Biomarkers of Cadmium, Lead and Selenium Toxicity in the Marine Bivalve Molluscs *Tellina deltoidalis* and *Anadara trapezia*: Linking Exposure, Dose and Response

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Abstract

The relationships between metal exposure, dose and response were investigated in two sediment dwelling marine bivalves: a deposit feeder *Tellina deltoidalis* and a filter feeder *Anadara trapezia*. The bivalves were exposed in the laboratory to individual metal spiked sediments: Cadmium 10 and 50 µg/g; lead 100 and 300 µg/g; selenium 5 and 20 µg/g dry mass, *T. deltoidalis* for 28 days *A. trapezia* for 56 days. *A. trapezia* was also exposed in the laboratory for 56 days to sediments from three sites along a metal contamination gradient of cadmium, lead, selenium, zinc and copper from Lake Macquarie, NSW. Metal total tissue dose was measured in whole tissue of *T. deltoidalis* over 28 days and in gill, hepatopancreas and haemolymph tissues in *A. trapezia* over 56 days. Subcellular metal distribution, biologically active metal (BAM) versus biologically detoxified metal (BDM) was measured in whole tissues of *T. deltoidalis* at day 28 and in gill and hepatopancreas tissues of *A. trapezia* at day 56. Biomarkers of response measured in spiked sediment exposed, at day 28 *T. deltoidalis* and day 56 *A. trapezia* were: total antioxidant capacity (TAOC); glutathione peroxidase enzyme activity (GPx); total glutathione concentration (GSH+2GSSG); reduced to oxidised glutathione ratio (GSH:GSSG); lipid peroxidation (TBARS); lysosomal membrane stability and micronuclei frequency. Response indices measured in *A. trapezia* exposed to Lake Macquarie sediments were: TAOC, TBARS, lysosomal membrane stability, micronucleus frequency and condition index. Native *A. trapezia* and sediments were also collected from Lake Macquarie and measured for sediment and tissue metal concentrations, TAOC, TBARS, lysosomal membrane stability and condition index to allow comparison between chronically exposed and previously unexposed organisms.

T. deltoidalis and *A. trapezia* accumulated metal over time in all sediment metal exposures with most reaching equilibrium tissue metal concentrations by the end of the exposure period. *T. deltoidalis* generally reached equilibrium with the exposure concentration for cadmium and lead but had significantly higher selenium tissue concentrations than the sediment metal at the 5 µg/g exposure. *A. trapezia* tissue lead was below the sediment concentration for all exposures including in the native organisms. A high proportion of accumulated lead and copper in *A. trapezia* was in the haemolymph, probably associated with haemoglobin which has a high affinity for these metals`. *A. trapezia* cadmium tissue concentrations were higher than the sediment metal in the 10 µg/g spiked sediment exposure and between half and one eighth the sediment concentrations in other treatments, including in native organisms.

A. trapezia including the native organisms exposed to selenium sediment concentrations at or below 5 µg/g in the Lake Macquarie mixed metal sediments accumulated significantly higher than ambient selenium tissue concentrations while those exposed to 5 and 20 µg/g selenium spiked sediments had lower than ambient selenium tissue concentrations. The majority of accumulated cadmium, selenium and zinc was associated with the gill/mantle tissues. *A. trapezia* hepatopancreas contributed significant selenium concentrations in the later part of the exposure period indicating and increased contribution from dietary derived selenium. Native *A. trapezia* had significantly lower tissue concentrations of selenium, copper and zinc, higher cadmium and approximately equal lead compared to organisms exposed to similar sediment metal concentrations in the laboratory.

T. deltoidalis detoxified around 50 % of accumulated cadmium and 70 % of lead while *A. trapezia* detoxified around 70 % of accumulated cadmium and 60 % of lead. Much of *T. deltoidalis* BDM cadmium was converted to metal rich granules (MRG), while *A. trapezia* had most in the metallothionein like proteins (MTLP) fraction. The conversion of lead to MRG was 75 % of the total BDM in *T. deltoidalis* while *A. trapezia* had an even distribution between MRG and MTLP. The majority of recovered selenium in both species was associated with the nuclei+cellular debris fraction, probably as protein bound selenium associated with plasma and selenium bound directly to cell walls. Selenium exposed organisms had increased BDM selenium burdens which were associated with both MRG and MTLP fractions, indicating selenium detoxification. The majority of BAM cadmium, lead and selenium was associated with the mitochondrial fraction in both species with increases in cadmium burden in this organelle of *T. deltoidalis* up to 7200 fold; lead 154 fold; and selenium 7 fold and in *A. trapezia* up to 84 fold cadmium, 50 fold lead and selenium 7 fold in exposed organisms compared to controls. The subcellular distribution of all three metals in *T. deltoidalis* and *A. trapezia* indicates active metal detoxification processes which at these exposure concentrations were unable to prevent significant metal burdens from accumulating in sensitive organelles.

A contamination gradient of zinc, lead, copper, cadmium and selenium was established in Lake Macquarie sediments which emanated from the same source. *A. trapezia* accumulated all metals in each sediment exposure. Accumulation and tissue distribution patterns of cadmium, lead and selenium were similar to those of the single metal spiked sediment exposures. Cadmium and lead BAM burdens increased at all exposures while selenium, zinc and copper did not.

T. deltoidalis and *A. trapezia* in the spiked sediment metal exposures generally had reduced GPx activity. This resulted in an increase in total glutathione concentrations which the reduced GSH:GSSG ratios indicated was due to a build up of oxidised glutathione. *T. deltoidalis* and *A. trapezia* had reduced TAOC in all laboratory sediment metal exposures which corresponded with increased TBARS concentrations, lysosomal destabilisation and micronucleus frequency. *A. trapezia* exposed to Lake Macquarie metal contaminated sediments also had a reduction in physiological condition, indicated by the reduced condition index, after 56 days at the higher metal exposures.

Clear exposure – dose – response relationships have been demonstrated for *T. deltoidalis* and *A. trapezia* exposed to single cadmium, lead and selenium spiked sediments and for *A. trapezia* exposed to Lake Macquarie mixed metal contaminated sediments. Detoxification of all metals was evident in both *T. deltoidalis* and *A. trapezia* but detoxification capacity was exceeded for cadmium, lead and selenium leading to significant accumulation of these metals in sensitive organelles. The significant relationships, in the laboratory exposed *T. deltoidalis* and *A. trapezia*, between TAOC reduction with increased TBARS, lysosomal destabilisation and micronuclei frequency and between increased TBARS with lysosomal destabilisation and micronuclei frequency indicates that increased tissue metal dose and BAM burdens caused significant impairment of the antioxidant reduction capacity which resulted in a cascade of effects from lipid peroxidation to cellular perturbation and genotoxic damage. The reduction in physiological condition in the organisms with the highest tissue metal doses suggests the response goes beyond subcellular perturbations to whole organism and potentially population effects.

Chronically metal exposed native Lake Macquarie *A. trapezia* did not show a clear metal exposure – dose - response relationship. Accumulation of the essential elements zinc, copper and selenium appeared to be regulated while cadmium and lead were not. TAOC was significantly reduced and TBARS significantly increased compared to reference organisms but lysosomal stability and condition were not significantly affected.

The suite of interrelated biomarkers used offers a weight of evidence approach for demonstrating adverse effects of metal tissue accumulation in *T. deltoidalis* and *A. trapezia.*

Table of Contents

List of Tables

List of Figures

List of Appendices

Appendix 1

Appendix 2

Appendix 3

1 Rationale

1.1 Introduction to the Study

1.1.1 Background

Coastal waters are exposed to anthropogenically derived metal contaminants, which are persistent and can be bioaccumulated (Phillips and Rainbow, 1994). Since the early 1970's there has been concern over the deleterious effects of contaminant loads to aquatic ecosystems (Luoma, 1996). Contaminants have also been associated with the decline in marine mammals, fish and bivalves (Luoma, 1996). Hence, there is a need to establish and monitor the links between contaminants in aquatic environments, their bioaccumulation in aquatic organisms and any consequent effects that occur, in order to protect valuable living natural resources and human health.

Monitoring gross effects of environmental pollution has traditionally been carried out by chemical analysis of water, sediments and biota. Biological assessment has included observation and quantification of ecological assemblages and routine assessment of contaminant toxicity, typically using LC_{50} lethality tests (Chapman, 1995; Taylor, 1996). Aquatic organisms have long been known to accumulate significant quantities of metals in their tissues. The degree to which organisms take up and retain metals varies markedly between phyla, and may also differ significantly between individual species within the different phyla. These variations are thought to be a reflection of different evolutionary strategies for detoxifying metals (Phillips and Rainbow, 1994). The accumulation and sequestration of biologically available metals by aquatic organisms has led to their use as biomonitors of contamination, as it this portion which is of interest in pollution effects assessments. They are also considered to provide a time-integrated measurement of contamination, reflecting the average of short term temporal fluctuations in contaminant abundance in the environment (Phillips, 1990). Molluscs, particularly bivalves have been extensively used and studied (Phillips, 1990). Molluscs are effective models for environmental toxicological studies because they are ubiquitous, have highly conserved control and regulatory pathways that are often homologous to vertebrate systems, and are extremely sensitive to anthropogenic inputs (Rittschof and McClelland-Green, 2005).

Metals and organic contaminants released into aquatic systems bind to particles and accumulate in estuarine sediments, which become the main repositories and therefore potential sources of contaminants (Byrne and O'Halloran, 2001). Burrowing and feeding by benthic organisms resuspends contaminants, increasing their biological availability both to the benthic fauna and flora, and to the higher order organisms which feed on them. Sediment toxicity tests, using sediment dwelling bivalves, aid in determining the potential for sediment toxicants to cause adverse effects to the sediment infauna and the potential for these effects to be transferred up food chains.

The history of pollution control and monitoring has been one of slowly evolving standards and techniques, with assessment of the potential effects of metal contaminants on the health of aquatic organisms being given progressively higher priority by many nations (Taylor, 1996). Estimating the extent of biological exposure to metal contaminants in aquatic environments is subject to uncertainties, as is attributing, let alone predicting, the adverse health or ecological effects that result from the exposure. The presence of a contaminant in a segment of an aquatic environment does not; by itself indicate injurious effects, connections must be established between external levels of exposure, internal levels of tissue contamination and early adverse effects.

Exposure to metals is difficult to assess because of the range of exposure routes, (water, sediments and food), differences in the biological availability of metals associated with the different environmental media, and individual and species differences in the metabolic pathways used to sequester or eliminate metals depending on their value or toxic potential. All these processes affect the amount of metal which enters organisms and reaches critical molecular targets. Seasonal changes, feeding habits, reproductive status, or metabolic activity can modify the nature and extent of exposure. Effects of exposure to metals cannot be readily quantified by measuring body burdens because the relationship between body burden and toxic response is complex and not fully understood (McCarthy, 1990). The presence of complex mixtures of metals and other contaminants creates further uncertainty. There is relatively little known about the toxicity of the majority of the tens of thousands of chemicals released to the environment and almost no information on the action of well characterised chemicals when they are in complex mixtures (Phillips and Rainbow, 1994).

It is now recognised that chronic exposure to sub lethal levels of toxicants, with the risk of their accumulation over time and magnification through the food chain, can have severe affects on the survival and reproductive capacity of individual organisms and on species and community diversity. The assessment of metal contamination has therefore broadened to include chronic exposure to sub lethal levels of metals and their effects at physiological, cellular and molecular levels (Huggett *et al.*, 1992). This approach uses biological markers of exposure and effect that can be defined as measurements of body fluids, cells or tissues that indicate in biochemical or cellular terms the presence of contaminants or the magnitude of the host response (McCarthy and Shugart, 1990). The physiological, cellular and molecular emphasis in aquatic toxicology is consistent with a biomarker approach, with such studies being likely to lead to detection of effects prior to changes at the organism and higher levels of organisation. Since these changes are early and sensitive, they may serve as markers of both exposure and effects (Hinton, 1994).

Cadmium and lead are metals which are accumulated in high concentrations by a range of marine organisms and which have no known biological function. They are extremely toxic to aquatic organisms at high concentrations and even at low concentrations may adversely affect physiology (Ercal *et al.*, 2001; Sokolova, 2004; Sokolova *et al.*, 2004). Selenium is an essential element involved in the reduction of peroxide in the glutathione cycle which at elevated concentrations is toxic (Hodson, 1988; Hoffman, 2002; Micallef and Tyler, 1987). Toxic effects of selenium include tetrogenicity (Hoffman, 2002). Copper and zinc are both essential elements which are required by molluscs for a range of metabolic processes, such as oxygen transportation, the formation of metalloenzymes and redox activities. The metals may only be available in trace amounts and may be difficult to obtain in sufficient quantities to maintain body processes. Storage systems have therefore evolved to ensure that cellular deficiencies of these metals do not occur (Rainbow, 1990). Excess zinc and copper are frequently toxic therefore detoxification is as important as storage and excretion is necessary to balance uptake (Simkiss and Taylor, 1989).

1.2 Aim

The aim of this research was to establish *exposure-dose-response* relationships for benthic marine bivalve molluscs exposed to metal contamination with particular emphasis on cadmium, lead and selenium.

1.2.1 Exposure-Dose-Response Framework

To determine the biological/ecological effects resulting from exposure to chemicals and other environmental stressors Widdows and Donkin (1992) suggest that it is necessary to establish:

a. relationships between the concentrations of chemical contaminants in the environments (exposure);
b. in the tissues of biota (dose); and
c. cause-effect relationships (response) between tissue contaminant concentrations and the resultant biological effects, based on an understanding of their mode of toxic action.

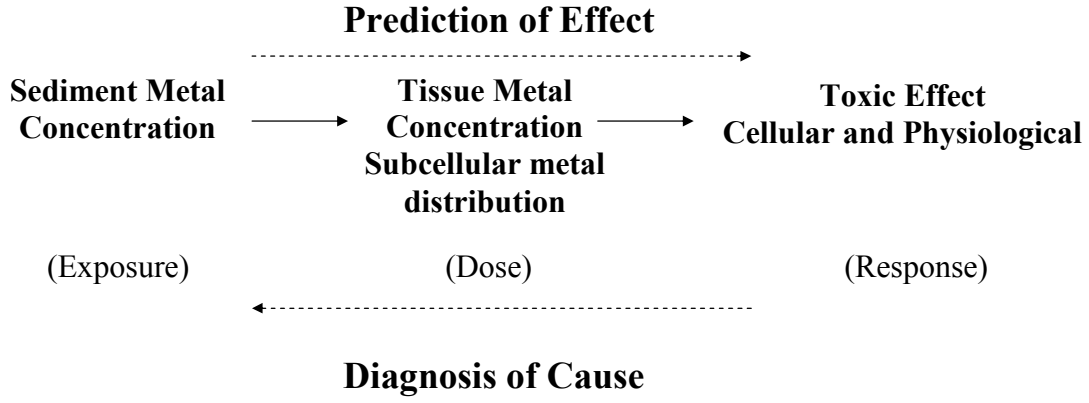


Figure 1.1: Ecotoxicological framework enabling the prediction and diagnosis of effects resulting from exposure to metals (modified from Widdows & Donkin (1992)).

1.2.2 Biomarkers in Environmental Assessment

To integrate biomarkers into environmental monitoring programs a research strategy is required to develop the data to both understand exposure and dose, and to provide the scientific basis necessary to interpret biomarker responses in selected species. McCarthy (1990) proposes a research plan for achieving the implementation of biomarker monitoring programs, consisting of five major tasks which lead from a preliminary proof-of-principle demonstration of the use of biomarkers to indicate exposure, to eventual linking of biomarker responses to individual and ecosystem effects. The present study addresses the second of the five tasks proposed:

Development, standardisation and validation of key biomarkers.

The main objectives of this task are:

- To standardise protocols for existing biomarker measurements;
- Develop and modify new biomarkers as needed; and
- To acquire a fundamental understanding of the dose-dependent relationships between exposure, biomarker responses and toxic effects.

The present study while addressing these objective also goes beyond it in that it aims to first establish the links between external metal exposure and internal dose and then to link these to biomarker responses, at molecular and cellular levels in order to address the task II objectives of McCarthy (1990) for developing biomarkers. McCarthy (1990) recommends an approach utilising laboratory exposures with single contaminants, or rationalised mixtures of contaminants, to establish dose-dependent relationships between exposure, biomarker responses and toxic effects on sentinel species coupled with ongoing interaction with field monitoring programs to resolve difficulties in interpreting biomarker responses.

1.3 Study Structure

This study is structured to follow the framework outlined in Figure 1.1. There are two parts to the study.

1.3.1 Part 1 – Metal Spiked Sediments Studies

To establish relationships between levels of exposure, organism dose and biological response to cadmium, lead and selenium, two species of benthic marine bivalve molluscs *Tellina deltoidalis* and *Anadara trapezia* were exposed to different concentrations of single metal spiked sediments in laboratory aquaria and compared to unexposed organisms. Organism internal exposure was measured by total tissue metal burden. The internal dose was examined by subcellular fractionation of whole tissue to determine what fraction of the total metal taken up was in a metabolically available form. Marker enzymes specific for mitochondria and lysosomes were measured in subcellular fractions to further understand intracellular localisation of metals in these organelles (Sokolova *et al.*, 2005a). Molecular measurements of oxidative stress, which are good general effects biomarkers for metal exposure (Stegeman *et al.*, 1992; van der Oost *et al.*, 2003; Winston and Di Giulio, 1991), selected were the total antioxidant scavenging capacity of cells, cellular concentrations of oxidised and reduced glutathione, glutathione peroxidase activity and the extent of lipid peroxidation. Lysosomal membrane stability was measured as a cellular effect biomarker, while the micronucleus assay was used to assess genotoxic damage.

1.3.2 Part 2 - Lake Macquarie Metal Contamination Gradient Sediments Study

A metal contamination gradient was established on the basis of sediment metal concentrations in sediments from Lake Macquarie NSW, a 125 km^2 barrier estuarine lake 90 km north of Sydney NSW, which has been subject to a variety of industrially derived metal inputs and has been shown to have concentrations of metals in sediments well above sediment quality guidelines (Batley, 1987; 1991; Roach, 2005). Sediment was sampled from three sites along an established metal gradient (Figure 3.1) and the effects of metals established using the same experimental set up as used previously for the sediment spiking experiments. Emphasis in this study was on cadmium, lead, selenium, copper and zinc as these metals were found to be elevated in the Lake Macquarie sediments collected, synergistic and antagonistic effects can therefore be considered.

One of the mollusc species *Anadara trapezia* tested in the metal spiked sediment experiments was selected, on the basis of the part 1 experimental results, and transplanted into the three sediment treatments, following the same protocol as the spiked sediment experiment, and compared to that of unexposed organisms. Organism internal exposure was measured using the same techniques as in the sediment spiking experiment. Molecular oxidative stress biomarker responses, determined from the sediment spiking experiment, were total antioxidant capacity, and lipid peroxidation. Lysosomal membrane stability, micronuclei frequency and condition index were also measured in a weight of evidence approach to determining the response of the organisms to metal contaminant exposure along the gradient.

1.4 Specific objectives

1.4.1 Metal Spiked Sediments Studies

- Determine if any relationships exist between cadmium, lead or selenium concentrations in the tissues of selected marine benthic bivalve molluscs *Tellina deltoidalis* and *Anadara trapezia*, and in single metal laboratory spiked sediments.
- Examine cadmium, lead and selenium uptake over time into the tissues of *T. deltoidalis* and *A. trapezia* through, 28 and 56 day respectively, single metal laboratory spiked sediment exposure experiments.
- Establish whether a relationship exists between gill/mantle, hepatopancreas, haemolymph and whole tissue metal concentrations in *A. trapezia*.
- Determine the subcellular tissue distribution of cadmium, lead and selenium in single metal laboratory spiked sediment exposed *T. deltoidalis* and *A. trapezia*, to establish the biologically active metal (BAM), versus biologically detoxified metal (BDM) tissue fractions.
- Investigate the dynamics of cadmium, lead and selenium accumulation from single contaminant spiked metal sediments in different organelles of *T. deltoidalis* and *A. trapezia* to gain an improved understanding of the mechanisms of cadmium, lead and selenium toxicity. Specifically in mitochondria, the primary site of ATP production and a key target for cadmium toxicity (Sokolova, 2004) and in lysosomes another important organelle for metal toxicity (Ringwood *et al.*, 2002).
- Determine molecular and cellular responses using biomarkers of oxidative stress, lysosomal membrane stability and micronucleus frequency of *T. deltoidalis* and *A. trapezia*, exposed for 28 to 56 days respectively, under controlled laboratory conditions to sediments spiked at environmentally realistic concentrations of cadmium, lead or selenium.
- Relate any changes in physiological condition (biological effects) to the metal concentrations and subcellular metal distribution in the tissues of exposed molluscs (dose) and to concentrations of metal in the spiked laboratory sediments (exposure).

1.4.2 Lake Macquarie Metal Contamination Gradient Sediments Study

- Establish whether a contamination gradient of cadmium, lead, zinc, copper and/or selenium exists in sediments emanating from Cockle Creek into Cockle Bay, in Lake Macquarie NSW.
- Examine cadmium, lead, selenium, copper and zinc uptake over time into the tissues of *A. trapezia* through 56 day laboratory exposures to mixed metal contaminated Lake Macquarie sediments.
- Determine if any relationships exist between cadmium, lead, selenium, copper, or zinc concentrations in the tissues of *A. trapezia*, and in mixed metal contaminated Lake Macquarie sediments.
- Determine the subcellular tissue distribution of cadmium, lead, selenium, copper and zinc in mixed metal contaminated Lake Macquarie sediment exposed *A. trapezia*, to establish the biologically active metal (BAM), versus biologically detoxified metal (BDM) tissue fractions.
- Investigate the dynamics of cadmium, lead, selenium, copper and zinc accumulation in different organelles of *A. trapezia* following exposure to mixed metal contaminated sediments from Lake Macquarie, NSW.
- Determine whether single metal contaminant exposure in laboratory spiked sediments results in significantly different tissue metal uptake patterns for *A. trapezia* for the same metal in a mixed metal contaminated sediment from Lake Macquarie, NSW.
- Determine molecular and cellular responses using biomarkers of oxidative stress, lysosomal membrane stability and micronucleus frequency of *A. trapezia* exposed for 56 days to metal contaminated sediments collected along a cadmium, lead, selenium, zinc and copper contamination gradient in Lake Macquarie, NSW.
- Determine whether exposure for 56 days to metal contaminated sediments from Lake Macquarie, NSW affect *A. trapezia* growth using a condition index measure.
- Relate any changes in physiological condition (biological effects) to the metal concentrations and subcellular metal distribution in the tissues of exposed molluscs (dose) and to concentrations of metal in the sediments of Lake Macquarie, NSW (exposure).

2 Literature Review

2.1 Introduction

2.1.1 Exposure Dose Response Pathways

An understanding of the fate and effects of metals in aquatic environments requires that the causal relationships between chemical exposure, internal dose and associated biological effects be established (Widdows and Donkin, 1992). Traditionally monitoring programs have measured metal contaminant concentrations in the physical environment, water, sediments and suspended matter. These measurements provide only momentary information giving no information on concentrations in and effects on aquatic organisms (Bervoets and Blust, 2003). The effects of metals in natural ecosystems are diverse, complex and often unpredictable (Depledge *et al.*, 1995). Internal dose can differ while the external dose does not as a result of varying exposure routes. If uptake is through food, for example, different feeding habits greatly influence uptake (Chapman, 1995). An ecotoxicological approach enables the differentiation between contamination defined in terms of chemicals, as an artificial increase above back ground levels; from contamination, which implies harm to living resources or risks to human health (Preston, 1989). Biomarkers help bridge the gap between exposure and effects, providing necessary information to evaluate subtle long term effects. Biomarkers comprise biochemical, physiological and histological endpoints and may be categorised as either (a) biomarkers of exposure, which reflect an organism's attempt to compensate for or tolerate, stressors in their environment; or (b) biomarkers of effect, which reflect deleterious sub lethal organism effects which may be causally linked to one of the four levels of biological organisation; biochemical and cellular; whole organism; population; and community (Chapman, 1995). If an organism that is a bioaccumulator (reaches equilibrium with its environment by detoxification and storage) shows adverse effects and a contaminant in its tissues shows a dose-response relationship then this is a strong indication of cause and effect (Chapman, 1995).

The term "biomarker" has been defined by Koeman *et al.* (1993) as a change in a biological response that can be related to an exposure to, or toxic effect of, an environmental chemical or chemicals. The term 'biological response' can range from molecular, biochemical, and physiological responses to behavioural responses through to species abundance and composition. In this review the focus will be on responses at the molecular to physiological level.

2.2 External Exposure

External exposure is traditionally characterised through the chemical analysis of discrete water and sediment samples (Salazar and Salazar, 2003). This can provide information on the environmental compartments into which the contaminants are distributed, at what concentrations and the chemical form. Metals are distributed among solution, suspended particles, sediments, pore waters and living and non-living food sources within all of these. Each species' exposure to metals is determined by how the species samples this complex mix and by the accessibility of metals within each compartment of the mix. In biological terms, consideration of a single environmental compartment is artificial and inhibits understanding of bioavailability processes (Luoma, 1996).

Metal bioavailability from water and sediments is influenced by physical and chemical factors acting outside the organism, which affect most biota in the same way. These include metal speciation, i.e. complexation of metal ions by inorganic and organic chelating agents, adsorption to particulate matter, precipitation and binding within insoluble matrices, sulphides, and sediment grain size and mineralogy (Borgmann, 2000; Campbell, 1995; Depledge and Rainbow, 1990). Environmental conditions such as pH, temperature, salinity and redox potential, that can cause a shift in the metal species from one form to another can affect both the bioavailability of the metal and the uptake pathways (Roesijadi and Robinson, 1994; Widdows and Donkin, 1992). Biological factors acting within or on the surface of organisms, such as exchange surfaces, growth, biochemical composition, reproductive condition, metabolism and elimination can also affect metal bioavailability and can be very species specific (Campbell, 1995; Widdows and Donkin, 1992).

Food and ingested organic particulate matter have been shown to be important metal exposure routes for both molluscs and fish (Handy, 1996; Luoma, 1996; Phillips and Rainbow, 1994). Metal uptake via the gut depends on similar chemical and physical factors to those operating outside the organism, as well as feeding rate, gut transit times, and digestion efficiency (Depledge and Rainbow, 1990). The total metal concentration of the food ingested is not necessarily itself a measure of metal availability, its bioavailability will be subject to the physiochemical conditions in the gut (Depledge and Rainbow, 1990). Although there is limited evidence of biomagnification of metal concentrations along food chains, amplification of a metal concentration can occur if food intake is high, the metal is assimilated efficiently and is not significantly excreted (Rainbow, 1990). Selenium is a notable exception to this. The high efficiency of selenium transfer from one trophic level to the next means biomagnification of selenium between trophic levels occurs more often than not (Luoma and Rainbow, 2008). Assimilation of ingested trace elements differs with food type, and availability from solution differs with geochemical conditions. The two pathways, are additive, therefore neither can be excluded from consideration in bioaccumulation studies (Luoma, 1996).

Sediment dwelling organisms differ in a number of ways from pelagic ones in terms of their exposure to contaminants. Sediment dwelling species are exposed continuously when in contaminated sediments. Burrowing invertebrates may be bathed in the interstitial water of the sediment, or by their own irrigation currents, interacting to varying degrees with the interstitial water. The interstitial water contains dissolved metals in equilibria with sediment-associated metals. Interstitial waters often have redox and other physiochemical conditions very different from those of overlying oxygenated waters (Rainbow, 1990). Pelagic organisms may only be exposed intermittently when foraging in sediments. Ultimately, it is where and how an organism lives, that dictates the way in which it is most likely to be affected by contamination (Chapman, 1995). Identifying chemical exposure pathways is becoming increasingly important as it is clear that dietary exposure may be controlling toxicity under many exposure conditions, this is particularly so for bivalves where particulate food in sediment can make a substantial contribution to toxicity (Salazar and Salazar, 2003).

2.3 Internal Dose

Dose is the concentration of a metal that appears in an organism's tissues and is the starting point for adverse effects (Luoma, 1996). Bioavailability determines dose; it is the relationship between metal concentrations in the environment and uptake into tissues from all sources. Bioaccumulation may not be related to total metal exposure concentrations; nor is it possible to measure one chemical fraction that is universally and exclusively the bioavailable fraction of any chemical (Luoma, 1996).

Once metals have been taken up by biota, systems for transport, storage and detoxification will determine their ultimate fate. The significance of different organisms' tissue concentrations is related to metal accumulation strategies. These accumulation strategies fall along a gradient from the accumulation of all metal taken up, to homeostasis of the body metal concentration at an approximately constant level by balancing metal uptake with excretion; intermediate strategies include degrees of net accumulation when uptake exceeds excretion, and partial homeostasis (Rainbow, 1993). Homeostasis as an accumulation strategy seems to be restricted to the essential metals, zinc and copper, however, if the internal metal dose exceeds the detoxification and excretion capacity of the organism the extra accumulated metal remains biologically available to play a toxic role (Rainbow, 1993). Chapman (1995) suggests that depending on the organism and the contaminant there are two basic accumulation patterns, which are related to contaminant toxicity. The first, are non-regulated metals such as cadmium, lead and mercury which are not essential for growth and may be more predictable from body burden data than from concentrations in external media, (water and sediment). The second, regulated metals such as the essential elements zinc, copper and selenium may be less predictable from internal dose measurements.

Internal dose can be characterised by chemically analysing the different tissues of organisms from different environmental compartments from the areas of concern e.g. sediment dwellers and water column. The tissue distribution of metals in fish indicates that they are preferentially accumulated in liver, kidney, spleen and gills (Phillips and Rainbow, 1994). Molluscs have been found to accumulate metals in the gills, liver, gut and in mussels also the byssal gland/threads (Widdows and Donkin, 1992).

2.3.1 Metal Metabolism

The gills of fish and molluscs are the most important tissue for the uptake of the water soluble form of metals and this is mainly a passive-transport process (Livingstone and Pipe, 1992). The digestive gland is the major site for particulate-bound metal uptake via endocytosis, an active-transport mechanism requiring adenosine triphosphate; the endocytotic vesicles subsequently fuse with primary lysosomes (Livingstone and Pipe, 1992).

Accumulation may occur as a result of physiological handling mechanisms in the case of essential metals, where the metal is delivered to tissues requiring the metal for some metabolic function. Metals may be transported to tissues in the haemolymph either dissolved or bound to proteins. Protein binding may be non-specific involving the dominant blood protein haemocyanin, or a specific metal transport protein such as transferrin-like proteins involved in the transport of iron. These proteins will also in addition to iron bind copper, zinc, manganese and plutonium (Depledge and Rainbow, 1990). How metals are transferred from the haemolymph to specific tissues remains obscure. Passive transfer along a cascade of ligands in cell membranes and cytoplasm exhibiting increasing metal affinity is a possibility, but active processes cannot be excluded (Depledge and Rainbow, 1990; Rainbow and Dallinger, 1993). On entering cells, metals are primarily complexed by thiol-containing molecules such as amino acids, glutathione and in particular the metal binding detoxification proteins, metallothioneins. In addition, part of the metal may be compartmentalised in the lysosomal vacuolar system, or trapped in different types of specialised inorganic granules (Livingstone and Pipe, 1992).

2.3.2 Subcellular Tissue Metal Distribution

Physiological effects and toxicity of metals strongly depend on their intracellular localisation and binding to organelles and ligands (Sokolova *et al.*, 2005a). Cadmium, for example, affects the bioenergetics of oyster mitochondria in vitro and in vivo at low concentrations, leading to reduced coupling and impaired ability to produce ATP (Sokolova, 2004). Cadmium accumulation in mitochondria may therefore result in serious disturbances of tissue energy balance and eventually cell death (Sokolova, 2004; Sokolova *et al.*, 2004; Sokolova *et al.*, 2005a). Lead is thought to interact with a variety of cellular lipids thus altering the lipid composition of cellular membranes. This results in perturbations in membrane integrity, permeability and function, thereby increasing susceptibility to lipid peroxidation (Ercal *et al.*, 2001).

Selenium is an essential element within a fairly narrow concentration range, above which it is toxic and below which selenium deficiency occurs (Hodson, 1988; Hoffman, 2002). Selenium appears to bind differently according to the selenium species. Selenide tends to bind to plasma proteins while selenate and selenomethionine occur in protein-free plasma (Ewan, 1989). Selenomethionine also binds to glutathione peroxidase intra and extracellularly (Burk, 1991) and directly to cell walls (Hortensia *et al*., 2006).

2.3.2.1 *Mitochondria*

Mitochondria are small intracellular organelles which are responsible for energy production and cellular respiration. The provision of energy through the coupling of oxidation to energy transfer via the phosphorylation of adenosine diphosphate (ADP) occurs in the mitochondrial electron transport chain (Winston and Di Giulio, 1991). Adenosine diphosphate respiration in the mitochondria uses pyruvate, formed by glycolysis from glucose, the carrier molecules nicotinamide adenine dinucleotide (NAD+), flavin adenine dinucleotide (FAD+) and oxygen to produce citric acid which is then broken down releasing hydrogen ions and carbon molecules. The carbon molecules are used to make carbon dioxide and the hydrogen ions are picked up by NAD and FAD and recycled. The hydrogen ions produced by the cycle are used to drive pumps that produce adenosine triphosphate (ATP). The release of energy from ATP is achieved via oxygen reduction metabolism, where ATP loses one of its phosphate groups and is converted back to adenosine diphosphate (ADP). The cycling of these two molecules releases energy which is used for cellular functions such as movement, transport, entry and exit of products and cell division (Winston and Di Giulio, 1991). Exposure of oysters *Crassostrea virginica* to cadmium has been shown to result in considerable cadmium accumulation in the mitochondria and a significant impairment of the ATP production capacity and a strong inhibition of the ADP-stimulated respiration (Sokolova, 2004; Sokolova *et al.*, 2005b).

2.3.2.2 *Heat Stable Proteins - Metallothioneins*

Metallothioneins are heat stable, low molecular weight, soluble (generally cytosolic), thiol-rich (high cysteine content) proteins with a high metal content (Roesijadi, 1992; Viarengo, 1989). Induction of metallothioneins by metals is specific and metal dependant (Roesijadi, 1996). While their primary role in marine organisms is the homeostasis of the essential metals zinc and copper (Cosson *et al.*, 1991), they can also bind non-essential metals such as cadmium and mercury (Livingstone and Pipe, 1992). There is increasing evidence that metallothioneins are turned over rapidly in cells.

Turnover involves lysosomal breakdown and associated production of residual bodies such as metal rich granules which may be stored or excreted. Whether metals handled in this way are available for metabolic utilisation remains unknown (Depledge and Rainbow, 1990).

2.3.2.3 *Microsomes*

Microsomes are small vesicles found in the endoplasmic reticulum which contain the cell's cytochrome P450 enzymes involved in oxidative metabolism. Microsomal electron transport of aquatic organisms have been studied in detail with respect to oxyradical production. Possible loci of electron transfer to oxygen to produce O_2^- in microsomes are the autoxidation of reduced oxycytochrome P450 and/or autoxidation of flavoprotein reductases (Winston and Di Giulio, 1991).

2.3.2.4 *Lysosomes*

Metal accumulation in lysosomes of the digestive gland and kidney of mussels has been described (Viarengo, 1989). Tertiary lysosomes accumulate undegradable end-products of lipid peroxidation, oxidised lipid and protein polymers known as lipofuschin. In kidneys, lipofuschin granules have been shown to bind metals in two ways; weakly bound by acidic groups in the outer region of the granules, and so able to dissociate and be in equilibrium with cations in the cytoplasm; and sterically 'trapped' in a non-toxic form in the centre of the developing granules (George, 1983a). Active excretion of these residual bodies by exocytosis enables metal elimination. A second method of elimination has been indicated for copper in the digestive gland, involving the accumulation of copper rich thionein-like proteins in lysosomes, followed by elimination of residual bodies (Carpene, 1993; Viarengo, 1989). Cadmium is not removed via either of these biochemical pathways in mussels and is consequently present in tissues for considerably longer once it is taken up (Viarengo, 1989).

2.3.2.5 *Inorganic Granules and Vesicles*

A variety of marine molluscs, both bivalves and gastropods, have been shown to sequester metals in inorganic granules as a means of detoxification (Carpene, 1993; Taylor, 1998; Viarengo, 1989). There are two major types identified, copper-sulphur-containing granules and calcium containing granules (Viarengo, 1989). Cells lining the digestive tract of invertebrates (e.g. midgut diverticula, hepatopancreas or caeca) may release metals detoxified in granules into the gut lumen when the epithelial cells complete their cell cycle. The kidney cells of bivalve molluscs also have the ability to excrete metal rich granules (Rainbow, 1990).

Metals in granules are inert and not available biologically therefore total metal burden measurements while they may give high concentrations do not give any information about adverse biological effects.

Whatever the handling mechanisms it is clear that metal ions in excess of metabolic requirements are potentially toxic and must be removed from the vicinity of important biological molecules by excretion from the specific tissue. The metal may then be eliminated from the organism or biotransformed prior to storage in specific tissues in inert non-toxic forms.

2.4 Biological Response - Biomarkers

Biological response to external metal exposures and consequent internal dose can be assessed at an organism or sub-organism level using a range of biochemical, physiological and histological biomarkers to estimate either internal exposure to toxicants or resultant effects. Metals exert their influence by interaction with a range of biochemical receptors and therefore measurement of these interactions can provide early indication of internal exposure and effects. Toxic effects of metals are influenced by bioavailability, routes of exposure and the level and time of exposure (Koeman *et al.*, 1993). The initial reactions of organisms to toxic compounds is at the molecular and cellular levels of target organs and tissues. Sub lethal compensatory and repair responses may prevent damage during prolonged exposure until cell regulatory systems fail. The effectiveness of specific biomarkers relies on the ability to link them to higher level effects (Figure 2.1) and thus show that they provide early warning of adverse effects, not just internal exposure (Chapman, 1995).

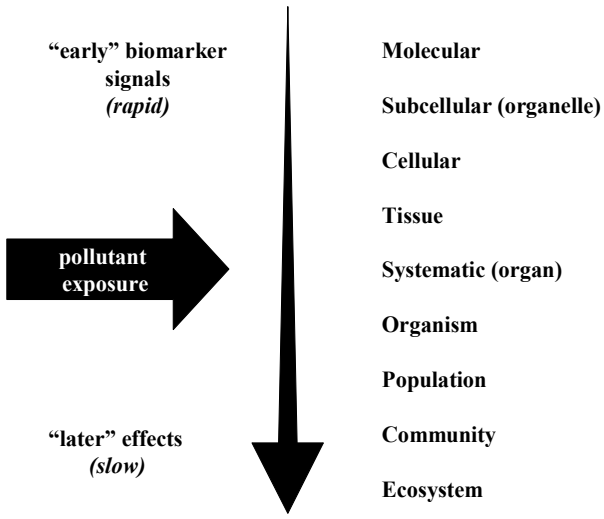


Figure 2.1: Sequential order of biological responses to toxic stress (modified from (Bayne *et al.*, 1985))**.**

Biomarker measurements can provide information which cannot be obtained through measurements of contaminants in the environmental media or tissue concentrations. They have the potential to provide evidence that organisms have been exposed to contaminants at levels that exceed their detoxification and repair capacity. This can provide evidence for establishing the link between toxicant exposure and ecologically relevant effects (Koeman *et al.*, 1993). The bioaccumulation of certain persistent environmental chemicals in animal tissues may be seen as a biomarker of exposure to these chemicals, however, these body burdens are not considered to be biomarkers or bioindicators since they do not provide information on deviations related to 'health' (van der Oost *et al.*, 2003). Biomarkers in this review will refer to biological, biochemical, physiological, histological and morphological parameters measured inside an organism or its products.

2.4.1 Biomarkers of Exposure

Biomarkers of exposure may represent either general or specific responses and have the advantage of quantifying only biologically available toxicants (Mayer *et al.*, 1992). General markers include those that are non-specific for a compound or chemical class but indicate that exposure to some exogenous chemical has occurred. Changes in some general biomarkers may be caused by environmental variables not related to toxic exposure, such as temperature increases stimulating the production of stress proteins (Stegeman *et al.*, 1993). Specific biomarkers of exposure may be used to demonstrate exposure response to a particular class of compound (Table 2.1). Biomarkers of exposure include the detection and measurement of an exogenous substance or its metabolite or the product of an interaction between the xenobiotic agent and a target molecule or cell that is measured in a compartment within an organism (van der Oost *et al.*, 2003).

Table 2.1: Common biomarkers of exposure and effect, and the compounds they respond to.

Category	Biomarker	Toxicant	Response	Examples of Use
Enzymatic / Biochemical				
Phase 1	**Cytochrome P450**	**PAH, PCB, pesticides**	+ / -	(Arun *et al.*, 2006; Kim *et al.*, 2004a; Kim *et al.*, 2004b; Peters *et al.*, 1998; Shaw *et al.*, 2004; Stegeman and Hahn, 1994; Watson, 2004)
	Ethoxyresorufin O-deethylase	**PAH, PCB, pesticides**	+	(Cristina Fossi *et al.*, 2004; Fouchecourt *et al.*, 1999; Kirby *et al.*, 2004; Miller *et al.*, 2004; Whyte *et al.*, 2000)
	Aryl hydrocarbon hydroxylase	**PAH, PCB, pesticides**	+	(Bogovski *et al.*, 1998)
Phase 2	**Glutathione S-transferase**	**metals, PAH, PCB, pesticides**	+ / -	(Hoarau *et al.*, 2004; Leaver and George, 1998; Lee, 1988)
Antioxidant				
Enzymes	**Glutathione peroxidase & reductase**	**metals, PAH, PCB**	+ / -	(Cossu *et al.*, 2000; de Almeida *et al.*, 2004; Maity *et al.*, 2008)
	Catalase / Superoxide dismutase	**metals, PAH, PCB**	+ / -	(Company *et al.*, 2004; Pedrajas *et al.*, 1995; van der Oost *et al.*, 2003)
Cofactors	**Total Glutathione GSH+2GSSG**	**metals, PAH, PCB**	+ / -	(Canesi *et al.*, 1999; Frenzilli *et al.*, 2004; Regoli *et al.*, 2004)
	Reduced :oxidised glutathione GSH:GSSG	**metals, PAH, PCB**	-	(Cossu *et al.*, 2000; Hoffman, 2002; Maity *et al.*, 2008; Tandon *et al.*, 2003)
Activity	**Total Antioxidant Capacity**	**metals, PAH, PCB**	-	(Gorinstein *et al.*, 2005; Moncheva *et al.*, 2004)
	Total Oxygen Scavenging Capacity	**metals, PAH, PCB**	-	(Camus *et al.*, 2004; Regoli, 2000; Regoli *et al.*, 2002; Regoli and Winston, 1999)
Damage	**Lipid Peroxidation**	**metals, PAH, PCB**	+	(Charissou *et al.*, 2004; Domouhtsidou and Dimitriadis, 2001)
Haematological	**Aspartate & alanine aminotransferases**	**metals, Cd, Cu Hg**	+	(Benson *et al.*, 1988; Beyer *et al.*, 1996; Blasco and Puppo, 1999; de Aguiar *et al.*, 2004)
	δ-aminolevulinic acid dehdratase	**metals Pb, Zn**	+	(Burden *et al.*, 1998; Campana *et al.*, 2003; Perottoni *et al.*, 2005; Rodriguez *et al.*, 1989)
Proteins	**Heat Shock Proteins**	**heat, metals**	+	(Cruz-Rodriguez and Chu, 2002; Feng *et al.*, 2003; Urani *et al.*, 2003)
	Metallothioneins	**metals**	+	(Amiard *et al.*, 2006; Lecoeur *et al.*, 2004; Marie *et al.*, 2006)
Neurotoxic	**Acetylcholinesterase**	**organophosphate & carbomate pesticides**	-	(Corsi *et al.*, 2004; Dellali *et al.*, 2001; Lionetto *et al.*, 2003; Pfeifer *et al.*, 2005; Rickwood and Galloway, 2004)
Genotoxic	**Micronuclei frequency**	**metals, PAH, PCB**	+	(Bolognesi *et al.*, 2004; Burgeot *et al.*, 1996; Koukouzika and Dimitriadis, 2008; Scarpato *et al.*, 1990; Williams and Metcalfe, 1992)
	DNA strand breaks	**metals, PAH, PCB**	+	(Akcha *et al.*, 2004)
	DNA adducts	**metals, PAH, PCB**	+	(Fouchecourt *et al.*, 1999; Kurelec *et al.*, 1990; Pisoni *et al.*, 2004)
Reproductive	**Vitellogenin**	**dioxin, endosulphan, pesticides, metals**	+	(Depledge and Billinghurst, 1999; Funkenstein *et al.*, 2004; Riffeser and Hock, 2002)
Cellular	**Lysosomal stability**	**metals**	-	(Castro *et al.*, 2004; Domouhtsidou and Dimitriadis, 2001; Moore *et al.*, 2006)
Physiological	**Histopathology**	**All xenobiotics**	-	(Au, 2004; Farley, 1988; Sunila, 1988; Wedderburn *et al.*, 2000; Zorita *et al.*, 2006)
	Cellular Energy Allocation	**All xenobiotics,**	-	(Cherkasov *et al.*, 2006; Smolders *et al.*, 2004)
	Scope for Growth	**All xenobiotics**	-	(Burt *et al.*, 2007; Goldberg and Bertine, 2000; Smolders *et al.*, 2004; Wo *et al.*, 1999)
	Condition Index	**All xenobiotics**	-	(Leung and Furness, 2001a; Lundebye *et al.*, 1997)

+ = increased; - = decreased expression

2.4.2 Biomarkers of Toxic Effect

As with biomarkers of exposure biomarkers of effect can be categorised as general or specific responses. While these biomarkers may also demonstrate exposure they can be used to further reveal a toxic effect resulting from that exposure. General biomarkers of toxic effect include measurable biochemical, physiological or other alterations within tissues or body fluids of an organism that can be recognised as associated with an established or possible health impairment or disease (van der Oost *et al.*, 2003). These include indicators of cellular and genetic damage such as increase of antioxidant enzyme activity, chromosomal aberrations, and histopathological lesions (Table 2.1). Chemical or class specific indicators of toxic effect can be used when the mode of action of the chemical is known, such as inhibition of brain acetylcholinesterase by organophosphates and carbamates (Mayer *et al.*, 1992).

The division of biomarkers into categories of exposure or effect is to some extent arbitrary since they are divided according to how they are used rather than by an inherent dichotomy (Suter, 1993). The responses of biomarkers can be seen as biological or biochemical effects after a certain toxicant exposure, which makes them theoretically useful as indicators of both exposure and effects (van der Oost *et al.*, 2003). Biomarkers of exposure can be used to confirm and assess the exposure of individuals or populations to a particular substance group (metals, hydrocarbons, pesticides etc.), providing a link between external exposure and internal dose. Biomarkers of effect can be used to document either preclinical alterations or adverse health effects due to external exposure and internal adsorption of a toxicant. Biomarkers of susceptibility have also been defined as a separate category by the (WHO, 1993). These help to elucidate variations in the degree of responses to toxicant exposure observed between different individuals and include genetic factors and changes in the receptors which alter the susceptibility of an organism to a specific toxicant exposure.

2.4.3 Biomarker Selection

As with other aspects of study design, biomarker selection depends on the question to be answered. Biological responses and therefore biomarker choice also depends on the mode of action of the chemical of interest and the level of biological organisation being examined. It is necessary to determine whether the study requires biomarkers of exposure to a chemical or group of chemicals, a biomarker of toxic effect, or whether a combination of these is preferred.

In most cases, the objectives of studies require or benefit from analysis of multiple biomarkers at several levels of organisation. A combination of sensitive early changes (e.g. molecular) and later changes (e.g. histological) may be particularly useful (Stegeman *et al.*, 1993). The selection of biomarker also depends on the sentinel species used and techniques selected may require laboratory verification before application to field studies (Stegeman *et al.*, 1993).

The following seven criteria for the selection and development of useful biomarkers are suggested based on ideas formulated by (Mayer *et al.*, 1992; Stegeman *et al.*, 1992; van der Oost *et al.*, 2003).

1. The assay to quantify the biomarker should be reliable (with quality assurance), relatively cheap and easy to perform, allowing quantification of multiple individuals
2. The biomarker response should be sensitive to pollutant exposure and/or effects in order to serve as an early warning parameter.
3. Baseline data of the biomarker should be well defined in order to distinguish between natural variability (noise) and contaminant induced stress (signal).
4. The underlying mechanism of the relationships between biomarker response and pollution exposure in a dose or time-dependent manner should be established so the magnitude of the exposure or effect can be determined.
5. The impacts of confounding factors (i.e., season, gender, weight, and handling) to the biomarker response should be understood and within acceptable limits.
6. The measure must have biological significance. Only biomarkers that can be linked to important biological processes and for which changes can be interpreted should be used.
7. Ideally a suite of interrelated biomarkers based on a cascade of effects should be selected to ensure robustness.

2.5 Molecular / Biochemical Biomarkers

Changes at the biochemical level offer specific advantages as biomarkers for two major reasons:

1. Biochemical or molecular alterations are usually the first detectable, quantifiable responses to environmental change, including changes in the chemical environment. Further, biochemical alterations can serve as markers of both exposure and effect. A chemically induced change in biochemical systems, by definition, represents an effect of the chemical (Stegeman *et al.*, 1993).
2. Biochemical system alterations are often more sensitive indicators than effects at higher levels of biological organisation as they usually precede higher order effects and may therefore indicate whether additional effects are likely to occur. Additionally these alterations are a more rapid measurable response to toxicity than the higher order effects which may follow, therefore, allowing remedial intervention to be implemented earlier in the process.

Biochemical systems which are involved in specific responses to toxic chemicals include a number of enzymes and proteins. Many responses are adaptive, but the same systems may be involved in reactions leading to toxic effects (Stegeman *et al.*, 1992). The main systems are:

2.5.1 Biotransformation Enzymes

Alterations in levels or activity of biotransformation enzymes are generally the most sensitive effect biomarkers (van der Oost *et al.*, 2003). Their activity may be enhanced or inhibited in response to contaminant exposure.

2.5.1.1 *Phase I Enzymes*

The initial phase of metabolism of organic compounds involves the addition of polar groups to the molecule through, oxidative, reductive or hydrolytic reactions (Buhler and Williams, 1988). Oxidative reactions are the most important category of phase I reactions (Buhler and Williams, 1988). They are catalysed primarily by cytochrome P450 dependant mixed function oxidase enzymes (MFOs; also referred to as monooxygenases) (van der Oost *et al.*, 2003). These enzymes comprise a large and expanding family of heme proteins which are membrane-bound and predominantly are located in the endoplasmic reticulum of the liver (Stegeman *et al.*, 1992). They metabolise a wide variety of substrates including endogenous molecules (e.g. fatty acids, prostaglandins, steroids) and xenobiotics (e.g. hydrocarbons, pesticides, drugs) (Snyder, 2000).

The toxicity of organic chemicals can be significantly altered by structural transformation. By affecting chemical structures cytochrome P450 enzymes may render a compound non-toxic or may drastically increase its toxicity (Stegeman *et al.*, 1992). The levels of some forms of cytochrome P450 can be increased in response to an organism's exposure to many types of chemicals and as a result the rate of chemical transformation catalysed by these enzymes is altered. Cytochrome P450 can also serve as a highly sensitive indicator of an organism's toxic burden, or the extent to which it has been exposed to chemical inducers in the environment (Stegeman *et al.*, 1992). Ethoxyresorufin O-deethylase (EROD) and aryl hydrocarbon hydroxylase (AHH) are two catalytic probes commonly used for determining the inductive response of the cytochrome P450 system to chemical exposure (van der Oost *et al.*, 2003). Increases in both AHH and EROD catalytic enzyme activity have been measured in many species of fish liver after exposure to organic pollutants and are considered to be sensitive biomarkers of organic chemical exposure which may also precede effects at various levels of biological organisation (Whyte *et al.*, 2000). The phase I biotransformation enzymes, particularly cytochrome P450 are the most sensitive fish biomarkers known at present for indicating exposure to organic compounds (van der Oost *et al.*, 2003).

2.5.1.2 *Phase II Enzymes*

Phase II (conjugating) enzymes aid in the detoxification and excretion of foreign compounds, including reactive metabolites formed by the phase I cytochrome P450 monooxygenase system, by linking them to various water soluble endogenous compounds present in the cell in high concentrations. These reactions generally result in further increases in solubility and elimination rates, and reduced toxicity of the compound (Buhler and Williams, 1988). The most widely studied and probably the most important of the phase II enzymes are glutathione S-transferases (GST), UDP-glucuronosyltransferases (UDPGT), and sulphotransferases (ST), which link metabolites to glutathione, glucuronic acid, and sulphate, respectively (Buhler and Williams, 1988; Stegeman *et al.*, 1992). Some xenobiotic compounds possess the required functional groups (e.g. COOH, -OH or $-NH_2$) for direct metabolism by conjugative phase II enzymes, while others are metabolised by an integrated process involving prior action of phase I enzymes (George, 1994). The major pathway for electrophilic compounds and metabolites is conjugation with GST while the major route for nucleophilic compounds is glucuronic acid (GA) conjugation (George, 1994).

Phase I and phase II biotransformation reactions usually work together in a sequential way to convert xenobiotics to more easily excreted metabolites. The different phase I and II enzymes may also compete with each other for the parent xenobiotic or its metabolites. Xenobiotics, therefore, generally undergo several types of biotransformation reactions simultaneously, often resulting in the formation of a large number of metabolites or conjugates (Buhler and Williams, 1988). Compared to phase I enzymes the induction reaction of phase II enzymes is generally less pronounced (George, 1994). They may be more useful in an integrated biomarker approach using a combination of biomarkers such as the biotransformation index (BTI, reflecting the ratio between phase I and II activities), as this reflects a balance between bioactivation and detoxification (van der Oost *et al.*, 1998).

2.6 Oxidant and Antioxidant Responses

All aerobic life has the potential to experience oxidative stress, when antioxidant defences are overwhelmed by activated oxygen species, also referred to as oxygen free-radicals, reactive oxygen species (ROS), reactive oxygen intermediates (ROIs) or oxyradicals (Winston, 1991). Many environmental contaminants or their metabolites have been shown to enhance the production of reactive oxygen species within cells (Andersen, 1994). There are many endogenous sources of oxyradical production, the MFO system, for example, in addition to metabolically activating/detoxifying polycyclic hydrocarbons and other xenobiotics, is also involved in oxyradical generation (Andersen, 1994), but from an environmental biomarker perspective the ability of a number of exogenous compounds, particularly metals, to enhance intracellular oxyradical production through the process of redox cycling is of particular interest (Stegeman *et al.*, 1992; van der Oost *et al.*, 2003). Oxidant-mediated effects with the potential for use as biomarkers include either adaptive responses through increased activities of antioxidant enzymes and concentrations of non-enzymatic compounds, or evidence of oxidant-mediated toxicity such as oxidation of proteins, lipids and nucleic acids, as well as perturbed tissue redox status (Stegeman *et al.*, 1992; van der Oost *et al.*, 2003; Winston and Di Giulio, 1991).

2.6.1 Oxygen Reduction Metabolism

Molecular oxygen is required by all aerobic organisms for the provision of energy through the coupling of oxidation to energy transfer via the phosphorylation of adenosine diphosphate (ADP). In aquatic organisms this process is managed by the mitochondrial electron transport system; in which O_2 undergoes a concerted four electron reduction to water (Figure 2.2) (Winston and Di Giulio, 1991). The first reaction is a one electron reduction of O_2 to superoxide (O_2^-). Superoxide anions are then converted to hydrogen peroxide (H_2O_2) by a further one electron reduction. Superoxide and to a large extent hydrogen peroxide are highly reactive and toxic ROIs; H_2O_2 in conjunction with myeloperoxidase and a halide, forms the basis of a potent antibacterial system (Andersen, 1994). The reduction of H_2O_2 to the hydroxyl radical ($^{\cdot}OH + OH^-$) and then to H_2O is achieved by the addition of a further electron at each step (Figure 2.2).

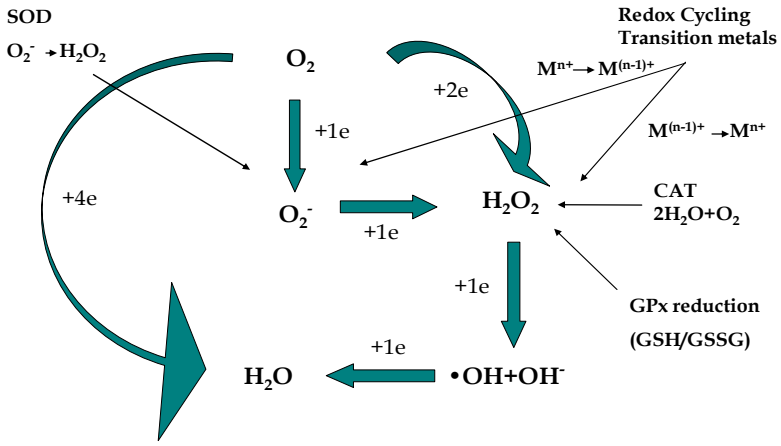


Figure 2.2: Oxygen reduction metabolism showing the 4 step electron-transfer reactions in the conversion of oxygen to water during energy transfer and the major enzymatic reduction mechanisms (modified from (Winston and Di Giulio, 1991)).

2.6.2 Redox Cycling

The hydroxyl radical is among the most potent oxidants known, capable of reacting kinetically indiscriminately with virtually all organic chemicals, including critical cellular macromolecules, possibly leading to protein degradation and enzyme inactivation, lipid peroxidation, DNA damage and ultimately cell death (Winston and Di Giulio, 1991).

The production of $^{\cdot}OH + OH^-$ may be significantly enhanced through redox cycling, via the Fenton and Haber-Weiss reactions, using transition metal chelates such as iron, copper, chromium (III), (IV), (V), and (VI), vanadium (V) and cobalt (I) (Leonard *et al.*, 2004; Winston and Di Giulio, 1991) (Figure 2.2). Other redox-active compounds include aromatic diols and quinones, nitroaromatics, aromatic hydroxylamines and bipyridyls. In redox cycles where these organic xenobiotics are univalently reduced, the parent compound is typically first enzymatically reduced by a nicotinamide adenine dinucleotide phosphate (NADPH) dependent reductase, (such as cytochrome P450 reductase) to produce a xenobiotic radical. The radical donates its unshared electron to molecular O_2, producing O_2^- and the parent compound. In this way at each turn of the cycle two potentially deleterious events have occurred: a reductant has been oxidised and an oxyradical has been produced (Winston and Di Giulio, 1991). These redox cycles produce O_2^- at the expense of cellular reducing equivalents such as NADPH (Winston and Di Giulio, 1991). In addition to the Fenton and Haber-Weiss mechanisms, redox inactive metal ions such as cadmium and lead can indirectly influence the oxidative system by reacting directly with cellular molecules to generate ROIs, inducing cell signalling pathways (Leonard *et al.*, 2004) or depleting the cell's major sulfhydryl reserves (Ercal *et al.*, 2001). Free radicals are not always harmful, singly or collectively, ROIs can participate in the cell mediated destruction of bacteria, fungi and protozoa by specialised blood cells called phagocytes (Andersen, 1994; Langseth, 1995).

2.6.3 Antioxidant Defence Mechanisms

The proliferation of ROIs is mediated by a number of antioxidant defence mechanisms. These specially adapted enzymes tend to inhibit the formation of ROIs by scavenging and reducing them to non-reactive molecules. Defence systems include the cytoplasmic enzyme superoxide dismutase (SOD) which catalyses the conversion of O_2^- to H_2O_2. The reduction of H_2O_2 to molecular oxygen and water is catalysed by either; the antioxidant enzyme catalase (CAT); or via the glutathione peroxidase (GPx) enzyme system (Winston, 1991) (Figure 2.2). It is likely that overproduction of ROIs via redox cycling, phagocytosis and general MFO activity could exhaust the inducible protective antioxidant defence system, contributing to pollutant-mediated toxicological responses (Andersen, 1994; Stegeman *et al.*, 1992; van der Oost *et al.*, 2003; Winston, 1991). Exposure of blood cells of a variety of aquatic animals to sub lethal levels of selected metals, pesticides and other organic compounds has also been shown to lead to a reduction in their production of ROIs. This form of immunosuppression may increase susceptibility to disease (Andersen, 1994).

2.6.3.1 *Superoxide Dismutase*

Superoxide dismutases (SOD) are a group of metalloenzymes that catalyse the reaction where O_2^- is disproportioned to produce H_2O_2 (Figure 2.2). Three distinct types with different metal centres have been identified:

- CuZnSODs - typically associated with the cytosol of eukaryotes and chloroplasts of higher plants.
- MnSODs - found in bacteria and organelles such as mitochondria and chloroplasts of higher organisms.
- FeSODs - found in bacteria and a few higher plants.

They are considered to play a pivotal antioxidant role; their importance being indicated by their presence in all aerobic organisms examined. Further, the rate of O_2^- dismutation by SOD approximates the diffusion limit making it the most active of the antioxidant enzymes described (Stegeman *et al.*, 1992). The highly inducible nature of SODs is the basis for their potential as biomarkers (Stegeman *et al.*, 1992). Significant SOD induction has been noted in field surveys of exposed fish (van der Oost *et al.*, 2003). The study of responses of SOD isoenzymes associated with particular organelles may be of particular value for monitoring oxidative responses at the subcellular level in organisms exposed invivo, including field studies (Stegeman *et al.*, 1992).

2.6.3.2 *Catalases*

Catalases (CAT) are haematin-containing enzymes that facilitate the removal of H_2O_2 by reducing it to water and free oxygen (Figure 2.2). Unlike other peroxidases which can also reduce various lipid peroxidases CAT can only reduce H_2O_2 (Stegeman *et al.*, 1992). CAT occurs in the peroxisomes of most cells where it scavenges the H_2O_2 produced during fatty acid metabolism. It is also present in erythrocytes independent of peroxisomes in most vertebrates where is appears to act in concert with GPx and methemoglobin reductase to counter the oxidative stress to which these cells are prone (Stegeman *et al.*, 1992). CAT activities in these cells may have potential as a biomarker of oxidative stress (Stegeman *et al.*, 1992), however, as both induction and inhibition of CAT activity has been measured in fish after exposure to environmental contaminants its usefulness as a biomarker is not yet clear (van der Oost *et al.*, 2003).

2.6.3.3 *Glutathione Peroxidases and Reductases*

Peroxidases (GPx) reduce peroxides to their corresponding alcohols using a range of reductants. In animals the main peroxidase, which is a selenium-dependent tetrameric cytosolic enzyme, uses reduced glutathione (GSH) as a cofactor to reduce H_2O_2 to $2H_2O$ (Stegeman *et al.*, 1992) (Figure 2.2). Also of interest is the ability of GPx to reduce organic hydroperoxides to their corresponding alcohols, as this is considered an important mechanism for halting lipid peroxidation chain reactions. Reductases (GR) are not as active as GPx in antioxidant defences, however, they play an important role in maintaining appropriate GSH:GSSG ratios in response to oxidative stress (Winston and Di Giulio, 1991). GR catalyses the transformation of the GSSG to its reduced form, GSH, with the concomitant oxidation of NADPH to $NADP^+$ and can be measured spectrometrically by following the decrease in NADPH levels (van der Oost *et al.*, 2003).

2.6.3.4 *Reduced and Oxidised Glutathione*

Reduced glutathione (GSH), a tripeptide made up of glutamic acid, cystine and glycine (George, 1994), has two contrasting roles in detoxification; 1) as a key conjugate of electrophilic intermediates, principally by glutathione S-transferase (GST) activities in phase II metabolism, and 2) as an important antioxidant enzyme (Stegeman *et al.*, 1992). In addition to its antioxidant function in the activities of GPx and GR already discussed, GSH can also act as a nonenzymatic scavenger of oxyradicals (Stegeman *et al.*, 1992). Increased fluxes of oxyradicals have been shown to alter GSH status with the most obvious direct effect being a decrease in the ratio of GSH to oxidised glutathione (GSSG), (Stegeman *et al.*, 1992) brought about by increased peroxidase and scavenging activities or indirectly due to reduced availability of NADPH following oxidations from the first step of the redox cycle (Figure 2.2). In healthy cells the GSH:GSSG ratio is typically high, greater than 10:1 (Stegeman *et al.*, 1992). If GSSG accumulates, thiol-containing enzymes can be inactivated through the formation of mixed disulphides. GSSG has also been shown to inhibit protein synthesis through an interaction with one of the initiation factors for translation (Melancon *et al.*, 1992). Increased synthesis of GSH in response to increased oxyradical generation might also result in the maintenance of the GSH:GSSG ratio and / or an increase in GSH levels (Stegeman *et al.*, 1992). The existence of effective feed back mechanisms for the maintenance of GSH levels in response to contaminant induced effects may mean that GSH levels alone are not useful as biomarkers of oxidative stress (Stegeman *et al.*, 1992).

