generally significant ($p < 0.05$), or near significant ($p < 0.091$), from t = 74 d onwards (including the second spring/summer season through to t = 500 d) (Table A4.6). The H treatment was only significantly different from C/VL/L treatments at some of the sampling times (Table A4.6). The taxa most sensitive to copper treatment (i.e. with species weights; < -2) were Chironominae, Cladocera and Ostracod Sp. 2 (Figure 4.4b).

(a)



(b)

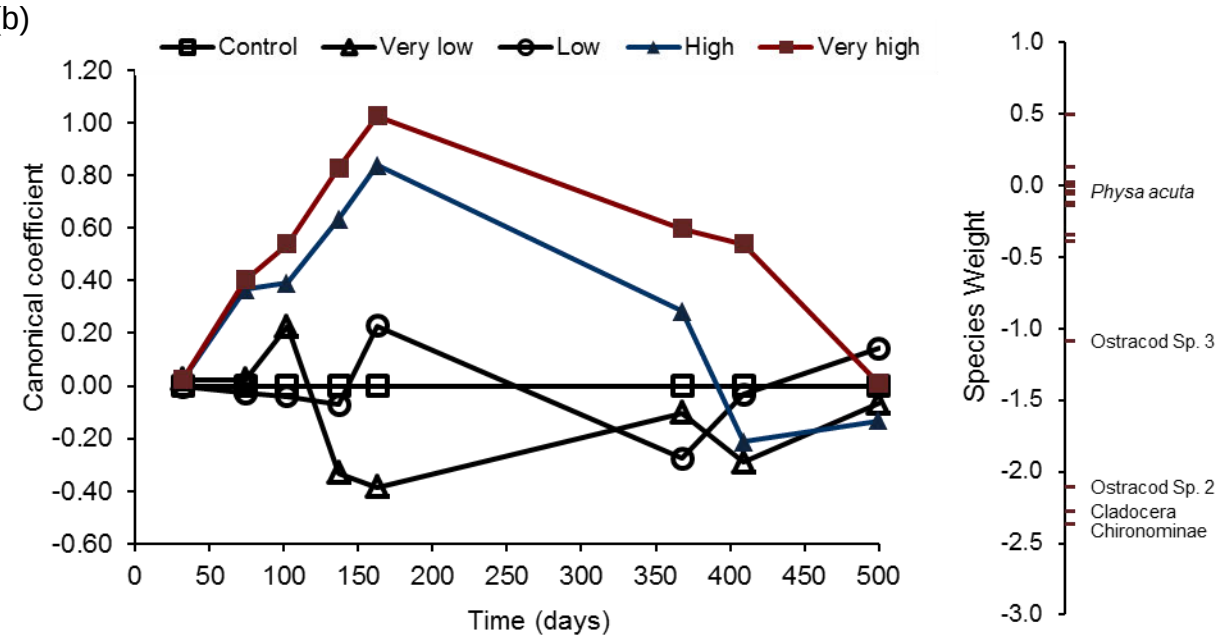


Figure 4.4: (a) dbRDA plot of the correlation between the benthic community and sediment and pore water copper concentrations and (b) Principal response curves with species weights showing the effect of copper on establishment of the benthic invertebrate community. Key for (a): C-□; VL-△; L-○; H-◆; and VH-■ and gradient of light⇢ dark blue is t = 74 ⇢ t = 497 d. For (b): red tick marks on the species weights axis indicate other taxa with calculated species weights (labels have been removed for clarity).

Chironominae increased in abundance over the first spring/summer season (up to t = 161 d) as the benthic communities in the mesocosms established – during this time Chironominae abundance was consistently lower in the H/VH treatments compared to the C/VL/L treatments (Figure 4.5a). By the second spring/summer season (t = 365 d), overall Chironominae abundance was lower than at the end of the first spring/summer season (t = 161 d), and was also more similar among treatments. There was a significant interaction between time and treatment on the abundance of Chironominae ($F_{28,105} = 2.9$, $p < 0.001$). Within the C, VL, L and H treatments the effect of time was significant ($p < 0.01$; Table A4.4), but in the VH treatment, where the abundance of Chironominae was consistently low, there was no effect of time ($F_{7,21} = 1.4$, $p = 0.28$). Within most sampling points (75%), treatment affected Chironominae abundance (Figure 4.5a; Table A4.4); generally the abundance of Chironominae in the VH treatment was significantly lower than the C/VL/L treatments. The abundance of Chironominae in the H treatment was also lower than the C/VL/L treatments, apart from at the last two sampling time points (t = 407 d, t = 497 d), where the abundance of Chironominae in the H treatment exceeded all other treatments.

The abundance of Ostracoda Sp. 2 increased in the C/VL/L treatments during the first spring/summer season (up to t = 161 d) but remained low in the H/VH treatments (Figure 4.5b). By the second spring/summer season (t = 365 d) the abundance of Ostracod Sp. 2 was lower in the C/VL/L treatments (compared to t = 161 d) and more similar to the H/VH treatments. There was a significant interaction between time and treatment on the abundance of Ostracoda Sp. 2 ($F_{11,40} = 2.6$, $p = 0.014$). The change in abundance of Ostracoda Sp. 2 over time was only significant in the C treatment ($F_{7,21} = 4.8$, $p = 0.002$) and the effect of treatment within each sampling time was only significant 40% of the time (Table A4.4).

During the first spring/summer season (up to t = 161 d) the abundance of Cladocera increased in the C/VL/L treatments but remained low in the H/VH treatments (Figure 4.5c). By the second spring/summer season (t = 365 d) the abundance of Cladocera in the H treatment had increased to be similar to the

C/VL/L treatments, then at t = 497 d the abundance of Cladocera was low across all treatments. There was a significant interaction between time and treatment on the abundance of Cladocera ($F_{14,53} = 2.1$, $p = 0.029$). The fluctuations in Cladocera abundance over time were significant (or near significant) in the C, VL, L and H treatments ($p < 0.055$; Table A4.4). Within each sampling point there was no significant effect of treatment on Cladocera abundance, despite the biological effect that was especially evident in the first spring/summer season (t = 74 d – t = 161 d).

P. acuta colonised the mesocosms between the first and second spring/summer seasons (between t = 161 d and t = 365 d) (Figure 4.5d). Corresponding to this, the effect of time was significant ($F_{7,105} = 5.6$, $p < 0.001$). In the benthic samples, the abundance of *P. acuta* was not affected by treatment ($F_{4,15} = 1.3$, $p = 0.3$). There was no interaction between treatment and time ($F_{28,105} = 0.72$, $p = 0.84$). Although there was no significant effect of treatment on abundance of *P. acuta* in the benthic samples, there is a clear biological effect, in which abundance was greater in the C/VL/L treatments compared to the H/VH treatments (Figure 4.5d); this was also evident from field observations, where the gastropod proliferated in the C/VL/L treatments and was rare or absent in the H/VH treatments (personal observation), and is supported by the sweep net data (see below).

In summary, sediment and pore water copper concentrations were both significant drivers of the benthic community composition. There was a clear difference in the composition of the benthic communities between the H/VH treatments and the C/VL/L treatments. During the establishment of the benthic communities in the first spring/summer season, the different abundances of Chironominae, Ostracod Sp. 2 and Cladocera among treatments were the main drivers of the community response. Although, according to the PRC, the benthic communities appeared to become more similar among treatments during the second/spring summer season, differences in community composition remained.

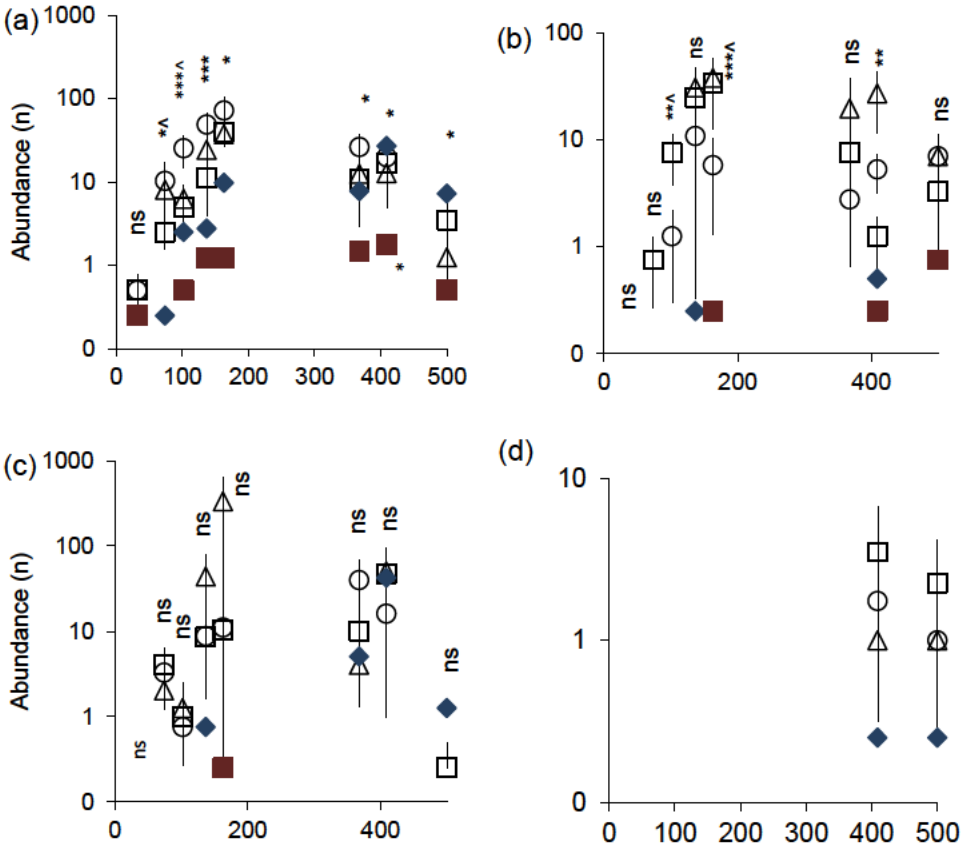


Figure 4.5: Population responses of individual benthic taxa: (a) Chironominae; (b) Ostracoda Sp. 2; (c) Cladocera; (d) *P. acuta* (which appeared in the mesocosms during the second spring/summer season) over time. Key: C (□), VL (△), L (○), H (◆) and VH (■) treatments. Values are mean ± S.E.M., (n = 4). The main results of the one-way ANOVAs performed at each time point on the abundances of Chironominae, Ostracoda Sp. 2 and Cladocera (due to a significant interaction between time and treatment for those data) are displayed above each respective time point: ns = not significant, * = p<0.05, ** = p<0.01, *** = p<0.001, ^ = homogeneity of variance p < 0.05.

Water column community response

The total abundance of invertebrates in the water column increased until t = 136 d (Figure 4.6a). During this time, the abundance of invertebrates in the VH treatment always appeared lower than the C/VL/L/H treatments. After t = 136 d, the total abundance of invertebrates in the water column decreased, and it remained low during the second spring/summer season (t = 365 d – t = 497 d), with no obvious biological difference among treatments. The effect of time on the total abundance of invertebrates in the water column was significant ($F_{8,120} = 59$, $p < 0.001$) however there was no significant effect of treatment ($F_{4,15} = 1$, $p = 0.44$), nor was there a significant interaction between time and treatment ($F_{32,120} = 1.2$, $p = 0.26$).

The taxonomic richness of the invertebrates in the water column increased until t = 72 d and then appeared to plateau (Figure 4.6b); this change over time was significant ($F_{8,120} = 9.5$, $p < 0.001$). There was no effect of treatment on taxonomic richness ($F_{4,15} = 2.4$, $p = 0.098$), nor was there a significant interaction between treatment and time ($F_{32,120} = 1.2$, $p = 0.22$).

The diversity of the invertebrates in the water column was low throughout the experimental period, but generally it was higher in the second spring/summer season (t = 365 d – t = 497 d) compared to the first (up to t = 161 d) and there was a significant effect of time ($F_{8,120} = 3.6$, $p < 0.001$) (Figure 4.6c). There was no significant difference in diversity of the invertebrates in the water column among

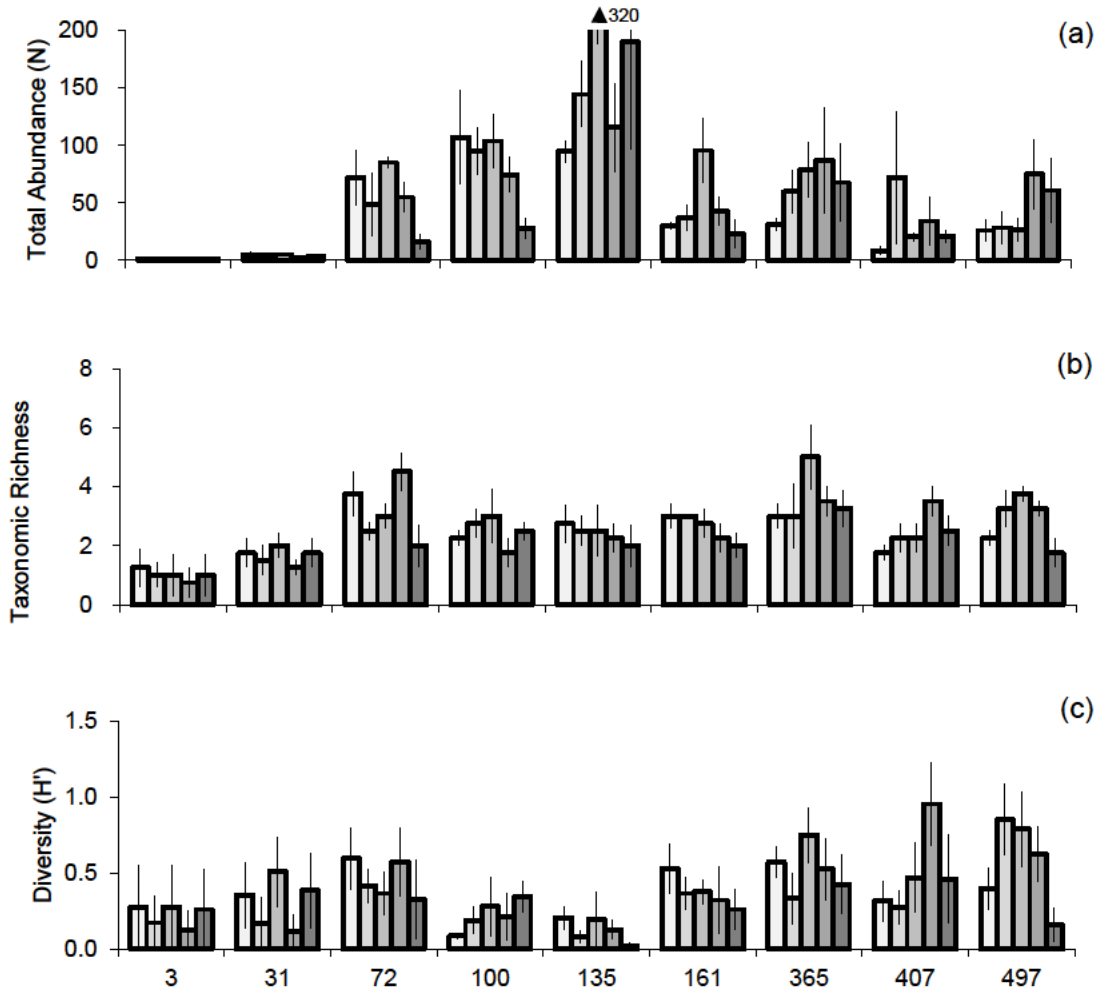


Figure 4.6: Water column community (a) total abundance, (b) taxonomic richness and (c) diversity in over time. Key: C (□), VL (▢), L (▤), H (▥) and VH (■).

treatments ($F_{4,15} = 1.3$, $p = 0.32$), nor was there an interaction between treatment and time ($F_{32,120} = 0.82$, $p = 0.74$).

There was no significant difference in the water-column invertebrate assemblages between treatments at the start of colonisation (Pseudo-$F_{4,15} = 0.86$, P(perm) = 0.60). Although, the 1st axis of the dbRDA plot shows an apparent separation of samples by treatment (Figure 4.7a), there was no significant correlation between the invertebrate assemblages in the water column and concentrations of copper in the overlying water ($p = 0.40$) or sediment ($p = 0.13$) (Table A4.7). There was no clear pattern as a result of treatment on the development of the invertebrate community in the water column over time (Figure 4.7b); the 1st axis of the PRC for the water column community accounted for 36% of the variance explained by the treatment regime, but was not significant ($p = 0.66$). However, there were significant differences in the invertebrate assemblages among treatments as they developed (Pseudo-$F_{4,135} = 1.8$, $p = 0.017$); there was no significant interaction between treatment and time (Pseudo-$F_{32,135} = 1$, $p = 0.41$). Pairwise comparisons at each time point found few cases where the community was significantly different among treatments; in all cases where a significant difference was found, it was between VL/L and H/VH treatments ($p < 0.05$) (Table A4.8).

In summary, there was no clear population or community level response in the water column to increasing copper concentrations, although some tests revealed differences in community composition between VL/L and H/VH treatments.

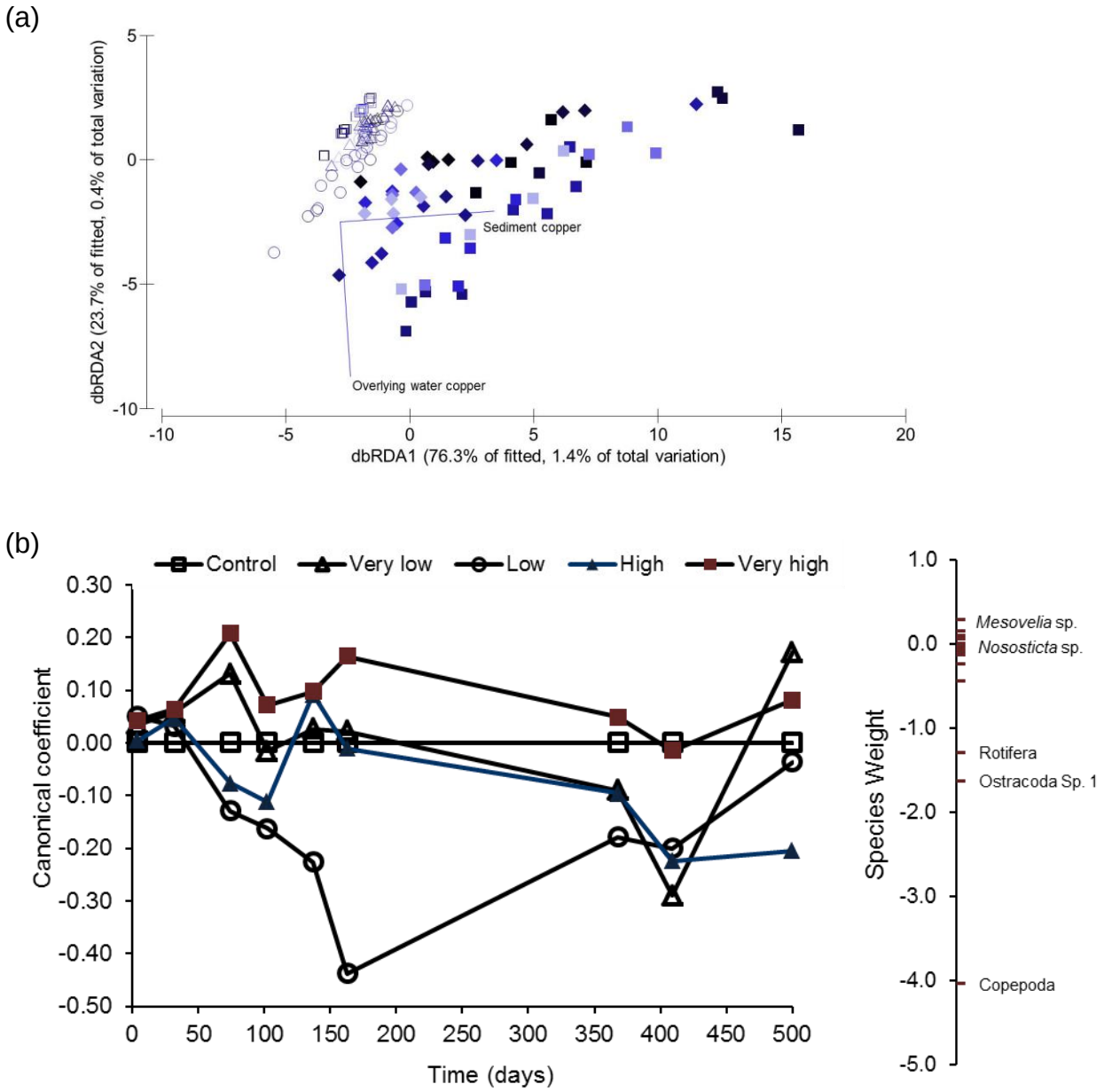


Figure 4.7: (a) dbRDA plot of the correlation between the water column invertebrate community and sediment and overlying water copper concentrations and (b) Principal response curves with species weights showing the effect of copper on establishment of the invertebrate community in the water column. Key for (a): C-□; VL-△; L-○; H-◆; and VH-■ and gradient of light⇢ dark blue is t = 74 ⇢ t = 497 d); in (b) red tick marks points indicate other taxa with calculated species weights (labels have been removed for clarity).

Sweep Net Samples

There was no significant effect of treatment on total abundance ($F_{4,15} = 0.33$, $p = 0.85$), taxonomic richness ($F_{4,15} = 0.78$, $p = 0.56$) or diversity ($F_{4,15} = 2.5$, $p = 0.086$) of the large invertebrate assemblages collected with sweep nets. Similarly, there was no significant relationship between the large invertebrate assemblages and the concentrations of copper in the sediments ($p = 0.08$), pore waters (p = 0.13) or overlying waters ($p = 0.15$) (Table A4.9). Although the dbRDA appears to show a separation of samples by treatment, it only accounts for 10.3% of the variation in the data set, thus the pattern shown does not represent a strong relationship between treatment and community composition. (Figure 4.8).

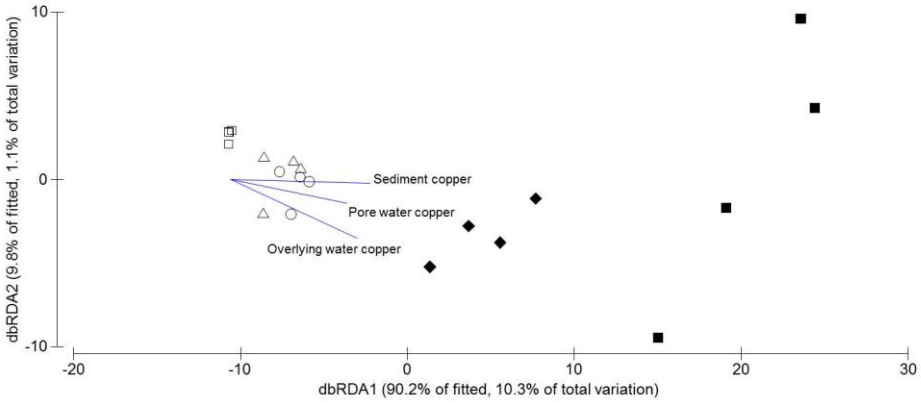


Figure 4.8: dbRDA plot identifying the influence of sediment, pore water and overlying water copper concentrations on the invertebrate communities collected in the sweep net samples. Key: C (□); VL (△); L (○); H (◆); and VH (■).

The abundance of *P. acuta* collected in the sweep net samples was analysed because field observations indicated that the gastropod was abundant in the C/VL/L treatments and rare in the H/VH treatments. Although there was no significant difference among treatments ($F_{4,15} = 1.9$, $p = 0.17$), *P. acuta* abundance was clearly higher in the C treatment than all other treatments (Figure 4.9).

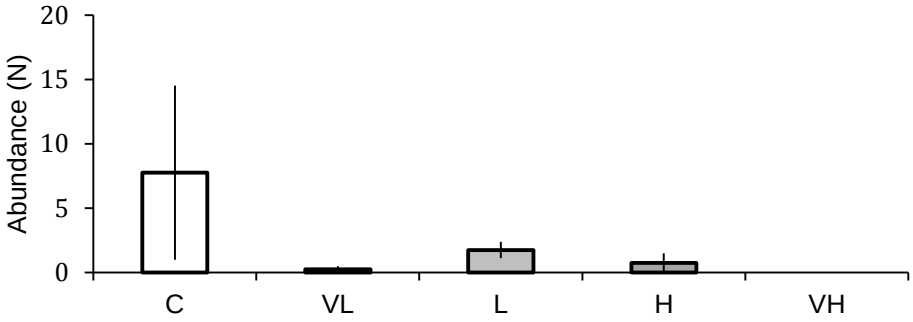


Figure 4.9: Total abundance of *P. acuta* among treatments in the sweep net samples.

Discussion

There was a clear, and significant, difference in the invertebrate communities that established within the highly copper contaminated mesocosms compared to the lower copper contaminated and control mesocosms. The richness, abundance and structure of the benthic invertebrate assemblages, in particular, were strongly affected by copper contamination. Benthic Chironominae, a species of Ostracoda, Cladocera and *P. acuta* were sensitive to increases in copper. Contrasting the benthos, invertebrate assemblages in the water column showed little response to copper contamination. The biotic response observed in this study demonstrates the importance of environmentally realistic exposure scenarios in manipulative studies that aim to characterise 'real world' responses to contaminants. In comparing this study with similar previous research, the importance of interspecies variation in sensitivity, interspecies interactions and the stage of ecological succession in driving the biotic response is demonstrated; showing that these factors must be taken into account to appropriately assess the effect that a contaminant has, or will have, on an aquatic ecosystem.

The importance of an environmentally realistic exposure scenario

In contrast to previous lentic mesocosm studies that considered the effect of dissolved copper in the overlying water on benthic communities (Shaw & Manning, 1996; Winner et al., 1990; Hedtke, 1984), this study used an environmentally realistic exposure scenario, with high concentrations of copper in the sediment and low dissolved concentrations of copper in the pore waters and overlying waters (Chapter 2). As a result, the concentrations of copper in sediments in this study were much higher than those reported by Hedtke (1984) and Shaw & Manning (1996) (711±4.6 mg/kg in the VH treatment compared to 5.3 mg/kg and 37 mg/kg respectively), despite having similar concentrations of copper in the overlying water (11±0.9 µg/L in the VH treatment compared to 9.3 µg/L and 20 µg/L respectively). It follows that the concentration of copper in the pore waters were also higher in this study compared to those previous studies, but this cannot be tested because pore water concentrations were not measured in previous studies.

With higher sediment (and likely pore water) copper concentrations, effects were observed on the benthic community when overlying water concentrations of copper were lower than reported in previous studies. Changes in the benthic community were evident in the H and VH treatments, in which average overlying copper concentrations were 7.8 ± 0.6 and 11 ± 0.9 µg/L respectively. In comparison, Hedtke (1984) and Shaw & Manning (1996) observed effects on the benthic community at overlying water copper concentrations of ≥30 µg/L (not ≤9.3 µg/L) and >20 µg/L, respectively. This demonstrates that, in agreement with laboratory studies (e.g. Campana et al. 2012; Strom et al. 2011), the major route of exposure to biota in the benthos is via the pore waters and sediments. In terms of risk assessment, this is extremely important, as based on previous studies, it may be assumed from overlying water copper concentrations that there would be no effect of copper on the benthic community, when in fact concentrations in the pore waters and sediments are causing an effect.

As the copper partitioning in this study is representative of historically contaminated sites (Chapter 2), it is concluded that chronic copper exposure may affect the invertebrates present in the benthos at such sites. However, because effects on the water column invertebrate community were not evident, the invertebrates in the water column at historically contaminated sites may not be affected. This lack of response in the water column invertebrate community is in line with previous research. Two studies considered the effects of copper on water column invertebrate communities during chronic (5 week and 32 week) exposures with overlying water copper concentrations in the same range as those used here (Winner et al., 1990; Hedtke, 1984); Winner et al. (1990) observed changes in the zooplankton community at concentrations of 20 µg Cu/L in the overlying waters, while Hedtke (1984) observed changes occurring between 9.3 µg Cu/L and 30 µg Cu/L. Therefore, in this study the concentrations of copper in the highest treatment (VH: average of 11±0.9 µg/L, maximum of 15 ± 0.5 µg/L at t = 135 d), were between the ‘no-effect’ and ‘effect’ concentrations reported by Winner et al. (1990) and Hedtke (1984).

This study emphasises the importance of carrying out truly chronic exposures to understand the long-term effects of a persistent contaminant, like copper, in the field. The strong response of the benthic community to copper in this study, carried out over 71 weeks, is similar to other long term studies: Hedtke (1984), also recorded severe effects of copper on snails (*Viviparus*, *Physa* sp. and *Helisoma campanulata*) during a 32-week overlying water copper exposure and Shaw & Manning (1996) observed a pronounced response of the benthic community to copper during 19 week exposures. In contrast, during their five-week lentic exposures, Winner et al. (1990) observed a weak response of the benthic community to copper; the densities of small chironomids responded differently depending on season and the only consistent response in the benthic community to copper was a reduction in the densities of small caenid mayflies at 40 µg/L (compared to 0 and 20 µg/L treatments). Specifically Winner et al. (1990) also noted that snails were unaffected by copper. Even based on overlying water exposures, Winner et al. (1990) should have seen a response (as noted earlier, Hedtke (1984) and Shaw & Manning (1996) observed strong effects on the benthic community at overlying water copper concentrations of ≥30 µg/L (not ≤9.3 µg/L) and ≥20 µg/L respectively). Winner et al. (1990) supposed that the weak response in the benthos to copper that they observed could have occurred because the five-week experiments did not incorporate effects on reproduction and recruitment, a theory supported by this study and the other truly chronic exposures previously performed (Shaw & Manning, 1996; Hedtke, 1984).

The importance of inter-species variation in sensitivity

Within the benthic response, some taxa responded differently compared to previous studies. For example, Tanypodinae were negatively affected by copper (Shaw & Manning, 1996), but in this study, no effect of copper was identified. Other studies that have considered the effects of copper on Tanypodinae in the field have also found species within the sub-family to be copper tolerant, which supports the lack of response in the present study (Montz et al., 2010; Clements et al., 1988). There was also inter-species variation in copper susceptibility of Ostracoda within this study, as one species of benthic Ostracoda was not affected

by copper but another declined in abundance. In constrast, Shaw & Manning (1996) showed an increase in benthic Ostracoda. This demonstrates that organisms with similar life histories may respond differently to contaminants and confirms that it is important to identify organisms to an appropriate taxonomic level to allow contaminant specific responses to be identified.

The importance of inter-species interactions

Interspecific interactions within the mesocosms influenced the response of the benthic community to copper. In the first spring/summer season (up to t = 161 d), the abundance of Chironominae, the Ostracoda species and Cladocera were lower in the H/VH treatments compared to the control. However, in the second spring/summer season (from t = 365 d) the abundances of each taxa generally declined in the C/VL/L treatments to become more similar to the H/VH treatments. It is possible that the decrease in abundance of these taxa was due to interspecific competition in the low copper treatments between these taxa and the gastropod *P. acuta* (which colonised the mesocosms and proliferated in those treatments between t = 161 d and t = 365 d). Interspecific competition between these taxa has been documented elsewhere (e.g. Cuker, 1983; Devereaux & Mokany, 2006; Gresens, 1995; Harvey & Hill, 1991). For Ostracoda and Cladocera it may be due to competition for food, but for Chironominae, there is evidence to suggest that reproductive interference may occur. Devereaux & Mokany (2006) showed that *Chironomus oppositus* avoided *P. acuta* during site selection for oviposition. Clearly, the interspecific interactions between taxa were important in driving the biotic response to copper, demonstrating that understanding these interactions is necessary when characterising the potential effects a contaminant has, or will have, on the community.