The measurement of elevated GSSG levels, however, suggest that the hepatic GSH:GSSG ratio may be a potential biomarker for oxidative stress (van der Oost *et al.*, 1996). The drain imposed on intracellular reducing equivalents such as NADPH by oxyradical-generating compounds can influence the redox status of cells with potentially profound consequences on a variety of metabolic processes (Stegeman *et al.*, 1992). Measurements of pyridine nucleotide ratios NAD(P):NAD(P)$^+$ may also be useful in assessing effects on redox status (Stegeman *et al.*, 1992).

2.6.4 Oxidative Damage

A failure of the antioxidant defence system to prevent ROI proliferation may result in a variety of oxyradical induced perturbations, including; lipid peroxidation, DNA oxidation, methemoglobinemia and a reduced capacity to neutralise reactive oxygen species.

2.6.4.1 *Total Antioxidant Capacity*

The total antioxidant capacity (TAOC) assay provides an overall measure of the ability of the reactive oxygen species reduction system to neutralise reactive oxygen species (ROS). One specific assay developed for measuring and quantifying the capability of biological samples to neutralise ROS is the total oxygen scavenging capacity (TOSC) (Regoli, 2000; Winston *et al.*, 1998). The TOSC assay has been standardised for measuring the scavenging capacity of cellular antioxidants with respect to various ROS (Regoli and Winston, 1999). While this assay, like the TAOC assay, is not a specific measure of oxidative damage it provides information on the antioxidant capacity of specific chemical scavengers and their activities with different oxidants which is fundamental to understanding and predicting the susceptibility of biological tissues to oxidative stress (Regoli and Winston, 1999).

2.6.4.2 *Lipid Peroxidation*

Lipid peroxidation is a widely recognised consequence of oxyradical production (Winston and Di Giulio, 1991). The process of lipid peroxidation proceeds in a chain reaction and like the redox cycle has the ability to propagate a number of deleterious biochemical reactions (Stegeman *et al.*, 1992). Lipid peroxidation has potential as a biomarker, however, it can occur due to cellular damage resulting from a range of insults other than chemically induced oxidative stress (Melancon *et al.*, 1992). A commonly used assay for lipid peroxidation is thiobarbituric acid reactive substances (TBARS) test for malonaldehyde (MDA), a byproduct of lipid peroxidation (Pedrajas *et al.*, 1995; Romeo *et al.*, 2003a).

2.6.4.3 *Methemoglobin*

Methemoglobin (MetHb) is a form of haemoglobin in which the heme iron is in the oxidised state and which is unable to bind and transport O_2, makes up only a small proportion (< 1-2%) of total haemoglobin under normal conditions. Transition metals have been shown to enhance the production of MetHb by the production of oxyradicals that facilitate the oxidation of heme bound Fe^{2+}. Increased levels of MetHb provide a relatively sensitive early indication of oxidative damage in red blood cells (Stegeman *et al.*, 1992). Reliable measurements of MetHb require freshly drawn, unfrozen whole blood samples and can be measured by a fairly straightforward spectrophotometric technique developed by (Hegesh *et al.*, 1970).

2.6.4.4 *DNA Oxidation*

The oxidation of DNA may produce hydroxylated DNA bases as result of alterations from ·OH attack at various DNA base sites (Stegeman *et al.*, 1992). Recently developed methods for measuring these products in biological samples, which show promise, use HPLC separation and electrochemical detection of hydroxylated bases, such as thymine glycol or 8-hydroxy deoxyguanosine. These methods are very sensitive but fairly involved, method refinements would enhance the feasibility of this promising biomarker (Stegeman *et al.*, 1992).

2.6.4.5 *Micronucleus Frequency*

Micronuclei are small intracytoplasmic masses of chromatin resulting from chromosomal breakage or aneuploidy during cell division (Bolognesi *et al.*, 2004). They resemble the main nucleus and are easily observed in interphasic cells (Scarpato *et al.*, 1990). The micronucleus assay is one of the most promising techniques to identify genetic alterations in organisms exposed to toxicants (Bolognesi *et al.*, 2004). As an index of chromosomal damage the micronucleus test is based on the enumeration of downstream aberrations after DNA damage and shows a time-integrated response to pollutants. It is thought to be a fast and sensitive test since it is able to detect genomic damage due to both clastogenic effects and alterations of the mitotic spindle (Migliore *et al.*, 1987). The micronucleus test has proved suitable for application to aquatic invertebrates and is simpler and more rapid to perform than other measurements of chromosomal aberration (Burgeot *et al.*, 1996). Micronuclei frequency has been studied in fish, (Castano *et al.*, 1998; Williams and Metcalfe, 1992) and invertebrates, (Bolognesi *et al.*, 2004; Kalpaxis *et al.*, 2004; Majone *et al.*, 1987; Scarpato *et al.*, 1990; Wrisberg *et al.*, 1992).

2.6.5 Stress Proteins

Stress proteins are a group of proteins which include two major groups of gene products: the 7–90 kDa *heat shock proteins* (hsp) induced by exposure to heat and a variety of other chemical and physical stressors; and the 78–100 kDa *glucose-regulated proteins*, (grp) synthesised in response to glucose and oxygen deprivation, and exposure to lead or agents which inhibit calcium and protein homeostasis (Locke, 2002). Each stress protein is made up of a multigene family in which some proteins are constitutively expressed and are present in cells under normal conditions, playing a role in basic cellular physiology while others are highly inducible in response to environmental stressors (Stegeman *et al.*, 1992). The term heat shock proteins was originally used to describe this family of proteins as they were originally studied in relation to heat shock response, it is now known that they can be induced by a number of environmental perturbations including metals (Agell *et al.*, 2004; Bauman *et al.*, 1993; Del Razo *et al.*, 2001; Werner *et al.*, 2004) and organics (Ait-Aissa *et al.*, 2000; Werner *et al.*, 2004). In aquatic species members of the hsp70 and hsp60 groups are highly conserved and exhibit measurable increases in synthesis in response to environmental contaminants (Stegeman *et al.*, 1992). In particular the hsp72 is only synthesised in response to environmental stressors and is not found in most cells under normal conditions, making it an excellent candidate as an exposure biomarker for chemical contamination (Stegeman *et al.*, 1992).

2.6.6 Heme Oxygenase

Heme oxygenase is a 32kDa stress protein which has been isolated and identified is inducible by metals (cadmium, zinc, copper and lead), sodium arsenite, oxidative stress and thiol-reactive agents (Sanders, 1990). It is described as a rate limiting enzyme which catabolises heme into three products: carbon monoxide (CO), biliverdin (which is rapidly converted to bilirubin) and free iron (which leads to the induction of ferritin, an iron-binding protein) (Otterbein *et al.*, 2003). It is thought that since these breakdown products of heme can react readily with peroxyl radicals they may play a significant role in protecting cells from oxidative damage as free radical scavengers in concert with glutathione (Otterbein *et al.*, 2003; Rivera and Zeng, 2005; Sanders, 1990; Stegeman *et al.*, 1992).

Jorgensen *et al*., (1998) examined the effect of a variety of stressors on heme oxygenase activity in Atlantic salmon and mackerel liver and spleen and concluded that heme oxygenase may be suitable for developing as a biomarker for certain metals and oxidative stress in fish but the application is reliant on the development of fish specific antibodies for the enzyme.

2.6.7 Metallothioneins -(see also Section 2.3.2.2.)

Metallothionein (MT) is a low molecular weight (≥ 10 kDa) cysteine rich metal binding protein synthesised in response to metal exposure (Roesijadi, 1996), which may have potential as biomarkers of exposure to toxic metals (Garvey, 1990; Petering *et al.*, 1990; Sanders, 1990; Stegeman *et al.*, 1992; van der Oost *et al.*, 2003). Its induction is slower than that of other "classic" stress proteins in response to transition metals, 24 hrs as opposed to 30 min (Sanders, 1990). Measurement of MTs does not necessarily reflect the degree of exposure to metals as physiological and environmental factors can affect mobilisation and partitioning of metals by MT (Roesijadi, 1996; Stegeman *et al.*, 1992). MTs may also be induced under many other conditions besides metal exposure, for example, glucocorticoid hormones (progesterone and glucagon) and peptide hormones (interleukin I and interferon) (Sanders, 1990; Stegeman *et al.*, 1992). The study of metal binding to MTs rather than measuring total tissue metal concentrations may be useful as it is increasingly clear that knowledge of intracellular compartmentalisation is essential to understanding mechanisms of metal-induced cell injury, as it aids in determining the extent to which organisms are able to sequester metals in forms which are not biologically reactive (Fowler, 1987; Vijver *et al.*, 2004). Since the normal physiological function of MT is presently not fully understood there is no way to determine if MT itself plays a direct role in the pathophysiology of cell injury. The current data suggest the reverse is true, the non MT bound fractions of these metals participate in the cell injury process, and MT induction appears to be a protective cellular response (Viarengo *et al.*, 1998). MT induction and metal binding appear to be a cellular defence mechanism against injury. Metal toxicity seems to occur only after this capacity has been exceeded (Roesijadi, 1996; Stegeman *et al.*, 1992). The use of MTs to assess organism health or fitness in response to toxic metal exposure requires extensive knowledge of their normal physiological function and the factors which control the levels of MT in selected organisms also needs to be established (Stegeman *et al.*, 1992).

2.7 Haematological Parameters

Haematological parameters provide a non-destructive method for effect assessment which are typically non-specific in their response to chemical stress (van der Oost *et al.*, 2003).

2.7.1 Serum Enzymes

Increased serum enzyme concentrations can result from: enzyme leakage from a cell with a damaged cell membrane; increased enzyme production and leakage from the cell; or decreased enzyme clearance from the blood (Mayer *et al.*, 1992). *Serum transaminases*, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are enzymes that catalyse the inter-conversion of amino acids and α-ketoacids by transfer of amino groups (van der Oost *et al.*, 2003). Increased levels of these enzymes in intracellular fluids may be a sensitive indicator of cellular damage as levels in cells usually exceed those in the intracellular fluids by more than three orders of magnitude (van der Oost *et al.*, 2003). Metals have been found to affect the activities of transaminases in fish. Fish exposed to acutely toxic concentrations of cadmium, copper or mercury had increased transaminase activities, alternatively chronic exposure to copper resulted in decreased AST activities and chronic exposure to cadmium had no effect on transaminase activity in fish serum (Mayer *et al.*, 1992). *Serum lysosomal enzymes* have been suggested as potential indicators of effect following exposure to organics, pesticides and metals. In particular N-acetyl-β-D-glucosaminidase (NAG) activity in spleen and liver dysfunction, and leucine amino naphthylamidase (LAN) enzyme activity for quantifying tissue damage in fish (Mayer *et al.*, 1992). The mechanism responsible for increased serum levels of lysosomal enzymes is not know but it is thought to differ from other serum enzymes. Lysosomes contain increased concentrations of metals and may be active in the degradation of metal binding proteins (Fowler and Nordberg, 1978). Metals have also been known to increase lysosome numbers and reduce lysosomal membrane stability possibly leading to enzyme leakage (Castro *et al.*, 2004; Domouhtsidou *et al.*, 2004; Nicholson, 2003; Petrovic *et al.*, 2001; Versteeg and Giesy, 1986). Serum enzymes have been demonstrated as useful biomarkers of tissue damage, use of a suite of serum enzymes may prove useful for understanding population-level effects (Mayer *et al.*, 1992).

2.7.2 Heme/Porphyrin Pathway **(see also section 2.5.1.1)**

The heme/porphyrin pathway is essential for synthesis of hemoproteins (e.g. haemoglobin) and various cytochromes (e.g. cytochrome P-450). A number of metals, metalloids and organics have been shown to induce enzymatic disturbances in this pathway, which correlate with overt cell injury (Stegeman *et al.*, 1992). In particular the activity of the δ-aminolevulinic acid hydratase (ALAD), a cytosolic enzyme found in many tissues and active in the synthesis of haemoglobin by catalysing the formation of porphobilinogen, a precursor of heme, has been shown to be inhibited by exposure to lead in mammals (Flora and Seth, 1999; Mayer *et al.*, 1992; Pande and Flora, 2002; Perottoni *et al.*, 2005). It has been suggested that the determination of ALAD activity in fish might be a useful biomarker of lead exposure and some studies have shown ALAD inhibition in fish blood and liver following water-borne exposure to lead (Conner and Fowler, 1994; Rodriguez *et al.*, 1989). Conner and Fowler (1994) found that although fish hepatic ALAD was inhibited by lead exposure the sensitivity of the fish reaction was lower than that reported for mammals, requiring a 40-fold increase in lead concentration exposure to produce the same IC_{50}. Further kinetic studies indicated major differences between fish and mammalian hepatic ALAD. They suggest the absence of a chelatable metal cofactor or greater binding affinity at the active binding site of the fish hepatic enzyme compared to that described for mammals may be responsible for the difference in sensitivity to lead. The use of this pathway may be applicable to the marine bivalve *Anadara trapezia* as it has haemoglobin as a respiratory pigment (Sullivan, 1961).

2.7.3 Ion Levels

Ion levels in aquatic organisms must be maintained through active regulation of water and ion influx and efflux. Exposure to metals can effect the ion regulatory organs, internal and external osmotic sensory receptors, endocrine system, metabolism or active transport processes, leading to alterations in the plasma ion levels of K^+, Na^+ and Cl^- ATPhase activity (Mayer *et al.*, 1992). Decreased levels of K^+, Na^+ ATPase activity have been measured in eel gills and intestines following exposure to cadmium (Lionetto *et al.*, 2000), fish (de la Torre *et al.*, 2000; Wong and Wong, 2000) and invertebrates exposed to silver (Bianchini *et al.*, 2005) and copper (Bianchini *et al.*, 2004). However, other studies of fish exposed to elevated levels of cadmium (Benson *et al.*, 1988) and mercury (Jagoe *et al.*, 1996) have failed to show significant alterations in haemolymph ionic composition.

Effects of stressors on osmoregulation have not been related conclusively to higher order and population level effects and this combined with difficulties of inherent variability, accessory factors and data interpretation, limits the potential for this technique as a biomarker for metal induced stress in field studies (Mayer *et al.*, 1992).

2.7.4 Neurotoxic Measures

The principle neurotoxic enzyme identified in aquatic organisms is acetylcholinesterase (AchE), which is involved in the deactivation of acetylcholine at nerve endings, preventing continuous nerve firings, which are vital for normal sensory and neuromuscular function. AchE activity is inhibited by organophosphate and carbamate pesticides and has been used in fish studies as an exposure biomarker for these xenobiotics (de Aguiar *et al.*, 2004; Eder *et al.*, 2004). It has been measured in mussels exposed to organophosphates but for these organisms it was not found to be a reliable indicator (Cajaraville *et al.*, 2000; Rickwood and Galloway, 2004), however, measurements in the clam and a polychaete worm showed it to be a sensitive biomarker along a pollution gradient (Perez *et al.*, 2004). It has been suggested that AchE may also be a sensitive biomarker in fish for a range of other chemicals including compounds in complex mixtures of combustion hydrocarbons and natural wood leachate (Payne *et al.*, 1996).

2.7.5 Endocrine System

Physiological and biochemical stress resulting from contaminant exposure must be compensated for in order to maintain homeostasis. The measurement of the synthesis, secretion, metabolism and clearance of hormonal concentrations in blood may be used to gauge the impact of contaminants on metabolism, growth and reproduction (Mayer *et al.*, 1992). Effective use of plasma concentrations of hormones as biomarkers requires knowledge of production, and clearance rates as well as seasonal, age, gender, reproductive and nutritional status influences. The following hormones have been considers as potential biomarkers: corticosteroids; catecholamines; thyroid hormones; reproductive steroids; insulin; glucagon and growth hormone (Mayer *et al*., 1992).

2.7.6 Other Metabolic Products

2.7.6.1 *Reproductive Hormone*

Levels of reproductive hormones have been shown to be altered and reproductive function impaired in response to a variety of chemical stressors both organic and inorganic (Chen, 1988; Depledge and Billinghurst, 1999; Siah *et al.*, 2003; Thomas, 1988). The complexity of the interactions among various parts of the reproductive system is a limiting factor in their use as biomarkers (Melancon *et al.*, 1992).

2.7.6.1.1 Vitellogenin

Vitellogenin is a large molecular weight lipophosphoprotein synthesised by the liver in vertebrates regulated by β-estradiol. The analogous compound in invertebrates is lipoprotein which is synthesised by a variety of tissues. These molecules, which are precursors, of yolk proteins are released into the blood stream and sequestered in the developing oocyte in response to gonadotrophin and other hormones. Impaired reproductive function due to decreased plasma vitellogenin levels has been demonstrated in rainbow trout exposed to cadmium (Haux *et al.*, 1988), Florida largemouth bass exposed to organics and metals (Sepulveda *et al.*, 2002), and increased levels were measured in shore crabs exposed to cadmium (Martin-Diaz *et al.*, 2004). Riffeser and Hock (2002) found no significant induction (or supression) of haemolymph vitellogenin in mussels exposed to estrogenic compounds. Very little is known about the effect of chemicals on invertebrate vitellogenin. A better understanding is required before plasma vitellogenin concentrations can be reliably used as biomarkers in invertebrates (Melancon *et al.*, 1992).

2.8 Cellular Biomarkers

Cell and tissue perturbations may serve as biomarkers of both exposure and effect of environmental contaminants. While changes at the molecular level in the expression of the various enzymes involved in the conjugation, detoxification and excretion of toxins are sensitive first order measures of exposure and in some instances effect, measures of cell integrity and tissue morphology offer a second order measure of exposure and more particularly of effect. Being at a higher order of biological organisation the cellular and tissue effect response is less likely to be specific for a particular contaminant but rather a general response often indicative of chronic toxicity.

Linkage of molecular responses to damage at the cell and higher levels of organisation is only beginning to emerge, and more focus is being brought to it in an effort to determine the significance of molecular events to subsequent forms of cell and tissue injury and response (Hinton, 1994).

2.8.1 Immunological Responses

A highly developed cell-mediated immune system, involving non-specific immune mechanisms and humoral antibody systems, has been demonstrated in teleosts (Weeks *et al.*, 1990). Assays of these immune responses, in their capacity to destroy foreign material and protect the host against disease can serve as useful sentinels of the health status of environmentally stressed organisms (Weeks *et al.*, 1990). Since immunocompetent cells are required for host resistance, measurement of increasing susceptibility to infectious agents or tumour cells can provide insight into the biological significance of immune alterations induced by xenobiotics (Weeks *et al.*, 1992). Immunicological biomarkers in fish which have been found to react to experimental stress include:

- Decreased haemocyte count and viability (Liu *et al.*, 2009; Mirella da Silva *et al.*, 2008; Vijayavel *et al.*, 2009);
- Macrophage function (Zelikoff, 1998);
- Increased phagocytosis (Brousseau *et al.*, 1997; Luengen *et al.*, 2004) or decreased phagocytosis (Hannam *et al.*, 2009);
- Increased susceptibility to bacterial infections (Zelikoff, 1998);
- Enlarged lysosomes and the loss of lysosomal structures (Regoli *et al.*, 1998b);
- Decreased respiratory burst (Rice *et al.*, 1996).

A three tiered approach to the application of immune system responses to the assessment of environmental impact is described in detail by Weeks *et al*., (1990). Briefly the relatively simple Tier I assays provide a general effect screening while the more specific and sensitive Tier II assays provide a comprehensive evaluation of the various components of the immune response, finally a Tier III host resistance challenge study can be used to complete an immune function assessment.

2.8.2 Lysosomal Membrane Stability

Lysosomes play an important role in sequestration and detoxification of metals (Viarengo, 1989). High metal concentrations may induce synthesis of metallothioneins which enter lysosomes for degradation and normal protein turnover thus overloading their storage and detoxification capacity (Viarengo, 1989). Destabilisation of the lysosomal membrane following exposure to metals may be a result of direct interaction of metals with the lysosomal membrane and from metal induced oxidative stress (Regoli *et al.*, 1998b). Lysosome membrane destabilisation has proven a useful effect biomarker of metal exposure in both field and laboratory exposures (Kalpaxis *et al.*, 2004; Nicholson, 1999a; b; Regoli *et al.*, 1998b; Ringwood *et al.*, 2002; Romeo *et al.*, 2000; Werner *et al.*, 2004). Winston *et al* (1996) demonstrated that lysosomal destabilisation in mussels is affected by production of oxyradicals generated from contaminant exposure both internally and externally to the lysosomal membrane.

2.8.3 Histopathological Alterations

Histopathological alterations are effects measures of exposure to environmental stressors which can be used to examine organ, tissue or cellular perturbations of both acute and chronic exposure to contaminants (Hinton *et al.*, 1992). Histopathological lesions can result from exposure to a wide range of toxic agents and it is only possible to make broad generalisations about the toxin responsible for a particular lesion, however, they provide useful evidence of the magnitude of toxic impairment to the individual and in some cases the potential impact on the population (Hinton *et al.* 1992).

2.8.4 Physiological Biomarkers

An optimal assessment of the altered health of on organism or ecosystem requires the selection of a range of indicators from different levels of biological organisation. While measurements at the cellular level provide the greatest sensitivity the overall fitness can be better assessed using physiological responses since these represent an integration of individual cellular effects (Duquesne *et al.*, 2004). Physiological biomarkers are non-specific. They can be used as indicators of both exposure to and effect of xenobiotics and may be useful in integrating the effects of a number of stressors by quantifying organism health (Mayer *et al.*, 1992).

Organism health has been defined by Bayne *et al*., (1985) as the residual capacity to withstand stress; the more stressed the less capable the organism is of withstanding further stress. General physiological biomarkers which are useful in assessing organism health and which can give some indication of the potential for population level effects are described below. These types of measurements can be highly variable, however, when linked with other evidence of perturbation at the subcellular and cellular level can provide further evidence for potential higher order effects (Figure 2.1).

2.8.4.1 *Energetics*

There are many energetic responses that may be initiated in response to toxicant exposure. Acute responses, which are generally controlled by the enzyme and hormone systems, are considered to be the initial response to a stressor and often involve an increase in energy related substrates in the haemolymph (Mayer *et al.*, 1992). Chronic stress can initiate compensatory physiological adjustments, so that energy metabolism changes may be needed to maintain normal physiological function, and this can result in reduced growth or reproduction (Mayer *et al.*, 1992). An assessment of the energy status of an organism can, therefore, give an indication of its overall condition.

2.8.4.1.1 Adenylate Energy Charge

Adenylate energy charge (AEC) is a measure of the metabolic energy available to an organism from the adenylate pool and is a direct calculation based on the concentrations of adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP) (Sokolova *et al.*, 2005b).

Equation 1: $$EC = \frac{ATP + \frac{1}{2} ADP}{ATP + ADP + AMP}$$

A decrease in the concentrations of ATP, the primary source of cellular energy, based on the increased use of energy by organisms under stress can result in reduced AEC values (Mayer *et al.*, 1992). Oysters *Crassostrea virginica* experimentally exposed to cadmium had reduced AEC values compared to control oysters (Cherkasov *et al.*, 2006; Sokolova *et al.*, 2005b) . Cellular metabolic supply and demand of ATP, ADP and AMP in a wide range of energy-stressed animals is reviewed by Staples and Buck (2009).

2.8.4.1.2 Cellular Energy Allocation

When organisms are exposed to suboptimal conditions there is a cost of dealing with stress in terms of metabolic resources. Energy available for growth based on energy budget analysis rather than direct measurements of growth itself may, therefore, provide a sensitive measure of stress in organisms (Smolders *et al.*, 2004). The cellular energy allocation (CEA) methodology was developed as a biomarker technique to assess the effect of toxic stress on the cellular energy budget of test organisms (De Coen and Janssen, 1997). The assay is based on the biochemical measurement of changes in energy reserves (glucose, protein, and lipid content) and the energy consumption (electron transport system activity (ETS)). The difference between the energy reserves and energy consumption represents the net cellular energy budget, expressed as CEA, of the test organism (De Coen and Janssen, 1997).

Equation 2: (E_a available energy) E_a = glucose + lipid + protein (mJ mg^{-1} wet wt.)
(E_c energy consumed) E_c = ETS activity (mJ mg^{-1} wet wt. h^{-1})
CEA (cellular energy allocation) = E_a/E_c

The utility of CEA as a stress biomarker, with links to higher order effects, has been shown for daphnids (De Coen and Janssen, 1997; 2003), mussels (Smolders *et al.*, 2004) and freshwater gastropods (Moolman *et al.*, 2007).

2.8.4.1.3 Scope for Growth

Growth as a process represents an integration of major physiological responses and specifically the balance between processes of energy acquisition (feeding and assimilation) and energy expenditure (metabolism and excretion) (Widdows and Donkin, 1992). This physiological energetics-based approach is usually referred to as Scope for Growth (SFG).

Equation 3: SFG = $A - (R + U)$ (J mg^{-1} dry wt h^{-1})
A = energy absorbed, R = energy respired, and U = energy excreted

SFG has been used extensively and found to be a sensitive growth biomarker of stress in marine mussels (Goldberg and Bertine, 2000), cockles *A. trapezia* exposed to metals (Burt *et al*, 2007) and marine gastropods *Nassarius festivus* exposed to cadmium (Wo *et al.*, 1999). Bayne *et al*., (1985) has reviewed the methods available for measuring the endpoints required to calculate SFG.

2.8.4.2 *Condition Index*

A reduction in AEC, CEA or SFG values can ultimately lead to reduced growth and reproductive capability. Body condition index can provide information on the impact of pollutants on individual organism health through relatively simple growth measures, such as determining what proportion of bivalve internal shell volume is occupied by the organs (Mayer *et al.*, 1992). A widely used condition index (CI) measure for bivalves is based on the measurement of the ratio of soft tissue weight to valve weight.

Equation 4: $CI = \frac{\text{Tissue Mass (g)} \times 100}{\text{Valve Mass (g)}}$

A high ratio indicates good physiological condition and reduced ratios indicate poor physiological condition. This CI has been used extensively with marine bivalves and gastropods exposed to metal and other contaminants and has proved to be a useful indicator of both severe and chronic stress (Andral *et al.*, 2004; Duquesne *et al.*, 2004; Leung and Furness, 2001a; Leung and Furness, 2001b; Li *et al.*, 2009; Lundebye *et al.*, 1997; Mubiana *et al.*, 2006; Romeo *et al.*, 2003b).

2.9 Summary and Conclusions

The preceding sections have dealt with the ways in which organisms are exposed to metals, the mechanisms they employ to manage their exposure, the consequent dose resulting from this and finally the myriad of molecular, cellular and physiological responses which may be measured as indicators that exposure to a toxicant has occurred and the dose has exceeded the organisms detoxification and repair capacity.

2.9.1 External Exposure

Exposure under laboratory conditions with single contaminants enables potentially confounding factors to be controlled and the exposure concentration set. This approach is needed to establish relationships between exposure, dose and response of the test species to the contaminant of interest (McCarthy, 1990). Exposure in the field can be assessed by measuring metals in the sediments and overlaying waters this gives a measure of total metal availability but not of the bioavailable fraction. By relating the water and sediment total metal concentrations with that of exposed organisms' metal body burdens an estimate of the bioavailable metal fraction can be gained.

2.9.2 Internal Dose

Further investigation of the partitioning of the metal in the various tissue subcellular fractions gives information on the proportion of the metal which is metabolically available, the biologically active metal (BAM), and therefore able to participate in effects and the proportion which is sequestered in the heat stable MT and MT like proteins and the granule fraction, the biologically detoxified metal (BDM), and therefore not metabolically available (Vijver *et al.*, 2004; Wallace *et al.*, 2003).

2.9.3 Response

A multi-biomarker approach at several levels of biological organisation has advantages over the use of a single biomarker and offers an effective early warning system of adverse effects in biomonitoring of aquatic environments (Adams *et al.*, 1988; Adams *et al.*, 1989; Brown *et al.*, 2004; Galloway *et al.*, 2004; Romeo *et al.*, 2003a; Smolders *et al.*, 2004). The biomarkers which derive from cytochemistry, cytophysiology, cytogenetics and pathology can detect early responses and prepathological alterations before other disturbances such as disease, mortality, or population changes occur (Adams *et al.*, 1989).

Effects of environmental stressors such as the toxic metals cadmium, lead and selenium on the health of aquatic organisms may involve a series of biological responses ranging from the molecular/biochemical to the cellular and physiological levels. As suggested by McCarthy, (1990), development of biomarker protocols are best done under controlled laboratory conditions with single contaminants. Once processes are well understood assessments using mixed contaminants should be carried out to determine whether biomarker responses can be quantified under more realistic conditions.

2.9.4 Biomarker Selection

It is important to select an appropriate suite of biomarker measurements which demonstrate the relationship between exposure dose and response. Thus biomarkers must be selected which have a demonstrable link to exposure or dose.

The oxidative system offers a range of general response and effect biomarkers which have been shown to be sensitive to metals through perturbations in the redox cycle and other oxidative pathways. The measurement of a suite of biomarkers within this system, from ROIs, through catalysing and phase II enzymes to oxidative damage indices offers a weight of evidence approach to assessing molecular level exposure and effects. As lysosomes are involved in metal management and are also susceptible to oxidative damage the measurement of their integrity offers a useful biomarker of effect at the cellular level. Impairment of the lysosomal membrane can be considered a second order effect which would follow perturbations at the molecular level. The frequency of micronuclei occurrence offers a measure of DNA damage which may aid in completing the picture of a cascade of reactions resulting from exposure to toxic levels of metals for individual organisms. Linking this to organism physiological condition, using a measure of condition index, gives an indication of the potential for these effects to result in reproductive and ultimately population level effects. An interrelated cascade of biomarker measurements which demonstrate the relationship between exposure dose and response is necessary for the molecular perturbations measured to be interpretable and therefore able to be linked to potential higher order effects (Figure 2.3). By identifying and establishing relationships between exposure, dose and response to toxicants at these levels of biological organisation we should be better able to understand the mechanisms of stress responses in ecological systems that could ultimately result in improved predictive capability of ecological risk assessment and also allow for more informed decisions regarding remedial actions.

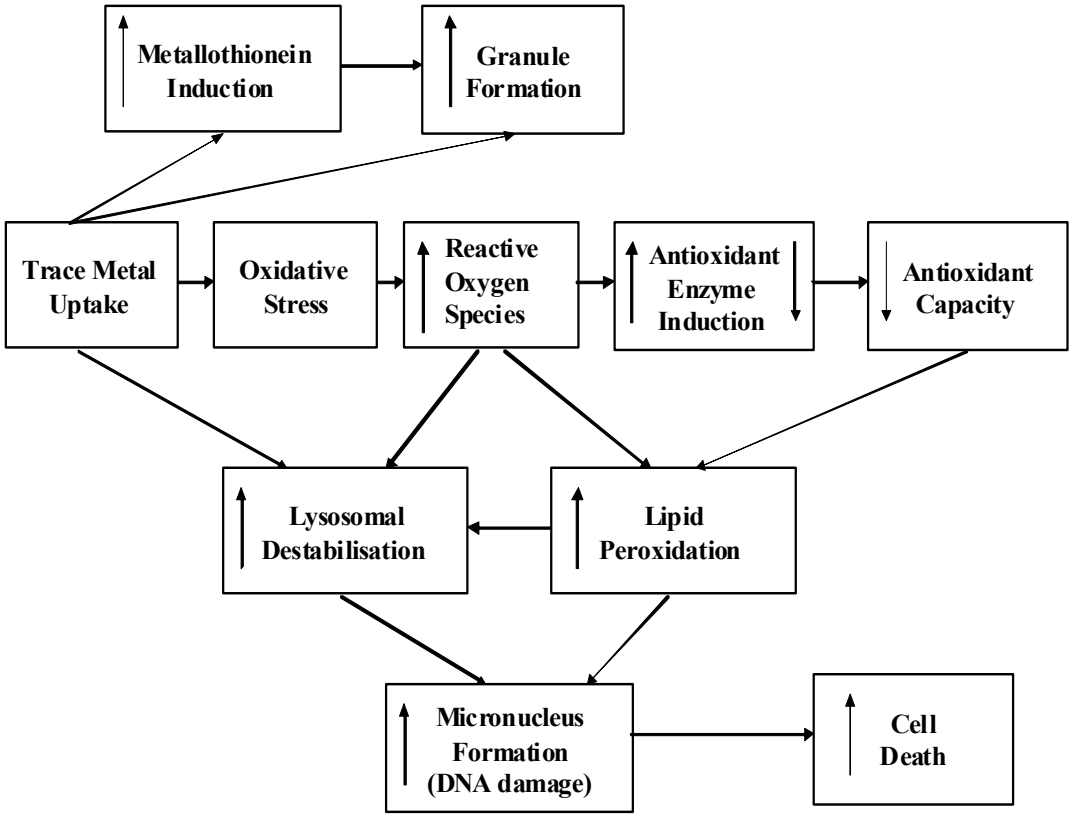


Figure 2.3: Flow diagram demonstrating a possible cascade of interlinked cellular reactions which may occur in response to metal exposure.

3 Methods

3.1 Organism Selection

Tellina deltoidalis and *Anadara trapezia* were selected as test organisms for metal exposure – dose - response studies as both are sediment dwelling bivalves which satisfy most of the basic requirements to be effective biomonitors (Phillips and Rainbow, 1994). They are:

- Sedentary and therefore representative of the study site of interest;
- Hardy and tolerant of high levels of metals (King *et al.*, 2005; Scanes, 1993);
- Abundant in estuarine environments along the eastern seaboard of Australia (Beesley *et al*., 1998);
- Easy to identify and collect and provide sufficient tissue for analysis of metal concentrations and biomarkers;
- Accumulate metals in tissues relative to sediment concentrations (Burt *et al.*, 2007; King *et al.*, 2005).

3.1.1 *Tellina deltoidalis* (Linnaeus, 1758)

Classification

Phylum:	Mollusca
Class:	Bivalvia
Subclass:	Heterodontia
Order:	Veneroida
Superfamily:	Tellinoidea
Family:	Tellinidae
Genus:	*Tellina*
Species:	*deltoidalis*

Figure 3.1: *Tellina deltoidalis*

Description

Tellina deltoidalis has a thin shell with a smooth surface and margins, which is white to cream with darker banding around the outer edge (Figure 3.1). The shell is laterally compressed and with equilateral valves and adult shell length is generally between 20 - 30 mm and shell The mantle has a gape at the antero-posterior end through which the triangular shaped muscular foot is extended. The foot is used to rapidly bury and anchor the animal in place in the sediment.

Live animals lie obliquely in the sediment on their left valve at a depth several times their shell length with their siphons extending up to the sediment surface. The inhalant siphon rotates with its tip touching the sediment surface and ingests deposited organic material and sand grains. The exhalent siphon expels faeces and water while extended more or less horizontally below the sediment surface (Beesley *et al.*, 1998).

3.1.2 *Anadara trapezia* (Deshayes, 1840)

Classification

Phylum:	Mollusca
Class:	Bivalvia
Subclass:	Pteriomorphia
Order:	Arcoida
Superfamily:	Arcoidea
Family:	Arcidae
Genus:	*Anadara*
Species:	*trapezia*

Figure 3.2: *Anadara trapezia*

Description

Anadara trapezia has a cream to white shell, partially covered with a dense brown fibrous periostracum (Figure 3.2). The shell is heavy, elongate, inflated and equivalve with broad flat radial ribs which interlock at the margins to aid in securing the valves and in mature organisms ranges in length from 300 – 800 mm (Figure 3.2). The mantle is large and flat without fusion or siphons and is composed mainly of connective tissue, with large blood spaces between it and the shell. Unlike other marine bivalves the haemolymph of *A. trapezia* contains haemoglobin as a respiratory pigment (Sullivan, 1961). The mantle lobes are opposed at the edges except at the posterior end where the primary inhalant and exhalant apertures are and anteriorly where the aperture for the foot is formed. The elongate foot is used to burrow into sediment and anchor the animal in place. It can also be used to move the animal along the sediment surface and is capable of being flexed rapidly against the substratum to allow it to jump distances of up to 20 cm (Sullivan, 1961). As *A. trapezia* has no siphon to extend beyond its shell it never buries entirely below the sediment surface but must keep its posterior end exposed to enable feeding.

A. trapezia is a filter feeder, the inhalant current draws water over the large gills and microscopic particles such as microalgae, phytoplankton, diatoms and flagellates are filtered out and passed to the mouth via labial palps. Waste material passes out with the exhalant current (Beesley *et al.*, 1998).

3.1.3 Organism Collection

Bivalves used in the laboratory exposure experiments were all collected from uncontaminated estuaries on the south coast of NSW. *Tellina deltoidalis* were collected from Durras Lake and Lake Tabourie and *Anadara trapezia* from Burrill Lake St. Georges Basin and Batemans Bay, NSW in July 2005, January and November 2006 and July 2007. Nine *A. trapezia* were also collected from a contaminated site in Lake Macquarie NSW in July 2007 for comparison of resident, naturally metal exposed with laboratory metal exposed organisms. All bivalves were placed in Eskies® with sediment and water from the collection sites. Aerators were used to oxygenate the overlying water during transportation to the laboratory. Organisms used in exposure experiments were maintained for a maximum of two weeks in uncontaminated sediments, depth 15 cm, in glass aquaria with filtration and aeration to allow acclimation before experimentation. Overlying water used in aquaria was collected from coastal waters near Murramurrang National Park, NSW and adjusted from 35 ‰ to 30 – 28 ‰ with deionised water depending on the salinity of the estuarine water from which organisms were collected. A water temperature of 22^{o}C and a day / night cycle of 14 / 10 hours was maintained to reflect spring / summer conditions. Organisms were fed every three days with a commercial powdered complete food suitable for marine bivalves (Sera Micron, Germany), made up in seawater. Half water changes were done weekly.

3.2 Sediments

3.2.1 Sediment Collection – Metal Spiked Sediments Studies

Sediments for use in metal spiking experiments were collected from a NSW Department of Environmental and Climate Change reference site in Durras Lake NSW. Sediments were placed in plastic barrels and stored in a cool room at 4^{o}C until use. Sediments were sieved through a 2 mm stainless steel sieve to remove large pieces of organic matter and organisms prior to the addition of metals. Sub samples of the collected sediments were measured for moisture content and grain size.

To ensure the sediment matrix was suitable for organism burrowing and feeding, sediment was mixed with clean beach sand so that the 63 µm fraction was not greater than 20 %.

3.2.1.1 *Cadmium Spiking*

The addition of metals to sediments causes the pH to drop and the redox potential to rise due to the hydrolysis of added metals, the displacement of protons from particulate organic matter and mineral metal-binding sites and the oxidative hydrolysis of displaced iron (II) (Simpson *et al.*, 2002). To ensure added cadmium was rapidly adsorbed and strongly bound to the sediment particles a method developed by (Simpson *et al.*, 2004) was followed. Briefly, wet sediment was added to 3 glass mixing containers. Container 1 had nothing added, $CdCl_2$, (AR grade Merck), was added to container 2 to a concentration of 10 mg/kg dry mass of sediment and to container 3 at 50 mg/kg dry mass of sediment. All containers were topped up with clean deoxygenated sea water and the final mixture was completely deoxygenated by bubbling with nitrogen for 2 hours. Head spaces of containers were filled with nitrogen prior to sealing. Any pH adjustments were made immediately after the addition of the $CdCl_2$ using 1M NaOH, (AR grade BDH), prepared in seawater. pH was checked weekly and maintained at 7 - 8.2 pH. Sediments were mixed on a Cell-production Roller Apparatus (Belco, USA) for several hours each day. Sediments were maintained at room temperature 22 - 25^{o}C. The time required for equilibration of added metals will be affected by the sediment properties, equilibration pH and the concentration and properties of the metal (Simpson *et al.*, 2004). To determine when the added $CdCl_2$ was completely bound to sediment particles pore waters were collected weekly by centrifuging a 20 ml sub sample from each container at 300 g x 15 min. Following centrifugation pore waters were removed from the tubes, acidified to 1 % with nitric acid (AristaR, BDH) and cadmium was measured using an ELAN® 6000 ICP-MS (PerkinElmer SCIEX). Once pore water cadmium concentrations had fallen below instrument detection limits the sediment was ready for use. Time to full absorption was 4 to 6 weeks for all metals.

3.2.1.2 *Selenium and Lead Spiking*

The protocol outlined above was also used for spiking sediments with selenium and lead. Selenium spiked sediments were prepared at concentrations of 5 and 20 mg/kg dry mass of sediment using Na_2SeO_3, (AR grade Sigma-Aldrich). Lead spiked sediments were prepared at concentrations of 100 and 300 mg/kg dry mass of sediment using $PbCl_2$, (AR grade Sigma-Aldrich).

Metal concentrations were chosen to reflect realistic metal contamination concentrations. The lower concentrations for cadmium and lead are based on the ANZECC and ARMCANZ (2000) Interim Sediment Quality Guidelines high values for sediment metal concentrations and the high values reflect concentrations which have previously been measured in contaminated estuarine sediments from Cockle Bay in Lake Macquarie NSW. There are currently no selenium sediment guideline concentrations, so selenium concentrations are based on sediment selenium concentrations previously measured in contaminated sediments from the same Lake (Peters, 1997).

3.2.2 Sediment Collection – Lake Macquarie Metal Contamination Gradient Sediments Study

Sediments for use in the metal contaminated gradient sediment exposure experiments were collected from each of three sites along a metal contamination gradient in Cockle Creek and Cockle Bay, Lake Macquarie, NSW. Cockle Creek was selected for this study as the lead/zinc smelter, established in 1897, is a point source of metal contamination discharge into Cockle Creek (Carroll, 1996). The source of the selenium contamination is fly ash from a coal fired power station situated in the same area (Peters, 1997). The sites selected show a north-south contamination gradient for zinc, lead, cadmium, copper and selenium that decreases in concentration from the smelter down Cockle Creek and into Cockle Bay. (Batley, 1987; 1991; Roach, 2005; Roy and Crawford, 1984). The zinc/lead smelter is at the top right section of figure 3.3. Sites 1 and 2 are in Cockle Creek (CC1 & CC2) and site 3 is in Cockle Bay (CB), as indicated. Sediments were also collected from a fourth site (LM cockle site) (Figure 3.3). This site was not included in the metal contamination gradient, but was sampled as native *A. trapezia* were found to be present at this location and this presented an opportunity to compare sediment tissue metal accumulation relationships as well effects in chronically exposed *A. trapezia* with those of previously unexposed *A. trapezia* exposed for 56 days to the metal contamination gradient sediments. Sediments were also collected from the sites in the uncontaminated estuaries St. Georges Basin and Batemans Bay (reference sites) where the *A. trapezia* used in the metal contamination gradient laboratory exposures were collected to establish that these sites were uncontaminated.

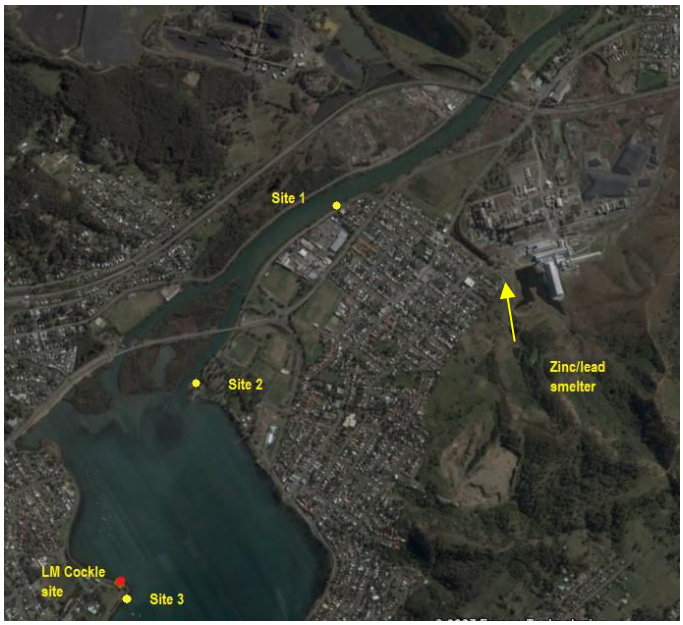


Figure 3.3: Lake Macquarie **s**ediment collection sites; modified from Google Earth, 2007, Europa Technologies, Image NASA, National Geographic Society.

All sediments were sieved through a 2 mm stainless steel sieve to remove large pieces of organic matter and organisms, placed in individual Eskies® for transportation and stored in a cool room at 4°C until use. Sub samples of the collected sediments were measured for grain size and metal concentration.

3.2.3 Metal Analysis

3.2.3.1 *Sediment Preparation*

Sediment sub samples of approximately 50 g were placed in glass petri dishes and oven dried at 60°C for 72 hours. Wet/dry weights were obtained for sediment moisture content used for calculating metal sediment spiking concentrations. Dried sediments were homogenised with a plastic spatula. Homogenised sediment was dry sieved through a 63 µm mesh and the resulting 2 fractions weighed to obtain the proportions of silt/clay (< 63 µm) and sand (> 63 µm) fractions in the sediments.

3.2.3.2 *Digestion*

The < 63 µm sediment fraction was sub sampled for metal analysis according to the international standard for sediment metal analysis (de Groot, 1995). Approximately 0.2 g dry wt. of sediment in 3 ml of nitric acid (AristaR, BDH) was digested in polyethylene 50 ml centrifuge tubes. Each 50 ml tube was capped, with lids pre-drilled with 3 mm holes. A glass thermo-well and fibreoptic probe was placed in one sample to monitor temperature.

Samples were microwaved in sets of 36 in a 630 watt oven (CEM MDS-2000) for 60 minutes at 400 W which produced a stable temperature of 115°C. Digested samples were cooled to room temperature in a fume cupboard to allow gases formed during digestion to vent. The digested samples were diluted to 30 ml with deionised water.

3.2.3.3 *Quality Assurance*

A quality assurance program was used to verify accuracy and precision of analysis. NRCC Certified Reference Materials, BCSS-1 marine sediment and NIST 1566a oyster tissue and acid blanks were routinely digested and diluted in the same way as the samples and analysed along with them. Mean CRM recoveries, concentrations µg/g dry mass, were:

BCSS-1 (n = 10)	Cadmium	Lead	Selenium	Zinc	Copper
Certified	0.25 ± 0.04	22.7 ± 3.4	0.43 ± 0.06	119 ± 12	18.5 ± 2.7
Recovered	0.22 ± 0.1	21 ± 4	0.41 ± 0.1	78 ± 13	15 ± 1
NIST 1566a (n = 50)					
Certified	4.15 ± 0.38	0.37 ± 0.014	2.21 ± 0.24	830 ± 57	66.3 ± 4.3
Recovered	4.4 ± 0.6	0.36 ± 0.02	2.1 ± 0.3	886 ± 98	70 ± 1

3.2.3.4 *Metal Determination*

Prior to analysis samples were further diluted with deionised water and an ICP-MS mixed 7-element internal standard (EM Science) was added to monitor for variations due to instrument drift and/or matrix effects. Cadmium, selenium, lead zinc and copper were measured using an ELAN® 6000 ICP-MS (PerkinElmer, SCIEX). External calibration standards used for quantitation were made up from a 10 mg/L Reference Standard, ICP-MS Calibration Multi-Element Standard 2 (AccuTrace™) in 1 % (v/v) HNO_3 acid as 1, 0.1, 0.01 and 0.001 mg/L solutions, recalibration was performed every 15 samples during analysis.

3.3 Experimental designs

3.3.1 *Tellina deltoidalis* - Metal Spiked Sediments Studies

The experimental design was as shown in (Figure 3.4). Procedures for conducting the exposures were adapted from the test method for conducting 28 day sediment bioaccumulation tests (Ingersoll *et al.*, 2000). Spiked and control sediments in the glass mixing containers were allowed to settle for 24 hours and the overlying water carefully removed. Sediment (500 g wet wt.) was placed in each of three replicate 750 ml poly propylene containers per treatment.

The containers were filled with fresh seawater adjusted to 28 ‰. Water was added by carefully pouring down a baffle to minimise disturbance to the sediments. Lids with small holes drilled in were used to minimise evaporation and allow the introduction of aerators to the water surface. Containers were placed in random order on a tray in an incubator set at 22°C with a day / night light cycle of 14 / 10 hours to reflect spring / summer conditions. Aeration was introduced and the treatments were left for 24 hours to allow them settle and the temperature to equilibrate. Twelve *T. deltoidalis* per treatment container were then introduced. Organisms were not given supplementary food and surface water was not changed during the 28 day exposure period.

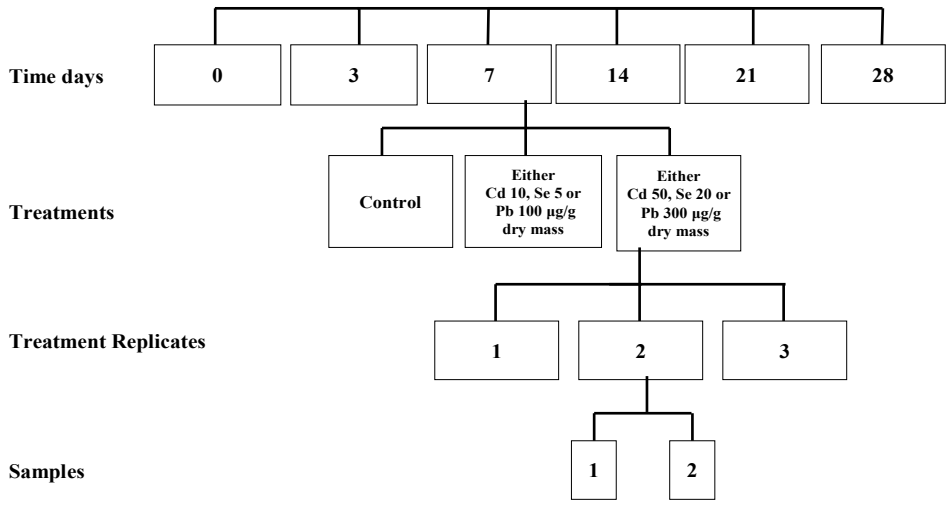


Figure 3.4: Experimental design for *T. deltoidalis* exposure to metal spiked sediments.

3.3.2 *Anadara trapezia* – Metal Spiked Sediments Studies

The experimental design was as shown in (Figure 3.5). Procedures for conducting the spiked sediment exposures were the same as those used for the *T. deltoidalis* exposures with the following modifications. Spiked or control sediment (1000 g wet wt.) was placed in each of three replicate 3 L polystyrene aquaria per treatment. The containers were filled with fresh seawater adjusted to 30 ‰. Water was added by carefully pouring down a baffle to minimise disturbance to the sediments. Lids with small holes were used to minimise evaporation and allow the introduction of aerators to the water surface. Containers were placed in random order on a Perspex tray filled with water cooled to 22°C by recirculation through a cooling unit (Resun CL650).

A day/night light cycle of 14/10 hours was produced using daylight fluorescent tubes to reflect spring / summer conditions. Aeration was introduced and the treatments were left for 24 hours to allow them settle and the temperature to equilibrate. Twelve *A. trapezia* per treatment aquarium were then introduced. As *A. trapezia* is larger more robust species than *T. deltoidalis* it was exposed for twice as long (56 days) as the standard laboratory toxicity test time (28 days) with a view to assessing its potential as a suitable organism for field exposures which are normally run over 2 to 3 months. Organisms were fed every three days with a commercial powdered complete food suitable for marine bivalves (Sera Micron, Germany), made up in seawater. Half water changes were done weekly.

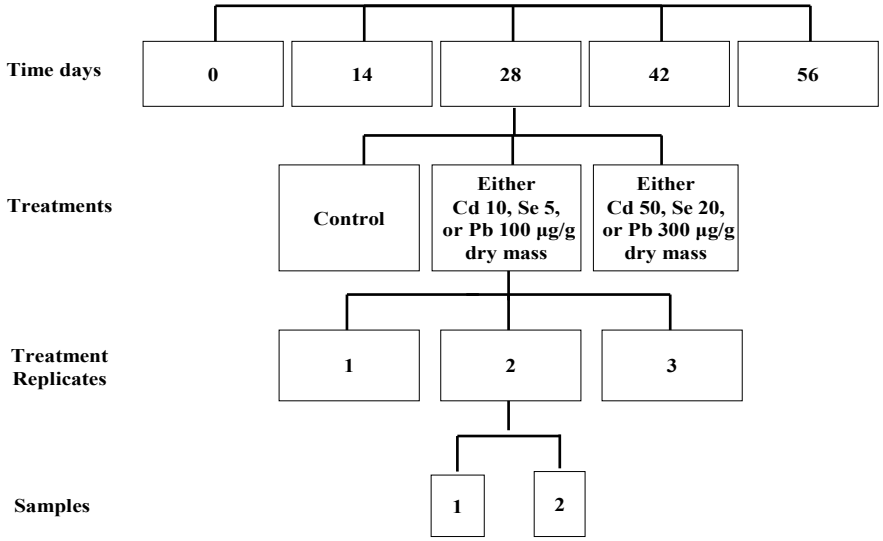


Figure 3.5: Experimental design for *A. trapezia* metal spiked sediment exposures

3.3.3 *Anadara trapezia* – Lake Macquarie Metal Contamination Gradient Sediments Study

The experimental design was as shown in (Figure 3.6). Procedures for conducting the exposures were the same as those used for the *A. trapezia* spiked sediment exposures with the following modifications. Sediment (3000 g wet wt.) collected from each of three sites in Cockle Creek and Cockle Bay (Figure 3.3) was placed in each of 3 replicate 12 L polystyrene aquariums per treatment. Twenty *A. trapezia* were added to each treatment aquarium.

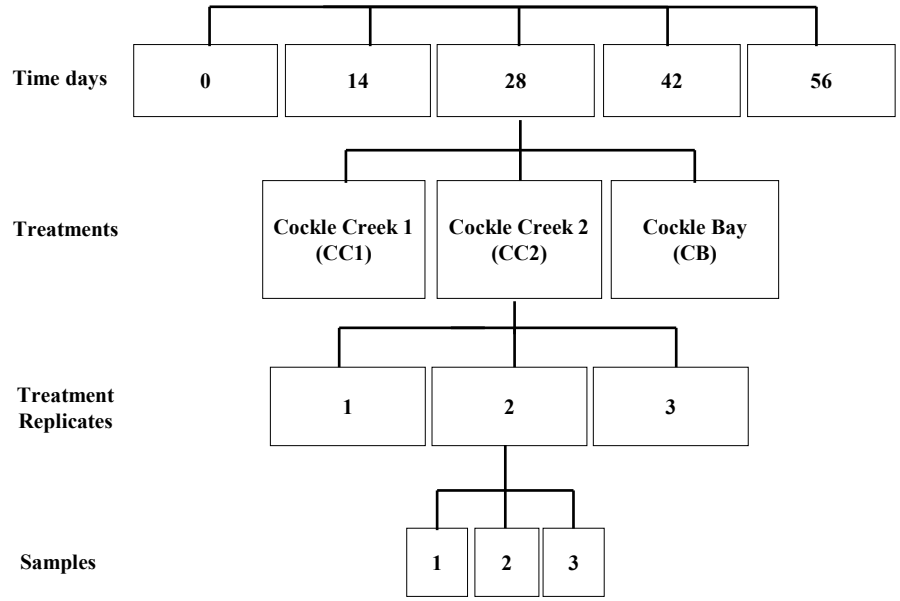


Figure 3.6 Experimental design for *A. trapezia* natural metal contaminated sediment exposures

3.4 Metal Bioaccumulation

3.4.1 *Tellina deltoidalis* - Metal Spiked Sediment Studies

To measure metal bioaccumulation over time, two organisms were removed from each treatment container at intervals of 3, 7, 14, 21 and 28 days. Six organisms from the acclimation tanks (day 0) were analysed at the start of the exposure experiment to give the background metal concentration. All organisms were placed in fresh seawater 28 ‰ with no sediment for 24 hours to allow depuration of ingested sediment particles. Shells were opened with a scalpel, tissue was removed and placed in acid washed polypropylene vials. Tissue was freeze-dried (Labconco, Freezone plus 6) and dry mass recorded. Freeze-dried tissue was reduced to a powder using stainless steel scissors and spatulas.

3.4.2 *Anadara trapezia* – Metal Spiked Sediment Studies

Procedures for bioaccumulation were the same as those used for the *T. deltoidalis* bioaccumulation with the following modifications. Two organisms were removed from each treatment container at intervals of 14, 28, 42 and 56 days. Five organisms from the acclimation tanks (day 0) were analysed at the start of the exposure experiment to give the background metal concentration. All organisms were placed in fresh seawater 30 ‰ with no sediment for 24 hours to allow depuration of ingested sediment particles.

Shells were opened with a scalpel and tissue was divided into 3 parts: haemolymph; gill / mantle and hepatopancreas and placed in acid washed polyethylene vials.

3.4.3 *Anadara trapezia* –Lake Macquarie Metal Contamination Gradient Sediments Study

Procedures for bioaccumulation were the same as those used for the *A. trapezia* spiked sediment bioaccumulation with the following modifications. Three organisms were removed from each treatment container at intervals of 14, 28, 42 and 56 days. Nine organisms from the acclimation tanks (day 0 - reference organisms) were analysed at the start of the exposure experiment to give the background metal concentration. Nine native *A. trapezia* collected from a contaminated site in Lake Macquarie, NSW (LM organisms) were analysed at the start of the experiment to allow a comparison with native versus laboratory exposed organisms to be made.

3.4.4 Bivalve Tissue Metal Analysis

3.4.4.1 *Digestion*

A procedure for microwave digestion from (Baldwin *et al.*, 1994) was used for the preparation of bivalve tissues for metal analysis. Approximately 0.07 g of ground tissue in 1 ml of nitric acid (AristaR BDH) was digested in polytetra-fluroacetate (PFA) closed 7 ml digestion vessels. Each 7 ml PFA vessel was capped, tightened to 2.3 N•m and placed in 120 ml screw-top pressure vessels containing 10 ml of deionised water (Sartorius-Arium 61316RO/611). Lids of the 120 ml vessels were tightened to 16.3 N•m. The 120 ml vessels were microwaved in sets of 12 in a 630 watt oven (CEM MDS-2000) for 2 minutes at 630 W, 2 minutes without power, and 45 minutes 315 W. Digested samples were cooled to room temperature in a fume cupboard to allow gases formed during digestion to vent. The digested samples were then transferred to acid washed polyethylene vials and diluted to 10 ml with deionised water. Details of CRM and metal determination are as previously reported (sections 3.2.3.3 & 3.2.3.4 above).

3.5 Subcellular Metal Distribution

3.5.1 Subcellular Fractionation

The subcellular tissue metal distribution was examined in whole organisms collected on day 28 *T. deltoidalis* and gill and hepatopancreas tissues of day 56 *A. trapezia* from the seven exposure experiments using a procedure adapted from (Sokolova *et al.*, 2005a; Wallace *et al.*, 2003). The dissected tissues were placed in polypropylene vials, snap frozen in liquid nitrogen and stored at -80^{o}C until processed. The tissue was thawed on ice and minced with scissors into small pieces. A sub sample, approximately 0.1 g wet wt., was taken for total tissue metal analysis. The remainder, approximately 0.5 g wet wt., was homogenised in Ca^{2+} / Mg^{2+} Free Saline buffer (CMFS, containing 20mM HEPES, 360mM NaCl, 12.5mM KCl and 5mM tetrasodium EDTA adjusted to pH 7.35 with NaOH). The tissues were homogenised on ice using an IKA® Labortechnick Ultra-turrax-T25 homogeniser equipped with an S25-UT dispersing tool at 9,500 $rpmin^{-1}$ (Janke & Kunkel). Homogenised tissue was subjected to differential centrifugation and tissue digestion procedures according to the following protocol (Figure 3.7), using an Eppendorf 5804R centrifuge and a Himac CP90WX preparative ultracentrifuge (Hitachi, Japan). Two organelle and one enzyme pellet, P3, P4 and P5 were grouped as biologically active metal (BAM) fractions while the granule pellet, P2 and final supernatant, S5 were grouped as biologically detoxified metal (BDM) fractions (Figure 3.7). The supernatant, S2 contained the cellular debris (Figure 3.7).

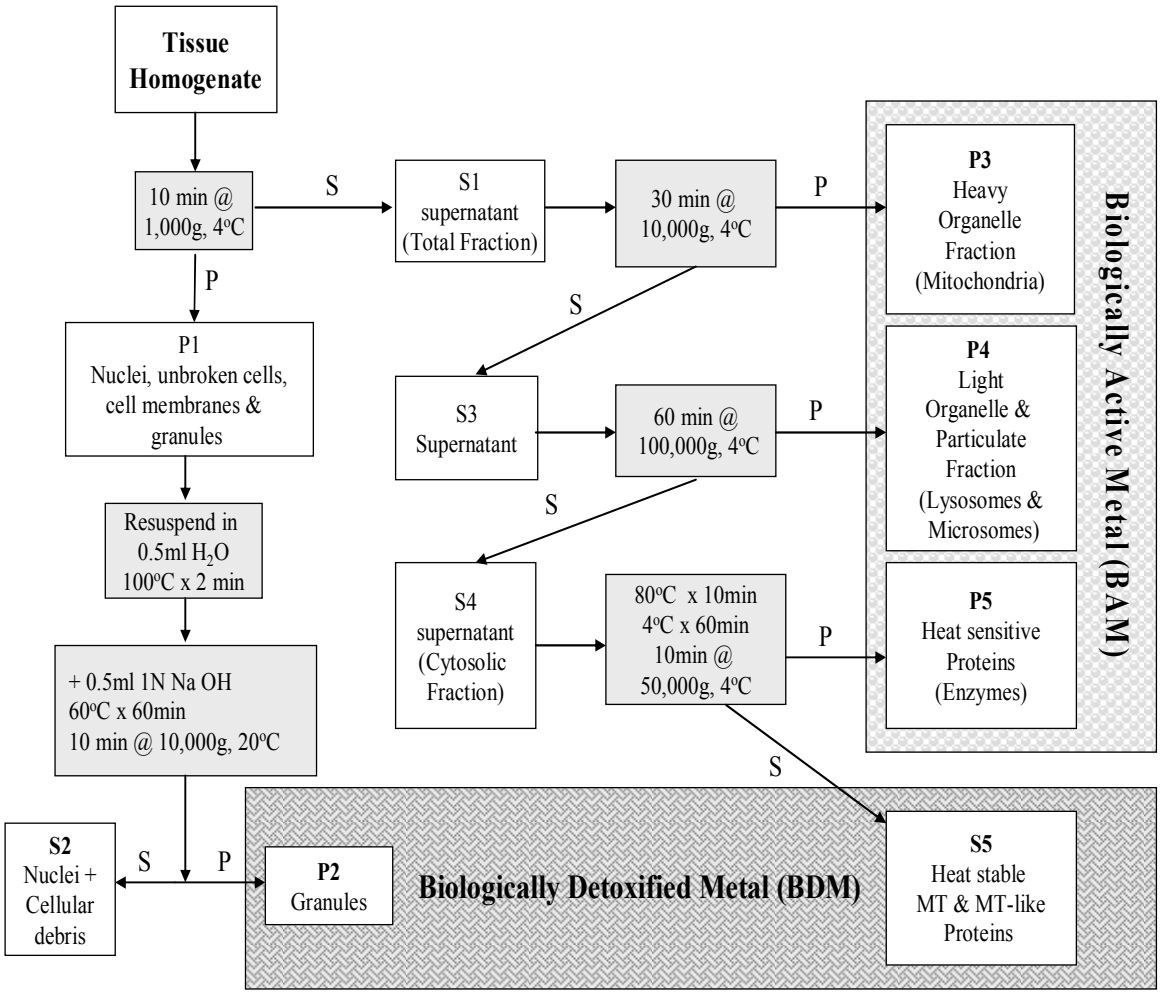


Figure 3.7: Procedure for subcellular fractionation of bivalve tissues by differential centrifugation. The shaded boxes show details of the centrifugation and digestion / heating steps used to obtain the specific fractions. The final fractions, four pellets P2, P3, P4 & P5 and two supernatants S2 & S5 are grouped as: biologically detoxified (BDM) P2 & S5; biologically active (BAM) P3, P4 & P5 metals or S2 which contains metal associated with dissolved tissues.

3.5.2 Metal Determination

A 40 µl aliquot of nitric acid (AristaR BDH) was added to each of the final fractions and the volume brought up to 0.5 ml with deionised water. Samples were placed in a water bath at 80^{o}C for 4 hours. Pyrex drip beads were placed on the top of each tube to limit evaporation. Samples were cooled to room temperature and diluted to 4 ml with deionised water containing internal standard. NIST CRM 1566a oyster tissue, buffer and acid blanks were digested and diluted in the same way as the samples and analysed along with them. Analysis of metals was as previously described (section 3.2.3.4).

3.5.3 Activity of Marker Enzymes

In order to determine mitochondrial and lysosomal content of the fractions obtained from the differential centrifugation the activity of enzymes specific for these organelles, Cytochrome c Oxidase and Acid Phosphatase respectively, were measured in each of the following fractions, P1, P3 and P4. Sub samples of each of these fractions were taken during processing, resuspended in 0.5 ml CMFS buffer and stored at -80^{o}C until analysis. Prior to analysis samples were defrosted on ice vortexed and centrifuged, in a 5804R centrifuge (Eppendorf, Germany), for 15 minutes at 10,000 x g at 4^{o}C. Supernatant was used for enzyme assays.

3.5.3.1 *Cytochrome c Oxidase Assay*

Cytochrome c Oxidase [EC 1.9.3.1.] is located in the inner mitochondrial membrane that divides the mitochondrial matrix from the intermembrane space and is commonly used as a marker for this membrane. The colourimetric assay used, (CYTOC-OX1 Sigma-Aldrich, USA), is based on observation of the decrease in absorbance of ferrocytochrome c caused by its oxidation to ferricytochrome c by cytochrome c oxidase. Assay buffer (50mM Tris-HCL, pH 7.0, containing 600 mM KCl) was added to a flat bottomed 96 well plate (Nunc). Sample was added (1:10 v/v) and the mixture shaken for 2 minutes. The reaction was started by the addition of a 0.22 mM ferrocytochrome c substrate solution (reduced with 0.1 M DTT) (1:20 v/v). The decrease in absorption was read immediately at 550 nm on a Benchmark Plus Microplate Spectrophotometer (Bio-Rad, USA) at room temperature (25^{o}C), using a kinetic program: 5 second delay; 10 second interval; 6 readings. The activity of the samples was calculated as units/ml. Samples were compared with the activity of a cytochrome c oxidase positive control and all absorbance reactions were corrected using a buffer blank. Unit definition: 1 unit will oxidise 1.0 µmole of ferrocytochrome c per minute pH 7.0 at 25^{o}C.

3.5.3.2 *Acid Phosphatase Assay*

Acid Phosphatase is one of the acid hydrolases that normally reside in lysosomes and is a classical marker for the identification of lysosomes in subcellular fractions. The colourimetric assay used (CS0740 Sigma-Aldrich, USA), determines the activity of acid phosphatase following reaction with 4-nitrophenyl phosphate in citrate. The reaction is stopped with NaOH and measured spectrophotomically. The 4-nitrophenyl phosphate in citrate buffer solution, 0.09 M pH 4.8, was added to a flat bottomed 96 well plate (Nunc). Sample was added (1:1 v/v) and the mixture shaken and incubated at 37^{o}C for 5-10 minutes. Reactions were stopped by adding 0.5 N NaOH (2:1 v/v) and the resulting coloured solution read at 405 nm on a microplate spectrophotometer. Samples were compared with the activity of an acid phosphatase positive control enzyme. The absorbance of a p-Nitrophenol standard solution, 10 mM, made up in 0.5 N NaOH stop solution (1:100 v/v) and that of a buffer blank were used to calculate sample activity in Units/ml. Unit definition: one unit of acid phosphatase will hydrolyse 1.0 µmole of 4-nitrophenyl phosphate per minute at pH 4.8 at 37^{o}C.

3.6 Enzymatic and Oxidative Damage Biomarkers

The following enzymatic and oxidative damage biomarkers were measured in whole organisms collected on day 28 *T. deltoidalis* and gill / mantle tissues of day 56 *A. trapezia* from the metal spiked sediment exposure experiments. Day 56 *A. trapezia* from the Lake Macquarie contaminated sediment exposure experiment, nine unexposed reference *A. trapezia* and six native *A. trapezia* collected from a contaminated site in Lake Macquarie, NSW (Figure 3.3) were only assayed for total antioxidant capacity and the oxidative damage biomarker lipid peroxidation (TBARS).

3.6.1 Reduced:Oxidised Glutathione Ratio & Glutathione Peroxidase

3.6.1.1 *Tissue Preparation*

Dissected tissue was homogenised in a 50 mM Tris-HCl buffer containing 5 mM EDTA and 1 mM DTT, pH 7.5 (1:5 w/v). The tissue was homogenised on ice using a Kontes motorised microcentrifuge pellet pestle (Sigma-Aldrich), sonicated for 15 seconds at 40 v and centrifuged, in a 5804R centrifuge (Eppendorf, Germany), at 10,000 x g for 15 minutes at 4^{o}C. Aliquots of 50 µl of supernatant for reduced glutathione (GSH) and glutathione peroxidise (GPx) were removed to separate vials and stored at -80^{o}C until analysis.

A 100 µl aliquot of supernatant was added to vials containing 10 µl of 1-methyl-2-vinyl-pyridium trifluoromethane sulfonate (M2VP) for oxidised glutathione (GSSG) and stored at -80^{o}C until analysis. The remaining tissue pellet and supernatant was reserved for protein analysis and stored at -80^{o}C until analysis.

3.6.1.2 *Reduced:Oxidised Glutathione Ratio Assay*

The ratio of reduced to oxidised glutathione (GSH:GSSG) was measured using an enzymatic method developed by (Tietze, 1969). The method uses Ellman's reagent (5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB)) which reacts with GSH to form a colour which is detected at 412 nm. In the Calbiochem®, GSH:GSSG ratio assay used, (371757-Merck, Germany) for GSSG analysis the GSH is first removed from the sample using the thiol scavenging agent (M2VP). The remaining GSSG is then reduced to GSH and determined by the reaction with Ellman's reagent. The rate of change in colour development over the course of the reaction is proportional to the GSH and GSSG concentrations.

Supernatants were thawed, vortexed and incubated at room temperature for 5 minutes. Samples were acidified by the addition of a 5 % solution of metaphosphoric acid (MPA) (290 µl GSSG; 350 µl GSH). Acidified samples were vortexed for 15 seconds and centrifuged at 1000 x g for 10 minutes at room temperature. A 50 µl aliquot of the MPA extract was added to 700 µl of sodium phosphate buffer for GSSG and to 350 µl for GSH. The diluted extracts were mixed at room temperature in 1 ml cuvettes with DTNB and glutathione reductase (GR) enzyme at (1:1:1 v/v/v). The reaction was initiated with ß - nicotinamide adenine dinucleotide phosphate (NADPH) and absorbance read at 412 nm for 3 minutes at intervals of 15 seconds on a Unicam Helios Gamma UV-Vis spectrophotometer (Spectronic, UK). Absorbance rates were calculated and GSH and GSSG concentrations calculated using a 6 point GSH calibration curve. A GSSG buffer blank was run for interference correction.

3.6.1.3 *Glutathione Peroxidase*

The activity of glutathione peroxidise (GPx) was measured using a Cayman Chemical assay (Sapphire Bioscience #703102). This assay measures GPx activity indirectly by a coupled reaction with glutathione reductase (GR). GSSG, produced on reduction of hydroperoxide by GPx, is recycled to its reduced state by GR and NADPH. The oxidation of NADPH to $NADP^{+}$ is accompanied by a decrease in absorbance at 340 nm. Under conditions where GPx activity is rate limiting, the rate of decrease in the A_{340} is directly proportional to the GPx activity in the sample.

Supernatants were thawed at room temperature. Assay buffer (50 mM Tris-HCl, pH 7.6, 5 mM EDTA), 100 µl, was added to sample wells of a flat bottomed 96 well plate with 50 µl of a co-substrate mixture (NADPH, glutathione and GR) (2:1 v/v). Aliquots of 20 µl of sample were added and the reaction initiated by the addition of 20 µl of cumene hydroperoxide. The plate was shaken briefly and the decrease in absorbance read at 340 nm for 5 minutes at intervals of 30 seconds on a microplate reader at 25^{o}C. Rates were calculated and samples were compared with a bovine erythrocyte GPx positive control. Buffer blanks run with the samples were used to correct for interferences and GPx activity was calculated using the NADPH extinction coefficient, adjusted for the pathlength of the solution, of 0.00373 μM^{-1}. One unit is defined as the amount of enzyme that will cause oxidation of 1.0 nmol of NADPH to $NADP^{+}$ per minute at 25^{o}C.

3.6.2 Total Antioxidant Capacity & Lipid Peroxidation

3.6.2.1 *Tissue Preparation*

Dissected tissue was homogenised in a 5 mM potassium phosphate buffer containing 0.9 % sodium chloride and 0.1 % glucose, pH 7.4 (1:5 w/v). The tissue was homogenised on ice using a motorised microcentrifuge pellet pestle, sonicated for 15 seconds at 40 v and centrifuged, in a 5804R centrifuge (Eppendorf, Germany), at 10,000 x g for 15 minutes at 4^{o}C. A 50 µl aliquot of supernatant for total antioxidant capacity (TAOC) and 100 µl for lipid peroxidation (TBARS) was removed to a separate vial and stored at -80^{o}C until analysis. The remaining tissue pellet and supernatant was reserved for protein analysis and stored at -80^{o}C until analysis.

3.6.2.2 *Total Antioxidant Capacity*

The total antioxidant capacity (TAOC) of tissue lysates was measured using a Cayman Chemical assay (Sapphire Bioscience #709001). This assay is based on the ability of the antioxidants in the samples to inhibit the oxidation of 2,2'-Azino-di-[3-ethylbenzthiazoline sulphonate] (ABTS®) to $ABTS^{®\bullet+}$ by metmyoglobin. The amount of $ABTS^{®\bullet+}$ produced is measured at 750 nm. The suppression of absorbance under the reaction conditions is proportional to the concentration of the combined antioxidants in the sample.

The samples were thawed at room temperature and 10 µl of each pipetted into a 96 well plate with 10 µl of metmyoglobin and 150 µl of ABTS®. Reactions were initiated with 40 µl of a 441 µM solution of hydrogen peroxide. The plate was shaken for 5 minutes at 25^{o}C and absorbance read at 750 nm on a microplate spectrophotometer.

The capacity of the antioxidants in the sample to prevent ABTS® oxidation was compared to Trolox, a water-soluble tocopherol analogue. Sample antioxidant capacity is quantified as millimolar Trolox equivalents calculated from a 7 point Trolox standard curve.

3.6.2.3 *Lipid Peroxidation*

Lipid peroxidation was determined by measuring the thiobarbituric acid reactive substances (TBARS) present in the tissue lysates. The Oxitek® TBARS assay (Zepometrix Corporation, #0801192) used is based on the specificity of malondialdehyde (MDA), which is a by-product of lipid peroxidation, for TBARS. The MDA present in the sample forms a 1:2 adduct with the thiobarbituric acid in the reaction reagent producing a colour which can be read spectrophotomically at 532 nm.

All reagents were at room temperature. Tissue lysates were thawed at room temperature and 100 µl of each was pipetted into labelled glass test tubes. One hundred microlitres of sodium dodecyl sulphate solution was added and tubes swirled to mix. A 2.5 ml aliquot of TBA / Buffer Reagent, (0.5 g thiobarbituric acid dissolved in 50 ml acetic acid and 50 ml sodium hydroxide), was added to each tube. Glass marbles were placed on the top of each tube to prevent evaporation and samples were incubated in a water bath at 95^{o}C for 60 minutes. Samples were cooled for 10 minutes in an ice bath and centrifuged at 3000 rpm for 15 minutes at room temperature. Supernatant absorbance's were read at 532 nm on a LKB Novaspec II Biochrom UV-Vis Spectrophotometer (Pharmacia, Sweden) zeroed against a buffer blank. Sample absorbance's were compared to those of an MDA standard prepared from a 100 nmol/ml Malondialdehyde Bis (dimethyl acetal) made up in buffer. Sample TBARS were calculated as MDA nmol/ml equivalents from a 5 point MDA standard curve.

3.7 Protein

All tissue lysates used for enzymatic assays were analysed for protein concentration and enzyme concentration / activity is expressed as mg^{-1} of protein. The FluoroProfile™ (Sigma #FP0010, Sigma-Aldrich, USA) protein assay used is a fluorescent assay based on Epiccoconone, a biodegradable natural product. The fluorescence intensity produced is directly proportional to the total protein concentration of the sample within an excitation and emission maxima of 510 nm and 620 nm respectively. The reaction is stable at room temperature for 6 hours.

Tissue lysates were thawed at room temperature and diluted at 1:100 v/v with deionised water to reduce the concentration of interfering compounds in the buffers.

A 50 µl volume of each of the diluted samples was pipetted into a black F96 MicroWell™ plate Nunc™ (Thermo Fisher Scientific, Denmark) and an equal volume of the fluorescent reagent added. Fluorescence was read at 485 excitation and 620 emission, on a Luminoskan Ascent Fluorescence Plate Reader (Thermo Electrical Corp., USA). Protein concentrations were calculated as ng/ml from an 8 point bovine serum (BSA) calibration curve. BSA standards were made up in buffer of the same type at the same dilution as those of the samples.

3.8 Cellular and Genotoxic Biomarkers

The cellular biomarker lysosomal stability and and genotoxic biomarker micronuclei frequency were measured in whole tissue of *T. deltoidalis* collected on day 28 from the metal spiked sediment experiments. Lysosomal stability was measured in the hepatopancreas and micronuclei frequency in gill tissues of *A. trapezia* collected on day 56 from the metal spiked and the Lake Macquarie metal contaminated sediment experiments, and in nine unexposed reference *A. trapezia*. Six native *A. trapezia* collected from a contaminated site in Lake Macquarie, NSW were analysed for lysosomal stability only.

3.8.1 Lysosomal Stability

Lysosomal stability was assessed using a method developed by (Ringwood *et al.*, 2003) for oysters. The assay uses neutral red (NR) dye retention to asses the integrity of the lysosomal membrane. Cells incubated in neutral red accumulate the lipophilic dye in the lysosomes. Healthy cells retain the dye in the lysosomes (Figure 3.8a) whereas in cells with damaged lysosomal membranes it leaks out into the cytoplasm (Figure 3.8b).

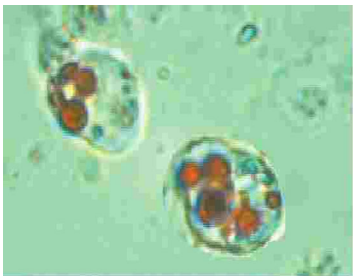
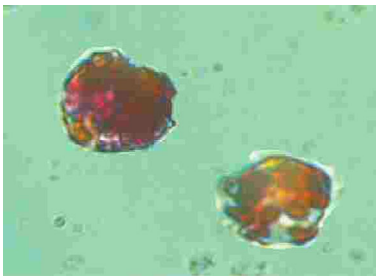
a)  b) 

Figure 3.8: *T. deltoidalis* cells scored as; a) Stable - dye present in the lysosome; b) Destabilised - dye present in the cytosol. Magnification x100.

Fresh CMFS buffer at pH 7.35 - 7.4 and salinity 25 – 35 ‰ was filtered through a 0.45 µm screen, oxygenated by bubbling and kept cool on ice . Dissected whole tissue of *T. deltoidalis* and hepatopancreas tissue of *A. trapezia* was rinsed in CMFS buffer minced into small pieces, and rinsed again with buffer. Samples were then placed into individual wells of a 24-well cell culture plate Nunc™ (Thermo Fisher Scientific, Denmark) with 600 µl of CMFS buffer. The plate was covered, placed on ice in a plastic container and shaken on a reciprocating shaker at 100 rpm for 20 minutes. Trypsin (Sigma, T4799), 325 µl at 1 mg/ml in CMFS buffer, was added to each sample well and the plate shaken for a further 20 minutes. Samples were then transferred to a 20 µm filter apparatus consisting of a 1.5 ml centrifuge tube a square of 20 µm nylon filter and a cut off 1 ml pipette tip. Samples were centrifuged at 250 - 500 g at 15^{o}C for 5 - 15 minutes. Filters with remaining tissue debris were removed and the supernatant discarded. One to two rinses were performed by resuspending pellets in 0.5 ml CMFS and centrifuging at 250 g. A neutral red (Sigma, N-7005) stock solution was made up in dimethyl sulphoxide (DMSO) (BDH 10323) at a concentration of 4 mg/ml and used to prepare the NR working solution, 20 µl of the stock in 2 ml of CMFS (final NR concentration 0.04 mg/ml). NR solutions were made up fresh prior to the assay and kept at room temperature in the dark. The final pellet was resuspended in 50 µl of CMFS buffer and an equal volume of NR working solution added. Cells in the NR solution were incubated in a dark humidified chamber for 60 minutes. A drop of the cell suspension was placed on a microscope slide and a cover-slip added. One hundred cells per slide were counted using a light microscope with 40 x lens and scored as stable or unstable. Two slides per sample were counted and the percentage of stable and unstable cells of each individual sample calculated.

3.8.2 Micronuclei Frequency

The micronuclei assay used was based on a technique developed on the mussel *Mytilus galloprovincialis* (Gorbi *et al.*, 2008). The assay uses DAPI (4',6-diamidine-2'-phenylindole dihydrochloride), a fluorescent dye specific for nucleic material, to stain the nuclei. Micronuclei are defined as small round structures less than one third the diameter and in the same optical plan as the main nucleus, with a boundary distinct from the nuclear boundary (Figure 3.9).

Tissue preparation for the collection of cells was the same as that used for the neutral red retention assay. Following the rinse step the pellet was resuspended in Carnoy's solution (methanol:acetic acid 3:1) and stored at 4^{o}C until counted.

A drop of the cell suspension was placed on a slide and air dried. A DAPI (# 32670 Sigma, USA) stock solution was made up in water at a concentration of 1 mg/ml and a working solution prepared fresh prior to analysis, 10 µl of stock in 100 ml PBS. A drop of the DAPI working solution was added to each slide and a cover-slip added. Slides were incubated in the dark for 5 minutes and observed under an inverted epifluorescent microscope (Nikon, Eclipse TE 300) with the appropriate filter for DAPI, excitation wavelength 350 nm magnification x 40. Two slides per sample were counted with 1000 cells per slide scored as micronuclei present or absent and the percentage of cells with micronuclei calculated for each sample.

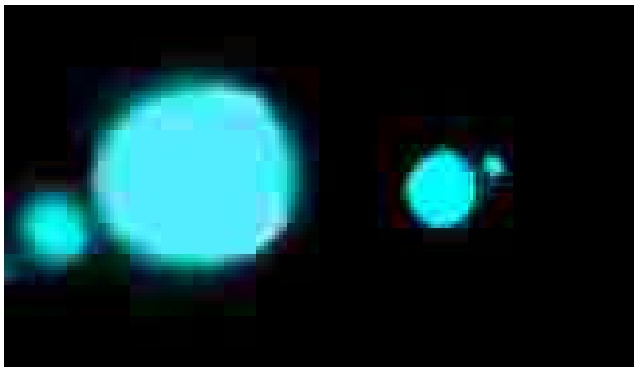


Figure 3.9: Examples of DAPI stained nuclei with associated micronuclei from gill tissue of *Anadara trapezia*. Magnification x 1000.

3.9 Physiological Biomarker

3.9.1 Condition Index

Condition index (CI) was measured in unexposed reference *A. trapezia*, LM native *A. trapezia* and at fortnightly intervals for the CC1 & 2 and CB metal contamination gradient exposed *A. trapezia.* The CI was calculated (CI = dry tissue mass (g) x 100/dry valve mass (g)) modified from (Duquesne *et al.*, 2004). Tissue was freeze dried (Labconco, Freezone plus 6) and valves were oven dried at 60 ^{0}C for 24 hours.

3.10 Data Analysis

A Mixed Linear Model analysis of variance (ANOVA) (SPSS *v* 14.0) was used to simultaneously analyse the effects of time (day) and treatment/site (metal exposure concentration) on the measurement variables cadmium, lead, selenium, copper or zinc in whole organism tissue of *T. deltoidalis* and *A. trapezia* (Quinn and Keough, 2002).

The calculations were performed on μg/g dry mass tissue concentrations so mass could be eliminated from the calculation. Multiple pair-wise comparisons were also performed using this model to determine where the significant differences lay between and within the treatments/sites on each of the analysis days. As a high number of tests were performed a Bonferroni correction for experiment wise error rate was applied to all calculations to determine the significance level (Sokal and Rohlf, 1995). A preliminary investigation of the data was done to examine the distribution of residuals using a non-parametric Kolmogorov-Smirnov Z Test. Where the population distribution was found to be non-normal a $\log_{10}$ transformation was performed prior to analysis. The individual tissues, of *A. trapezia* were simultaneously analysed for the effects of time (day), treatment/site (metal exposure concentration) and tissue (gill, hepatopancreas and haemolymph) on the measurement variables cadmium, lead, selenium, copper or zinc using a Repeated Measures Mixed Linear Model ANOVA (SPSS *v* 14.0). After trialling a range of repeated covariance error structures, compound symmetry heterogeneous was selected, as it gave the lowest AIC value. Pair-wise comparisons with Bonferroni adjustment were used to determine where the significant differences lay between and within tissues at each collection time. Where a Kolmogorov-Smirnov Z Test indicated that the population distribution was non-normal a $\log_{10}$ transformation was performed prior to analysis. A Mixed Linear Model ANOVA (SPSS *v* 14.0) was used analyse the effect of treatment/site (metal exposure concentration) on the effect measurement variables total antioxidant capacity (TAOC), glutathione peroxidase, total glutathione, reduced and oxidised glutathione ratio, thiobarbituric acid reactive substances (TBARS), lysosomal stability, micronuclei frequency and condition index. Where population distribution was shown to be non-normal $\log_{10}$ transformations were applied. Arsine transformation was used for non-normal lysosomal stability and micronuclei frequency data as these values were percentages. Tank(treatment) was run as a covariance parameter in all analysis to determine whether the tank replicates were contributing to the treatment variance, in all cases the variance estimate was found to be ≤ 0.01. Regressions of sediment metal and mean tissue metal concentrations and means of effects variables TAOC, TBARS, lysosomal stability and micronuclei frequency were calculated using EXCEL™ *v* 2003.

4 *Tellina deltoidalis* Metal Spiked Sediment Studies

4.1 Introduction

Sediment dwelling bivalves are commonly used as biomonitors of metals as they are hardy, representative of the area of interest and accumulate bioavailable metals (Phillips, 1990). In order to acquire sufficient essential metals from low ambient concentrations aquatic organisms have evolved highly efficient uptake mechanisms, coupled with detoxification storage and excretion strategies varying from regulation through to no-regulation of metal (Phillips and Rainbow, 1989). Metals may be accumulated by sediment dwelling deposit feeding bivalves from the water, directly ingested from sediments or from food (Luoma and Rainbow, 2005). The route of uptake may influence the organisms metal handling and therefore the toxicity (Rainbow, 2007). This study looked at the bioaccumulation of cadmium, lead and selenium by the deposit feeding bivalve *T. deltoidalis* exposed to single metal spiked sediments, at environmentally realistic concentrations, at intervals of 3, 7, 14, 21 and 28 days. The metal handling strategy and cellular responses to increased metal concentration were examined after 28 days exposure.

4.2 Aim

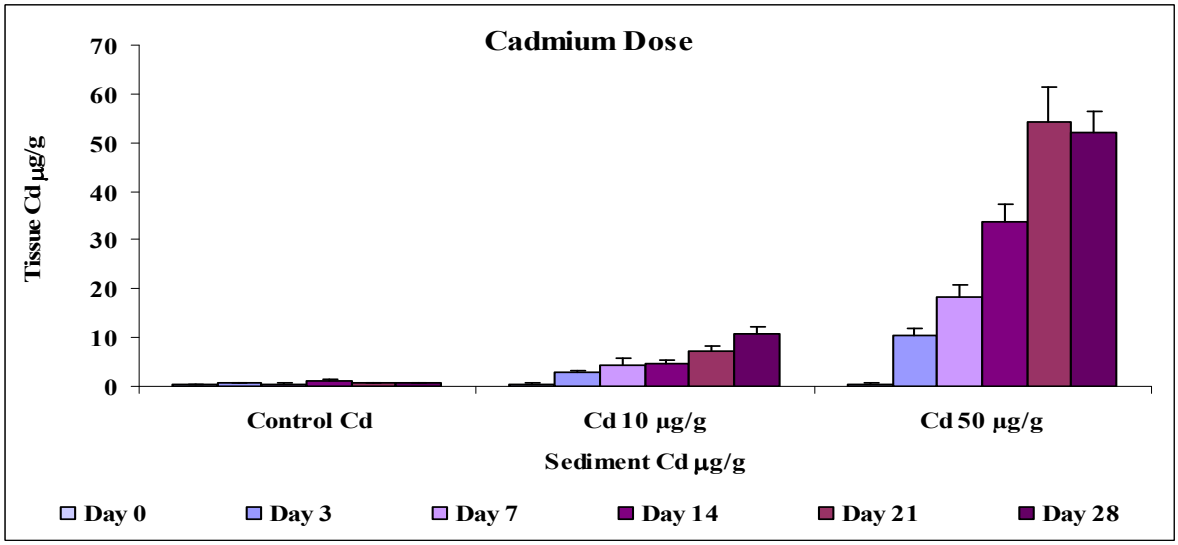
The purpose of these studies was to examine the exposure - dose - response relationship to cadmium, lead and selenium in *T. deltoidalis* using 28 day sediment bioaccumulation tests (Ingersoll *et al.*, 2000), to develop useful biomarkers of effect, with a view to determining whether they would be a suitable organism to collect from the field and use in laboratory sediment metal toxicity tests. The studies examine the pattern of metal uptake, subcellular tissue metal distribution and the associated effects of the individual metals. The uptake and effects of the different metals are not compared except in general terms as in the "real world" it is acknowledged that synergistic or antagonistic interactions will occur and other physicochemical variables, which have not been replicated here, will be important.

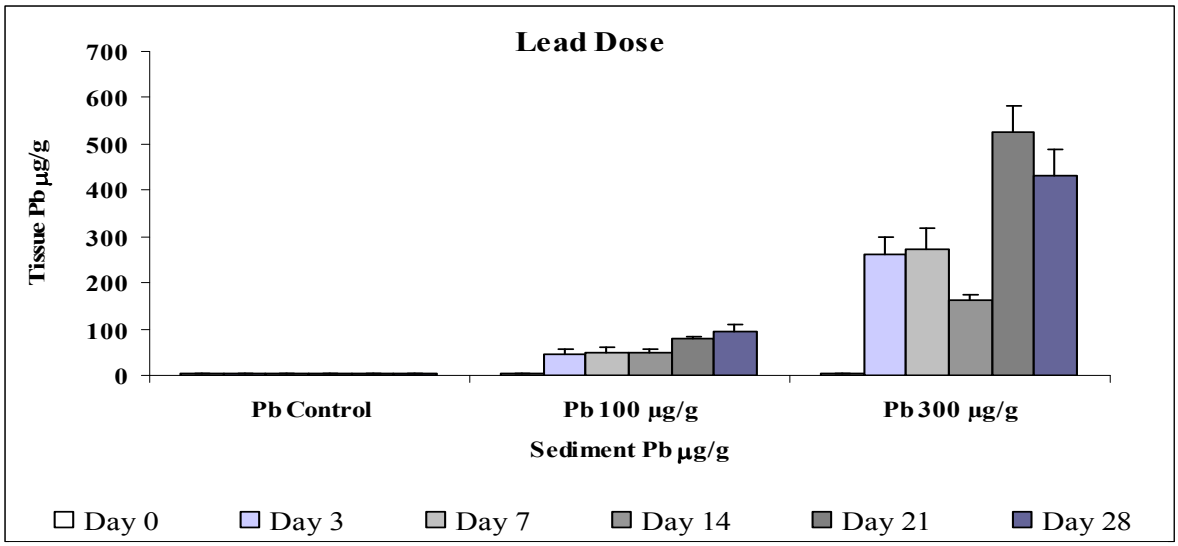
4.3 Results

4.3.1 Metal Accumulation

4.3.1.1 *Cadmium*

Tellina deltoidalis accumulated cadmium from both the 10 and 50 µg/g spiked sediments (Figure 4.1). ANOVA showed that the factors, time and sediment cadmium concentration, were both highly significant in cadmium accumulation and there was a significant interaction between time and sediment cadmium concentration (Appendix 1.1). Cadmium tissue concentrations were in the order 50 µg/g > 10 µg/g > control for each analysis time (Figure 4.1). Bonferroni pair-wise comparisons showed that the cadmium tissue concentrations of the control organisms did not differ significantly from day 0, unexposed organisms (Appendix 1.2). Organisms from both the cadmium treatments differed significantly from the unexposed and control organisms and from each other at all collection times (Appendix 1.2 & 1.3). At day 28, cadmium tissue concentrations in both treatments were equal to that of the cadmium spiked sediment concentrations (Figure 4.1). The 10 µg/g treatment organisms had the highest cadmium concentration at day 28, while the 50 µg/g treatment organisms had the highest cadmium concentration at day 21 and then a decrease to day 28 (Figure 4.1). The regression between cadmium sediment concentration and organism tissue cadmium concentration after 28 days shows a significant positive linear relationship (Figure 4.2). Analysis of the within treatment differences between collection days showed that the gradual pattern of accumulation of the 10 µg/g cadmium exposed organisms (Figure 4.1) resulted in a significant increase in tissue cadmium every 14 days (Appendix 1.4). The 50 µg/g cadmium exposed organisms had a significant cadmium increase in the first 3 days and then the same pattern of significant accumulation at 14 day intervals up to day 21 (Appendix 1.4). The decrease in concentration from day 21 to day 28 (Figure 4.1) was not significant (Appendix 1.4). There was no significant difference over time in the cadmium concentrations of the control organisms (Appendix 1.4).





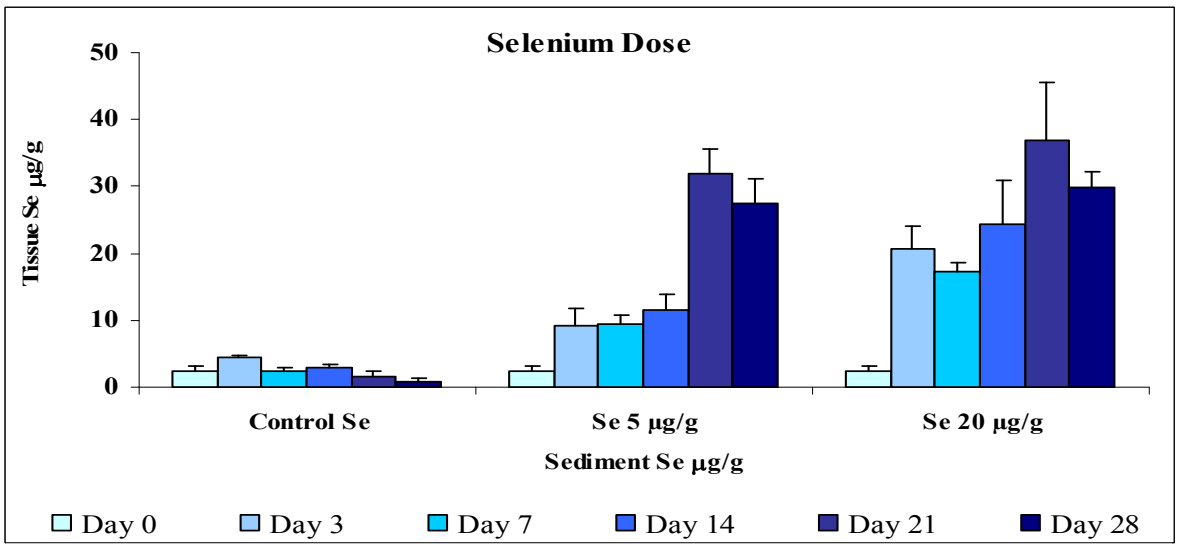


Figure 4.1: *T. deltoidalis* tissue metal concentrations (µg/g dry mass) of cadmium, lead and selenium, with standard error, at 3, 7, 14, 21, and 28 day intervals exposed to metal spiked sediments of Cd 10, 50; Pb 100, 300;and Se 5, 20 µg/g dry mass. Accumulation in metal exposed organisms is compared to that of organisms from clean sediments (control). Mean ± SE, n = 12. Day 0 are unexposed organisms n = 6.

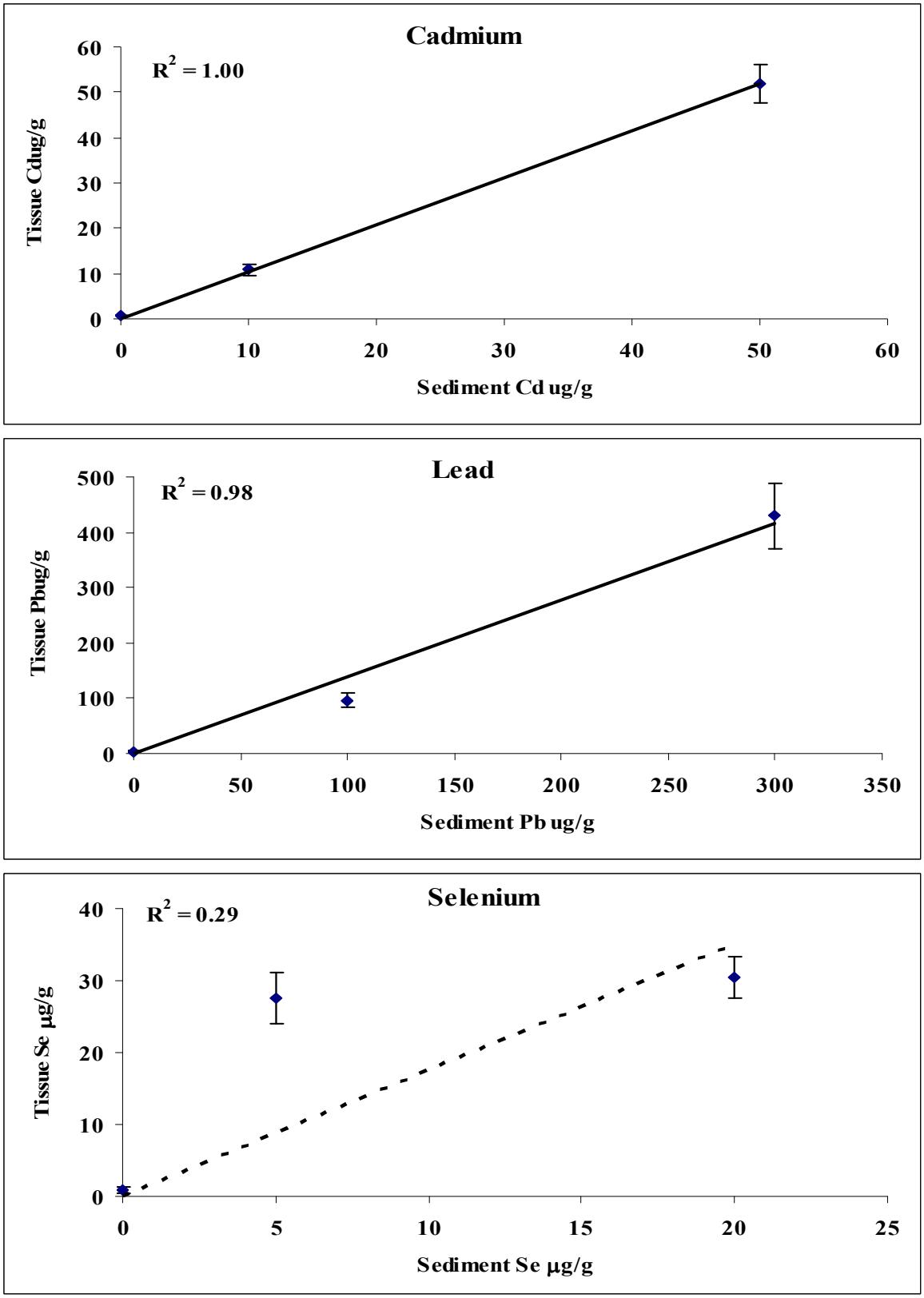


Figure 4.2: Regressions of *T. deltoidalis* tissue metal concentrations after 28 days exposure against sediment exposure concentrations of Cd 10, 50; Pb 100, 300; Se 5, 20 µg/g dry mass and clean sediments (control). Dotted regression for selenium is to indicate there was no significant relationship. Mean ± SE, n = 12.

4.3.1.2 *Lead*

Tellina deltoidalis accumulated lead from both the 100 and 300 µg/g spiked sediments (Figure 4.1). ANOVA showed that the factors time and sediment lead concentration were both highly significant in lead accumulation and there was no significant interaction between time and sediment lead concentration (Appendix 1.1). Lead tissue concentrations were 300 µg/g > 100 µg/g > control for each analysis time (Figure 4.1). Bonferroni pair-wise comparisons showed that the lead tissue concentrations of the control organisms did not differ significantly from day 0 unexposed organisms (Appendix 1.2). Organisms from both the lead treatments differed significantly from the unexposed and control organisms and from each other at all collection times (Appendix 1.2 & 1.3). At day 28, lead tissue concentrations in the 100 µg/g treatment were equal to that of the lead spiked sediment concentration, while the 300 µg/g treatment organisms had a higher lead tissue concentration than the lead spiked sediment (Figure 4.1). The regression between lead sediment concentration and organism tissue lead concentration after 28 days exposure shows a significant positive polynomial relationship (Figure 4.2). As sediment lead concentration increased from 100 µg/g to 300 µg/g the tissue lead accumulation was not proportional (Figure 4.2). The 100 µg/g lead exposed organisms had the highest lead concentration at day 28. The 300 µg/g treatment organisms had a rapid accumulation to day 3 which then decreased from day 7 to 14, a further uptake then occurred to the highest lead concentration at day 21 with a further decrease to day 28. Pair-wise analysis of the within treatment differences between collection days showed that the majority of the lead accumulation in the 100 µg/g exposed organisms occurred in the first 3 days with all days differing significantly from the day 0 unexposed organisms but not from each other (Appendix 1.4). The pattern is similar for the 300 µg/g exposed organisms, however, the decrease in lead concentration at day 14 and rapid increase to the highest concentration at day 21 (Figure 4.1), resulted in significant differences in lead between days 14 and 21 and 14 and 28 (Appendix 1.4). The decrease in lead concentration between day 21 and day 28 (Figure 4.1), was not significant (Appendix 1.4). There was no significant difference over time in the lead concentrations of the control organisms (Appendix 1.4).

4.3.1.3 *Selenium*

The tissue selenium accumulation pattern of *T. deltoidalis* was similar in the 5 and 20 µg/g selenium spiked sediment treatments (Figure 4.1). ANOVA showed that sediment selenium concentration was a highly significant factor in selenium accumulation while time (collection day) was not and there was a significant interaction between time and sediment selenium concentration (Appendix 1.1). Selenium tissue concentrations were in the order 20 µg/g > 5 µg/g > control for each analysis time (Figure 4.1). Bonferroni pair-wise comparisons showed that the selenium tissue concentrations of the control organisms did not differ significantly from day 0, unexposed organisms (Appendix 1.2). Organisms from both the selenium treatments differed significantly from the unexposed and control organisms but not from each other (Appendix 1.2). The pair-wise comparisons between treatments for each collection day shows that the tissue concentrations of the 5 µg/g selenium exposed organisms did not differ significantly from the control at day 3 but did at all other times. The 20 µg/g selenium exposed organisms differed significantly from the control at all days and the two selenium treatments were never significantly different from each other (Appendix 1.3). The highest selenium concentrations for both treatments were at day 21 with a subsequent decrease to day 28. At day 28, selenium tissue concentrations in the both treatments were higher than the selenium spiked sediment with 5 µg/g treatment organisms having 5 times and the 20 µg/g 1.5 times the sediment selenium concentration (Figure 4.1). The regression between selenium sediment concentration and organism tissue selenium concentration after 28 days was not significant (Figure 4.2). As sediment selenium concentration increased from 5 µg/g to 20 µg/g the tissue selenium accumulation was not proportional (Figure 4.2). There was a significant within treatment difference in the selenium concentrations of the control organisms between days 3 and 28 and 14 and 28 (Appendix 1.4). The graph indicates that this was due to the day 28 organisms having a lower selenium concentration than the organisms sampled on the other days (Figure 4.1). A within treatment pair-wise analysis of the two selenium treatments shows that the 5 µg/g selenium exposed organisms had a significant increase in tissue selenium concentration about every 14 days while the majority of the selenium accumulation in the 20 µg/g selenium exposed organisms occurred in the first 3 days with all days differing significantly from the day 0 unexposed organisms but not from each other (Appendix 1.4).

4.3.2 Subcellular Tissue Metal Distribution

4.3.2.1 *Cadmium*

Approximately half of the total cadmium was recovered in the fractions (Table 4.1). Of the metal recovered in the fractions around one half was in the biologically detoxified metal (BDM) fractions for all treatments (Table 4.1). The percentage of metal recovered in the biologically active metal (BAM) fractions of each of the 10 and 50 µg/g cadmium treatments was less than half that of the control, however, despite this the total cadmium burden (µg) within these fractions was 100 and 280 times respectively, greater in the cadmium exposed organisms (Table 4.1). Of the BDM, the percentage of cadmium in the MTLP fraction was greater than that of the MRG fraction with a similar distribution pattern for the control and 10 µg/g cadmium exposed organisms (Figure 4.3; Table 4.2). The BDM of the 50 µg/g cadmium exposed organisms had a greater percentage of metal in the MRG fraction than the MTLP fraction compared to the other treatments (Figure 4.3; Table 4.2). The BAM of the control organisms had the majority of the cadmium equally distributed between the heat sensitive proteins (HSP) and lysosome/microsome fractions with very little (5 %) in the mitochondrial fraction (Figure 4.3; Table 4.2). The 10 and 50 µg/g cadmium exposed organisms had 59 and 72% respectively of the recovered BAM in the mitochondrial fraction with the remainder fairly equally distributed between the lysosome+microsome and HSP fractions (Figure 4.3; Table 4.2).

Table 4.1: Cadmium, lead and selenium concentrations (µg wet mass) in whole tissue and subcellular fractions with the percentage of total metal recovered in all fractions of *T. deltoidalis* after 28 days exposure to metal spiked sediments. Metal subcellular concentrations (µg wet mass) and percentage distribution of total recovered metal fractions are grouped as debris and biologically active and biologically detoxified metal (Figure 3.7), mean ± SD, n = 2.

	Sediment Treatments		
	Cd control	Cd 10 µg/g	Cd 50 µg/g
Total Tissue Cadmium (µg)	**0.04 ± 0.01**	**5.9 ± 0.02**	**26 ± 7**
Total Recovered Cadmium (µg)	**0.01 ± 0**	**3 ± 0.4**	**14 ± 2**
Proportion of total recovered in fractions (%)	38 ± 8	50 ± 7	56 ± 21
Cadmium Subcellular Distribution			
Nuclei + Cellular debris (µg)	**0.002**	**0.8 ± 0.1**	**5.2 ± 0.9**
Nuclei + Cellular debris (%)	14 ± 3	28 ± 0.5	37 ± 2
Biologically Active Metal (BAM) (µg)	**0.005**	**0.5 ± 0.1**	**1.4 ± 0.2**
Biologically Active Metal (%)	36 ± 12	16 ± 5	11 ± 2.4
Biologically Detoxified Metal (BDM) (µg)	**0.007**	**1.6 ± 0.2**	**7.2 ± 1**
Biologically Detoxified Metal (%)	50 ± 5	56 ± 2	52 ± 3
	Pb control	Pb 100 µg/g	Pb 300 µg/g
Total Tissue Lead (µg)	**0.3 ± 0.08**	**20 ± 3**	**46 ± 6**
Total Recovered Lead (µg)	**0.1 ± 0.04**	**10 ± 4**	**24 ± 1**
Proportion of total recovered in fractions (%)	40 ± 4	48 ± 13	53 ± 4
Lead Subcellular Distribution			
Nuclei + Cellular debris (µg)	**0.02 ± 0.01**	**2 ± 0.8**	**5 ± 0.2**
Nuclei + Cellular debris (%)	13 ± 3	22 ± 1	21 ± 1
Biologically Active Metal (BAM) (µg)	**0.02 ± 0.01**	**0.6 ± 0.3**	**2.3 ± 0.03**
Biologically Active Metal (%)	17 ± 3	6 ± 2.6	10 ± 0.4
Biologically Detoxified Metal (BDM) (µg)	**0.1 ± 0.02**	**6.8 ± 2.7**	**17 ± 1**
Biologically Detoxified Metal (%)	70 ± 9	72 ± 8	69 ± 3
	Se control	Se 5 µg/g	Se20 µg/g
Total Tissue Selenium (µg)	**0.9 ± 0.1**	**1.8 ± 0.5**	**3.4 ± 0.7**
Total Recovered Selenium (µg)	**0.2 ± 0.03**	**0.9 ± 0.3**	**1.6 ± 0.1**
Proportion of total recovered in fractions (%)	20 ± 1	51 ± 1	47 ± 8
Selenium Subcellular Distribution			
Nuclei + Cellular debris (µg)	**0.07 ± 0.01**	**0.4 ± 0.1**	**0.7 ± 0.1**
Nuclei + Cellular debris (%)	36 ± 1	44 ± 5	48 ± 4
Biologically Active Metal (BAM) (µg)	**0.1 ± 0.01**	**0.3 ± 0.1**	**0.5 ± 0.05**
Biologically Active Metal (%)	53 ± 4	36 ± 3	33 ± 7
Biologically Detoxified Metal (BDM) (µg)	**0.02**	**0.2 ± 0.1**	**0.3 ± 0.03**
Biologically Detoxified Metal (%)	11 ± 1	20 ± 4	19 ± 6

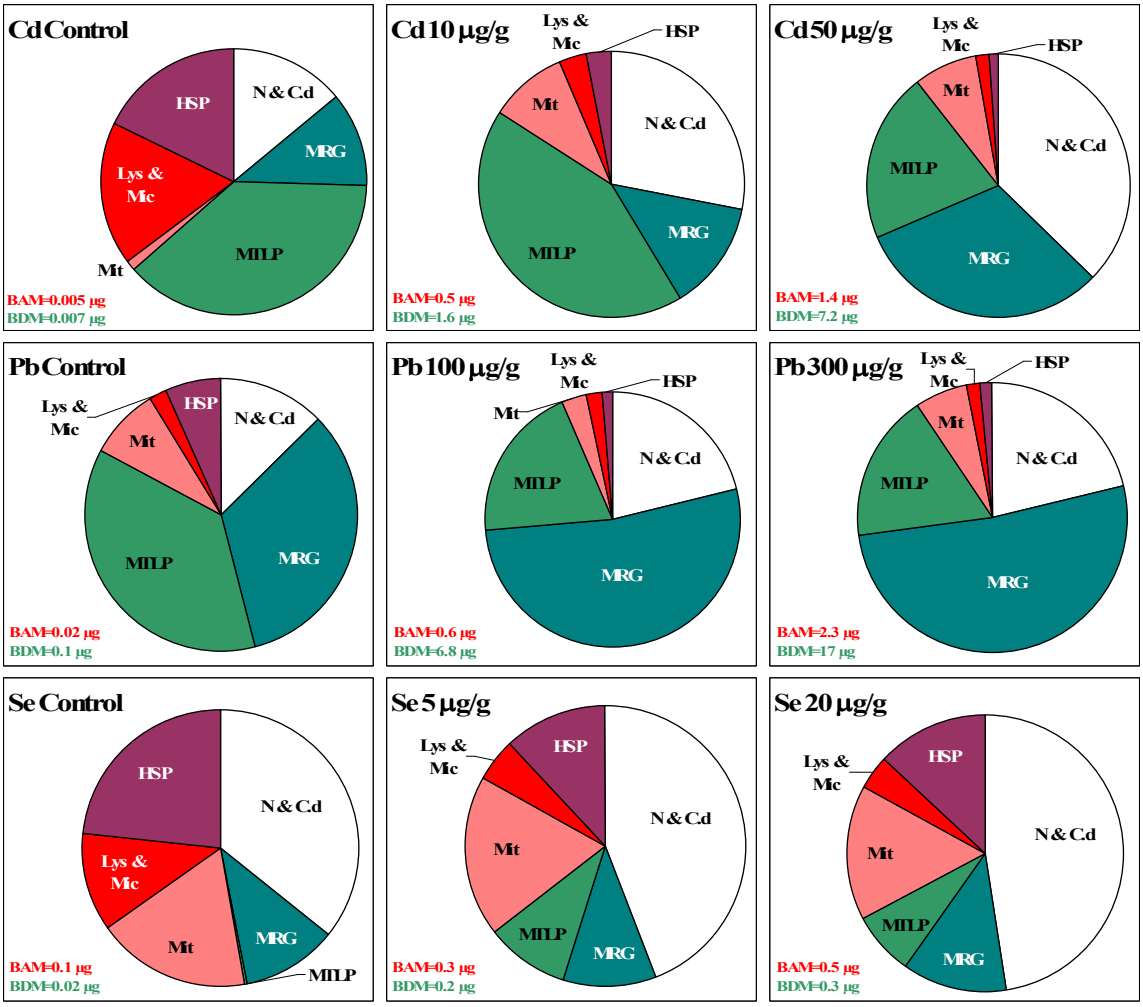


Figure 4.3: Distribution (%) of cadmium, lead and selenium in each of the subcellular fractions of *T. deltoidalis* following 28 days exposure to metal spiked sediments. Subcellular fractions are: nuclei+cellular debris (N & C.d); metal rich granules (MRG); heat stable, metallothionein like proteins (MTLP); mitochondria (Mit); lysosomes+ microsomes (Lys & Mic); heat sensitive proteins (HSP). Red fractions ( ) make up the biologically active metal (BAM), green fractions ( ) make up the biologically detoxified metal (BDM), n = 2.

Table 4.2: Mean percentage of metal in the nuclei+cellular debris, biologically detoxified metal (BDM) and biologically active metal (BAM) with the percentage of metal each of the fractions within contributes to BDM or BAM of *T. deltoidalis* subcellular tissue fractions after 28 days exposure to metal spiked sediments. n = 2.

	Sediment Treatments		
	Cd control	**Cd 10 µg/g**	**Cd 50 µg/g**
Nuclei + Cellular debris % of total	**14**	**28**	**37**
BDM % of total	**50**	**56**	**52**
Metal Rich Granules % of BDM	23	24	60
Heat Stable MT Like Proteins % of BDM	77	76	40
BAM % of total	**36**	**16**	**11**
Mitochondria % of BAM	3	60	72
Lysosomes + Microsomes % of BAM	48	22	17
Heat Sensitive Proteins % of BAM	49	18	11
	Pb control	**Pb 100 µg/g**	**Pb 300 µg/g**
Nuclei + Cellular debris % of total	**13**	**22**	**21**
BDM % of total	**70**	**72**	**69**
Metal Rich Granules % of BDM	47	73	74
Heat Stable MT Like Proteins % of BDM	53	27	26
BAM % of total	**17**	**6**	**10**
Mitochondria % of BAM	50	50	67
Lysosomes + Microsomes % of BAM	11	30	18
Heat Sensitive Proteins % of BAM	39	20	15
	Se control	**Se 5 µg/g**	**Se 20 µg/g**
Nuclei + Cellular debris % of total	**36**	**44**	**48**
BDM % of total	**11**	**20**	**19**
Metal Rich Granules % of BDM	99	51	62
Heat Stable MT Like Proteins % of BDM	1	49	38
BAM % of total	**53**	**36**	**33**
Mitochondria % of BAM	34	53	48
Lysosomes + Microsomes % of BAM	22	14	12
Heat Sensitive Proteins % of BAM	44	34	40

4.3.2.2 *Lead*

Approximately half of the total lead was recovered in the fractions (Table 4.1). Of the metal recovered in the fractions around 70 % was in the BDM fractions for all treatments (Table 4.1). The percentage of metal recovered in the BAM fractions of each of the 100 and 300 µg/g lead treatments was less than half that of the control, however, the total lead burden (µg) within these fractions was 30 and 115 times respectively, greater in the lead exposed organisms (Table 4.1). The lead in the BDM component of the control organisms was fairly equally distributed between the MTLP and MRG fraction (Figure 4.3; Table 4.2). In the 100 and 300 µg/g lead exposed organisms the MRG fraction contained three quarters of the BDM with the remaining quarter in the MTLP fraction (Figure 4.3; Table 4.2). The control and 100 µg/g lead exposed organisms had 50 % of BAM in the mitochondrial fraction. This fraction increased to 67 % in the 300 µg/g lead exposed organisms (Figure 4.3; Table 4.2). The HSP contained the majority of the remaining lead in the control organisms BAM with a minor amount in the lysosome+microsome fraction (Figure 4.3; Table 4.2). The 100 µg/g lead exposed organisms had most of the remaining BAM in the lysosomal+microsome fraction while in the 300 µg/g lead exposed organisms it was distributed fairly evenly between the HSP and lysosomal+microsome fractions (Figure 4.3; Table 4.2).

4.3.2.3 *Selenium*

Approximately half the total selenium from the organisms exposed to sediment concentrations of 5 and 20 µg/g selenium was recovered in the fractions with only 20 % recovered from the controls (Table 4.1). Of the recovered metal, almost half was in the nuclei+cell debris fraction in all treatments (Table 4.1). The percentage of metal recovered in the BAM fractions of each of the 5 and 20 µg/g selenium treatments was less than that of the control, however, the total selenium burden (µg) within these fractions was 3 and 5 times respectively, greater in the selenium exposed organisms (Table 4.1). The majority, 99 %, of selenium in the BDM component of the control organisms was in the MRG fraction with almost none in the MTLP fraction. In the 5 µg/g selenium exposed organisms it was evenly distributed between the two fractions, while in the 20 µg/g selenium exposed organisms the MRG fraction increased to 62 % (Figure 4.3; Table 4.2). The percentage of BAM in the control organisms was highest in the HSP, lower in the mitochondrial fractions with the lysosome+microsome fraction containing the least (Figure 4.3; Table 4.2).

The mitochondrial fraction contained the highest percentage of selenium of the BAM component in the 5 and 20 µg/g selenium exposed organisms followed by the HSP fraction with only a small percentage in the lysosome+microsome fraction (Figure 4.3; Table 4.2).

4.3.3 Activities of Marker Enzymes

4.3.3.1 *Total Tissue Enzyme Activity*

Cadmium

The activity in whole tissue of the acid phosphatase (AP) lysosomal enzyme and cytochrome c oxidase (CcO) mitochondrial enzyme of both the cadmium treatments was greater than that of the control organisms with the 50 µg/g cadmium exposed organisms having the highest activity for both enzymes (Figure 4.4).

Lead

The 100 µg/g lead exposed organisms had higher AP enzyme activity and lower CcO activity than the 300 µg/g lead exposed organisms (Figure 4.4). Both lead treatments had higher enzyme activity than the control organisms (Figure 4.4).

Selenium

The AP activity of the 5 µg/g selenium exposed organisms was lower than the controls and the 20 µg/g selenium exposed organisms. Their CcO activity was higher than the controls but lower than the 20 µg/g selenium exposed organisms (Figure 4.4). The activity of both enzymes of the 20 µg/g selenium exposed organisms was higher than that of the other two treatments (Figure 4.4).

4.3.3.2 *Subcellular Fraction Enzyme Activity*

Enzyme activities in the subcellular fractions of all treatments indicate that the CP 3 fractions are enriched in mitochondria while the CP 4 fractions were enriched with lysosomes (Figure 4.5). There was some carry over of mitochondria into the CP 4 fractions and also some lysosomal enzyme activity present in the CP 3 fraction (Figure 4.5).

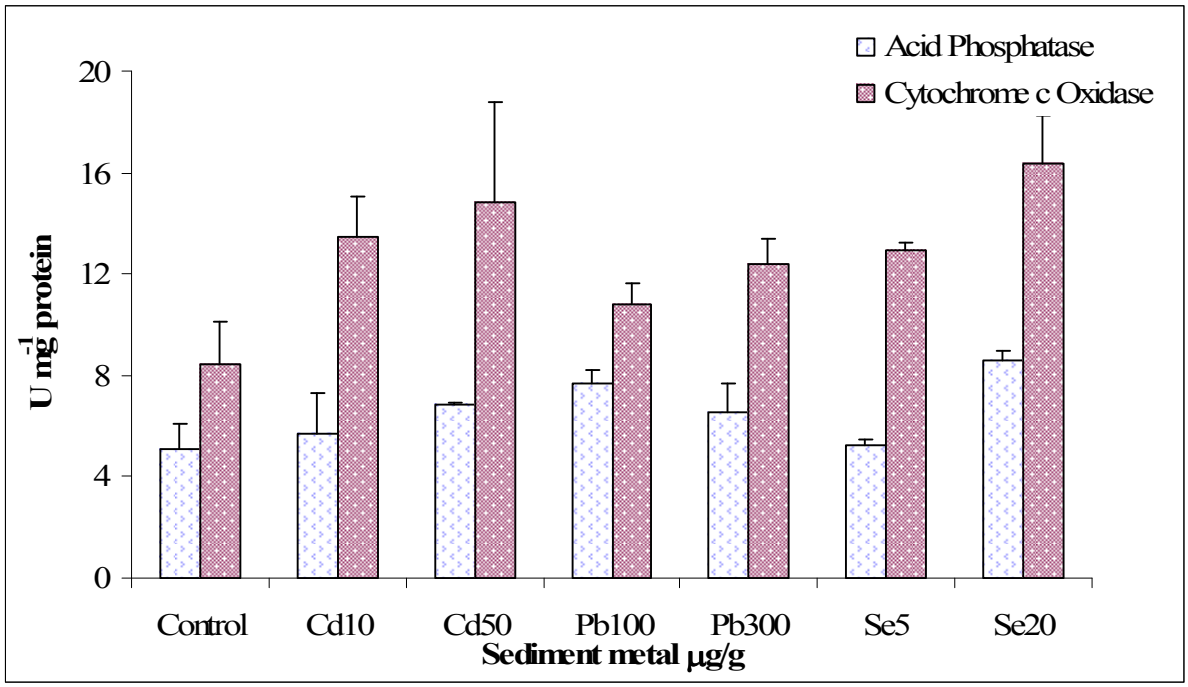


Figure 4.4: Specific activity of mitochondrial (cytochrome c oxidase) and lysosomal (acid phosphatase) marker enzymes, in whole tissue of *T. deltoidalis*. Mean ± SD, n = 2.

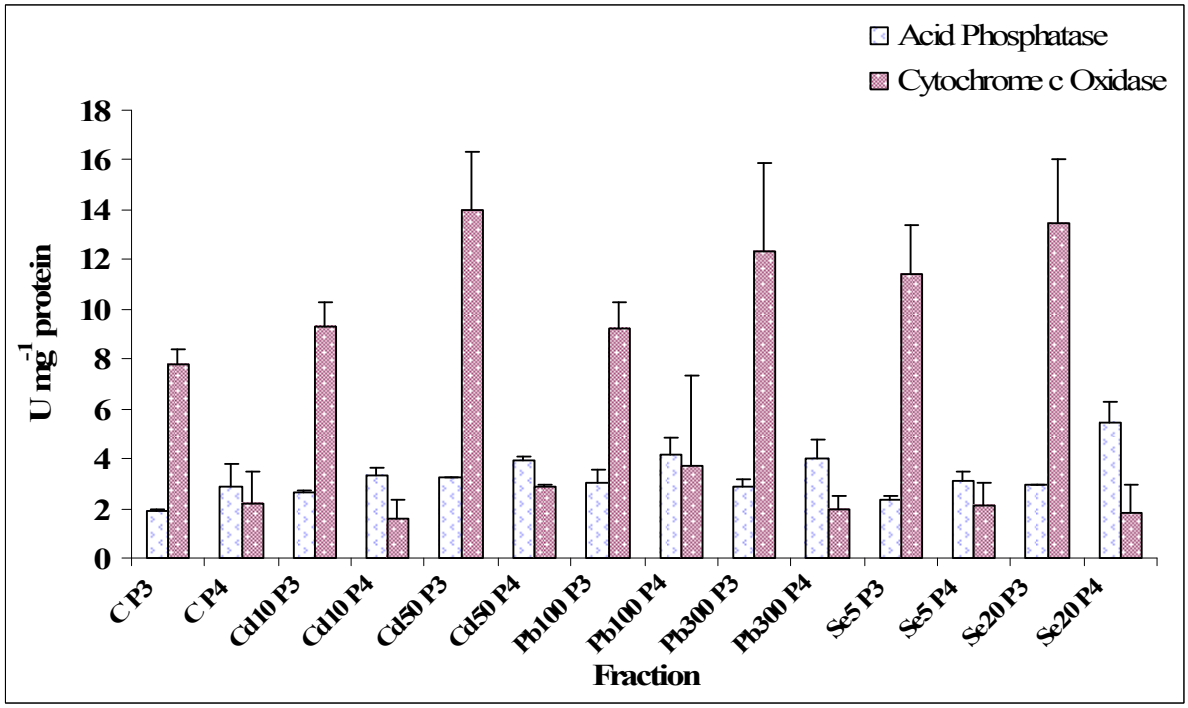


Figure 4.5: Enzyme activity of lysosomal enzyme (acid phosphatase) and mitochondrial enzyme (cytochrome c oxidase) in the mitochondrial fraction (P3) and lysosomal+microsomal fraction (P4), following subcellular fractionation. Mean ± SD, n = 2.

4.3.4 Enzymatic Biomarkers

4.3.4.1 *Total Antioxidant Capacity*

The total antioxidant capacity (TAOC) was reduced in organisms from all metal treatments compared to that of unexposed organisms (Figure 4.6). ANOVA showed that the factor sediment metal concentration was significant for TAOC (Appendix 1.5). The 10 µg/g cadmium exposed organisms had a higher TAOC than those exposed to 50 µg/g cadmium (Figure 4.6). The reverse was found for lead with the 100 µg/g having a lower TAOC than the 300 µg/g exposed organisms (Figure 4.6). The TAOC of the selenium exposed organisms was similar for both treatments (Figure 4.6). Bonferroni pair-wise comparisons showed that the TAOC of the metal exposed organisms were significantly reduced compared to the control organisms for all metal treatments, however, the TAOC of each of the high and low metal treatments were not significantly different to each other (Figure 4.6; Appendix 1.6).

4.3.4.2 *Glutathione Peroxidase Activity*

The activity of the glutathione peroxidase enzyme (GPx) was reduced in both cadmium treatments and the highest lead treatment and enhanced in both selenium treatments compared to the control organisms (Figure 4.6). The lower lead treatment had similar activity to the control (Figure 4.6). The enzyme activity was higher in the lower metal treatments compared to the corresponding high metal treatments for lead and selenium and similar in both cadmium treatments (Figure 4.6). ANOVA with Bonferroni pair-wise comparison showed that the GPx activity of the metal exposed organisms was not significantly different to the control organisms and each of the high and low metal treatments were not significantly different to each other (Figure 4.6; Appendix 1.5 & 1.6).

4.3.4.3 *Total Glutathione Concentration*

The total glutathione concentration (GSH+2GSSG) was lowest in the cadmium treatments and also reduced in the highest lead treatment compared to the control while it was enhanced in both selenium treatments (Figure 4.6). The GSH+2GSSG concentration of the lower lead treatment was similar to the control concentration (Figure 4.6). The GSH+2GSSG concentration was higher in the lower lead and selenium treatments compared to the high treatments and similar between both cadmium treatments (Figure 4.6).

ANOVA with Bonferroni pair-wise comparison showed that the GSH+2GSSG concentration of the metal exposed organisms was not significantly different to the control organisms and each of the high and low metal treatments were not significantly different to each other (Figure 4.6; Appendix 1.5 & 1.6).

4.3.4.4 *Reduced : Oxidised Glutathione Ratio*

The ratio of reduced and oxidised glutathione (GSH:GSSG) was lower in organisms from all sediment metal treatments compared to that of unexposed organisms (Figure 4.6). ANOVA showed that the factor sediment metal concentration was significant for GSH:GSSG ratio (Appendix 1.5). The ratio of the lead sediment treatments was higher than that of the cadmium and selenium sediment treatments (Figure 4.6). Bonferroni pair-wise comparison showed that GSH:GSSG ratio of both treatments of the cadmium and selenium exposed organisms was significantly reduced compared to the control organisms, however, the GSH:GSSG ratios of each of the high and low metal treatments were not significantly different to each other (Figure 4.6; Appendix 1.6). The organisms from the two lead treatments did not have significantly different GSH:GSSG ratios from the control or from each other (Figure 4.6; Appendix 1.6).