Stage of ecological succession

This study indicates that the effect of copper on taxonomic richness is most prominent when benthic communities are being established in new, or recently disturbed, freshwater habitats. An effect of higher taxonomic richness in C/VL/L

treatments compared to H/VH treatments was only observed during the establishment of the benthic communities within the mesocosms. Consistent with this, Shaw & Manning (1996) observed no effect on taxonomic richness on already established benthic communities. Here, there are implications for environmental management. When aquatic habitats are disturbed, contaminants are often remobilised from sediments, increasing their bioavailability so further influencing the re-establishment of healthy aquatic communities.

Final conclusions

There was a strong difference in the benthic communities that developed within the highly copper contaminated sediments compared to those in the controls. However, there was little effect of copper on the invertebrates in the water column. This study has demonstrated the importance of using environmentally realistic exposure scenarios – including both realistic partitioning of contaminants between phases in the environment and environmentally relevant exposure times – the latter being particularly important with regard to persistent contaminants in the field. Further, there was evidence to suggest that communities may be particularly sensitive during colonisation, with implications for environmental management when sites are disturbed. This study has demonstrated the importance of considering interspecific interactions and interspecies variation in sensitivity to contaminants in driving the community response. These community level responses would have been impossible to elucidate from single (or few) species laboratory toxicity studies due to their complex nature, demonstrating the clear benefit of carrying out outdoor mesocosm studies open to natural colonisation.

Ultimately, this study has shown that different communities will respond to contaminants in different ways through both direct and indirect effects. As such, although guidelines are useful, site specific assessments on the health of aquatic ecosystems are paramount in understanding the effects of contaminants at those sites.

Chapter 5. Environmental DNA metabarcoding meets ecotoxicology: the response of the eukaryote community to copper exposure in an empirical outdoor mesocosm study

The aim of the work presented in this chapter was to characterise the effect of copper on the whole of the eukaryote community. The development of the community from micro- to macro- fauna was assessed by environmental DNA (eDNA) metabarcoding. This is a new technique of community-level analysis and is still in the early stages of development as a bioassessment tool. Therefore, a further aim was to compare eDNA metabarcoding with traditional techniques of optical analysis (Chapter 4). Although the technique can currently only provide presence/absence data, eDNA metabarcoding proved to be more sensitive than analysis of the invertebrate community because it incorporated a larger proportion of the biotic community.

Authors

	Problem formulation	Experimental design/ methodology	Statistical analysis	Results interpretation	Paper preparation
Stephanie Gardham[1,2]	85	85	90	85	90
Grant C. Hose[1]	7.5	5	2.5	2.5	2.5
Anthony A. Chariton[2]	7.5	10	7.5	12.5	7.5

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Abstract

Environmental DNA (eDNA) metabarcoding has the potential to revolutionise ecosystem health assessments, incorporating a much larger proportion of the biotic community than current methods of analysis allow. Here, eDNA metabarcoding was performed to assess the effects of copper on the establishment of the benthic eukaryote community within a series of environmentally relevant copper contaminated freshwater mesocosms. The organisms present from the micro- to macro- scale were characterised by this method. Taxonomic richness of the whole community (and dominant phyla) was reduced in the high compared to the control and low copper contaminated treatments. The composition of the benthic eukaryote community showed more subtle changes, with significant differences apparent between all treatments, even the control and low copper contaminated treatments. All the taxa identified to be sensitive to copper were micro- and meio- fauna, including several Chlorophyta, Nematoda and Bacillariophyta. This study emphasises the need to incorporate micro- and meio- fauna into bioassessment processes and demonstrates the potential to create ‘eDNA metabarcoding signatures’ and identify novel indicator species for toxicity studies.

Introduction

Bioassessment is an essential tool in the management and conservation of ecosystem health. In freshwater ecosystems, benthic macroinvertebrates are generally monitored as indicators of ecosystem health, a time-consuming, labour intensive and expensive procedure, which requires taxonomic expertise (Chariton et al., 2010a; Lear et al., 2009). Further, macroinvertebrates comprise just a small fraction of the total biodiversity in an ecosystem, so by protecting the macroinvertebrate community, the assumption is made that the macroinvertebrates are representative of the whole ecosystem. This is flawed logic, as meio- and micro- fauna are generally more sensitive than macrofauna (e.g. Ogilvie & Grant 2008; Kennedy & Jacoby 1999). Yet, eukaryote microbes are essential players in many globally important biogeochemical cycles, for example primary production and nitrogen assimilation (Gadd & Raven, 2010). Thus the meio- and micro- fauna should be incorporated into bioassessment methods for ecosystem health assessment.

Evolving from the molecular sciences, a new tool has emerged in recent years, which allows a greater understanding of functional and ecological biodiversity (Shokralla et al., 2012). Environmental DNA (eDNA) metabarcoding involves the analysis of DNA extracts from environmental samples allowing the identification of multiple taxa at the same time (Yoccoz, 2012). Amplicons (informative fragments of DNA) obtained via PCR of the eDNA extract are sequenced by Next Generation Sequencing (NGS) technology and compared to online or custom reference libraries, like GenBank, to assign taxonomy to biota present in the environmental sample (Shokralla et al., 2012). The taxonomic level that can be assigned is dependent on the length of the sequenced gene, whether the amplified target region has previously been sequenced for the relevant taxa (at an appropriate quality), and the homology of that gene to the annotated sequence (Chariton et al., 2010a). For eukaryotes, the gene encoding 18S rRNA is often sequenced when investigating divergence rates and establishing phylogenetic information for individual species, so there is a large quantity of appropriate data available (Chariton et al., 2010a). As a result, most eukaryote surveys use the 18S rRNA

gene as a marker (Tang et al., 2012). Ultimately, metabarcoding enables the identification of a much greater proportion of the biotic community present than traditional techniques and has the potential to be a reliable, rapid and cost-effective form of assessment (Chariton et al., 2010a).

Environmental DNA metabarcoding techniques have been used to assess changes in biotic communities along environmental gradients from alveolate turnover in lakes (Medinger et al., 2010), to root-associated fungal communities along a primary succession gradient in glacier foreland (Blaalid et al., 2012) and transitional changes in eukaryotic biodiversity on floodplains (Baldwin et al., 2013) amongst many other studies (e.g. Creer et al. 2010; Chariton et al. 2010). These studies have demonstrated that through eDNA metabarcoding, extensive data from a standard environmental sample can be extracted, which are ecologically relevant at the whole ecosystem scale (Baird & Hajibabaei, 2012; Chariton et al., 2010a).

Chariton et al. (2010a) identified that there is an opportunity to create 'eDNA metabarcoding signatures' of the biotic response to changing environmental conditions like changes in metal concentration. However, the eDNA metabarcoding studies carried out to date have focused on characterising the changes in communities across natural gradients in the field. As such, they have inherently been correlative as unmeasured environmental variables or contaminants may have been present causing the changes in community composition observed. This precludes identification of a signature for a particular contaminant from field datasets. Mesocosm studies may be important in this endeavour as they allow true cause and effect between contaminants and biotic responses at the community level to be determined.

To explore the potential of the 'eDNA metabarcoding signature' approach, the effects of copper on changes in the establishment of benthic eukaryote communities in a series of outdoor mesocosms over 1.5 years were assessed. Copper is a key metal contaminant, which enters the aquatic environment through urban waste water, industrial and mine effluents, agricultural run off and

atmospheric deposition (Serra & Guasch, 2009). Effects on the benthic community were explored as sediments act as a sink for copper present in surface waters, so benthic biota are generally exposed to higher concentrations of copper in comparison to those present in the water column.

Methods

Study design

Twenty pond mesocosms were established on the Macquarie University campus (33.76946 S, 151.11496 E), in northern Sydney, NSW, Australia. The facility is described in detail in Chapter 2, and briefly below. Each mesocosm had a volume of 1500 L, was sunk into the ground, shaded by 70% shade-cloth, aerated and inputs from rainwater were sufficient to maintain water depth. Prior to being opened to the environment to allow biotic colonisation on the 1st November 2010 (t = 0 d), sediments were spiked *in situ* with copper and allowed two months to equilibrate and create environmentally relevant copper contaminated sediment (Chapter 2). The experimental design consisted of a control (C) and four sediment copper concentrations (very low, VL; low, L; high, H; and very high, VH), each with four replicates. Mean concentrations of copper in the sediments, pore waters and overlying waters following t = 74 d (once the sediments appeared to have reached pseudo-equilibration; Chapter 2) are presented in Table 5.1. Analysis of the changes in conductivity, pH, turbidity, dissolved oxygen and oxidation-reduction potential of the overlying water and copper concentrations in the sediments, pore waters and overlying waters showed that the predominant abiotic difference between the mesocosms was copper concentration (see Chapter 4).

Table 5.1: Mean ± S.E.M. (n=4) copper concentrations in the sediment, pore waters and overlying waters by treatment.

Treatment	Sediment copper (mg/kg)	Pore water copper (µg/L)	Overlying water copper (µg/L)
Control	4.6 ± 0.7	1.5 ± 0.2	1.3 ± 0.3
Very low	71 ± 4.6	2.8 ± 0.2	2.2 ± 0.2
Low	99 ± 7.1	4.6 ± 1.2	3.8 ± 0.5
High	410 ± 41	18 ± 3.1	7.8 ± 0.6
Very high	711 ± 46	30 ± 2.5	11 ± 0.9

Sampling strategy

Sediment samples were taken from each mesocosm four times during the first spring/summer season (t = 15 d, t = 31 d, t = 135 d and t = 161 d) and three times during the second spring/summer season (t = 365 d, t = 407 d and t = 497 d). Three cores (6 cm diameter) were randomly collected from the surficial layer of the sediment (top 1 – 2 cm) using a plastic corer. They were combined in a plastic sample jar, which was then capped and inverted six times to mix the cores together. A 50 ml falcon tube was filled with a sub-sample of the sediment and stored on ice in the field. On return to the laboratory (within 2 hours), the samples were frozen at -80ºC until processing. All equipment was sterilised by soaking in 15% bleach solution for 24 hours followed by a thorough rinse with tap water and/or UV sterilisation (10 minutes). Separate sampling equipment was used for each mesocosm to prevent cross-contamination.

Molecular analysis

DNA was extracted and purified from a 10 g subsample of each homogenised surficial sediment sample using PowerMax Soil DNA Isolation Kits (MO BIO Laboratories Inc, Carlsbad, CA). After performing a gradient PCR to establish the optimal annealing temperature (58ºC) and template DNA dilution (1:6), PCR was performed on each of the eDNA extracts using the primers All18SF (5'-TGGTGCATGGCCGTTCTTAGT-3') and All18SR (5'-CATCTAAGGGCATCACAGACC-3'). This primer pair has high coverage, theoretically able to amplify 18S target sequences from >85% of species across all Kingdoms (Baldwin et al., 2013), and targets between 200 and 500 bp in the 3' region of the gene encoding 18S rRNA. PCR amplifications were performed in 50 µl volumes with Phusion High Fidelity (HF) Master Mix (Thermo Fisher Scientific Inc, Pittsburgh, PA) in the buffer supplied and 0.5 µM All18SF, 0.5 µM All18SR, nuclease free water, DMSO and 2 µl of template DNA. Amplification was performed in an Eppendorf thermocycler (Eppendorf, Hamburg, Germany) with the following cycling: one cycle of 98ºC for 1 minute, then 35 cycles of 98ºC for 10 s, 58ºC for 20 s, and 72ºC for 30 s, followed by one cycle of 72ºC for 5 minutes.

All PCRs were run with a positive artificial assemblage control and a negative water control to detect contamination and to test bioinformatics parameters. PCR integrity and amplicon bp size was assessed using MultiNA (Shimadzhu, Japan) digital gel analysis. The 18S amplicons were purified using an AMPure XP purification kit (Agencourt, Beverly, MA) and quantified by NanoDrop spectrophotometry (NanoDrop 2000, Thermo Fisher Scientific Inc). Custom designed fusion primers were ligated to the 18S amplicons to incorporate 454 pyrosequencing adapter A and B sequences and unique 10 base barcode sequences were attached to each sample for multiplexing to the ends of the 18S target sequences, as required for 454 pyrosequencing. This was achieved using a four cycle ligation PCR, performed in 50 µl volumes with Phusion HF Master Mix and 15 µl template DNA (which comprised 200 ng of the 18S amplicons – ensuring equimolar concentrations and even ligation of fusion primers across amplicons within samples). The PCR cycle was: one cycle of 98ºC for 2 minutes, and then four cycles of 98ºC for 30 s, 54ºC for 1 minute and 72ºC for 1 minute, followed by one cycle of 72ºC for 3 minutes. These amplicons were purified, quantified and pooled to achieve a target of 300 ng/µL then purified again using QIAquick PCR purification kit (Qiagen, Valencia, CA). Pyrosequencing of the pooled 18S amplicon libraries was performed using a two region large gasket format on a Roche 454 GS FLX sequencer with titanium chemistry at the Australian Genome Research Facility (Brisbane, Queensland, Australia).

Sequence analysis

The raw sequence reads were processed using the Amplicon Pyrosequence Denoising Program (APDP) (Morgan et al. in press). Through APDP, sequences were blasted against the NCBI server to find valid 18S rDNA GenBank accession numbers, referred to as operational taxonomic units (OTUs). Within each sample, three way alignments of all possible combinations of sequences were conducted to identify potential chimeric and other residual error-derived sequences (these were only removed if their relative read abundances fell below thresholds set for chimera, indel and point errors by 454 sequencing of predefined mixtures containing reference DNAs amplified under identical PCR conditions). ‘Singleton’

OTUs, that were present in only one sample and OTUs that were represented by <10 reads overall were removed. To account for differences in sampling effort (number of reads produced in each sample), read counts were normalised to the sample with the lowest total number of reads. Where available, taxonomic information for each sequence from NCBI's Taxonomy database was obtained and OTUs that were potentially obtained from sources other than the targeted media (e.g. terrestrial debris) were filtered from the analysis. In downstream analyses percentage similarity (homology) cut-offs were applied to OTUs for accurate classification: Genus, 100%; Family, >99%; Order, > 97%; Class 92%; Phyla, >90%; Kingdom, >80%; and, unassigned Eukaryote <80% (Baldwin et al., 2013).

Statistical analysis

The effect of time and treatment on total OTU richness and the OTU richness of dominant phyla within each sample were assessed by repeated measures (RM) ANOVAs (between-subjects factor: treatment, within-subjects factor: time) followed by least significant difference (LSD) pair-wise multiple comparison tests using PASW Statistics 18.0.3. Where a significant interaction between treatment and time was found, a one-way ANOVA followed by LSD pair-wise multiple comparison was performed on the data from within each time point to identify where a significant difference in OTU richness among treatments occurred, and a separate RM ANOVA was performed for the data of each treatment to identify changes in OTU richness within treatments over time. Untransformed data generally met assumptions of homogeneity of variance (assessed by Levene's test) and normality (assessed by Shapiro Wilk's test), where data failed Levene's test, transformations of the data were considered but did not change the outcome of the test, so the significance level (α) was reduced to 0.01 (for all other comparisons α was 0.05) (Underwood, 1997). If the assumption of sphericity (assessed by Mauchly's test) was not met during the RM ANOVA analyses, the Greenhouse-Geisser correction was used.

To investigate differences in community composition amongst samples, a two-dimensional plot with non-metric multidimensional scaling (nMDS) was generated

from the Jaccard's similarity matrix of the presence/absence data in Primer 6+ (PrimerE Ltd, Plymouth, UK). The presence/absence data were then analysed using principal response curves (PRC) (Van den Brink & Ter Braak, 1999) with Canoco version 4.5; recommended software settings were used. PRC is a multivariate method of analysis, which shows differences in community composition between treatments and the control at each time point. The species weights enable consideration of the effect at the species level, the higher the weight (positive or negative), the more similar the response to that identified in the figure; taxa with negative weights show the opposite pattern to the PRC. The significance of the PRC was tested using Monte Carlo permutation tests (using α 0.05). To further interrogate the pattern observed in both the nMDS plot and PRC, the variation in community composition between treatments at each time point was analysed using Permutational Multivariate Analysis of Variance (PERMANOVA) (Anderson, 2001) followed by post hoc pair-wise comparisons. The PERMANOVA analysis was based on the Jaccards similarity matrix of the presence/absence OTU data and performed in Primer 6+.

Distanced-based linear modeling (distLM) (McArdle & Anderson, 2001) was performed to identify the significance of the sediment and pore water copper concentrations in driving community compositional change, producing a distance-based RDA (dbRDA) plot. The analysis was based on the Jaccards similarity matrix of the biological data and normalised environmental data. The presence/absence data from all time points were interrogated against both sediment and pore water copper concentrations (as both were identified to be significant drivers) using Theshold Indicator Taxa ANalysis (TITAN), performed in R 2.15.2 (Baker & King, 2010), with the recommended settings. TITAN distinguishes negative (z-) and positive (z+) taxa (in this case OTU) responses to a changing environmental variable and identifies threshold change point(s) of that variable for each taxa. The analysis then calculates the cumulative responses of declining (sum[z-]) and increasing (sum[z+]) taxa to identify threshold change points for the community. It employs bootstrapping to estimate the reliability, purity and uncertainty of identified change points. The threshold concentrations of

individual taxa plotted in the final figures had default purity and reliability criteria (pur.cut = 0.95, pval.cut = 0.05, rel05.cut = 0.90).

Results

The filtered data contained 995,000 reads. The presence absence data derived from these data represented 1397 OTUs which were recorded as present 18,443 times across all time points and samples.

Total richness increased over time in the C, VL and L treatments (C: $F_{6,18} = 4.2$, $p = 0.008$; VL: $F_{6,18} = 12$, $p < 0.001$l; L $F_{6,18} = 20$, $p < 0.001$) (Figure 5.1). In the H treatment there was no change in total richness over time ($F_{6,18} = 0.57$, $p = 0.075$), while in the VH treatment it decreased over time ($F_{6,18} = 4.6$, $p = 0.005$). Corresponding to these different trajectories in richness over time, there was a significant interaction between time and treatment (interaction: $F_{14,57} = 4.9$, $p < 0.001$). Within each time point, there was a significant effect of treatment 60% of the time; at the start of the experimental period total richness in the VH treatment was higher than all other treatments, but by the final two time points the pattern had reversed (Figure 5.1; Table A5.1).

The most “OTU rich” phyla present in the mesocosms were Cercozoa (12%), Chlorophyta (10%), Nematoda (9.1%), Ciliophora (8.9%), Ascomycota (6.6%), Chytridiomycota (5%), and Rotifera (3%) (Figure 5.1). For all of these phyla, OTU richness generally increased, or was constant over time in the C/VL/L treatments (Figure 5.2a-g). In the H/VH treatments the OTU richness of each phyla generally decreased over time, apart from for the Nematoda, where the OTU richness of the H/VH treatments increased. By the final timepoints, generally the OTU richness of each phyla in the C/VL/L treatments was higher than that in the H/VH treatments, except for Ascomycota, where there was no significant difference among treatments (Figure 5.2a-g).

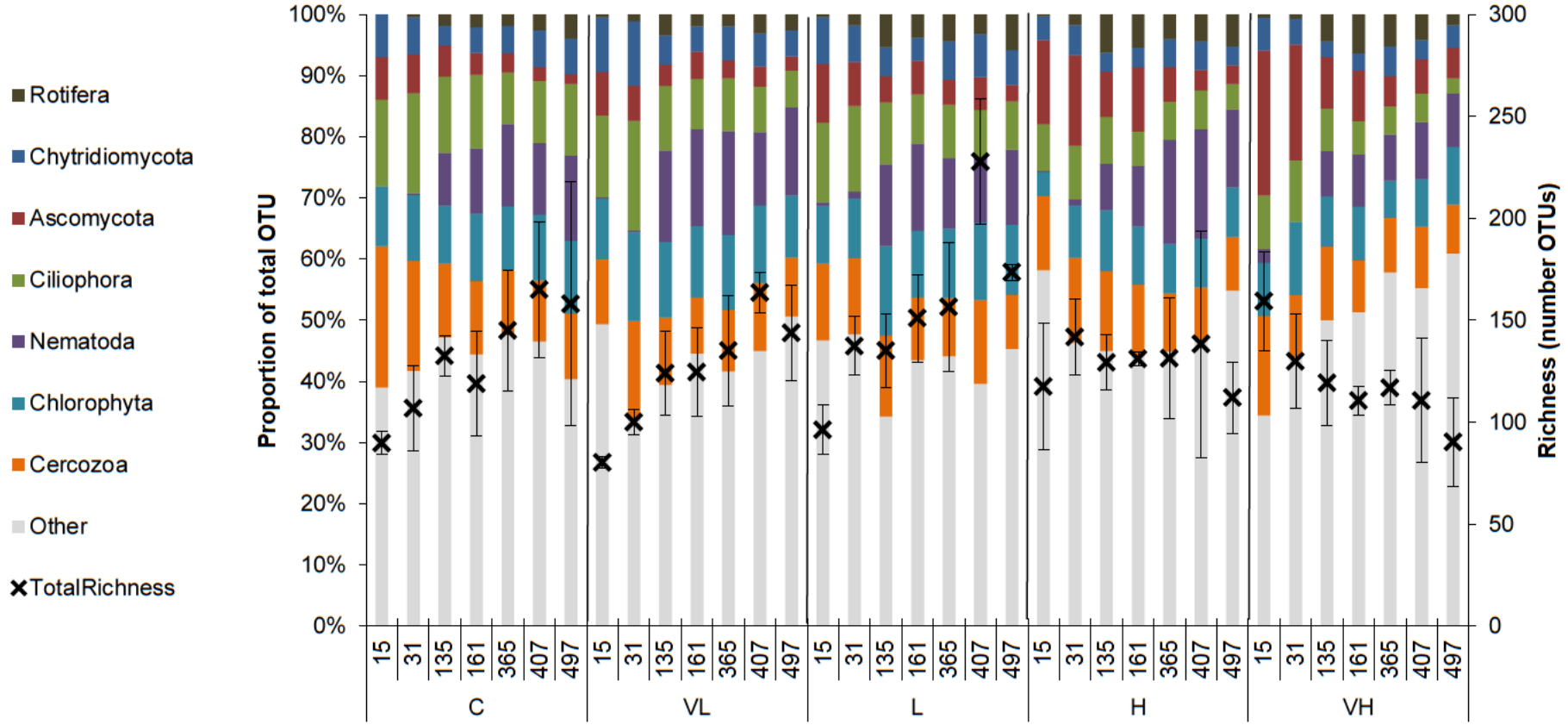

Figure 5.1: The relative proportions of eukaryote OTUs grouped by Phyla and the change in total richness of OTUs over time within each treatment.

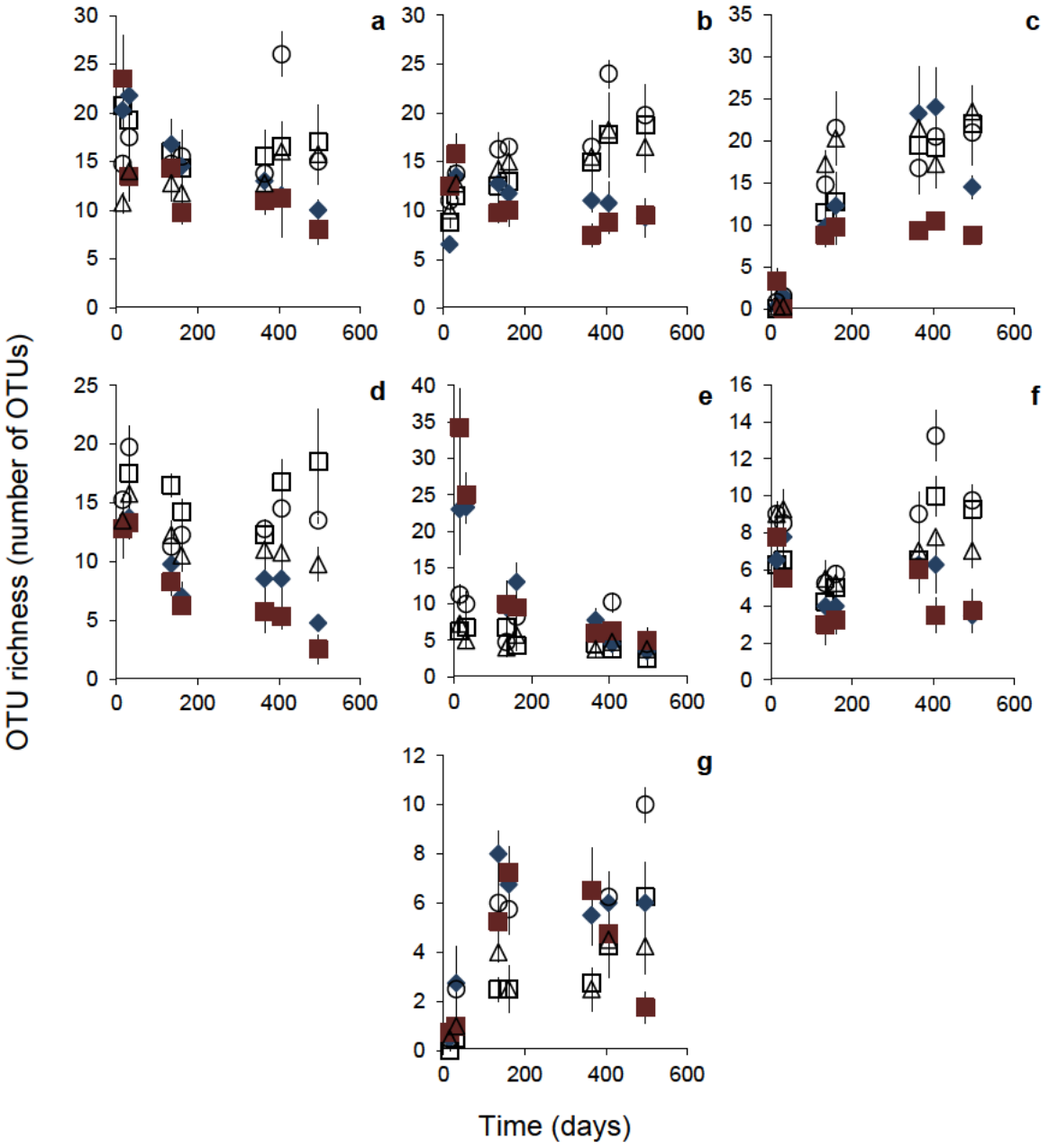

Figure 5.2: Change in richness of the dominant phyla (a) Cercozoa (b) Chlorophyta (c) Nematoda (d) Ciliophora (e) Ascomycota (f) Chytridiomycota (g) Rotifera over time. Key: C (□), VL (△), L (○), H (◆) and VH (■) treatments.

The eukaryote communities present within each treatment were significantly different from each other on the first sampling occasion (t = 15 d; two months after spiking; Chapter 2) (Table A5.2). Sediments for each mesocosm were sourced from the same batch of homogenised soil, so it is assumed that they each had similar microbial assemblages prior to spiking. While the spiking method

successfully excluded invertebrates (Chapter 4), it was not possible to exclude microbiota. The microbial assemblages present at t = 15 d reflect the development of aquatic biota in response to copper as well as a transition from soil (terrestrial) to aquatic sediment assemblages.

The benthic eukaryote communities within each treatment clearly changed over time as they became established within the mescocosms (Figure 5.3a), the changes over time were different depending on treatment (interaction: Pseudo-$F_{24,105} = 1.5$, $p = 0.001$). Although, the eukaryote communities in the C/VL/L treatments appear to be quite similar across time points according to the PRC (Figure 5.3b), in the nMDS plot slight differences in community composition can be seen (Figure 5.3a). In fact, the benthic eukaryote communities were only similar between the C and VL treatments, and VL and L treatments on the final two time points (407 d and 497 d) respectively (Table A5.2).

Within the H/VH treatments, the community composition changed significantly between time points up to t = 135 d, this can clearly be seen in Figure 5.3b, where the communities in the H/VH treatments diverge from those in the control from the start of sampling (t = 15 d) until t = 135 d. At the start of the second spring/summer season (t = 365 d), although over time the communities had changed according to the nMDS (Figure 5.3a), the community in the H treatment does not appear to have diverged further from the control (in comparison to the end of the first spring/summer season (t = 161 d)). In contrast, the VH treatment does appear to have diverged further from the C treatment. However, by the end of the second spring/summer season (t = 497 d) there appears to have been some convergence between the VH and C communities, although they are still very different from each other. Within each time point, the eukaryote communities present in the H and VH treatments were significantly different from those in the C/VL/L treatments, and from each other (Table A5.2).