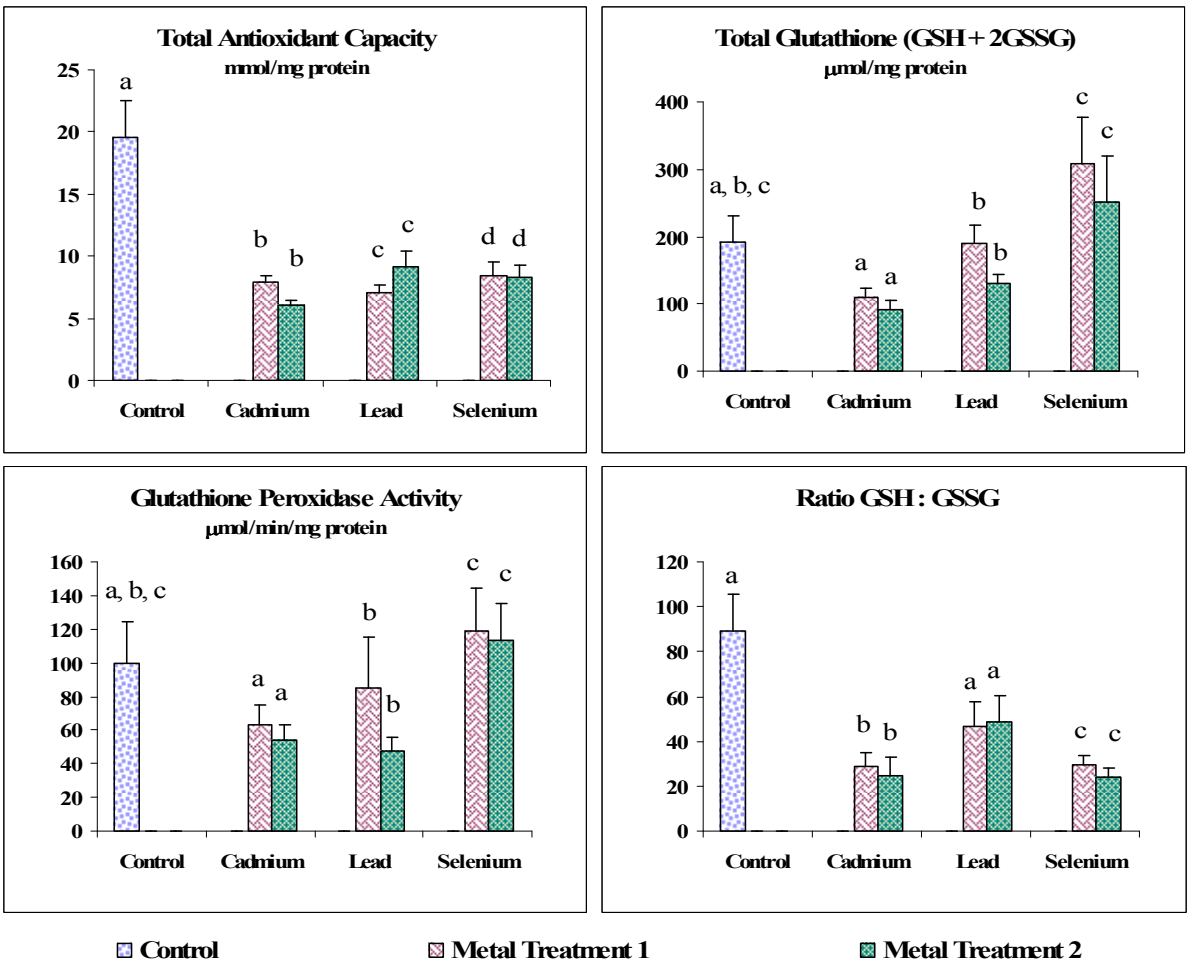


Figure 4.6: Antioxidant enzyme biomarkers of *T. deltoidalis* after 28 days: Control - (unspiked sediment); Metal Treatment 1 - (Cd 10 µg/g; Pb 100 µg/g; Se 5 µg/g) and Metal Treatment 2 - (Cd 50 µg/g; Pb 300 µg/g; Se 20 µg/g). Mean ± SE, n = 12 for all except Cd GPx n = 6. Ratio GSH:GSSG – (Ratio of reduced to oxidised glutathione) Different letters indicate significant differences betweens means (Bonferroni test; $p < 0.05$).

4.3.5 Oxidative Damage Biomarker – Thiobarbituric Acid Reactive Substances

Thiobarbituric acid reactive substances (TBARS) were higher in all metal exposed organisms than in unexposed organisms (Figure 4.7). ANOVA showed that sediment metal concentration was a significant factor for TBARS concentration (Appendix 1.5). The higher sediment metal treatments had similar TBARS concentrations to each other and all were higher than the TBARS concentrations of the lower sediment metal treatments (Figure 4.7). Bonferroni pair-wise comparison showed that the 10 µg/g cadmium exposed organisms did not have significantly higher TBARS than the control while the 50 µg/g cadmium exposed organisms did and the treatments were not significantly different to each other (Figure 4.7; Appendix 1.6). The 100 µg/g lead exposed organisms did not have significantly higher TBARS than the control while the 300 µg/g had significantly higher TBARS than both the control and 100 µg/g treatment (Figure 4.7; Appendix 1.6). The organisms from the two selenium exposures both had significantly higher TBARS than the control but not from each other (Figure 4.7; Appendix 1.6).

4.3.6 Cellular Biomarker – Lysosomal Stability

Lysosomal stability was reduced in all metal exposed organisms compared to the controls (Figure 4.7). ANOVA showed that the factor sediment metal concentration was significant for lysosomal stability (Appendix 1.5). The higher sediment metal treatments all had a higher percentage of unstable lysosomes than the lower sediment metal treatments (Figure 4.7). The highest instability was in the high selenium treatment and the lowest in the low selenium treatment compared to the lead and cadmium treatments (Figure 4.7). Bonferroni pair-wise comparison showed that both sediment treatments of the cadmium and lead exposed organisms had significantly more unstable lysosomes than the control organisms but the metal treatments were not significantly different to each other (Figure 4.7; Appendix 1.6). The selenium exposed organisms both had significantly more unstable lysosomes than the control organisms and the 20 µg/g selenium exposed organisms had significantly more unstable lysosomes than the 5 µg/g selenium exposed organisms (Figure 4.7; Appendix 1.6).

4.3.7 Genotoxic Biomarker – Micronuclei Frequency

The frequency of micronuclei was higher in all metal exposed organisms compared to the controls (Figure 4.7). ANOVA showed that the factor sediment metal concentration was significant for micronuclei frequency (Appendix 1.5). The higher metal treatments all had a higher percentage of micronuclei than the lower metal treatments (Figure 4.7). The highest frequency was in the high cadmium treatment (Figure 4.7). The frequency of the lower cadmium treatment was similar to that of the high selenium treatment (Figure 4.7). The lead treatments had the lowest percentage of micronuclei compared to the cadmium and selenium treatments (Figure 4.7). The organisms exposed to cadmium and selenium spiked sediment both had significantly more micronuclei than the control organisms and the 50 µg/g cadmium and 20 µg/g selenium exposed organisms each had significantly more micronuclei than the 10 µg/g cadmium and 5 µg/g selenium exposed organisms respectively (Figure 4.7; Appendix 1.6). The lead exposed organisms had significantly more micronuclei than the control organisms but were not significantly different to each other (Figure 4.7; Appendix 1.6).

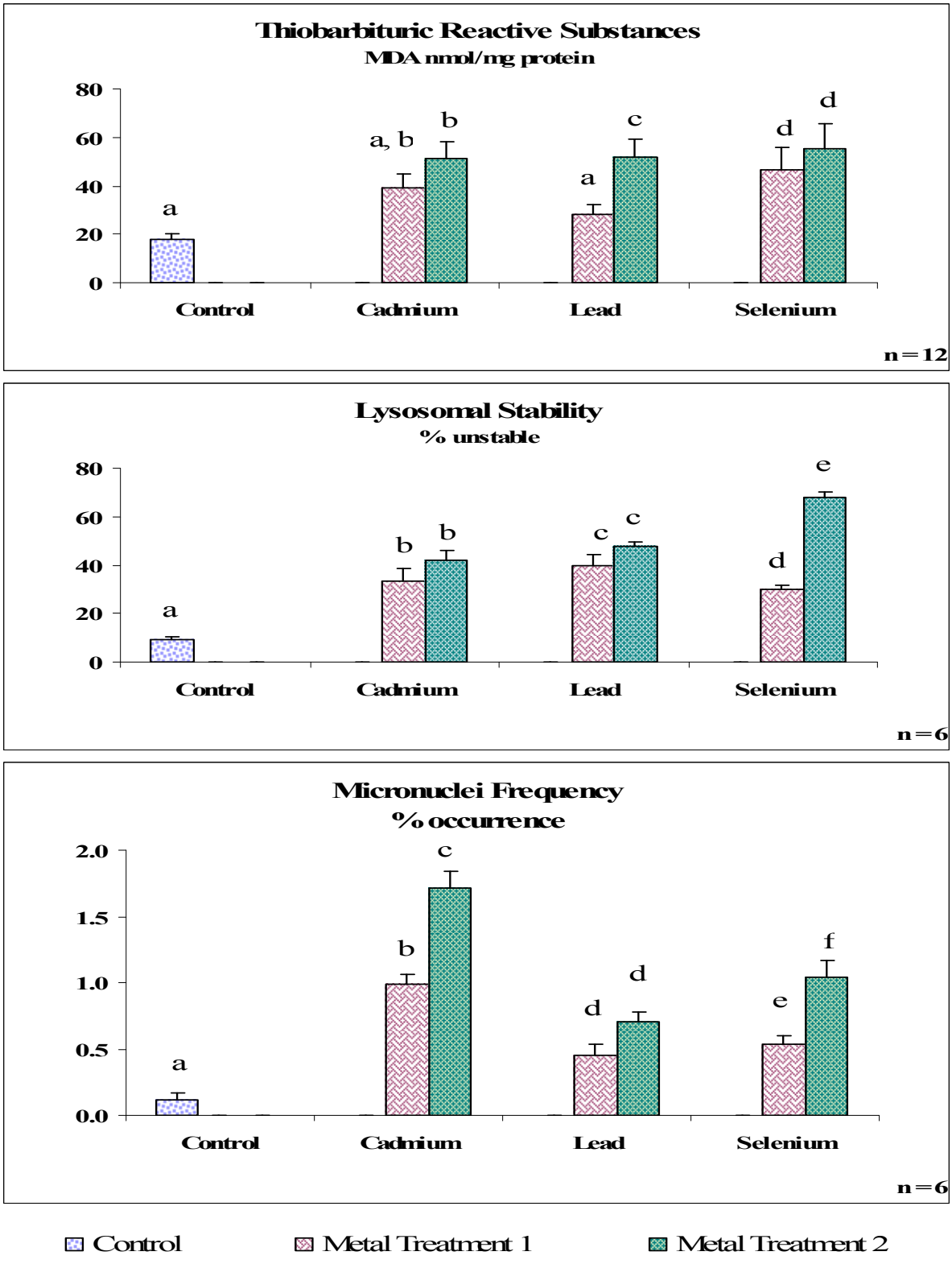


Figure 4.7: Changes in oxidative damage, cellular and genotoxic biomarkers of *T. deltoidalis* after 28 days. Control (unspiked sediment); Metal Treatment 1 (Cd 10 µg/g; Pb 100 µg/g; Se 5 µg/g); and Metal Treatment 2 (Cd 50 µg/g; Pb 300 µg/g; Se 20 µg/g). Mean ± SE. Different letters indicate significant differences betweens means (Bonferroni test; $p < 0.05$).

4.3.8 Relationship Between Biomarkers

4.3.8.1 *Enzymatic with Oxidative Damage, Cellular and Genotoxic Effects*

Regression analysis shows that the reduced total antioxidant capacity (TAOC) within cells had a negative relationship with the effects measures TBARS, lysosomal stability and micronuclei frequency for cadmium, lead and selenium exposed organisms (Figure 4.8). The relationship between TAOC and TBARS for lead exposed organisms and TAOC and lysosomal stability for selenium was of low significance (Figure 4.8). Generally as the metal exposure increased the capacity of the cells to neutralise reactive oxygen was reduced with a consequent increase in cell damage.

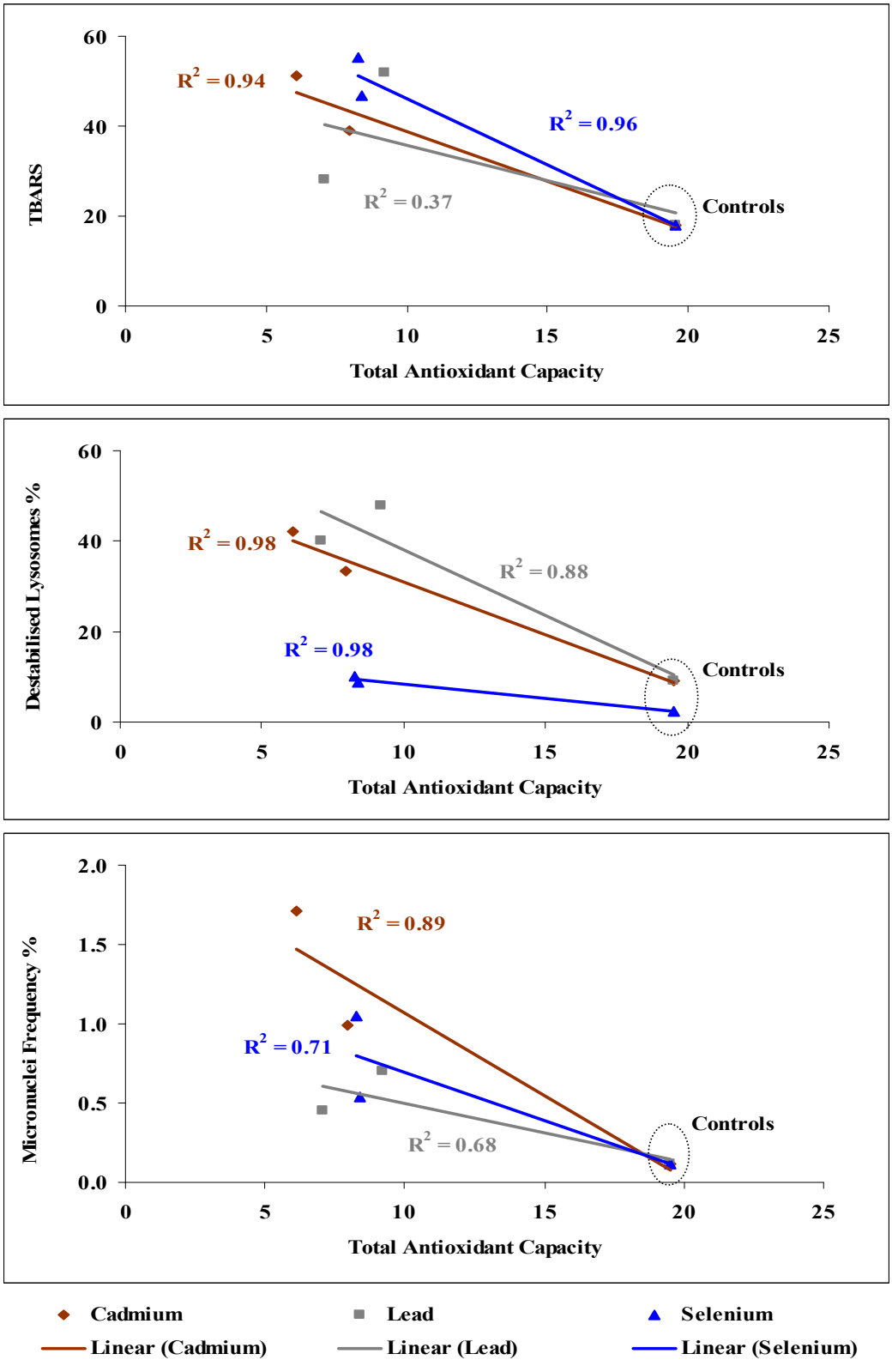


Figure 4.8: Regressions of total antioxidant capacity means, (n=12); with TBARS, (n=12), lysosomal stability and micronucleus frequency means (n=6) for sediment metal treatments. Control (unspiked sediment); Metal Treatments Cd 10 & 50 µg/g; Pb 100 & 300µg/g; Se 5 & 20µg/g.

4.3.8.2 *Oxidative Damage with Cellular and Genotoxic Effects*

There was a positive relationship between TBARS and the cellular effects measure of lysosomal stability and genotoxic measure of micronuclei frequency for all metals (Figure 4.9). Generally increased metal exposure increased the TBARS and the regression analysis indicates that this was associated with an increase in the percentage of unstable lysosomes and the frequency of micronuclei (Figure 4.9).

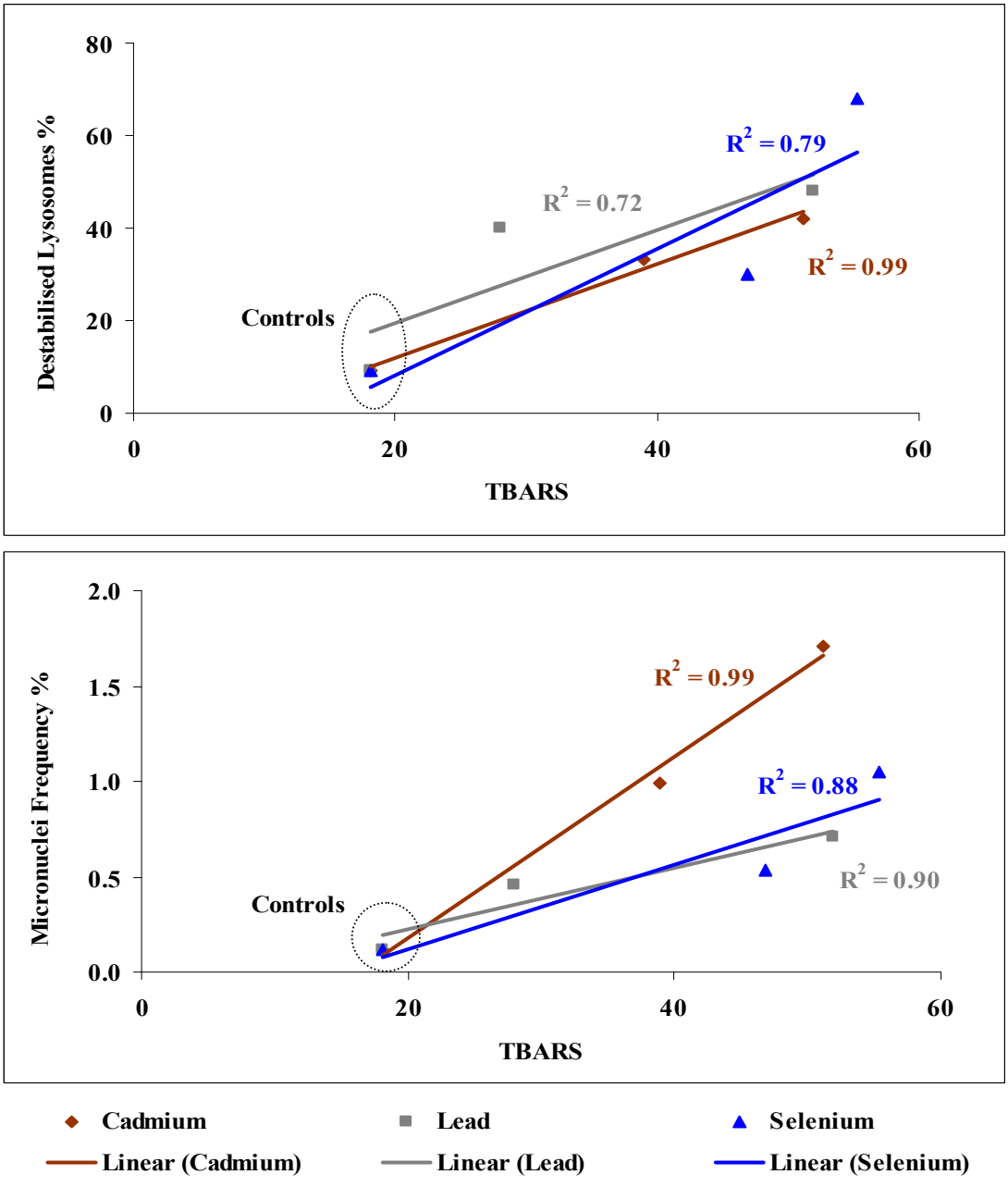


Figure 4.9: Regressions of TBARS means (n=12); with lysosomal stability and micronucleus frequency means (n=6). For sediment treatments: Control (unspiked sediment); Metal Treatments Cd 10 & 50 µg/g; Pb 100 & 300µg/g; Se 5 & 20µg/g.

4.4 Discussion

4.4.1 Metal Accumulation and Subcellular Distribution

4.4.1.1 *Cadmium Accumulation*

The *T. deltoidalis* exposed to 10 and 50 µg/g of cadmium both showed a steady increase in tissue cadmium over the 28 day exposure with final tissue concentrations equal to that of the spiked sediments (Figure 4.1). Bivalve molluscs which accumulate metals in direct proportion to their ambient concentrations and can be considered weak accumulators of these metals (Luoma and Rainbow, 2008). *T .deltoidalis* cadmium uptake in these experiments fits this pattern. The slight decrease in tissue cadmium between day 21 and 28 in the 50 µg/g exposed organisms suggests that they may have reached equilibrium with their exposure environment by day 21. This is at odds with modelling work on cadmium accumulation in *T. deltoidalis* tissues by King *et al* (2005) which concluded that > 40 days would be required for *T. deltoidalis* to reach a steady-state concentration with their cadmium exposure environment. Bioturbation of sediments by deposit feeders leads to dispersal of metal oxides by mechanical actions through burrowing as well as via bioresuspension when fluid faecal pellets are ejected into the water column (Meysman *et al.*, 2006). Atkinson (2007) showed that *T. deltoidalis* caused bioturbation of sediments which increased metal release compared to unmixed sediments. Surprisingly their study which exposed *T. deltoidalis* to sediment containing concentrations of 25 µg/g of cadmium for 21 days achieved a final cadmium tissue concentration of only 5 µg/g dry mass. The sediment used was highly silty < 63 µm = 73 % compared to this experiment < 63 µm = 30 % which may have affected feeding and burrowing behaviour. Their sediment also had significant concentrations of copper, lead and zinc so metal interactions may have affected the individual metal bioavailability (Moolman *et al.*, 2007). In estuarine and marine environments cadmium is relatively soluble due to strong complexation by chloride (Stumm and Morgan, 1996). The even pattern of cadmium accumulation over time (Figure 4.1, Appendix 1.4) and positive linear sediment tissue cadmium concentration relationship for the two treatments (Figure 4.2) suggests the sediment bound cadmium was readily resuspended and bioavailable and the major exposure route remained the same over the course of the experiment.

King *et al* (2005) concluded this was largely via sediment ingestion, although, the model developed, using *T. deltoidalis,* was based on 24 hour exposure and depuration experiments and the authors felt that sediment retained in the gut and shell may have resulted in an overestimation of the tissue concentrations from sediment exposure compared to water and food exposures used.

4.4.1.2 *Cadmium Subcellular Distribution*

Once accumulated around 50 % of the cadmium was detoxified (BDM) in organisms from all treatments (Figure 4.3, Table 4.1). The control and 10 µg/g exposed organisms only converted a quarter of their total BDM into metal rich granules (MRG) with the remaining 75 % in the metallothionein like proteins (MTLP) fraction. The 50 µg/g exposed organisms in contrast converted 60 % of BDM cadmium to MRG with 40 % in the MTLP fraction (Table 4.2). The formation of MRG has been associated with increased metal tolerance in marine organisms (Wallace *et al.*, 1998). George, (1983b) found that granules of cadmium exposed *Mytilus edulis* contained high concentrations of protein, calcium and sulphur which he postulated may be due to an increase in lysosomal protein degradation, due to enzyme inactivation by intracellular cadmium, causing an increase in intracellular protein turnover. The reduction in the percentage of cadmium in the lysosomal fraction of the exposed versus control organisms and the increase in cadmium in the MRG fraction of the 50 µg/g expose *T. deltoidalis* tends to support the route of cadmium transfer from the BAM lysosomal fraction to the MRG fraction (Table 4.2). MT plays a key role in metal detoxification and the relationship between MT and transport of metal to sites of MRG production is also likely to be important (Wallace *et al.*, 1998). Cadmium bound to MT in the kidney of the mussel *Mytilus edulis* became incorporated in the granules (George, 1983a). Cadmium bound to MT in the digestive gland of *Mytilus galloprovincialis* becomes incorporated into lysosomes and is transformed into insoluble thionein polymers, a likely precursor of MRG (Viarengo *et al.*, 1987). It is likely that a combination of these two transfer routes are operating in *T. deltoidalis* as seen in the change in the percentage distribution of cadmium in the lysosomal, MTLP and MRG fractions with increasing cadmium exposure (Figure 4.3, Table 4.2).

The activity of marker enzymes cytochrome c oxidase (CcO), for mitochondria and acid phosphatase (AP), for lysosomes in the total homogenates shows an increase in activity of both organelles with increased cadmium exposure, particularly in the mitochondria (Figure 4.4). This is also seen in the mitochondria and lysosomal fractions (Figure 4.5) and suggests that both organelles are responding to the accumulation of cadmium within the cells. The majority of cadmium in the BAM fractions of the cadmium exposed *T. deltoidalis* was in the mitochondrial fraction (Figure 4.3; Table 4.2). Cadmium burdens in the freshwater bivalve *Pyganodon grandis* were also found to be higher in the mitochondria than the lysosome+microsome fraction with increased cadmium exposure (Bonneris *et al.*, 2005). Li *et al,* (2003) showed that cadmium could directly lead to dysfunction of mitochondria including inhibition of respiration, loss of transmembrane potential and the release of CcO. In the BAM portion of the cadmium exposed *T. deltoidalis* the percentage of cadmium in the mitochondrial fraction of the 10 µg/g and 50 µg/g organisms was 60 and 72 % respectively compared to only 3 % in the controls (Table 4.2). In terms of the total cadmium recovered in the mitochondrial fractions of the 10 µg/g and 50 µg/g organisms this represents a 2000 and 7200, respectively, fold increase in mitochondrial cadmium, compared to the control organisms. Extensive Cd^{2+} accumulation in mitochondria mediated by Ca^{2+} voltage dependant channels has previously been reported by Li *et al.* (2000; 2003). The increased mitochondrial cadmium observed in *T. deltoidalis* is also in agreement with studies on cadmium subcellular distribution following increased exposure in oysters *Crassostrea virginica* (Sokolova *et al.*, 2005a). Mitochondrial function was also found to be highly sensitive to cadmium at physiological and environmentally relevant low concentrations in oysters (Sokolova, 2004). Cadmium has been shown to have a high affinity for mitochondria, it is capable of inhibiting respiration and oxidative phosphorylation and interfering with the 1-hydroxylation of vitamin D (Fowler and Mahaffey, 1978). Lysosomes are involved in numerous functions including, nutrition, tissue repair, cellular defence, turnover of membranes, organelles and proteins as well as in the sequestration and metabolism of toxins, such as organic xenobiotics and metals and are an important target organelle for metal toxicity (Ringwood *et al.*, 1998a). While the percentage of cadmium in the lysosomal+microsomal fraction of the 10 µg/g and 50 µg/g cadmium exposed organisms was slightly less than half that of the controls (Table 4.2) the cadmium associated with them was 46 and 100, respectively, times greater than that of the controls. Lysosomes have been identified as the metal cation homeostasis mechanism which sits between soluble binding ligands such as MT and the formation of insoluble precipitates such as MRG (Viarengo and Nott, 1993).

While lysosomal cadmium uptake may reflect sequestration and detoxification of the metal, it can also lead to adverse effects when the handling capacity of the lysosomes is overwhelmed (Sokolova *et al.*, 2005a; Viarengo *et al.*, 1987). This fraction also contained the microsomal component of the cell. Since this includes fragmented endoplasmic reticulum, which is generally responsible for protein synthesis and transport; if cadmium in this fraction was associated with microsomes rather than lysosomes then this could be indicative of toxicity (Bonneris *et al.*, 2005). Similar amounts of cadmium were also associated with the heat sensitive proteins (HSP) of the 10 µg/g and 50 µg/g cadmium exposed organisms compared to the controls as those found in the lysosome+microsome fraction (Table 4.2). The HSP fraction contains enzymes, high and low molecular weight proteins and other target molecules which are sensitive to metals. (Wallace *et al.*, 2003) The increased binding of cadmium to this fraction may therefore have implications for toxicity. The percentage of cadmium within the nuclei+cellular debris fraction of 10 µg/g cadmium exposed organisms was double, and in the 50 µg/g two and a half times the control cadmium. This fraction was not included in the BAM and BDM compartments as it contains tissue fragments, cell membranes and other cellular components of unknown consequence in terms of function, as well as the nucleic material (Wallace *et al.*, 2003). Binding of cadmium to cell membranes could result in toxicity, however, if the cadmium in this fraction was bound to less sensitive fractions within the cellular debris this could reduce binding to more sensitive cell components (Lucu and Obersnel, 1996). MT has been shown to be present in the nucleus of gill, digestive gland, gonad and posterior adductor muscle of *Mytilus edulis* in equal or lower concentrations than the corresponding tissue cytosol (del Castillo and Robinson, 2008). If this is also the case for *T. deltoidalis*, cadmium in the nuclei+cellular debris fraction may be associated with nuclear MT and so detoxified. The effectiveness of cadmium detoxification relies on binding to MTs transfer across cell membranes, compartmentalisation within lysosomes and the formation of MRG. The presence of increased cadmium associated with these fractions shows that these processes were occurring, however, increases in cadmium within the mitochondrial fraction and to a lesser extent the HSP fraction indicates the detoxification and storage mechanisms were overwhelmed and unable to process the excess cadmium suggesting the potential for cadmium toxicity.

4.4.1.3 *Lead Accumulation*

The *T. deltoidalis* exposed to 100 µg/g lead showed a rapid accumulation of lead in the first 3 days of exposure followed by small increases over the remainder of the 28 day exposure to reach a final tissue concentration equal to that of the spiked sediment (Figure 4.1). This suggests *T. deltoidalis* is a weak accumulator of lead (Rainbow, 2007). Like cadmium, lead as Pb^{2+} acts as a Ca^{2+} analogue, and is taken up presumably through high affinity Ca uptake mechanisms (Macdonald *et al.*, 2002). Mussels *Mytilus galloprovincialis* transplanted in a lead polluted area reached a steady state of tissue lead after 4 weeks exposure (Regoli and Orlando, 1993). The organisms exposed to 300 µg/g of lead also accumulated lead rapidly over the first 3 days and reached a tissue concentration very close to that of the spiked sediment in this time (Figure 4.1). The subsequent drop in tissue lead between day 7 and day 14 (Figure 4.1) in these organisms suggests a regulatory response involving excretion. By day 21 the lead tissue concentration was almost double the exposure concentration but had again decreased to a concentration which was 1.5 times greater than the exposure concentration by day 28 (Figure 4.1). Rapid accumulation of lead and slow excretion has been observed in mussels, *Mytilus galloprovincialis* (Regoli and Orlando, 1994) and clams, *Ruditapes philippinarum* (Blasco and Puppo, 1999). This pattern of pulses of uptake and loss could be due to binding sites being temporarily saturated, following which lead is bound to MT and transferred into lysosomes with some excretion then occurring, this in turn would 'free up' binding sites for further accumulation (Marigómez *et al.*, 2002; Rainbow, 2002; Rainbow, 2007). That the tissue concentrations of the 300 µg/g lead exposed organisms were far greater than ambient concentrations by 3 weeks of exposure (Figures 4.1; 4.2) points to the regulatory mechanisms being progressively overwhelmed at this exposure concentration. A study by Atkinson *et al.* (2007) which exposed *T. deltoidalis* to sediment containing concentrations of 314 µg/g of lead for 21 days achieved a final lead tissue concentration of only 55 µg/g dry mass. The zinc, copper and cadmium which the sediment also contained may have competed for binding sites, with preferential binding of zinc and copper reducing the lead binding (Rainbow, 2007). Alternatively, despite their observation that bioturbation by *T. deltoidalis* increased lead bioavailability compared to undisturbed sediments, lead in their experiment may have been less bioavailable than in the present study due to different physicochemical properties of the sediment and overlying waters used.

4.4.1.4 *Lead Subcellular Distribution*

Of the accumulated lead around 70 % was in the BDM fractions of organisms from all treatments (Figure 4.3, Table 4.1). In the control organisms, the distribution in this compartment was fairly even between the MTLP and MRG fractions whereas in the lead exposed organisms the MRG contained nearly three quarters of the BDM with the remaining quarter associated with the MTLP fraction (Table 4.2). A high proportion of lead was also found to be associated with the MRG of lead exposed mussels, *Mytilus galloprovincialis* (Regoli and Orlando, 1994), and the variegated scallop, *Chlamys varia* (Bustamante and Miramand, 2005). Simkiss and Taylor, (1989) describe two pathways of granule formation in marine organisms, one based on orthophosphate and the other on pyrophosphate. They suggest that these are derived from ATP during normal cell metabolism and that many marine organisms have the ability to switch between the two metabolic pathways of granule production which is related to the physical properties of the cell membranes. Some metals, such as lead, appear to enter the deposits by displacing calcium into the cytoplasm which in extreme conditions can result in cell death, but in less severe treatments it is presumed that small amounts of calcium are released inducing exocytosis and excretion of the granules. This mechanism may explain the fluctuation in lead tissue concentrations over the course of the exposure (Figure 4.1). MT are a class of inducible proteins which have a high metal binding capacity and can detoxify excess metals that have penetrated cells (Viarengo and Nott, 1993). The turnover rate of MTs in the oyster *Crassostrea virginica* is about 4 - 5 days (Roesijadi and Robinson, 1994). It has been shown in mussels that MT binding of cellular metal can reduce interaction of metals with lysosomal membranes thus protecting them from destabilisation (Viarengo *et al.*, 1987), however, it has also been demonstrated that MT bound metals can be internalised into the lysosomes (Viarengo and Nott, 1993). As was seen for cadmium it is likely that a combination of the two transfer routes of MTLP and lysosomes to MRG are operating in *T. deltoidalis* as seen in the change in the distribution of lead in the lysosomal, MTLP and MRG fractions with increasing lead exposure (Figure 4.3, Table 4.2).

The activity of mitochondrial and lysosomal marker enzymes CcO and AP, in the total homogenates shows an increase in activity of both organelles with increased lead exposure (Figure 4.4). This is also seen in the mitochondria and lysosomal fractions (Figure 4.5) and suggests a response in both organelles to the accumulation of lead within the cells.

The percentage of lead in the BAM compartment of the exposed organisms was quite low, 10 % in the 300 µg/g and 6 % in the 100 µg/g compared to 17 % in the controls (Table 4.1), a further clear indication that detoxification processes were operating at these lead exposures. Within the BAM compartment the mitochondrial fraction of control and 100 µg/g *T. deltoidalis* contained 50 % and the 300 µg/g *T. deltoidalis* 67 % of the lead (Table 4.2), which in the 100 µg/g and 300 µg/g lead exposed organisms corresponded to a 30 and 154, respectively, fold increase in mitochondrial lead burden, compared to the control organisms. Lead is known to accumulate in mitochondria causing swelling indicative of nephrotoxicity. It is thought to inhibit the synthesis of a variety of enzymes in the mitochondria and may cause cell death by impairment of energy production in this organelle system (Fowler and Mahaffey, 1978). The percentage of lead in the lysosomal+microsomal fraction of the 100 µg/g lead exposed organisms was close to three times that of the controls while the 300 µg/g lead exposed organisms had only a slightly higher percentage than that of the controls (Table 4.2). The lead associated with them, however, was 82 and 188, respectively, times greater than that of the controls. It is thought that metals in the lysosomes may bind to lipofuscins, which are lipid peroxidation end products in the lysosomes, as insoluble lipoprotein granules (George, 1983a; b). In most cells of marine invertebrates these lipofuscin granules are usually excreted by exocytosis (George, 1983b). Thus, lead associated with lysosomes may be in the process of detoxification, however, unbound lead associated with this fraction can lead to the formation of oxygen derived free radicals in the cells which if not fully detoxified can start a sequence of lipid peroxidation reactions (Viarengo and Nott, 1993). The percentage of lead associated with the HSP fraction and nuclei and cell debris in the lead exposed organisms was fairly consistent with that found in the cadmium exposed organisms (Table 4.2) and is indicative of a detoxification system struggling with the excess lead. The high percentage of lead in the BDM fraction is clear evidence that an efficient system of lead binding and detoxification was present and active, however, the increased lead associated with the BAM fractions indicates that the metal sensitive organelles were exposed to potentially toxic concentrations of lead as the tissue concentrations increased.

4.4.1.5 *Selenium Accumulation*

The day 0 organisms, which were taken from the collected organisms as a measure of pre-exposure tissue metal concentrations, had around 2.5 µg/g of selenium (Figure 4.1). The control organisms showed an increase at day 3 and then gradually lost selenium over the remainder of the exposure time, suggesting that depuration may have occurred (Figure 4.1). The variation in tissue concentration over time in the control organisms was only in the order of a few micrograms per gram and as selenium is an essential element some background concentration is expected (Hamilton, 2004), so alternatively this may be indicative of natural variation. The accumulation of selenium was rapid during the first 3 days of exposure in organisms from both selenium treatments, with the 5 µg/g selenium exposed organisms accumulating twice the exposure concentration and the 20 µg/g equalling it in this time (Figure 4.1). Organisms from both treatments had a decrease in selenium concentration at day 7 followed by a increase to day 21 (Figure 4.1). An equilibrium concentration appears to have been reached after 4 weeks in both treatments, however, this was at a very similar tissue concentration for organisms from both treatments and was five times the exposure concentration for the 5 µg/g exposed organisms (Figures 4.1; 4.2). Peters *et al.,* (1999b) found native *T. deltoidalis* exposed to 3.4 µg/g of selenium in Lake Macquarie NSW accumulated tissue concentrations of 32 µg/g which is a considerably higher exposure to tissue selenium ratio than observed for the *T. deltoidalis* in this experiment. Selenite is taken up rapidly by aquatic microflora and fauna and may be consumed directly by deposit feeding bivalves from the sediment surface or as part of the detritus. In addition selenite adsorbed to sediment particles may be ingested (Fan *et al.*, 2002; Hamilton, 2004). *T. deltoidalis* in these experiments may have had a change in selenium exposure route from an initial direct absorption from sediment particles to a later additional dietary exposure as microfauna and flora, present in the natural sediments and water used, absorbed selenium and were consumed. The major route of selenium uptake in aquatic systems is via food rather than as the free ion in solution (Luoma and Rainbow, 2008), therefore the final greater than ambient selenium tissue concentrations observed in this experiment after 21 days exposure may be related to the addition of dietary selenium.

4.4.1.6 *Selenium Subcellular Distribution*

A large proportion of the selenium recovered in the subcellular fractions was in the nuclei+cellular debris fraction, increasing from 36 % in the controls to 44 and 48 %, respectively, in the 5 and 20 µg/g exposed organisms (Table 4.1; Figure 4.3).

Selenite is bound to plasma proteins for transport to tissues. It has been suggested that selenite is taken up by haemolymph, reduced to selenide, released into the plasma and rapidly bound by plasma proteins (Ewan, 1989). The majority of accumulated selenate and selenomethionine occurs in the protein-free plasma (Ewan, 1989). Selenomethionine has been shown to bind to glutathione peroxidase (GPx) extracellularly as well as intracellularly (Burk, 1991). Mycelia of the fungus *Pleurotus ostreatus* enriched with selenium had 56 % of accumulated selenium associated with the cell wall (Hortensia *et al.*, 2006). A combination of protein bound selenium associated with plasma and selenium bound directly to cell walls would account for the high proportion of selenium associated with this fraction and therefore it would be comprised of both BDM and BAM. The BAM selenium in the nuclei+cellular debris fraction can be considered as detoxified, that is, like metal bound to MT it is effectively removed from active sites within the cell. Of the remaining selenium recovered in the fractions the control organisms had 11 % and the exposed organisms 20 % in the BDM fractions (Table 4.1; Figure 4.3), but the distribution within this portion differed. The control organisms had most of in the MRG fraction while the 5 µg/g exposed organisms had an even distribution between the MTLP and MRG fraction and the 20 µg/g had 62 % in the MRGs and 38 % in the MTLP (Table 4.2). Selenium associated with MTs has not previously been reported, however, the majority of selenium not associated with selenoproteins of the GPx family has been found bound to selenoamino acids and other low molecular weight proteins and it is presumed that these act as storage and transport proteins and intermediaries in the synthesis of selenoproteins (Akesson and Srikumar, 1994). The MTLP fraction may therefore represent a pool of detoxified selenium. Like MT, selenium associated with MRG has not previously been described. The operational fraction defined as MRG in this procedure has been examined in fractions obtained from cadmium exposed oligochaetes by Wallace *et al.* (1998), with a compound microscope, and shown to contain numerous MRG of varying sizes. The fraction obtained in the present study using the same fractionation technique as described by Wallace *et al.* (1998) was not examined visually for MRG so it can only be assumed that the fraction contained detoxified selenium rich concretions of some sort. Using a similar fractionation procedure Zhang and Wang (2006) found 40 % in crustaceans and 60 % in bivalves of accumulated selenium was associated with the MRG fraction, while Dubois and Hare (2009) obtained only 1 - 2 % of selenium in the MRG fraction of the oligochaete *Tubifex tubifex* and insect *Chironomus riparius*. George, (1983b) showed that MRG of cadmium exposed *Mytilus edulis* contained high concentrations of protein, calcium and sulphur.

Selenium is known to substitute for sulphur in organic compounds and has some similarities with the chemistry of sulphur (Ewan, 1989). The presence of selenium in a protein is always related to the presence of sulphur, the selenium atom is either incorporated in the place of a sulphur atom in a sulphur amino acid, or it is attached to the sulphur atoms of cysteine residues (Ganther, 1974). It is possible that selenium is incorporated into MRG like structures via a similar process to that postulated for cadmium by George (1983b), due to an increase in lysosomal protein degradation, following enzyme inactivation by intracellular selenium, causing an increase in intracellular protein turnover. Alternatively the MRG fraction may represent selenium associated with incompletely digested tissue and cell debris in the Na OH digestion step of the fractionation procedure (Figure 3.7). The fractionation procedure used by Zhang and Wang (2006) which found 40 % - 60 % in crustaceans and bivalves of accumulated selenium associated with the MRG fraction used a shorter Na OH digestion step, 10 minutes rather than the 60 minutes used in my study, so incomplete digestion of the tissue and cell debris fraction is also a possibility here. If this is the case then it is still a reasonable assumption that a fair proportion of this fraction represents detoxified selenium. The increased percentage of selenium associated with the BDM fractions (Figure 4.3) demonstrates that selenium detoxification processes are present.

The activity of the mitochondrial enzyme CcO was increased in the total homogenate of the selenium exposed organisms (Figure 4.4) indicating an increased response in this organelle to selenium. This was also evident in the mitochondrial fractions (Figure 4.5). This supports the organelle selenium distribution results (Table 4.2; Figure 4.3) which show that while the total percentage of selenium in the BAM fraction was only 35 and 33 % respectively in the 5 and 20 µg/g treatments compared to 53 % in the controls, the percentage in the mitochondrial fraction within this component increased from 34 % in the controls to 53 and 48 % respectively in the 5 and 20 µg/g treatments. This increase corresponded to a 5 and 7 fold increase, respectively, in mitochondrial selenium in the exposed organisms. As selenium is an essential component of the glutathione peroxidase enzyme it is expected that it will be present in the mitochondria where oxygen reduction and cellular energy production occurs, however, selenium toxicity can arise at concentrations only slightly greater than those that are required (Palace *et al.*, 2004). The percentage of selenium in the HSP fraction was only slightly less than that of the mitochondria in the selenium exposed organisms and higher in the controls (Table 4.2). The HSP fraction contains enzymes, high and low molecular weight proteins and other target molecules which are sensitive to metals. (Wallace *et al.*, 2003)

The increased binding of selenium in this fraction is not unexpected as selenium is largely associated with protein complexes (Ganther, 1974). The activity of the lysosomal enzyme AP was only increased in the 20 µg/g exposure while it was suppressed in the 5 µg/g (Figures 4.4; 4.5). The percentage of BAM selenium in the lysosomal+microsomal fraction of the selenium exposed organisms was around half that of the controls and the increase in selenium was only 2 and 3 times, respectively, higher in the 5 and 20 µg/g treatments than the controls (Table 4.2). The microsomal component of the cell includes fragmented endoplasmic reticulum, which is generally responsible for protein synthesis and transport, selenium in this fraction may be associated with microsomes rather than lysosomes which could be indicative of essential activity but equally could have implications for toxicity (Bonneris *et al.*, 2005).

4.4.2 Enzymatic Biomarkers – Oxidative Enzymes

Marine invertebrates exposed to elevated concentrations of metals are susceptible to intracellular fluxes of reactive oxygen species (ROS) mainly produced by the Fenton reaction in the redox cycling of transition metals (Winston, 1991) or by redox inactive metal ions such as cadmium, lead and selenium which can indirectly influence the oxidative system by reacting directly with cellular molecules to generate ROS, inducing cell signalling pathways (Leonard *et al.*, 2004) or depleting the cell's major sulfhydryl reserves (Ercal *et al.*, 2001).

Cadmium

The capacity to reduce reactive oxygen species (TAOC) was significantly reduced in *T. deltoidalis* in both cadmium treatments compared to the control organisms (Figure 4.6; Appendix 1.6). While the TAOC of the *T. deltoidalis* in the 50 µg/g cadmium treatment was lower than those in the 10 µg/g cadmium treatment the difference was not significant (Figure4.6; Appendix 1.6). A reduced oxyradical scavenging capacity in mussels exposed to a range of contaminants has also been reported: *Mytilus galloprovincialis*: sewage, agricultural, industrial and oil tanker effluents (Camus *et al.*, 2004), metals and PAHs (Frenzilli *et al.*, 2004; Regoli, 2000; Regoli *et al.*, 2004); *Modiolus modiolus*: cadmium (Dovzhenko *et al.*, 2005). A TAOC reduction in cadmium exposed *T. deltoidalis* indicates a breakdown in the reactive oxygen species detoxification pathway with the potential for higher order effects.

An investigation of glutathione cycling and the glutathione peroxidase enzyme (GPx) which are involved in the reduction of oxyradicals was undertaken to further investigate the mode of action of accumulated metal on the ROS reduction pathway of *T. deltoidalis*. Glutathione peroxidase activity and the total glutathione (GSH+2GSSG) were reduced in the cadmium treated organisms compared to the control organisms (Figure 4.6) but the reduction was not significant (Appendix 1.6). When the ratio of reduced glutathione (GSH) to oxidised glutathione (GSSG) is considered, a significant reduction was seen in the cadmium exposed organisms compared to the controls (Figure 4.6; Appendix1.6). Reduced glutathione (GSH) is a tripeptide of glutamine, cysteine and glycine which provides reducing equivalents for the GPx catalysed reduction of hydrogen peroxide to water and the respective alcohol. During this process GSH becomes oxidised glutathione (GSSG) which is recycled into GSH by glutathione reductase and NADPH (Tietze, 1969). In healthy cells the ratio is usually high, however, when cells are exposed to increased oxidative stress the ratio of GSH to GSSG decreases as a consequence of GSSG accumulation (Wataha *et al.*, 2000). The responsiveness of antioxidants to specific toxicants is difficult to predict and a high degree of variability has been reported in early work on marine bivalves relating to the class of chemical, exposure, organism type and phase in biological cycle (Regoli and Principato, 1995; Ribera *et al.*, 1989; Viarengo *et al.*, 1991; Viarengo *et al.*, 1989). The use of single antioxidant enzyme measurements to interpret exposure effects, therefore, may give inconclusive or contradictory results. The total glutathione concentration (GSH+2GSSG) has been investigated in marine bivalves exposed to metal contamination in numerous studies and has been reported as both increasing (Camus *et al.*, 2004; Frenzilli *et al.*, 2004; Regoli *et al.*, 2004; Regoli *et al.*, 1998a) and decreasing (de Almeida *et al.*, 2004; Regoli, 1998; Regoli *et al.*, 1998b; Regoli and Principato, 1995), indicating that glutathione may be up-regulated in response to metal contamination but at different concentrations and varying physiochemical conditions may be depleted when the system is overwhelmed. The suppression of total glutathione concentrations in *T. deltoidalis* by cadmium may be related to the high affinity of this molecule for metals, resulting in either a stable coordination complex or the oxidation of GSH to GSSG which may be subsequently excreted from the cell more rapidly than it can be reconverted to the reduced form by glutathione reductase (Regoli *et al.*, 2002). GSH cadmium complexes are not detectable with the methods used here. Despite the tendency for excess GSSG to be actively excreted from cells the decreased ratio of reduced to oxidised glutathione detected in the cadmium exposed organisms indicates that despite reduced GPx activity oxidation of glutathione was occurring.

Although not measured, it is probable that glutathione reductase activity was also reduced as evidenced by the increased concentrations of GSSG relative to GSH. The freshwater bivalve *Unio tumidus* exposed to a wide mix of contaminants including PAHs, persistent organics and metals showed a decrease in the GSH:GSSG ratio (Cossu *et al.*, 2000; Cossu *et al.*, 1997). The GSH:GSSG ratio has not been used widely in environmental toxicological work but may be a more useful indicator of the oxidative status than total glutathione concentrations. Regoli *et al.*, (2002) recommends an integrative approach to the use of antioxidant measurements in ecotoxicology where the individual antioxidant parameters are used in understanding modes of toxic action of a stressor and integrated with the total antioxidant capacity to provide a more holistic assessment of the overall biological significance of the variations. The suppression of GPx, GSH and the GSH:GSSG ratio, together with the significantly reduced TAOC, in response to cadmium exposure clearly indicates cadmium induced impairment of the antioxidant system.

Lead

The TAOC of the lead exposed *T. deltoidalis* was significantly reduced in both treatments compared to the control organisms (Figure 4.6; Appendix 1.6). The reduction in TAOC of the 100 µg/g lead exposed organisms was similar to those exposed to cadmium. While the 300 µg/g lead exposed organisms had a slightly higher TAOC than the 100 µg/g lead exposed organisms, the difference was not significant (Figure 4.6; Appendix 1.6). Even so, this is a surprising result as the 300 µg/g lead exposed organisms had four times the lead burden of the 100 µg/g lead exposed organisms in the BAM fraction, of which 67 % was associated with the mitochondria (Table 4.1; Figure 4.3). *Mytilus edulis* exposed to lead accumulated it as granules in the lysosomes and nucleus causing impairment of lysosomal function, a reduction of the mitochondrial cristae and deformation of the nuclear envelope (Einsporn and Koehler, 2008). There was some suppression of the total GSH concentration and GPx activity in the 300 µg/g lead exposed *T. deltoidalis* but this was not significant. The GSH concentration and GPx activity in the 100 µg/g lead exposed organisms were no different to the control organisms and neither lead exposure resulted in a significant reduction in the GSH:GSSG ratio (Figure 4.6; Appendix 1.6). Studies of lead induced oxidative stress in rats showed lead was bound to glutathione leading to a decreased in the GSH:GSSG ratio (Tandon *et al.*, 2002). Lead exposure in the brown mussel *Perna perna* decreased the GSH:GSSG ratio (Dafre *et al.*, 2004). Exposure of *Perna perna* to 200 mg/L of lead caused the depletion of GSH after 12 hours and increased GPx activity after 120 hours (de Almeida *et al.*, 2004).

It has been suggested that GSH may protect cells from accumulating lead by forming insoluble sulphide adducts with lead and excreting these complexes (Alcutt and Pinto, 1994). A response such as this would be expected to deplete GSH, reducing antioxidant potential thereby increasing susceptibility to oxidative stress. This may have occurred in the early part of the *T. deltoidalis* lead exposure but not been apparent at the end of the 28 days. Earthworms, *Lampito mauritii*, exposed to 75, 150 and 300 mg/kg Pb^{2+} spiked soil showed a significant decrease in the GSH:GSSG ratio at day 2 and 7 of the exposure but returned to pre-exposure GSH:GSSG levels by day 14 and remained so until the end of the exposure at day 28 (Maity *et al.*, 2008). An adaptive response by *L. mauritii* to lead exposure in the Maity *et al.,* (2008) study is possible, however, the lead tissue dose was not measured so it is unclear whether the oxidative response was linked to an increased metal dose or if the return to the pre-exposure concentrations of glutathione was due to a cessation of lead uptake. Although lead has the capacity to indirectly influence the oxidative system by reacting directly with cellular molecules to generate ROS my results indicate that the glutathione ROS reduction system of *T. deltoidalis* was not significantly affected by tissue lead accumulation. The reduced TAOC, however, shows that the overall antioxidant system was impaired indicating a probable increase in ROS which were not able to be completely reduced.

Selenium

Selenium is an essential element involved in the reduction of peroxide in the glutathione cycle which at elevated concentrations is toxic (Hodson, 1988; Hoffman, 2002; Micallef and Tyler, 1987). TAOC was significantly reduced in the selenium exposed *T. deltoidalis* compared to the control organisms but there was no difference in TAOC between selenium treatments (Figure 4.6; Appendix 1.6). The similarity in the TOAC response between selenium treatments may be explained by the similarity in the final selenium tissue concentrations between the two treatments (Figure 4.2). The activity of the GPx enzyme was enhanced in the *T. deltoidalis* from both selenium exposures although the activity was not significantly higher than the activity in the control organisms (Figure 4.6; Appendix 1.6). The increase in GPx activity appears to have increased the rate of oxidation of GSH as seen in the increased GSH+2GSSG concentration and the significantly reduced GSH:GSSG ratio (Figure 4.6; Appendix 1.6).

Studies in mallard ducks showed that increased dietary and subsequent selenium tissue concentrations resulted in increases in plasma and hepatic GPx activity and GSH concentrations, followed by a dose-dependant decrease in the ratio of hepatic GSH to GSSG concentrations which ultimately led to increased hepatic lipid peroxidation (Hoffman, 2002). Excess GSSG can react with protein sulfhydryls, contributing to the total thiol and protein bound thiol depletions, by the formation of mixed glutathione:protein disulphides. Formation of mixed disulphides may be part of a significant mechanism in regulating metabolic activity as well as the integrity of the cell membranes in response to oxidative stress (Hoffman, 2002). An examination of selenomethionine metabolism in embryos of rainbow trout *Oncorhynchus mykiss* showed oxidative stress which appeared to be generated by methioninase enzyme activity liberating methylselenol from L-Selenomethionine. The methylselenol is able to redox cycle in the presence of glutathione producing superoxide and likely accounts for oxidative stress measured in aquatic organisms environmentally exposed to excess selenomethionine (Palace *et al.*, 2004). Although the sediment in my study was spiked with sodium selenite which is readily bioaccumulated by bivalves and bound to proteins following assimilation into cells they do not have the capacity to transform it into selenomethionine (Wrisberg *et al.*, 1992). Marine algae and bacteria, however, are known to convert selenite mainly into selenomethionine (Wrisberg *et al.*, 1992) and this secondary pathway of dietary derived selenium may have resulted in significant selenomethionine exposure for *T. deltoidalis* during the course of the exposure.

4.4.3 Oxidative Damage Biomarker – Thiobarbituric Acid Reactive Substances

Thiobarbituric acid reactive substances TBARS are a measure of lipid peroxidation which is a widely recognised consequence of excess oxyradical production (Winston and Di Giulio, 1991). Like the oxygen reduction system the lipid production process is a complex sequence of biochemical reactions, broadly defined as oxidative deterioration of polyunsaturated fatty acids, which results in the production of highly reactive and unstable lipid radicals and a variety of lipid degradation products, the most abundant of which is malondialdehyde, which can alter the structure of cell membranes (Viarengo, 1989). The process of lipid peroxidation destabilises cell membranes which can lead to loss of lysosomal integrity and the leaking of the lysosomal contents into the cytoplasm (Winston *et al.,* 1991).

Cadmium

The TBARS concentration was increased in *T. deltoidalis* from both cadmium treatments compared to the control organisms, however, the difference was only significant in the 50 µg/g cadmium exposed organisms (Figure 4.7; Appendix 1.6). The 10 µg/g cadmium exposed organisms were not significantly different to the control or the 50 µg/g cadmium exposed organisms (Figure 4.7; Appendix 1.6). Other cadmium exposed marine bivalves have also shown reduced antioxidant enzyme activity and a consequent increase in lipid peroxidation (Chelomin *et al.*, 2005; Company *et al.*, 2004; de Almeida *et al.*, 2004; Legeay *et al.*, 2005). The TBARS concentration was highly negatively correlated with the TAOC (Figure 4.8), indicating that the progressive reduction in the capacity to reduce ROS and their subsequent increase directly influenced the build up of lipid peroxidation by-products.

Lead

The TBARS concentration was increased in *T. deltoidalis* from both lead treatments compared to the control organisms, however, the difference was only significant in the 300 µg/g lead exposed organisms which had significantly higher TBARS than both the control and the 100 µg/g lead exposed organisms (Figure 4.7; Appendix 1.6). A study of the effects of lead on the liver, kidneys and blood of the toadfish *Halobatrachus didactylus* over seven days did not show significant increases in TBARS although lysosomal integrity had been impaired and a variety of histological and histochemical changes were seen (Campana *et al.*, 2003). The livers of mallard ducks fed on diets containing 2 g/kg lead for three weeks had increased lipid peroxidation with no important histological changes observed (Mateo *et al.*, 2003). The marine bivalve *Perna viridis* exposed to 13 µg/L of lead over 30 days had a linear accumulation of lead into the tissues and enhanced TBARS concentrations relative to controls were seen in all tissues but this response was not linear over the 30 days (Prakash and Rao, 1995). The brown mussel *Perna perna* exposed to 200 mg/L of lead showed no significant increase in TBARS over 120 hours despite significant changes to antioxidant enzymes (de Almeida *et al.*, 2004). De Almeida *et al*., (2004) suggest that this may be due to elevated concentrations of the enzyme phospholipid hydroperoxide glutathione peroxidase, which is highly reactive towards peroxidised phospholipids, having a protective effect against lipid peroxidation. *T. deltoidalis* TBARS concentrations were poorly correlated with the TAOC (Figure 4.8) an increase in tissue lead resulted in a significant reduction in the TAOC (Figure 4.6) but the response was not linear for lead concentration while the TBARS concentration increase was linear for lead concentration (Figures 4.7).

Ercal *et al*., (2001) suggest that as lead cannot initiate lipid peroxidation on membrane lipids directly it might induce oxidative stress by interacting with oxyhaemoglobin, leading to peroxidative hemolysis in red blood cell membranes. As *T. deltoidalis* do not have haemoglobin, this pathway for lipid peroxidation is not available thereby reducing the total lipid peroxidation potential of lead in these organisms. It is appears from the range of different TBARS responses seen for lead exposure that lipid peroxidation alone may not be strong indicator for lead toxicity in the majority of marine invertebrates, however, when viewed together with the TAOC a pattern of lead induced perturbation in *T. deltoidalis* is apparent.

Selenium

The concentration of TBARS increased significantly in *T. deltoidalis* from both selenium treatments compared to the control organisms (Figure 4.7; Appendix 1.6). The 20 µg/g selenium exposed organisms had slightly higher TBARS than the 5 µg/g selenium exposed organisms (Figure 4.7; Appendix 1.6). Increased hepatic lipid peroxidation related to effects of accumulated selenium on glutathione metabolism have been measured in a number of wild aquatic birds, including their hatchlings and eggs (Hoffman, 2002). The TBARS concentration of the selenium exposed organisms was highly negatively correlated with the TAOC (Figure 4.8), indicating that increased tissue selenium resulted in a reduction in the capacity to reduce ROS and it is likely their subsequent increase directly influenced the build up of lipid peroxidation by-products.

4.4.4 Cellular Biomarker – Lysosomal Stability

Lysosomes are intracellular organelles that contain acid hydrolases for the digestion of cellular waste: including excess or damaged organelles; food particles; viruses and bacteria. The lysosomal interior is more acidic (pH ≈ 4.8) than the cytosol (pH ≈ 7.2) and it is enclosed in a single membrane which stabilises the low pH by pumping protons from the cytosol via proton pumps and ion channels. Metals can also enter lysosomes via these channels, or more usually as protein complexes with metallothioneins, and it is thought that from here they are then formed into granules for storage or excretion (Marigómez *et al.*, 2002). Metal accumulation in the lysosomes can induce lipid peroxidation through redox cycling or by direct reaction with cellular molecules to generate ROS. This can destabilise the lysosomal membrane causing the contents to leak out into the cytosol thereby reducing the cells capacity to remove waste which will ultimately lead to cell death (Viarengo *et al.*, 1987).

Cadmium

The 10 µg/g cadmium exposed *T. deltoidalis* had 33 % and the 50 µg/g 43 % lysosomal destabilisation. This was significantly higher than the control organisms which had less than 10 % of lysosomes destabilised (Figure 4.7; Appendix 1.6). Using the same assay technique for oyster hepatopancreas cells, Ringwood *et al*., (2003) developed the following criteria for the percentage of destabilised lysosomes that constitute cellular stress: < 15 – 30 % was considered the normal range; 30 – 40 % concern range; and > 40 % stressed. Given that the unexposed *T. deltoidalis* in the present study had less than 10 % lysosomal destabilisation this puts the cadmium exposed organisms well into the concern / stressed range. Cadmium accumulation has been linked to lysosomal destabilisation in other marine bivalves: mussels *Mytilus galloprovincialis* (Regoli *et al.*, 2004; Viarengo and Nott, 1993) and oysters *Crassostrea virginica* Ringwood *et al*., (2004; 2002). The lysosomal destabilisation was strongly negatively correlated with TAOC (Figure 4.8) and positively correlated with TBARS (Figure 4.9). This suggests that increased cadmium accumulation in metal sensitive tissue fractions initiated a reduction in the capacity to reduce ROS which may have both directly damaged lysosomal membrane but also induced lipid peroxidation which also induced lysosomal membrane destabilisation.

Lead

The lead exposed *T. deltoidalis* had significantly higher lysosomal destabilisation than the control organisms (Figure 4.7; Appendix 1.6). The 100 µg/g lead exposed *T. deltoidalis* had 40 % and the 300 µg/g 48 % lysosomal destabilisation which was higher than that of the cadmium exposed organisms and by the Ringwood *et al*., (2003) criteria they would be classed as stressed. *Mytilus galloprovincialis* transplanted in a lead contaminated area of the North Tyrrhenian Sea accumulated lead over time and showed a severe disturbance in lysosomal membrane stability with increased tissue lead (Regoli and Orlando, 1993). The *T. deltoidalis* lysosomal destabilisation was negatively correlated with TAOC (Figure 4.8) and positively correlated with TBARS (Figure 4.9). Despite the limited increase in TBARS of the 100 µg/g lead exposed *T. deltoidalis* the lysosomal destabilisation of organisms from both treatments was at a similar level (Figure 4.7), suggesting that lead interaction with the lysosomal membrane leading to its destabilisation was probably occurring via more than just the lipid peroxidation pathway. Ercal *et al*., (2001) suggests that lead alters the lipid composition of cellular membranes which may alter membrane integrity, permeability and function.

Selenium

The selenium exposed *T. deltoidalis* had significantly higher lysosomal destabilisation than the control organisms (Figure 4.7; Appendix 1.6). The 5 µg/g selenium exposed *T. deltoidalis* had 30 % destabilised lysosomes, which is in the concern range of the Ringwood *et al*., (2003) criteria. The 20 µg/g selenium exposed organisms had extremely high levels of lysosomal destabilisation, 68 %, which was higher than that of organisms from any of the other metal exposures and would be classed as stressed by the Ringwood *et al*., (2003) criteria. The selenium exposed organisms also had a significantly reduced GSH:GSSG ratio (Figure 4.6) and elevated TBARS concentrations (Figure 4.7) so a high degree of lysosomal destabilisation is not surprising. The very high instability in the 20 µg/g selenium exposed *T. deltoidalis* compared to the 5 µg/g selenium exposed organisms is surprising as the day 28 selenium tissue concentrations of organisms from both treatments were similar (Figure 4.1). The BAM selenium burden of the 20 µg/g selenium exposed organisms was ≈ 1.7 times that of the 5 µg/g selenium exposed organisms and this may account for the significantly higher lysosomal membrane damage. The lysosomal destabilisation in the selenium exposed *T. deltoidalis* was negatively correlated with TAOC although the correlation was not particularly strong (Figure 4.8) and positively correlated with TBARS (Figure 4.9). Despite there being no significant difference between the TAOC and TBARS concentrations between the 5 and 20 µg/g selenium exposed *T. deltoidalis*, the lysosomal destabilisation of organisms from the 20 µg/g selenium treatment was significantly higher than the 5 µg/g exposed organisms (Figures 4.6 & 4.7). The lysosomal fraction of the selenium exposed organisms did not have a marked selenium burden increase, the majority of BAM was associated with the mitochondrial and HSP fractions (Table 4.2). Selenium binding to metal sensitive molecules present in the HSP of the cytosol may contribute to the total thiol and protein bound thiol depletions, which may be part of a significant mechanism in regulating metabolic activity as well as the integrity of the cell membranes in response to oxidative stress (Hoffman, 2002).

4.4.5 Genotoxic Biomarker – Micronuclei Frequency

Micronuclei are small, intracytoplasmic masses of chromatin resulting from chromosomal breakage or aneuploidy during cell division. As an index of chromosomal damage the micronucleus test is based on the enumeration of downstream aberrations after DNA damage and gives a time-integrated response to toxic exposure. The MN test is a fast and sensitive test to detect genomic damage due to both clastogenic effects and alterations to the mitotic spindle (Migliore *et al.*, 1987). It has been used in bivalves to examine the genotoxicity of a range of chemicals including metals (Bolognesi *et al.*, 2004; Burgeot *et al.*, 1996; Scarpato *et al.*, 1990; Williams and Metcalfe, 1992).

Cadmium

The 10 µg/g cadmium exposed *T. deltoidalis* had significantly more micronuclei than the control organisms and the 50 µg/g cadmium exposed *T. deltoidalis* had significantly more micronuclei than both the control and the 10 µg/g cadmium exposed organisms (Figure 4.7; Appendix 1.6). Increased micronuclei frequency has been observed in wild and, after 30 days, in caged mussels *Mytilus galloprovincialis* exposed to PAHs, cadmium and mercury along a pollution gradient on the Ligurian coast of Italy (Bolognesi *et al.*, 2004) and to a mix of metals near an offshore platform in the Adriatic sea, Italy (Gorbi *et al.*, 2008). The frequency of micronuclei in the cadmium exposed *T. deltoidalis* was negatively correlated with TAOC (Figure 4.8) indicating that an increase in ROS resulted in an increase in genotoxic damage. DNA in cellular nuclei is a key cellular component that is particularly susceptible to oxidative damage by ROS (Cerutti, 1985). The frequency of micronuclei in the cadmium exposed *T. deltoidalis* was positively correlated with TBARS (Figure 4.9) suggesting that an increase in lipid peroxidation products may have contributed to an increase in genotoxic damage. Gorbi *et al*., (2008) found zinc and cadmium bioaccumulation only slightly increased oxidative stress, intracellular accumulation of neural lipids and lysosomal destabilisation but micronuclei frequency increased significantly, particularly during winter. Micronuclei frequency appears to be a sensitive indicator of cadmium toxicity in *T. deltoidalis*.

Lead

T. deltoidalis exposed to both lead treatments had a significantly higher micronuclei frequency than the control organisms but they were not significantly different to each other (Figure 4.7; Appendix 1.6). The frequency of micronuclei in the lead exposed *T. deltoidalis* was much lower than that of the cadmium exposed organisms (Figure 4.7) which is generally in keeping with the pattern seen for the other stress indices (Figures 4.6 & 4.7). This suggests that cadmium is a genotoxic agent exerting a direct influence on the induction of micronuclei, perhaps in concert with ROS, while lead had a more indirect influence perhaps via ROS alone. Lead, for example, has been shown to affect the expression of 2 nuclear transcription factors: NF-kB and HIF-1 which may lead to cell cycle arrest, apoptosis or interruption to gene transcription, while cadmium affects 3: NF-kB, and AP-1 and p53 which help protect cells from carcinogenesis (Leonard *et al.*, 2004). The frequency of micronuclei in the lead exposed *T. deltoidalis* was negatively correlated with TAOC (Figure 4.8) and positively correlated with TBARS (Figure 4.9) indicating that an increase in ROS contributed to an increase in genotoxic damage.

Selenium

The 5 µg/g selenium exposed *T. deltoidalis* had significantly more micronuclei than the control organisms and the 20 µg/g selenium exposed *T. deltoidalis* had significantly more micronuclei than both the control and the 5 µg/g selenium exposed organisms (Figure 4.7; Appendix 1.6). Induction of micronuclei in response to selenium bioaccumulation has not previously been investigated in bivalves, however, these results fit the pattern of metal induced genotoxic damage as increased frequency of micronuclei shown for: *Mytilus galloprovincialis* (Bolognesi *et al.*, 2004; Dailianis *et al.*, 2003; Gorbi *et al.*, 2008; Kalpaxis *et al.*, 2004). Increased micronuclei frequency in response to selenium exposure has been observed in fish erythrocyte cells (al Sabti, 1994) and mice bone marrow (Itoh and Shimada, 1996). The frequency of micronuclei in the selenium exposed *T. deltoidalis* was negatively correlated with TAOC (Figure 4.8) and positively correlated with TBARS (Figure 4.9) indicating that an increase in ROS contributed to an increase in genotoxic damage, either through interaction of reactive oxygen intermediates and lipid peroxidation products with DNA or direct interaction of selenium with cellular macromolecules forming adducts, alkaline labile sites and strand breaks (Regoli *et al.*, 2004).

4.5 Summary and Conclusions

4.5.1 Cadmium

Cadmium exposed *T. deltoidalis* accumulated cadmium over 28 days and reached equilibrium tissue concentrations which were equal to that of the sediment cadmium exposure concentrations. Approximately 50 % of accumulated cadmium was detoxified. *T. deltoidalis* exposed to 10 µg/g of cadmium converted 25 % and those exposed to 50 µg/g of cadmium converted 60 % of BDM to MRG with the remainder in the MTLP fraction. The majority of BAM cadmium in exposed organisms was in the mitochondrial fraction. Mitochondrial cadmium burdens of the 10 and 50 µg/g cadmium exposed *T. deltoidalis* were 2000 and 7200, respectively, greater than the control organisms and this was associated with an increase in the activity of the mitochondrial cytochrome c oxidase enzyme. The TAOC and ratio of GSH:GSSG of cadmium exposed *T. deltoidalis* was significantly reduced compared to control organisms. The impairment of the oxidative system initiated a cascade of cellular damage. Lipid peroxidation increased, contributing to significant lysosomal destabilisation and increased frequency of micronuclei. The significant exposure – dose – response relationships for cadmium established in this study indicate that sediment cadmium at these concentrations has the potential to lead to increased BAM burdens and impairment of individual *T. deltoidalis* at a cellular and subcellular level. This has implications for higher order effects which may impact on population viability in the long term.

4.5.2 Lead

The response of *T. deltoidalis* to lead exposure differed between sediment lead exposure concentrations. Accumulation was rapid at both lead exposure concentrations in the first 3 days of the exposure, however, the 100 µg/g lead exposed organisms continued to accumulate slowly over the remaining exposure time reaching an equilibrium tissue lead concentration which was equal to that of the sediment lead exposure concentration, while the 300 µg/g lead exposed organisms had a pattern of pulses of uptake and loss over the remainder of the exposure suggesting binding site saturation may have occurred. Final tissue concentrations of lead in the 300 µg/g lead exposed organisms were 1.5 times the sediment lead and this points to the lead regulatory mechanisms being overwhelmed. Around 70 % of accumulated lead was detoxified.

T. deltoidalis from both lead exposures converted about 75 % of BDM lead to MRG with the remainder in the MTLP fraction. *T. deltoidalis* exposed to 100 µg/g of lead had 50 % and those exposed to 300 µg/g of lead 67 % of BAM in the mitochondrial which corresponded to a 30 and 154, respectively, fold increase in lead burden in this organelle. An increased lead burden in the lysosomal+microsomal fraction was also seen in the lead exposed organisms with 82 and 188 fold increases in the 100 µg/g and 300 µg/g, respectively, lead exposed organisms compared to the controls. The TAOC of lead exposed *T. deltoidalis* was significantly reduced compared to control organisms but this was not associated with significant changes in the glutathione cycling or GPx enzyme activity. TBARS concentration was only significantly higher in the 300 µg/g lead exposed organisms compared to both the control and the 100 µg/g lead exposed organisms, however, the lysosomal destabilisation and frequency of micronuclei was significantly increased in organisms from both lead treatments. The exposure – dose showed a strong relationship for lead with significant increases in BAM lead burdens. The dose - response relationship was mixed, with a poor correlation between TAOC and TBARS but strong negative relationships between TAOC and lysosomal destabilisation and increased micronuclei and positive correlations between TBARS and lysosomal destabilisation and increased micronuclei frequency. The exposure – dose – response relationship demonstrated in this experiment for lead in *T. deltoidalis* indicates that lead exposure leads to significant tissue lead accumulation and increased BAM lead burdens with associated cellular perturbations. The mixed response to lead observed for the effects biomarkers supports the multibiomarker approach, to both investigate the mechanisms of toxicological effect and by using interlinked biomarkers demonstrate a cascade of effects.

4.5.3 Selenium

Accumulation was rapid at both selenium exposure concentrations in the first 3 days of the exposure. There was a pattern of loss and uptake in both exposures over the remainder of the exposure suggesting binding site saturation may have occurred. An equilibrium concentration appears to have been reached in organisms from both exposures after 4 weeks, however, this was at a similar selenium concentration for organisms from both treatments which for the 5 µg/g selenium exposed organisms was 5 times the exposure concentration. This may be due to an increase in dietary exposure over the course of the exposure. Forty four to 48 % of the accumulated selenium was in the nuclei+cellular debris fraction of the selenium exposed organisms.

This was probably comprised of a combination of protein bound selenium associated with plasma and selenium bound directly to cell walls and was therefore effectively removed from active sites within the cell. The percentage of selenium increased in the BDM fraction exposed organisms compared to the controls. The 5 μg/g selenium exposed organisms had an even distribution between the MRG and MTLP fractions, while the 20 μg/g selenium exposed organisms had more than 60 % in the MRG fraction with the remainder in the MTLP. Selenium associated with MT and granules has not previously reported for marine bivalves. Selenium has been associated with low molecular weight proteins and it is likely that these act as storage and transport and intermediaries in the synthesis of selenoproteins and therefore may represent a pool of detoxified selenium. The strong association of selenium with sulphur is a likely mechanism for the incorporation of selenium into granules as has been demonstrated for cadmium. Around half of the BAM selenium was in the mitochondrial fraction of the selenium exposed organisms with a 5 and 7 fold increase, respectively, in mitochondrial selenium burden in the 5 and 20 μg/g selenium exposed organisms. This is not unexpected as selenium is an essential component of GPx which is located in the mitochondria. The TAOC and ratio of GSH:GSSG of selenium exposed *T. deltoidalis* was significantly reduced compared to the control organisms. This initiated an increase in lipid peroxidation, lysosomal destabilisation and micronuclei frequency. This study has demonstrated a significant exposure – dose – response relationship for selenium in *T. deltoidalis*. Dietary selenium uptake contributed to higher than ambient selenium tissue concentrations. BAM selenium burdens were increased with selenium exposure and this lead to impairment of the antioxidant system with associated lipid peroxidation and damage to cellular membranes and DNA.

5 *Anadara trapezia* Cadmium Spiked Sediment Study

5.1 Aim

The purpose of this study is to determine the exposure - dose - response of *A. trapezia* to sediments with known concentrations of cadmium. The exposure time was extended to 56 days and the development of useful biomarkers of effect undertaken with a view to determining whether they would be a suitable organism to collect from the field and use in field metal exposures in cages.

5.2 Results

5.2.1 Cadmium Accumulation

5.2.1.1 *Whole Organism Cadmium Accumulation*

ANOVA showed that the factors time and sediment cadmium treatment were both highly significant in cadmium accumulation and there was a significant interaction between time and cadmium treatment (Appendix 2.1). Tissue concentrations were in the order 50 µg/g > 10 µg/g > control for each analysis time (Figure 5.1). The tissue cadmium concentration of the control organisms remained the same over the course of the exposure (Figure 5.1). Bonferroni pair-wise comparisons showed that the cadmium tissue concentrations of the control organisms did not differ significantly from day 0 unexposed organisms (Appendix 2.2). The pattern of cadmium accumulation was similar in organisms from both cadmium treatments (Figure 5.1). The over-all statistical comparison between treatments showed organisms from both the cadmium treatments differed significantly from the unexposed and control organisms and from each other at all collection times (Appendix 2.2). The tissue cadmium concentrations of the 50 µg/g treatment organisms were close to those of the 10 µg/g organisms for the first 28 days with a rapid accumulation to day 42 resulting in a final concentration which was nearly double that of the 10 µg/g treatment. Further analysis between treatments showed that the 50 µg/g exposed organisms had significantly more tissue cadmium than the 10 µg/g at day 14 but not at day 28 and on days 42 and 56 the difference was of low significance (Appendix 2.3). The cadmium exposed organisms reached the highest tissue concentrations at day 42 with a plateau to day 56 suggesting that an equilibrium tissue cadmium concentration was reached (Figure 5.1).

The 50 µg/g exposed organisms had a final tissue cadmium concentration half that of the exposure concentration, while the 10 µg/g organisms had a concentration 1.2 times that of the sediment (Figure 5.1). The regression between cadmium sediment concentration and organism tissue cadmium concentration after 56 days shows a significant positive relationship (Figure 5.2), as sediment cadmium concentration increased from 10 µg/g to 50 µg/g the tissue cadmium accumulation increased, but not proportionally (Figure 5.2). Analysis of the within treatment differences between collection days showed that there was no significant difference over time in the cadmium concentrations of control organisms (Appendix 2.4). The 10 µg/g cadmium exposed organisms had no significant increase in tissue cadmium for the first 14 days of exposure but had significant uptake between the 14^{th} and 42^{nd} days while the 50 µg/g cadmium exposed organisms had a significant cadmium increase at each collection day up to day 42 (Appendix 2.4). The decrease in cadmium concentration from day 42 to day 56 (Figure 5.1) was not significant for either treatment (Appendix 2.4).

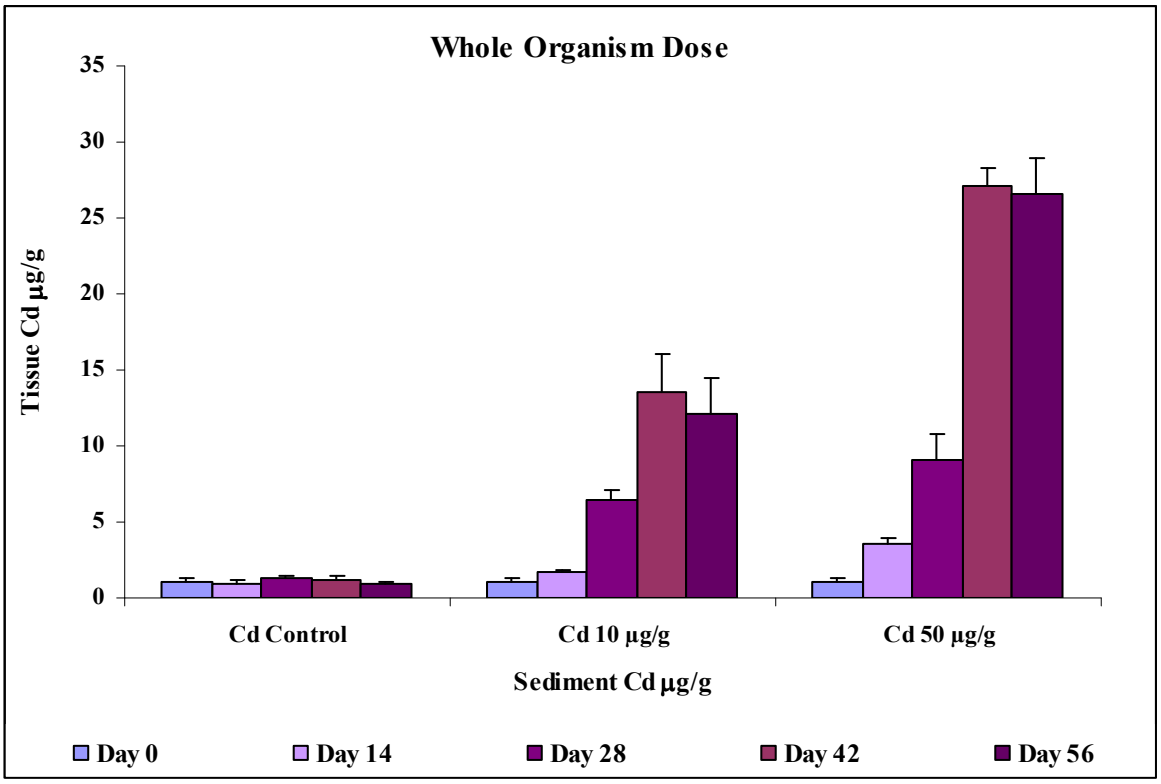


Figure 5.1: Cadmium accumulation in whole tissue of *A. trapezia* at 2 weekly intervals over 56 days of exposure to sediments containing cadmium at 0 (control), 10 & 50 µg/g dry mass. Mean ± SE, n = 8, 7 and 7 respectively. Day 0 are unexposed organisms n=5.

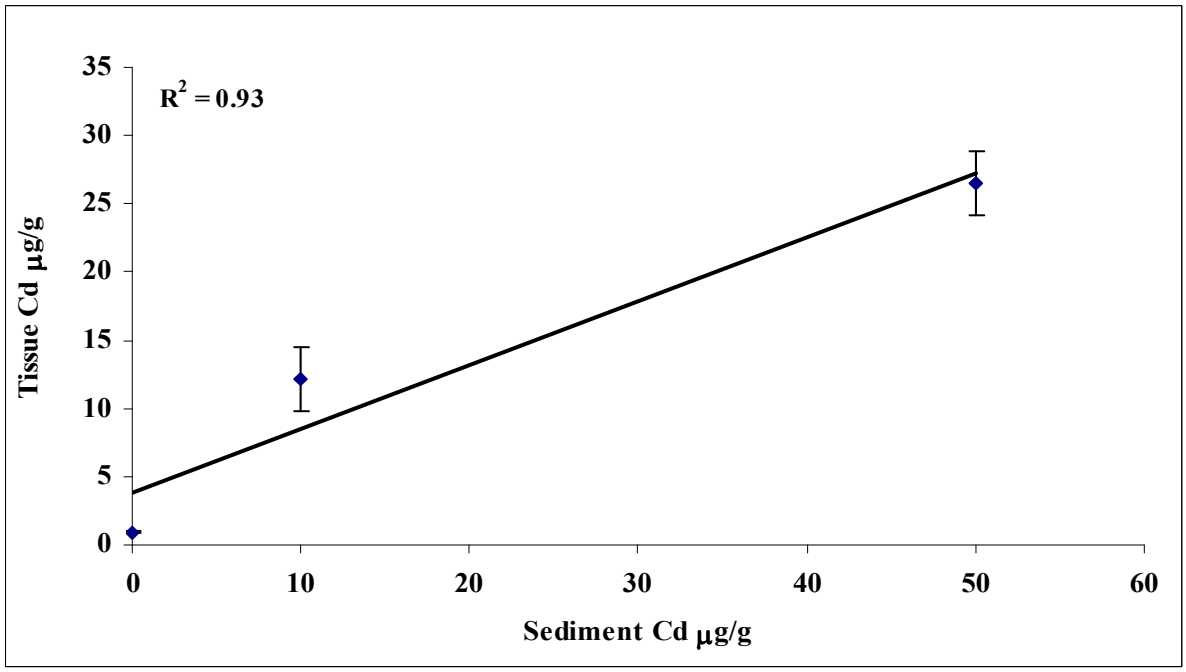


Figure 5.2: Regression of *A. trapezia* tissue cadmium concentration after 56 days exposure against sediment cadmium exposure concentrations of 0, 10 and 50 µg/g dry mass. Mean ± SE, n = 8, 7 and 7 respectively.

5.2.1.2 *Individual Tissue Cadmium Accumulation*

ANOVA showed that cadmium accumulation was significantly different between tissues and there was a significant interaction between the factors tissues, time and cadmium treatment (Appendix 2.5). Bonferroni multiple pair-wise comparisons showed significant differences between all tissues for all treatments (Appendix 2.6). Analysis of the between tissue differences at each collection time showed that cadmium concentrations were significantly different between all tissues at all times except for the hepatopancreas and haemolymph tissues of the 50 µg/g treated organisms on day 14 (Appendix 2.7).

Gill

Tissue cadmium concentrations were higher in gills than the other two tissues for all treatments at all analysis times (Figure 5.3). Accumulation was greatest between days 28 and 42 for both cadmium treatments (Figure 5.3). Analysis of the within gill tissue differences between collection days showed that the 10 µg/g cadmium exposed organisms had a significant uptake between the 14^{th} and 42^{nd} days while the 50 µg/g cadmium exposed organisms had a significant cadmium increase in gill tissue at each collection day up to day 42 (Appendix 2.8). The control organisms had no significant increase in gill cadmium concentration over the course of the experiment (Appendix 2.8). Cadmium concentrations were similar in the gills of organisms at day 14 and 28 in both cadmium treatments, however, the cadmium concentration of 50 µg/g treatment gill tissues was about twice that of the lower treatment gills by day 42 (Figure 5.3). Analysis of the within tissue differences between collection days showed that the decrease in gill tissue cadmium concentration of the 50 µg/g exposed organisms from day 42 to day 56 (Figure 5.3) was not significant (Appendix 2.8). The pattern of cadmium accumulation in the gills is reflected in the whole organism dose (Appendix 2.4 & 2.8).

Hepatopancreas

Tissue cadmium concentrations were generally lower in the hepatopancreas than in the gills and higher than the haemolymph (Figure 5.3). The cadmium accumulation pattern of the hepatopancreas appears similar to that of the gills in both cadmium treatments (Figure 5.3), however, pair-wise comparisons of the within hepatopancreas tissue cadmium concentrations over time showed that while the control organisms had no significant change in cadmium over the course of the exposure and uptake in the 50 µg/g cadmium exposed organisms was significant between each collection time for the first 42 days the 10 µg/g cadmium exposed organisms only had a significant increase in hepatopancreas tissue cadmium between the 14th and 28th day of exposure (Appendix 2.8). The total cadmium concentration in this tissue was around half that of the gills for the 14 to 56 day analysis times in the 10 µg/g treatment, this was also the case for day 14 and 28 for the 50 µg/g treatment, however, by day 42 the cadmium concentration of the hepatopancreas was only a quarter of that of the gills. There was no difference in hepatopancreas cadmium concentration between organisms from the 10 and 50 µg/g cadmium treatments during the course of the experiment (Figure 5.3).

Haemolymph

Tissue cadmium concentrations were generally lower in the haemolymph than in the other two tissues (Figure 5.3). The cadmium concentrations were similar in the haemolymph of both treatments and remained relatively stable during the course of the experiment (Figure 5.3). Pair-wise comparisons of the within haemolymph cadmium concentrations over time showed that the slightly higher cadmium concentration at day 42 in the control organisms was significantly different to days 14 and 56, however, the significance was low (Appendix 2.8). The pattern of cadmium accumulation in the haemolymph of the 10 µg/g exposed organisms was the same as that of the hepatopancreas tissue (Appendix 2.8). The haemolymph of the 50 µg/g exposed organisms showed a significant increase in cadmium after the first 14 days, following a drop in concentration at day 28 (Figure 5.3), then another increase of lower significance to day 42 (Appendix 2.8).

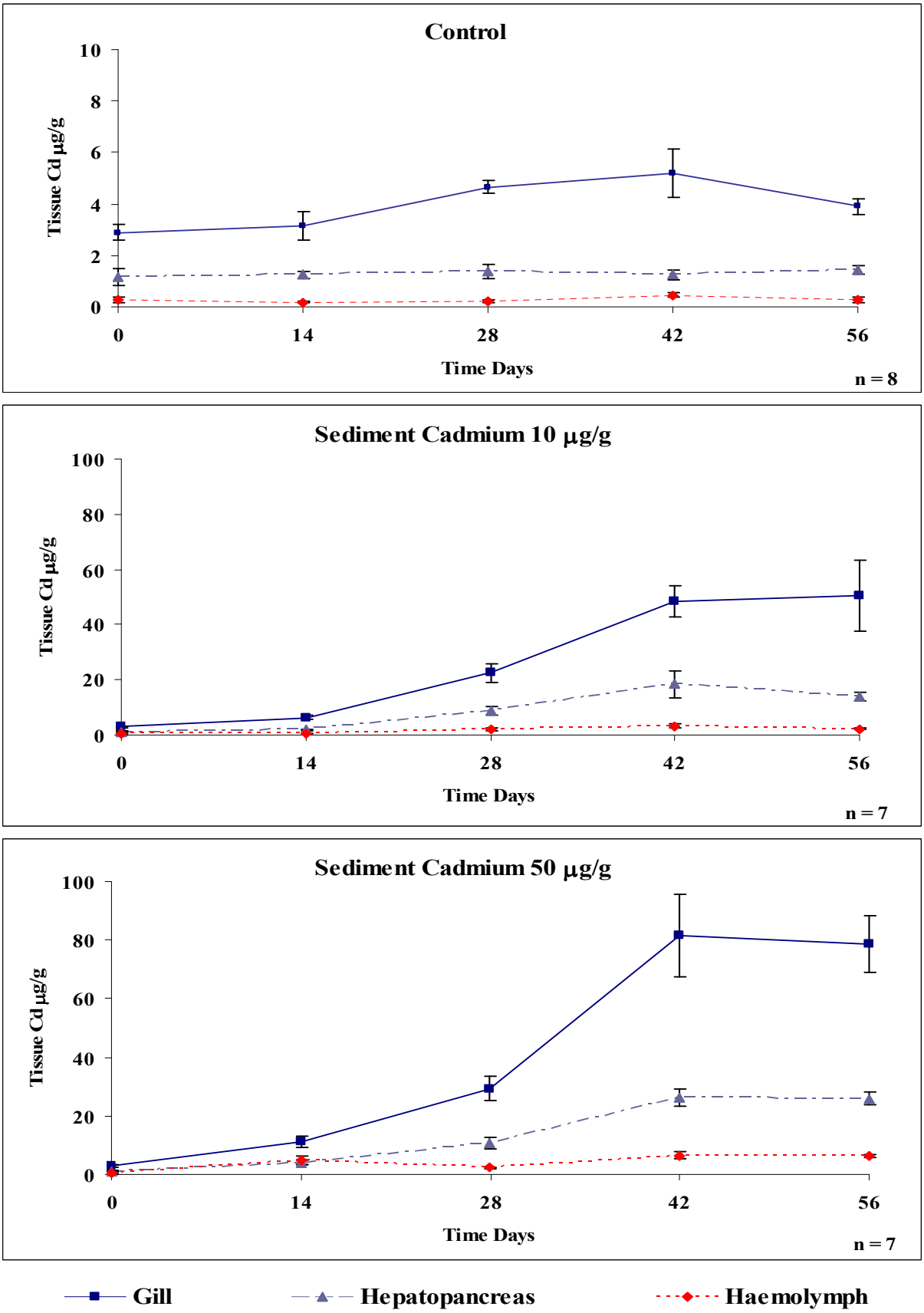


Figure 5.3: Cadmium accumulation in gill, hepatopancreas and haemolymph tissues of *A. trapezia* at two weekly intervals for 56 days exposure to sediments spiked with cadmium at; 0 (control), 10 and 50 µg/g dry mass. Mean ± SE. Day 0 are unexposed organisms, n=5.

5.2.2 Subcellular Tissue Cadmium Distribution

5.2.2.1 *Gill*

Between 45 and 70 % of the total cadmium was recovered in the fractions (Table 5.1). Of the metal recovered in the fractions between 66 and 73 % was in the biologically detoxified metal (BDM) fraction (Table 5.1). The percentage of metal recovered in the biologically active metal (BAM) fraction of each of the 10 and 50 µg/g cadmium treatments was less than that of the control, however, the total cadmium burden (µg) within these fractions were 10 and 100 times respectively, greater than the unexposed organisms (Table 5.1). The majority of BDM was in the MTLP fraction for all treatments (Figure 5.4), although the percentage of cadmium in the MRG fraction increased with the cadmium exposure concentration in the order, control < 10 µg/g < 50 µg/g treated organisms (Table 5.2). The highest percentage of cadmium in the BAM fractions of all treatments was in the mitochondria (≈ 50 %) with the remainder equally distributed between the heat sensitive proteins (HSP) (≈ 25%) and lysosome+microsome fractions (≈ 25 %) (Figure 5.4; Table 5.2).

5.2.2.2 *Hepatopancreas*

The distribution of cadmium in the hepatopancreas fractions was similar to that in the gills (Table 5.1; Figure 5.4). Between 42 and 82 % of the total cadmium was recovered in the fractions (Table 5.1). Of the metal recovered in the fractions the control organisms had 46 % in the BDM fraction (Table 5.1). This increased to 66 and 67 % respectively in the 10 and 50 µg/g cadmium exposed organisms (Table 5.1). The percentage of metal recovered in BAM fraction of each of the 10 and 50 µg/g cadmium treatments was less than half that of the control, however, the total cadmium burden (µg) within this fractions was 20 and 30 times respectively, greater in the cadmium exposed organisms (Table 5.1). The majority of BDM was in the MTLP fraction for all treatments (Figure 5.4), although, the percentage in the MRG fraction increased in the cadmium exposed organisms in the order, control < 50 µg/g < 10 µg/g treated organisms (Table 5.2). The highest percentage of cadmium in the BAM fractions of the cadmium treatments was in the mitochondria (≈ 50 %) with the remainder equally distributed between the heat sensitive proteins (HSP) (≈ 25 %) and lysosome+microsome fractions (≈ 25 %) (Figure 5.4: Table 5.2). The cadmium in the BAM fractions of the control was fairly equally distributed among the three fractions (Figure 5.4; Table 5.2).

Table 5.1: Cadmium concentrations (µg wet mass) in gill and hepatopancreas tissues and the total cadmium, with percentage, recovered from subcellular fractions of *A. trapezia* after 56 days exposure to cadmium spiked sediments. Metal subcellular concentrations (µg wet mass) and percentage distribution of total recovered cadmium fractions are grouped as nuclei+cellular debris and biologically active and detoxified metal (Figure 3.7). Mean ± SD, n = 2.

	Gill			**Hepatopancreas**		
	Cd control	**Cd 10 µg/g**	**Cd 50 µg/g**	**Cd control**	**Cd 10 µg/g**	**Cd 50 µg/g**
Total Tissue Cadmium (µg)	**0.1 ± 0.01**	**2.6 ± 0.3**	**13 ± 1.3**	**0.04 ± 0.03**	**1.9 ± 1**	**3.1 ± 0.7**
Total Recovered Cadmium. (µg)	**0.1 ± 0**	**1.2 ± 0.03**	**9 ± 4**	**0.03 ± 0.03**	**0.8 ± 0.4**	**1.6 ± 0.2**
Proportion of total recovered in fractions (%)	69 ± 9	45 ± 6	70 ± 38	82 ± 16	42 ± 2	51 ± 4
Cadmium Distribution						
Nuclei + Cellular debris (µg)	**0.005 ± 0**	**0.1 ± 0**	**0.8 ± 0.3**	**0.003 ± 0**	**0.1 ± 0.1**	**0.2 ± 0.1**
Nuclei + Cellular debris (%)	8 ± 1	8 ± 0.6	10 ± 2	8 ± 4	13 ± 3	15 ± 2
Biologically Active Metal (µg)	**0.02 ± 0**	**0.2 ± 0.01**	**2 ± 1**	**0.01 ± 0.01**	**0.2 ± 0.1**	**0.3 ± 0.03**
Biologically Active Metal (%)	26 ± 7	19 ± 1	19 ± 2	46 ± 15	19 ± 1	19 ± 3
Biologically Detoxified Metal (µg)	**0.04 ± 0.01**	**0.9 ± 0.1**	**6 ± 3**	**0.02 ± 0.02**	**0.5 ± 0.3**	**1 ± 0.1**
Biologically Detoxified Metal (%)	66 ± 8	73 ± 2	71 ± 0	46 ± 11	66 ± 1	67 ± 1

Table 5.2: Mean percentage of cadmium in the debris, biologically detoxified metal (BDM) and biologically active metal (BAM) with the percentage of metal that each of the fractions within contributes to the BDM or BAM fraction of *A. trapezia* subcellular tissue fractions after 56 days exposure to cadmium spiked sediments., n = 2.

	Gill			**Hepatopancreas**		
	Cd control	**Cd 10 µg/g**	**Cd 50 µg/g**	**Cd control**	**Cd 10 µg/g**	**Cd 50 µg/g**
Nuclei + Cellular debris % of total	**8**	**8**	**10**	**8**	**13**	**15**
BDM % of total	**66**	**73**	**71**	**46**	**67**	**66**
Metal Rich Granules % of BDM	5	9	12	5	16	13
Heat Stable MT Like Proteins % of BDM	95	91	88	95	84	87
BAM % of total	**26**	**19**	**19**	**46**	**19**	**19**
Mitochondria % of BAM	47	50	44	37	46	47
Lysosomes + Microsomes % of BAM	26	26	29	33	28	26
Heat Sensitive Proteins % of BAM	27	24	27	31	26	27

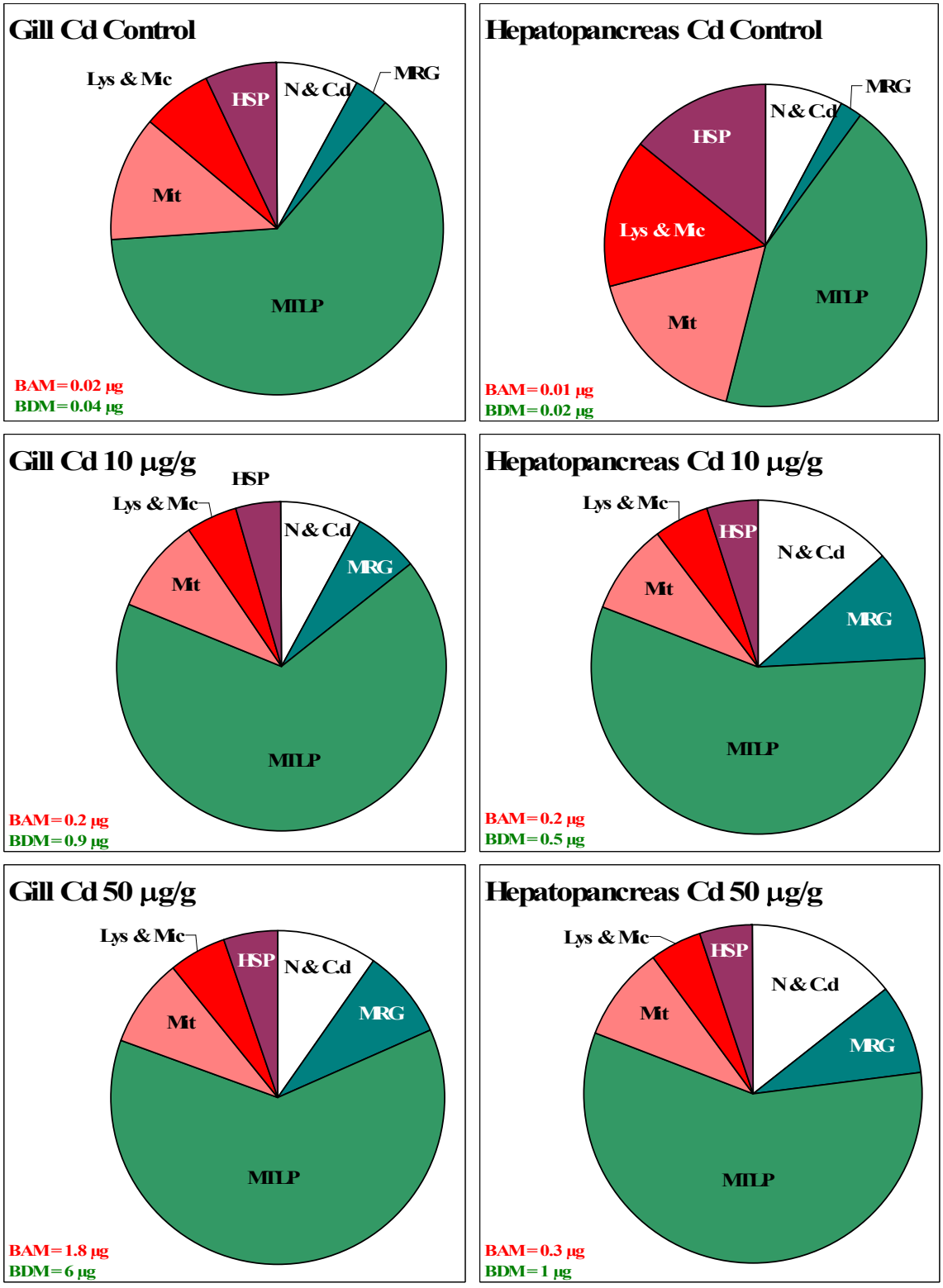


Figure 5.4: Distribution (%) of cadmium in the subcellular fractions of *A. trapezia* gill and hepatopancreas tissues following 56 days of exposure to cadmium spiked sediments. Subcellular fractions are: nuclei+ cellular debris (N & Cd); metal rich granules (MRG); heat stable metallothionein like proteins (MTLP); mitochondria (Mit); lysosomes+ microsomes (Lys & Mic); heat sensitive proteins (HSP). Red fractions ( ) make up the biologically active metal (BAM), green fractions ( ) make up the biologically detoxified metal (BDM), n = 2.

5.2.3 Activity of Marker Enzymes

5.2.3.1 *Whole Tissue Enzyme Activity*

The activity in whole tissue of the acid phosphatase (AP) lysosomal enzyme was greater in the hepatopancreas tissues of the control and 50 µg/g cadmium exposed organisms while in the 10 µg/g cadmium exposed organisms it was equal to that of the gill (Figure 5.5). Cytochrome c oxidase (CcO) mitochondrial enzyme activity was greater in the gill than in the hepatopancreas for all treatments (Figure 5.5). AP activity was reduced in the gill of 50 µg/g cadmium exposed organisms compared to the control and 10 µg/g exposed organisms (Figure 5.5). CcO was greater in the gills and hepatopancreas of cadmium exposed organisms than in the tissues of the control organisms (Figure 5.5).

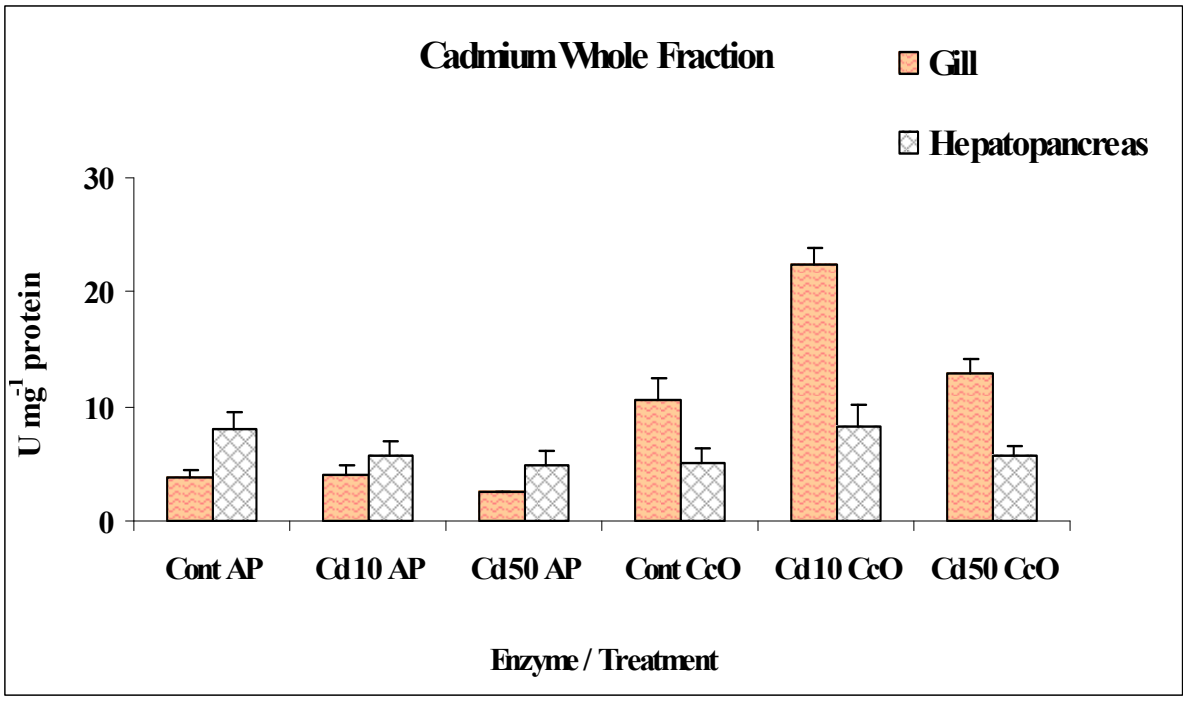


Figure 5.5: Activity of specific marker enzymes for lysosomes (acid phosphatase (AP)) and mitochondria (cytochrome c oxidase (CcO)) in whole gill and hepatopancreas tissue of *A. trapezia* exposed to cadmium spiked sediments at 0, 10 and 50 µg/g dry mass. Mean ± SD, n = 2.

5.2.3.2 *Subcellular Fraction Enzyme Activity*

Gill and Hepatopancreas

Enzyme activities in the subcellular fractions of all treatments indicate that the CP 3 fractions of both tissues were enriched in mitochondria (Figure 5.6). The CP 4 fractions of the gills were enriched with lysosomes compared to the CP3 fractions but had some carry over of mitochondria (Figure 5.6). There was also some carry over of mitochondria into the hepatopancreas CP 4 fractions. The lysosomal activity of the hepatopancreas CP4 fractions generally higher those of the gill. There was some lysosomal enzyme activity present in the CP 3 fractions (Figure 5.6).

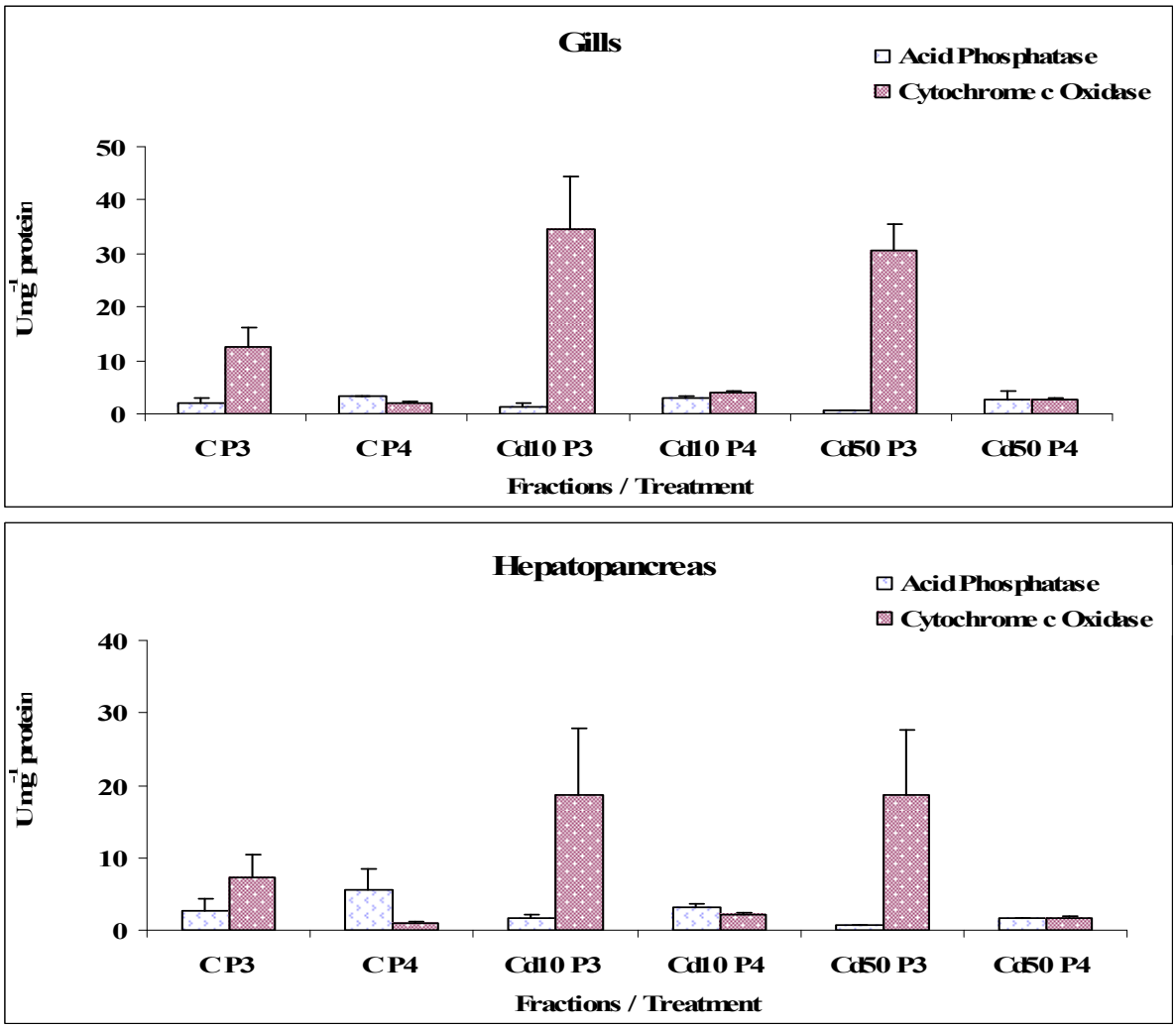


Figure 5.6: Activity of specific marker enzymes for lysosomes (acid phosphatase) and mitochondria (cytochrome c oxidase) in the mitochondrial fraction (P3) and lysosomal+microsomal fraction (P4) following subcellular fractionation of gill and hepatopancreas tissue of *A. trapezia* exposed to cadmium spiked sediments at 0 (C), 10 and 50 µg/g dry mass. Mean ± SD, n = 2.

5.2.4 Enzymatic Biomarkers

5.2.4.1 *Total Antioxidant Capacity*

The total antioxidant capacity (TAOC) of cells was reduced in both the cadmium treatments, relative to the control organisms (Figure 5.7). ANOVA showed that the factor sediment cadmium concentration was highly significant in TAOC (Appendix 2.9). Bonferroni pair-wise comparison showed that while organisms from both cadmium treatments had significantly lower TAOC than those of the control they were not significantly different from each other (Figure 5.7, Appendix 2.10).

5.2.4.2 *Glutathione Peroxidase Activity*

Glutathione peroxidase activity was reduced in both of the cadmium treatments compared to the control organisms (Figure 5.7). ANOVA showed that glutathione peroxidase activity was not significantly different between treatments (Figure 5.7, Appendix 2.9).

5.2.4.3 *Total Glutathione Concentration*

Mean total glutathione (GSH+2GSSG) concentration was the same for the control and 10 µg/g cadmium exposed organisms, but increased in the 50 µg/g treated organisms (Figure 5.7). ANOVA showed that although the GSH+2GSSG concentration increased in the 50 µg/g exposed organisms it was not significantly different to the concentration of the control or 10 µg/g cadmium exposed organisms (Figure 5.7, Appendix 2.9).

5.2.4.4 *Reduced : Oxidised Glutathione Ratio*

The mean ratio of reduced to oxidised glutathione in the 10 µg/g cadmium exposed organisms was half that of the controls and the 50 µg/g treated organisms had a ratio one quarter of the control, indicating that concentrations of oxidised glutathione were increased in the cadmium exposed organisms (Figure 5.7). ANOVA showed that sediment cadmium concentration significantly influenced the ratio of GSH:GSSG (Appendix 2.9). Bonferroni pair-wise comparisons showed that the organisms from the 50 µg/g cadmium treatment had a significantly lower GSH:GSSG ratio than the control organisms while organisms from the 10 µg/g treatment were not significantly different from either the control or the higher cadmium treatment (Figure 5.7, & 2.10).

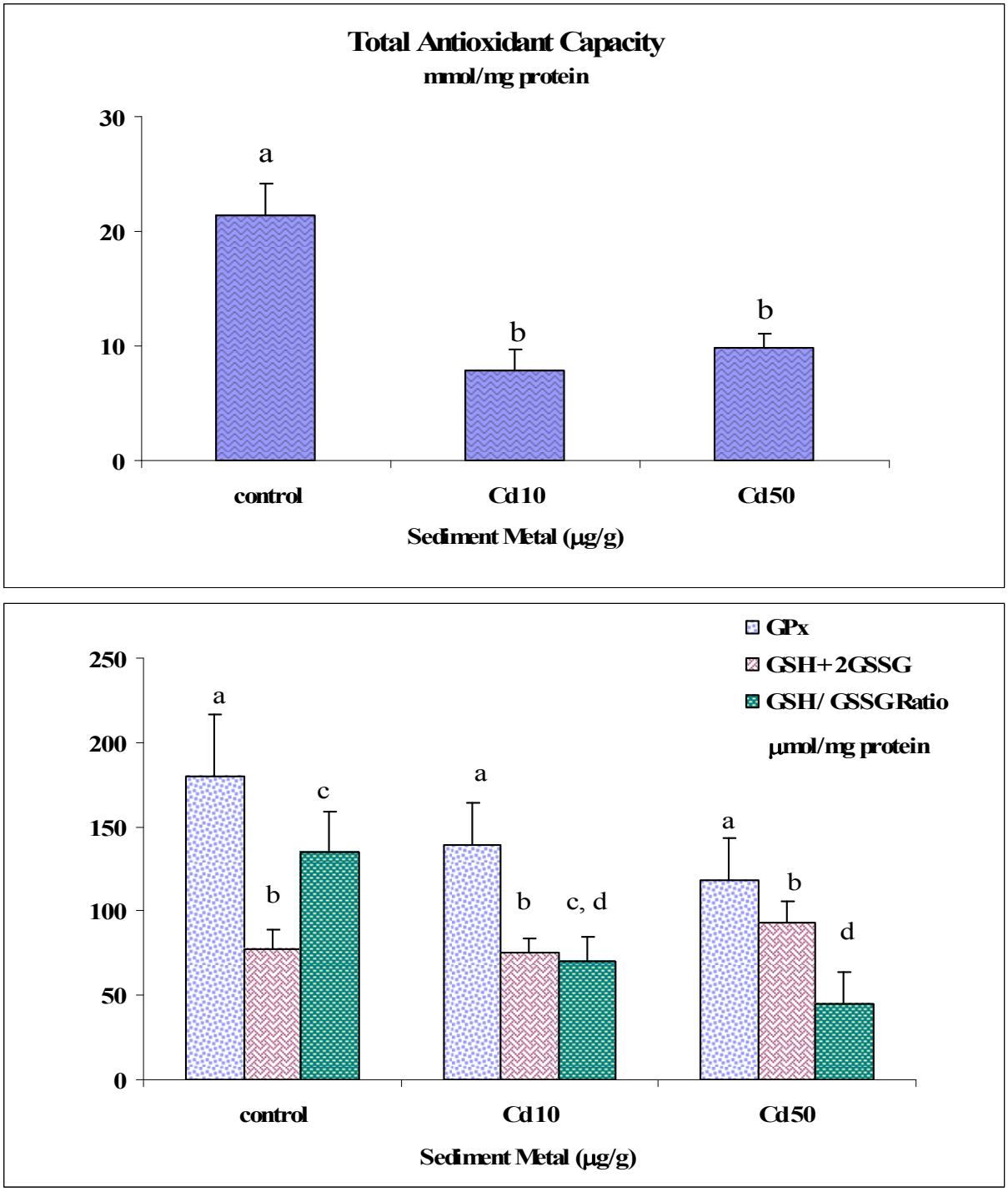


Figure 5.7: Antioxidant enzyme biomarkers: total antioxidant capacity; glutathione peroxidase (GPx); total glutathione (GSH+2GSSG); and ratio of reduced to oxidised glutathione (GSH/GSSG Ratio) of *A. trapezia* following 56 days of exposure to cadmium spiked sediments: 0 Cd (control), Cd 10 µg/g; and Cd 50 µg/g dry mass. Mean ± SE, n = 8, 7 and 7 respectively. Different letters indicate significant differences betweens means (Bonferroni test; $p < 0.05$).

5.2.5 Oxidative Damage Biomarker – Thiobarbituric Acid Reactive Substances

TBARS increased with exposure to increased sediment cadmium concentrations (Figure 5.8). ANOVA showed that TBARS were not significantly different between cadmium treatments (Figure 5.8, Appendix 2.9).

5.2.6 Cellular Biomarker – Lysosomal Stability

Lysosomal stability decreased with exposure to increased cadmium concentrations (Figure 5.8). ANOVA with Bonferroni pair-wise comparison showed that cadmium treatment was highly significant in lysosomal stability with organisms from both cadmium treatments having significantly more unstable lysosomes than those from the control, however, the lysosomal stabilities of cadmium exposed organisms were not significantly different from each other (Figure 5.8, Appendix 2.9 & 2.10).

5.2.7 Genotoxic Biomarker – Micronuclei Frequency

Micronuclei frequency increased with exposure to increased cadmium concentrations (Figure 5.8). ANOVA with Bonferroni pair-wise comparison showed that cadmium treatment was highly significant in micronuclei frequency with organisms from both cadmium treatments having significantly more micronuclei than those from the control, however, the frequency of micronuclei in cadmium exposed organisms were not significantly different from each other (Figure 5.8, Appendix 2.9 & 2.10).

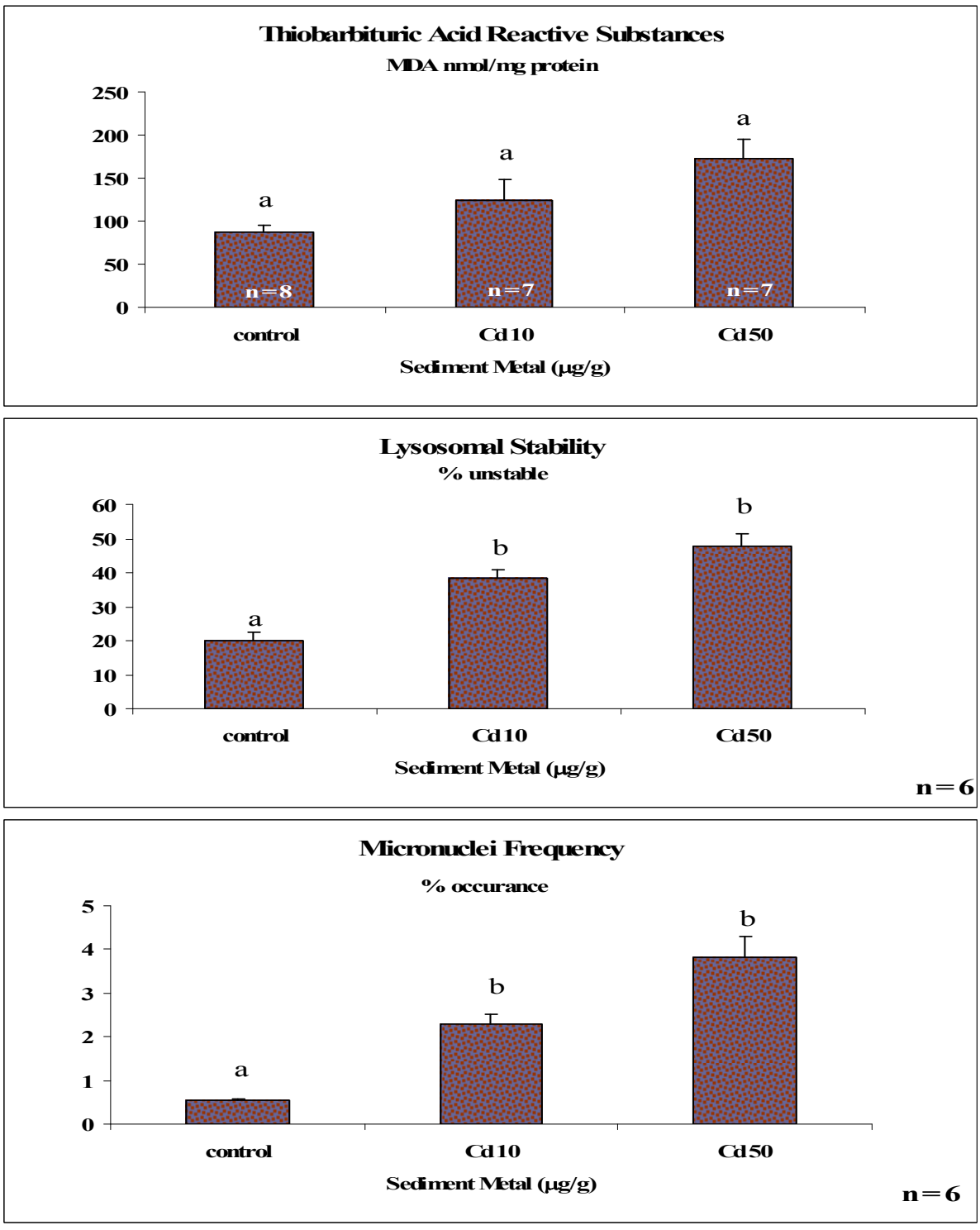


Figure 5.8: Changes in oxidative damage, cellular and genotoxic biomarkers of *A. trapezia* following 56 days exposure to cadmium spiked sediments, Cd 0 (control),Cd 10 µg/g and Cd 50 µg/g; dry mass. Mean ± SE. Different letters indicate significant differences betweens means (Bonferroni test; $p < 0.05$).

5.2.8 Relationship Between Biomarkers

5.2.8.1 *Enzymatic with Oxidative Damage, Cellular and Genotoxic Effects*

Regression analysis shows that the reduced TAOC within cells had a negative relationship with the effects measures of TBARS, lysosomal stability and micronuclei frequency for cadmium exposed organisms (Figure 5.9). As the sediment cadmium concentration increased the capacity of the cells to neutralise reactive oxygen was reduced with a consequent increase in lipid peroxidation, cell and genotoxic damage.

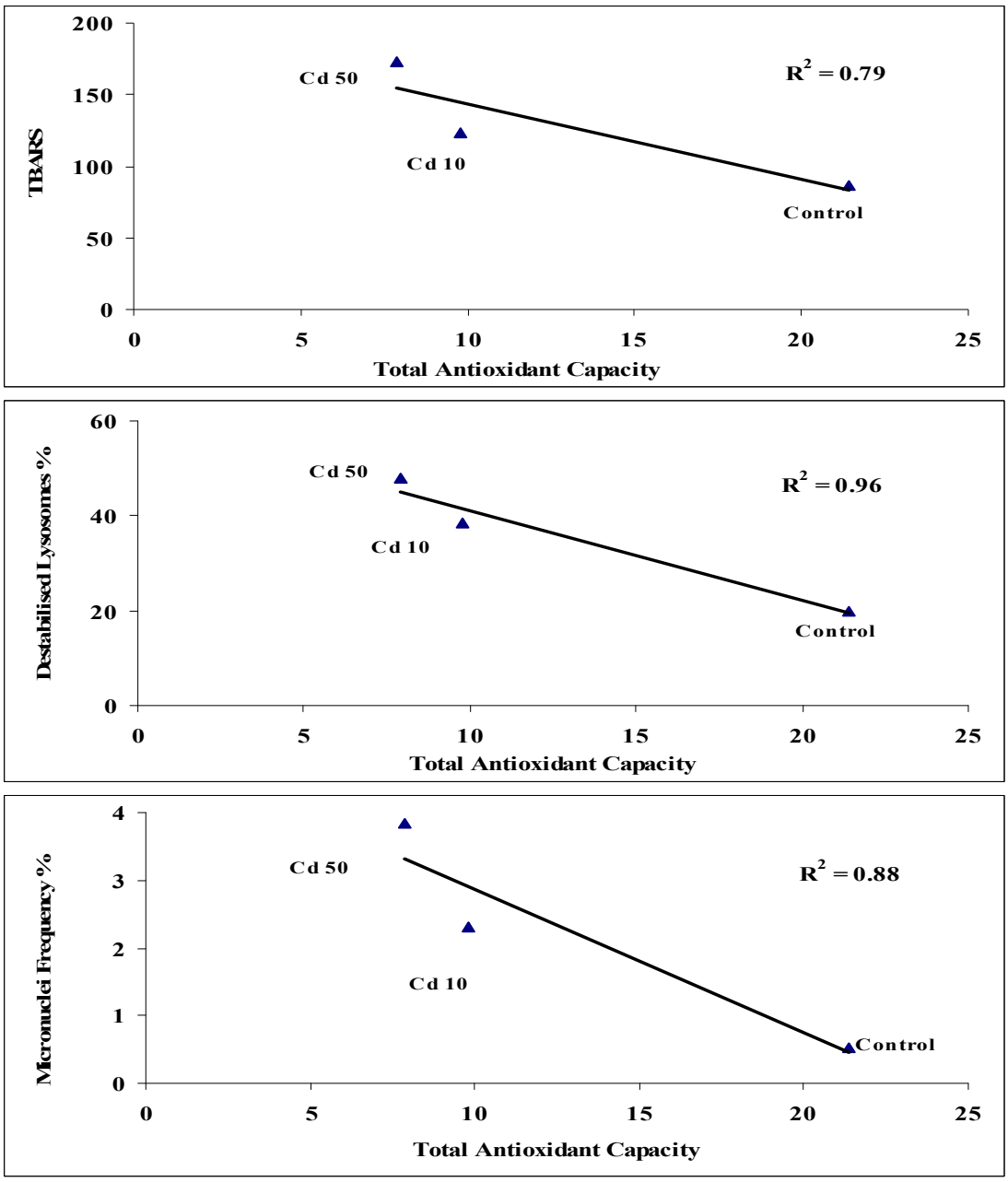


Figure 5.9: Regressions of mean total antioxidant capacity, (control; n=8: Cd 10 & 50; n=7), with TBARS, (control; n=8: Cd 10 & 50; n=7); lysosomal stability and micronucleus frequency (n=6) for sediment metal treatment means.

5.2.8.2 *Oxidative Damage with Cellular and Genotoxic Effects*

There was a positive relationship between TBARS and lysosomal stability and micronuclei frequency (Figure 5.10). Increased sediment cadmium concentration increased the TBARS and the regression analysis indicates that this was associated with an increase in the percentage of unstable lysosomes and the frequency of micronuclei (Figure 5.10).

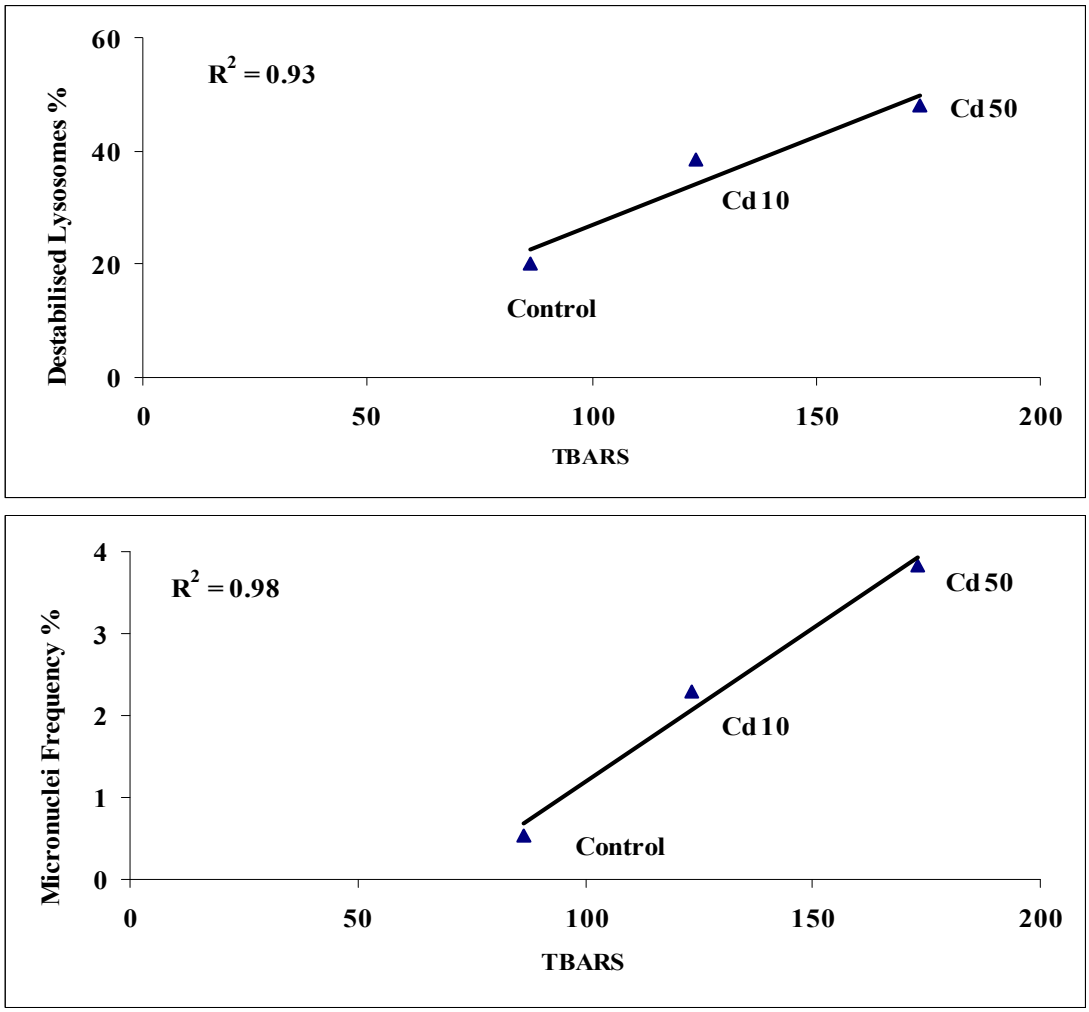


Figure 5.10: Regressions of treatment mean TBARS (control; n=8: Cd 10 & 50; n=7); with lysosomal stability and micronucleus frequency (n=6) for sediment metal treatment means.