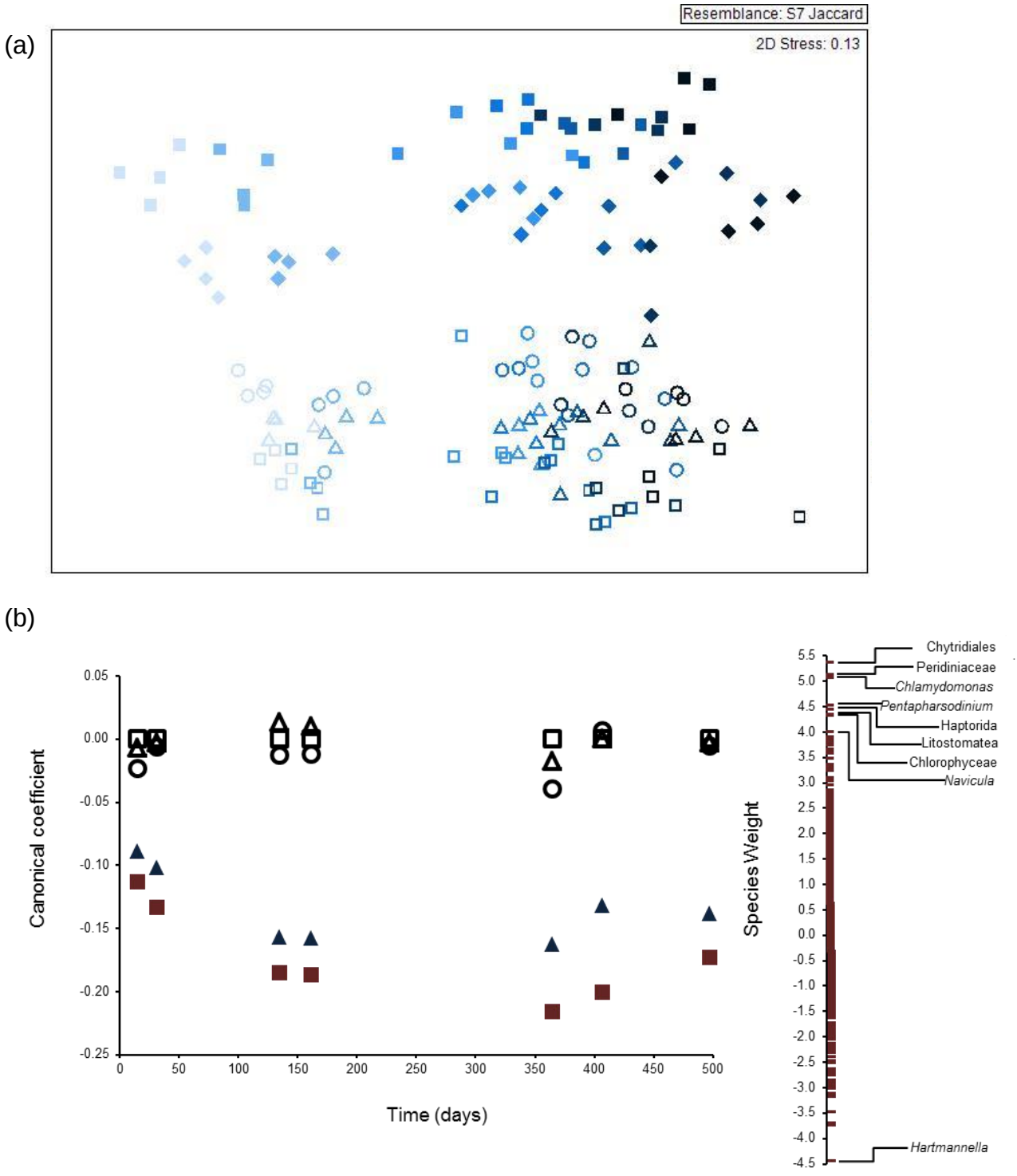

Figure 5.3: (a) nMDS plot showing differences in community composition among samples, where points closer together represent a more similar community composition than those further apart; and (b) Principal response curves with species weights showing the effect of copper on establishment of the benthic invertebrate community; Key: for (a): C-□; VL-△; L-○; H-◆; and VH-■ and gradient of light⇢ dark blue is t = 74 ⇢ t = 497; for (b) red tick marks points indicate other taxa with calculated species weights (labels have been removed for clarity) - the higher the weight (positive or negative), the more similar the response to that identified in the figure; taxa with negative weights show the opposite pattern to the PRC.

The differences in community composition among sampling times described accounted for 16% of the total variance in community composition while 30% of the variance was explained by the differences in community composition due to treatment. The first axis of the PRC was significant (p = 0.002) and explained 28% of the variance captured by the treatment regime. The 2nd axis was also significant (p = 0.005) but only explained 11% of the variance, therefore only the first PRC axis has been described.

The OTUs that most strongly followed the response identified by the first axis of the PRC were all microfauna (Figure 5.3b). Those that responded negatively to copper included two dinoflagellate (both of the family Peridiniaceae, of which one was *Pentapharsodinium* sp.) and two ciliate (both of the class Litostomatea, of which one was Haptorida) alveolates. One diatom (*Navicula* sp.), one aquatic fungi of the order Chytridiales and two green algae of the class Chlorophyceae (one of which was *Chlamydomonas* sp.) were also identified to respond negatively to increasing copper. One Amoebozoa (*Hartmannella* sp.), was identified to respond positively to the increase in copper concentrations.

Sediment and pore water copper concentrations were significant drivers of the changes in community composition (p = 0.001) (Figure 5.4a; Table A5.3). At the final time point (t = 497 d), there was a single community threshold of 180 mg/kg for both sum(z+) and sum(z-) OTUs with respect to sediment concentrations (Figure 5.4b). For the pore water copper, the community thresholds were 8.4 µg/L for negatively affected OTUs and 14 µg/L for positively affected OTUs (Figure 5.4c).

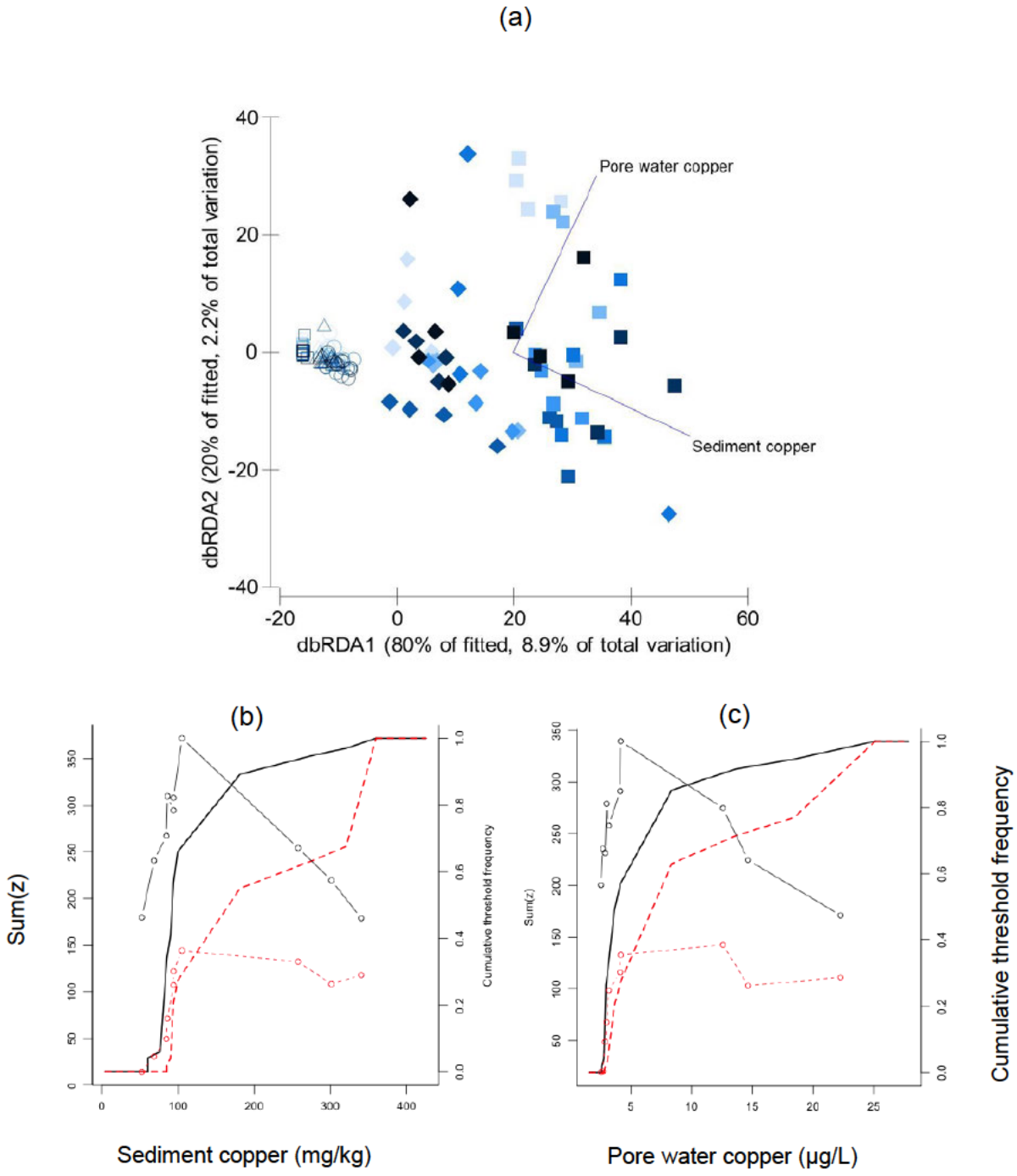

Figure 5.4: (a) dbRDA plot of the correlation between the benthic community and sediment and pore water copper concentrations; and TITAN sum(z) plots against (b) sediment and (c) pore water copper concentrations. Key: for (a): C-□; VL-△; L-○; H-◆; and VH-■ and gradient of light→ dark blue is t = 74 →t = 497 d); for (b) and (c): black = Sum(z-) and red = Sum(z+).

At the final time point (t = 497 d), 23 OTUs were negatively affected and 5 OTUs were positively affected by increasing concentrations of copper in the sediment and/or pore water (with appropriate reliability and purity). The OTUs identified all represented meio- and micro- fauna (Figure 5.5). The OTUs that were positively affected by increasing concentrations of copper in the sediment and/or pore waters represented two Cercozoa (Rhizaria), one Fungi of an unknown phylum, one flagellate protozoa (Rigifilida) and one Amoebozoa belonging to Flabellinea (Figure 5.5a,b).

Of the OTUs that were negatively affected by increasing copper concentrations, seven belonged to the kingdom Viridiplantae; one was of an unknown phylum but the remaining six belonged to Chlorophyta. Of these six, one belonged to the class Trebouxiophyceae and the remaining five were Chlorophyceae, of which two belonged to the family Chlamydomonadaceae (one was *Chlamydomonas* sp.), one represented the genus *Oedogonium* and one represented the genus *Tetraedron*. Metazoans were represented by seven of the OTUs that responded negatively, comprised of five nematodes (one of unknown class and the other four belonging to the family Monhysteridae) and two ostracods (one unknown order and one *Strandesia* sp.). Four fungi were negatively affected (one of an unknown phylum and three Chytridiales) and three alveolates were negatively affected, including a species of *Sterkiella* and one each of the order Urostylida and family Peridiniaceae respectively. There were also two diatoms negatively affected by increasing copper concentrations, both belonged to the genus *Navicula* (Figure 5.5a,b).

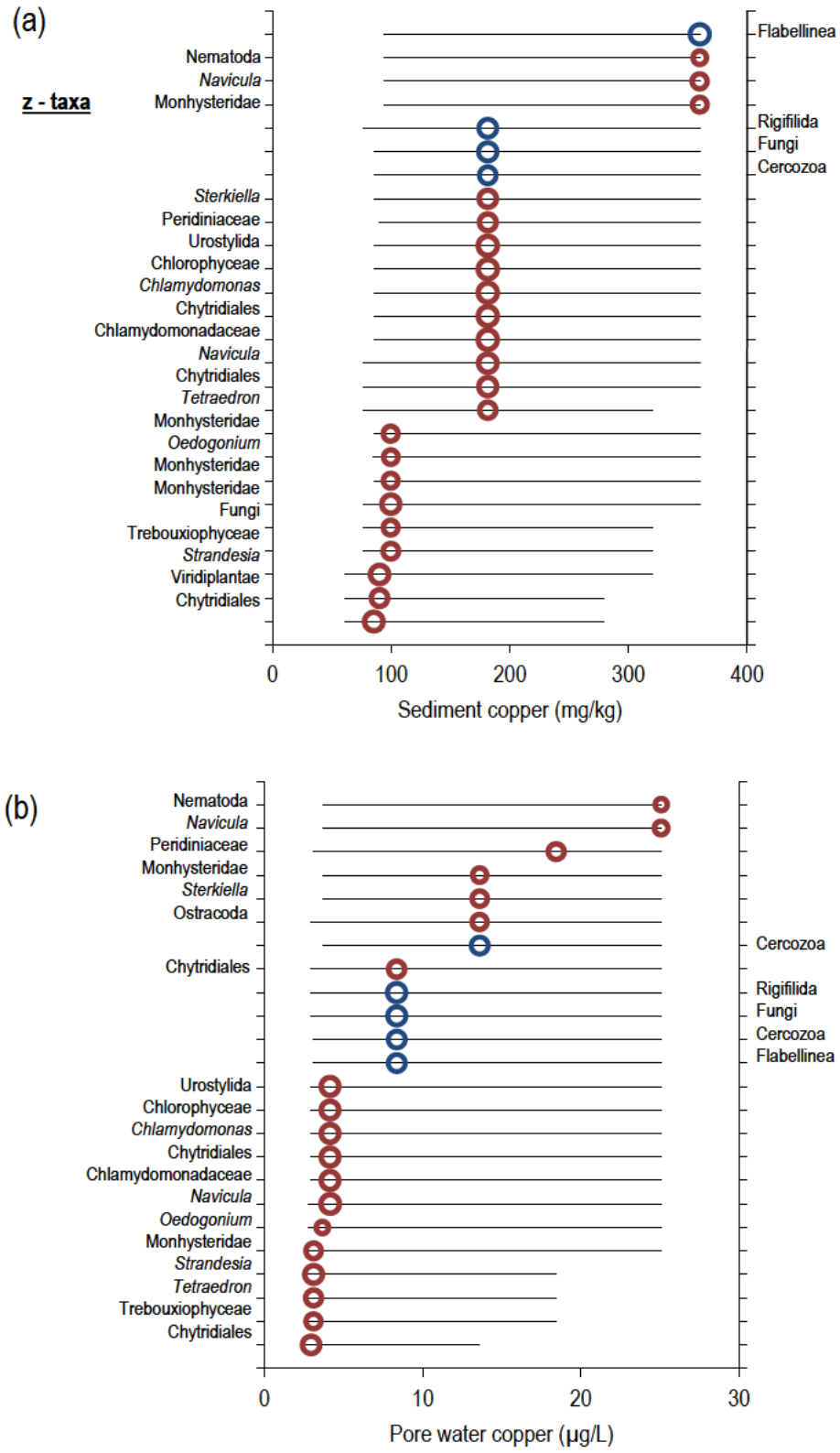

Figure 5.5: (a) Sediment and (b) pore water thresholds for OTUs with appropriate purity and reliability. OTUs have been identified to their lowest level based on homology.

Discussion

Through eDNA metabarcoding the effect of copper on eukaryotes from the micro- to macro- scale has been demonstrated. By characterising a greater proportion of the biotic community than ever before, subtle effects of copper at low levels of contamination have been observed. The results complement and increase understanding of the effects of copper gained from traditional invertebrate analyses, demonstrate the potential of creating eDNA metabarcoding signatures for contaminants and provide opportunities to identify novel indicator species for toxicity studies with copper.

The biotic response

The biotic response characterised by eDNA metabarcoding in this study was driven by micro- and meio- fauna. There has been limited research into the effects of copper on the structure and function meio- and micro- fauna within freshwater communities (Massieux et al., 2004). Studies that consider the effects of copper on microfauna in the benthic layer have focused on phototrophic species within the biofilm. It is well known that photosynthesis and growth in algae is inhibited by copper at low concentrations (Garvey et al., 1991). Overall richness of phototrophic species decreased in response to copper in Leland & Carter (1984) and Kaufman (1982), consistent with this study where Chlorophyta richness was lower, particularly in the second spring/summer season (from t = 365 d). Further, several of the OTUs sensitive to copper according to the PRC and TITAN analyses were identified as Chlorophyta and Bacillariophyta. Bacillariophyta have similarly been shown to reduce in richness, due to replacement by cyanobacteria (Barranguet et al., 2003), but in contrast, an increase in richness in response to copper has also previously been reported (e.g. Serra et al., 2009).

Considering the response of Chlorophyta further, although seven sensitive OTUs belonging to the phylum were identified to be sensitive to copper, there were 137 different OTUs identified as Chlorophyta in the community across all time points. Further, in relation to the PRC, 72 of the OTUs belonging to the phyla had species

weights between 0.5 and -0.5 (data not shown), meaning they show no response to copper (or one that is unrelated to the pattern shown by the PRC). This shows that overall, there were many taxa of green algae tolerant of copper, which is consistent with previous research where green algae have become the dominant algae in both plankton and periphyton communities (Soldo & Behra, 2000; Effler et al., 1980). Further, of the thirteen OTUs identified as species of *Chlamydomonas* across the community, two were sensitive; in some studies *Chlamydomonas* spp. have been considered tolerant to copper (Knauer et al., 1997), while in others sensitivity to copper has been observed, for example Garvey et al. (1991) observed deflagellation of *Chlamydomonas reinhardtii* at 6.7 µg/L.

Interspecies variation in sensitivity can also be demonstrated by further consideration of the diatoms present in the current study. Ten Bacilliarophyceae were identified within the community, all belonged to the family Naviculaceae and five could be identified as *Navicula* spp. (data not shown). Of the OTUs identified as *Navicula* spp., one was identified as sensitive to copper in both the long term response (PRC analysis) and at the final time point (TITAN analysis), where a second OTU of *Navicula* was also shown to be sensitive. Species of *Navicula* have often been shown to be sensitive to metals, for example, in response to copper the growth of *Navicula incerta* was reduced (Rachlin et al., 1983). However, five of the OTUs had species weights between -0.5 and 0.5 (data not shown); this indicates that some species were not sensitive, consistent with community level analyses of biofilms exposed to copper, where some tolerant *Navicula* spp. have been identified (Guasch et al., 2002; Leland & Carter, 1985).

Although most studies that have considered the effects of copper on aquatic microfauna have focused on phototrophic species, one comprehensive study considered taxonomic changes in both heterotrophic and phototrophic microfauna within plankton exposed to copper (Le Jeune et al., 2007). They showed a decrease in the biomass *Chlamydomonas* and *Haemotococcus* within the pigmented flagellate community and a decrease in Haptorida, Prostomatida and Oligotrichida in the ciliate community (Le Jeune et al., 2007). Similarly in this

study, both *Chlamydomonas* and Haptorida were identified to be sensitive to the increase in copper.

Within the meiofauna identified to be most sensitive to copper, five OTUs represented Monhysteridae, a family of Nematoda. Nematodes have previously shown sensitivity to copper (Boyd & Williams, 2003) and are often considered as potential indicators for biomonitoring due to their ubiquitous nature and interspecies variability in sensitivity (Ekschmitt et al., 2001; Boyd et al., 2000; Bongers & Ferris, 1999). This interspecies variability was demonstrated in this study, as 77 Nematoda in total were identified. However, considering five (out of sixteen) OTUs classified as Monhysteridae were sensitive to copper, certain species of this family may be good potential indicator taxa of copper (or metal) contamination.

The other meiofauna identified as sensitive included two species of Ostracoda. Here, the invertebrate data (Chapter 4) and eDNA metabarcoding data may support each other. A species of Ostracoda was found to be sensitive to copper in the invertebrate community analysis (Ostracod Sp. 2; Chapter 4) and based on taxonomic guides, the species of Ostracoda could be *Strandesia* sp. (identified as sensitive by eDNA metabcaroding). However, this identification of the sensitive ostracod in Chapter 4 is not confirmed. The effect of copper on *Strandesia* has not previously been described, only one study characterised the response of benthic ostracoda within a freshwater lentic community (Shaw & Manning, 1996); in contrast to this study they found Ostracoda to increase in abundance in response to copper. However, nine OTUs represented Ostracoda in this study, and of those seven had species weights between 0.5 and -0.5 indicating that the majority showed no response to copper.

Implications for biomonitoring

A much greater proportion of the biotic community was characterised by eDNA metabarcoding than that which could be examined using traditional optical techniques, which were also used to characterise the community present,

identifying 35 different taxa, in the mesocosms over the same time period (Chapter 4). In contrast, 1397 OTUs were distinguished, nearly 40 times the number of taxa identified by optical techniques. In fact, even when OTUs were clustered at the phylum level, there was greater resolution compared to the optical analysis, with 57 different taxon clusters identified. The number of taxa identified by traditional optical analysis is within the range of standard processing of river biomonitoring samples, which typically results in the identification of between 10 – 100 families of macroinvertebrates (Baird & Hajibabaei, 2012). The benefit of eDNA metabarcoding in characterising a greater proportion of the biotic community compared to traditional techniques of biomonitoring has been recognised previously (Baird & Hajibabaei, 2012; Hajibabaei et al., 2011; Chariton et al., 2010a).

With a greater proportion of the biotic community taken into account, the analysis of the effects of copper using eDNA metabarcoding was more sensitive than the analysis by traditional optical techniques (Chapter 4). Using metabarcoding, significant differences in the biological composition of the benthic community amongst all individual treatments were observed (apart from at the final two time points, where similar communities were found in the C/VL and VL/L treatments). In contrast, in the traditional optical analyses, only the C/VL/L treatments and H/VH treatments could be distinguished from each other (Chapter 4). Chariton et al. (2010a) were similarly able to discriminate differences in community composition between reference sites and sites with low levels of contamination. Current biomonitoring practices are often unable to identify sites which are only moderately impacted (Chariton et al., 2010a). Thus, if eDNA metabarcoding is incorporated into biomonitoring programmes, it could provide sought after evidence of moderately impacted sites. This would allow management measures to be implemented earlier, preventing further contamination.

Further comparison of the invertebrate and eDNA metabarcoding data sets indicates that the two methods of bioassessment should be used to support each other, rather than eDNA metabarcoding replacing the traditional technique. Firstly, although the invertebrate analysis identified nearly 40 x less taxa, many of the

macrofauna identified by invertebrate analysis were not found through eDNA metabarcoding – notable exceptions included five families of Hemiptera, five families of Diptera, two families of Trichoptera and a family of Ephemeroptera. This could be due to the relatively small amount of sample 10 g (around 10 ml) that the eDNA was extracted from, compared to the samples processed for invertebrate analysis (100 ml of sediment each). Nevertheless, these larger invertebrates are valuable components of the ecosystem and their characterisation should not be lost form the bioassessment process.

Additional support for the combination of the two bioassessment processes is found from comparison of the results for Nematoda. While the eDNA metabarcoding found that Nematoda were affected by increasing copper concentrations in terms of overall richness, there was no effect of copper on the overall abundance of Nematoda according to the invertebrate analysis. This indicates that the tolerant Nematoda were able to proliferate in the absence of competition from those Nematoda that were lost from the system. It would be ideal if abundance data could be obtained by eDNA metabarcoding. However, attaining the relative abundance of organisms from read counts is confounded by risks of bias from differential PCR amplification and a disparity in the number of copies of target regions within organisms (Baird & Hajibabaei, 2012; Yoccoz, 2012). Thus, it is currently unreliable to estimate abundance or biomass from eDNA metabarcoding and therefore optical analysis is essential to further explore patterns in terms of abundance of eukaryotes.

An aim of this metabarcoding study was to assess the potential of creating ‘eDNA metabarcoding signatures’. From the final time point, 28 specific OTUs were identified to respond either positively or negatively to increasing copper concentrations. Further analysis at other time points is likely to yield additional taxa that show a specific response to copper but were not present in the final time point, for example due to seasonal changes. Thus, this study provides a starting point to the eDNA metabarcoding signature approach. However, further research needs to be conducted with other major contaminants to identify if the biotic responses that are elicited by different contaminants can be distinguished. Further,

the biotic response to copper, and the other contaminants, should be tested under a variety of environmental conditions to identify ubiquitous taxa that consistently respond to each contaminant. Logistically, it would be impossible to create an individual 'eDNA metabarcoding signature' for all contaminants; there are too many, and studies on the scale of this current experiment require too high an investment of money and time. However, if a common biotic response to major groups of contaminants, which are known to have similar modes of action can be determined, eDNA metabarcoding signatures could potentially help identify the contaminants of most concern at complex field sites with poor ecosystem health.

For ecotoxicologists, eDNA metabarcoding also presents opportunities to identify novel indicator taxa for traditional toxicity studies (Chariton et al., 2010a). The OTUs shown to respond to copper represented meio- and micro- fauna and some could be classified to the genus level (e.g. *Navicula)*, providing accurate taxonomic information of possible indicator taxa that could be explored for toxicity studies. However, clearly there is interspecies variation even at the genus level and species level identification would be more informative. Nevertheless, the most promising aspect of eDNA metabarcoding for ecotoxicology studies is the identification of novel indicator taxa within the meio- and micro- fauna, that have previously been underrepresented, if considered at all, in bioassessment by toxicity assays.

Despite its evident potential, eDNA metabarcoding is still in the early stages of development and several limitations remain beyond the issue of obtaining abundance data. Firstly, bias in PCR and sequencing may obscure the presence of rare taxa, while conversely errors may generate artefactual sequences that could errantly be classified as rare taxa (Coissac et al., 2012). A large investment is also required to create high-quality taxonomic databases (Taberlet et al., 2012) and Dejean et al (2011) highlighted that DNA can persist beyond the life span of an individual, confounding the bioassessment of solely live organisms. However, Baldwin et al. (2013) more recently noted that DNA in dead organisms is normally rapidly degraded and only preserved under exceptional circumstances. Further, there is no consensus for a single target marker region for eukaryotic molecular

surveys (Meadow & Zabinski, 2012). Targeting of the gene encoding 18s rRNA has been shown to underestimate true species richness by Tang et al. (2012), who favoured the use of CO1. However, CO1 has poorly conserved priming sites (Tang et al., 2012) so an individual marker with wide coverage across all eukaryote kingdoms is hard to find. Some studies have developed metabarcoding markers for several specific organism groups (e.g. Epp et al. 2012), so multiple markers could be used to gain wider coverage of the biotic community and more accurately capture the biodiversity. However by using multiple markers, sequencing power is vastly reduced. Many of these issues in metabarcoding will be resolved as technologies develop, for example methods are currently being developed to remove the requirement of PCR, removing potential bias and error from that step (Taberlet et al., 2012).

Final conclusions

Ultimately, eDNA metabarcoding is probably not the 'silver bullet', but will add to the line of evidence rather than replace existing methods for biomonitoring (Yoccoz, 2012). It will enable the meio- and micro- fauna, at the base of the ecological food web, and critical to the biogeochemical functioning of the environment, to be routinely assessed. However, the charismatic megafauna, are likely to be underrepresented by this technique, yet still need to be assessed and conserved (Baird & Hajibabaei, 2012). This study is one of the first to provide empirical evidence of the toxic response of copper on the composition of the eukaryote community from the micro- to macro- scale within a freshwater ecosystem, the first using eDNA metabarcoding. It has highlighted the power of the technique and its potential for creating eDNA metabarcoding signatures. With more studies like this one, we may not only be able to identify that an ecosystem has been altered and detrimentally affected by anthropogenic impacts, but also confidently identify the stressor that is causing the most severe impact.

Chapter 6. Exploring the direct and indirect effects of copper at the ecosystem level

The aim of the work presented in this chapter was to characterise effects of copper on the functioning of the mesocosms. A further aim was to identify the importance of direct and indirect effects in driving the biological responses measured. Organic matter decomposition and primary production were assessed through in situ exposures of leaf litter and cotton strips and by quantifying chlorophyll a concentrations, periphyton cover and plant growth, respectively. A variety of direct and indirect effects were observed, which could be explained in part by the responses of P. acuta measured at the organism, population and community levels.

Authors

	Problem formulation	Experimental design/ methodology	Statistical analysis	Results interpretation	Paper preparation
Stephanie Gardham[1,2]	85	90	95	90	92.5
Anthony A. Chariton[2]	5	5	0	2.5	2.5
Grant C. Hose[1]	10	5	5	7.5	5

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Abstract

Copper has been shown to directly affect primary producers and decomposers, which are key players in essential ecosystem processes such as the nutrient cycle. However, even though indirect effects of any contaminant may be more common and more significant than direct effects, the indirect effects of copper on primary producers and decomposers are little known. The effects of copper on phytoplankton, macrophytes, periphyton and organic matter decomposition in an outdoor lentic mesocosm facility were assessed, and links between the responses observed explored. Direct, negative, effects of copper on macrophytes, phytoplankton and subsurface organic matter decomposition were observed. However, periphyton cover and organic matter decomposition on the surface of the sediment were positively affected by the presence of copper. These responses were attributed to indirect effects; they probably occurred because of a decrease in the abundance of snails, particularly *Physa acuta* in the higher copper contaminated mesocosms. This would have indirectly resulted in a reduction in grazing pressure, allowing growth of periphyton, and associated heterotrophs, and ultimately increased decomposition at the sediment surface. The research demonstrates the importance of indirect effects of contaminants, which may not be understood from traditional laboratory studies, in structuring biological communities and associated functioning of ecosystems.

Introduction

Nutrient cycling is essential for the maintenance of aquatic ecosystems and a key ecosystem service on which humans depend (Vanni, 2002; Costanza et al., 1997). Primary producers and decomposers are the two most important groups involved in the process (Daufresne & Loreau, 2001). Decomposers use carbon from organic sources, the majority of which is the detritus of primary producers, while primary producers obtain nutrients from inorganic sources – which have been predominantly mineralised by decomposers (Naeem et al., 2000).

Anthropogenic releases of copper into the aquatic environment have led to concentrations many times higher than background levels (Eisler, 1998), which have been shown to cause a direct effect on aquatic organisms. The direct effects of a contaminant relate to the sensitivity of an organism, or organisms, to that contaminant and are often assessed in the laboratory through toxicity studies (Brinke et al., 2011; Mayer-Pinto et al., 2010). For example, the growth rate of a primary producer, *Elodea canadensis,* was shown to decrease as a direct result of exposure to 1 mg/L copper sulphate (Mal et al., 2002). Direct effects of a contaminant can also be evaluated in mesocosm and field studies; Roussel et al. (2008) showed 75 µg/L copper to directly decrease the abundance of a shredder (*Gammarus pulex* L.) involved in leaf litter decomposition within their stream mesocosms.

In the field, indirect effects appear due to altered dynamics of interacting species, for example, changes in predator-prey relationships or competition (Brinke et al., 2011; Roussel et al., 2007b). A common indirect effect of contamination is an increase in primary production because of a release of grazing pressure following mortality, or reproductive inhibition, of grazing taxa (Mayer-Pinto et al., 2010). Such indirect effects may be more common and more significant than direct effects (Fleeger et al., 2003). Predictions from laboratory studies, for example, by food web modeling, can provide some understanding of indirect effects. However, it is often the case that, despite a wealth of knowledge on the direct effects of a

contaminant, the response of a community in the field to that contaminant can only be poorly predicted (Mayer-Pinto et al., 2010).

The indirect effects of copper on primary producers and decomposers in freshwater environments have only been considered in a handful of experiments (Roussel et al., 2007b, 2008; Le Jeune et al., 2006, 2007; Havens, 1994a; Winner & Owen, 1991; Leland & Carter, 1984). For example, Havens (1994b) attributed an increase in bacterial biomass in the presence of copper to be due to either reduced grazing pressure or nutrient release from dying plankton. Roussel et al. (2007b) unexpectedly found no significant effect of copper on the total abundance of phytoplankton, which they attributed to either the replacement of sensitive species by more tolerant ones, or a decrease in grazing pressure from zooplankton. These previous studies all carried out dissolved, overlying water, exposures of copper to assess the effects on primary producers and decomposers.

The aim of this study was to examine the long-term direct and indirect effects of copper on primary production and organic matter decomposition in model freshwater ecosystems with copper-spiked sediments. Here, a number of biological end-points were assessed: the growth of *Vallisneria spiralis* L., a rooted-submerged macrophyte; chlorophyll *a* concentrations in the phytoplankton (diatoms, cyanobacteria and green algae); periphyton biomass; and leaf litter and cotton strip assays to assess organic matter decomposition. Although, cotton strip decomposition is considered more standardised than leaf litter and has been proposed for use as a functional indicator of water quality (Imberger et al., 2010), the effects of copper on cotton strip decomposition have not been considered previously. The hypothesis was that the direct effects of copper would cause a decrease in abundance of primary producers and rates of organic matter decomposition. However, it was expected that indirect effects of copper might confound the observations of direct effects.

Methods

Study design

Twenty pond mesocosms were established on the Macquarie University campus (33.76946 S, 151.11496 E), in northern Sydney, NSW, Australia. Full details of the infrastructure are included in Chapter 2. Briefly, each mesocosm had a volume of 1500 L, was sunk into the ground, shaded by 70% shade-cloth, aerated and a drainage system along with inputs from rainwater maintained a consistent water depth.