5.3 Discussion

5.3.1 Cadmium Accumulation and Subcellular Distribution

5.3.1.1 *Whole Organism Cadmium Accumulation*

A. trapezia exposed to both 10 and 50 µg/g of cadmium showed a steady increase in tissue cadmium over the 56 day exposure with final tissue concentrations in the 10 µg/g cadmium exposed organisms higher than that of the spiked sediments (Figure 5.1). *A. trapezia* in both treatments appeared to reach an equilibrium cadmium tissue concentration by day 42 with slight decreases to day 56 (Figure 5.1). The 10 µg/g exposed organisms reached a final tissue concentration 1.2 times their exposure concentration while those exposed to 50 µg/g of cadmium had half the exposure concentration (Figure 5.2). This is somewhat different to cadmium concentrations of about 10 µg/g measured in *A. trapezia* exposed for 60 days in cages in Lake Macquarie NSW, to cadmium sediment concentrations of around 30 µg/g (Burt *et al.*, 2007). Sediments in this study also contained elevated concentrations of lead, zinc and selenium and strong correlations were found between cadmium tissue concentrations with both lead and zinc tissue concentrations suggesting co-accumulation (Burt *et al.*, 2007). If total metal burden of organisms from the Burt *et al.* 2007 study are considered they would be closer in µg/g terms to the cadmium results of the present study. Zinc accumulation, in particular, in the Burt *et al*., (2007) study was high which suggests it may have been preferentially accumulated. Competitive interactions between zinc and cadmium have been shown for *Mytilus edulis, Mytilus galloprovincialis* and *Mulina lateralis,* with the result that cadmium uptake was reduced (Simkiss and Mason, 1984). Physicochemical differences between lake exposures and aquaria exposures may have also influenced bioavailability and route of exposure. Cadmium is relatively soluble in estuarine and marine environments due to strong complexation of by chloride (Stumm and Morgan, 1996). The pattern of cadmium accumulation over time (Figure 5.1, Appendix 2.4) suggests the sediment bound cadmium in this experiment was readily resuspended and bioavailable. Bioturbation by sediment dwelling bivalves leading to oxidation and subsequent release of sediment bound metals into pore waters has been reported in a number of previous studies (Atkinson *et al.*, 2007; Ciutat and Boudou, 2003; King *et al.*, 2004; Meysman *et al.*, 2006).

A. trapezia, unlike a number of other filter feeding bivalves, lives buried in sediment with only the posterior end exposed to enable feeding, it has no siphon to extend beyond its shell (Beesley *et al.*, 1998), so when feeding the shell is partly open allowing water, food and sediment particles into the mantle cavity. Rapid opening and closing of the shell causes disturbance to sediments as evidenced by plumes of sediment pushed into the overlying water, as well, the foot muscle used in burrowing and to move the animal along the sediment surface further adds to bioturbation. This bioturbation will increase cadmium bioavailability by releasing sediment bound metal particles and by alteration of the metal redox state through oxidation. The positive sediment tissue cadmium concentration relationship for the two treatments and the higher than ambient cadmium tissue concentrations gained by the 10 µg/g cadmium exposed organisms (Figure 5.2) are indicative if *A. trapezia* being a net accumulator of cadmium, which includes organisms in which regulatory mechanisms have become saturated (Rainbow *et al.*, 1990).

5.3.1.2 *Individual Tissue Cadmium Accumulation*

The majority of whole organism cadmium accumulation is explained by the gills which had significantly more cadmium in all treatments than the hepatopancreas and haemolymph at all collection times (Figure 5.3; Appendix 2.7). Previous studies of cadmium accumulation by sediment dwelling filter feeding bivalves have identified the gill as the dominant tissue for cadmium accumulation (Bebianno *et al.*, 1993; Chan *et al.*, 2002; Riba *et al.*, 2004; Tessier *et al.*, 1993). *A. trapezia* like other filter feeding bivalves have large gills which are the sole means of feeding and respiration. Water is drawn past the gill filaments by cilia to the labial palps which capture and store food for transfer to the mouth (Purchon, 1977). Exposure from dissolved metal in water is therefore high for the gills. Bioturbation of sediments by *A. trapezia* during feeding and burrowing will likely increase the availability of free cadmium in the pore waters. Excess metal may be stored and detoxified in the gills or transported to the hepatopancreas for storage and detoxification (Bebianno *et al.*, 1994). The hepatopancreas also had significant cadmium accumulation over the course of the experiment (Appendix 2.7). The cadmium concentration in this tissue was half that of the gill in the 10 µg/g treatment and in the 50 µg/g exposed organisms was one quarter the gill concentration after 42 days (Figure 5.3). The difference in cadmium concentration between the two tissues may be related to the different exposure routes the gills being primarily exposed to dissolved metal and the hepatopancreas to cadmium in food and water (Sokolova *et al.*, 2005a).

The hepatopancreas tissue cadmium concentrations were similar in organisms from both treatments at each collection time (Figure 5.3). The accumulation in the hepatopancreas may be limited by the rate at which cadmium is transported from the gills or accumulated from food rather than the exposure concentration. The haemolymph contributed very little to the total cadmium burden (Figure 5.3). Cadmium accumulated by the gills is probably transported by the haemolymph to organs where, detoxification and storage occurs so its presence in haemolymph would be transitory. As the cockles were depurated for 24 hours before processing much of the metal associated with the haemolymph may have been transferred to the hepatopancreas during this time.

5.3.1.3 *Subcellular Cadmium Distribution*

Between 66 and 73 % of the accumulated cadmium in the gill and hepatopancreas of all cadmium treated organisms was in the BDM compartments, except for the control organism hepatopancreas which had only 46 % BDM cadmium (Table 5.1; Figure 5.4). Of the BDM, the majority of cadmium, 84 to 95 %, was in the MTLP fraction in both gill and hepatopancreas from all treatments with very little sequestered in the MRG fraction (Table 5.2; Figure 5.4). The induction of MTLP during the metabolism of cadmium by a range of bivalves has been reported (Bebianno *et al.*, 1994; Chan *et al.*, 2002; Giguere *et al.*, 2003; Roesijadi, 1996). Giguère *et al.* (2003) found that 80 % of accumulated cadmium in the cytosol of the mussel *Pyganodon grandis* from metal contaminated freshwater lakes was in the MTLP fraction and for these chronically exposed molluscs there was no threshold concentration at which cadmium spilled over from this fraction into the metal sensitive protein fraction. It has been shown for the oligochaete *Limnodrilus hoffmeisteri* that a previous history of chronic metal exposure resulted in a different pattern of cadmium detoxification (Wallace *et al.*, 1998). Previously exposed organisms produced both MTLP and MRG when exposed experimentally to cadmium, whereas those with no exposure history produced only MTLP. The organisms used in this study had no previous cadmium exposure, therefore the pattern of accumulated cadmium storage, from these exposures, in MTLP with only a small fraction in the MRG may be related to their previous exposure history.

Specific activity of cytochrome c oxidase (CcO) in the gill tissue was higher than in the hepatopancreas (Figures 5.5; 5.6) indicating greater mitochondrial activity in this tissue. There was also greater mitochondrial activity in the whole tissue, gills and hepatopancreas of the cadmium exposed organisms compared to the control tissues (Figures 5.5; 5.6) indicating activity was increased in response to cadmium accumulation.

About half the BAM cadmium was in the mitochondrial fraction of gill and hepatopancreas tissue in *A. trapezia* from all cadmium treatments (Table 5.2, Figure 5.4). Increased cadmium burdens in the gill and hepatopancreas of the freshwater bivalve *Pyganodon grandis* also resulted in higher mitochondrial cadmium compared to the lysosome+microsome and HSP fractions (Bonneris *et al.*, 2005). Extensive Cd^{2+} accumulation in mitochondria mediated by Ca^{2+} voltage dependant channels has previously been reported by Li *et al.* (2000; Li *et al.*, 2003), who showed that cadmium could directly lead to dysfunction of mitochondria including inhibition of respiration, loss of transmembrane potential and the release of CcO. While the percentage of cadmium in mitochondrial fraction of the 10 µg/g and 50 µg/g cadmium exposed *A. trapezia* BAM was similar to the controls (Table 5.2), in terms of the total cadmium recovered there was a 11 and 84, in gill and a 24 and 38 in hepatopancreas, respectively, fold increase in mitochondrial cadmium, compared to the control organisms. The increased mitochondrial cadmium observed is in agreement with studies on cadmium subcellular distribution following increased exposure in oysters *Crassostrea virginica* (Sokolova *et al.*, 2005a). The activity of the lysosomal marker enzyme acid phosphatase was greater in the hepatopancreas than in the gill tissue of all treatments reflecting an enrichment of hepatopancreas tissue with lysosomes (Figure 5.5). The percentage of cadmium in the lysosome+microsome fraction was similar for both tissues for all cadmium treated organisms (Table 5.2) and like the mitochondrial fraction there was an increase in cadmium burden in the lysosomal+microsome fraction of both tissues of the cadmium exposed organisms (Table 5.1). Cadmium saturated thioneins do not have a strong tendency to polymerise into the lysosomes, probably because Cd-thiolate complexes are unstable at the lysosomal acidic pH, which will cause the hydrolysis of the apoprotein and the metal release to the cytosol to be bound to newly synthesised thioneins (Viarengo and Nott, 1993). This continued cycling process is likely to lead to a weakening of the lysosomal membrane. It has been shown for other marine bivalves that cadmium exposure can result in destabilisation of lysosomes (Bolognesi *et al.*, 1999; Ringwood *et al.*, 1998a). Cadmium associated with the HSP fraction was similar among tissues and treatments and accounted for around one quarter of the BAM cadmium (Table 5.2; Figure 5.4). Increased cadmium in this fraction may include binding to metal sensitive enzymes and proteins which has implications for toxicity (Wallace *et al.*, 2003).

Only a small proportion of the recovered cadmium was in the nuclei and cell debris fraction with a slightly greater amount in the hepatopancreas of the cadmium exposed organisms compared to the control and the gill tissues (Table 4.1; Figure 5.4). This may be related to the presence of cadmium binding MTLP associated with the hepatopancreas nuclei (del Castillo and Robinson, 2008).

The increased BDM cadmium burden of the cadmium exposed *A. trapezia* indicates cadmium detoxification was occurring, however, the majority of cadmium in this fraction was associated with the MTLP and while this is classed as detoxified metal it also has the potential to be remobilised. As very little cadmium was associated with the MRG fraction which is a more stable form of detoxification and storage there is a greater likelihood that the MTLP may become saturated and cadmium spill over into active sites will result if cadmium exposure continues. The increased cadmium burden in the BAM fractions of the cadmium exposed *A. trapezia* indicates that this process was occurring and cadmium toxicity is therefore likely.

5.3.2 Enzymatic Biomarkers – Oxidative Enzymes

The TAOC of *A. trapezia* from both cadmium treatments was significantly reduced compared to the control organisms but they were not significantly different to each other (Figure 5.7; Appendix 2.10). Northern horse mussel *Modiolus modiolus* showed a gradual reduction in total oxyradical scavenging capacity over 21 days of laboratory exposure to dissolved cadmium (Dovzhenko *et al.*, 2005) and this has also been demonstrated in mussels *Mytilus galloprovincialis* exposed to cadmium and other metals in coastal regions around Italy (Regoli *et al.*, 2004; Regoli and Principato, 1995). An examination of components of the glutathione cycle, to determine the specifics of the cadmium effect on the total antioxidant system, showed that activity of the GPx enzyme was reduced in the cadmium exposed organisms from both treatments and the concentration of total glutathione (GSH+2GSSG) was increased slightly in the 50 µg/g cadmium exposed organisms, but the changes were not significant (Figure 5.7; Appendix 2.10). The ratio of GSH:GSSG was reduced in organisms from both cadmium treatments (Figure 5.7) indicating that recycling of glutathione from the oxidised back to the reduced form was affected by cadmium accumulation. The subcellular fractionation showed cadmium burdens in the mitochondrial fraction of the exposed *A. trapezia* increased up to 84 fold (Tables 5.1 & 5.2).

As the mitochondria is the primary site of ATP production, which includes the cycling of oxygen, the increased cadmium in this organelle has affected the glutathione cycling, particularly in the 50 µg/g cadmium exposed organisms, which has the potential for oxyradical build up and damage to cell membranes. Studies on oysters have shown cadmium concentrations as low as 5 µM can result in a significant decrease in antioxidant capacity and decreased coupling in mitochondria (Sokolova *et al.*, 2004). The pattern of altered glutathione status together with the reduced TAOC in *A. trapezia* indicates cadmium accumulation had a negative impact on the antioxidant system which may have the potential to affect population viability. It has been shown that oyster embryos derived from parents with reduced glutathione cycling were more susceptible to cadmium toxicity than embryos from parents with normal glutathione concentrations (Ringwood *et al.*, 2004).

5.3.3 Oxidative Damage Biomarker – Thiobarbituric Acid Reactive Substances

There was a trend of increasing TBARS concentration in the cadmium exposed organisms but this was not significantly higher than the TBARS concentration of the control organisms (Figure 5.7; Appendix 2.10). A study of the cadmium-handling strategy of the chronically exposed freshwater bivalve *Pyganodon grandis* linked higher cadmium exposure to increased cytosolic and mitochondrial cadmium with increased TBARS in these fractions of gill tissue but the digestive gland showed the opposite trend for TBARS (Bonneris *et al.*, 2005). A study of cadmium accumulation in the marine bivalve *Perna viridis* over 30 days exposure showed TBARS increased as tissue cadmium increased in both gill and digestive gland tissue (Prakash and Rao, 1995). TBAR measurements in *A. trapezia* were done in gill tissue which had the highest BAM cadmium burden and although it was not significant they also showed a similar trend for lipid peroxidation with increased cadmium exposure in the cytosolic and mitochondrial fractions (Figures 5.4 & 5.8). The TBARS concentration was negatively correlated with the TAOC (Figure 5.9), therefore, the cadmium induced perturbations in the antioxidant reduction system influenced the build up of lipid peroxidation by-products.

5.3.4 Cellular Biomarker – Lysosomal Stability

Lysosomes are the target for a wide range of toxic chemicals including metals which may effect these organelles directly and indirectly through the enhanced formation of oxygen radicals have been widely used as a general biomarker of effect (Cajaraville *et al.*, 2000; Moore *et al.*, 2006; Regoli, 2000; Regoli *et al.*, 2004).

There was a significant increase in the number of destabilised lysosomes in the cadmium exposed *A. trapezia* compared to the control organisms but no significant difference between the two cadmium treatments (Figure 5.8 Appendix 2.10). The control organisms had 20 % of lysosomes destabilised while the 10 and 50 µg/g cadmium exposed organisms had 38 and 48 %, respectively, destabilised lysosomes. Based on the Ringwood et al., (2003) criteria for oysters this percentage of destabilised lysosomes places the cadmium exposed *A. trapezia* in the concern / stressed range. Increased cadmium associated with the lysosomes following cadmium exposure has been shown for *Pyganodon grandis* and *Crassostrea virginica* (Bonneris *et al.*, 2005; Sokolova *et al.*, 2005a) and was also observed in the *A. trapezia* in this study (Table 5.2). Lysosomal destabilisation associated with the increased lysosomal cadmium burden and impairment of the antioxidant reduction system has also been observed in a number of bivalve studies (Chelomin *et al.*, 2005; Regoli *et al.*, 2004; Ringwood *et al.*, 2004; Ringwood *et al.*, 2002). Lysosomal destabilisation of the cadmium exposed *A. trapezia* was strongly negatively correlated with TAOC (Figure 5.9), and positively correlated with TBARS (Figure 5.10). This supports a cadmium induced reduction in the TAOC resulting in an increase in ROS which initiated an increase in the build up of lipid peroxidative products both of which would have contributed to the destabilisation of the lysosomal membranes. These results confirm the sensitivity of the lysosomal stability assay for detection of early adverse effects from cadmium in *A. trapezia*. The percentage of destabilised lysosomes seen in *A. trapezia* in response to cadmium exposure has the potential to reduce reproductive success. A study on oysters demonstrated that lysosomal destabilisation of > 35 % in parent oysters exposed to cadmium during gamete maturation had very low rates of normal embryonic development (Ringwood *et al.*, 2004).

5.3.5 Genotoxic Biomarker – Micronuclei Frequency

Micronucleus frequency increased with increased cadmium exposure and tissue dose with the cadmium exposed *A. trapezia* having significantly more micronuclei than the control organisms although they were not significantly different to each other (Figure 5.8 Appendix 2.10). Increased micronuclei frequency associated with reduced TAOC has also been observed in caged mussels *Mytilus galloprovincialis* exposed for 4 weeks to PAHs, cadmium lead, zinc and mercury during harbour sediment dredging activities (Bocchetti *et al.*, 2008) and to a mix of metals near an offshore platform in the Adriatic sea (Gorbi *et al.*, 2008).

The micronuclei frequency of the cadmium exposed *A. trapezia* was negatively correlated with TAOC (Figure 5.9) and positively correlated with TBARS (Figure 5.10) supporting the pathway of genotoxic damage via the increase in ROS resulting from cadmium inhibition of the ROS detoxification capacity.

5.4 Summary and Conclusions

Cadmium exposed *A. trapezia* accumulated cadmium over 56 days and reached tissue concentrations which at the 10 µg/g cadmium exposure were higher than that of the sediment exposure concentration and at the 50 µg/g sediment cadmium exposure were half the exposure concentration. *A. trapezia* in both treatments reached an equilibrium cadmium tissue concentration by day 42 with slight decreases to day 56. The majority of whole organism cadmium accumulation is explained by uptake by the gills which had significantly more cadmium in all treatments than the hepatopancreas and haemolymph at all collection times. Up to 73 % of accumulated cadmium in the gill and hepatopancreas of cadmium exposed *A. trapezia* was detoxified. The majority of BDM, 84 to 95 %, in both tissues of cadmium exposed organisms was in the MTLP fraction with only a small proportion sequestered in the MRGs. This cadmium detoxification pattern may relate to the organisms having had no previous exposure to cadmium, as previously exposed organisms have been shown to increase MRG formation when re-exposed. Approximately half of the BAM cadmium in both tissues of exposed organisms was in the mitochondrial fraction. Mitochondrial cadmium burdens of the 10 and 50 µg/g cadmium exposed *A. trapezia* were 11 and 84 in gill, and 24 and 38 in hepatopancreas, respectively, greater than the control organisms and this was associated with an increase in the activity of the mitochondrial cytochrome c oxidase enzyme. Mitochondrial cadmium burdens of this magnitude have the potential to cause dysfunction in mitochondrial activity. The TAOC of cadmium exposed *A. trapezia* was significantly reduced compared to control organisms. The ratio of GSH:GSSG of the 50 µg/g cadmium exposed *A. trapezia* was significantly reduced compared to the 10 µg/g cadmium exposed and control organisms. There was a trend of increased lipid peroxidation with increased cadmium exposure but this was not significant. Increased cadmium exposure resulted in significant lysosomal destabilisation and increased frequency of micronuclei. A significant exposure – dose – response relationship for cadmium has been established in this study which indicates that sediment cadmium at these concentrations has the potential to lead to increased BAM burdens and impairment of individual *A. trapezia* at a cellular and subcellular level.

6 *Anadara trapezia* Lead Spiked Sediment Study

6.1 Aim

The purpose of this study is to determine the exposure – dose - response of *A. trapezia* to sediments with known concentrations of lead. The exposure time was 56 days and the development of useful biomarkers of effect undertaken with a view to determining whether they would be a suitable organism to collect from the field and use for field metal exposures in cages.

6.2 Results

6.2.1 Lead Accumulation

6.2.1.1 *Whole Organism Lead Accumulation*

ANOVA showed that, the factor lead treatment was highly significant in lead accumulation, time (day) was not significant and there was a significant interaction between time and lead treatment (Appendix 2.1). The tissue lead concentration of the control organisms remained the same over the course of the exposure (Figure 6.1). Bonferroni pair-wise comparisons showed that the lead tissue concentrations of the control organisms did not differ significantly from day 0 unexposed organisms (Appendix 2.2). The pattern of lead accumulation was similar in both the lead treatments (Figure 6.1). Tissue concentrations were in the order, 300 µg/g > 100 µg/g > control for each analysis time, with the tissue lead concentrations of the 300 µg/g treatment organisms being 6 times higher than those of the 100 µg/g organisms at days 14 and 28, increasing to 9 and 15 times greater on days 42 and 56 respectively (Figure 6.1). The over-all comparison between treatments showed organisms from both the lead treatments differed significantly from the unexposed and control organisms and from each other at all collection times (Appendix 2.2). The lead exposed organisms reached the highest tissue concentrations at day 56 with a rapid increase from day 42 suggesting that lead uptake was not regulated. The 300 µg/g exposed organisms had a final tissue lead concentration of 0.04 that of the sediment concentration, while the 100 µg/g organisms had a concentration only 0.01 that of the sediment concentration (Figure 6.1). The regression between lead sediment concentration and organism tissue lead concentration after 56 days exposure shows a significant positive relationship (Figure 6.2).

As sediment lead concentration increased from 100 to 300 µg/g the tissue concentration increased but not proportionally (Figure 6.2). Pair-wise comparisons between treatments showed that the 100 µg/g lead exposed organisms were only significantly different from the control organisms at days 28 and 56 while the 300 µg/g lead exposed organisms had significantly more tissue lead than the control and 100 µg/g lead exposed organisms at all collection times (Appendix 2.3). Analysis of the within treatment differences between collection days showed that there was no significant difference over time in the lead concentrations of the control organisms. The 100 µg/g lead exposed organisms had no significant increase in tissue lead until day 56 compared to the day 0 and the 300 µg/g lead exposed organisms had significant lead increases in the first and final 14 days of exposure (Appendix 2.4).

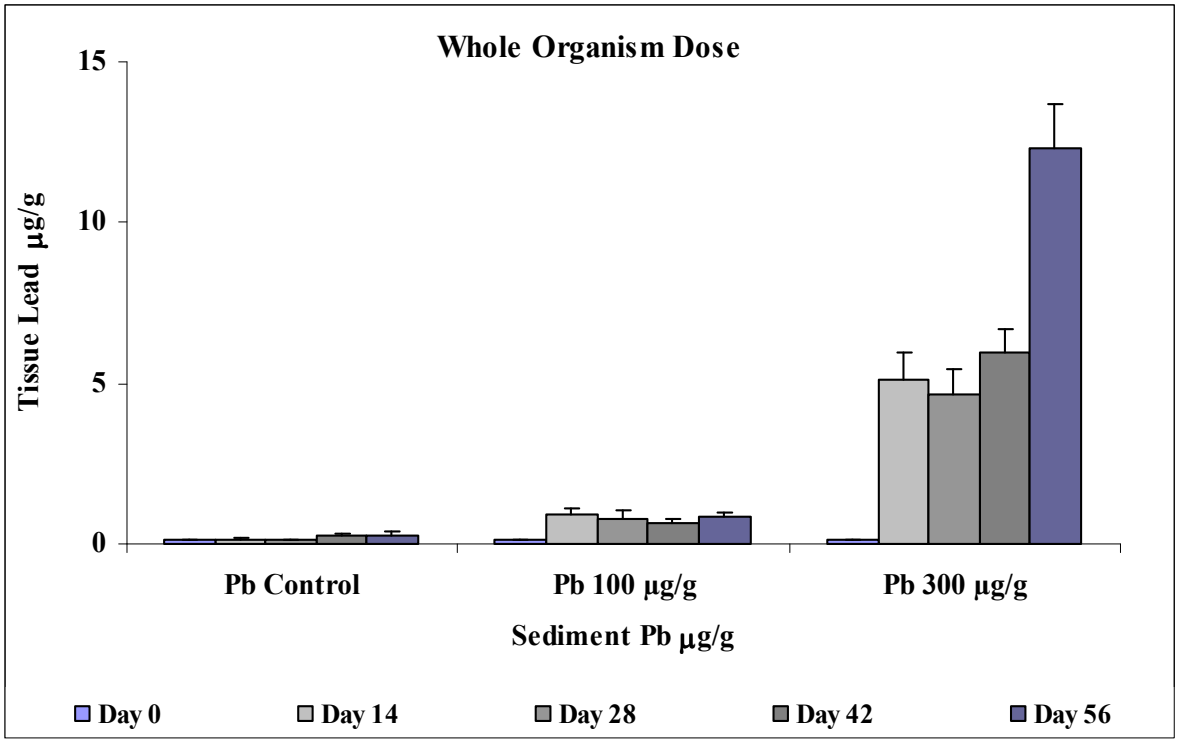


Figure 6.1: Lead accumulation in whole tissue of *A. trapezia* at 2 weekly intervals over 56 days of exposure to lead spiked sediments at: 0 (control), 100 and 300 µg/g dry mass, n = 8, 9 and 12 respectively. Mean ± SE. Day 0 are unexposed organisms n=5.

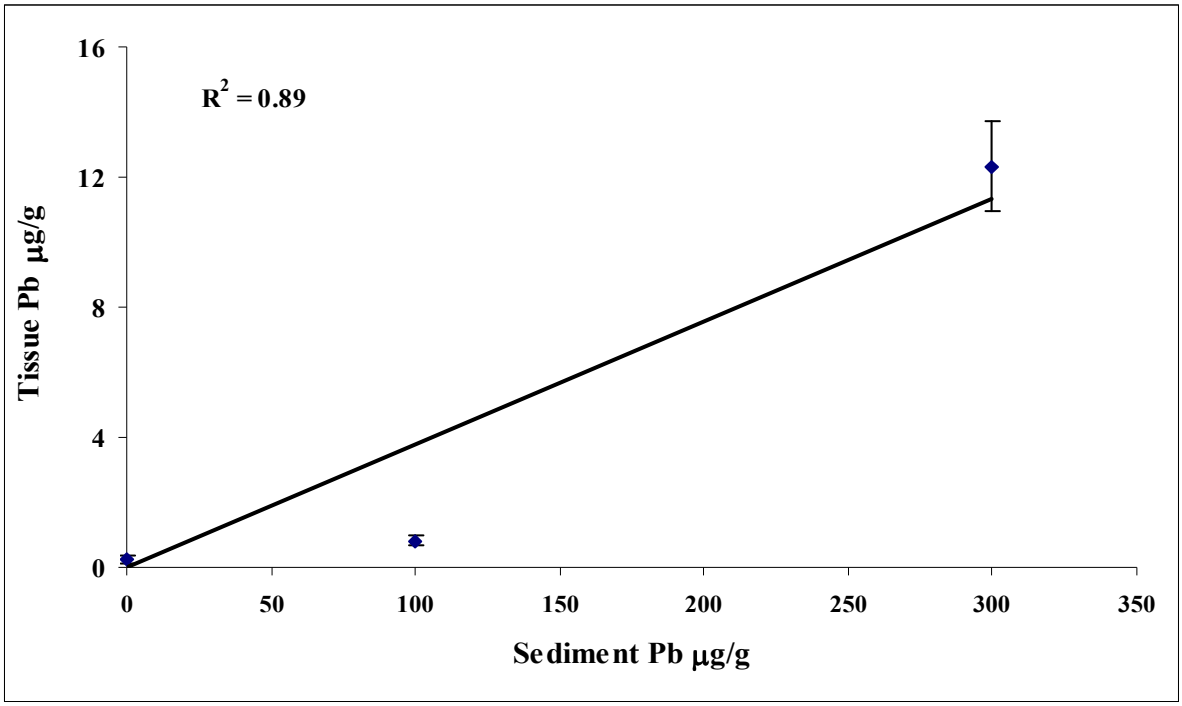


Figure 6.2: Regression of *A. trapezia* tissue lead concentration after 56 days of exposure against sediment lead exposure concentrations of 0, 100 and 300 µg/g dry mass. Means ± SE, n = 8, 9 and 12 respectively.

6.2.1.2 *Individual Tissue Lead Accumulation*

Lead accumulation was significantly different between tissues and there was a significant interaction between the factors, tissue time and lead treatment (Appendix 2.5). Bonferroni multiple pair-wise comparisons showed there was no significant difference in lead concentration between tissues of unexposed and control organisms (Appendix 2.6). The lead concentrations in the hepatopancreas tissues of the 100 µg/g lead exposed organisms were significantly different to the gill and haemolymph tissues but the lead concentrations in the gill and haemolymph were not significantly different from each other (Appendix 2.6). The 300 µg/g lead exposed organisms had significant differences between all tissues for all treatments (Appendix 2.6). Pair-wise analysis of the between tissue lead concentration differences at each collection time showed no significant differences between tissues of unexposed and control organisms. The 100 µg/g lead exposed organisms had significant differences in lead concentration between gill and hepatopancreas tissues at days 14, 42 and 56 and between hepatopancreas and haemolymph at day 14 (Appendix 2.7). The 300 µg/g lead exposed organisms had significant differences in lead concentration between gill and hepatopancreas tissues at all collection times and between hepatopancreas and haemolymph tissues on day 14, 42 and 56 (Appendix 2.7).

Lead concentrations of gill and haemolymph were only significantly different on day 56 in the 300 µg/g lead exposed organisms (Appendix 2.7).

Gill

The lead accumulation in the gill tissue was generally higher than that of the hepatopancreas in all treatments for each collection time (Figure 6.3). Gill lead accumulation was lower than that of the haemolymph for the first 28 days and higher over the final 28 days, (Figure 6.3), suggesting a change in metal processing or exposure route may have occurred. Analysis of the within tissue differences of lead concentrations between collection days showed that the 100 µg/g lead exposed organisms did not reach a gill tissue lead concentration which was significantly different to the unexposed organisms until day 42; there was also a significant lead increase between day 28 and 56 (Appendix 2.8). The 300 µg/g lead exposed organisms showed significant increases in gill lead concentration in the first and final 14 days of the exposure period (Appendix 2.8). The control organisms had no significant increase in gill lead concentration over the course of the experiment (Appendix 2.8). The pattern of lead accumulation in the gills of 300 µg/g lead exposed organisms is reflected in the whole organism dose (Appendix 2.4 & 2.8).

Hepatopancreas

The pattern of lead accumulation in the hepatopancreas was generally the same as that of the gill in the lead exposed organisms but lead concentrations were about half that of the gill at each collection time except for day 28 in the 100 µg/g exposed organisms (Figure 6.3). Analysis of the within tissue lead concentration differences between collection days showed that the control and 100 µg/g lead exposed organisms had no significant increase in hepatopancreas lead concentrations over the course of the experiment (Appendix 2.8). The 300 µg/g lead exposed organisms showed significant increases in hepatopancreas lead concentration in the first and final 14 days of the exposure period (Appendix 2.8). The pattern of lead accumulation in the hepatopancreas of 300 µg/g lead exposed organisms is the same as the gills and is therefore also reflected in the whole organism dose (Appendix 2.4 & 2.8).

Haemolymph

The accumulation of lead in the haemolymph was higher than that of the other two tissues for the first 28 days of the 300 µg/g lead exposure and then decreased to below that in the gill on day 42 and below both other tissues by day 56 (Figure 6.3). Haemolymph lead concentrations only decreased to below those of the gill on day 42 in the 100 µg/g lead exposed organisms and were always higher than the lead concentrations found in hepatopancreas in this treatment (Figure 6.3). Analysis of the within tissue lead concentration differences between collection days, showed that the control and 100 µg/g lead exposed organisms had no significant increase in haemolymph lead concentration over the course of the experiment (Appendix 2.8). The 300 µg/g lead exposed organisms showed significant increases in haemolymph lead concentration in the first 14 days of the exposure period (Appendix 2.8).

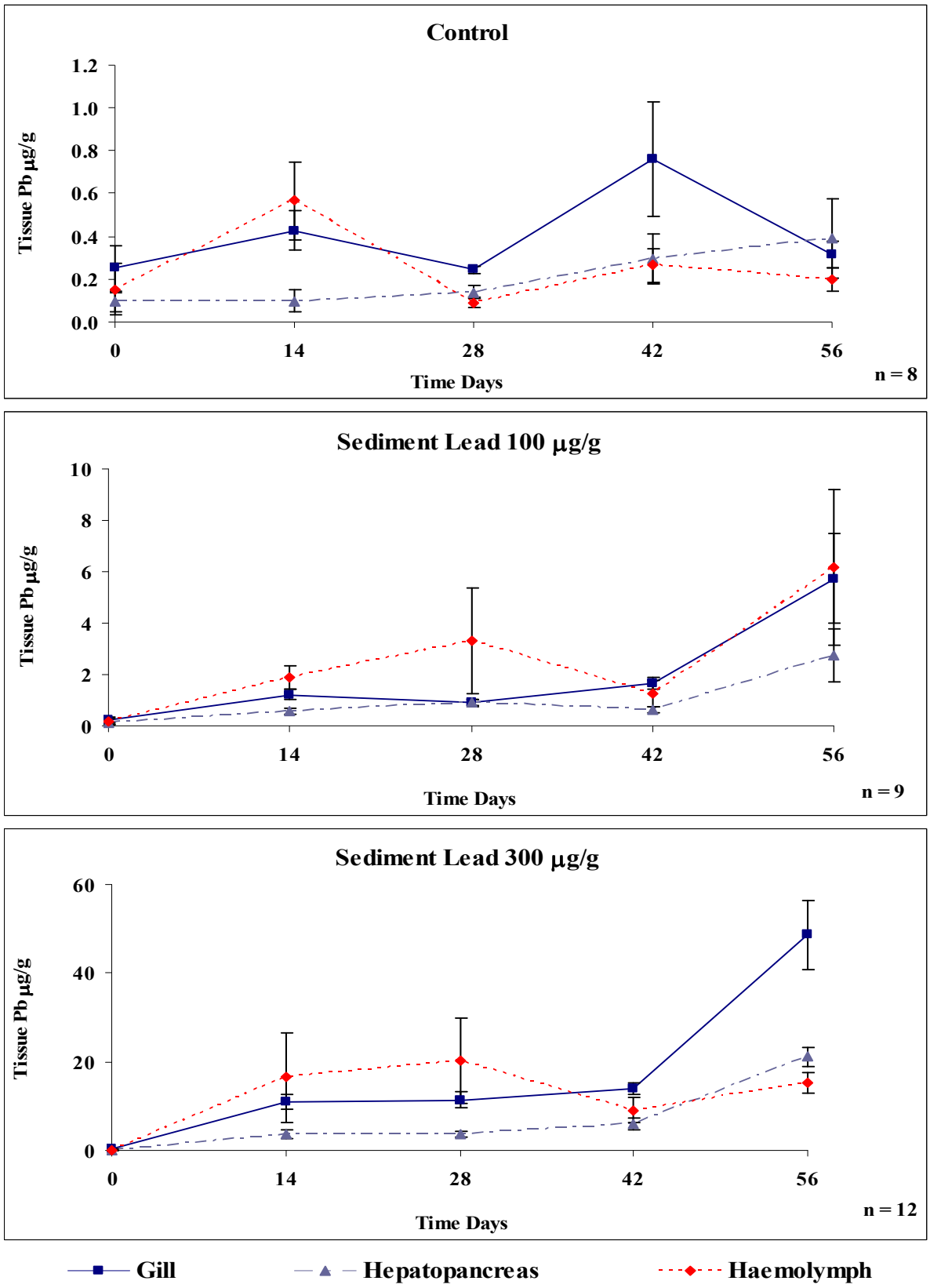


Figure 6.3: Lead accumulation in gill, hepatopancreas and haemolymph tissues of *A. trapezia* at two weekly intervals for 56 days of exposure to lead spiked sediments at: 0 (control), 100 and 300 µg/g dry mass. Mean ± SE, n = 8, 9 and 12. Day 0 are unexposed organisms, n=5.

6.2.2 Subcellular Tissue Lead Distribution

6.2.2.1 *Gill*

Between 44 and 88 % of the total gill lead was recovered in the fractions (Table 6.1). Of the metal recovered in the fractions, 30 % of the control and 66 and 69 % respectively of the 100 and 300 µg/g treatments was in the BDM fraction (Table 6.1). The percentage of metal recovered in the BAM fraction of each of the 100 and 300 µg/g lead treatments was less than one third that of the control, however, the total lead burden (µg) within these fractions was 10 and 50 times respectively, greater in the lead exposed organisms (Table 6.1). The majority of BDM was in the MTLP fraction for the control organisms while the lead treatments had more in the MRG fraction (Figure 6.4). The percentage of lead in the MRG fraction increased with the lead exposure, control < 100 µg/g < 300 µg/g organisms, while for MTLP the percentage decreased with lead exposure in the order, control > 100 µg/g > 300 µg/g organisms (Table 6.2). The highest percentage of lead in the BAM fraction of all lead treatments was in the mitochondria (≈ 50 %) with the remainder equally distributed between the heat sensitive proteins (HSP) (≈ 25%) and lysosome+microsome fractions (≈ 25 %) (Figure 6.4; Table 6.2).

6.2.2.2 *Hepatopancreas*

Between 62 and 90 % of the total hepatopancreas lead was recovered in the fractions (Table 6.1). Of the metal recovered in the fractions, 28 % of the control treatment and 49 and 56 % respectively of the 100 and 300 µg/g lead treatments was in the BDM fraction (Table 6.1). The percentage of metal recovered in the BAM fraction of the 100 µg/g lead treatment was 1.5 and the 300 µg/g lead treatment 5 times lower than that of the control, however, the total lead burden (µg) within these fractions was 4 and 50 times, respectively, greater in the lead exposed organisms (Table 6.1). The majority of BDM was in the MTLP fraction for the control organisms while the lead treatments had a fairly even distribution between the MTLP and MRG fractions (Figure 6.4). The percentage of lead in the MRG fraction increased with lead exposure in the order, control < 100 µg/g < 300 µg/g, while for MTLP the percentage decreased with lead exposure in the order, control > 100 µg/g > 300 µg/g (Table 6.2). The distribution of lead in the BAM fractions of all treatments was quite different with control organisms: mitochondria > heat sensitive proteins (HSP) > lysosome+microsome fractions; 100 µg/g organisms: mitochondria > HSP > lysosome+microsomes; 300 µg/g organisms: lysosome+microsome > mitochondria > HSP (Figure 6.4; Table 6.2).

Table 6.1: Lead concentrations (µg wet mass) in gill and hepatopancreas tissue and the total lead with percentage recovered from subcellular fractions of *A. trapezia* after 56 days exposure to lead spiked sediments. Metal subcellular concentrations (µg wet mass) and percentage distribution of total recovered lead in fractions are grouped as nuclei+cellular debris and biologically active and detoxified metal (Figure 3.7). Mean ± SD, n = 2.

	Gill			**Hepatopancreas**		
	Pb control	**Pb 100 µg/g**	**Pb 300 µg/g**	**Pb control**	**Pb 100 µg/g**	**Pb 300 µg/g**
Total Tissue Lead. (µg)	**0.04 ± 0.02**	**3.4 ± 0.6**	**11.5 ± 0.03**	**0.02 ± 0.01**	**0.2 ± 0.04**	**5.3 ± 0.2**
Total Recovered Lead (µg)	**0.03 ± 0.03**	**1.5 ± 0.3**	**7.3 ± 3.1**	**0.02 ± 0.01**	**0.1 ± 0.04**	**4.8 ± 0.1**
Proportion of total recovered in fractions (%)	88 ± 16	44 ± 15	64 ± 27	62 ± 41	74 ± 9	90 ± 2
Lead Distribution						
Nuclei + Cellular debris (µg)	**0.004 ± 0**	**0.3 ± 0.2**	**1 ± 0.3**	**0.001 ± 0**	**0.02 ± 0.01**	**1 ± 0.1**
Nuclei + Cellular debris (%)	15 ± 9	19 ± 7	17 ± 11	15 ± 14	15 ± 0.4	23 ± 1
Biologically Active Metal (µg)	**0.02 ± 0.01**	**0.2 ± 0.1**	**1 ± 0.2**	**0.01 ± 0.01**	**0.04 ± 0.01**	**1 ± 0.2**
Biologically Active Metal (%)	55 ± 22	15 ± 7	15 ± 3	57 ± 7	36 ± 5	21 ± 0.2
Biologically Detoxified Metal (µg)	**0.01 ± 0.02**	**1 ± 0.2**	**5 ± 3**	**0.01 ± 0.01**	**0.1 ± 0.03**	**3 ± 0.2**
Biologically Detoxified Metal (%)	30 ± 31	66 ± 0.5	69 ± 14	28 ± 21	49 ± 5	56 ± 4

Table 6.2: Mean percentage of lead in the nuclei+cellular debris, biologically detoxified metal (BDM) and biologically active metal (BAM) with the percentage of metal each of the fractions within contributes to BDM or BAM from subcellular fractions of *A. trapezia* after 56 days exposure to lead spiked sediments, n = 2.

	Gill			**Hepatopancreas**		
	Pb control	**Pb 100 µg/g**	**Pb 300 µg/g**	**Pb control**	**Pb 100 µg/g**	**Pb 300 µg/g**
Nuclei + Cellular debris % of total	**15**	**19**	**17**	**15**	**15**	**23**
BDM % of total	**30**	**66**	**69**	**28**	**49**	**56**
Metal Rich Granules % of BDM	28	55	58	33	44	52
Heat Stable MT Like Proteins % of BDM	72	45	42	67	56	48
BAM % of total	**55**	**15**	**15**	**57**	**36**	**21**
Mitochondria % of BAM	49	52	49	47	45	28
Lysosomes + Microsomes % of BAM	27	24	27	22	35	53
Heat Sensitive Proteins % of BAM	25	24	24	31	21	19

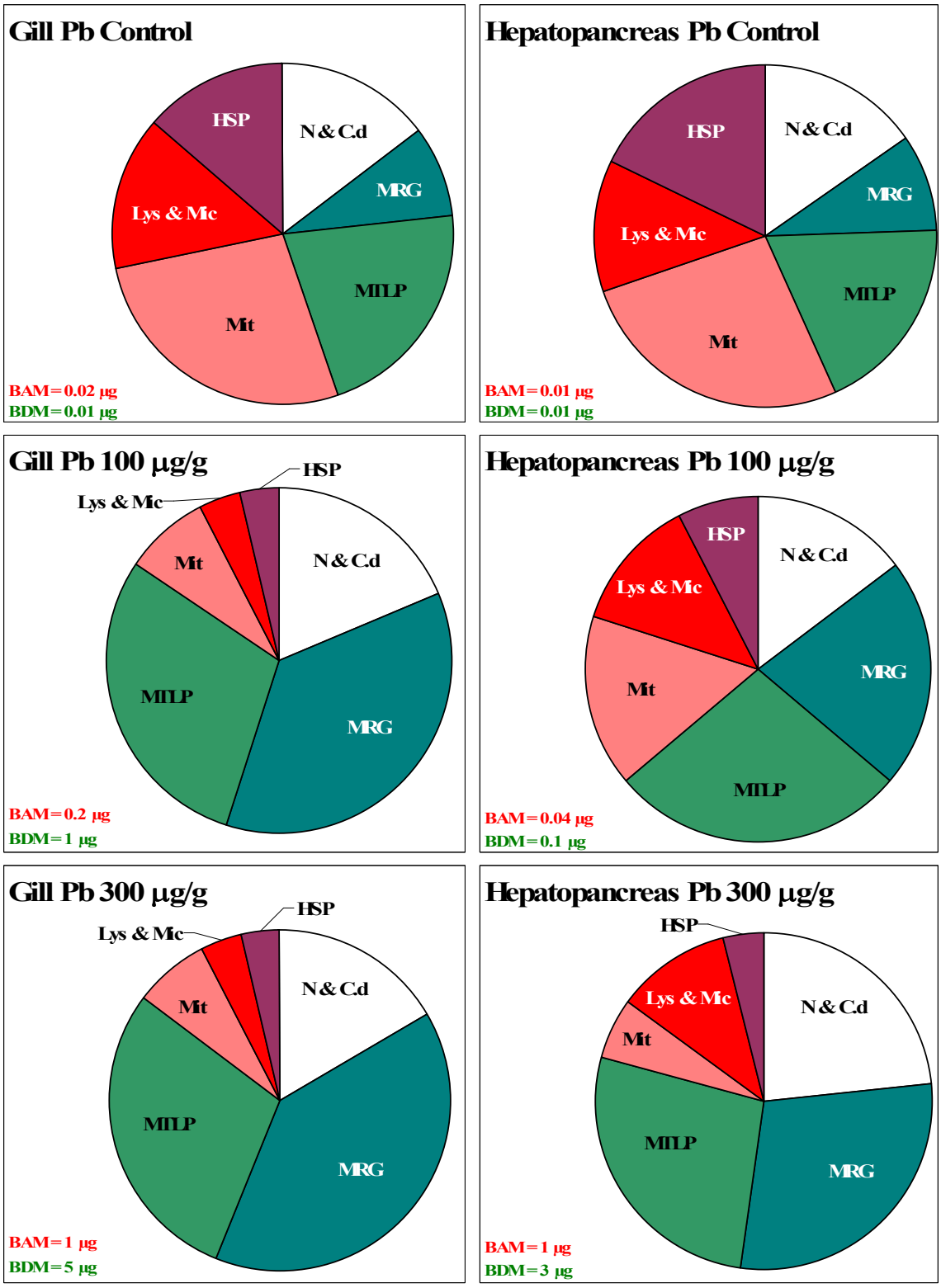


Figure 6.4: Distribution (%) of lead in each of the subcellular fractions of *A. trapezia* gill and hepatopancreas tissues following 56 days of exposure to lead spiked sediments. Subcellular fractions are: nuclei + cellular debris (N & C.d); metal rich granules (MRG); heat stable metallothionein like proteins (MTLP); mitochondria (Mit); lysosomes + microsomes (Lys & Mic); heat sensitive proteins (HSP). Red fractions ( ) make up the biologically active metal (BAM), green fractions ( ) make up the biologically detoxified metal (BDM), n = 2.

6.2.3 Activity of Marker Enzymes

6.2.3.1 *Whole Tissue Enzyme Activity*

The activity in whole tissue of the acid phosphatase (AP) lysosomal enzyme was greater in the hepatopancreas tissues than in the gill tissues for all treatments (Figure 6.5). Cytochrome c oxidase (CcO) mitochondrial enzyme activity was greater in the gill than in the hepatopancreas tissues for all treatments (Figure 6.5). AP activity was reduced in the gill of 300 µg/g lead exposed organisms compared to the control and 100 µg/g lead exposed organisms (Figure 6.5). CcO was greater in the gills and hepatopancreas tissues of lead exposed organisms than in the tissues of the control organisms (Figure 6. 5).

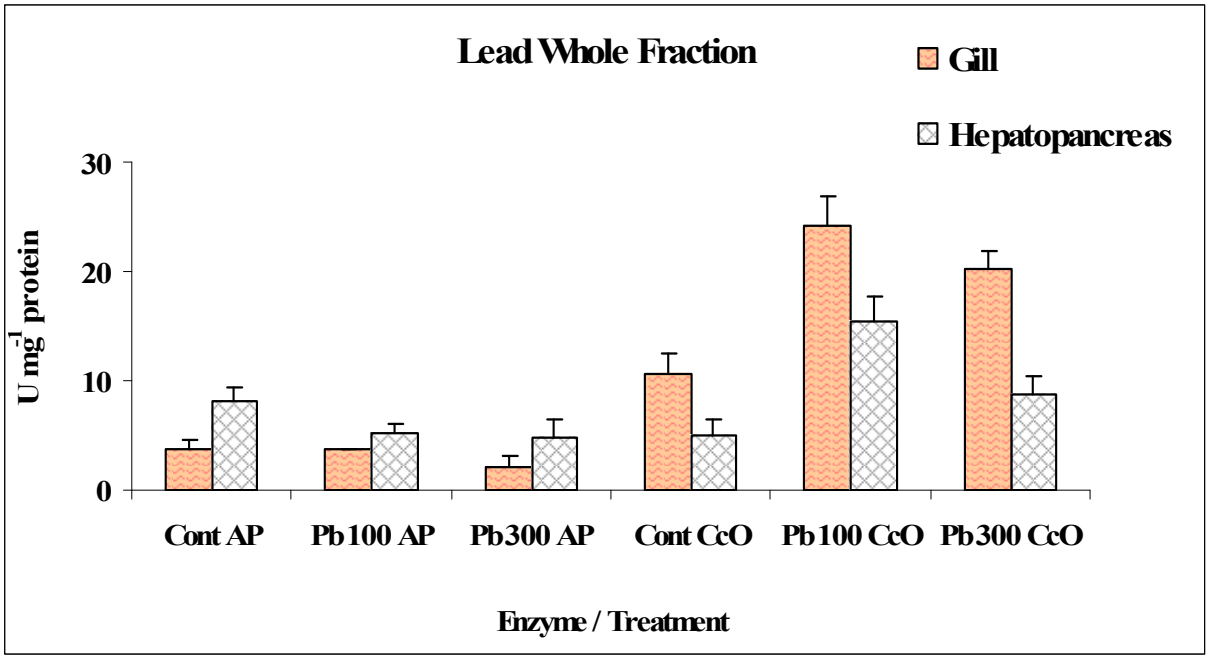


Figure 6.5: Activity of specific marker enzymes for lysosomes (acid phosphatase (AP)) and mitochondria (cytochrome c oxidase (CcO)) in whole gill and hepatopancreas tissue of *A. trapezia* exposed to sediments treated with lead at: 0 (cont), 100 and 300 µg/g dry mass. Mean ± SD, n = 2.

6.2.3.2 *Subcellular Fraction Enzyme Activity*

Gill and Hepatopancreas

Enzyme activities in the subcellular fractions of all treatments indicate that the CP 3 fractions of both tissues were enriched in mitochondria while the CP 4 fractions were enriched with lysosomes (Figure 6.6). There was some carry over of mitochondria into the CP 4 fractions and also some lysosomal enzyme activity present in the CP 3 fraction (Figure 6.6).

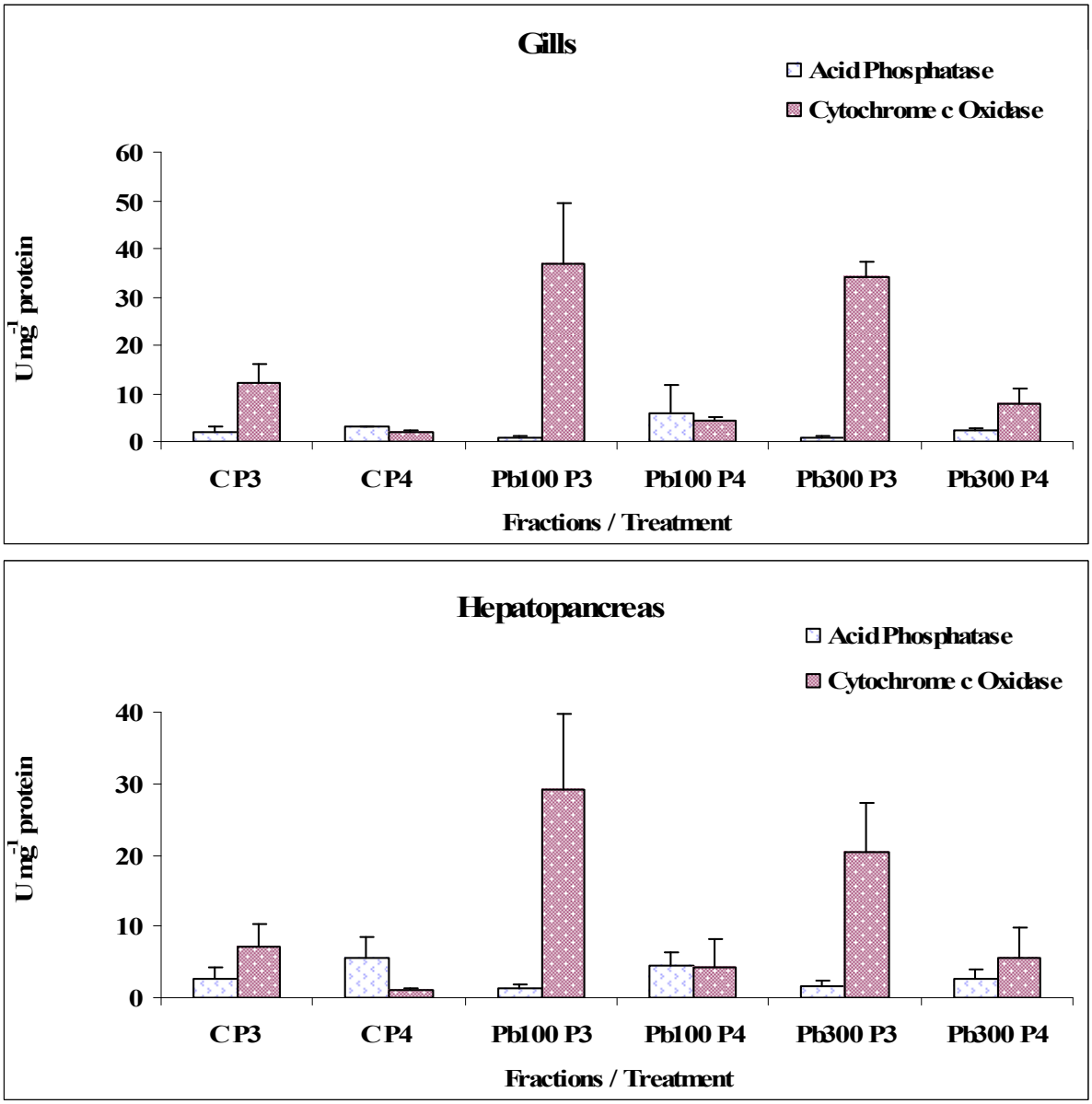


Figure 6.6: Activity of specific marker enzymes for lysosomes (acid phosphatase) and mitochondria (cytochrome c oxidase) in the mitochondrial fraction (P3) and lysosomal+microsomal fraction (P4) following subcellular fractionation of gill and hepatopancreas tissue of *A. trapezia* exposed to lead spiked sediments at: 0, 100 and 300 µg/g dry mass. Mean ± SD, n = 2.

6.2.4 Enzymatic Biomarkers

6.2.4.1 *Total Antioxidant Activity*

Total antioxidant capacity (TAOC) was reduced in lead exposed organisms compared to the control organisms (Figure 6.7). ANOVA with pair-wise comparisons showed that the factor lead sediment concentration was significant in describing TAOC with organisms from both lead treatments having significantly lower TAOC than the control organisms, however, the lead exposed organisms were not significantly different to each other (Figure 6.7, Appendix 2.9 & 2.10).

6.2.4.2 *Glutathione Peroxidase Activity*

Glutathione peroxidase (GPx) activity was reduced in lead exposed organisms compared to the control organisms, with activity in the order control > 300 µg/g > 100 µg/g (Figure 6.7). ANOVA showed that the factor lead sediment concentration was significant in determining glutathione peroxidase activity (Appendix 2.9). Bonferroni pair-wise comparison showed the 100 µg/g lead exposed organisms had significantly lower GPx activity than the control and 300 µg/g lead exposed organisms which were not significantly different to each other (Figure 6.7, Appendix 2.10).

6.2.4.3 *Total Glutathione Concentration*

Total glutathione concentration (GSH+2GSSG) increased with lead exposure in the order control < 100 µg/g < 300 µg/g (Figure 6.7). ANOVA showed the factor lead treatment did not significantly affect GSH+2GSSG concentration (Appendix 2.9).

6.2.4.4 *Reduced : Oxidised Glutathione Ratio*

The ratio of reduced to oxidised glutathione was reduced in lead exposed organisms (Figure 6.7). ANOVA with Bonferroni pair-wise comparisons showed that organisms from both lead treatments had significantly lower GSH:GSSG ratios than the control organisms but they were not different to each other (Figure 6.7, Appendix 2.9 & 2.10).

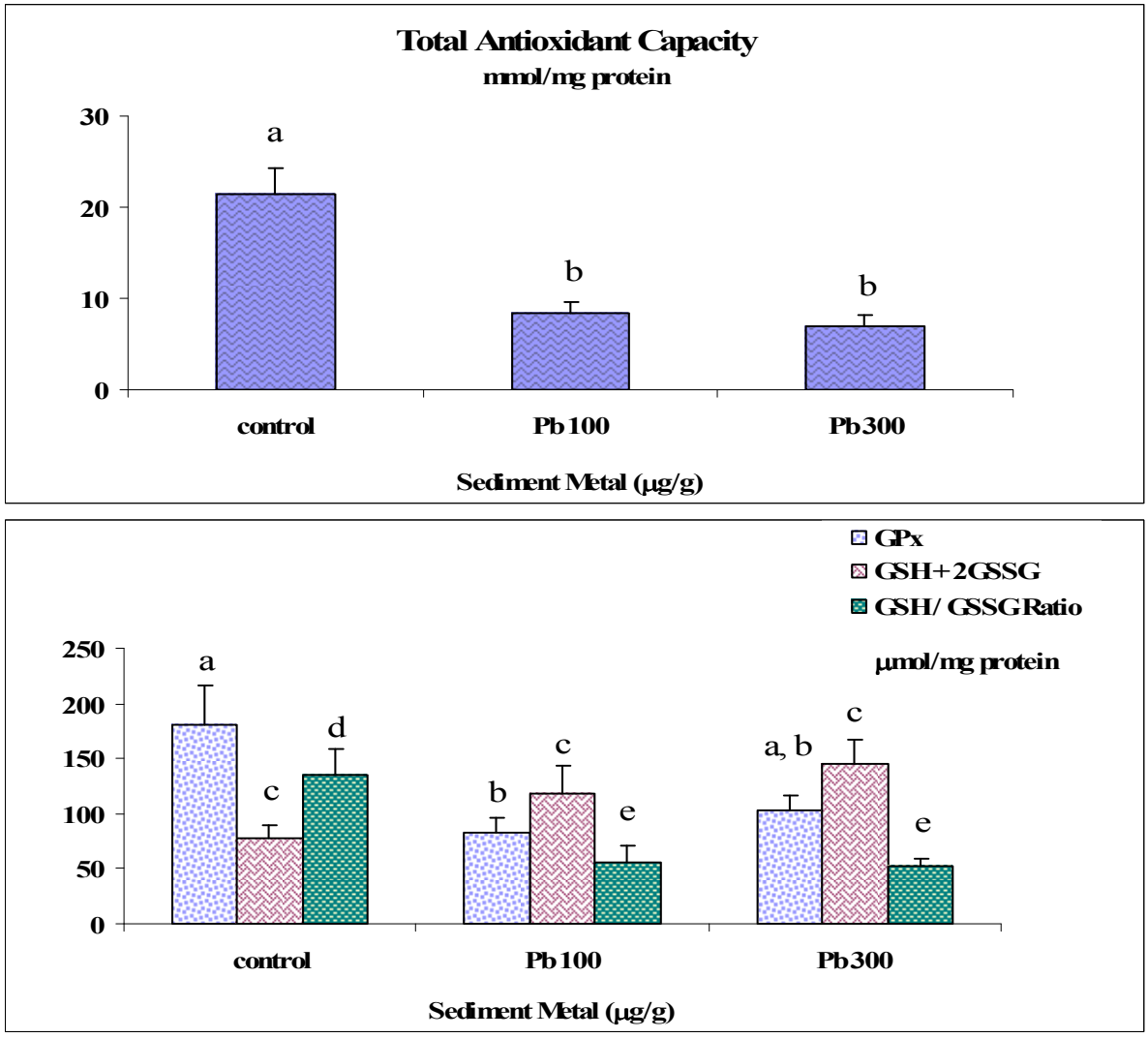


Figure 6.7: Antioxidant enzyme biomarkers: total antioxidant capacity, glutathione peroxidase (GPx), total glutathione (GSH+2GSSG), ratio of reduced to oxidised glutathione (GSH/GSSG Ratio) of *A. trapezia* (mean ± SE) following 56 days of exposure to: Pb 0 (control), n = 8, Pb 100 µg/g dry mass, n = 9 and Pb 300 µg/g dry mass, n = 12 lead spiked sediments. Different letters indicate significant differences betweens means (Bonferroni test; $p < 0.05$).

6.2.5 Oxidative Damage Biomarker – Thiobarbituric Acid Reactive Substances

Thiobarbituric acid reactive substances (TBARS) increased with exposure to higher sediment lead concentrations (Figure 6.8). ANOVA showed that the factor, lead sediment treatment had a significant influence on organism TBARS concentration (Appendix 2.9). Bonferroni pair-wise comparisons showed the lead exposed organisms had significantly higher TBARS than the control organisms but they were not significantly different to each other (Figure 6.8, Appendix 2.10).

6.2.6 Cellular Biomarker – Lysosomal Stability

Lysosomal instability increased with lead exposure (Figure 6.8). ANOVA showed that lead treatment was a significant factor in determining lysosomal destabilisation (Appendix 2.9). Bonferroni pair-wise comparisons showed that the organisms exposed to higher sediment lead concentrations had significantly more unstable lysosomes than the control organisms but they were not significantly different to each other (Figure 6.8, Appendix 2.10).

6.2.7 Genotoxic Biomarker – Micronuclei Frequency

Micronuclei occurred more frequently in lead exposed organisms than in control organisms, occurrence was in the order, control < 300 µg/g < 100 µg/g (Figure 6.8). ANOVA showed lead treatment was a significant factor in determining micronuclei frequency (Appendix 2.9). Bonferroni pair-wise comparisons showed that organisms exposed to increased sediment lead concentrations had significantly more micronuclei than the control organisms but were not significantly different to each other (Figure 6.8, Appendix 2.10).

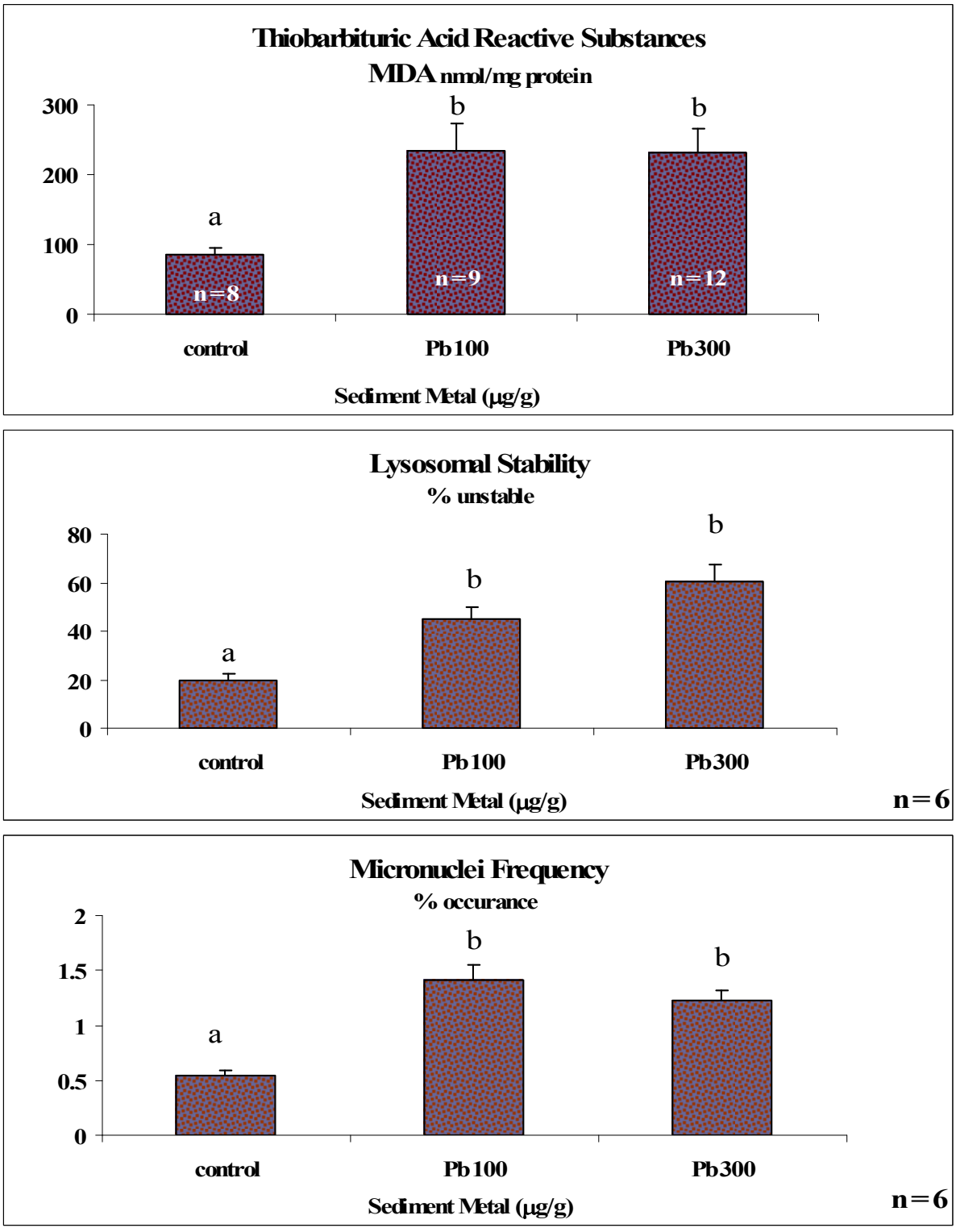


Figure 6.8: Changes in oxidative damage, cellular and genotoxic biomarkers of *A. trapezia* following 56 days exposure to lead spiked sediments, Pb 0 (control), Pb 100 µg/g and Pb 300 µg/g; dry mass. Mean ± SE. Different letters indicate significant differences between means (Bonferroni test; $p < 0.05$).

6.2.8 Relationship Between Biomarkers

6.2.8.1 *Enzymatic with Oxidative Damage, Cellular and Genotoxic Effects*

Regression analysis shows that antioxidant capacity within cells had a negative relationship with the effects measures of TBARS, lysosomal stability and micronuclei frequency (Figure 6.9). As the sediment lead exposure increased the capacity of the cells to neutralise reactive oxygen was reduced with a consequent increase in cell damage.

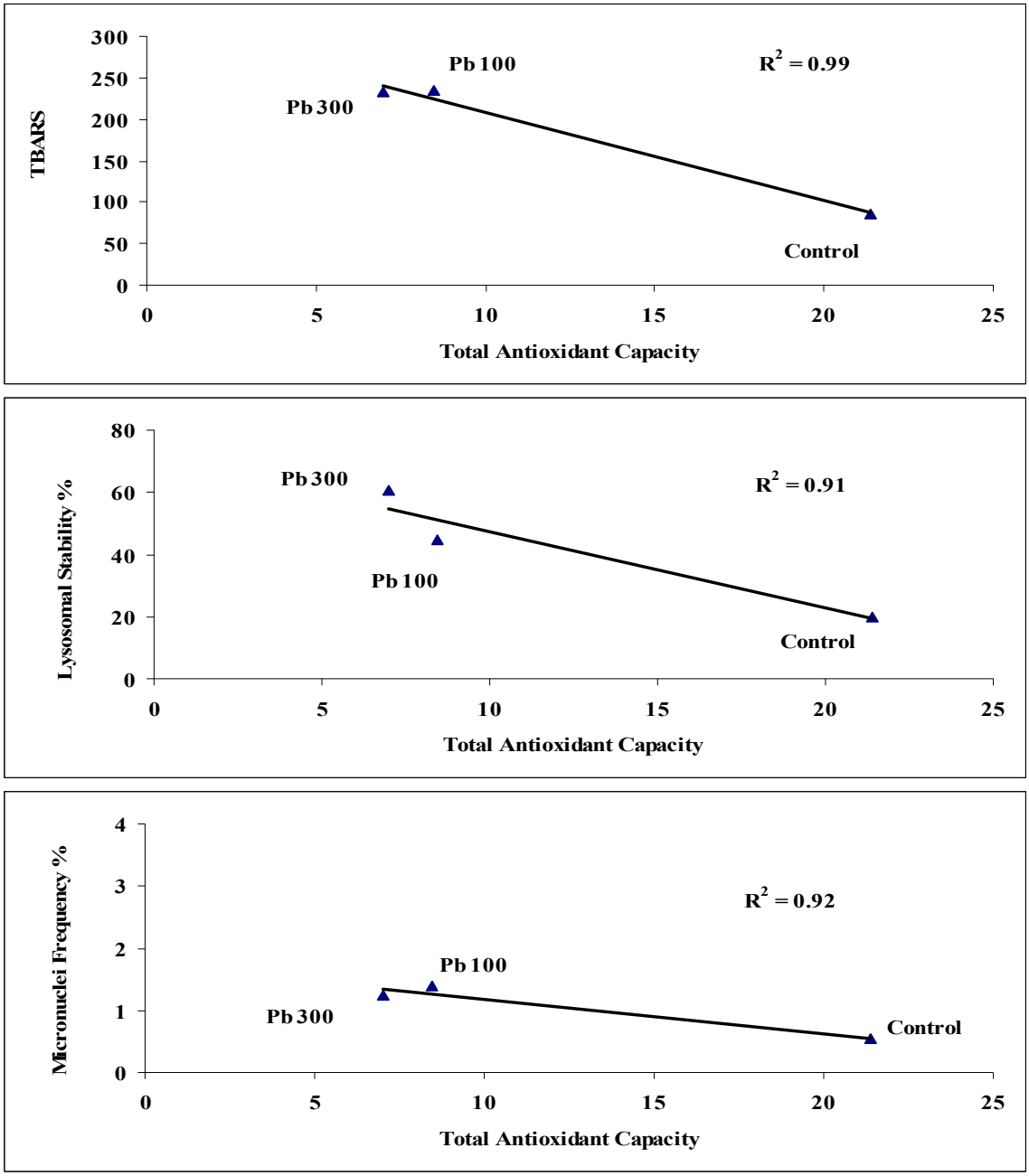


Figure 6.9: Regressions of treatment means of antioxidant capacity, (control; n=8: Pb 100, n=9 & 300; n=12), and TBARS, (control; n=8: Pb 100 n=9 & 300; n=12), lysosomal stability and micronucleus frequency (n=6).

6.2.8.2 *Oxidative Damage with Cellular and Genotoxic Effects*

There was a positive relationship between TBARS and lysosomal stability and micronuclei frequency (Figure 6.10). Increased lead exposure increased the TBARS and the regression analysis indicates that this was associated with an increase in the percentage of unstable lysosomes and the frequency of micronuclei (Figure 6.10).

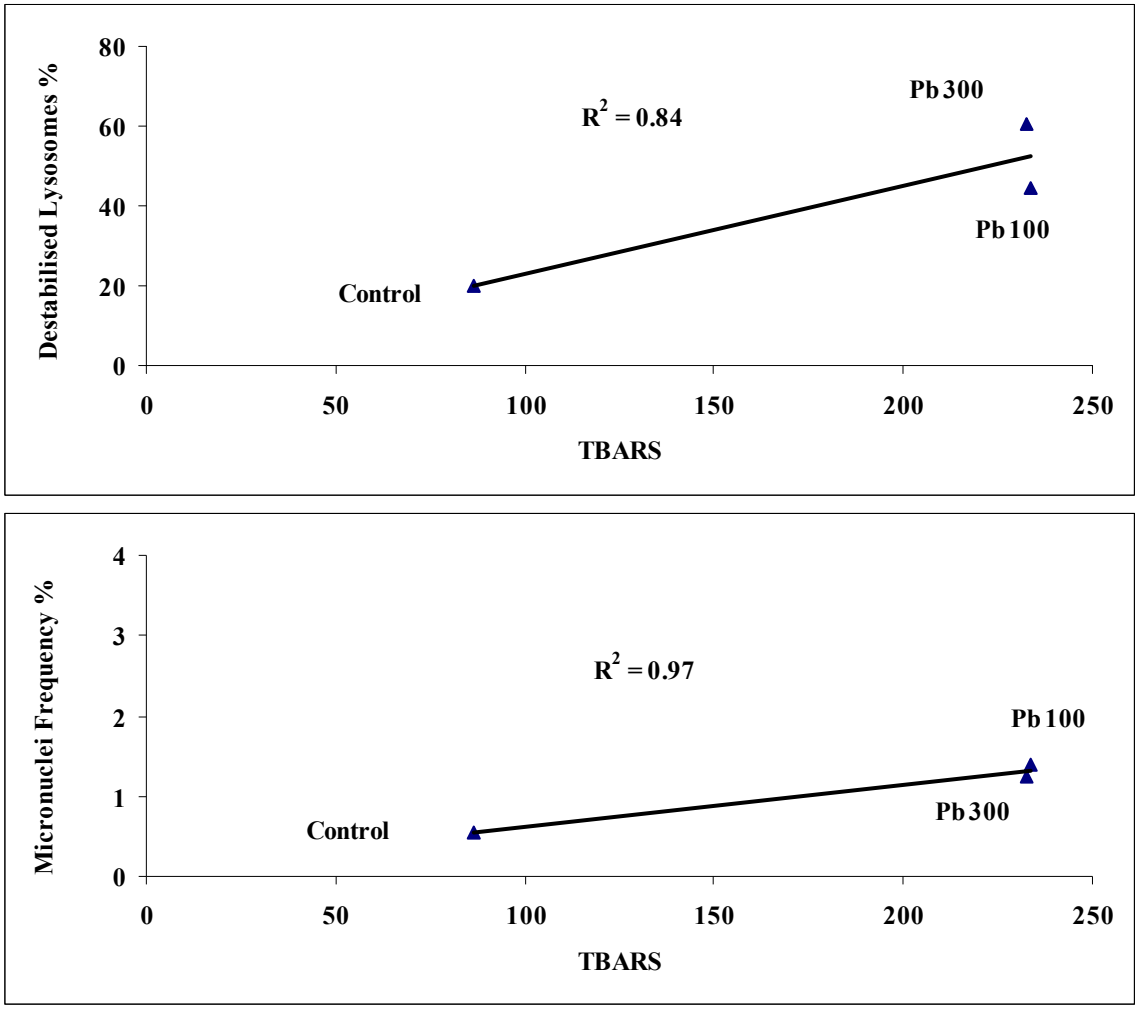


Figure 6.10: Regressions of treatment means of TBARS (control; n=8: Pb 100 n=9 & 300; n=12), with lysosomal stability and micronucleus frequency (n=6).

6.3 Discussion

6.3.1 Lead Accumulation and Subcellular Distribution

6.3.1.1 *Whole Organism Lead Accumulation*

The most striking feature of the lead accumulation pattern in this experiment is the doubling of lead tissue concentration between the 42nd and 56th day in organisms from both lead treatments (Figure 6.1). *A. trapezia* exposed to 300 µg/g of lead accumulated significantly more lead than the 100 µg/g exposed organisms (Figure 6.1; Appendix 2.3). By day 56, the tissue lead concentration of the 100 µg/g exposed organisms was one hundredth and the 300 µg/g one twenty fifth that of the exposure concentration (Figure 6.1). The lack of significant lead accumulation between the 14th and 42nd days and lower than ambient lead tissue concentrations gained (Figure 6.1) are indicative of *A. trapezia* being a weak accumulator of lead or alternatively that the lead bioavailability was low during this period. This pattern seems to hold true for the first 42 days of the exposure, however, the rapid accumulation seen in the final 2 weeks and the positive day 56 sediment tissue lead concentration relationship (Figure 6.2), for the two lead treatments, points to them being net accumulators of lead, indicating that the regulatory mechanisms became saturated after 42 days (Rainbow *et al.*, 1990). The pattern observed could relate to a change in exposure route from food and sediment to dissolved lead as the oxidation of the sediments over time released the sediment bound lead into the pore water. Whatever the cause there are important implications for exposure time when conducting experiments of this kind. A longer exposure time may have resulted in the organisms reaching equilibrium with their exposure concentration, whereas a shorter exposure may have resulted in an erroneous conclusion about their capacity to regulate lead uptake. Previous studies using bivalves have found that equilibrium lead concentrations are reached between 28 and 60 days. *Mytilus galloprovincialis,* for example, transplanted into a lead contaminated area reached a steady state of tissue lead after 4 weeks exposure (Regoli and Orlando, 1993). Burt *et al* (2007) found that after 60 days exposure *A. trapezia* reached the maximum tissue lead concentration and a steady state with the exposure environment. Their study measured concentrations of around 2 and 10 µg/g, respectively, in *A. trapezia* after 90 days of exposure in Lake Macquarie NSW, to lead sediment concentrations of 120 and 225 µg/g, which are similar to the tissue concentrations measured in the present study.

6.3.1.2 *Individual Tissue Lead Accumulation*

The pattern of lead accumulation within the individual tissues changed over the course of the experiment. Haemolymph lead concentrations were higher in both lead treatments for the first 42 days, the pattern changed between days 42 and 56 with the gill and haemolymph lead concentration being equal in the 100 µg/g lead treatment and the gill higher than the haemolymph in the 300 µg/g lead treatment (Figure 6.3). In mammals, blood is the initial site of lead absorption and distribution to other tissues. The half-life of lead in blood is estimated to be 28 - 36 days with 95 - 99 % found in haemoglobin (Jin *et al.*, 2008). *A. trapezia* haemolymph, unlike other bivalves, contains haemoglobin as a respiratory pigment (Sullivan, 1961). The high lead concentrations in the haemolymph relative to the other two tissues for the first 42 days of exposure may be related to the affinity for lead to bind to haemoglobin. The hepatopancreas generally had lower lead concentrations than the other tissues (Figure 6.3). Previous bivalve studies have found the gills to accumulate higher (Blasco and Puppo, 1999; Domouhtsidou *et al.*, 2004; Jing *et al.*, 2007) or equal (Riba *et al.*, 2004) concentrations of lead to the hepatopancreas. Jing *et al* (2007) found the mantle of the pearl oyster *Pinctada fucata* was secondary to the gill but higher than the hepatopancreas in lead accumulation and they suggest the mantle may play an important role in lead detoxification and storage in this oyster. The mantle was included with the gill in the present study so would have contributed to the gill lead concentrations measured. In particular the rapid increase in the gill tissue lead concentrations compared to the other tissues in the 300 µg/g lead exposed organisms during the last two weeks of the exposure may be related as much to the mantle as to the gill (Figure 6.3). As lead transportation is known to occur via analogous pathways to calcium ions it is feasible that in the process of shell formation where the transport of calcium ions are regulated via the mantle (Li, 2004), lead may be transported and accumulated in the mantle by the same pathway. Lead was found to be fairly evenly distributed between the gill and hepatopancreas tissues, in native populations of the scallop *Chamys varia* from a contaminated bay, but with increased size the pattern was reversed suggesting that with time lead is transported to the hepatopancreas for detoxification and storage thus increasing the lead burden in this tissue relative to the gills (Bustamante and Miramand, 2005). The present experiment was conducted with previously unexposed *A. trapezia* exposed to reasonably high lead concentrations over a relatively short period, so these longer term adaptive patterns to chronic lead exposure cannot be observed.

6.3.1.3 *Subcellular Lead Distribution*

The percentage of lead in the BDM fractions of organisms from both treatments more than doubled in the gill tissues and roughly doubled in the hepatopancreas tissues compared to these tissues in the control organisms from around 30 % to 50 – 69 % (Table 6.1; Figure 6.4). The MTLP fractions of both tissues of the control *A. trapezia* contained about 70 % of the BDM lead whereas the tissues of the lead exposed *A. trapezia* from both lead treatments had a fairly even distribution of lead between the two fractions with generally a slightly higher percentage of lead in the MRG (Table 6.2; Figure 6.4). The percentage of lead in the pellet fraction of terrestrial snails *Helix pomatia* fed lead enriched lettuce increased in a similar proportion to this study after 30 - 35 days compared to snails fed uncontaminated food and was presumed to be associated with MRG and cell debris (Dallinger and Wieser, 1984). The lead treated snails had only a small percentage of lead in the supernatant, comprised of cytosolic proteins and enzymes, which was bound to unspecified proteins (Dallinger and Wieser, 1984). MT induction in marine molluscs has been reported for zinc, copper, cadmium and mercury (George *et al.*, 2000; Hamza-Chaffai *et al.*, 1995; Langston *et al.*, 1989; Roesijadi, 1992; 1996), and it is generally assumed that other metals such as lead would be bound and transported by similar MTLP, however, no specific MTLP for lead has been described in molluscs. In mammals, MT biosynthesis is induced by and is a major cytosolic binding site for lead and it is thought to play an important role in regulating the intracellular toxicity of lead (Chu *et al.*, 2000). An increased lead burden in the tissues of the lead exposed organisms in this experiment was in the heat stable MTLP fraction so the presence and activity of a lead binding protein is demonstrated for *A. trapezia*. The increased lead associated with the MRG fraction in the hepatopancreas of the lead exposed *A. trapezia* is in agreement with a study of lead accumulation in the digestive gland of mussels transplanted to a lead contaminated area by Regoli and Orlando (1994). Lead associated with MRG in mussels *Mytilus edulis* is thought to be accumulated by endocytosis in a colloidal or particulate form and precipitated as a sulphur or phosphate salt inside the digestive cells as well as in the extracellular compartments (George, 1990). The higher percentage of lead in the MRG fraction in the gills compared to the hepatopancreas of *A. trapezia* in this study is in agreement with that found for copper zinc and cadmium in the freshwater bivalve *Pyganodon grandis* collected from metal contaminated lakes by Bonneris *et al* (2005). These MRG are likely to be extracellularly bound in the gill filaments or associated with storage in the mantle (Jing *et al.*, 2007) and reflect the higher accumulation of lead in the gill tissue.

Increased AP activity (Figure 6.5) in the hepatopancreas indicates that this tissue was enriched with lysosomes compared to the gills. Lysosomal enrichment in oyster hepatopancreas has previously been reported (Sokolova *et al.*, 2005a). The epithelium of the hepatopancreatic diverticula of bivalves contain digestive cells which are characterised by a well developed endo-lysosmal system (Marigómez *et al.*, 2002). These cells are primarily involved in intracellular food digestion but also accumulate metals coming from the internal medium via haemolymph or from food and sediment particles (Viarengo *et al.*, 1988; Viarengo and Nott, 1993). In *A. trapezia* in this experiment, internal transport of lead via haemolymph to lysosomal rich digestive cells is likely, given the high lead concentrations in the haemolymph relative to the other tissues during the early part of the exposure (Figure 6.3). A higher percentage of lead in the lysosomal+microsome fraction of the hepatopancreas of exposed organisms compared to the gill was also seen (Figure 6.4). Lead accumulation in lysosomes of both gill and hepatopancreas tissues of mussels *Mytilus edulis* has also been demonstrated (Einsporn and Koehler, 2008), after 10 days lead exposure. Lead in the microsomes of the lead exposed *A. trapezia*, which made up part of this fraction, may be associated with fragmented endoplasmic reticulum, which is generally responsible for the synthesis and transport of proteins (Bonneris *et al.*, 2005; Jarosch *et al.*, 2002).

The activity of CcO in the gill tissue was higher than that of the hepatopancreas tissue in all treatments and was enhanced in the lead exposed organisms compared to the control organisms (Figure 6.5). The mitochondrial fraction of both tissues in all treatments contained around half the BAM lead, with the exception of the hepatopancreas of the 300 µg/g where it was only 28 %. For the lead exposed organisms, this represents a considerable increase in lead concentration (Table 6.1) for this organelle and has the potential to result in adverse effects. Localisation of lead in mussel gill and hepatopancreas mitochondria after 10 days exposure, for example, resulted in a reduction in mitochondrial cristae (Einsporn and Koehler, 2008). The tendency for lead associated with the BAM fractions in the lead exposed *A. trapezia* to increase in line with the increase in whole tissue indicates that lead is not being completely detoxified and the potential for lead toxicity exists.

6.3.2 Enzymatic Biomarkers – Oxidative Enzymes

Increased lead burdens in the mitochondrial fraction of the lead exposed organisms significantly reduced the TAOC of *A. trapezia* (Figure 6.7). The TAOC of the lead exposed organisms was virtually the same, despite the gill mitochondrial lead burden being higher in organisms from the 300 µg/g lead exposure (Table 6.2). It is possible that there is a critical lead concentration at which TAOC is impaired to this level that may actually be lower than the concentrations measured here. The GPx activity was reduced in the lead exposed organisms, compared to the controls, and for the 100 µg/g lead exposed organisms the reduction was significant (Figure 6.7). The reduction in GPx is reflected in increased GSH+2GSSG concentrations which the significantly reduced GSH:GSSG ratios of the lead exposed organisms indicate was due to a build up of oxidised glutathione (Figure 6.7). Earthworms *Lampito mauritii* exposed for 28 days to soil spiked with 75 to 300 mg/kg Pb^{2+} also showed significantly increased GSH:GSSG ratios in response to increased tissue lead (Maity *et al.*, 2008). Mussels *Mytilus galloprovincialis* exposed for 4 weeks in cages in harbour waters following sediment dredging accumulated significant tissue lead over time with a subsequent decrease in the GPx activity and peroxyl and hydroxyl radical reduction capability (TAOC) (Bocchetti *et al.*, 2008). The mussels in the Bocchetti *et al*., (2008) study were also exposed to PAHs and other metals but only lead showed significant accumulation. The results for *A. trapezia* oxidative impairment in response to lead suggest that lead may have been the most significant toxicant in the Boccehetti *et al*., (2008) study. Lead bioaccumulation has been shown to be toxic to bivalve cells in other studies, localisation of lead in mussel gill and hepatopancreas mitochondria after 10 days exposure, for example, resulted in a reduction in mitochondrial cristae (Einsporn and Koehler, 2008).

6.3.3 Oxidative Damage Biomarker – Thiobarbituric Acid Reactive Substances

The reduction in TOAC in the lead exposed *A. trapezia* induced significant lipid peroxidation (Figure 6.8). As was the case for TOAC the concentration of TBARS was almost the same for organisms from both lead exposures which supports the link between increased ROS and lipid peroxidation. The TBARS concentration was highly negatively correlated with TAOC (Figure 6.9) which further supports an interaction between increased ROS and lipid peroxidation.

A lead induced increase in TBARS associated with increased GSSG concentrations has also been show in rat kidneys (Pande and Flora, 2002), mice brains (Flora and Seth, 1999), in the livers of mallard ducks (Mateo *et al.*, 2003) and in the gill, digestive gland and mantle of the marine bivalve *Perna viridis* (Prakash and Rao, 1995). Investigations into the toxic effects of lead on human cell membrane components have determined that lead induces ROS generation which reduces the antioxidant defence system of cells via glutathione depletion. This alters the lipid composition, changing membrane integrity, permeability and function, thereby increasing their susceptibility to lipid peroxidation (Ercal *et al.*, 2001; Gurer and Ercal, 2000). Ercal *et al*., (2001) suggests that as lead cannot initiate lipid peroxidation on membranes directly it might induce oxidative stress by interacting with oxyhaemoglobin, leading to peroxidative haemolysis in red blood cell membranes. As *A. trapezia* has haemoglobin as a respiratory pigment (Sullivan, 1961), this may have been a pathway for the production of the significantly higher TBARS measured in the lead exposed organisms (Figure 6.8).

6.3.4 Cellular Biomarker – Lysosomal Stability

The increase in TBARS in the lead exposed *A. trapezia* significantly reduced the stability of the lysosomes (Figure 6.8; Appendix 2.10). Lysosomal destabilisation was positively correlated with TBARS concentration (Figure 6.10). The percentage of destabilised lysosomes in the 100 µg/g and 300 µg/g lead exposed organisms was 45 % and 61 %, respectively, which puts them in the highly stressed range (Ringwood *et al*., 2003). Severe disturbance in lysosomal membrane stability has also been reported for mussels *Mytilus galloprovincialis* with increasing concentrations of tissue lead (Regoli and Orlando, 1993). Lead accumulation in the lysosomes of the digestive gland of the mussel *Mytilus edulis* caused specific alterations such as aggregation of residual bodies, proliferation of auto-phagosomes and an increase in multi-lamellated aggregates which all have the potential to contribute to impairment of lysosomal function (Einsporn and Koehler, 2008). Lysosomal destabilisation in the *A. trapezia* exposed to lead was negatively correlated with TAOC (Figure 6.9), suggesting that the destabilisation of the lysosomal membrane was probably two fold: direct attack from ROS; and through unstable lipid radicals, from excess oxyradical production, destabilising the lysosomal membrane.

6.3.5 Genotoxic Biomarker – Micronuclei Frequency

The significant increase in micronucleus frequency in the lead exposed *A. trapezia* compared to control organisms (Figure 6.8) indicates lead accumulation in cells had a genotoxic effect. This could be a direct effect of lead in the cell DNA or an indirect effect of oxygen radicals reacting with cellular macromolecules. Significant tissue lead accumulation in mussels *Mytilus galloprovincialis*, exposed to harbour waters following sediment dredging, showed significant impairment of the TAOC with a subsequent increase in both lysosomal destabilisation and micronuclei frequency over a period of 4 weeks (Bocchetti *et al.*, 2008). The micronuclei frequency of lead exposed *A. trapezia* was negatively correlated with TAOC (Figure 6.9) and positively correlated with TBARS (Figure 6.10) indicating both a direct and indirect influence of reduced antioxidant reduction capacity. Micronuclei frequency in chronically exposed *.M galloprovincialis* has shown stronger correlations with tissue chemical residues than short term caged exposures (Bolognesi *et al.*, 2004). *M. galloprovincialis* transplanted into metal polluted waters accumulated similar concentrations of all metals, after 30 days exposure, to those found in native mussels from the area, with the exception of lead which was twice that of native mussels (Nigro *et al.*, 2006). Despite this, the frequency of micronuclei, which doubled in the transplanted mussels after 30 days, did not equal that of the native mussels which were 4 times that of pre-exposure mussels (Nigro *et al.*, 2006). The differences in micronuclei frequency observed between chronically exposed and short term exposures supports the usefulness of the assay for time-integrated response to toxic exposure. That the lead exposed *A. trapezia* after 56 days exposure had significant genotoxic alterations indicates that long term exposure to lead has the potential to reduce population viability.

6.4 Summary and Conclusions

The most significant feature of the lead accumulation pattern of *A. trapezia* exposed to lead for 56 days in this experiment was the doubling of tissue lead concentrations between the 42nd and 56th day in organisms from both lead treatments. Final tissue concentrations were considerably lower than that of the sediment lead exposure concentrations the 100 µg/g had 0.01 and the 300 µg/g lead exposed organisms 0.04 that of the sediment lead. A lack of significant lead accumulation during the first 42 days of exposure and the lower than ambient tissue lead concentrations at day 56 are indicative of *A. trapezia* being a partial regulator of lead.

The rapid lead accumulation in the final 2 weeks of the exposure suggests detoxification mechanisms were overwhelmed. The haemolymph contained a significant proportion of the accumulated lead throughout most of the exposure. As *A. trapezia* contains haemoglobin as a respiratory pigment the high concentrations in the haemolymph relative to the other two tissues may be related to the affinity for lead binding to haemoglobin. Between 50 to 70 % of accumulated lead in the gill and hepatopancreas of lead exposed *A. trapezia* was detoxified. The BDM was fairly evenly distributed between the MRG and MTLP fractions in both tissues of lead exposed organisms. BAM lead burdens of the 100 and 300 µg/g lead exposed *A. trapezia* were 10 and 50 in gill, and 4 and 50 in hepatopancreas, respectively, greater than the control organisms. Approximately half of the BAM lead in gill tissues of exposed organisms was in the mitochondrial fraction, while the hepatopancreas had the majority in the lysosome+microsome fraction. The higher lead burden in the gill mitochondria probably relates to dissolved lead exposure and rapid transport into cells via haemolymph while the higher proportion in the lysosomes in the hepatopancreas relates to it being the main absorption site of food associated lead which is normally enriched in lysosomes. The TAOC and GSH:GSSG ratio of lead exposed *A. trapezia* was significantly reduced compared to control organisms. The reduction was similar in organisms from both treatments and it is possible that there is a critical lead concentration at which TAOC is impaired to this extent that may be lower than the concentrations measured here. The GPx activity of the 100 µg/g lead exposed *A. trapezia* was significantly reduced compared to the 300 µg/g lead exposed and control organisms. There was a significant increase in lipid peroxidation, lysosomal destabilisation and micronuclei frequency with lead exposure. A significant exposure – dose – response relationship for lead has been established in this study which indicates that sediment lead at these concentrations leads to increased BAM burdens with significant impairment of the oxidative reduction pathway leading to a cascade of effects at a cellular and subcellular level which has the potential to influence *A. trapezia* reproductive success.

7 *Anadara trapezia* Selenium Spiked Sediment Study

7.1 Aim

The purpose of this study is to determine the exposure dose response of *A. trapezia* to sediments with known concentrations of selenium. The exposure time was 56 days and the development of useful biomarkers of effect undertaken with a view to determining whether they would be a suitable organism to collect from the field and use for field metal exposures in cages.

7.2 Results

7.2.1 Selenium Accumulation

7.2.1.1 *Whole Organism Selenium Accumulation*

ANOVA showed that, time and selenium treatment were both significant factors in selenium accumulation and there was no interaction between time and selenium treatment (Appendix 2.1). Tissue selenium concentrations were in the order 20 µg/g > 5 µg/g > control for each analysis time (Figure 7.1). The tissue selenium concentration of the control organisms remained the same over the course of the exposure (Figure 7.1). Bonferroni pair-wise comparisons showed that the selenium tissue concentrations of the control organisms did not differ significantly from day 0 unexposed organisms (Appendix 2.2). The pattern of selenium accumulation was similar in organisms from both selenium treatments (Figure 7.1). The over-all statistical comparison between treatments showed organisms from both the selenium treatments differed significantly from the unexposed and control organisms and from each other at all collection times (Appendix 2.2). The tissue selenium concentrations of the 20 µg/g selenium exposed organisms were around twice those of the 5 µg/g selenium exposed organisms by day 14 and remained so for day 28 and 56, at day 42 the difference was 1.5 times greater (Figure 7.1). Bonferroni pair-wise comparisons between treatments for each collection day showed that the control organisms had significantly less tissue selenium than the 5 and 20 µg/g selenium exposed organisms for each collection day and the 5 µg/g selenium exposed organisms had significantly less tissue selenium than the 20 µg/g selenium exposed organisms for each collection day except for day 42 (Appendix 2.3).

The selenium exposed organisms both accumulated selenium quite rapidly in the first 14 days with very slight increases over the next 28 days and then a slight decrease to day 56 suggesting that an equilibrium tissue selenium was reached (Figure 7.1). The 5 µg/g selenium exposed organisms had final tissue selenium concentrations 0.3 times and the 20 µg/g selenium exposed organisms, 0.2 times that of that of the exposure concentration (Figure 7.1). The regression between selenium sediment concentration and organism tissue selenium concentration after 56 days shows a significant positive relationship (Figure 7.2), however, as sediment selenium concentration increased from 5 µg/g to 20 µg/g the tissue selenium accumulation was not proportional (Figure 7.2). Analysis of the within treatment differences between collection days showed that organisms from both the selenium treatments had significant selenium accumulation after 14 days of exposure with no further significant accumulation at any other collection time (Appendix 2.4). The control organisms showed a significant difference in selenium concentration between day 28 and days 14 and 56 (Appendix 2.4). Figure 7.1 shows that this was due to day 28 organisms having a slightly higher selenium concentration than the other collection times.

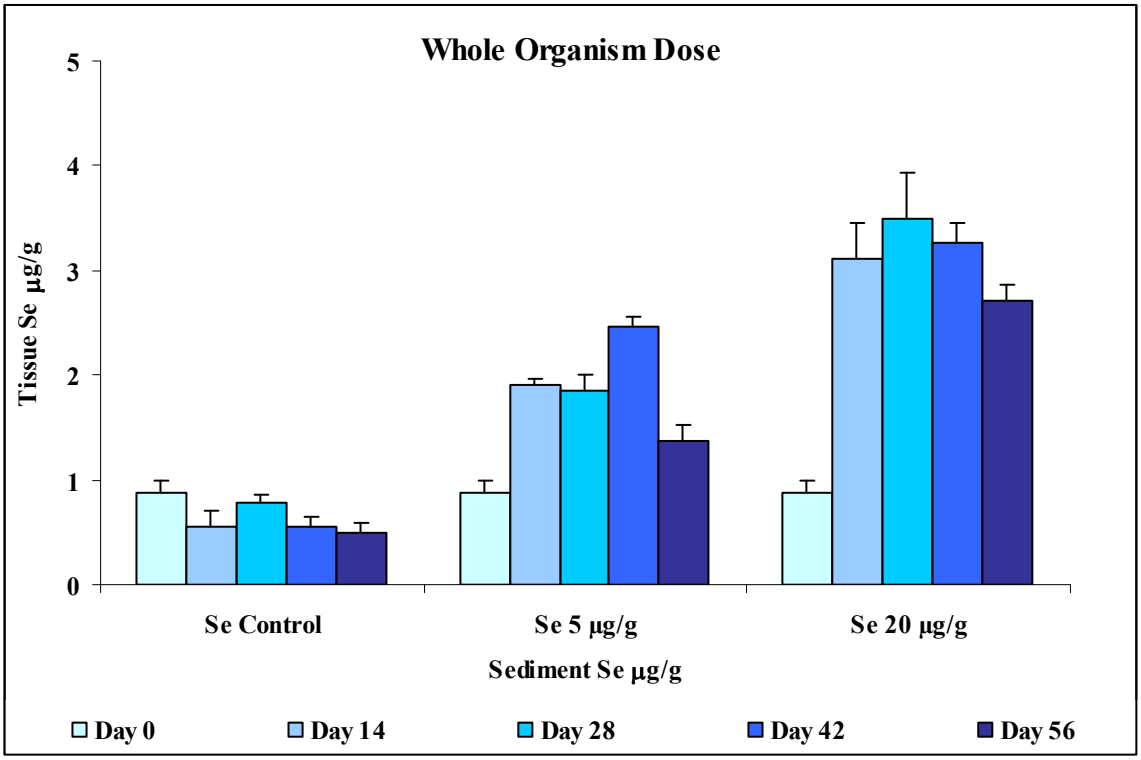


Figure 7.1: Selenium accumulation in whole tissue of *A. trapezia* at 2 weekly intervals over 56 days of exposure to selenium spiked sediments at: 0 (control), 5 and 20 µg/g dry mass. Mean ± SE, n = 8, 12 and 8 respectively.

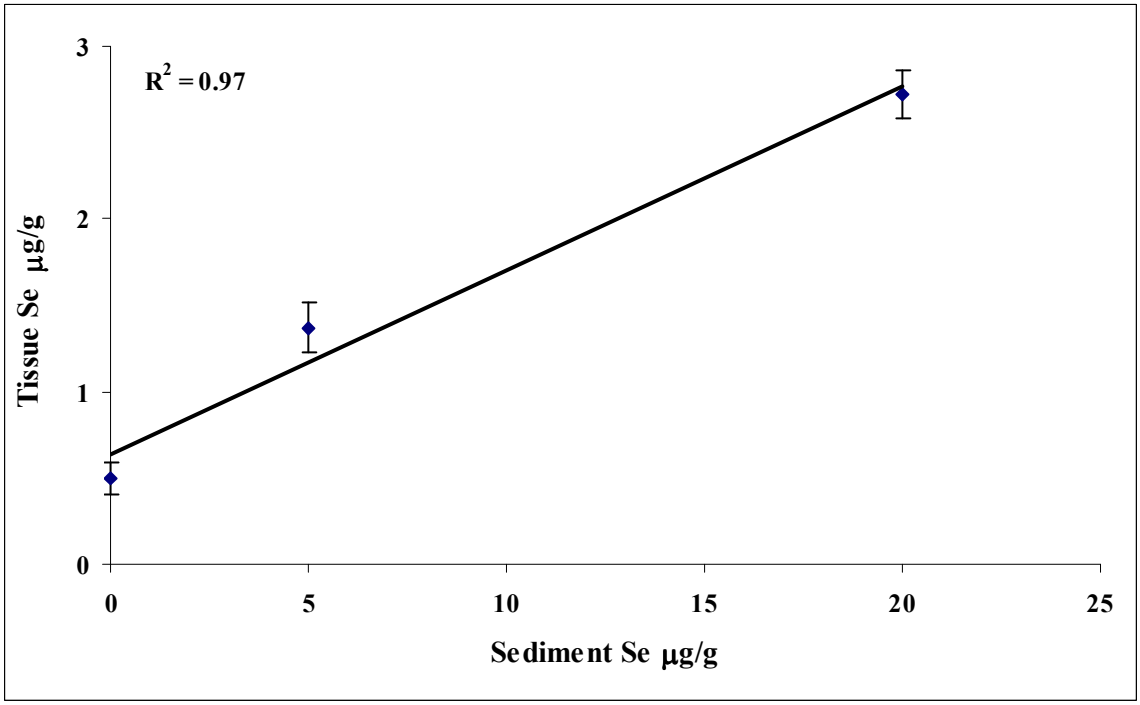


Figure 7.2: Regression of *A. trapezia* tissue selenium concentration after 56 days exposure and sediment selenium concentrations of 0, 5 and 20 µg/g dry mass. Mean ± SE, n = 8, 12 and 8 respectively.

7.2.1.2 *Individual Tissues Selenium Accumulation*

ANOVA showed that selenium accumulation was significantly different between tissues and there was a significant interaction between the factors, tissue and selenium treatment and tissue and day but not between the factors, tissue, day and treatment (Appendix 2.5). Bonferroni multiple pair-wise comparisons showed significant differences in selenium concentrations between gill and haemolymph tissues for all treatments and in selenium concentrations between gill and hepatopancreas tissues for all but the unexposed organisms (Appendix 2.6). The hepatopancreas and haemolymph tissues of the unexposed, control and 5 µg/g selenium exposed organisms had significantly different selenium concentrations (Appendix 2.6). Analysis of the between tissue differences at each collection time showed that selenium concentrations were significantly different between gill and the other two tissues for all treatments at all collection times (Appendix 2.7). The selenium concentrations of the hepatopancreas and haemolymph tissues of the control organisms were significantly different at all collection times while the 20 µg/g selenium treated organisms were not and the 5 µg/g selenium treated organisms only on day 28 (Appendix 2.7).

Gill

Tissue selenium concentrations were higher in gills than the other two tissues for all treatments at all analysis times (Figure 7.3). Selenium accumulation was greatest in the first 14 days for both selenium treatments (Figure 7.3). Analysis of the within tissue differences between collection days showed that this was a significant selenium accumulation difference (Appendix 2.8). The control organisms had a significant selenium increase in gill tissue between the 14^{th} and 28^{th} day (Appendix 2.8). Selenium concentrations in the gill tissues of the 20 µg/g selenium treatment was about 1.5 times that of the gills of the lower selenium treatment by day 14 and this pattern was the same for the remaining days (Figure 7.3). Analysis of the within tissue differences between collection days showed that the decrease in gill tissue selenium concentration of the selenium exposed organisms from day 42 to day 56 (Figure 7.3) was not significant for either treatment (Appendix 2.8). The pattern of selenium accumulation in the gills of the selenium exposed organisms is reflected in the whole organism dose (Appendix 2.4 & 2.8).

Hepatopancreas

Tissue selenium concentrations were generally lower in the hepatopancreas than in the gills and higher than the haemolymph (Figure 7.3). The selenium accumulation pattern of the hepatopancreas was similar to that of the gills in both selenium treatments (Figure 7.3), however, Bonferroni pair-wise comparisons of the within hepatopancreas tissue selenium concentrations over time showed that while uptake in the 20 µg/g selenium exposed organisms was significant between day 0 and the 14th day the 5 µg/g selenium exposed organisms only had a significant increase in hepatopancreas tissue selenium concentration after the 28th day of exposure and the control organisms had no significant change in selenium concentration over the course of the experiment (Appendix 2.8). The total selenium concentration in this tissue was around half that of the gill tissues for the selenium exposed organisms throughout the exposure time (Figure 7.3).

Haemolymph

The accumulation of selenium in the haemolymph was higher in the 20 µg/g selenium exposure and equal in the 5 µg/g selenium exposure to the hepatopancreas after 14 days and then decreased to below it for the remainder of the exposure time (Figure 7.3), suggesting a change in metal processing or exposure route may have occurred. Analysis of the within tissue selenium concentration differences between collection days showed that the control organisms had no significant increase in haemolymph selenium concentration over the course of the experiment (Appendix 2.8). The selenium exposed organisms in both treatments showed significant increases in haemolymph selenium concentration in the first 14 days of the exposure (Appendix 2.8).

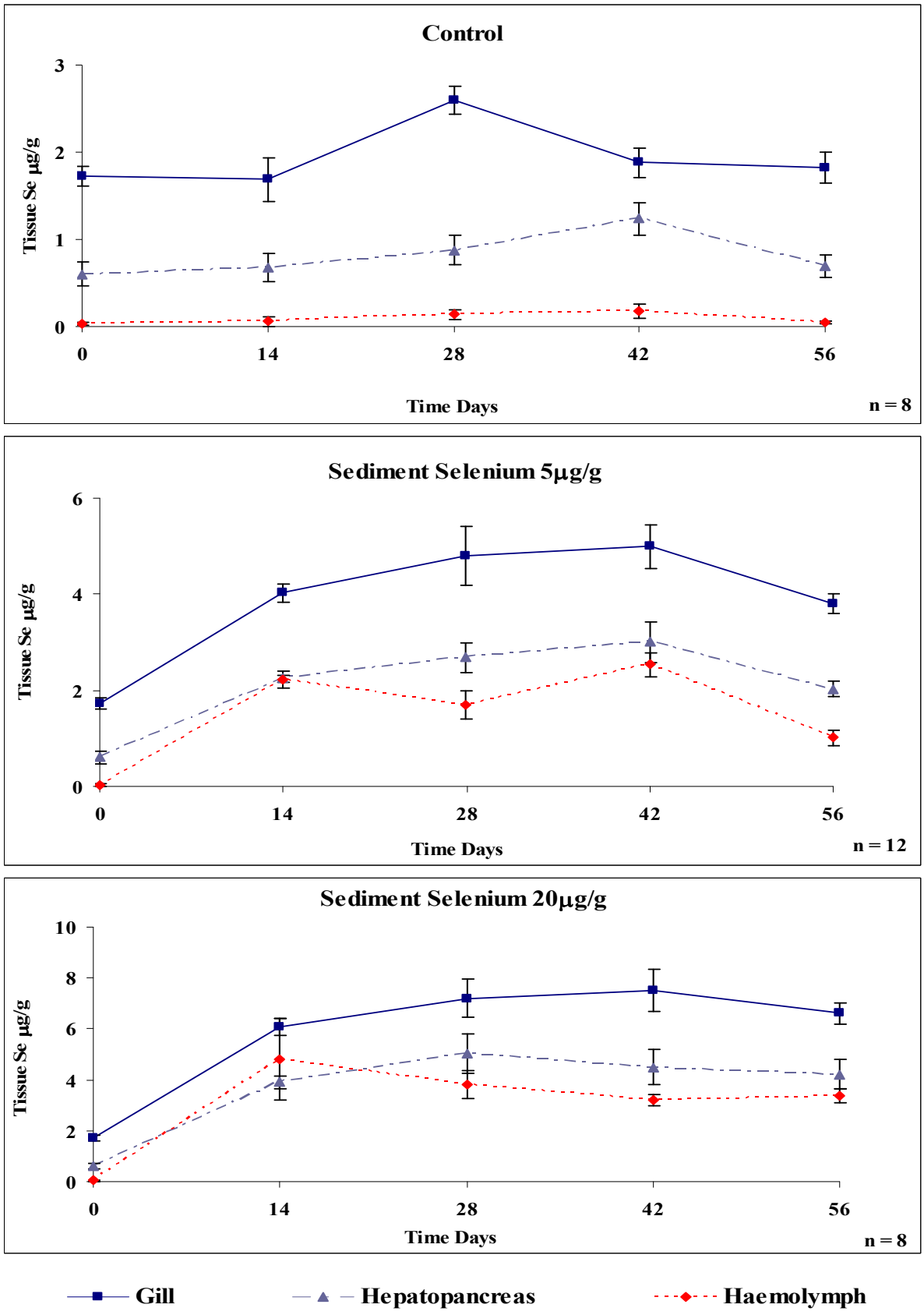


Figure 7.3: Selenium accumulation in gill, hepatopancreas and haemolymph tissues of *A. trapezia* at two weekly intervals over 56 days of exposure to selenium spiked sediments 0 (control), 5 and 20 µg/g dry mass. Mean ± SE, n = 8, 12 and 8 respectively.

7.2.2 Subcellular Tissue Selenium Distribution

7.2.2.1 *Gill*

Between 35 and 50 % of the total gill tissue selenium was recovered in the fractions (Table 7.1). Of the metal recovered in the fractions, 23 % of the selenium in the controls, increasing to 42 and 35 % respectively, in the 5 and 20 µg/g selenium treatments was in the nuclei+cellular debris fraction. The combined BDM fractions contained 10 % of the control treatment and 21 and 16 % respectively, of the 5 and 20 µg/g selenium treated organisms' selenium (Table 7.1). The percentage of metal recovered in the BAM fractions of each of the 5 and 20 µg/g selenium treatments was half and two thirds respectively, that of the control, however, the total selenium burden (µg) within these fractions was 1.5 and 7 times respectively, greater in the selenium exposed organisms (Table 7.1). All of the BDM was in the MRG fraction for the control organisms while the 5 µg/g selenium exposed organisms had more in the MRG fraction than MTLP fraction and the 20 µg/g selenium exposed organisms had an even distribution between the two fractions (Figure 7.4, Table 7.2). The highest percentage of selenium in the BAM fractions of all treatments was in the mitochondria (≈ 60 %) with the remainder fairly evenly distributed between the heat sensitive proteins (HSP) and lysosome+microsome fractions (Figure 7.4, Table 7.2).

7.2.2.2 *Hepatopancreas*

Between 31 and 59 % of the total hepatopancreas tissue selenium was recovered in the fractions (Table 7.1). Of the metal recovered in the fractions around half was in the nuclei+cellular debris fraction of the selenium exposed organisms, while the controls had only 14 % in this fraction (Table 7.1). The percentage in the BDM fractions was similar for all treatments, between 12 and 16% (Table 7.1). The percentage of metal recovered in the BAM fractions of each of the 5 and 20 µg/g selenium exposed organisms was half that of the control organisms, however, the total selenium burden (µg) within these fractions was 1.2 and 1.6 times respectively, greater in the selenium exposed organisms (Table 7.1). All of the BDM was in the MRG fraction of the control organisms (Figure 7.4, Table 7.2). The 5 and 20µg/g selenium exposed organisms had more selenium in the MRG fraction than in MTLP fraction, however, the 20 µg/g selenium exposed organisms had a lower percentage in the MRG fraction and a higher percentage in the MTLP fraction than the 5 µg/g selenium exposed organisms (Figure 7.4, Table 7.2).

The distribution of selenium in the BAM fractions of the control organisms was in the order, mitochondria > HSP > lysosome+microsome fraction while in the 5 and 20µg/g selenium exposed organisms it was in the order, HSP ≈ mitochondria > lysosome+microsome fraction (Figure 7.4, Table 7.2).

Table 7.1: Selenium concentrations (µg wet mass) in gill and hepatopancreas whole tissue and the total selenium with percentage recovered from all subcellular fractions of *A. trapezia* after 56 days exposure to selenium spiked sediments. Metal subcellular concentrations (µg wet mass) and percentage distribution of total recovered selenium fractions are grouped as nuclei+cellular debris and biologically active and detoxified metal (Figure 3.7). Mean ± SD, n = 2.

	Gill			**Hepatopancreas**		
	Se control	**Se 5 µg/g**	**Se20 µg/g**	**Se control**	**Se 5 µg/g**	**Se20 µg/g**
Total Tissue Selenium (µg)	**0.3 ± 0.2**	**0.5 ± 0.1**	**1.7 ± 0.1**	**0.3 ± 0.1**	**0.4 ± 0.05**	**0.4 ± 0.1**
Total Recovered Selenium (µg)	**0.1 ± 0.02**	**0.2 ± 0.1**	**0.8 ± 0.05**	**0.1 ± 0.01**	**0.1 ± 0.01**	**0.2 ± 0.01**
Proportion of total recovered in fractions (%)	35 ± 14	45 ± 0.3	50 ± 1	31 ± 5	34 ± 7	59 ± 12
Selenium Distribution						
Nuclei + Cellular debris (µg)	**0.02 ± 0.01**	**0.1 ± 0.01**	**0.3 ± 0**	**0.01 ± 0.01**	**0.06 ± 0.01**	**0.1 ± 0**
Nuclei + Cellular debris (%)	23 ± 10	42 ± 6	35 ± 2	14 ± 6	47 ± 3	51 ± 2
Biologically Active Metal (µg)	**0.06 ± 0**	**0.09 ± 0.04**	**0.4 ± 0.02**	**0.05 ± 0**	**0.06 ± 0**	**0.08 ± 0.01**
Biologically Active Metal (%)	67 ± 13	37 ± 7	49 ± 1.4	70 ± 13	37 ± 6	37 ± 5
Biologically Detoxified Metal (µg)	**0.01 ± 0**	**0.05 ± 0.01**	**0.13 ± 0.03**	**0.01 ± 0.01**	**0.02 ± 0**	**0.02 ± 0.01**
Biologically Detoxified Metal (%)	10 ± 4	21 ± 2.5	16 ± 2.5	16 ± 5	16 ± 3	12 ± 2

Table 7.2: Mean percentage of selenium in the nuclei+cellular debris, biologically detoxified metal (BDM) and biologically active metal (BAM) with the percentage of selenium each of the fractions within contributes to BDM or BAM, from subcellular fractions of *A. trapezia* after 56 days exposure to selenium spiked sediments, n = 2.

	Gill			**Hepatopancreas**		
	Se control	**Se 5 µg/g**	**Se 20 µg/g**	**Se control**	**Se 5 µg/g**	**Se 20 µg/g**
Nuclei + Cellular debris % of total	**23**	**42**	**35**	**17**	**47**	**51**
BDM % of total	**10**	**22**	**16**	**14**	**16**	**12**
Metal Rich Granules % of BDM	100	56	50	100	77	67
Heat Stable MT Like Proteins % of BDM	0	44	50	0	23	33
BAM % of total	**67**	**37**	**49**	**70**	**37**	**37**
Mitochondria % of BAM	57	58	62	45	35	39
Lysosomes + Microsomes % of BAM	14	19	18	23	26	20
Heat Sensitive Proteins % of BAM	28	24	20	32	38	42

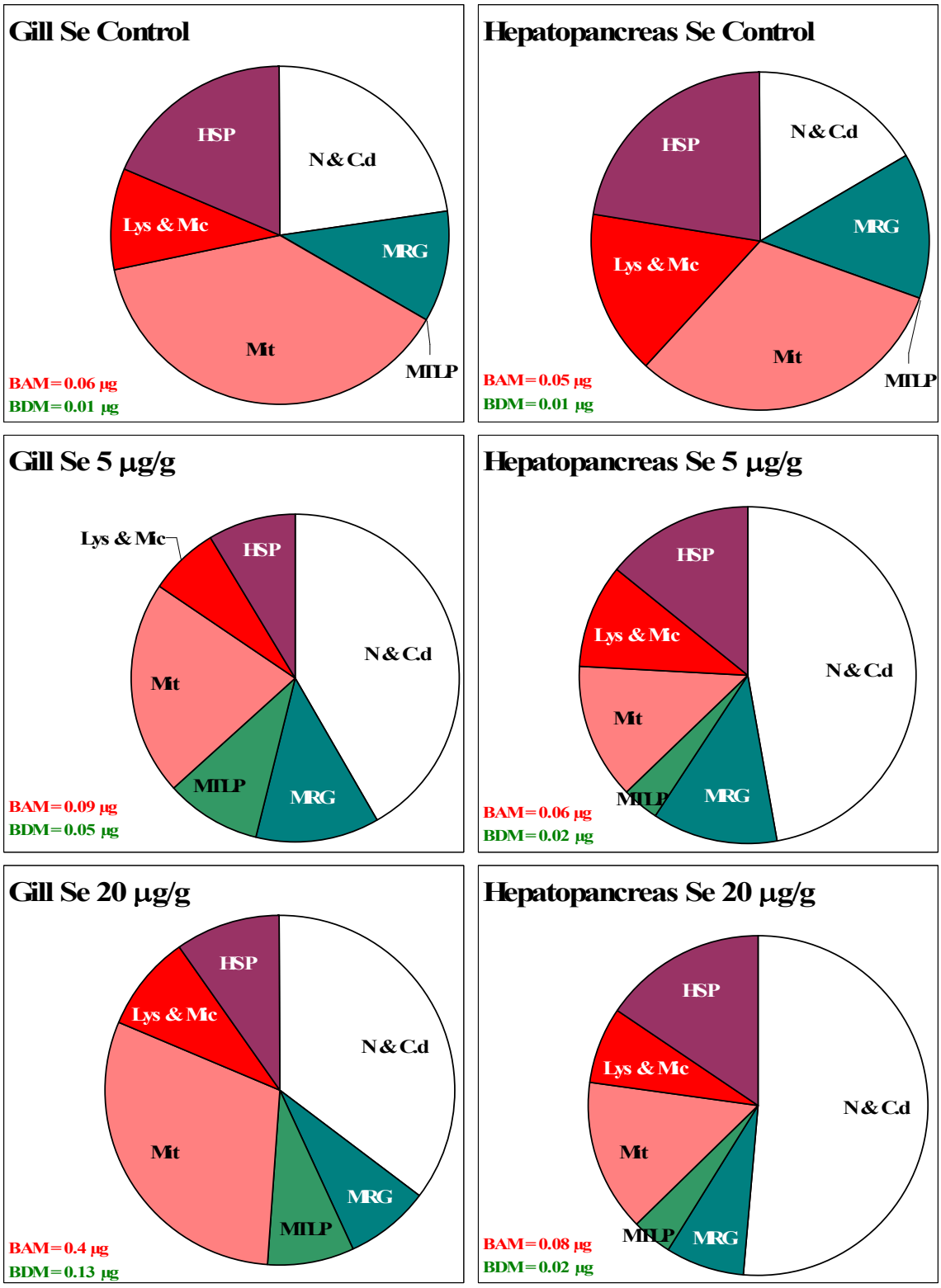


Figure 7.4: Distribution (%) of selenium in each of the subcellular fractions of *A. trapezia* gill and hepatopancreas tissues following 56 days of exposure to selenium spiked sediments. Subcellular fractions are: nuclei + cellular debris (N & C.d); metal rich granules (MRG); heat stable metallothionein like proteins (MTLP); mitochondria (Mit); lysosomes + microsomes (Lys & Mic); heat sensitive proteins (HSP). Red fractions ( ) make up the biologically active metal (BAM), green fractions ( ) make up the biologically detoxified metal (BDM), n = 2.

7.2.3 Activity of Marker Enzymes

7.2.3.1 *Whole Tissue Enzyme Activity*

The activity in whole tissue of the acid phosphatase (AP) lysosomal enzyme was greater in the hepatopancreas tissues than in the gill tissues of the control organisms, equal to that of the gill tissues in the 5 µg/g selenium exposed organisms and lower than the gill tissues in the 20 µg/g selenium exposed organisms (Figure 7.5). Cytochrome c oxidase (CcO) mitochondrial enzyme activity was greater in the gill tissues than in the hepatopancreas tissues for all treatments (Figure 7.5). AP activity was reduced in the gill tissues of 5 µg/g selenium exposed organisms compared to the control and 20 µg/g selenium exposed organisms (Figure 7.5). CcO was greater in the gill and hepatopancreas tissues of selenium exposed organisms than in the tissues of the control organisms (Figure 7.5).

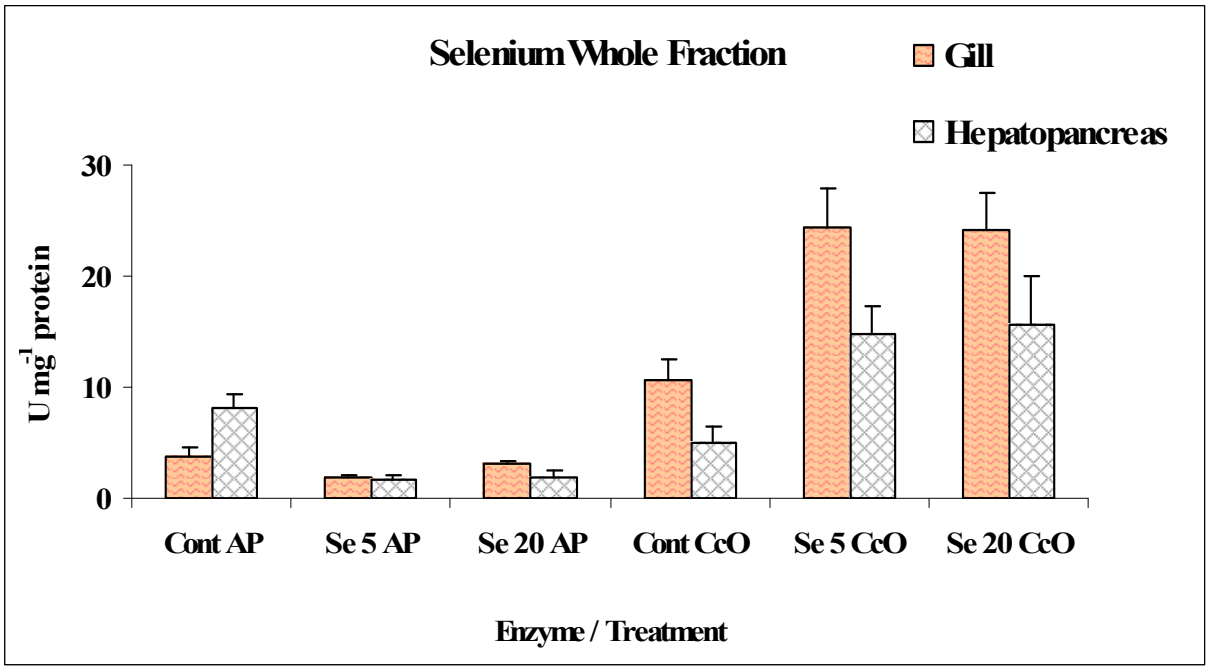


Figure 7.5: Activity specific marker enzymes for lysosomes (acid phosphatase (AP)) and mitochondria (cytochrome c oxidase (CcO)) in whole gill and hepatopancreas tissues of *A. trapezia* exposed to selenium spiked sediments: 0 (Control), 5 and 20 µg/g dry mass. Mean ± SD, n = 2.

7.2.3.2 *Subcellular Fraction Enzyme Activity*

Gill and Hepatopancreas

Enzyme activities in the subcellular fractions of all treatments indicate that the CP 3 fractions of both tissues were enriched in mitochondria while the CP 4 fractions were enriched with lysosomes (Figure 7.6). There was some carry over of mitochondria into the CP 4 fractions and also some lysosomal enzyme activity present in the CP 3 fraction (Figure 7.6).

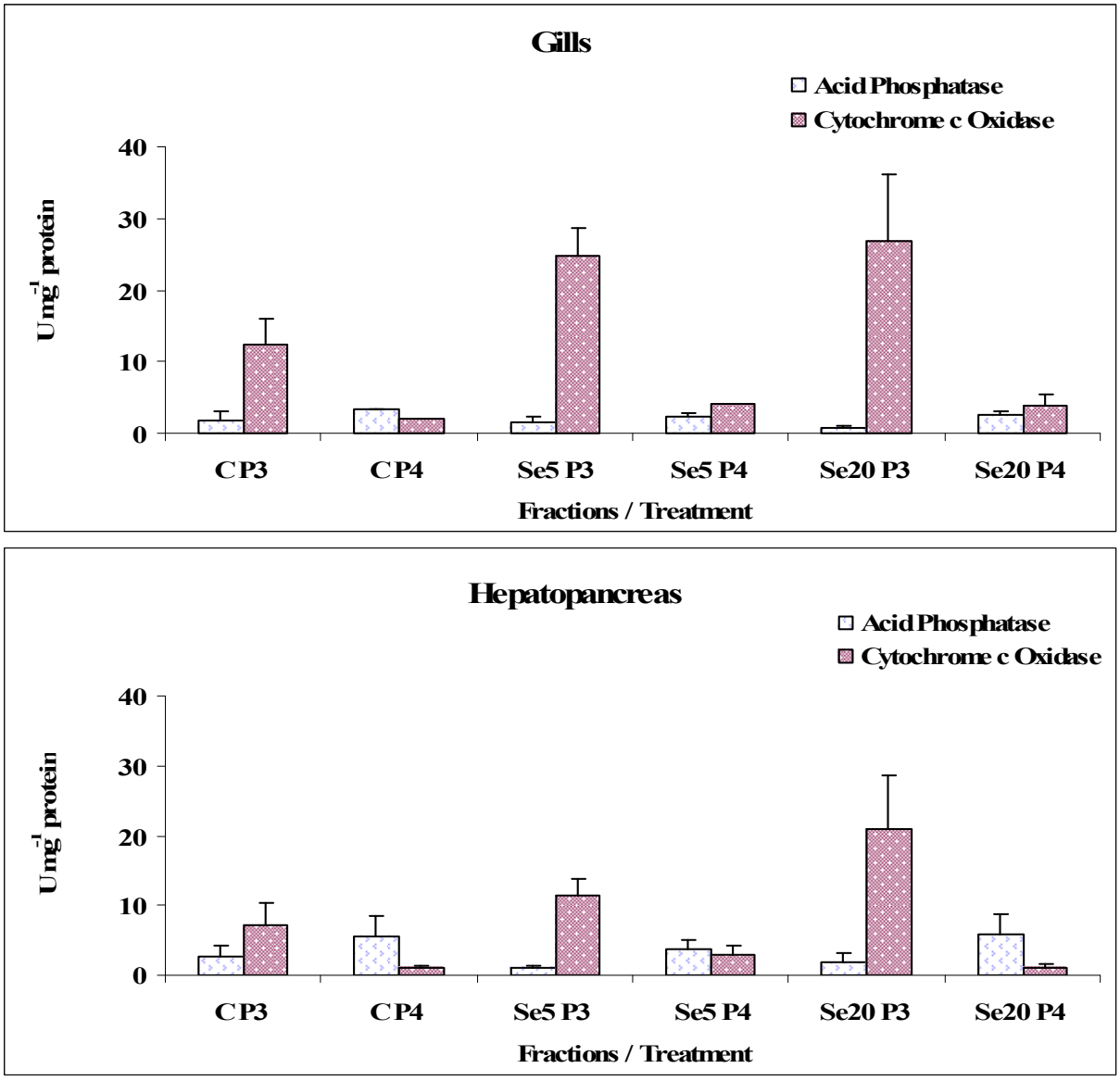


Figure 7.6: Activity of specific marker enzymes for lysosomes (acid phosphatase) and mitochondria (cytochrome c oxidase) in the mitochondrial fraction (P3) and lysosomal/microsomal fraction (P4) following subcellular fractionation of gill and hepatopancreas tissue of *A. trapezia* exposed to selenium spiked sediments at:0 (Control), 5 and 20 µg/g dry mass. Mean ± SD, n = 2.

7.2.4 Enzymatic Biomarkers

7.2.4.1 *Total Antioxidant Capacity*

Total antioxidant capacity was reduced in selenium exposed organisms compared to the control organisms (Figure 7.7). ANOVA with Bonferroni pair-wise comparisons showed that the factor selenium treatment was significant in determining antioxidant capacity with the capacity to reduce antioxidants of organisms from both selenium treatments being significantly lower than that of the control organisms, however, the selenium exposed organisms were not significantly different to each other (Figure 7.7, Appendix 2.9 & 2.10).