Sediments were spiked with copper prior to opening the mesocosms up to colonisation on 1st November 2010 (t = 0 d). The experiments described in this study were performed once the mesocosms were established and pseudo-equilibration of copper had been achieved, between 5th July 2011 (t = 256 d) and 7th November 2012 (t = 737 d). The experimental design consisted of a control (C) and four sediment copper concentrations (very low (VL), low (L), high (H) and very high (VH)), each with four replicates. The copper partitioning between sediments, pore waters and overlying waters was measured during the present study in November 2011 (t = 365 d), December 2011 (t = 407 d), March 2012 (t = 497 d) and November 2012 (t = 737 d) and average concentrations for each treatment over the exposure period are shown in Table 6.1. Details of the treatment design and establishment of environmentally relevant partitioning of copper in the mesocosms are described in detail in Chapter 2.

Plant growth

On 5th July 2011, 44.5 ± 0.7 g (n = 20), wet weight, of *V. spiralis* was planted in each mesocosm; there was no significant difference in the average mass of plants between treatments at the start of the study ($F_{4,15} = 0.71$, $p = 0.60$). Plant growth was assessed in December 2011 and October 2012 by calculating the shoot-density (no. shoots/m^2) within each mesocosm.

Table 6.1: Copper concentrations in sediments, pore waters and overlying waters during the experimental period (mean ± S.E.M (n = 4)).

Treatment	Sediment copper (mg/kg)	Pore water copper (µg/L)	Overlying water copper (µg/L)
Control (C)	5.8 ± 0.7	3.6 ± 2.2	0.88 ± 0.12
Very low (VL)	62 ± 3.9	5.0 ± 2.2	2.7 ± 2.5
Low (L)	97 ± 10	3.3 ± 0.92	3.7 ± 0.61
High (H)	310 ± 19	16 ± 4.3	10 ± 2.7
Very high (VH)	650 ± 61	31 ± 4.5	14 ± 2.7

Phytoplankton

Chlorophyll *a* was analysed on five occasions during Spring 2012 (September – October). Water samples (500 mL) were collected 15 cm below the surface, stored in the dark (so the phytoplankton became dark adapted) and analysed within three hours using a PHYTO-PAM phytoplankton analyser with an Emitter-Detector Unit PHYTO-ED (Heinz Walz, Effeltrich, Germany). The PHYTO-PAM emits light pulses at four wavelengths (470, 535, 620 and 659 nm) to excite chlorophyll. Fluorescence pulses are detected by a photomultiplier and the signals are analysed using PhytoWin software. An MF32 estimation was carried out, based on standard reference excitation spectra from pure algal cultures in Germany, which allowed a coarse differentiation between chlorophyll *a* concentrations of cyanobacteria, green algae and diatoms (Schreiber, 1998).

Periphyton cover

Periphyton cover on the walls of the mesocosms was assessed on the 7th November 2012. Six open squares 5 × 5 cm were randomly placed against the wall of each mesocosm at a depth of 15 cm. Photos of the squares were taken underwater with a camera (Pentax W90, Tokyo) (Figure 6.1) and image analysis software (ImageJ) was used to analyse the percentage cover of periphyton in each 5 × 5 cm square. Note that this technique measured the periphyton that was clearly visible and of a high biomass (as opposed to that that was of low biomass and less easy to see).

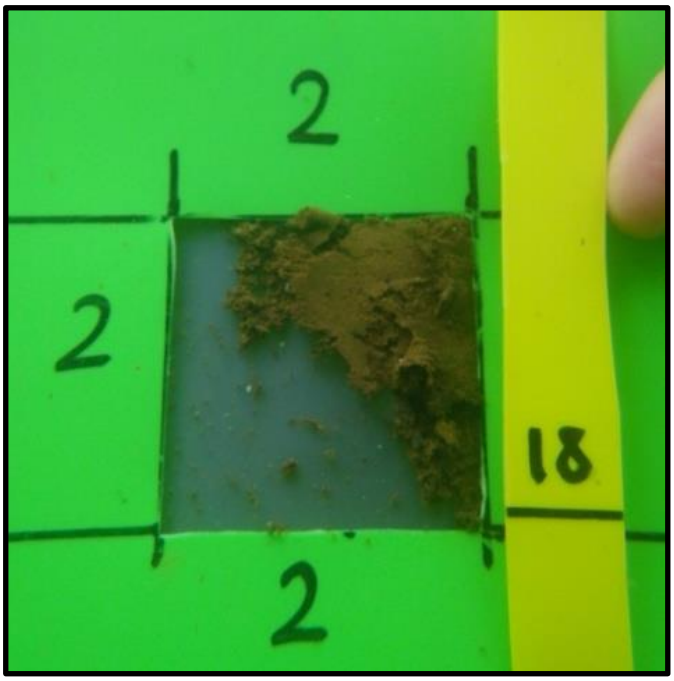


Figure 6.1: Example photo used for periphyton cover analysis (yellow number = mesocosm number, green number = replicate number, square size = 5 × 5 cm).

Organic matter decomposition

Pre-exposure treatments

For cotton strip exposures, a single sheet of calico cotton was obtained from Lincraft Pty Ltd, Miranda, New South Wales. The material was soaked overnight in 10% bleach solution and rinsed ten times using tap water. Cotton strips (5 × 20 cm) were cut from the sheet in the same orientation to minimise variability between strips. Individual strips were placed into plastic mesh cages (mesh size 5 mm).

For the leaf litter exposures, *Eucalyptus* spp. leaves were collected from leaf litter on Macquarie University campus grounds and dried for 3 days at 80°C. Leaves (6 g) were enclosed in plastic mesh cages (5 mm mesh).

Exposure period

Eight cotton strips and four leaf litter parcels were deployed into each mesocosm on the 7th September 2011. The leaf litter parcels and four cotton strips were left on the surface of the sediment (Figure 6.2), while the other four cotton strips in

each meosocm were buried 1 – 2 cm below the sediment surface. Following 70, 105, 140 and 175 days of exposure, one surface cotton strip, one buried cotton strip and one leaf parcel was removed from each mesocosm and stored on ice until processing in the laboratory (1 – 2 hours maximum).

Figure 6.2: Leaf litter parcel placed on the surface of the sediment in a mesocosm.

Post-exposure treatments

In the laboratory, the cotton strips were taken out of the cages, placed into a tray containing water and gently rubbed (to remove soil and debris), then immersed in 70% ethanol (to inhibit further decay), air dried and stored individually in a zip lock bag until analysis. To analyse the extent of decomposition, three 1 × 10 cm sub-samples were cut from the centre of each cotton strip and individually extended using an Instron 5542 at a rate of 50 mm/min until they broke (software: Bluehill 2). The tensile strength was measured as the load (mN) required to break the sub-samples. Two unexposed cotton strips were processed at each time point to account for any potential differences in post-exposure treatment (e.g. differences in humidity). The mean load to break across all unexposed cotton strips at all sampling times was 52000 ± 880 mN; they did not change significantly over time

($F_{3,13}$ = 2.7, p = 0.10). The mean standard deviation (variability) of the three sub-samples from each cotton strip was 2800 ± 130 mN; thus differences less than 2800 mN may be due to methodological variation rather than differences in decomposition.

In the laboratory, leaves were removed from their cages and placed into a tray. The cages and each leaf were washed with tap water over a 106 µm sieve to remove adhering debris and collect small leaf parts. Cleaned leaves were dried in an 80°C oven overnight. The leaves were allowed to cool and then weighed.

Statistical analysis

The mass loss of leaf litter is expressed as the percentage of final weight relative to the initial weight (%IW). To calculate decay rates, leaf litter %IW data and cotton strip final tensile strength data from each individual mesocosm was fitted to an exponential decay curve following the model, as per Tiegs et al. (2007):

$$X_t = X_o e^{-kt}$$

where –
t = exposure time (in days)
X_t = leaf dry weight or cotton strip tensile strength when removed at time t
X_o = initial leaf dry weight or cotton strip tensile strength
k = decay rate.

Statistical analyses were performed in PASW Statistics 18.0.3 (IBM Corp, USA). Data are reported as mean ± standard error (n = 4), unless otherwise stated. Data were transformed to meet assumptions of homogeneity of variance (tested by Levene's test) and approximate normality (tested by Shapiro-Wilk's test) (Table A6.1). For statistical comparisons the significance level α was 0.05, apart from where transformed data failed Levene's test, in which case the significance level was reduced to 0.01 (Underwood, 1997). Endpoints measured at one time point were analysed using a one-way ANOVA with LSD post hoc tests (Table A6.1).

Endpoints measured on multiple occasions were analysed using a repeated measures ANOVA (RM-ANOVA) (between-subjects factor: treatment, within-subjects factor: time) with LSD post hoc tests (Table A6.1). Leaf litter mass loss, and cotton strip tensile strength data were analysed using a two-way ANOVA with LSD post hoc tests. Where an interaction between treatment and time occurred, one-way ANOVAs followed by LSD post hoc tests were performed to test the effect of treatment at individual time points and the effect of time for individual treatments.

Results

Copper concentrations

The sediment copper concentrations followed the expected pattern (C<VL<L<H<VH) ($F_{4,15}$ = 580, p < 0.001) (Figure 6.3a, Table A6.2). At t = 409 d sediment copper concentrations were higher than those measured at other time points ($F_{3,45}$ = 4.5, p = 0.008). There was no interaction between time and treatment ($F_{12,45}$ = 0.62, p = 0.81).

The concentrations of copper in the pore waters changed differently within each treatment over time (Figure 6.3b) (interaction: $F_{12,45}$ = 4.4, p < 0.001). Within each time point, there was always an effect of treatment (Figure 6.3b, Table A6.2), although individual treatments were not always different from each other – generally the pore water concentrations of the C/VL/L treatments were lower than the H/VH treatments. In general earlier time points had lower pore water copper concentrations than later time points (Figure 6.3b).

The overlying water copper concentrations followed the expected pattern (C<VL<L<H<VH) ($F_{4,15}$ = 150, p < 0.001) (Figure 6.3c). Copper concentrations in the overlying waters decreased between t = 368 d and t = 409 d, then remained stable to t = 500 d but were higher at 737 d ($F_{6.4,24}$ = 0.92, p = 0.50) (Table A6.2). There was no interaction ($F_{1.6,24}$ = 15, p < 0.001).

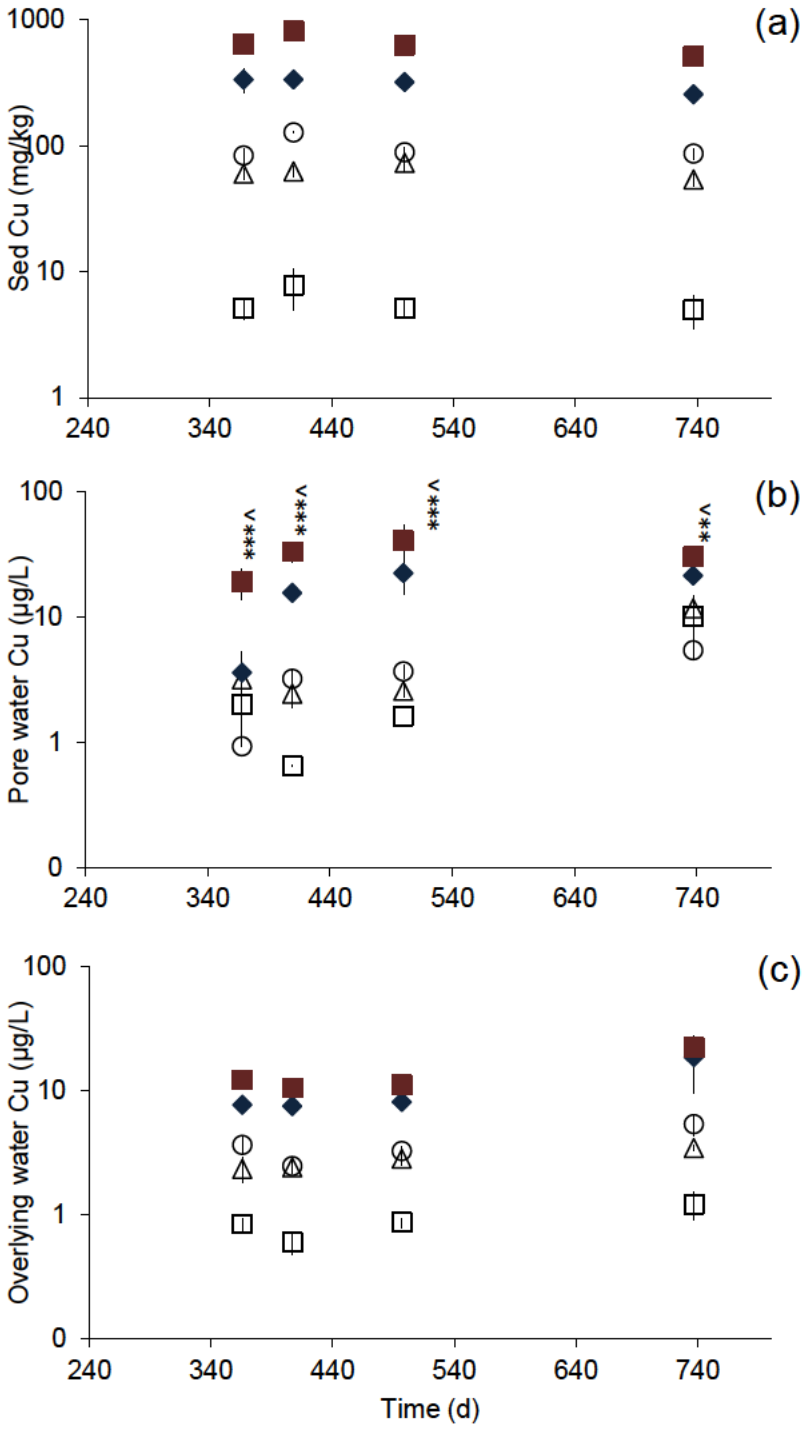


Figure 6.3: Changes in copper concentrations within (a) sediments (b) pore waters and (c) overlying waters over time. Key: control (□), very low (△), low (○), high (◆) and very high (■). The main effect of treatment based on the one-way ANOVAs performed at each time point on the pore water data (due to a significant interaction between time and treatment) are displayed above each respective time point: ** = p<0.01, *** = p<0.001, ^ = homogeneity of variance p < 0.05.

Primary producers

Shoot density generally increased over time and was lower in higher copper treatments, but there was an interaction between time and treatment ($F_{4,10}$ = 16, p < 0.001) (Figure 6.4). In December 2011, there was a minimal effect of shoot density ($F_{4,14}$ = 2.9, p = 0.06); shoot density was only higher in VL/L treatments compared to the VH treatment. By October 2012 the effect of treatment was clear ($F_{4,10}$ = 19, p < 0.001); C/VL treatments had higher shoot densities than the L treatment, which in turn had a higher shoot density than the H/VH treatments (Figure 6.4, Table A6.3). The shoot densities in the C/VL/L/H treatments increased over time, but this did not occur in the VH treatment ($F_{1,3}$ = 3.5, p = 0.160).

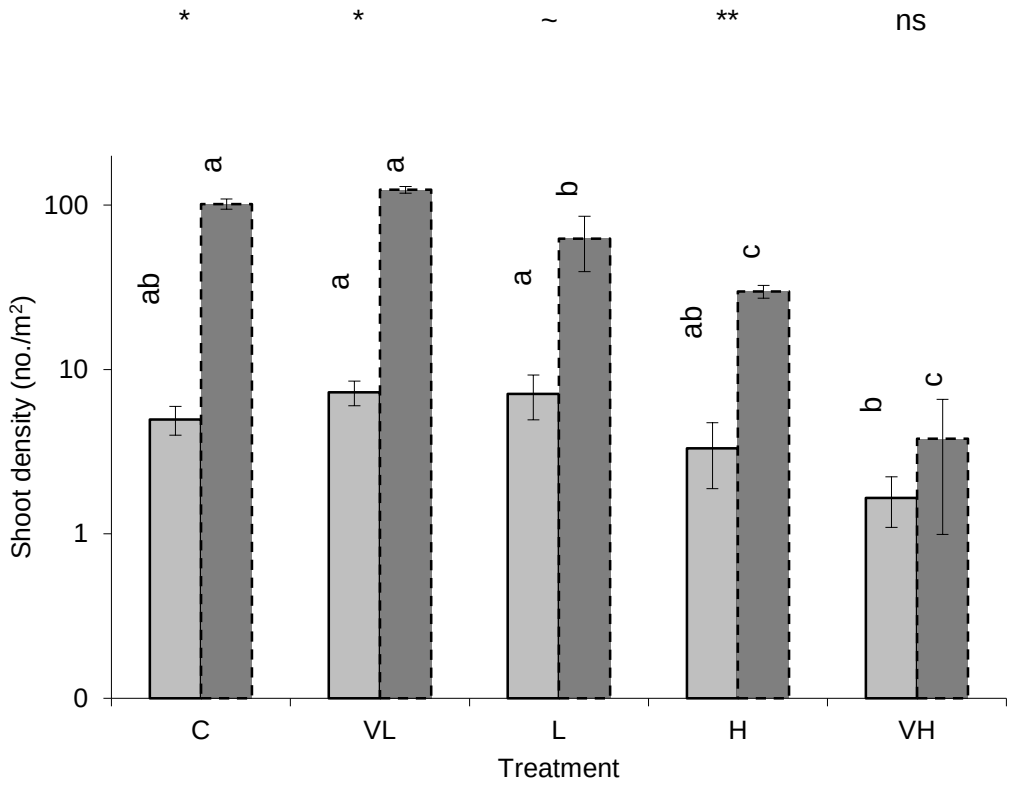


Figure 6.4: Shoot density of *V. spiralis* in December 2011 and October 2012. Treatments that do not share a letter (directly above the bars) within each time point are significantly different from each other. The effect of time for each treatment is also displayed: * = p<0.05; ** = p<0.01; ~ = near significant; ns = not significant.

Periphyton cover was positively affected by treatment ($F_{4,15} = 7.9$, $p = 0.001$); it was highest in the H/VH treatments compared to the C/VL/L treatments (Figure 6.5, Table A6.3).

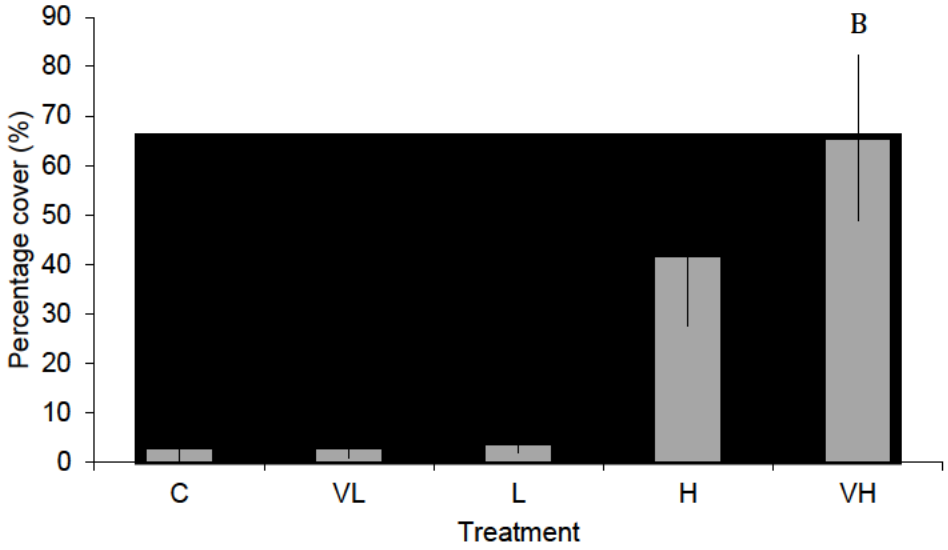


Figure 6.5: Periphyton cover on the mesocosm walls. Letters represent the post hoc LSD analysis, where different letters denote a significant difference between treatments.

The highest chlorophyll *a* concentrations were found in the diatoms, followed by the cyanobacteria; very little chlorophyll *a* was attributed to the green algae (<1.3 µg/L). Total, diatom, cyanobacteria, and green algae chlorophyll *a* concentrations were not different among copper treatments ($p > 0.05$) and did not change over time ($p > 0.05$) (Figure 6.6, Table A6.3). There were no interactions between time and treatment ($p > 0.05$) (Table A6.3). However, there were large variations in total, diatom and cyanobacteria chlorophyll *a* concentrations in the C/VL/L treatments, indicating that some mesocosms had high chlorophyll *a* concentrations. In contrast, the variation in the H/VH treatments was low as chlorophyll *a* concentrations were always low (Table A6.3).

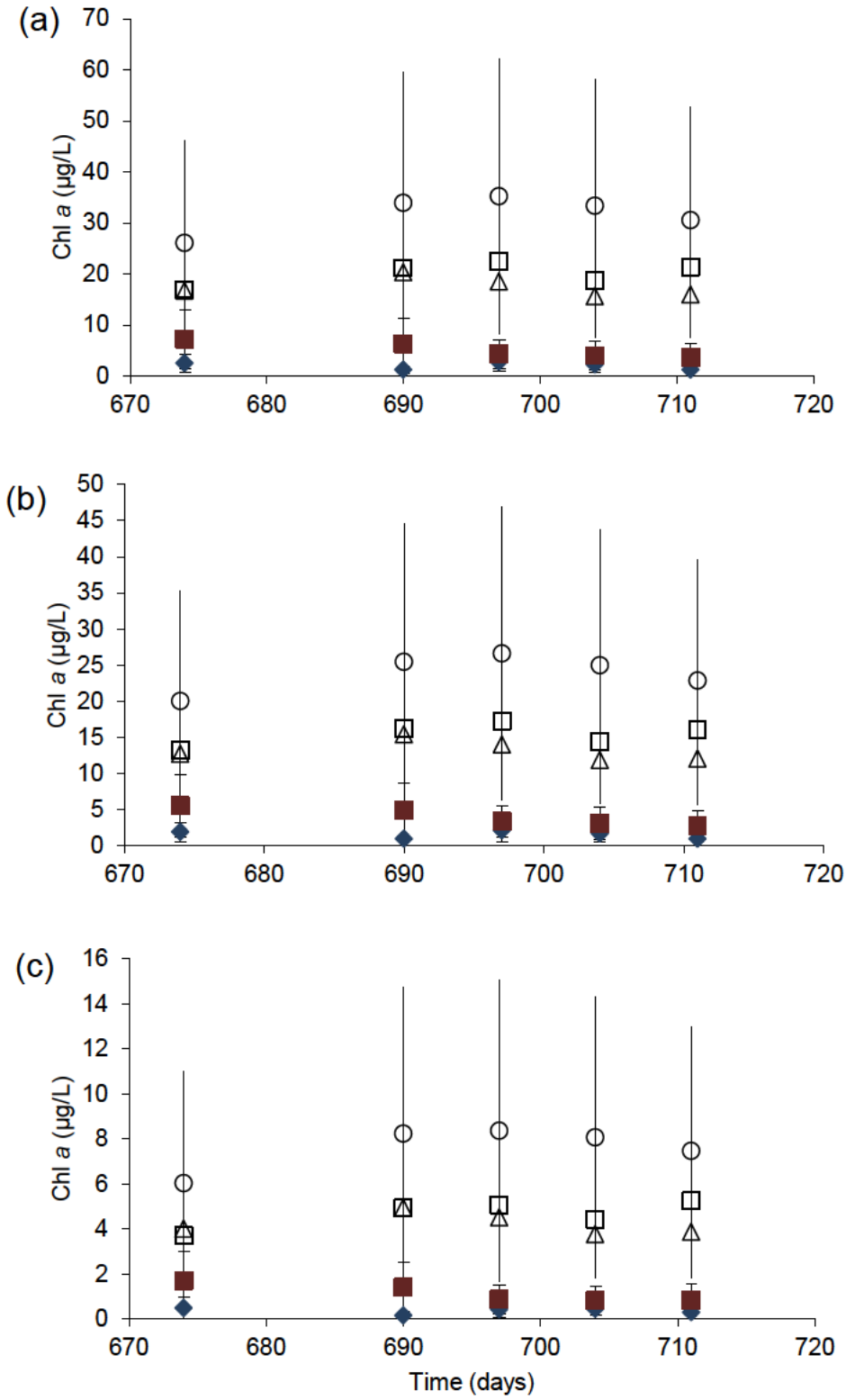


Figure 6.6: Change in (a) total; (b) diatom; and (c) cyanobacteria chlorophyll *a* concentrations in the phytoplankton over time. Key: control (□), very low (△), low (○), high (◆) and very high (■). H/VH treatments have capped error bars, showing their low variation. Green algae data are not shown.

Organic matter decomposition

The weight of leaf litter decreased over time ($F_{3,60} = 52$, $p < 0.001$) (Figure 6.7a, Table A6.4). However, copper treatment did not affect the loss of leaf litter mass ($F_{4,60} = 1.1$, $p = 0.37$); there was no interaction between time and treatment ($F_{12,60} = 0.83$, $p = 0.62$). Leaf litter decay rates were also unaffected by treatment ($F_{4,15} = 0.36$, $p = 0.84$).

The tensile strength of cotton strips on the surface of the mesocosms decreased with time ($F_{3,60} = 34$, $p < 0.001$) and unexpectedly decreased with increasing copper treatment ($F_{4,60} = 5.1$, $p = 0.001$) (Figure 6.7b, Table A6.4). The control had a higher tensile strength (i.e. was less decomposed) compared to all other treatments ($p < 0.01$). There was no interaction between treatment and time ($F_{12,60} = 0.77$, $p = 0.68$). However, treatment did not affect decay rates of the surface cotton strips ($F_{4,15} = 1.6$, $p = 0.22$).

The tensile strength of the subsurface cotton strips decreased over time ($F_{3,60} = 25$, $p < 0.001$) and increased with increasing treatment, as expected ($F_{4,60} = 14$, $p < 0.001$) (Figure 6.7c, Table A6.4). The pattern was C < VL/L < H/VH (i.e. C treatments the most decomposed) ($p < 0.01$). There was no interaction between treatment and time ($F_{12,60} = 0.53$, $p = 0.89$). Treatment did not affect the decay rates of the buried cotton strips ($F_{4,15} = 1.1$, $p = 0.38$).

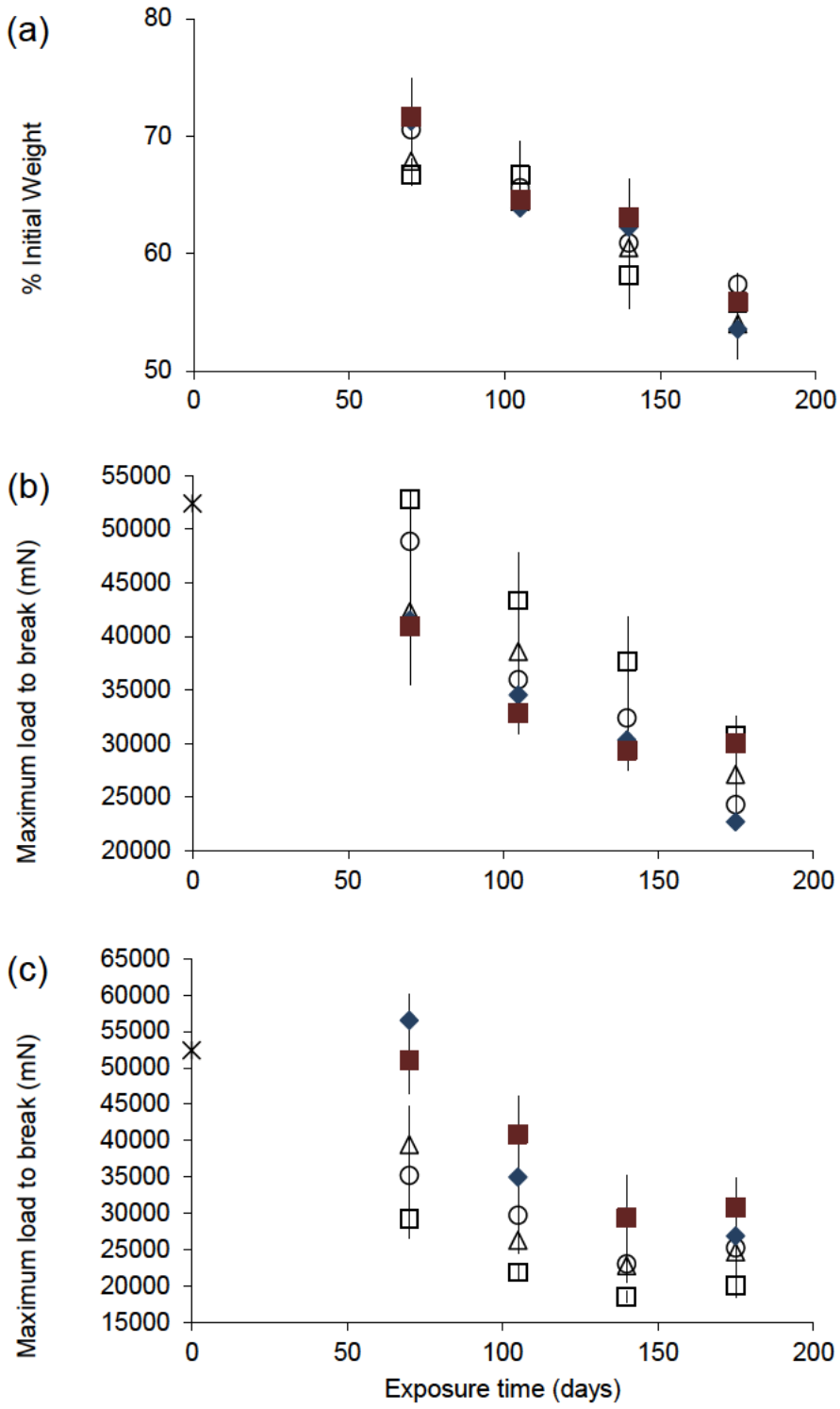


Figure 6.7: Change in (a) %IW of leaf litter; (b) tensile strength of surface cotton strips and (c) tensile strength of buried cotton strips during the 25 week exposure period. Key: control (□), very low (△), low (○), high (◆) and very high (■); × = tensile strength of unexposed cotton strips.

Discussion

The results show that indirect effects of copper contamination under field conditions can mask direct effects, producing a response at the community and ecosystem level entirely different to that observed through toxicity testing in the laboratory. For example, all previous research indicates that the direct effect of copper causes a decrease in periphyton growth (Serra & Guasch, 2009; Serra et al., 2009a; Barranguet et al., 2003; Guasch et al., 2002; Soldo & Behra, 2000) and similarly organic matter decomposition on the sediment surface (Duarte et al., 2008; Roussel et al., 2008; Leland & Carter, 1985). However, it is likely that due to the loss of a dominant grazer, *Physa acuta* (Chapter 4), in higher treatments, copper indirectly reduced grazing pressure on the periphyton and associated heterotrophs and, as a result, an increase in periphyton and surface organic matter decomposition with increasing copper concentrations was observed. While this response may be predicted from laboratory studies by, for example, food web modeling, the precise community response is dependent on the site-specific composition and environmental factors influencing that community.

Effects of copper on primary producers

The presence of copper inhibited the growth of *V. spiralis* with biological effects observed in the H and VH treatments. This response was attributed to the direct effects of copper as it is consistent with previous research (e.g. Wang et al., 2011). Possible mechanisms of action have previously been explored; decreases in growth rate were observed to occur in conjunction with a reduction in total chlorophyll within leaves and shoots, a decrease in nitrate reductase activity and protein content and an increase in the induction of active oxygen species (Wang et al., 2011; Vajpayee et al., 2005; Sinha et al., 1994). These effects of copper on *V. spiralis* were observed during 24 – 168 hour laboratory exposures where the minimum dissolved copper concentrations that produced an effect were between 100 – 1900 µg/L (Wang et al., 2011; Vajpayee et al., 2005; Sinha et al., 1994). The pore water and overlying water concentrations of copper in the H and VH treatments of this study were much lower (between 10 ± 2.7 and 31 ± 4.5 µg/L).

However, the plants were exposed for 472 days, thus it is likely that effects at lower concentrations were observed due to the longer exposure period.

Similar to inhibiting vascular plant growth, the presence of copper in the H and VH treatments (10 ± 2.7 and 14 ± 2.7 µg/L) reduced total chlorophyll *a* concentrations in the phytoplankton, due to a decrease in chlorophyll *a* concentrations of the diatom and cyanobacteria communities (chlorophyll *a* concentrations of the green algae were consistently low). Previous short term (4 – 15 day) studies have also found phytoplankton biomass to be reduced as a direct effect of copper exposure (Barranguet et al., 2003; Havens, 1994ac). However, in more environmentally realistic scenarios, similar to this one, copper exposure has caused a variety of effects on phytoplankton biomass. For example, in spring, Winner & Owen (1991) found copper concentrations of 20 and 40 µg/L to reduce algal densities in pond enclosures, however no effect on algal density was found during summer, and in autumn algal densities were only significantly reduced on the final day of exposure (after 5 weeks) (Winner & Owen, 1991). Inconsistent effects of copper were also found by Le Jeune et al. (2006), when they exposed natural spring and summer phytoplankton communities to copper over 27 days in glass aquaria; although they observed a decrease in chlorophyll *a* concentrations at 160 µg/L of copper, at 80 µg/L chlorophyll *a* concentrations increased in spring, but decreased in summer. In addition, Roussel et al. (2007) found no significant effect of copper (4 ± 0.2, 20 ± 0.4 and 57 ± 0.6 µg/L) on the total abundance of phytoplankton during an 18 month exposure in stream mesocosms.

Even though copper has caused a variety of effects on phytoplankton biomass, the community composition always changed as a result of copper exposure. Le Jeune et al. (2006) found the diatom *Rhizosolenia ereinsis* dominated the community at 80 µg/L in spring (when phytoplankton biomass exceeded that of the control). While in Roussel et al. (2007), copper caused a change in the dominance from three species of diatom (*Amphora* sp., *Cocconeis* spp. and *Epithemia* sp.) to green algae (*Mougeiotia* sp. and *Scenedesmus* spp.) and a different diatom species (*Nitzschia* spp.). When Winner & Owen (1991) found no effect of copper on phytoplankton biomass in summer and autumn, *Cryptomonas* and *Peridinium*

(summer) or *Cyclotella* (autumn) were replaced by *Uroglena* (summer) or *Dinobryon* (autumn), while, in the spring (when a reduction in biomass was observed) *Cryptomonas, Uroglena* and *Peridinium* all decreased in abundance and no organisms replaced them (Winner & Owen, 1991).

Clearly, there is diversity in susceptibility to copper within the phytoplankton community and even within algal species of the same genus (e.g. *Uroglena*). Therefore, when a phytoplankton community is exposed to copper, the effect that copper will have is dependent on the species that are present; the direct effect of copper will decrease the biomass of sensitive species and indirectly reduce competition for tolerant species, so if present they could proliferate and an overall increase in algal biomass would be observed. In this study, there must have been few or no copper tolerant species of diatom, green alga or cyanobacteria in the mesocosms, leading to the decrease in phytoplankton biomass observed as a result of copper.

The presence of copper led to an increase in periphyton biomass in the H and VH treatments, which was not expected based on the perceived direct effects of copper on primary producers. This response is probably due to an indirect effect; the overlying water copper concentrations of the highest copper treatment (VH) was 14 ± 2.7 µg/L, and past studies exploring the effects of copper on periphyton in this dissolved concentration range (6 – 317 µg/L), have either found no effect, or a reduction in periphyton biomass as a result of copper exposure (Serra & Guasch, 2009; Serra et al., 2009a; Barranguet et al., 2003; Guasch et al., 2002; Soldo & Behra, 2000). For example, exposure to 32.5 µg/L copper for four weeks resulted in a lower periphyton algal biomass of 0.80 ± 0.17 µg chl *a*/cm^2, compared to 1.37 ± 0.14 µg chl *a*/cm^2 in control treatments. Therefore, given that the overlying water copper concentrations were low in this study, there was probably no, or a minimal, direct effect of copper on periphyton biomass. However, in this study, the gastropod *Physa acuta*, was present, but the growth of individuals, and population abundance was lower in higher treatments due to the direct effect of copper (Chapter 3, Chapter 4). Thus it is possible that a high grazing pressure in low treatments prevented development of the periphyton, while

in the H and VH treatments, because copper was directly affecting gastropod abundances, grazing pressure was reduced and the periphyton biomass could develop. Further evidence of this is that in the first spring/summer season, the periphyton cover appeared similar across treatments (personal observation), and it only decreased in the C, VL and L treatments as the populations of snails in those treatments proliferated during the second spring/summer season (the snails were introduced in between the spring/summer seasons via the introduction *V. spiralis* for the plant growth experiment). Roussel et al. (2007b) also described a similar indirect effect of copper leading to an increase in periphyton biomass in their copper contaminated stream mesocosms.

Effects of copper on decomposition

The leaf litter assay did not reveal any differences in organic matter decomposition between treatments. This finding contrasts with those of previous studies (Duarte et al. 2008; Roussel et al. 2008; Leland & Carter 1985). This difference in results may be due to the random use of multiple species of *Eucalyptus* leaves in this study. There are a wide range of decomposition rates across tree species due to leaf quality (Tiegs et al., 2007). Thus an increase in the variability of decomposition may have concealed differences in leaf litter decomposition between treatments. However, the variation observed in this study was not inconsistent with other studies, for example, the largest variation in percentage mass loss reported by Duarte et al. (2008) was 6% (S.E.) while the largest variation in this study was 3% (S.E.).

The contrast in results of the leaf litter assay between this study and previous studies may have been because decomposition rates in this study were much lower than previous studies; with lower decomposition rates, the test could be less sensitive. For example Duarte et al. (2008) recorded a maximum percentage mass loss of 80% over just 40 days, compared to this study, where the most decomposed leaf litter was reduced in mass by just 46% after 175 days. The lower decomposition rates observed in this study may be attributed to the choice of leaf litter; previous studies exploring the effects of copper have used leaves of alder,

willow, aspen, oak or maple, which are generally broken down more readily than *Eucalyptus* spp. (Canhoto & Graca 1996). Perhaps, as there were no external sources of nutrients to the mesocosms in this study, their oligotrophic status meant that decomposition rates in the mesocosms were so low that there was not a real difference between treatments. However, a clear biological effect using cotton strip assays was observed. A more standardised method of using leaf discs in each parcel so the surface area to volume ratio was the same across leaf litter bags (as per Duarte et al. (2008)) may have increased the potential to differentiate decomposition in leaf litter between treatments in this study.

Similar to Tiegs et al. (2007), the cotton strip assay proved to be more sensitive than the leaf litter assay. Although there was little difference in decay rates, there were clear differences in the tensile strength of cotton strips among treatments both on the surface and buried within the sediment. Interestingly, opposite patterns were observed on the sediment surface compared to below; subsurface cotton strips were more decomposed in treatments with lower copper concentrations, whereas, the cotton strips exposed on the surface of the sediment were generally less decomposed in the control treatments compared to copper contaminated ones.

The inhibition of subsurface cotton strip decomposition is consistent with previous studies (Duarte et al., 2008; Roussel et al., 2008; Leland & Carter, 1985). Although these studies are not directly comparable as they considered decomposition of leaf litter (not cotton strips) on the surface (not buried below the sediment surface) they clearly identified that copper directly causes a reduction in organic matter decomposition. The studies showed the inhibition of organic matter decomposition at copper concentrations of 2.5 – 15 µg/L (Leland & Carter, 1985), 75 µg/L (Roussel et al., 2008) and 6350 µg/L (Duarte et al., 2008). The large variation in effect concentrations across studies, demonstrates that the susceptibility of microbial communities and therefore organic matter decomposition rates to copper is highly dependent on multiple factors which likely include substrate type and other physical characteristics of the location.

The pattern observed with the cotton strips on the surface of the sediment contrasts previous findings of the direct effect of copper on organic matter decomposition (Duarte et al., 2008; Roussel et al., 2008; Leland & Carter, 1985). Because cellulose is almost exclusively broken down by microbiological processes (Lategan et al., 2010), it is likely that the patterns observed are due to differences in the microbial community. One possible reason for the observed pattern is linked with the higher biomass of periphyton cover in the H and VH treatments compared to the control. With a higher biomass of periphyton, it is reasonable to assume that there would be a higher biomass of heterotrophs within the biofilm, leading to increased decomposition within the H and VH treatments. Alternatively, the overlying water concentrations of copper in the H and VH treatments were lower than the pore water concentrations (10±2.7 – 14±2.7 instead of 16±4.3 – 31±4.5 µg/L). In addition, previous research has shown that iron and manganese oxides in surface sediments have resulted in a decrease in the bioavailability of nickel due to increased complexation (Costello et al., 2011). Thus it is possible that the bioavailability of copper was lower on the surface than subsurface and did not inhibit decomposition. Further, the presence of copper at lower concentrations may have improved the ability of the cellulose-degrading microorganisms to break down cellulose through inducing additional enzyme pathways; for example, Quinlan et al. (2011), demonstrated that the glycoside hydrolase family of enzymes (found within fungi) are copper-dependent oxidases that can enhance the initial disruption of cellulosic structure. In addition (Burton et al., 1987), observed a significant correlation between stream concentrations of copper and both gglucosidase and galactosidase activity in a stream that received point a nonpoint metals from a mine and city.

Final conclusions

Copper directly decreased macrophyte growth, chlorophyll *a* concentrations and subsurface organic matter decomposition. However, a higher periphyton biomass in copper treatments was also observed, possibly due to a reduced grazing pressure by gastropods. This increase in periphyton biomass in the higher copper treatments may have been associated with higher abundances of heterotrophs

and the slight increase in organic matter decomposition observed on the surface of the sediments in the H and VH treatments. This study has confirmed that the direct effects of copper on dominant grazers can lead to indirect effects within aquatic ecosystems, which shape the communities present and ultimately modify the functions that the biota perform.

Chapter 7. Discussion

The discussion summarises the findings of this research and integrates the responses observed across levels of biological organisation. Within the chapter future opportunities and directions for the research are also identified.

There is an ever-pressing question in ecotoxicology as to whether knowledge of the effects of contaminants at low levels of biological organisation (i.e. molecular, biochemical, physiological and organism level effects; see Chapter 1) can be used to predict effects at high levels of biological organisation (population, community and ecosystem level effects). In their review of the current understanding of the effects of metals on aquatic assemblages, Mayer-Pinto et al. (2010) identified that, although metals can be toxic from laboratory studies, few studies have assessed the effects of metals under natural conditions at higher levels of biological organisation. Chapter 1 showed that the generalisations Mayer-Pinto et al. (2010) made for metals are true for copper: 96% of the records held within the U.S. EPA Ecotoxicology database on freshwater copper toxicity were derived from laboratory studies. There were just 34 field based artificial ecosystem studies, of which only four considered effects at both lower and higher levels of biological organisation, and of those, links between the levels of biological organisation were tenuous, if considered at all. However, from this data, guidelines for copper have been made, and risk assessments based on projected or current copper concentrations in aquatic ecosystems are performed – is this right?

In lieu of this question, the main aim of this study was to identify relationships between the effects of copper at low levels of biological organisation and the responses observed at high levels of biological organisation. With effects characterised at organism, population, community and ecosystem levels, relationships across levels of biological organisation were identified (Figure 7.1). The most prominent relationship identified was driven by the presence/absence of snails, which appeared to play a critical role in structuring the ecosystems within the control and low copper treatments. Their abundance decreased with increasing copper concentrations (Chapter 4), which could, in part, explain the observed increase in periphyton cover (due to lower grazing pressure) and organic matter decomposition (due to increased periphyton cover) in the high copper treatments (Chapter 6). Further, during the *in situ* exposures (Chapter 3), the inhibitory effect of copper on the growth of *P. acuta* demonstrated that individual snails in the higher treatments had a reduced fitness and so direct effects of copper on the population of snails within those treatments were plausible.

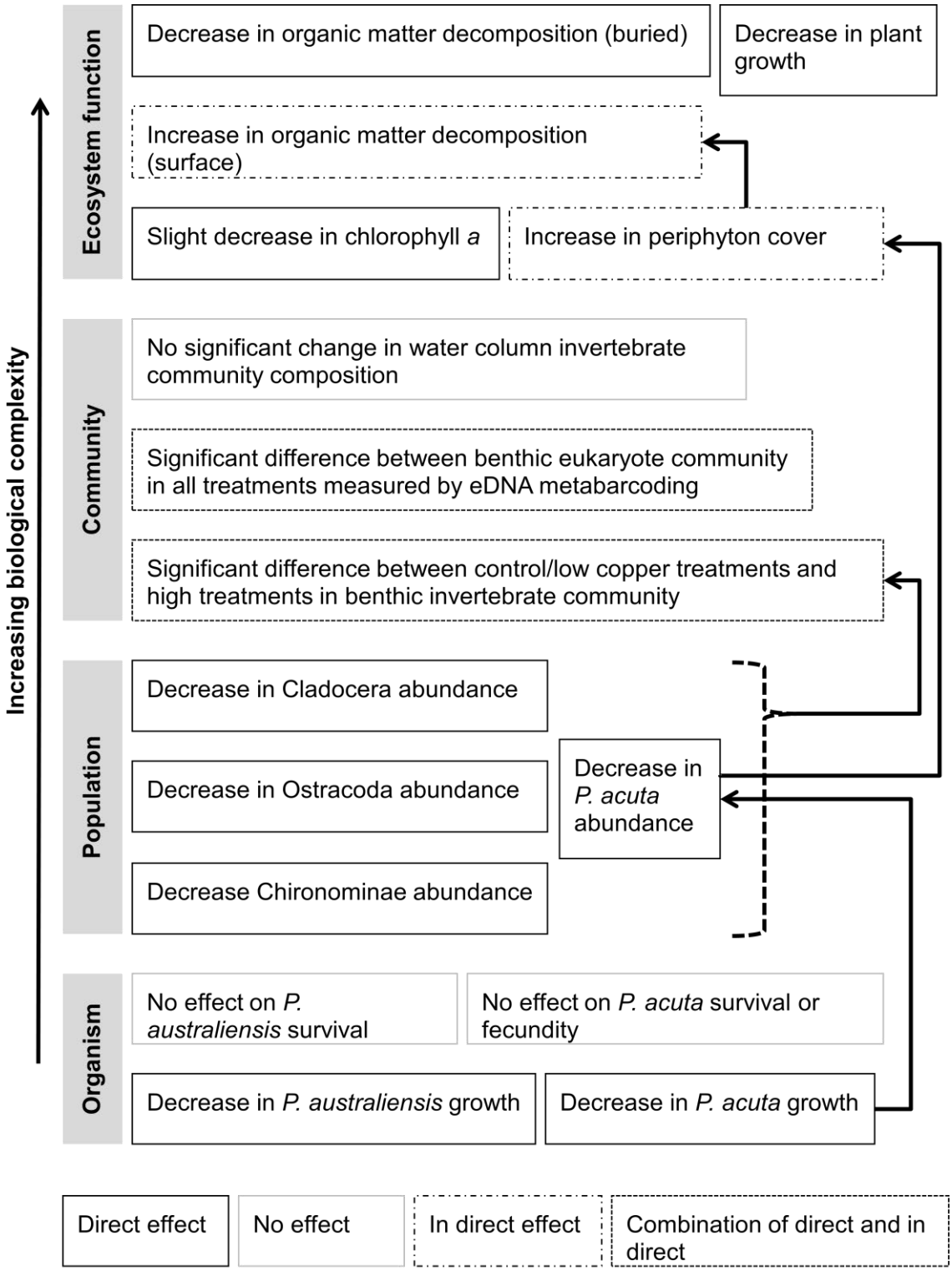


Figure 7.1: Summary of responses to copper observed at each level of biological organisation and potential links between each level. NB: responses at the community level are simplified to just community composition.

To consider the underlying question of this research, 'whether effects at low levels of biological organisation can be used to predict effects at population, community and ecosystem levels'; effects on the snails at the population level were predictable based on the decreased fitness under copper exposure. However, both periphyton cover and organic matter decomposition were expected to decrease based on previous knowledge of the direct effects of copper on both end points (Serra et al., 2009a; Duarte et al., 2008; Roussel et al., 2008; Barranguet et al., 2003). In fact, the increase in primary production because of a reduction in the abundance of grazers is a common response to contamination in the field (Mayer-Pinto et al., 2010). However, this response could only be predicted by considering how snails and periphyton interact. The research clearly demonstrates that indirect effects of a contaminant can be more influential in an ecosystem than individual direct effects. Thus, I agree with Fleeger et al. (2003), in concluding that currently, the meaningful extrapolation of data from low to high levels of biological organisation, without considering the biological interactions occurring within an ecosystem, is impossible.

In order to enable prediction of effects at high levels of biological organisation from those at low levels, a greater understanding of how contaminants like copper affect the interactions between organisms and the relative importance of direct and indirect effects is needed. In this regard, within this study, much knowledge has been gained by assessing the effects at multiple levels of biological organisation (Chapter 3 – Chapter 6) and indirect effects of copper have successfully been identified (Figure 7.1). However, further research at the mesocosm facility would help identify more indirect effects that are occurring. In particular, analysis of the food web structure would be useful: it would allow predator-prey relationships within each treatment to be identified, and therefore show where changing interactions between predators and prey have occurred due to copper. Such models have rarely been applied in risk assessments (De Laender et al., 2013), so this would be an exciting avenue for future research at the facility.

Ultimately, if the direct and indirect effects of contaminants can be characterised and their importance in structuring ecosystems understood, the data could be

used to create more robust ecosystem models (Fleeger et al., 2003). Recent modeling based methods for assessing the effects of contaminants on natural communities assume overall impacts represent an accumulation of direct effects and there have been limited attempts to incorporate indirect effects (Rohr et al., 2006). If more accurate ecological models, which incorporate direct and indirect effects of contaminants, could be made for aquatic ecosystems, such models could be used to predict effects of novel contaminants (with similar modes of action) on ecosystems without the need for large scale, mesocosm studies. Thus, returning to the original research question, a large amount of research is needed, but in the future the prediction of effects at population, community and ecosystem levels based on effects at low levels of biological organisation may be possible.

A note on the use of mesocosms

The only way to assess population and community level effects of a contaminant in a realistic environment, that incorporates indirect and direct effects, is by using mesocosms (Van den Brink, 2013). However, despite allowing analyses of ecological interactions, there have been several criticisms of previous mesocosm experiments. For example, often manipulative studies that have explored the effects of contaminants at higher levels of biological organisation have still held environmental variables (e.g. temperature and light) constant (Mayer-Pinto et al., 2010). As a result, even though intra- and inter- specific interactions can occur, they may still be different to those occurring in the field (because maintaining organisms under these constant conditions may affect organism behaviour and cause physiological stress) (Mayer-Pinto et al., 2010). In addition, many previous studies were not designed with adequate replication or controls and therefore robust conclusions could not be drawn (Mayer-Pinto et al., 2010).

Further to these issues, a major flaw common in mesocosm studies is the use of unrealistic exposure scenarios when assessing the effects of contaminants that are predominantly adsorbed by the sediments in the field (this is particularly true with respect to previous mesocosm studies exploring the effects of copper; Chapter 1). Most historic contaminated sites contain the majority of such

contaminants, including copper, in the sediments, with low concentrations in the pore waters and even lower concentrations in the overlying waters (de Deckere et al., 2011). As such, sediments are now considered a major source of contamination, which may be transferred into benthic organisms and further up the food chain (Van Geest et al., 2010). For example, biofilms containing copper may be a source of metals to grazing animals (Chapman et al., 2003). However, only one previous study considered sediments as a source of contamination (Hoang et al., 2011), and that study, confounded in its experimental design, could not identify cause-effect relationships between copper and the biotic response (Chapter 1).

This is probably the first study that has truly identified the effects of copper at higher levels of biological organisation using a realistic exposure scenario representative of historically contaminated sites. However, the study is a static mesocosm study and, while it has increased our understanding of the effects of copper, there should still be caution in extrapolating these results to real sites due to the influences of multiple stressors and changing environmental variables. For example, the pH of the overlying waters was too high for most field sites (Chapter 2) and the water was hard (where hardness is a key determinant of metal bioavailability), which should be considered if extrapolating to reals sites where the water is soft. Nevertheless, by using an environmentally realistic contaminant exposure scenario, changes in the benthic community and effects on the growth of both snails and shrimp were observed (Chapter 3 – Chapter 5). This is extremely important, because to model the potential ecological effects of contaminants in natural environments appropriately, data that is representative of those natural environments is needed.

Implications for the current assessment of ecosystem health

The incorporation of ecological models into environmental risk assessment is a great challenge for the future (Van den Brink, 2013). However, we are far from understanding effects at the population and ecosystem levels (Chapman et al., 2003), and incorporating these effects into ecosystem models will take even

longer. In the mean time we still need to be able to assess ecosystem health, and how it may change due to contamination, as contamination is a current, not a future, problem.

This study was performed in Australia, therefore, nominal sediment concentrations were based on the Australia and New Zealand guidelines for fresh and marine water quality (ANZECC & ARMCANZ, 2000) (Chapter 2). By comparing the measured concentrations in this study against these chemical guidelines an assessment of ecosystem health based on copper concentrations can be made: there would be the expectation that clear effects on the biota within the water column of the H/VH treatments, a possible effect on biota in the L treatment and no effect on biota within the VL treatment would occur (Table 7.1). Bioassessments performed in relation to the water column included analysis of the invertebrate community, where no effect was observed (Chapter 4), and phytoplankton chlorophyll *a* concentrations, which appeared lower in the H/VH treatments compared to the C/VL/L treatments, although the effects were not significant (Chapter 6). This indicates therefore that the trigger values for dissolved concentrations of copper in the overlying waters are protective, predicting severe effects when none were observed.

In relation to the benthic community, based on the copper guidelines, a clear effect on the biota within the benthos of the H/VH treatments, and an effect, though perhaps not as evident, in the L treatment would be expected (Table 7.1). In the VL treatment, copper bioavailability should be low and effects minimal – although possible due to the high sediment copper concentrations. For the benthic biota, there was nearly always a significant effect of copper on the measured response for the H/VH treatments (Table 7.1). For the L treatment, a response to copper was only really evident at higher levels of biological organisation, through assessments of the effects on organic matter decomposition, primary production (through plant growth) and by incorporating the meio- and micro- fauna into the benthic eukaryote community response (Chapter 5 and Chapter 6). Further for the VL treatment, effects on primary production through plant growth were not evident, but the responses observed on organic matter decomposition and the benthic

eukaryote communities (Chapter 5 and Chapter 6), indicate that the microbial component of the community was affected.

Table 7.1: Identification of response variation* from the control treatment

				Very low	Low	High	Very high
Exceedance of Australian and New Zealand guidelines for copper^			Sediment				
			Pore water				
			Overlying water				
Organism	*Physa acuta*		Survival				
			Reproduction				
			Growth				
	Parataya australiensis		Survival				
			Growth				
Population	Benthic Chironominae		Abundance				
	Benthic Cladocera		Abundance				
	Benthic Ostracoda Sp 2		Abundance				
	Benthic *Physa acuta*		Abundance				
	Benthic Copepoda		Abundance				
Community	Benthic invertebrates		Abundance				
			Richness				
			Diversity				
			Composition				
	Water column invertebrates		Abundance				
			Richness				
			Diversity				
			Composition				
	Sweep net invertebrates		Abundance				
			Richness				
			Diversity				
			Composition				
	Benthic eukaryotes (eDNA metabarcoding)	Cercozoa	Richness				
		Chlorophyta	Richness				
		Nematoda	Richness				
		Ciliophora	Richness				
		Ascomycota	Richness				
		Chytridiomycota	Richness				
		Rotifera	Richness				
		Total community	Richness				
			Composition				
Ecosystem function	*Valisneria spiralis*		Growth				
	Periphyton		Biomass				
	Phytoplankton		Chlorophyll *a*				
	Leaf litter (surface)		Decomposition				
	Cotton strip (surface)		Decomposition				
	Cotton strip (buried)		Decomposition				

*green = response similar to control, orange = response appears different but not significant, red = significant difference in response compared to control.

^Red = highest sediment quality guideline exceeded / derived HMTV to protect 80% species exceeded. Orange = low sediment quality guideline exceeded / derived HMTV to protect 99% of species exceeded. Green = concentrations below guideline levels.

Overall, the benthic response indicates that the current chemical guidelines are in the right range for the protection of benthic biota as they did highlight that effects may occur, even in the lowest copper treatment. However, the guidelines would have only triggered further assessments into factors controlling bioavailability and acute and chronic toxicity testing to consider the risks to the ecosystem and potential management actions required (ANZECC & ARMCANZ, 2000). Although standard acute and chronic toxicity tests were not performed, based on the *in situ* exposure results (Chapter 3), these assessments may have concluded that there was no risk to the biota, when in fact effects on the microbial components and ecosystem functioning did occur. This highlights the need for assessment at multiple levels of biological organisation, which includes the microbial component of the ecosystem, and the functions that the ecosystem performs.

Bioassessments must rely on a weight of evidence approach, incorporating both chemical guidelines and biomonitoring (Adams et al., 2005). Assessment at each level of biological organisation within this study provided different, useful, information which when combined enabled identification of the key drivers causing a change in the biota and allowed a reasonable assessment of ecosystem health. However, no individual response assessed could provide a true understanding of the overall biotic response on its own. The weight of evidence approach is gaining momentum and both chemical and biological parameters are now incorporated into guidelines in many countries.

Although the weight of evidence approach is being adopted in many countries, little work has been conducted on the use of microorganisms in environmental risk assessment, particularly of metals (Chapman et al., 2003). Further, biomonitoring is still dominated by characterising the macroinvertebrate community (Friberg et al., 2011). Use of macroinvertebrate data to assess ecosystem health assumes that changes in the macroinvertebrate community represent those occurring in the whole ecosystem. Yet, in this study the eukaryote meio- and micro- fauna were more sensitive to contamination than the invertebrates: eDNA metabarcoding showed differences between each individual copper treatment, while optical analysis of the invertebrates present could only distinguish differences between

the high copper treatments and control (Chapter 4 and Chapter 5). This is extremely important, as microbial components of aquatic ecosystems drive ecosystem processes (Friberg et al., 2011).

This study has demonstrated that, eDNA metabarcoding could be used to incorporate micro- and meio- fauna into the assessment process. The benefits of eDNA metabarcoding in allowing a greater proportion of the biotic community to be assessed (compared to traditional techniques of optical analysis) were demonstrated in Chapter 5. There is clear potential for the technique to be a reliable, rapid and cost-effective form of assessment (Chariton et al., 2010a). Further, creation of eDNA metabarcoding signatures could allow identification of the stressors that are of most concern within an ecosystem subject to multiple impacts (Chapter 5). However, the comparison of the invertebrate and eDNA metabarcoding data sets indicated that they should be used to support each other (Chapter 5). Many of the larger invertebrate taxa present were not identified by eDNA metabarcoding (probably due to a low sample size). Further, as eDNA metabarcoding currently produces presence/absence data, the invertebrate dataset was still important in characterising the biotic response; for example, although nematode richness was affected by copper according to eDNA metabarcoding, the invertebrate analysis showed no change in abundance, indicating tolerant species were able to proliferate in the absence of competition from those lost from the system. Thus, there is still great value in conducting traditional invertebrate sampling and the techniques complement each other well.

Conclusions

The historical and current release of persistent and toxic chemicals contaminates sediments and is a major environmental concern for aquatic ecosystems (Van Geest et al., 2010). The ultimate aim of ecotoxicologists is to preserve and restore the integrity of populations, communities and ecosystems by studying the effects of anthropogenic contaminants on ecological systems (Fleeger et al., 2003; Maltby, 1999). However, historically ecotoxicologists have predominantly focused on effects on individual organisms to define potential effects in the real world.

The overall aim of this research was to assess how effects of contaminants at low levels of biological organisation translate into those at higher levels of biological organisation in the field. The creation of semi-field artificial pond ecosystems (Chapter 2) allowed the causal effects of copper to be investigated under natural environmental conditions. To achieve the overall aim, the main aim of each chapter of work was to characterise the effects of copper within the pond ecosystems at a different level of biological organisation – on individual organisms (Chapter 3), populations (Chapter 4), the developing aquatic communities (Chapter 4 and Chapter 5) and the functions that the ecosystems performed (Chapter 6).

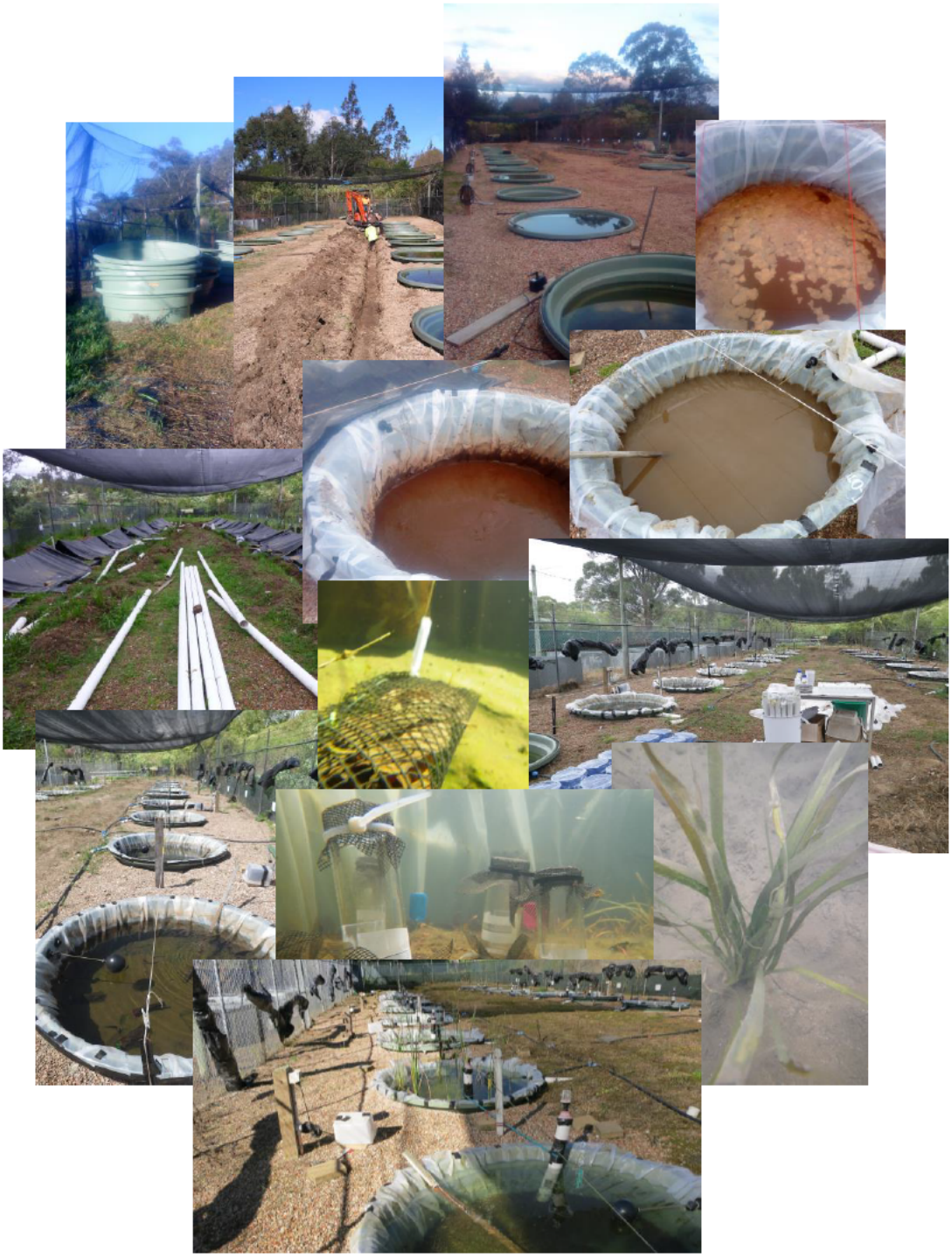
Increasing copper concentrations significantly decreased the growth of snails (*Physa acuta*) and shrimp (*Parataya australiensis*), but did not affect survival in either species or fecundity in the snails (fulfilling the aim of Chapter 3). Copper caused lower abundances within the benthic populations of Chironominae, a species of Ostracoda, Cladocera and *Physa acuta*. While the benthic invertebrate community composition changed significantly between C/VL/L treatments and H/VH treatments, there was little effect of copper on the invertebrate community in the water column (fulfilling the aim of Chapter 4). The biotic community from the micro- to macro- scale was analysed using eDNA metabarcoding and significant differences in benthic community composition between all individual treatments were found (fulfilling the aim of Chapter 5). Finally, direct, negative effects of copper on macrophyte cover, phytoplankton chlorophyll *a* concentrations and organic matter decomposition below the sediment surface and in contrast, positive responses of periphyton cover and organic matter decomposition on the sediment surface were observed (fulfilling the aim of Chapter 6).

By assessing the effects across levels of biological organisation, clear links between each level were determined (Figure 7.1). Overall the importance of indirect effects in driving the community response was demonstrated; this included the direct effect of copper on changes in the snail population between treatments indirectly causing an increase in periphyton cover and organic matter decomposition on the sediment surface. Beyond the overall aim, this study has

shown the importance of using realistic exposure scenarios when considering the effects of contaminants in manipulative studies and using multiple lines of evidence to assess ecosystem health. The study has highlighted the potential of eDNA metabarcoding, which will enable the incorporation of changes in microbial communities within ecosystem health assessments. This discussion has highlighted the need for a greater understanding of ecological interactions; it is only with this knowledge that we might be able to predict effects at population, community and ecosystem scales from those measured at low levels of biological organisation.

A final note

In the real world, ecosystems are rarely contaminated by just one stressor, but may be influenced by several chemical stressors, organic enrichment and elevated nutrient levels (Fleeger et al., 2003). Yet, environmental risk assessments are still typically based on individual contaminants (Chapman et al., 2003). Ultimately, there needs to be a greater emphasis on simultaneously comparing the effects of multiple contaminants at standardised and relevant concentrations under ecologically applicable conditions that include both direct and indirect effects (Boyd, 2010; Rohr et al., 2006; Fleeger et al., 2003). This response needs to be characterised across multiple ecozones and climate zones, as natural populations and communities are spatially heterogeneous and may respond differently to contaminants (Van den Brink, 2008).



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Appendix 1. Supporting information to Chapter 1

Table A1.1: Description of sites from literature with measured copper concentrations used to create Figure 1.4.

SED Cu (mg/kg)	Water Cu* (µg/L)	Description	Reference
0.9-3.4	<3	*A reference site (South Florida, USA)*	(Frakes et al., 2008)
1.9-13.1	1.5-7.6	*A site impacted with copper via agricultural runoff (South Florida, USA)*	(Frakes et al., 2008)
5	2.8	*A reference site upstream of city sparsely populated, Irai River (Brazil)*	(Sodré et al., 2012)
6	2	*Hazar Lake (Turkey)*	(Ozturk et al., 2009)
6.8-20.1	1.5-13	*A site impacted with copper via agricultural runoff (South Florida, USA)*	(Frakes et al., 2008)
7.8	1.5-2	*Reference (specifically no historical mining) (Montana, USA)*	(Farag et al., 2007)
13	2	*Reference (specifically no historical mining) (Montana, USA)*	(Farag et al., 2007)
13.7-110	2.0-15.7	*A site impacted with copper via agricultural runoff (South Florida, USA)*	(Frakes et al., 2008)
15	20	*Demirkopru Dam Lake (Turkey)*	(Ozturk et al., 2009)
15	<LOD	*A site impacted by industry (Italy)*	(Santoro et al., 2009)
15.6-302	1.3-2.1	*A site impacted with copper via agricultural runoff (South Florida, USA)*	(Frakes et al., 2008)
16	1.6-3.3	*Reference (specifically no historical mining) (Montana, USA)*	(Farag et al., 2007)
23	10	*Avsar Dam Lake (Turkey)*	(Ozturk et al., 2009)
23	220	*Ataturk Dam Lake (Turkey)*	(Ozturk et al., 2009)
25	4.6	*A site impacted by industry (Italy)*	(Santoro et al., 2009)
30	10	*Avsar Dam Lake (Turkey)*	(Ozturk et al., 2009)
30	5	*A site impacted by heavy urbanisation, Iguacu River (Brazil)*	(Sodré et al., 2012)
35.8-133	3.8-9.5	*A site impacted with copper via agricultural runoff (South Florida, USA)*	(Frakes et al., 2008)
38	7.2-7.9	*Site impacted by historical mining (Montana, USA)*	(Farag et al., 2007)
53	<LOD	*Urban river site (Italy)*	(Santoro et al., 2009)
68-70	11	*Reference site (Turkey)*	(Karadede-Akin & Unlü, 2007)
84	8.9-12	*Site impacted by historical mining (Montana, USA)*	(Farag et al., 2007)
98	3.0-10	*Site impacted by historical mining (Montana, USA)*	(Farag et al., 2007)
101-140	75	*Urban river site (Turkey)*	(Karadede-Akin & Unlü, 2007)
110	4.5-7.2	*Site impacted by historical mining (Montana, USA)*	(Farag et al., 2007)
110	9.2-11	*Site impacted by historical mining (Montana, USA)*	(Farag et al., 2007)
112-113	58	*Urban river site (Turkey)*	(Karadede-Akin & Unlü, 2007)
117-195	92	*Urban river site (Turkey)*	(Karadede-Akin & Unlü, 2007)
140	3.1-5.8	*Site impacted by historical mining (Montana, USA)*	(Farag et al., 2007)
156	94	*Urban river site (Italy)*	(Santoro et al., 2009)
180	32-69	*Site impacted by historical mining (Montana, USA)*	(Farag et al., 2007)
257	34	*Gediz River (Turkey)*	(Ozturk et al., 2009)
440	20-33	*Site impacted by historical mining (Montana, USA)*	(Farag et al., 2007)

450	33-53	*Site impacted by historical mining (Montana, USA)*	(Farag et al., 2007)
550	11-12	*Lake Pangua floodplain below a mine, Fly River (Papua New Guinea)*	(Apte, 2009)
700-900	16-17	*Lake Pangua channel (off river water body down stream of copper mine) (Papua New Guinea)*	(Apte, 2009)
744	38	*Site contaminated from nuclear power plant cooling system, Mirgenbach lake*	(Apte, 2009)
800-1100	18-20	*A floodplain downstream of mine, Fly River (Papua New Guinea)*	(Apte, 2009)
20365	90	*Site downstream of copper smelting operation*	(Suedel et al., 1996)
34	2	*Site above a copper mine, Le An River (China)*	(Liu et al., 1999)
290	11	*Site below copper mine, Le An River (China)*	(Liu et al., 1999)
310	10	*Site below copper mine, Le An River (China)*	(Liu et al., 1999)
439	15	*Site below copper mine, Le An River (China)*	(Liu et al., 1999)
506	19	*Site below copper mine, Le An River (China)*	(Liu et al., 1999)
700-900	5-65	*Lake Pangua channel (off river water body down stream of copper mine) (Papua New Guinea)*	(Apte, 2009)
800-1100	1-25	*A floodplain downstream of mine, Fly River (Papua New Guinea)*	(Apte, 2009)
914	24	*Site below copper mine, Le An River (China)*	(Liu et al., 1999)
950	19	*Site below copper mine, Le An River (China)*	(Liu et al., 1999)
1790	10	*Site below copper mine, Le An River (China)*	(Liu et al., 1999)
20365	350	*Site downstream of copper smelting operation*	(Suedel et al., 1996)

*Water concentrations are either overlying wter (x) or pore water (x) copper concentrations.

Table A1.2: Review of artificial field studies the have explored the effects of copper on freshwater ecosystems[$].

Description of endpoints	**No. endpoints in EPA database**	**Level of biological organisation***	**System (conditions^) *substrate***	**Exposure concentration**	**Exposure time**	**Reference**
Control of filamentous algae, phytoplankton and submerged macrophytes by copper	3	Po	Ponds (size unknown) (NT, NL) *Earth*	1 mg/L	Several weeks	(Anderson & Dechoretz, 1984)
Effect of copper on composition and functioning of photosynthetic riverine biofilms.	1	C, E	60 L artificial stream channels fed with river water (CT, AL) *etched glass plates*	1 and 2 µM	15 days	(Barranguet et al., 2003)
Effect of cuprous chloride and copper sulphate on survival of *Bulinus truncates* (snails)	14	O	Ponds fed with garden drain water (NT, NL) *Earth*	5, 20, 30, 40 mg/L	3 days	(Chu et al., 1968)
Effect of copper on seasonal changes of structure and protein, carbohydrate and organic content of periphyton communities (in terms of food quality for higher trophic levels).	12	C, E	546 L artificial streams fed with river water (NT, NL) *glass slides*	50 µg/L	Colonisation over 10-25 days of glass slides in streams over 9 months	(Clark et al., 1982)
Macroinvertebrate community response to copper and the influence of water quality (alkalinity, hardness, conductivity, temperature, pH, dissolved oxygen).	7	Po, C	94 L artificial stream channels fed with river water (NT, NL) and 37 L artificial stream channels fed with river water (CT, AL) *10x10x6 trays of pebbles/small cobbles naturally colonised*	Control, 12 and 25 µg/L; and Control, 6, 12 and 25 µg/L	4 and 10 days	(Clements et al., 1989)

Description of endpoints	No. endpoints in EPA database	Level of biological organisation*	System (conditions^) *substrate*	Exposure concentration	Exposure time	Reference
Comparison of copper bioaccumulation in *Sialis velata* through prey and contaminated water	2	MB	53 L artificial channels fed with lake water with plankton removed (CT, UL) *no substrate*	Uncontaminated/contaminated lake water (50/200 nM) and/or contaminated prey:uncontaminated prey ratio of 23	6 days	(Croisetière et al., 2006)
Celluloytic activity in a freshwater clam exposed to copper.	4	MB	20 L artificial stream channels fed with river water (NT, NL) *coarse sand sediment*	Control (5), 16, 21 and 26 µg/L	96 hr and 30 days	(Farris et al., 1988)
Effect of copper on several organism, population, community and ecosystem function endpoints in stream and pond mesocosms.	29	O, Po, C, E	440 L stainless steel stream mesocosms fed with filtered stream water (CT, NL) *shingle and sand*	6, 60, 200, 600, 2000 µg/L as $CuSO_4$	Up to 35 days	(Girling et al., 2000)
		O, Po, C, E	1000 L enclosures within 180,000 L pond (NT, NL) *pond sediment*	150 µg/L as $CuSO_4$	Up to 29 days	
Bioaccumulation of copper in freshwater clams	2	MB	735 L artificial channels fed with stream water (NT, NL) *60% pebble, 40% sand with some clay and silt*	0, 16 and 57 µg/L	28 days	(Graney et al., 1977)
Induction and activity of oxidative stress-related proteins by copper	5	MB	100 L tanks with flow through fed by river water (NT, NL)	0.6 and 16 µg/L	15 days	(Hansen et al., 2006)
Sensitivity of glochidial stages of unionid mussels to copper.	1	Ph	27 L artificial streams fed with river water (NT, NL) *rock filled basket*	3.2 (control), 10.6 and 19.1 µg/L	30 days	(Jacobson et al., 1997)

Description of endpoints	No. endpoints in EPA database	Level of biological organisation*	System (conditions^) *substrate*	Exposure concentration	Exposure time	Reference
Control (mortality) of *Australorbis glabratus* by copper sulphate in still water (SW) and flowing water (FW).	4	O	Natural swamp/ponds and streams (NT, NL) *earth*	SW 1 mg/L; FW 3.5 and 36 mg/L	6 hours	(Jobin & Unrau, 1967)
Chlorosis, necrosis, tissue browning, growth, bioaccumulation of copper.	18	MB, Ph	~3L artificial 'ponds' filled with tap water, liquid nutrient and microelement solution (NT, NL) *no substrate*	0, 500, 1000, 2500, 5000 µg/L	6 weeks	(Kay et al., 1984)
Effects of copper on growth of *Hydrilla verticillata*	1	O	803 L concrete tank filled with well water (NT, NL) *Hydrilla potted in commercial top soil*	1 µg/L	Up to 24 days	(Langeland et al., 2002)
Effect of copper on phytoplankton, periphyton, macroinvertebrate and macrophyte community structure, microbial degradation, and individual and population responses in sticklebacks	135	O, Po, C, E	6000 L artificial stream channels fed with tap water (NT, NL) *pebbles upper section then fine grain sediment (natural/artificial mix) lower section*	0 (control), 5, 25 and 75 µg/L	18 months	(Roussel et al., 2007ab; 2008)
Survival, growth, metabolism, bioaccumulation of copper (and other metals) in grass shrimp (*Palaemonetes paludosus*)	1	MB, Ph, O	20 L mesh enclosures within contaminated and uncontaminated ponds (NT, NL) *pond sediment*	Copper levels in contaminanted vs uncontaminated: sediment 18mg/kg vs 4 mg/kg, water 3 µg/L vs 1 µg/L	8 months	(Rowe, 1998)
Mortality rates, overall abundance and repopulation capacity of snails exposed to copper.	8	Po	Static tanks (NT, NL) *sediment*	0 (control), 10 µg/L	Single exposure, up to 3 months	(Shukla & Roy, 1975)

Description of endpoints	No. endpoints in EPA database	Level of biological organisation*	System (conditions^) *substrate*	Exposure concentration	Exposure time	Reference
Photosynthesis, metal tolerance and community structure of periphyton.	5	C, E	Flow through glass aquaria fed with river water (NT, NL) *microscope slides*	0 (control), 0.05, 0.1, 0.5, 1, 5 µM	16 weeks	(Soldo & Behra, 2000)
Copper accumulation in hydrilla and southern naiad.	2	MB	Outdoor pools (NT, NL) *peat/sand soil mixture*	1000 µg/L	35	(Sutton et al., 1970)
Effects of copper on algal growth and plankton community structure.	12	Po, C, E	3L nutrient water stocked with artificial community (NT, NL) *sand sediment*	500 µg/L	35	(Swartzman et al., 1990)
Effect of copper on aquatic insect community structure over three seasons (winter, spring, summer).	Not included	C	13 L artificial stream channels fed with dechlorinated tap water drawn from a river (CT, AL) *10x10x6 trays of pebbles/small cobbles naturally colonised*	0 (control), 15-32 and 135-178 µg/L	96 hr	(Clements et al., 1988)
Effect of copper on macroinvertebrate community structure in the laboratory (receiving dechlorinated tap water) and field (receiving unfiltered natural river water).	Not included	C	50 L artificial stream channels fed with river water (NT, NL) and 37 L artificial stream channels fed with dechlorinated tap water drawn from a river (CT, AL) *10x10x6 trays of pebbles/small cobbles naturally colonised*	Field: <3 (control), 11, 19, 30 µg/L Laboratory: <3 (control), 11, 22, 31 µg/L	10 days	(Clements et al., 1990)

Description of endpoints	No. endpoints in EPA database	Level of biological organisation*	System (conditions^) *substrate*	Exposure concentration	Exposure time	Reference
Effect of copper on zooplankton assemblages.	Not included	Po, C	100L enclosures within a 27 ha lake containing lake filtrate (200µm) inoculated with zooplankton (NT, NL), *no substrate*	0 (control), 2, 5, 7, 10, 20, 50, 70, 100, 200 µg/L	4 days	(Havens, 1994b)
Effect of copper on plankton assemblages, including structural and functional responses.	Not included	Po, C, E	100L enclosures within a 27 ha lake containing lake filtrate (200µm) inoculated with zooplankton (NT, NL), *no substrate*	0-20 µg/L (controls), 110-140 µg/L (treated)	14 days	(Havens, 1994c; a)
Effect of copper on primary production, decomposition, phytoplankton, zooplankton and benthic macroinvertebrates.	Not included	Po, C, E	33 L lentic microcosms fed by water from an outdoor reservoir with a turnover time of 3.5 days (AT, AL) *natural pond sediment*	1.4, 4.0 9.3, 30. 90, 420 µg/L	32 weeks	(Hedtke, 1984)
Bioaccumulation of copper and effects on reproduction, growth and survival in *Pomacea paludosa* (snail) and *Gambusia affinis* (mosquito fish)	Not included	MB, O	4m^3 tanks fed by rainwater (NT, NL) *top soil from agricultural sites with different copper concentrations due to agricultural use*	7, 55, 99 mg/kg (particulate) Overlying water initially: 3, 82 and 43 µg/L, after 3 months reduced to be <9 µg/L	430 days	(Hoang et al., 2011)
Biomass accumulation, adenosine triphophate and chlorophyll *a* content, diatom community structure of periphyton under pulses of copper exposure to assess stress resistance & resilience.	Not included	Po, C, E	480 L artificial streams fed by river water (NT, NL) *glass slides*	0, 30 µg/L pulses for 10 days then 60 µg/L for 1 day then 0 or 30 µg/L pulses for 7 days	18 days	(Kaufman, 1982)

Description of endpoints	No. endpoints in EPA database	Level of biological organisation*	System (conditions^) *substrate*	Exposure concentration	Exposure time	Reference
Phytoplankton community structure and direct and indirect effects of copper on natural microbial loop communities (in spring and summer).	Not included	Po, C, E	85 L glass aquaria containing lake water with natural planktonic communities (<200µm) (CT, AL) *no substrate*	<1 (control), 63/65 and 115/123 µg/L (spring/summer experiments respectively)	27 days	(Le Jeune et al., 2006, 2007)
Effect of copper on net community metabolism, community structure, lipid and protein content of periphyton, and growth rate and reproduction of *Stagnicola vulnerata*.	Not included	O, Po, C	17 L artificial stream channels fed with well water (CT, AL) *sandblasted glass plates*	44 µg/L	14 day	(Real et al., 2003)
Structure and function of periphyton communities during colonisation under different exposure regimes of copper (initial chronic (C) and pulse exposures (P)) and the influence of the exposure regimes on bioaccumulation capacity with a copper bioaccumulation study (CBS).	Not included	C, E	2.3 L artificial stream channels fed with dechlorinated tap water (CT, AL) *etched glass plates*	Initial: <2 µg/L (control), 20 µg/L (P), 26 µg/L (C). CBS: 100 µg/L	Initial: 5 weeks CBS: 6 hours	(Serra et al., 2009a)
Retention of copper, photosynthetic activity and algal biomass in a fluvial biofilm under different flow conditions.	Not included	E	2.3 L artificial stream channels fed with dechlorinated tap water (CT, AL) *etched glass plates*	1.2 (control), 35 µg/L	Analysis occurred once conducitivity had reached a plateau	(Serra et al., 2009b)

Description of endpoints	No. endpoints in EPA database	Level of biological organisation*	System (conditions^) *substrate*	Exposure concentration	Exposure time	Reference
Structure, physiology and copper tolerance of periphyton communities, through an initial chronic exposure (ICC), copper retention study (CRS) and then short term toxicity study (STS)	Not included	Ph, C, E	2.3 L artificial stream channels fed with dechlorinated tap water (CT, AL) *etched glass plates*	ICC: 0.88 (control), 33 µg/L; CRS: 30 µg/L (both); STS: 30 µg/L (controls), 92-95µg/L	ICC: 4 weeks CRS: 1 week STS: 6 hours	(Serra & Guasch, 2009)
Effect of copper on macroinvertebrate community structure and populations of species of Ephemeroptera (*Caenis* sp.), Amphipoda (*Hyalella azteca*) and Hemiptera (Notonectidae).	Not included	Po, C	17000 L enclosures within 400 m3 ponds (NT, NL) *pond sediment*	2-6 (control), 20, 40, 90, 160, 260 µg/L	74 days	(Shaw & Manning, 1996)
Effect of copper on planktonic and benthic community structure and primary production.	Not included	Po, C, E	100 L enclosures within a 6000m^2 pond containing pond water - not refreshed (NT, NL) 225cm^2 of benthic sediments from the pond	0, 20, 40 µg/L	3*5 week periods over 3 seasons	(Winner et al., 1990; Moore & Winner, 1989)

[$] Rows coloured green assess effects at multiple levels of biological complexity; rows coloured blue assess effects at both low and high levels of biological organisation.

* MB – molecular/biochemical, Ph = physiological, O = organism, Po = population, C = community, E= ecosystem

^ CT = constant temp, AL = artificial light cycle, NT = naturally fluctuating temp, NL = naturally fluctuating light, UL = unknown light regime

Studies identified by EPA database as artificial field studies but not included in this review either due to access issues or due to them not being a mesocosm study:

Harrison FL, Lam JR (1986) Copper-binding proteins in liver of bluegills exposed to increased soluble copper under field and laboratory conditions. *Environmental Health Perspectives* 65:125–132. [not a mesocosm study]

Kalita MC (1997) Impact of copper on biomass yield, chlorophyll metabolism and total nitrogen accumulation of *Azolla pinnata*. *Environment and Ecology* 15:756–760 [no access to journal]

Guseynova VP (2005) Combined influence of some pollutants and ultraviolet radiation on phytoplankton photosynthesis. *Hydrobiological Journal* 41:80–94. [no access to journal]

Gindy EL (1957) Laboratory studies on the effect of copper sulphate on *Biomphalaria boissyi* , the snail vector of *Mansoni schistosomiasis* in Egypt. *Journal of the Egyptian Medical Association* 40:45–53. [no access to journal]

Abdel-Sabour MF, Ismail AS, Radwan RM (1996) Tolerance index and transfer factor coefficient of Zn, Cu, and Co for water hyacinth plant, *Eichhornia crassipes*. *Egyptian Journal of Soil Science* 36:355–364. [no access to journal]

Table A1.3: In depth review of papers that consider the effects of copper at low and high levels of biological complexity

Real et al. (2003)	
Examined:	Effect of copper on net community metabolism, community structure, lipid and protein content of periphyton, and growth rate and reproduction of *Stagnicola vulnerata*.
Found:	Grazers laid fewer egg masses, with fewer eggs per egg mass when exposed to copper, which was attributed to a lower investment in reproduction due to a lower growth rate (gained less weight). Embryo hatching success was also reduced. Copper reduced net community metabolism and chlorophyll content of the biofilm (though not statistically significant), but grazers were more influential, significantly reducing both parameters. Grazing and copper together did not affect these parameters more than copper alone. Copper caused an increase in lipid content of the biofilm but this had no effect on the lipid content of the snails (perhaps because the experiment was too short). Copper caused a decrease in protein content of the biofilm, but an increase in protein content of snails (this could be related to the induction of metalloprotein synthesis). No significant effect of copper on algal composition was found. *Copper was not found to significantly affect net community metabolism or algal composition, so although effects were observed at the organism level on the snail Stagnicola vulnerata, these were not linked to community or ecosystem level responses, as there was no response.*
Ecological relevance:	Although a natural periphyton community from an unpolluted stream was used, the experiment was carried out indoors with controlled temperature and artificial light system and sandblasted glass was used as a substrate.
Serra & Guasch (2009)	
Examined:	Structure, physiology and copper tolerance of periphyton communities, through an initial chronic exposure (ICC), copper retention study (CRS) and then short term toxicity study (STS)
Found:	Chronic copper exposure was found to cause an increase in extracellular polymeric substances (EPS) in the biofilm, which were potentially acting as detoxification agents against metals. However, intracellular concentrations of copper were higher in the copper exposed community – it was suggested that this copper was sequestered and immobilised within the cells. Chronic copper exposure caused a reduction in algal growth, leading to a lower accumulation of biomass. A shift in the dominant algal group from diatoms to green algae was also found. Ultimately copper retention efficiency was reduced after chronic copper exposure, which, it was suggested, could be attributed to the saturation of binding sites. Copper exposure did not have a marked influence on the stream phosphate retention efficiency, nor did it affect photosynthetic capacity. *Links were made between the high intracellular copper concentrations as a result of copper exposure and the reduced copper retention efficiency of the biofilm and also between reduced algal growth and reduced overall biomass of the periphyton.*
Ecological relevance:	Although the periphyton communities originated from field biofilm inocula, the source of water during exposure was tap water and the experiment was conducted indoors, with controlled temperature and light regime and etched glass plates were used as a substrate.

Girling et al. (2000)	
Examined:	Effect of copper on several organism, population, community and ecosystem function endpoints in stream and pond mesocosms.
Found:	Stream mesocosms: NOECs or LOECs were calculated for growth rate of *Lemna minor*, biomass of *Tubifex* spp., population density, drift and precopula separation in *Gammarus pulex*, population density in *Chironomus riparius*, hatching, juvenile survival and growth, adult survival and growth (shell length, biomass) in *Lymnea stagnalis* and population density in *Polycelis* sp., abundance of algae, chlorophyll *a* concentrations and photosynthesis rates and detritivore feeding rates. Pond mesocosms: NOECs and/or LOECs were calculated for long term population dynamics of *Mallomonas* spp, *Planktosphaeria* spp., *Cryptomonas* spp., *Dinobryon* spp. and *Phacus* spp., *Daphnia longispina, Chydorus sphaericus, Chaoborus flavicans, Anureopsis* spp., *Keratella quadrata*, and *Polyarthra* spp., frond numbers and root length in *Lemna minor*, length, dry weight, root numbers and oxygen production in *Elodea Canadensis*, 2nd instar mortality in *Chironomus riparius*, the shift in dominant taxa of phytoplankton community and cell density of the phytoplankton community. *The research was carried out to determine a range of NOECs and LOECs for different end points to use in risk assessment; as the patterns observed in the mesocosms were not described, no links were made between levels of biological organisation.*
Ecological relevance:	Streams: Overall this system was quite ecologically relevant - invertebrates were stocked from the local watercourses and periphyton were established prior to stocking, decaying leaf litter was also added for detritivores and a stream bed substrate was provided consisting of sand and pea shingle. However, water depth was kept constant and temperature fluctuations were minimised by using heat exchangers – but as the streams were not insulated in the ground, these may have made the temperature fluctuations more ecologically relevant – fine particles (<1mm) were also removed, which would affect the copper binding capacity of the substrate. In addition there were relatively short exposure times (28 days). Ponds: these were also quite ecologically relevant as they were carried out in enclosures within a pond that had been established over several years with a natural zooplankton community and the enclosures spanned the water column and were exposed to the sediment of the larger pond.
Roussel et al. (2007a,b; 2008)	
Examined:	Effect of copper on phytoplankton, periphyton, macroinvertebrate and macrophyte community structure, microbial degradation, and individual and population responses in sticklebacks
Found:	High copper accumulation in the liver of stickleback in copper treated mesocosms was correlated with fish size, liver weight, condition factor and organosomatic indices of the fish. However, no direct effect on population abundance was found. In fact the population was highest in the highest copper treatment; the authors hypothesised that this could be due to a reduction in predation pressure of stickleback eggs and juveniles as large invertebrates (the predators) were reduced in abundance in the highest copper exposure level. Copper was not found to affect the aquatic hyphomycetes associated with the decomposition of leaf litter in the mesocosms, but the invertebrate community was affected – in particular a dominant shredder in the upper part of the mesocosm systems was lost. The lower decomposition rates in the copper treated mesocosms was attributed to the loss of the shredder. A less strong effect on decomposition was observed in the lower sections of the mesocosms, this was also attributed to a different community structure, present due to the different habitat in the lower mesocosm sections. With time, the periphyton in the copper treatments were found to have a higher biomass – attributed to an indirect effect whereby gastropod abundance declined dramatically in the high treatments leading to a reduced grazing pressure on the periphyton. Periphyton community structure also changed from diatom domination to blue green algae. Copper also affected the community structure of the phytoplankton

	where sensitive species (diatoms) were replaced by green algae, but did not affect total phytoplankton abundance – the change in community structure with more tolerant species was attributed to have compensated for the lack in change in phytoplankton abundance observed, an alternative hypothesis related to the decrease in grazing pressure. Over the experimental period, there was a lower biomass of macrophytes in copper treated mesocosms. The macrophyte community was also affected due to direct effects on macroalgae and *Lemna minor,* which colonised all controls but not the treated mesocosms. *Although effects at lower levels of biological organisation were measured in the stickleback, they could not be related to population level changes in the fish. This study demonstrates the importance of indirect effects, which can confound elucidation of direct effects. However, they are a key part of the aquatic environment and should be incorporated into studies (as discussed in the section above).*
Ecological relevance:	This study was quite ecologically relevant with an environmentally relevant dissolved copper exposure, natural temperature and light fluctuations, and a substrate representative of aquatic systems.

Appendix 2. Supporting information to Chapter 2

Table A2.1: A review of copper partitioning in freshwater environments between sediments, pore waters and overlying waters and the corresponding K_d values.

OW/PW	SED Cu (mg/kg)	Water Cu (µg/L)	K_d (**reported**) (calculated) (L/kg)	Description	Reference
			Mode: 15858 (range: 5 - 1584893)	*Literature review of Kd values between sediment and water in freshwater environments (n=12)*	(Allison & Allison, 2005)
OW	-	-	**25000-100000**	*Partition values between sediment and surface waters in urbanised catchment Irai and Iguacu rivers (Brazil)*	(Sodré & Grassi, 2006)
OW	-	-	**10000-1600000**	*Partition values between surface waters and suspended sediments in rivers with broad spectrum of development/contamination (Southern New England, USA)*	(Benoit & Rozan, 1999)
OW	0.9-3.4	<3	300-1100	*A reference site (South Florida, USA)*	(Frakes et al., 2008)
OW	1.9-13.1	1.5-7.6	250-8700	*A site impacted with copper via agricultural runoff (South Florida, USA)*	(Frakes et al., 2008)
OW	5	2.8	1800	*A reference site upstream of city sparsely populated, Irai River (Brazil)*	(Sodré et al., 2012)
OW	6	2	3000	*Hazar Lake (Turkey)*	(Ozturk et al., 2009)
OW	6.8-20.1	1.5-13	523-13000	*A site impacted with copper via agricultural runoff (South Florida, USA)*	(Frakes et al., 2008)
OW	7.8	1.5-2	3900-5200	*Reference (specifically no historical mining) (Montana, USA)*	(Farag et al., 2007)
OW	13	2	6500	*Reference (specifically no historical mining) (Montana, USA)*	(Farag et al., 2007)
OW	13.7-110	2.0-15.7	870-55000	*A site impacted with copper via agricultural runoff (South Florida, USA)*	(Frakes et al., 2008)
OW	15	20	750	*Demirkopru Dam Lake (Turkey)*	(Ozturk et al., 2009)
OW	15	<LOD	n/a	*A site impacted by industry (Italy)*	(Santoro et al., 2009)
OW	15.6-302	1.3-2.1	7400-230000	*A site impacted with copper via agricultural runoff (South Florida, USA)*	(Frakes et al., 2008)
OW	16	1.6-3.3	4800-10000	*Reference (specifically no historical mining) (Montana, USA)*	(Farag et al., 2007)
OW	23	10	2300	*Avsar Dam Lake (Turkey)*	(Ozturk et al., 2009)
OW	23	220	100	*Ataturk Dam Lake (Turkey)*	(Ozturk et al., 2009)

OW/PW	SED Cu (mg/kg)	Water Cu (µg/L)	K_d (**reported**) (calculated) (L/kg)	Description	Reference
OW	25	4.6	5400	*A site impacted by industry (Italy)*	(Santoro et al., 2009)
OW	30	10	3000	*Avsar Dam Lake (Turkey)*	(Ozturk et al., 2009)
OW	30	5	6000	*A site impacted by heavy urbanisation, Iguacu River (Brazil)*	(Sodré et al., 2012)
OW	35.8-133	3.8-9.5	3800-35000	*A site impacted with copper via agricultural runoff (South Florida, USA)*	(Frakes et al., 2008)
OW	38	7.2-7.9	4800-5200	*Site impacted by historical mining (Montana, USA)*	(Farag et al., 2007)
OW	53	<LOD	n/a	*Urban river site (Italy)*	(Santoro et al., 2009)
OW	68-70	11	6200-6400	*Reference site (Turkey)*	(Karadede-Akin & Unlü, 2007)
OW	84	8.9-12	7000-9400	*Site impacted by historical mining (Montana, USA)*	(Farag et al., 2007)
OW	98	3.0-10	9800-33000	*Site impacted by historical mining (Montana, USA)*	(Farag et al., 2007)
OW	101-140	75	1300-1900	*Urban river site (Turkey)*	(Karadede-Akin & Unlü, 2007)
OW	110	4.5-7.2	15000-24000	*Site impacted by historical mining (Montana, USA)*	(Farag et al., 2007)
OW	110	9.2-11	10000-12000	*Site impacted by historical mining (Montana, USA)*	(Farag et al., 2007)
OW	112-113	58	1900	*Urban river site (Turkey)*	(Karadede-Akin & Unlü, 2007)
OW	117-195	92	1200-2100	*Urban river site (Turkey)*	(Karadede-Akin & Unlü, 2007)
OW	140	3.1-5.8	24000-45000	*Site impacted by historical mining (Montana, USA)*	(Farag et al., 2007)
OW	156	94	1700	*Urban river site (Italy)*	(Santoro et al., 2009)
OW	180	32-69	2600-5600	*Site impacted by historical mining (Montana, USA)*	(Farag et al., 2007)
OW	257	34	7600	*Gediz River (Turkey)*	(Ozturk et al., 2009)
OW	440	20-33	13000-22000	*Site impacted by historical mining (Montana, USA)*	(Farag et al., 2007)
OW	450	33-53	8500-14000	*Site impacted by historical mining (Montana, USA)*	(Farag et al., 2007)
OW	550	11-12	46000-50000	*Lake Pangua floodplain below a mine, Fly River (Papua New Guinea)*	(Apte, 2009)
OW	700-900	16-17	41000-56000	*Lake Pangua channel (off river water body down stream of copper mine) (Papua New Guinea)*	(Apte, 2009)

OW/PW	SED Cu (mg/kg)	Water Cu (µg/L)	K_d (**reported**) (calculated) (L/kg)	Description	Reference
OW	744	38	20000	*Site contaminated from nuclear power plant cooling system, Mirgenbach lake*	(Apte, 2009)
OW	800-1100	18-20	40000-61000	*A floodplain downstream of mine, Fly River (Papua New Guinea)*	(Apte, 2009)
OW	20365	90	230000	*Site downstream of copper smelting operation*	(Suedel et al., 1996)
PW	34	2	17000	*Site above a copper mine, Le An River (China)*	(Liu et al., 1999)
PW	290	11	26000	*Site below copper mine, Le An River (China)*	(Liu et al., 1999)
PW	310	10	31000	*Site below copper mine, Le An River (China)*	(Liu et al., 1999)
PW	439	15	29000	*Site below copper mine, Le An River (China)*	(Liu et al., 1999)
PW	506	19	27000	*Site below copper mine, Le An River (China)*	(Liu et al., 1999)
PW	700-900	5-65	11000-18000	*Lake Pangua channel (off river water body down stream of copper mine) (Papua New Guinea)*	(Apte, 2009)
PW	800-1100	1-25	32000-1100000	*A floodplain downstream of mine, Fly River (Papua New Guinea)*	(Apte, 2009)
PW	914	24	38000	*Site below copper mine, Le An River (China)*	(Liu et al., 1999)
PW	950	19	50000	*Site below copper mine, Le An River (China)*	(Liu et al., 1999)
PW	1790	10	179000	*Site below copper mine, Le An River (China)*	(Liu et al., 1999)
PW	20365	350	58000	*Site downstream of copper smelting operation*	(Suedel et al., 1996)

Table A2.2 Quality assurance/quality control analyses; values are mean ± 1 standard deviation (number of analyses).

Analyte	Procedural reproducibility (Relative % difference)	Procedural spike recovery*
Dissolved Cu (OW)	5±6 (29)	
Dissolved Cu (PW)	10±13 (26)	
Total Cu	4±4 (48)	99±4 (11)
Dissolved Ca (OW)	3±4 (23)	
Dissolved Ca (PW)	4±6 (22)	
Total Ca	3±2 (36)	
Dissolved Mg (OW)	2±3 (23)	
Dissolved Mg (PW)	4±6 (22)	
Total Mg	2±2 (36)	
Total NH_4-N	5.3±4.6 (4)	
Extractable P	2.9±0.3 (4)	
Total NO_3-N	All below <LOD	
NH_4-N (OW)	All below <LOD	
PO_4-P (OW)	2.2±2.5 (3) (1 <LOD)	
NO_3-N (OW)	2.6 (1) (3 <LOD)	

Table A2.3: pH of overlying waters (unless otherwise noted) measured in the freshwater environments reviewed in Table S1.

Reference	Site descriptions	pH range (overlying water unless otherwise noted)
(Sodré & Grassi, 2006)	*Partition values between sediment and surface waters in urbanised catchment Irai and Iguacu rivers (Brazil)*	6.7 – 6.9
(Benoit & Rozan, 1999)	*Partition values between surface waters and suspended sediments in rivers with broad spectrum of development/contamination (Southern New England, USA)*	4.8 – 7.9.
(Sodré et al., 2012)	*A reference site upstream of city sparsely populated, Irai River (Brazil)* *A site impacted by heavy urbanization, Iguacu River (Brazil)*	6.5; 7.0
(Farag et al., 2007)	*3 x Reference (specifically no historical mining) (Montana, USA)* *8 x Site impacted by historical mining (Montana, USA)*	7.0 – 8.5
(Santoro et al., 2009)	*1 x A site impacted by industry (Italy)* *2 x Urban river site (Italy)*	7.0 – 8.0
(Apte, 2009)	*A floodplain downstream of mine, Fly River (Papua New Guinea)* *Lake Pangua floodplain below a mine, Fly River (Papua New Guinea)* *Lake Pangua channel (off river water body down stream of copper mine) (Papua New Guinea)*	7.2 – 8.2
(Vinot & Pihan, 2005)	*Site contaminated from nuclear power plant cooling system, Mirgenbach lake*	8.2 – 9.3
(Suedel et al., 1996)	*Site downstream of copper smelting operation*	6.6 (sediment)

Table A2.4: Concentrations of total organic carbon, nitrate (as NO_3-N), ammonia (as NH_4-N) and extractable phosphorus measured in the original batch of soil mix and mesocosm sediments.

	Mesocosm sediments	
	March 2011 (n=20)	March 2012 (n=20)
Total organic carbon (%)	3.8±1.5	3.4±0.9
Nitrate (mg/kg)	<1.0	<1.0
Ammonia (mg/kg)	8.0±2.6	10±4.0
Extractable phosphorus (mg/kg)	9.0±2.2	9.0±1.3

Table A2.5: Copper hardness modified trigger values by treatment in overlying waters and pore waters based on mean hardness* from t = 74 d onwards. Trigger values highlighted in red are exceeded by the mean copper concentration from t = 74 d onwards.

	Treatment	Mean hardness (mg/L as $CaCO_3$)*	Hardness classification	Level of protection (% species)				Mean copper (µg/L)
				99%	95%	90%	80%	
				Trigger value at hardness of 30 mg/L as $CaCO_3$ (µg/L)				
				1	1.4	1.8	2.5	
				Hardness modified trigger values (µg/L)**				
Overlying waters	Control	83.1	Moderately hard	**2.4**	**3.3**	**4.3**	**5.9**	1
	Very low	97.6	Moderately hard	**2.7**	**3.8**	**4.9**	**6.8**	2
	Low	94.7	Moderately hard	**2.7**	**3.7**	**4.8**	**6.6**	4
	High	91.8	Moderately hard	**2.6**	**3.6**	**4.7**	**6.5**	8
	Very high	109	Moderately hard	**3.0**	**4.2**	**5.4**	**7.5**	11
Pore waters	Control	147	Hard	**3.9**	**5.4**	**6.9**	**9.7**	1
	Very low	167	Hard	**4.3**	**6.0**	**7.7**	**10.8**	3
	Low	163	Hard	**4.2**	**5.9**	**7.6**	**10.5**	5
	High	169	Hard	**4.3**	**6.1**	**7.8**	**10.9**	18
	Very high	204	Very hard	**5.1**	**7.1**	**9.2**	**12.8**	30

*calculated from measured concentrations of calcium and magnesium

**algorithm used to calculate hardness-modified trigger values was (ANZECC & ARMCANZ, 2000):

$$HMTV = TV\left(\frac{H}{30}\right)^{0.85}$$

where:

HMTV = Harness-modified trigger value (µg/L)

TV = trigger value (µg/L) at a hardness of 30 mg/L as $CaCO_3$

H = measured hardness (mg/L as $CaCO_3$) of a fresh surface water (≤2.5‰).

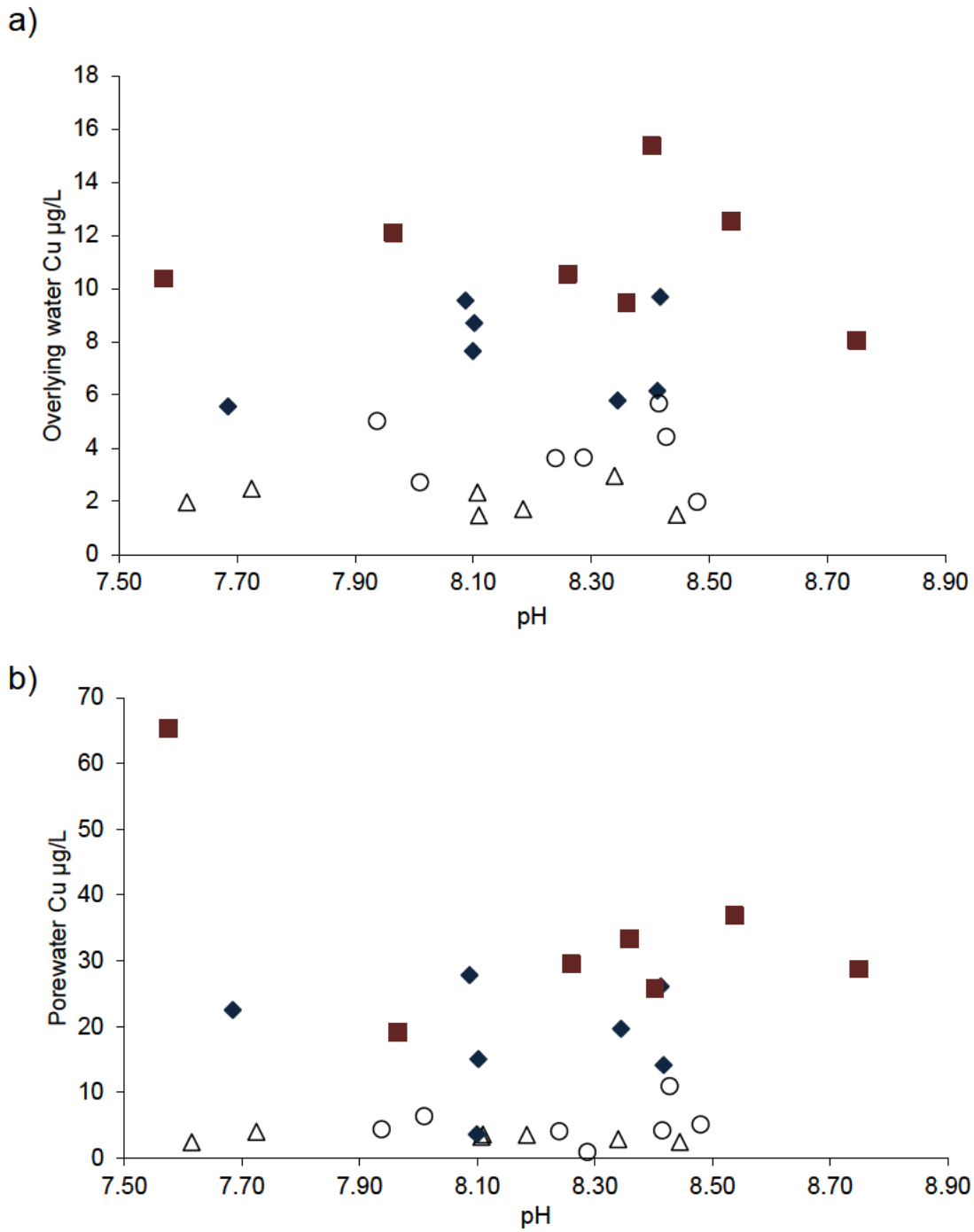

Figure A2.1: Overlying water pH versus (a) overlying water copper concentrations and (b) pore water copper concentrations in very low (△), low (○), high (◆) and very high (■) treatments (n=4).

Appendix 3. Supporting information to Chapter 3

Table A3.1: Results of the statistical analyses performed on *P. australiensis* exposure data.

Variable	Treatment	Time	Interaction	LSD posthoc (treatment)	LSD posthoc (time)
***P. australiensis* exposure 1: Survival**					
df	4,15	1.8,27	7,27	n/a	n/a
F	0.75	2	0.75		
p	0.57	0.16	0.64		
SS	0.39	0.34	0.51		
***P. australiensis* exposure 2: Survival**					
df	4,15	3,45	12,45	(2d = 5d) > (7d = 9d) > 12d/14d/16d/19d/23d/28d 12d = 14d 12d > 16d/19d/23d/28d 14d = 16d 14d > 19d/23d/28d 16d = 19d 16d > 23d/28d (19d = 23d) > 28d	
F	2	23	1.3		
p	0.15	<0.001	0.28		
SS	40	73	16		
***P. australiensis* exposure 2: Growth**					
df	4,19	n/a	n/a	C>VH (p = 0.014) L>VH (p = 0.019)	n/a
F	2.7				
p	0.068				
SS	0.12				

Table A3.2: Results of the statistical analyses performed on *P. acuta* data

Variable	Treatment	Time	Interaction	LSD posthoc (treatment)	LSD posthoc (time)
***P. acuta* exposure 1: Survival**					
df	4,15	9,135	36,135	n/a	(2d = 5d = 7d = 9d = 12d = 14d = 16d = 19d = 23d) > 32d
F	1.9	2.8	1.2		
p	0.17	0.004	0.23		
SS	61	13	22		
***P. acuta* exposure 1: Egg mass production (cumulative)**					
df	4,15	9,135	36,135	n/a	2d < 5d < 7d < 9d/12d/14d/16d/19d/23d/32d 9d = 12d 9d <14d/16d/19d/23d/32d 12d = 14d/16d 12d < 23d/32d 14d = 16d =19d 14d/16d < 23d/32d 19d = 23d < 32d
F	0.57	70	0.94		
p	0.69	<0.001	0.58		
SS	140	1200	63		
***P. acuta* exposure 2: Survival**					
df	4,15	6,90	24,90	n/a	7d =10d = 13d 7d > 16d/19d/22d/29d 10d = 13d = 16d = 19d = 22d = 29d
F	0.73	3.6	0.36		
p	0.59	0.003	1		
SS	6.4	2.2	0.9		
***P. acuta* exposure 2: Egg mass production**					
df	4,15	1.7,25	6.7,25	C>VH *p* = 0.069	7d < 10d < 13d < 16d < (19d = 22d = 29d)
F	1	23	0.73		
p	0.42	<0.001	0.64		
SS	1.9	0.95	0.12		
***P. acuta* expolsure 2: Egg mass size**					
df	4,15	n/a			
F	0.11				
p	0.98				
SS	0.047				
***P. acuta* exposure 2: Eggs per egg mass**					
df	4,15	n/a			
F	0.29				
p	0.88				
SS	0.037				

Variable	Treatment	Time	Interaction	LSD posthoc (treatment)	LSD posthoc (time)
***P. acuta* exposure 2: Egg density**					
df	4,15	n/a			
F	1				
p	0.42				
SS	0.001				
***P. acuta* exposure 2: Growth**					
df	4,15	C>VH (p = 0.009); VL>VH (p = 0.007), L>VH (p <0.001), H>VH (p = 0.005)			
F	5.5				
p	0.006				
SS	0.19				

Appendix 4. Supporting information to Chapter 4

Table A4.1: Data transformations used for each analysis

Analysis	Data set*	Variable	Transformation
RM ANOVA	WQ	pH	$Log_{10}(x+1)$
RM ANOVA		Oxidation-reduction potential	$Log_{10}(x+1)$
RM ANOVA		Conductivity	Untransformed
RM ANOVA		Dissolved oxygen	Untransformed
RM ANOVA		Turbidity	$Log_{10}(x+1)$
RM ANOVA		Sediment copper	$Log_{10}(x+1)$
RM ANOVA		Pore water copper	$Log_{10}(x+1)$
RM ANOVA		Overlying water copper	$Log_{10}(x+1)$
RM ANOVA	BI	Total abundance	$Log_{10}(x+1)$
RM ANOVA		Taxa richness	Untransformed
RM ANOVA		Diversity	Untransformed
PRC		Community assemblage	$Log_{10}(x+1)$
PERMANOVA		Community assemblage	$Log_{10}(x+1)$
DistLM	BI	Community assemblage	$Log_{10}(x+1)$
	WQ	Sediment copper	Untransformed
	WQ	Pore water copper	Untransformed
TITAN Analysis	BI	Community assemblage	$Log_{10}(x+1)$
	WQ	Pore water copper	Untransformed
		Sediment copper	Untransformed
RM ANOVA	BI	Chironiminae abundance	$Log_{10}(x+1)$
RM ANOVA		Ostracoda Sp. 2 abundance	$Log_{10}(x+1)$
RM ANOVA		Cladocera abundance	$Log_{10}(x+1)$
RM ANOVA		*Physa acuta* abundance	Sqrt(x)
RM ANOVA		Copepoda abundance	Sqrt(x)
RM ANOVA	WCI	Total abundance	$Log_{10}(x+1)$
RM ANOVA		Taxa richness	Untransformed
RM ANOVA		Diversity	Untransformed
PRC		Community assemblage	$Log_{10}(x+1)$
PERMANOVA		Community assemblage	$Log_{10}(x+1)$
DistLM	WCI	Community assemblage	$Log_{10}(x+1)$
	WQ	Sediment copper	Untransformed
	WQ	Overlying water copper	Untransformed
TITAN Analysis	WCI	Community assemblage	$Log_{10}(x+1)$
	WQ	Overlying water copper	Untransformed
		Sediment copper	Untransformed
One way ANOVA	SNI	Total abundance	Untransformed
One way ANOVA		Taxa richness	Untransformed
One way ANOVA		Diversity	Untransformed
DistLM	SNI	Community assemblage	$Log_{10}(x+1)$
	WQ	Sediment copper	Untransformed
	WQ	Pore water copper	Untransformed
	WQ	Overlying water copper	Untransformed
One way ANOVA	SNI	Sweep net *P. acuta* abundance	$Log_{10}(x+1)$

WQ = Water quality dataset; BI = Benthic invertebrate data set; WCI = Water column invertebrate data set; SNI = Sweep net invertebrate data set

Table A4.2: Results of RM ANOVAs and further analyses performed on water quality and copper data.

	Treatment	Time	Interaction	Further analysis^	
				Treatment	Time
pH*					
df	4,15	4,61	16,61	@ 4 d: $F_{4,15} = 20$, $p < 0.001$ @ 15 d: $F_{4,15} = 0.67$, $p = 0.62$ @ 42 d: $F_{4,15} = 0.67$, $p = 0.62$ @ 72 d: $F_{4,15} = 5.1$, $p = 0.008$ @ 100 d: $F_{4,15} = 13$, $p < 0.001$ @ 135 d: $F_{4,15} = 1.5$, $p = 0.24$ @ 161 d: $F_{4,15} = 14$, $p < 0.001$ @ 247 d: $F_{4,15} = 17$, $p < 0.001$ @ 310 d: $F_{4,15} = 1.8$, $p = 0.18$ @ 365 d: $F_{4,15} = 0.91$, $p = 0.48$ @ 407 d: $F_{4,15} = 0.56$, $p = 0.70$ @ 455 d: $F_{4,15} = 0.97$, $p = 0.45$ @ 497 d: $F_{4,15} = 2.2$, $p = 0.12$	Control: $F_{12,36} = 24$, $p < 0.001$ Very low: $F_{12,36} = 39$, $p < 0.001$ Low: $F_{12,36} = 14$, $p < 0.001$ High: $F_{12,36} = 5.5$, $p < 0.001$ Very high: $F_{12,36} = 12$, $p < 0.001$
F	6.8	59	4.6		
p	0.003	<0.001	<0.001		
SS	0.015	0.15	0.045		
Redox potential*					
df	4,15	2.4,37	9.8,37	n/a	4 d = 15 d/310 d 4 d < 365 d/497 d 4 d > 407 d/455 d 15 d < 365 d/497 d 15 d > 310 d/407 d/455 d 310 d < 365 d/497 d 310 d > 407 d/455 d 365 d > 407 d/455 d/497 d 407 d < 455 d/497 d 455 d < 497 d
F	1.1	293	2.5		
p	0.39	<0.001	0.021		
SS	0.002	0.86	0.03		

	Treatment	Time	Interaction	Further analysis^	
				Treatment	Time
Conductivity*					
df	4,15	1.8,27	7.3,27	n/a	15 d < 31 d/42 d/72 d/ 100 d/135 d/161 d/247 d 15 d = 310 d/365 d/ 407 d /455 d/497 d 31 d = 72 d/247 d 31 d < 42 d/100 d/135 d/ 161 d 31d > 310 d/365 d/ 407 d /455 d/497 d 42 d = 72 d = 161 d 42d/72 d < 100 d/135 d 42 d/72 d/100 d/161 d > 247 d/310 d/365 d/407 d/455 d/497 d 100 d = 161 d 100 d < 135 d 135 d > all others 247 d > 310 d/365 d/407 d/455 d/497 d 310 d = 407 d/455 d 310 d < 365 d 310 > 497 d 365 d = 407 d > 455 d = 497 d
p	0.67	<0.001	0.44		
SS	10,000	1,000,000	41,000		
Dissolved oxygen*					
df	4,15	3.7,55	15,55	n/a	4 d < 15 d < all others 31 d = 72 d = 497 d 31 d < 42 d/100 d/407 d/455 d 31 d > 310 d/365 d 42 d > 72 d/310 d/365 d/497 d 42 d/72 d < 100 d/407 d/455 d 72 d = 365 d/497 d 72 d > 310 d 100 d > all others 310 d < 365 d < 407 d/ 455 d/497 d 497 d < 407 d < 455 d
F	0.59	190	0.75		
p	0.68	<0.001	0.72		
SS	330	140,000	2,300		

	Treatment	Time	Interaction	Further analysis^	
				Treatment	Time
Turbidity*					
df	4,15	4.2,64	17,64	n/a	31 d = 42 d = 72 d > 100 d/ 135 d/161 d/310 d/365 d/ 407 d/497 d 100 d = 135 d = 161 d/310 d 100 d/135 d > 365 d/407 d/497 d 161 d > 310 d/365 d/ 407 d/497 d 310 d = 365 d/497 d 310 d > 407 d 365 d = 407 d/497 d 407 d < 497 d
F	0.13	60	1.2		
p	0.97	<0.001	0.28		
SS	0.094	22	1.8		
Sediment copper*					
df	4,15	4,64	17,64	@ 15 d: $F_{4,15} = 480$, $p < 0.001$ @ 31 d: $F_{4,15} = 270$, $p < 0.001$ @ 42 d: $F_{4,15} = 300$, $p < 0.001$ @ 72 d: $F_{4,15} = 300$, $p < 0.001$ @ 100 d: $F_{4,15} = 320$, $p < 0.001$ @ 135 d: $F_{4,15} = 240$, $p < 0.001$ @ 161 d: $F_{4,15} = 150$, $p < 0.001$ @ 365 d: $F_{4,15} = 200$, $p < 0.001$ @ 407 d: $F_{4,15} = 150$, $p < 0.001$ @ 497 d: $F_{4,15} = 280$, $p < 0.001$	Control: $F_{9,27} = 5.2$, $p < 0.001$ Very low: $F_{9,27} = 2$, $p = 0.073$ Low: $F_{9,27} = 3.8$, $p = 0.003$ High: $F_{9,27} = 2.6$, $p = 0.025$ Very high: $F_{9,27} = 5.1$, $p < 0.001$
F	2100	9.0	2.6		
p	<0.001	<0.001	0.003		
SS	120	0.95	1.1		

	Treatment	Time	Interaction	Further analysis^	
				Treatment	Time
Pore water copper					
df	4,15	3.6,53	14,53	@ 15 d: $F_{4,15}$ = 110, p < 0.001 @ 31 d: $F_{4,15}$ = 210, p < 0.001 @ 42 d: $F_{4,15}$ = 280, p < 0.001 @ 72 d: $F_{4,15}$ = 43, p < 0.001 @ 100 d: $F_{4,15}$ = 24, p < 0.001 @ 135 d: $F_{4,15}$ = 110, p < 0.001 @ 161 d: $F_{4,15}$ = 29, p < 0.001 @ 365 d: $F_{4,15}$ = 10, p < 0.001 @ 407 d: $F_{4,15}$ = 79, p < 0.001 @ 497 d: $F_{4,15}$ = 58, p < 0.001	Control: $F_{9,27}$ = 9.3, p < 0.001 Very low: $F_{9,27}$ = 0.99, p = 0.47 Low: $F_{9,27}$ = 7.5, p < 0.001 High: $F_{9,27}$ = 5.2, p < 0.001 Very high: $F_{9,27}$ = 4, p = 0.003
p	<0.001	<0.001	<0.001		
SS	35	2.3	2.5		
Overlying water copper					
df	4,15	3.2,48	13,48	@ 15 d: $F_{4,15}$ = 79, p < 0.001 @ 31 d: $F_{4,15}$ = 52, p < 0.001 @ 42 d: $F_{4,15}$ = 14, p < 0.001 @ 72 d: $F_{4,15}$ = 125, p < 0.001 @ 100 d: $F_{4,15}$ = 30, p < 0.001 @ 135 d: $F_{4,15}$ = 48, p < 0.001 @ 161 d: $F_{4,15}$ = 88, p < 0.001 @ 365 d: $F_{4,15}$ = 66, p < 0.001 @ 407 d: $F_{4,15}$ = 73, p < 0.001 @ 497 d: $F_{4,15}$ = 130, p < 0.001	Control: $F_{9,27}$ = 8.1, p < 0.001 Very low: $F_{9,27}$ = 3.4, p = 0.007 Low: $F_{9,27}$ = 2.5, p = 0.031 High: $F_{9,27}$ = 9, p < 0.001 Very high: $F_{9,27}$ = 2.7, p = 0.022
p	<0.001	0.007	<0.001		
SS	15	0.37	1.4		

^Where no significant interaction was found this is the results of the LSD posthoc analysis. Where a significant interaction was found, this is the main results of the one-way ANOVAs performed to test the effect of treatment at each time point and the RM ANOVAs to test the effect of time within each treatment.

*Significance level α 0.01

Table A4.3: Taxa recorded across all data sets

Taxa	Size class	Total count	Percentage of total abundance
Ostracoda Species 1	Me	8097	30
Nematoda	Me	7154	30
Cladocera	Me	2785	10
Rotifera	Me	2203	8
Copepoda	Me	1931	7
Ostracoda Species 2	Me	939	3
Ostracoda Species 3	Me	735	3
Acarina	Me	69	0.3
Collembola	Me	37	0.1
Diptera/ Chironomidae/ Chironominae	Ma	1840	7
Diptera/ Chironomidae/ Tanypodinae	Ma	822	3
Odonata/ Libellulidae	Ma	146	0.5
Diptera/ Culicidae	Ma	89	0.3
Gastropoda/ Physidae/ *Physa acuta*	Ma	86	0.3
Hemiptera/ Mesovellidae/ *Mesovelia*	Ma	63	0.2
Diptera/ Ceratopoginidae	Ma	23	0.09
Hemiptera/unknown	Ma	15	0.06
Odonata/ Coenagrionidae/ *Austroagrion watsoni*	Ma	13	0.05
Hemiptera/ Notonectidae/ *Martarega*	Ma	7	0.03
Diptera/ Simuliidae	Ma	5	0.02
Hempitera/ Corixidae/ *Agraptocoria*	Ma	3	0.01
Amphipoda	Ma	3	0.01
Diptera/ Chironomidae/ Podonomine	Ma	2	0.007
Gastropoda/ Planorbidae/ *Helicorbis*	Ma	2	0.007
Trichoptera/ Limnephilidae/ *Archaeophylax*	Ma	1	0.004
Trichoptera/ Ecnomidae/ *Ecnomus*	Ma	1	0.004
Odonata/ Lestidae/ *Austrolestes*	Ma	1	0.004
Odonata/ Coenagrionidae/ *Xanthagrion erythroneurum*	Ma	1	0.004
Odonata/ Coenagrionidae/ *Ischnura*	Ma	1	0.004
Odonata/ Protoneuridae/ *Nososticta*	Ma	1	0.004
Hemiptera/ Gerridae	Ma	1	0.004
Gastropoda/ Lymnaeidae/ *Pseudosuccinea*	Ma	1	0.004
Ephemeroptera/ Baetidae/ Baetid Genus 1	Ma	1	0.004
Decapoda/ Atyidae	Ma	1	0.004
Arachnida	Ma	1	0.004

Table A4.4: Results of RM ANOVAs and further analyses performed on benthic taxa data.

Variable	Treatment	Time	Interaction	Further analysis^	
				Treatment	Time
Benthic total abundance*					
df	4,15	7,105	28,105	C/VL/L/H > VH	31 d <72 d <100 d < 135 d < (161 = 365 d = 407 d = 497 d)
F	6.9	58	1.6		
p	0.002	<0.001	0.058		
SS	4.7	59	6.3		
Benthic taxa richness					
df	4,15	7,105	28,105	@ 31 d: $F_{4,15}$ = 0.2, p = 0.93 @ 72 d: $F_{4,15}$ = 3.8, p = 0.026 @ 100 d: $F_{4,15}$ = 3.6, p = 0.031 @ 135 d: $F_{4,15}$ = 3.6, p = 0.03 @ 161 d: $F_{4,15}$ = 4.1, p = 0.02 @ 365 d: $F_{4,15}$ = 3.1, p = 0.048 @ 407 d: $F_{4,15}$ = 87, p = 0.51 @ 497 d: $F_{4,15}$ = 19, p = 0.94	Control: $F_{7,21}$ = 5.6, p = 0.001 Very low: $F_{7,21}$ = 11, p < 0.001 Low: $F_{7,21}$ = 10, p < 0.001 High: $F_{7,21}$ = 7.9, p < 0.001 Very high: $F_{7,21}$ = 7.7, p < 0.001
F	5.3	73	1.6		
p	0.007	<0.001	0.047		
SS	88	510	95		
Benthic diversity					
df	4,15	7,105	28,105	@ 31 d: $F_{4,15}$ = 0.41, p = 0.8 @ 72 d: $F_{4,15}$ = 0.27, p = 0.069 @ 100 d: $F_{4,15}$ = 3.3, p = 0.04 @ 135 d: $F_{4,15}$ = 1.8, p = 0.17 @ 161 d: $F_{4,15}$ = 1.6, p = 0.22 @ 365 d: $F_{4,15}$ = 2.2, p = 0.11 @ 407 d: $F_{4,15}$ = 1.1, p = 0.41 @ 497 d: $F_{4,15}$ = 1.5, p = 0.25	Control: $F_{7,21}$ = 7.9, p < 0.001 Very low: $F_{7,21}$ = 4.1, p = 0.006 Low: $F_{7,21}$ = 4.6, p = 0.003 High: $F_{7,21}$ = 2.7, p = 0.036 Very high: $F_{7,21}$ = 3.8, p = 0.008
F	1.8	14	1.9		
p	0.18	<0.001	0.012		
SS	2.7	14	7.2		

Variable	Treatment	Time	Interaction	Further analysis^	
				Treatment	Time
Benthic chironominae abundance*					
df	4,15	7,105	28,105	@ 31 d: $F_{4,15} = 0.27$, $p = 0.9$ @ 72 d: $F_{4,15} = 4.8$, $p = 0.011$* @ 100 d: $F_{4,15} = 7.9$, $p = 0.001$* @ 135 d: $F_{4,15} = 14$, $p < 0.001$ @ 161 d: $F_{4,15} = 4.8$, $p = 0.011$ @ 365 d: $F_{4,15} = 3.2$, $p = 0.045$ @ 407 d: $F_{4,15} = 4.6$, $p = 0.012$ @ 497 d: $F_{4,15} = 4.6$, $p = 0.012$	Control: $F_{7,21} = 3.9$, $p = 0.006$ Very low: $F_{7,21} = 8.8$, $p < 0.001$ Low: $F_{7,21} = 9.7$, $p < 0.001$ High: $F_{7,21} = 5.9$, $p = 0.001$ Very high: $F_{7,21} = 1.4$, $p = 0.28$
F	23	21	2.9		
p	<0.001	<0.001	<0.001		
SS	12	18	10		
Benthic Ostracoda Sp. 2 abundance*					
df	4,15	2.7,40	11,40	@ 31 d: none present @ 72 d: $F_{4,15} = 2.7$, $p = 0.069$ @ 100 d: $F_{4,15} = 5.6$, $p = 0.006$ @ 135 d: $F_{4,15} = 4.3$, $p = 0.017$ @ 161 d: $F_{4,15} = 8.2$, $p = 0.001$ @ 365 d: $F_{4,15} = 2.4$, $p = 0.098$ @ 407 d: $F_{4,15} = 6.6$, $p = 0.003$ @ 497 d: $F_{4,15} = 2.2$, $p = 0.112$	Control: $F_{7,21} = 4.8$, $p = 0.002$ Very low: $F_{7,21} = 5.6$, $p = 0.073$ Low: $F_{7,21} = 1.4$, $p = 0.25$ High: $F_{7,21} = 0.84$, $p = 0.57$ Very high: $F_{7,21} = 0.75$, $p = 0.63$
F	14	8.8	2.6		
p	<0.001	<0.001	0.014		
SS	11	8	9.4		
Benthic Cladocera abundance					
df	4,15	3.5,53	14,53	@ 31 d: none present @ 72 d: $F_{4,15} = 2.7$, $p = 0.069$ @ 100 d: $F_{4,15} = 0.65$, $p = 0.64$ @ 135 d: $F_{4,15} = 2$, $p = 0.15$ @ 161 d: $F_{4,15} = 2.8$, $p = 0.063$ @ 365 d: $F_{4,15} = 1.9$, $p = 0.16$ @ 407 d: $F_{4,15} = 1$, $p = 0.17$ @ 497 d: $F_{4,15} = 0.83$, $p = 0.53$	Control: $F_{7,21} = 2.4$, $p = 0.055$ Very low: $F_{7,21} = 3.9$, $p = 0.007$ Low: $F_{7,21} = 2.3$, $p = 0.07$ High: $F_{7,21} = 5.8$, $p = 0.001$ Very high: $F_{7,21} = 1$, $p = 0.46$
F	2.3	8.8	2.1		
p	0.11	<0.001	0.029		
SS	6.4	11	11		

Variable	Treatment	Time	Interaction	Further analysis^	
				Treatment	Time
Benthic *P. acuta* abundance					
df	4,15	7,105	28,105	n/a	None found 31 d – 365 d 407 d = 497 d
F	1.3	5.6	0.72		
p	0.30	<0.001	0.84		
SS	1.4	8.6	4.4		
Benthic Copepoda abundance*					
df	4,15	2.7,40	11,40	n/a	31 d = 72 d = 100 d = 135 d = 161 d = 365 d 31 d/72 d/100 d < 407 d/497 d 135 d/161 d/365 d = all others 407 d = 497 d
F	0.99	5.8	1.0		
p	0.44	0.003	0.44		
SS	28	73	52		

^Where no significant interaction was found this is the results of the LSD posthoc analysis. Where a significant interaction was found, this is the main results of the one-way ANOVAs performed to test the effect of treatment at each time point and the RM ANOVAs to test the effect of time within each treatment.

Table A4.5 Outputs from distLM analysis of relationship between benthic community assemblages and sediment and pore water copper concentrations.

Variable	SS (trace)	Pseduo-F	p	Prop
SEDCU	19000	13	0.001	8.70E-02
PWCU	15000	10	0.001	6.80E-02

Table A4.6: PERMANOVA analysis with post hoc pairwise comparisons of benthic community assemblages at each time point

Factor	df	SS	MS	Pseudo-F	P(perm)
Treatment	4	24076	6019	6.49	0.001
Time	7	$1.1x10^5$	16079	17.3	0.001
Interaction	28	36460	1302	1.4	0.002
Res	120	$1.1x10^5$	930		
Total	159	$2.8x10^5$			

Pair-wise comparison *p* values

	C	VL	L	H
t = 32 d				
VL	0.915			
L	0.578	0.727		
H	0.796	n/a	0.558	
VH	0.775	0.819	0.681	1
t = 74 d				
VL	0.121			
L	0.882	0.45		
H	0.039	0.052	0.063	
VH	0.03	0.026	0.024	0.559
t = 102 d				
VL	0.227			
L	0.351	0.521		
H	0.111	0.375	0.059	
VH	0.075	0.067	0.026	0.247
t = 137 d				
VL	0.744			
L	0.21	0.587		
H	0.233	0.112	0.223	
VH	0.03	0.025	0.024	0.348

t = 163 d				
VL	0.796			
L	0.526	0.193		
H	0.026	0.032	0.192	
VH	0.021	0.03	0.056	0.615
t = 368 d				
VL	1			
L	0.624	0.737		
H	0.285	0.062	0.196	
VH	0.145	0.091	0.063	0.642
t = 409 d				
VL	0.126			
L	0.443	0.376		
H	0.063	0.057	0.033	
VH	0.033	0.025	0.059	0.026
t = 500 d				
VL	0.864			
L	0.175	0.329		
H	0.122	0.029	0.055	
VH	0.058	0.031	0.058	0.118

Table A4.7: Outputs from distLM analysis of relationship between water column community assemblages and sediment and overlying water copper concentrations.

Variable	SS (trace)	Pseduo-F	p	Prop
SEDCU	1700	1.8	0.133	1.30E-02
OWCU	950	1	0.402	7.20E-03

Table A4.8: PERMANOVA analysis with post hoc pairwise comparisons of water column community assemblages at each time point

Factor	df	SS	MS	Pseudo-F	P(perm)
Treatment	4	5500	1400	1.8	0.017
Time	8	$1.3x10^5$	16000	22	0.001
Interaction	32	24000	760	1	0.413
Res	135	$1.0x10^5$	750		
Total	179	$2.6x10^5$			

Pair-wise comparison *p* values				
	C	VL	L	H
t = 3 d				
VL	0.906			
L	1	0.977		
H	0.459	0.345	0.308	
VH	0.501	0.333	0.796	0.427
t = 31 d				
VL	0.171			
L	0.661	0.776		
H	0.361	0.697	0.5	
VH	0.155	0.399	0.521	0.602
t = 73 d				
VL	0.521			
L	0.338	0.432		
H	0.191	0.186	0.677	
VH	0.028	0.881	0.029	0.059
t = 101 d				
VL	0.346			
L	0.463	0.966		
H	0.626	0.179	0.617	
VH	0.151	0.049	0.092	0.267
t = 136 d				
VL	0.821			
L	0.743	0.77		
H	0.951	0.882	0.513	
VH	0.369	0.473	0.674	0.791
t = 161 d				
VL	0.894			
L	0.216	0.133		
H	0.583	0.726	0.204	
VH	0.515	0.31	0.027	0.39
t = 367 d				
VL	0.743			
L	0.421	0.147		
H	0.858	0.724	0.027	
VH	0.708	0.888	0.025	0.854

t = 408 d				
VL	0.623			
L	0.377	1		
H	0.061	0.355	0.682	
VH	0.097	0.792	0.177	0.156
t = 498 d				
VL	0.205			
L	0.621	0.296		
H	0.631	0.285	0.436	
VH	0.343	0.571	0.269	0.818

Table A4.9: Outputs from distLM analysis of relationship between sweep net community assemblages and sediment, pore water and overlying water copper concentrations.

Variable	SS (trace)	Pseduo-F	p	Prop
SEDCU	2700	2.1	0.08	1.00E-01
PWCU	2300	1.7	0.13	8.70E-02
OWCU	2300	1.7	0.15	8.60E-02

Appendix 5. Supporting information to Chapter 5

Table A5.1: Results of RM ANOVAs and further analyses performed on water quality and copper data.

	Treatment	Time	Interaction	Further analysis^	
				Treatment	Time
Total OTU Richness*					
df	4,15	4,57	15,57	@ 15 d: $F_{4,15} = 11$, $p < 0.001$ @ 31 d: $F_{4,15} = 4.5$, $p = 0.014$ @ 135 d: $F_{4,15} = 0.56$, $p = 0.70$ @ 161 d: $F_{4,15} = 2.8$, $p = 0.062$ @ 365 d: $F_{4,15} = 1.3$, $p = 0.33$ @ 407 d: $F_{4,15} = 6.1$, $p = 0.004$ @ 497 d: $F_{4,15} = 4.7$, $p = 0.012$	Control: $F_{6,18} = 4.2$, $p = 0.008$ Very low: $F_{6,18} = 12$, $p < 0.001$ Low: $F_{6,18} = 20$, $p < 0.001$ High: $F_{6,18} = 0.57$, $p = 0.075$ Very high: $F_{6,18} = 4.6$, $p = 0.005$
F	4	10	4.9		
p	0.021	<0.001	<0.001		
SS	20,000	31,000	58,000		
Cercozoa OTU Richness					
df	4,15	6,90	24,90	@ 15 d: $F_{4,15} = 4.6$, $p = 0.013$ @ 31 d: $F_{4,15} = 3.3$, $p = 0.038$ @ 135 d: $F_{4,15} = 0.36$, $p = 0.83$ @ 161 d: $F_{4,15} = 2.4$, $p = 0.28$ @ 365 d: $F_{4,15} = 0.58$, $p = 0.69$ @ 407 d: $F_{4,15} = 4.6$, $p = 0.013$ @ 497 d: $F_{4,15} = 2.7$, $p = 0.071$	Control: $F_{6,18} = 1.1$, $p = 0.38$ Very low: $F_{6,18} = 1.4$, $p = 0.27$ Low: $F_{6,18} = 5.4$, $p = 0.002$ High: $F_{6,18} = 3.4$, $p = 0.021$ Very high: $F_{6,18} = 6.2$, $p = 0.001$
F	1.7	5.2	3.1		
p	0.19	<0.001	<0.001		
SS	380	510	1200		
Chlorophyta OTU Richness					
df	4,15	3.6,54	14,54	@ 15 d: $F_{4,15} = 4.3$, $p = 0.017$ @ 31 d: $F_{4,15} = 1.3$ $p = 0.31$ @ 135 d: $F_{4,15} = 2.4$, $p = 0.094$ @ 161 d: $F_{4,15} = 3.4$, $p = 0.035$ @ 365 d: $F_{4,15} = 2.7$, $p = 0.073$ @ 407 d: $F_{4,15} = 7$, $p = 0.002$ @ 497 d: $F_{4,15} = 4.1$, $p = 0.02$	Control: $F_{6,18} = 1.9$, $p = 0.14$ Very low: $F_{6,18} = 4$, $p = 0.01$ Low: $F_{6,18} = 10$, $p < 0.001$ High: $F_{6,18} = 2.6$, $p = 0.055$ Very high: $F_{6,18} = 2.7$, $p = 0.05$
F	8	5.9	2.6		
p	0.001	0.001	0.005		
SS	800	430	770		

	Treatment	Time	Interaction	Further analysis^	
				Treatment	Time
Nematoda OTU Richness*					
df	4,15	3.7,56	15,56	@ 15 d: $F_{4,15} = 3.5$, $p = 0.033$ @ 31 d: $F_{4,15} = 1.6$ $p = 0.25$ @ 135 d: $F_{4,15} = 2.5$, $p = 0.09$ @ 161 d: $F_{4,15} = 3.2$, $p = 0.043$ @ 365 d: $F_{4,15} = 2.2$, $p = 0.11$ @ 407 d: $F_{4,15} = 2.8$, $p = 0.064$ @ 497 d: $F_{4,15} = 4$, $p = 0.021$	Control: $F_{6,18} = 9.3$, $p < 0.001$ Very low: $F_{6,18} = 22$, $p < 0.001$ Low: $F_{6,18} = 21$, $p < 0.001$ High: $F_{6,18} = 12$, $p < 0.001$ Very high: $F_{6,18} = 14$, $p < 0.001$
F	3.7	61	2.6		
p	0.028	<0.001	0.005		
SS	900	7500	1300		
Ciliophora					
df	4,15	6,90	24,90	@ 15 d: $F_{4,15} = 0.5$, $p = 0.73$ @ 31 d: $F_{4,15} = 3$ $p = 0.054$ @ 135 d: $F_{4,15} = 5.3$, $p = 0.007$ @ 161 d: $F_{4,15} = 11$, $p < 0.001$ @ 365 d: $F_{4,15} = 4.8$, $p = 0.01$ @ 407 d: $F_{4,15} = 3.9$, $p = 0.023$ @ 497 d: $F_{4,15} = 8.7$, $p = 0.001$	Control: $F_{6,18} = 1.4$, $p = 0.27$ Very low: $F_{6,18} = 2.3$, $p = 0.081$ Low: $F_{6,18} = 3.3$, $p = 0.022$ High: $F_{6,18} = 3.2$, $p = 0.024$ Very high: $F_{6,18} = 11$, $p < 0.001$
F	18	9.9	1.8		
p	<0.001	<0.001	0.029		
SS	1200	610	440		
Ascomycota					
C	4,15	2.8,42	11,42	@ 15 d: $F_{4,15} = 10$, $p < 0.001$ @ 31 d: $F_{4,15} = 20$, $p < 0.001$ @ 135 d: $F_{4,15} = 2.7$, $p = 0.070$ @ 161 d: $F_{4,15} = 3.6$, $p = 0.03$ @ 365 d: $F_{4,15} = 1.1$, $p = 0.41$ @ 407 d: $F_{4,15} = 5.5$, $p = 0.006$ @ 497 d: $F_{4,15} = 0.53$, $p = 0.72$	Control: $F_{6,18} = 2.2$, $p = 0.086$ Very low: $F_{6,18} = 1.6$, $p = 0.2$ Low: $F_{6,18} = 4$, $p = 0.011$ High: $F_{6,18} = 8.5$, $p < 0.001$ Very high: $F_{6,18} = 18$, $p < 0.001$
F	400	29	6.4		
p	<0.001	<0.001	<0.001		
SS	11,000	2600	2400		
Chytridiomycota					
df	4,15	6,90	24,90	@ 15 d: $F_{4,15} = 3.6$, $p = 0.029$ @ 31 d: $F_{4,15} = 2.6$, $p = 0.078$ @ 135 d: $F_{4,15} = 2.1$, $p = 0.13$ @ 161 d: $F_{4,15} = 4.3$, $p = 0.016$	Control: $F_{6,18} = 5.2$, $p = 0.003$ Very low: $F_{6,18} = 9.7$, $p = 0.052$ Low: $F_{6,18} = 9$, $p < 0.001$ High: $F_{6,18} = 3.8$, $p = 0.013$
F	12	14	2.8		
p	<0.001	<0.001	<0.001		

	Treatment	Time	Interaction	Further analysis^	
				Treatment	Time
SS	270	260	210	@ 365 d: $F_{4,15} = 0.81$, $p = 0.54$ @ 407 d: $F_{4,15} = 11$, $p < 0.001$ @ 497 d: $F_{4,15} = 8.5$, $p = 0.001$	Very high: $F_{6,18} = 4.6$, $p = 0.005$
Fungi (ukP)					
df	4,15	2.5,38	10,38	n/a	15d < 31d < (135d = 161d) < (365d < 407d) = 497d
F	1.4	45	1.1		
p	0.29	<0.001	0.42		
SS	56	1200	110		
Rhizaria (ukP)					
df	4,15	6,90	24,90	(C > VL) = L > (H = VH)	15d = 31d/365d/497d 15d < 135d/161d/407d 135d > 497d 31d = 135d = 161d/365d/407d 31d = 161d = 365d = 407d = 497d
F	8.6	3	1.2		
p	0.001	0.009	0.26		
SS	330	61	96		
Rotifera					
df	4,15	3.6,54	14,54	@ 15 d: $F_{4,15} = 0.58$, $p = 0.68$ @ 31 d: $F_{4,15} = 1.5$, $p = 0.26$ @ 135 d: $F_{4,15} = 3.9$, $p = 0.022$ @ 161 d: $F_{4,15} = 4.3$, $p = 0.017$ @ 365 d: $F_{4,15} = 2.8$, $p = 0.065$ @ 407 d: $F_{4,15} = 0.65$, $p = 0.64$ @ 497 d: $F_{4,15} = 9.1$, $p = 0.001$	Control: $F_{6,18} = 7.6$, $p < 0.001$ Very low: $F_{6,18} = 4.6$, $p = 0.005$ Low: $F_{6,18} = 8.8$, $p < 0.001$ High: $F_{6,18} = 6.4$, $p = 0.001$ Very high: $F_{6,18} = 7.7$, $p < 0.001$
F	6	25	2.7		
p	0.004	<0.001	0.004		
SS	180	510	220		

^Where no significant interaction was found this is the results of the LSD posthoc analysis. Where a significant interaction was found, this is the main results of the one-way ANOVAs performed to test the effect of treatment at each time point and the RM ANOVAs to test the effect of time within each treatment.

Table A5.2: PERMANOVA analysis with post hoc pairwise comparisons of benthic community assemblages at each time point

Factor	df	SS	MS	Pseudo-F	P(perm)
Treatment	4	76192	19048	8.4575	0.001
Time	6	90439	15073	6.6926	0.001
Interaction	24	82060	3419.2	1.5181	0.001
Res	105	2.36×10^5	2252.2		
Total	139	4.85×10^5			

Pair-wise comparison *p* values

	C	VL	L	H
t = 15 d				
VL	0.026			
L	0.025	0.033		
H	0.033	0.029	0.026	
VH	0.023	0.024	0.029	0.034
t = 31 d				
VL	0.019			
L	0.032	0.029		
H	0.026	0.027	0.025	
VH	0.024	0.03	0.029	0.021
t = 135 d				
VL	0.039			
L	0.023	0.034		
H	0.024	0.028	0.029	
VH	0.035	0.014	0.02	0.042
t = 161 d				
VL	0.025			
L	0.029	0.032		
H	0.033	0.02	0.032	
VH	0.027	0.03	0.029	0.032
t = 365 d				
VL	0.061			
L	0.03	0.045		
H	0.023	0.024	0.041	
VH	0.029	0.036	0.034	0.029
t = 407 d				
VL	0.179			
L	0.026	0.357		
H	0.024	0.039	0.037	

VH	0.034	0.031	0.033	0.036
t = 497 d				
VL	0.143			
L	0.052	0.109		
H	0.026	0.034	0.03	
VH	0.025	0.032	0.031	0.03
Control				
(15d = 31d) ≠ 135 = 161 d = 365 d = 407 d = 497 d 135/161 d ≠ 407 d/497 d 365 d ≠ 497 d				
Very low				
15d ≠ 31d ≠ 135 = 161 d = 365 d = 407 d = 497 d 135/161 d ≠ 407 d/497 d				
Low				
15d ≠ 31d ≠ 135 = 161 d = 365 d = 407 d = 497 d 135 ≠ 407 d/497 d				
High				
15d ≠ 31d ≠ 135 = 161 d = 365 d = 407 d = 497 d 135/161 d ≠ 407 d/497 d 365 d ≠ 497 d				
Very high				
15d ≠ 31d ≠ 135 = 161 d = 365 d = 407 d = 497 d 135 ≠ 365 d/407 d/497 d 161 d/365 d ≠ 497 d				

Table A5.3: Outputs from distLM analysis of relationship between benthic community assemblages and sediment and pore water copper concentrations.

Variable	SS (trace)	Pseduo-F	p	Prop
SEDCU	42936	13.4	0.001	8.85×10^{-2}

Table A5.4: Benthic community TITAN with sediment copper concentrations: community level results

	cp	0.05	0.1	0.5	0.9	0.95
sumz-	181.395	76.49	85.37	93.99	279.49	281.5615
sumz+	181.395	93.99	93.99	181.395	360.07	360.07
ncpa.bc	181.395	85.37	90.055	181.395	360.07	360.07
ncpa.euc	181.395	76.49	85.37	181.395	320.92	360.07

Table A5.5: Benthic community TITAN with pore water copper concentrations: community level results

	cp	0.05	0.1	0.5	0.9	0.95
sumz-	8.37	2.955	2.955	4.15	13.61	25.09
sumz+	13.61	3.66	3.66	8.37	25.09	25.09
ncpa.bc	8.37	2.955	3.66	8.37	18.45	25.09
ncpa.euc	8.37	2.955	3.105	8.37	18.45	25.09

Appendix 6. Supporting information to Chapter 6

Table A6.1: Transformations and analyses performed on endpoints considering ecosystem functions.

Endpoint	Transformation	Analysis performed
Sediment copper concentration	$Log_{10}(x+1)$	RM-ANOVA
Pore water copper concentration	$Log_{10}(x+1)$	RM-ANOVA
Overlying water copper concentration	$Log_{10}(x+1)$	RM-ANOVA
Initial wet weight of *V. spiralis*	Untransformed	One-way ANOVA
Shoot density of *V. spiralis*	Square root	RM-ANOVA
Total chlorophyll a concentration	$Log_{10}(x+1)$	RM-ANOVA
Diatom chlorophyll a concentration	$Log_{10}(x+1)$	RM-ANOVA
Cyanobacteria chlorophyll a concentration	$Log_{10}(x+1)$	RM-ANOVA
Green algae chlorophyll a concentration	$Log_{10}(x+1)$	RM-ANOVA
Periphyton cover	Arcsine	One-way ANOVA
Leaf litter mass loss (%IW)	Arcsine	Two way-ANOVA
Unexposed cotton strip tensile strength	Untransformed	One-way ANOVA
Surface cotton strip tensile strength	$Log_{10}(x+1)$	Two way-ANOVA
Buried cotton strip tensile strength	$Log_{10}(x+1)$	Two way-ANOVA
Leaf litter decay rate	Untransformed	One-way ANOVA
Surface cotton strip decay rate	Untransformed	One-way ANOVA
Buried cotton strip decay rate	Untransformed	One-way ANOVA

Table A6.2: Results from RM ANOVAs performed on sediment, pore water and overlying water copper concentrations, and subsequent analyses.

	Main effects			**Further analysis^**	
	Treatment	**Time**	**Interaction**	**Treatment**	**Time**
Sediment copper*					
df	4,15	3,45	12,45	C< VL< L< H< VH	t = 409 d > all other time points
F	580	4.5	0.62		
p	<0.001	0.008	0.81		
SS	37	0.17	0.094		
Pore water copper*					
df	4,15	3,45	12,45	@ 365 d: $F_{4,15}$ = 10, p < 0.001 @ 407 d: $F_{4,15}$ = 79, p < 0.001 @ 497 d: $F_{4,15}$ = 58, p < 0.001 @ 737 d: $F_{4,15}$ = 6.2, p = 0.004	Control: $F_{3,9}$ = 8, *p* = 0.007 Very low: $F_{3,9}$ = 39, *p* = 0.027 Low: $F_{3,9}$ = 21, *p* < 0.001 High: $F_{3,9}$ = 25, *p* < 0.001 Very high: $F_{3,9}$ = 3.5, *p* = 0.065
F	54	29	4.4		
p	<0.001	<0.001	<0.001		
SS	11	2.6	1.6		
Overlying water copper*					
df	4,15	1.6,24	6.4,24	C< VL< L< H< VH	t = 368 d > t = 409 d t = 737 d > all other time points
F	150	15	0.92		
p	<0.001	<0.001	0.50		
SS	8.0	0.54	0.13		

^Where no significant interaction was found this is the results of the LSD posthoc analysis. Where a significant interaction was found, this is the main results of the one-way ANOVAs performed to test the effect of treatment at each time point and the RM ANOVAs to test the effect of time within each treatment.

*Significance level α 0.01

Table A6.3: Results from ANOVAs performed on plant growth phytoplankton chlorophyll *a* and periphyton data.

	Main effects			Further analysis	
	Treatment	Time	Interaction	Treatment	Time
V. spiralis Shoot density*					
df	4,10	1,10	4,10	@ December 2011: $F_{4,18}$ = 2.9, p = 0.06 @ October 2012: $F_{4,14}$ = 19, p < 0.001	Control: $F_{1,1}$ = 300, *p* = 0.037 Very low: $F_{1,1}$ = 870, *p* = 0.022 Low: $F_{1,2}$ = 10, *p* = 0.085 High: $F_{1,3}$ = 72, *p* = 0.003 Very high: $F_{1,3}$ = 3.5, *p* = 0.160
F	22	120	16		
p	<0.001	<0.001	<0.001		
SS	15000	24000	12000		
Total chlorophyll *a*					
df	4,15	1.6,24	6.4,24	n/a	
F	1.5	1.9	1.1		
p	0.24	0.18	0.42		
SS	9.4	0.099	0.23		
Diatom chlorophyll *a*					
df	4,15	1.7,25	6.8,25	n/a	
F	1.5	2.0	0.87		
p	0.26	0.16	0.54		
SS	8.3	0.097	0.17		
Cyanobacteria chlorophyll *a*					
df	4,15	1.8,27	7.2, 27	n/a	
F	1.2	0.40	0.78		
p	0.35	0.66	0.61		
SS	4.4	0.012	0.097		
Green algae chlorophyll *a**					
df	4,15	2,30	8.1, 30	n/a	
F	0.72	1.5	0.65		
p	0.59	0.24	0.73		
SS	0.060	0.012	0.022		
Periphyton cover*					
df	4,15	One way ANOVA LSD posthoc (effect of treatment): C~<H (*p* = 0.027); C<VH (*p* = 0.001); VL~<H (*p* = 0.027); VL<VH (*p* = 0.001); L~<H (p = 0.03); L<VH (p = 0.001); H~<VH (p = 0.073)			
F	7.9				
p	0.001				
SS	1.8				

^Where no significant interaction was found this is the results of the LSD posthoc analysis. Where a significant interaction was found, this is the main results of the one-way ANOVAs performed to test the effect of treatment at each time point and the RM ANOVAs to test the effect of time within each treatment.

*Significance level α 0.01

Table A6.4: Results from two-way/one-way ANOVAs performed on decomposition end points.

Variable	Treatment	Time	Interaction	LSD posthoc (treatment)	LSD posthoc (time)
Leaf litter mass loss*					
df	4,60	3,60	12,60	n/a	10 wks < 15 wks < 20 wks < 25 wks
F	1.1	52	0.93		
p	0.37	<0.001	0.62		
SS	0.01	0.37	0.023		
Leaf litter decay rates					
df	4,15	n/a			
F	0.36				
p	0.84				
SS	0.000				
Surface cotton strip tensile strength*					
df	4,60	3,60	12,60	C>(VL=L=H=VH)	10 wks < 15 wks < 20 wks < 25 wks
F	5.1	34	0.77		
p	0.001	<0.001	0.68		
SS	0.11	0.54	0.049		
Surface cotton strip decay rates					
df	4,15	n/a			
F	1.6				
p	**0.22**				
SS	0.000				
Buried cotton strip tensile strength					
df	4,60	3,60	12,60	C<(VL=L)<(H=VH)	10 wks < 15 wks < (20 = 25 wks)
F	14	25	0.53		
p	<0.001	<0.001	0.89		
SS	0.50	0.66	0.056		
Buried cotton strip decay rates					
df	4,15	n/a			
F	1.1				
p	0.38				
SS	0.000				

*Significance level α 0.01