7.2.4.2 *Glutathione Peroxidase Activity*

Glutathione peroxidase activity was reduced in selenium exposed organisms compared to the control in the order, control > 5 μg/g > 20 μg/g (Figure 7.7). ANOVA showed the factor selenium treatment was not significant in determining glutathione peroxidase activity (Figure 7.7, Appendix 2.9).

7.2.4.3 *Total Glutathione Concentration*

Total glutathione concentration (GSH+2GSSG) was increased in the selenium exposed organisms compared to the control in the order, 5 μg/g > 20 μg/g > control (Figure 7.7). ANOVA showed the factor selenium treatment did not significantly influence total glutathione concentration (Figure 7.7, Appendix 2.9).

7.2.4.4 *Reduced : Oxidised Glutathione Ratio*

The ratio of reduced to oxidised glutathione was reduced in selenium exposed organisms (Figure 7.7). ANOVA showed that the differences in GSH:GSSG ratio between treatments were significant (Appendix 2.9), with pair-wise comparisons showing organisms from both selenium treatments had significantly lower ratios than the control organisms but were not different to each other (Figure 7.7, Appendix 2.10).

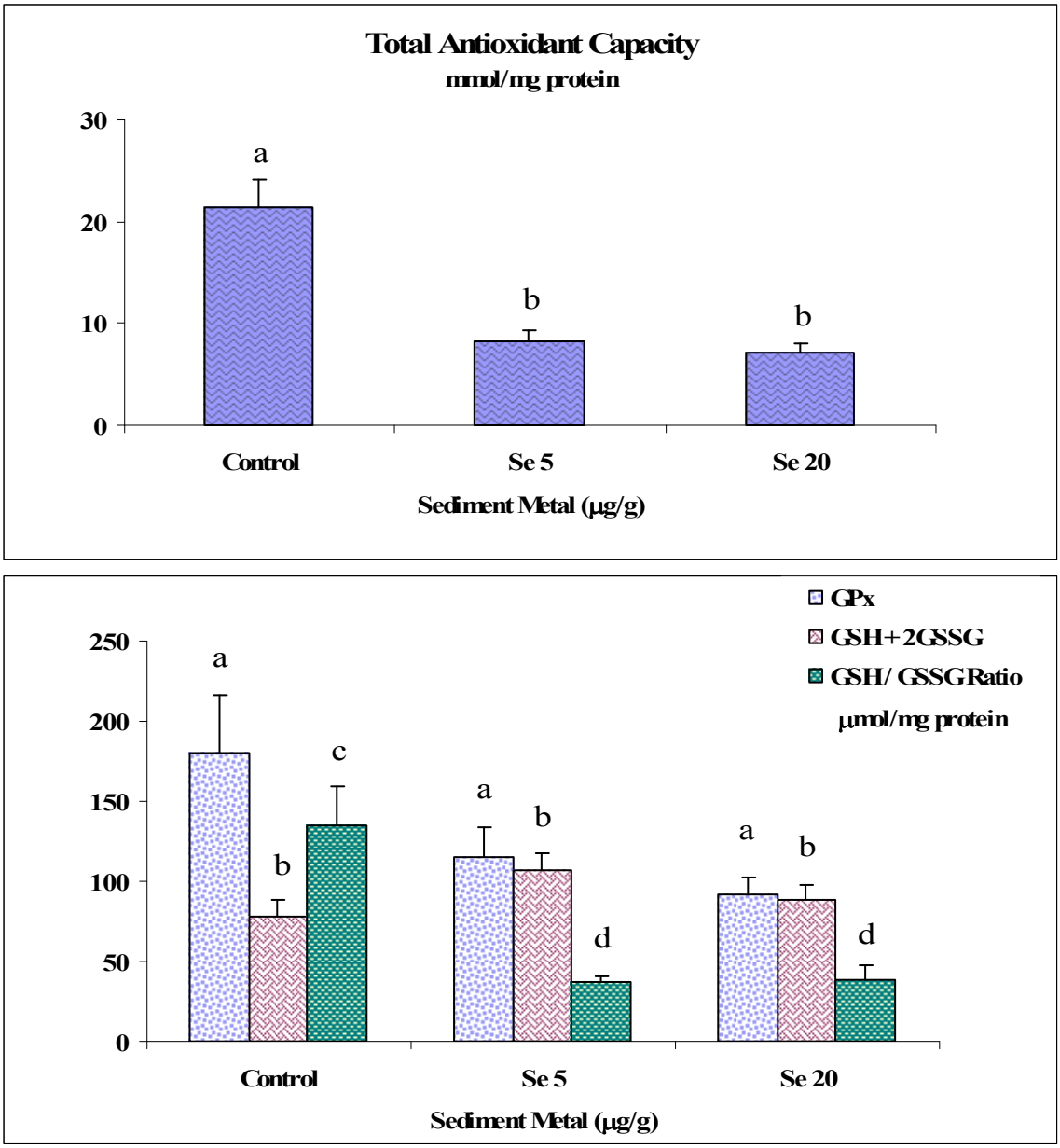


Figure 7.7: Activity of antioxidant enzyme biomarkers: total antioxidant capacity; glutathione peroxidase (GPx); total glutathione (GSH+2GSSG); ratio of reduced to oxidised glutathione (GSH/GSSG Ratio) in *A. trapezia* following 56 days of exposure to selenium spiked sediments: 0 Se (control) n = 8; Se 5 µg/g n = 12 and Se 20 µg/g n = 8 dry mass. Mean ± SE. Different letters indicate significant differences betweens means (Bonferroni test; $p < 0.05$).

7.2.5 Oxidative Damage Biomarker – Thiobarbituric Acid Reactive Substances

TBARS increased with exposure to increased selenium sediment concentrations (Figure 7.8). ANOVA showed that the factor selenium treatment significantly influenced TBARS concentration (Appendix 2.9). Pair-wise comparisons showed that the selenium exposed organisms had significantly higher TBARS than the control organisms but were not significantly different to each other (Figure 7.8, Appendix 2.10).

7.2.6 Cellular Biomarker – Lysosomal Stability

The percentage of unstable lysosomes increased with increased selenium exposure (Figure 7.8). ANOVA showed that the factor selenium treatment was highly significant in determining lysosomal stability (Appendix 2.9). Bonferroni pair-wise comparisons showed that organisms from both selenium treatments had significantly more unstable lysosomes than the control organisms and the 20 µg/g selenium exposed organisms had significantly more than the 5 µg/g selenium exposed organisms (Figure 7.8, Appendix 2.10).

7.2.7 Genotoxic Biomarker – Micronuclei Frequency

The occurrence of micronuclei increased with increased selenium exposure (Figure 7.8). ANOVA showed that the factor selenium treatment significantly influenced micronuclei frequency (Appendix 2.9). Bonferroni pair-wise comparisons showed that organisms from both selenium treatments had significantly more micronuclei than the control organisms and the 20 µg/g selenium exposed organisms had significantly more than the 5 µg/g selenium exposed organisms (Figure 7.8, Appendix 2.10).

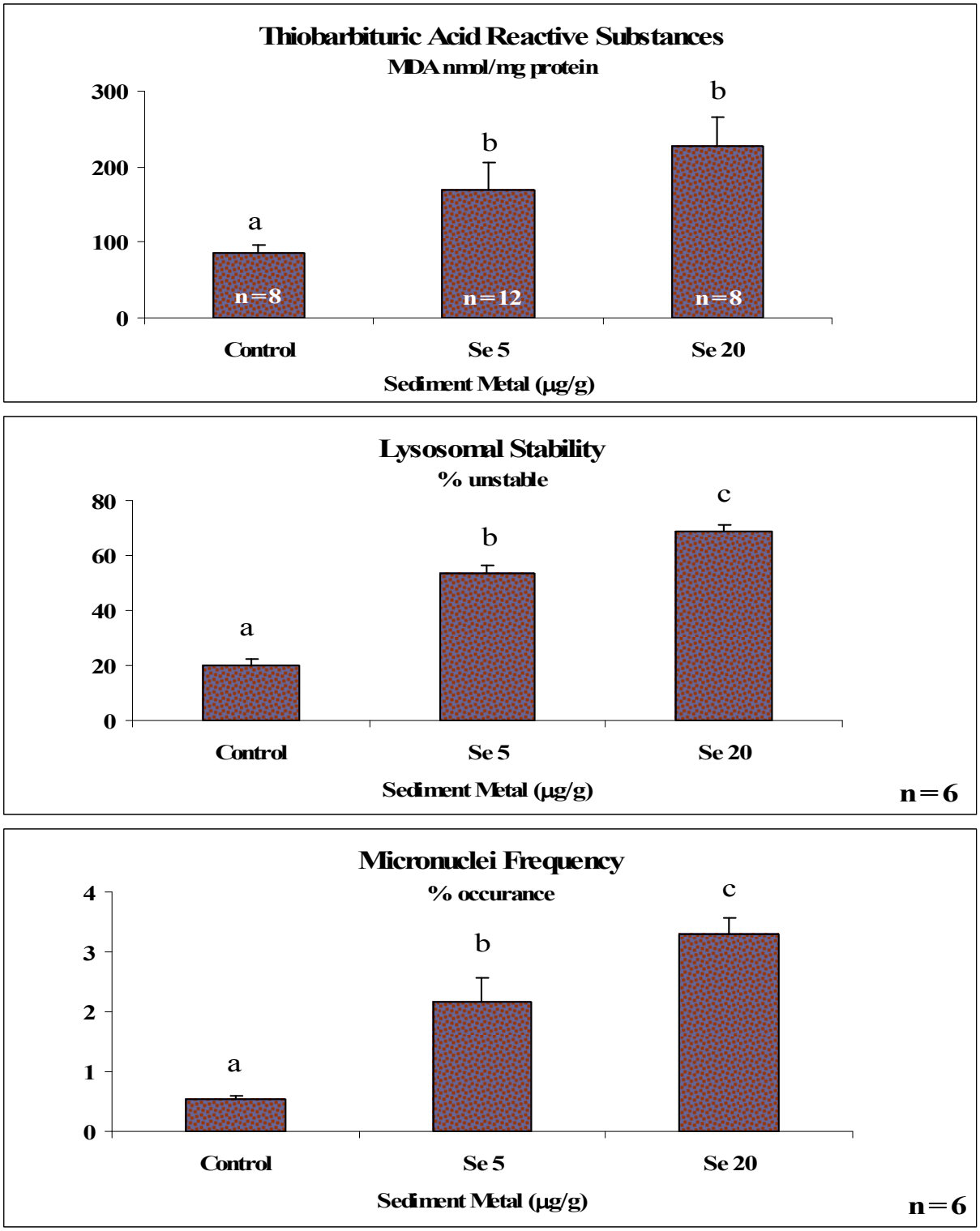


Figure 7.8: Changes in oxidative damage, cellular and genotoxic biomarkers of *A. trapezia* following 56 days of exposure to selenium spiked sediments, Se 0 (control), Se 5 µg/g and Se 20 µg/g; dry mass. Mean ± SE. Different letters indicate significant differences betweens means (Bonferroni test; $p < 0.05$).

7.2.8 Relationship Between Biomarkers

7.2.8.1 *Enzymatic with Oxidative Damage, Cellular and Genotoxic Effects*

Regression analysis shows that total antioxidant capacity within cells had a negative relationship with the effects measures TBARS, lysosomal stability and micronuclei frequency (Figure 7.9). As the selenium exposure increased the capacity of the cells to neutralise reactive oxygen was reduced with a consequent increase in cell damage.

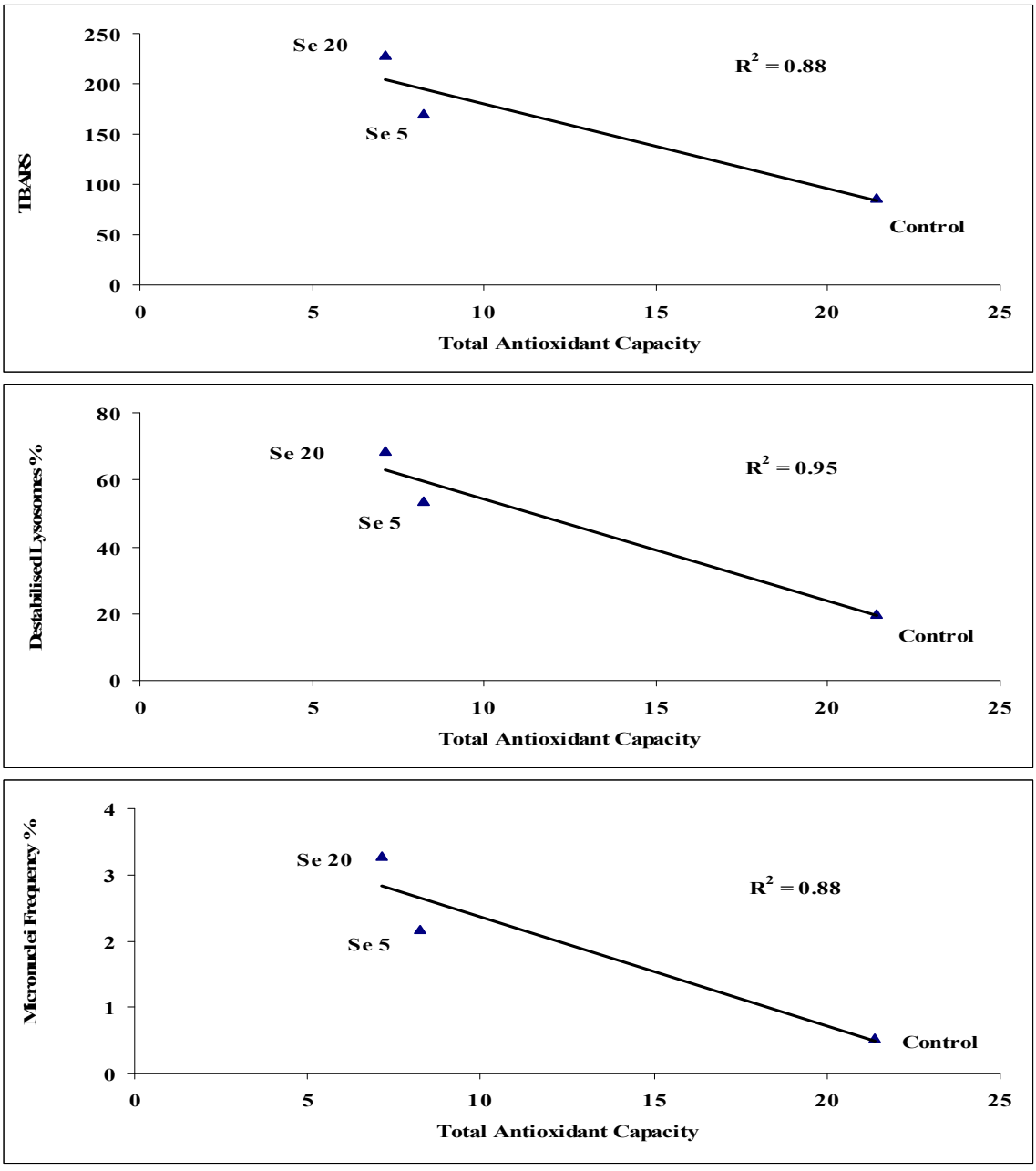


Figure 7.9: Regressions of treatment means of total antioxidant capacity, (control; n=8: Se 5 n=12 & 20; n=8), and TBARS, (control; n=8: Se 5 n=12 & 20; n=8), lysosomal stability and micronuclei frequency (n=6).

7.2.8.2 *Oxidative Damage with Cellular and Genotoxic Effects*

There was a positive relationship between TBARS and lysosomal stability and micronuclei frequency (Figure 7.10). Increased selenium exposure increased the TBARS and the regression analysis indicates that this was associated with an increase in the percentage of unstable lysosomes and the frequency of micronuclei (Figure 7.10).

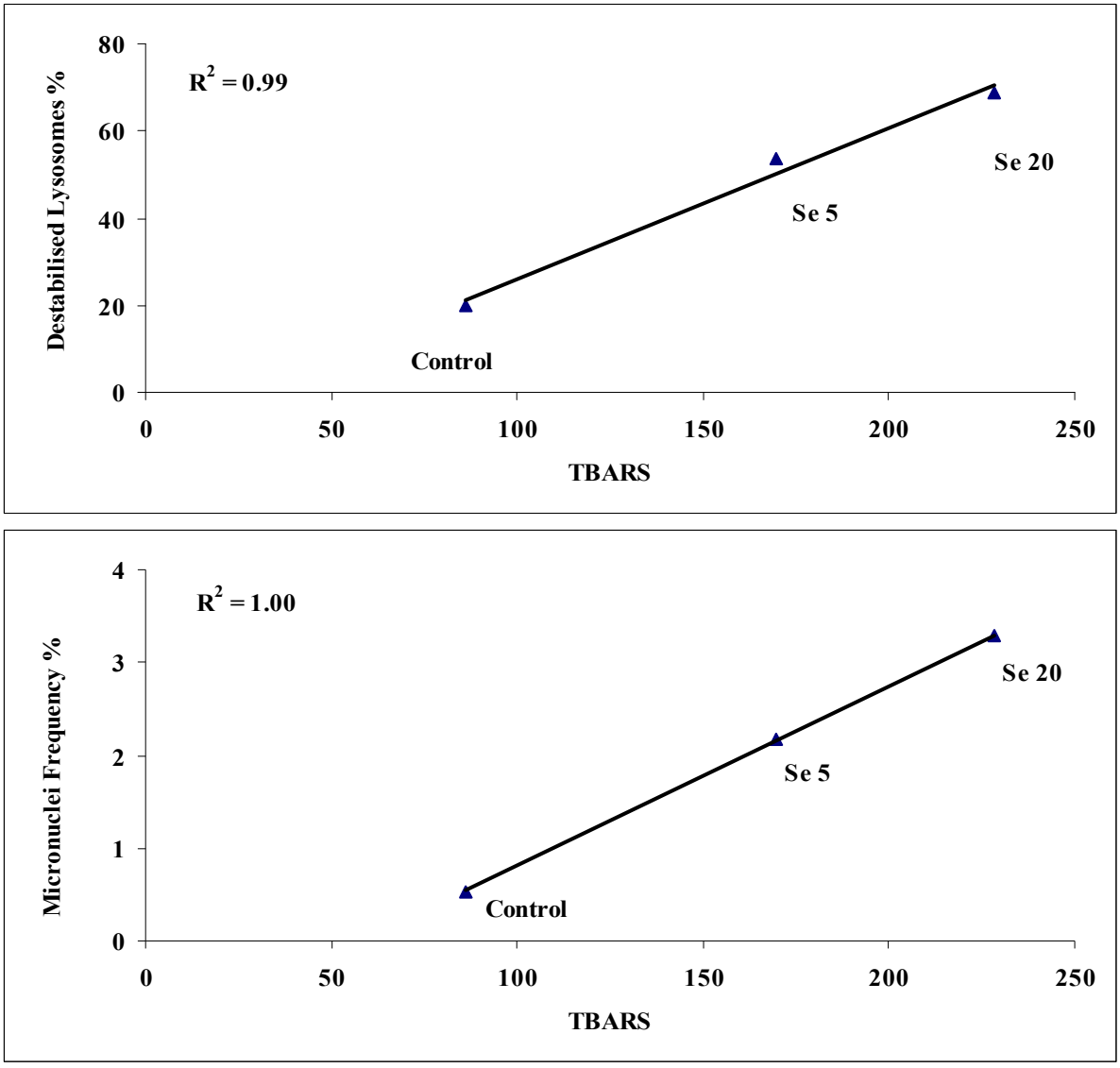


Figure 7.10: Regressions of treatment means of TBARS (control; n=8: Se 5 n=12 & 20; n=8), with lysosomal stability and micronuclei frequency (n=6).

7.3 Discussion

7.3.1 Selenium Accumulation and Subcellular Distribution

7.3.1.1 *Whole Organism Selenium Accumulation*

The background selenium concentration of the *A. trapezia* used was approximately 1 µg/g (Figure 7.1). Selenium tissue concentrations of 2 µg/g in *A. trapezia* from a relatively pristine environment were reported by (Jolley *et al.*, 2004). Selenium concentrations in this range presumably reflect the metabolic selenium requirement. *A. trapezia* exposed to both 5 and 20 µg/g of selenium accumulated selenium rapidly for the first 14 days with only slight increases over the next 28 days followed by a decrease in selenium concentration in the final 14 days of the exposure experiment (Figure 7.1). This accumulation pattern suggests an equilibrium tissue concentration was reached at both exposure concentrations. While tissue selenium of the 5 µg/g selenium exposed organisms was approximately one third that of the exposure medium and the 20 µg/g selenium exposed organisms about one seventh the ambient concentration the positive sediment tissue regression (Figure 7.2) indicates that *A. trapezia* is only a partial regulator of selenium. Jolley *et al.* (2004) found *A. trapezia* from a single population were able to maintain almost constant internal selenium concentrations but that this concentration depended on the individual populations sediment exposure concentration. Jolley *et al.,* (2004) found that selenium tissue concentration co-varied with the sediment concentration and populations from areas with higher selenium sediment exposure maintained higher tissue selenium concentrations. Burt *et al* (2007) found that *A. trapezia* transplanted from an uncontaminated environment to sites with elevated selenium in Lake Macquarie NSW, increased their selenium tissue concentrations from 2 µg/g to selenium concentrations to a concentration approaching that of the indigenous population (4 – 5 µg/g) within three months reaching equilibrium selenium tissue concentrations which were higher than that of their exposure environment after 60 days of exposure.

7.3.1.2 *Individual Tissue Selenium Accumulation*

The pattern of selenium accumulation in the selenium exposed organisms was the same for the gill and hepatopancreas tissues in both treatments throughout the experiment with the gill tissues having roughly double the hepatopancreas tissue concentration (Figure 7.3). The haemolymph contributed a significant amount to the total selenium concentration particularly in the first 14 days and had only slightly less selenium than the hepatopancreas tissues for the remainder of the time (Figure 7.3). It has been demonstrated that burrowing and feeding by benthic organisms, including *A. trapezia,* in selenium contaminated sediments causes oxidation of sediments which increases the selenium flux to interstitial waters, resulting in higher concentrations of selenium becoming available to these organisms (Peters *et al.*, 1999a). It has also been established that dietary exposure is a major route of selenium uptake in aquatic organisms (Hamilton, 2002; 2004; Lemly, 1999b; Luoma and Rainbow, 2008). The pattern of tissue accumulation observed in the 20 µg/g selenium exposed organisms indicates that during the initial 14 days the exposure route was probably via dissolved selenium in interstitial waters as the haemolymph and gill tissues both had high selenium concentrations relative to the hepatopancreas tissues while in the 5 µg/g selenium exposed organisms the haemolymph and hepatopancreas tissues had equal concentrations of selenium at this time. The gill tissues continued to have higher selenium concentrations for the remainder of the exposure period but the hepatopancreas tissues contributed more to the total selenium concentration than the haemolymph suggesting that dietary exposure may have been contributing to selenium exposure probably combined with internal transport of accumulated dissolved selenium to the digestive system (Viarengo and Nott, 1993). Growth of algae was observed during the course of the experiment and sediment associated bacteria would also have been present, these potential food sources would probably both take up selenium and subsequently become a source of dietary selenium to the *A. trapezia.* Bacteria and marine algae are also able to transform selenite to selenomethionine which is more readily absorbed via the gut and stored by bivalves than dissolved selenite (Wrisberg *et al.*, 1992).

7.3.1.3 *Subcellular Selenium Distribution*

A large proportion of the selenium recovered in the subcellular fractions of both the gill and hepatopancreas tissues was in the nuclei+cellular debris fraction, increasing in the gill tissues from control organisms at 23 % to 42 and 35 % and in the hepatopancreas tissues from control organisms at 14 % to 47 and 51 %, respectively, in the 5 and 20 µg/g selenium exposed organisms (Table 7.1; Figure 7.4). Ewan, (1989) suggests that selenite is taken up by haemolymph, reduced to selenide, released into the plasma and rapidly bound by plasma proteins for transport to tissues, while the majority of accumulated selenate and selenomethionine occurs in the protein-free plasma. Selenomethionine has also been shown to bind to glutathione peroxidase extracellularly as well as intracellularly (Burk, 1991). Fifty six percent of accumulated selenium in enriched mycelia of the fungus *Pleurotus ostreatus* was associated with the cell wall (Hortensia *et al.*, 2006). A combination of protein bound selenium associated with plasma and selenium bound directly to cell walls would account for the high proportion of selenium associated with the debris fraction and therefore it would be comprised of both BDM and BAM. Only a small percentage of the accumulated selenium was recovered in the BDM fractions in the selenium exposed organisms (Figure 7.4). The 5 µg/g selenium exposed organisms had a slightly higher proportion than the 20 µg/g selenium exposed organisms in the BDM component of both tissues (Table 7.1) but the distribution within the fractions of the BDM differed between tissues, with an even distribution in the gill tissues and a higher proportion in the MRG than the MTLP in the hepatopancreas tissues (Table 7.2). As discussed in (Section 4.4.1.6) selenium associated with MTs and MRGs has not previously been reported for marine molluscs. The association of selenium with selenoamino acids and other low molecular weight proteins which are presumed to act as storage and transport proteins not unlike MTs has been shown (Akesson and Srikumar, 1994). The strong relationship of selenium with sulphur (Ganther, 1974) is a possible pathway for the incorporation of selenium into MRG, as sulphur has been reported as a major component of zinc and cadmium granules in *M. edulis* (George, 1983a). The lack of selenium in the MTLP of the control organisms (Figure 7.4) is probably due to all of the available selenium being required for metabolic activity. The MRG component seen in the tissues of the control organisms may be a result of incompletely broken down tissue debris during the NaOH digestion step in sample preparation (Figure 3.7), or could represent a previous exposure history in which excess selenium was detoxified and stored.

If this is the case the fact that it has not been lost during the exposure to clean sediments raises the question of whether selenium excretion occurs in *A. trapezia* and if so over what timescale. Selenium half-lives varying between 19 and 42 days have been reported for juvenile fathead minnow, after oral administration of selenate, selenite and l-selenomethionine, depending on the form of the selenium and the fish tissue studied (Kleinow and Brooks, 1986). Mature fish chronically exposed to selenium in natural waters did not show any selenium loss from muscle tissue from year to year (Osmundson *et al.*, 2000). It is thought that selenium bound within selenomethionine would probably require more energy to eliminate due to its incorporation into proteins and tissue (Hamilton, 2004).

The majority of the recovered selenium in the gill and hepatopancreas tissues of the control organisms was in the BAM fraction (Table 7.1), which supports the view that the selenium measured in these organisms represents the metabolic requirement (DeNicola Cafferky *et al.*, 2006). While the percentage of BAM in the tissues of the selenium exposed organisms was lower than that of the controls, in terms of selenium concentration it was between 1.2 and 7 times greater than the unexposed *A. trapezia* BAM (Table 7.1). The percentage of selenium in the BAM fractions of organisms from all treatments was far greater than the percentage in the BDM fractions indicating that *A. trapezias'* capacity to detoxify or store selenium is limited. More than half of the selenium in the BAM component of the gill tissues was in the mitochondrial fraction (Figure 7.4; Table 7.2). This may represent dissolved selenium released to interstitial water following bioturbation (Lemly, 1999b) being incorporated rapidly via the large gill filaments across cell walls into the active sites of glutathione induction in the mitochondria. Increased activity of the mitochondrial enzyme CcO in the gill tissues compared to the hepatopancreas tissues (Figures 7.5; 7.6) shows this tissue is enriched in mitochondria and this was enhanced in the selenium exposed organisms indicating selenium induced mitochondrial activity. The hepatopancreas tissues of exposed organisms had 35 to 39 % of the selenium in the mitochondrial fraction with about the same amount in the HSP fraction (Figure 7.4; Table 7.2). The higher binding in the HSP in the hepatopancreas tissue may be due to the incorporation of selenium into the hepatopancreas via food sediment and haemolymph transfer as well as directly from water (Fan *et al.*, 2002). The increased selenium concentrations in the BAM of the exposed organisms has implications for adverse effects in *A. trapezia* as there is only a narrow concentration band at which selenium is required for metabolic processes and beyond this it is highly toxic (Hamilton, 2004).

7.3.2 Enzymatic Biomarkers – Oxidative Enzymes

Selenium's prooxidant activity arises from its ability to oxidise thiols, some forms of selenium complex with glutathione to form a selenopersulfide anion that ultimately generates superoxide radicals (Palace *et al.*, 2004). There was a significant reduction in the TAOC in *A. trapezia* from both selenium treatments to less than half that of the control organisms (Figure 7.7). This was also observed in the selenium exposed *T. deltoidalis* which had very similar final BAM selenium burdens (Table 4.1). The final gill BAM selenium burden of the 5 µg/g selenium exposed *A. trapezia* was 4.5 times lower than that of the 20 µg/g selenium exposed organisms. The pattern of equivalent TOAC reduction in organisms with very different BAM burdens was also seen for lead exposure in *A. trapezia* (Figure 6.7; Table 6.1) and like lead it is possible that there is a critical selenium concentration that impairs TAOC to this level which may be lower than these concentrations. The GPx enzyme activity, in which selenium plays a major role, was reduced in *A. trapezia* from both treatments, the effect was slightly greater in the 20 µg/g selenium exposed organisms but neither was significantly lower than control organisms (Figure 7.7). At optimal selenium mitochondrial concentrations the formation of Se-dependent GPx would be expected to be enhanced in response to an oxidative challenge thereby increasing the total GPx activity (Winston and Di Giulio, 1991). The reduced activity in the selenium exposed *A. trapezia* may relate to the increased BAM selenium burden having a toxic inhibitory effect on GPx formation. The GSH+2GSSG concentration was enhanced in *A. trapezia* from both treatments, the effect was slightly greater in the 5 µg/g selenium exposed organisms but neither was significantly higher than control organisms (Figure 7.7). The GSH:GSSG ratio by contrast was significantly reduced in *A. trapezia* from both treatments, indicating the a significant proportion of the increased total glutathione was comprised of oxidised glutathione. Selenite in high concentrations can also deplete GSH, inhibiting the release of selenium metabolites (Magos and Webb, 1980). Significantly reduced ratios of GSH:GSSG have been measured in selenium exposed mallard ducks (Hoffman, 2002). Excess GSSG can be excreted from cells more rapidly than it is converted back to the reduced form (Regoli *et al*., 2002) but equally can react with protein sulfhydryls, contributing to total and protein bound thiol depletions (Hoffman, 2002).

7.3.3 Oxidative Damage Biomarker – Thiobarbituric Acid Reactive Substances

There was a trend of increasing TBARS with increased selenium exposure which was significantly higher in organisms from both selenium treatments than in the control organisms (Figure 7.8). Increased lipid peroxidation was measured in the tissues of adult and hatchling tissues and in the eggs of aquatic birds which was directly related to the effects of selenium accumulation on the glutathione system (Hoffman, 2002). The strong negative correlation between TAOC and TBARS with increased selenium exposure (Figure 7.9) supports the existence of a link between increased ROS and the production of lipid peroxidative products.

7.3.4 Cellular Biomarker – Lysosomal Stability

The lysosomal destabilisation of selenium exposed *A. trapezia* was extremely high 54 and 69 %, respectively, in the 5 and 20 µg/g selenium exposed organisms (Figure 7.8). This level of lysosomal destabilisation indicates significant selenium toxicity and puts them well into the stressed range of the Ringwood *et al*., (2003) criteria. The increased lysosomal destabilisation with increased selenium burdens follows the same pattern as seen for lipid peroxidation (Figure 7.8) and the strong positive correlation between these two biomarkers (Figure 7.9) indicates peroxidative damage was a significant pathway for lysosomal destabilisation. TAOC was also strongly correlated with lysosomal destabilisation (Figure 7.10) suggesting that an increase in ROS was also a contributory factor, both directly and through lipid peroxidation, to the destabilisation of the lysosomal membrane. Heat sensitive cytosolic proteins of the selenium exposed *A. trapezia* had significantly increased selenium burdens (Table 7.2) which may have been bound to sensitive macromolecules. This may have contributed to the total protein thiol depletions, reducing the effectiveness of metabolic regulation, thereby contributing to the breakdown of the lysosomal membrane integrity (Hoffman, 2002).

7.3.5 Genotoxic Biomarker – Micronuclei Frequency

There was significant induction of micronuclei which increased with selenium exposure (Figure 7.8). This was also highly correlated with both TAOC reduction (Figure 7.9) and increased TBARS (Figure 7.10) indicating that an increase in ROS contributed to an increase in genotoxic damage, either through interaction of reactive oxygen intermediates and lipid peroxidation products with DNA or direct interaction of selenium with cellular macromolecules forming adducts, alkaline labile sites and strand breaks (Regoli *et al.*, 2004). An investigation of the bone marrow of selenium exposed mice reported increased micronuclei induction (Itoh and Shimada, 1996) and the erythrocyte cells of fish exposed to selenium showed significant increases in micronuclei frequency (al Sabti, 1994). The accumulation of selenium not only has implications for individual organism health, the primary point of impact can be the gamete which receives selenium via the female's diet and stores it until hatching when tetragenic deformity and death can occur. While mature organisms may appear outwardly unaffected reproductive failure may be occurring (Lemly, 1999a). The increased induction of micronuclei in the selenium exposed *A. trapezia* indicates that significant DNA damage occurred at the individual organism level and the tetragenic tendency of this element suggests there is a potential for this level of exposure to have consequences for population viability.

7.4 Summary and Conclusions

The metabolic selenium requirement of *A. trapezia* appears to be in the order of 1 to 2 µg/g, based on the background concentrations of unexposed organisms from this and previous studies. Selenium exposed organisms reached equilibrium tissue concentrations after 42 days which were below ambient selenium concentrations at both exposure concentrations indicating partial regulation of selenium by *A. trapezia.* The pattern of selenium distribution among tissues indicates that the exposure route changed over the course of the experiment. Gill selenium was always highest indicating dissolved selenium exposure was significant. The haemolymph had higher concentrations than the hepatopancreas for the first 14 days supporting an initial dominance of dissolved selenium exposure which was replaced by a significant contribution from the hepatopancreas for the remainder of the time indicating a dietary selenium contribution. A large percentage of the accumulated selenium was associated with the cellular debris and this was probably comprised of a combination of protein bound selenium associated with plasma and selenium bound to cell walls. Selenium exposed *A. trapezia* only converted a small percentage of accumulated selenium into BDM as MRG and MTLP although the high proportion in the nuclei+cellular debris potentially bound to cell walls would be effectively detoxified. It is unknown, however, whether selenium directly bound to cell walls could ultimately catalyse reactions which would lead to damage to the cell membrane. There was an increase in selenium burdens in the BAM fraction of the 5 and 20 µg/g exposed organisms of 1.2 and 7 times, respectively, the control organisms. The gill was enriched with mitochondria and the majority of BAM selenium of this tissue was located here, while the hepatopancreas had a greater percentage of selenium in the HSP. The differences in selenium distribution in the BAM fractions of the two tissues supports the proposal that dissolved selenium in the gill and dietary in the hepatopancreas were the major routes of selenium exposure. The considerably higher percentage of selenium in the BAM fractions compared to the BDM fractions of both gill and hepatopancreas tissues suggests *A. trapezia* has limited capacity to detoxify or store selenium. There was a significant reduction in the TAOC in *A. trapezia* from both selenium treatments to less than half that of the control organisms. The pattern of equivalent TOAC reduction in organisms with very different BAM burdens was also seen for lead exposure in *A. trapezia* and similar to lead, it is possible that there is a critical selenium concentration that impairs TAOC to this extent which may be lower than the concentrations measured here.

A reduction in GPx was reflected in an increased GSH+2GSSG concentrations which the significantly reduced GSH:GSSG ratios of the selenium exposed organisms indicates was due to a build up of oxidised glutathione. The concentration of TBARS increased with increased selenium exposure and the strong negative correlation between TAOC and TBARS with increased selenium exposure supports the existence of a link between increased reactive oxygen species and the production of lipid peroxidative products. Lysosomal membrane destabilisation and the frequency of micronuclei increased with selenium exposure and there were also strong relationships between reduced TOAC and increased TBARS with increased lysosomal destabilisation and micronuclei frequency associated with increased selenium exposure. These relationships support a significant exposure – dose – response relationship for selenium in *A. trapezia* where increased exposure resulted in an increased tissue selenium dose and more specifically an increase in BAM selenium burdens which in turn resulted in a cascade of interrelated effects from perturbations to the oxidative reduction system, membrane destabilisation and genotoxic effects.

8 Lake Macquarie Cadmium, Lead, Selenium, Zinc and Copper Contamination Gradient Sediment Study

8.1 Introduction

Since it was established in 1897, a zinc/lead smelter located on the Cockle Creek inflow to Cockle Bay in the northern area of Lake Macquarie has been a point source of metal input into the Lake (Carroll, 1996). Elevated concentrations of zinc, copper, lead, and cadmium have previously been measured in sediments in a decreasing gradient down the creek from the inflow and into the bay (Batley, 1987; 1991; Roach, 2005; Roy and Crawford, 1984). Metals in the sediments have been shown to be bioavailable (Batley, 1987; Burt *et al.*, 2007; Roach *et al.*, 2008). Contaminated sediments from Lake Macquarie were collected from each of three sites, Cockle Creek 1 & 2 (CC1 & CC2) and Cockle Bay (CB), along the identified metal gradient (Figure 3.3) and used in a laboratory exposure experiment in the same way as those in the single metal spiked sediment experiments. *A. trapezia* used in this experiment were collected from uncontaminated estuaries at St. Georges Basin and Batemans Bay, NSW. Metal dose, including subcellular distribution of metals were measured as in the previous experiments with *A. trapezia*. The control organisms used in the subcellular fractionation analysis were *A. trapezia* exposed to uncontaminated sediments for 56 days in the laboratory from the sediment spiking experiments (Figure 3.5). Response indices measured in this experiment were: total antioxidant capacity (TAOC), thiobarbituric acid reactive substances (TBARS), lysosomal stability, micronuclei frequency and condition index. Sediments from the uncontaminated *A. trapezia* collection sites (reference sites) were measured for metal concentrations and *A. trapezia* from the uncontaminated collection sites (reference organisms) were measured for tissue metal concentrations, TAOC, TBARS, lysosomal stability, micronuclei frequency and condition index to represent the pre-exposure condition. Native *A. trapezia* and sediments were also collected from a site (LM) along the metal contamination gradient in Lake Macquarie (Figure 3.3) and analysed for sediment and tissue metal concentrations and tissue TAOC, TBARS, lysosomal stability and condition index to represent chronically metal exposed organisms.

8.2 Aim

To examine the exposure-dose-response of *A. trapezia* to sediment contaminated with metals from a lead/zinc smelter in laboratory exposures over 56 days and compare them to unexposed and chronically metal exposed native *A. trapezia.* A comparison between the exposure-dose-response of *A. trapezia* to single metal spiked sediments and the mixed metal contaminated sediment will also enable antagonistic and or synergistic metal effects to be explored to determine whether the metal dose response relationships observed in individual metal spiked sediments hold for multi element exposures.

8.3 Results

8.3.1 Sediment Metal Gradient

A clear contamination gradient for cadmium, lead, selenium, zinc and copper is evident (Figure 8.1; Table 4.1). There is a reduction in metal concentrations from Cockle Creek site 1 (CC1) > Cockle Creek site 2 (CC2) > Cockle Bay (CB) for all metals except copper which had a higher concentration at CB than CC2 (Figure 8.1; Table 4.1). The metal concentrations in the sediments from the three sites along the gradient compared to those from the site where the native *A. trapezia* were collected from Lake Macquarie (LM) were, for zinc and lead CC1 > LM > CC2 > CB, for copper CC1 > LM > CB > CC2, for selenium CC1 > CC2 > LM > CB and for cadmium CC1 > CC2 > CB = LM (Figure 8.1; Table 4.1).

ANOVA showed site was significant for all metal concentration differences (Appendix 3.1). Bonferroni pair-wise comparisons between sites showed that all sites had significantly higher concentrations of all metals than the reference sites, where the uncontaminated *A. trapezia* used in the exposure experiment were collected, with the exception of selenium which was not significantly higher at the CB site than at the reference sites (Appendix 3.2). The CC1 site had significantly higher lead, selenium zinc and copper concentrations than the CC2 and CB sites, while cadmium concentrations at the CC1 site were significantly higher than at the CB site but not different to the CC2 site (Appendix 3.2). The CC2 site had significantly higher lead and selenium concentrations than the CB site but there were no significant differences in cadmium, zinc or copper concentrations between these sites. The zinc, lead and copper concentrations at the LM site, where the native chronically metal exposed *A. trapezia* were collected, were lower than the CC1 site and higher than the CC2 and CB site concentrations (Figure 8.1).

These differences were significant for all three metals for CC2 and CB but only the LM lead concentrations were significantly lower than the CC1 lead concentrations (Appendix 3.2). Cadmium concentrations at the LM site were significantly lower than both the CC1 and CC2 sites but were not different to the CB site, while LM selenium concentrations were significantly lower than at CC1 higher than CB and not different to CC2 (Appendix 3.2). Pearson's correlation (2-tailed) showed cadmium, lead, selenium, zinc and copper were all significantly positively correlated ($p < 0.01$) in sediments from the three contamination gradient sites, indicating a common source of contamination from the zinc/lead smelter is likely. Characterisation of the sediment showed the < 63 µm sediment fraction was CC1 = 10 %, CC2 = 6 %, CB = 2% and LM = 15 % of the total sediment.

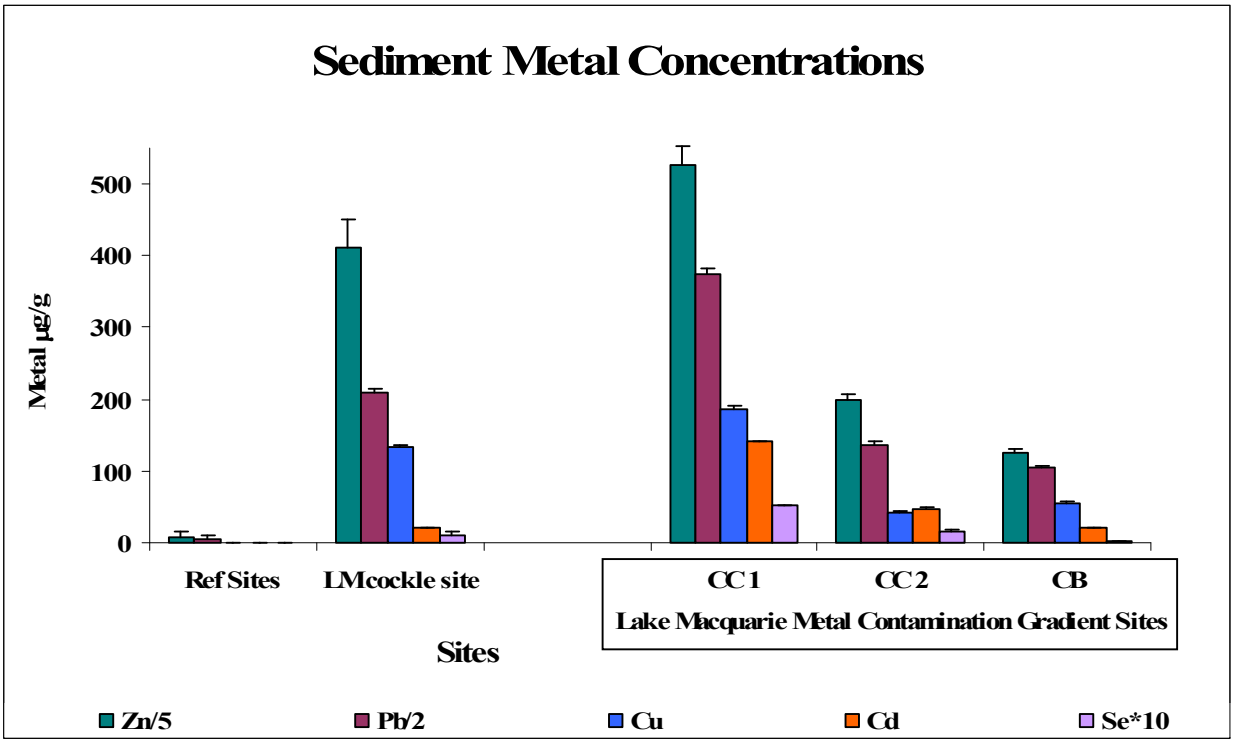


Figure 8.1: Metal concentrations (µg/g dry mass) in sediments collected along a metal gradient in Lake Macquarie, CC1 = Cockle Ck. Site 1, CC2 = Cockle Ck. Site 2, CB = Cockle Bay site. Sediment metal concentrations from unexposed organism collection sites (Ref) and the Lake Macquarie native metal exposed *A. trapezia* collection site (LM) are also shown. Mean ± SE, n = 9.

Metal concentrations measured in the sediment collected along the gradient from the lead/zinc smelter and the native *A. trapezia* collection site in Lake Macquarie in 2007 (Figure 3.3) and those from the reference sites where the test organisms were collected, are compared to the ANZECC and ARMCANZ (2000) interim sediment quality guidelines and to two previous studies of Lake Macquarie sediment metal concentrations in Cockle Bay (Table 8.1).

All metal concentrations at the reference sites were below the ANZECC / ARMCANZ (2000) low values (Table 8.1). Copper concentrations were above the ANZECC / ARMCANZ (2000) low value at the CC1 and LM sites sampled in 2007 but below the high value. The remaining sites were all below the ANZECC / ARMCANZ (2000) low value for copper. Cadmium and zinc concentrations exceeded the high ANZECC / ARMCANZ (2000) value at all other sites (Table 8.1). Lead concentrations were higher than the ANZECC / ARMCANZ (2000) high value at all other sites except at the 2007 Cockle Bay site which were higher than the low value (Table 8.1). The cadmium, lead, selenium, zinc and copper concentrations measured in the Cockle Creek site 1 Lake Macquarie sediments in 2007 were above those measured in Cockle Bay by (Roach, 2005) with the CC2 site falling between and the CB site below the concentrations measured by Burt *et al* (2007) and Roach (2005) for cadmium, lead, zinc and copper. The selenium concentrations at the LM and CB sites in this study were lower and at the CC1 site higher than those measured by Burt *et al* (2007) and Roach (2005). There are currently no sediment guideline values for selenium.

Table 8.1: Sediment metal concentrations (µg/g dry mass, mean ± SE) sampled in this study (n = 9), compared to previous sediment studies in Lake Macquarie and the ANZECC / ARMCANZ (2000) interim sediment guideline values. Ref = unexposed organism collection sites, CC1 = Cockle Ck. 1, CC2 = Cockle Ck. 2, CB = Cockle Bay, LM = Lake Macquarie native metal exposed *A. trapezia* collection site.

Metal	Ref	LM	CC1	CC2	CB	CB (Roach, 2005)	CB (Burt *et al.*, 2007)	ANZECC/ ARMCANZ (2000) Low	ANZECC/ ARMCANZ (2000) High
Cadmium	0.1 ± 0.1	21 ± 0.1	141 ± 1.3	48 ± 1	21 ± 1	65 ± 2	36 ± 0.8	1.5	10
Lead	9 ± 10	422 ± 9	750 ± 15	275 ± 9	211 ± 5	326 ± 11	257 ± 19	50	220
Selenium	0	1.1 ± 0.3	5.2 ± 0.1	1.5 ± 0.3	0.2 ± 0.1	4.1 ± 0.1	1.5 ± 0.2	n/a	n/a
Zinc	39 ± 47	2062 ± 193	2628 ± 137	997 ± 36	628 ± 24	1003 ± 11	650 ± 33	200	410
Copper	0.18 ± 0.4	134 ± 3	187 ± 5	41 ± 3	55 ± 3	64 ± 0.6	38 ± 4	65	270

n/a = not available

8.3.2 Tissue Metal Accumulation

8.3.2.1 *Whole Organism Metal Accumulation*

ANOVA showed site and day were significant in cadmium, lead and zinc accumulation differences, day was significant in copper and selenium accumulation but site was not and there was no significant interaction between site and day for any metal (Appendix 3.1). Tissue cadmium, lead and zinc concentrations were CC1 > CC2 > CB for each collection time (Figure 8.2). While selenium and copper tissue concentrations showed minor variations between sites over time by day 56 selenium concentrations were CC1 > CC2 > CB and copper concentrations were CC2 = CB > CC1 (Figure 8.2). The tissue metal accumulation of the laboratory exposed *A. trapezia* after 56 days compared to the native *A. trapezia* collected from Lake Macquarie were for copper CC2 = CB > CC1 > LM, for zinc and selenium CC2 > CC1 > CB > LM, for cadmium CC1 > CC2 > LM > CB and for lead CC1 > CC2 = LM > CB (Figure 8.2). The over-all statistical comparison of metal concentrations in tissues between sites showed organisms from the metal contaminated sediment exposures had significantly higher tissue concentrations than the reference organisms for all metals except selenium (Appendix 3.3). There was no significant difference in tissue metal concentrations between the contamination gradient sites for selenium or copper. The CC1 site organisms had significantly higher lead and zinc concentrations than organisms from the other two sites and higher cadmium concentrations than the CB site organisms. The only tissue metal concentration which was significantly different between sites CC2 and CB was lead (Appendix 3.3). Further analysis using Bonferroni pair-wise comparisons of differences in tissue metal concentrations between the metal contaminated sites for each collection day showed copper and selenium tissue concentrations were not significantly different between the sites on any collection day (Appendix 3.4). CC1 organisms had higher cadmium lead and zinc tissue concentrations than the CB organisms on all collection days, they did not have significantly more tissue cadmium than the CC2 organisms on any day but had significantly higher lead concentrations on days 14, 28 and 56 and higher zinc concentrations on days 28 and 56 (Appendix 3.4). The CC2 organisms did not have significantly higher zinc concentrations than the CB organisms, had significantly higher cadmium concentrations on days 42 and 56 and higher lead concentrations on days 28 and 42 (Appendix 3.4).

Within site metal accumulation between collection days showed that the majority of significant cadmium and zinc accumulation at site CC1 occurred within the first 14 days, the first 28 days for CC2 and within 42 days for CB. Significant lead accumulation for all sites occurred in the first 14 days while selenium did not show any significant accumulation at any site until day 28 (Appendix 3.5). Copper tissue concentrations were significantly higher than the reference organisms at all sites by day 42 (Appendix 3.5).

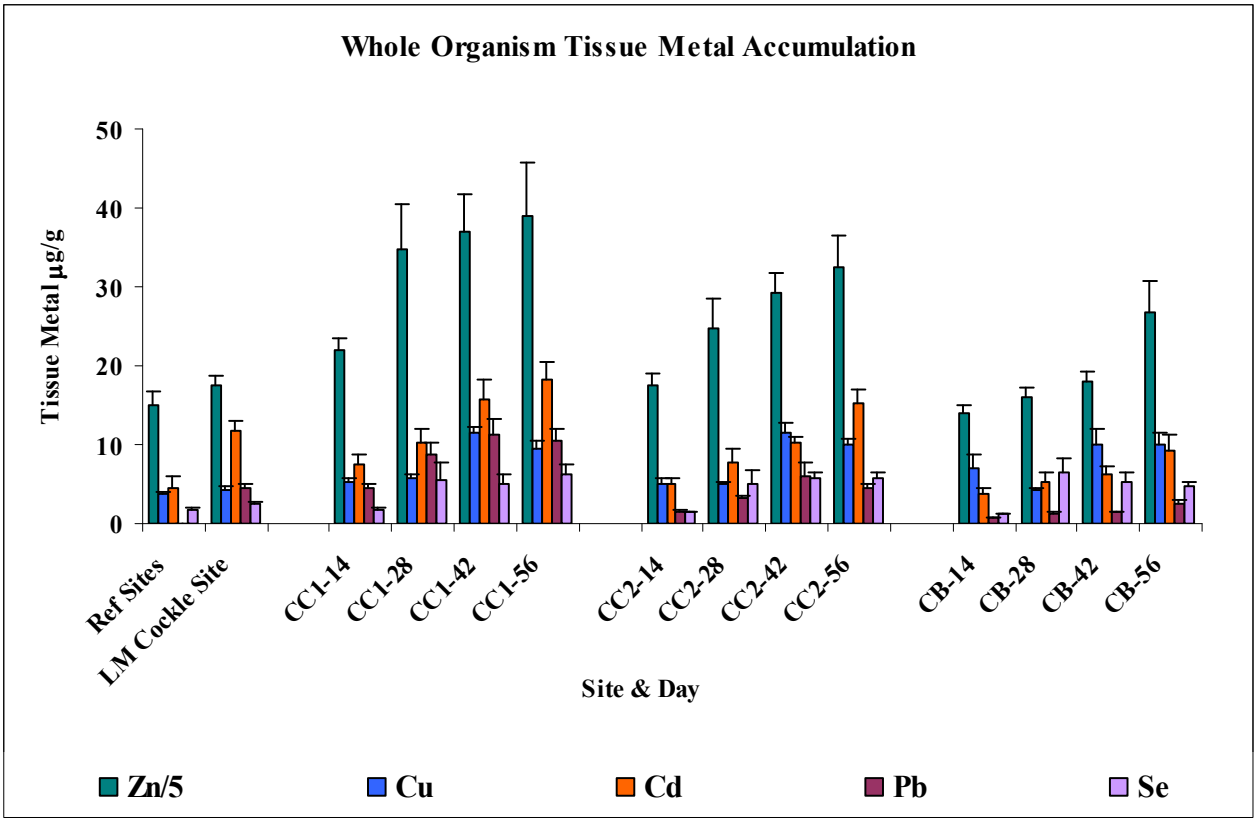


Figure 8.2: Metal accumulation in whole tissue of *A. trapezia* at 2 weekly intervals over 56 days of laboratory exposure to sediments from a metal contamination gradient in Lake Macquarie. CC1 = Cockle Ck. Site 1, CC2 = Cockle Ck. Site 2, CB = Cockle Bay site. Tissue metal concentrations from unexposed organisms (Ref) and the Lake Macquarie native metal exposed *A. trapezia* (LM) are also shown. Mean ± SE, n = 9.

The regression between total tissue and sediment metal concentrations shows a significant positive relationship (Figure 8.3a), as sediment metal concentration increased the tissue metal concentration increased but not proportionally. Regressions of individual tissue and sediment metal concentrations (Figure 8.3b-f) indicate that this is probably largely due to the accumulation patterns of selenium, which showed a weak positive sediment/tissue regression and copper which showed no positive regression (Figure 8.3d & f).

The tissue metal concentrations of the native *A. trapezia* collected from Cockle Bay in Lake Macquarie plotted against the sediment metal concentrations of the collection site show a very different metal exposure accumulation relationship to the organisms which had no previous metal exposure (Figure 8.3a). Plots of individual tissue versus sediment metal concentrations (Figure 8.3b-f) of these organisms show that they had accumulated higher tissue cadmium burdens relative to their exposure concentration than the other organisms, slightly less lead and considerably less selenium, zinc and copper. Regressions of the different tissue metal concentrations after 56 days of exposure showed a positive relationship for all metals with increasing tissue metal except for copper which had a weak relationship with lead and cadmium (Figure 8.4). The LM native *A. trapezia* are also shown but not included in the regressions. They fitted the regression well for the essential metals zinc, copper and selenium regressed together and also for cadmium and lead regressed together but not for the essential elements zinc, copper and selenium when they are compared with cadmium and lead (Figure 8.4).

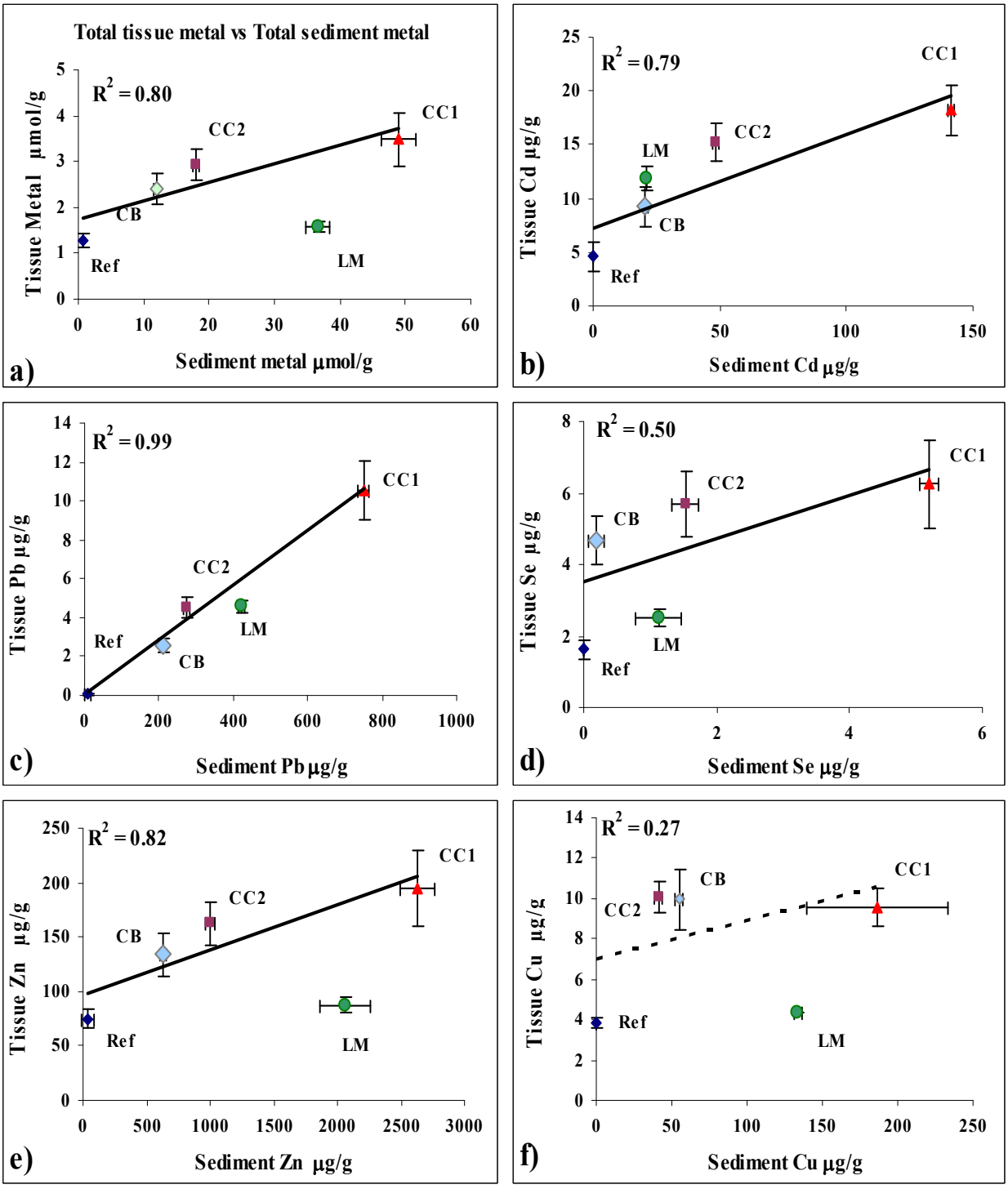


Figure 8.3: Regressions of sediment and tissue metal concentrations after 56 days of exposure for Ref (◆), CB (◇), CC2 (■) and CC1 (▲). LM (●) is shown but not included in the regression. Graph a) shows the total tissue and total sediment metal in µmol/g dry mass n = 54. Graphs b) – f) show the individual metals in µg/g dry mass, n = 9. Mean ± SE.

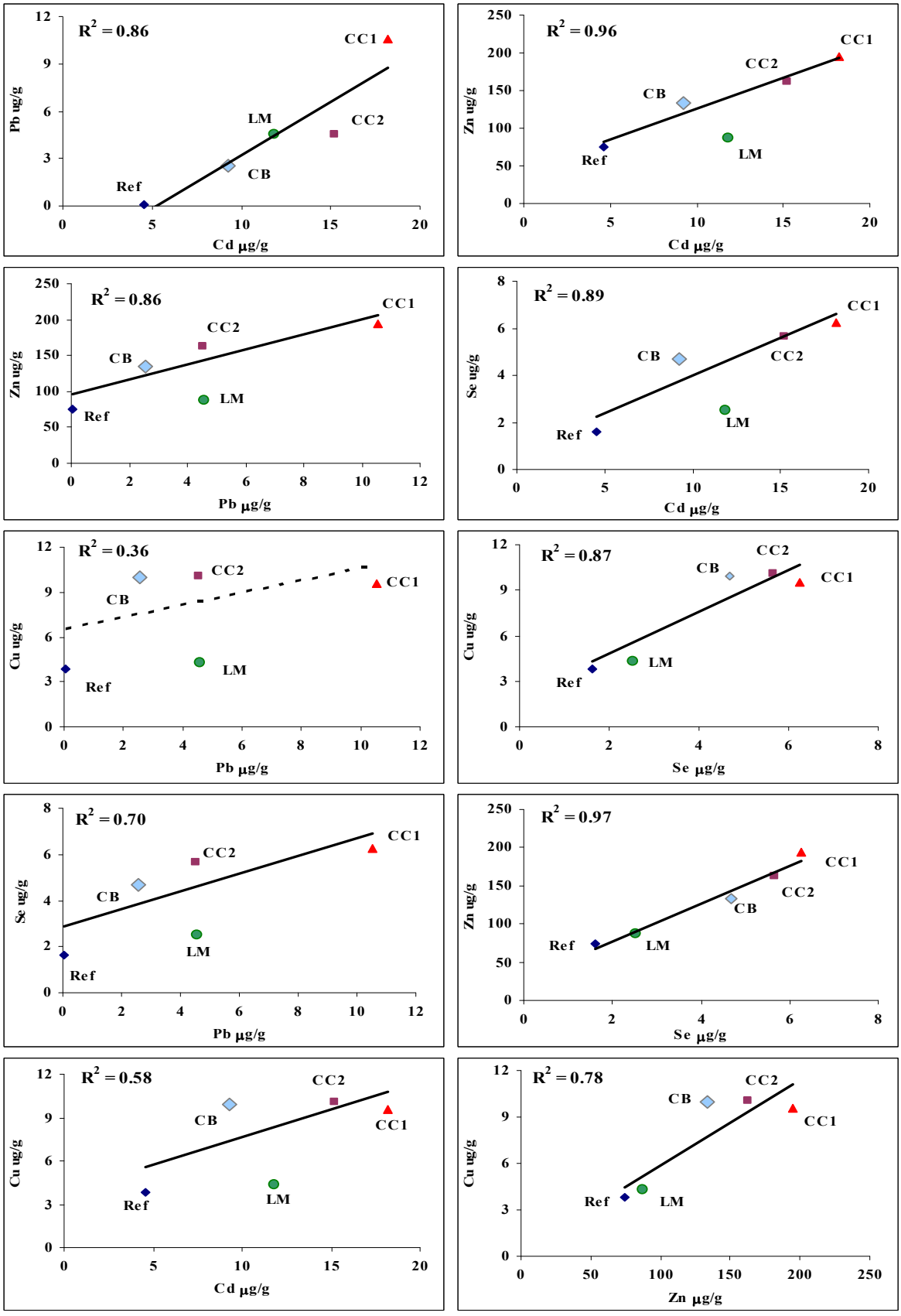


Figure 8.4: Regressions of tissue metal concentrations after 56 days of exposure for Ref (◆), CB (◇), CC2 (■) and CC1 (▲). LM (●) is shown but not included in the regression. Mean µg/g dry mass, n = 9.

8.3.2.2 *Individual Tissue Metal Accumulation*

ANOVA showed that metal concentrations were significantly different between tissues for all metals (Appendix 3.6). There was a significant interaction between site and tissue for cadmium and zinc concentration and between day and tissue for selenium, zinc and copper concentration but not between site, day and tissue for any metal concentration (Appendix 3.6). Bonferroni pair-wise comparisons between tissue metal concentrations at each site for each metal showed significant differences between all tissues for cadmium concentrations and between all tissues at sites CC1, CC2 and CB for lead concentrations but not for the reference organisms (Appendix 3.7). Gill was significantly different to the other two tissues for selenium and zinc concentrations at sites CC1, CC2 and CB. Zinc concentrations were significantly different between hepatopancreas and haemolymph at site CC2 and in the reference organisms but not at the other 2 sites (Appendix 3.7). Copper concentrations were significantly different between gill and hepatopancreas and hepatopancreas and haemolymph but not different between gill and haemolymph for all sites (Appendix 3.7). Further statistical analysis of metal concentration differences between tissues at each site for each collection day showed that cadmium concentrations differed between all tissues at all sites at all collection days in the order gill > hepatopancreas > haemolymph (Appendix 3.8; Figure 8.5). Bonferroni pair-wise comparisons of within tissue metal differences showed the majority of the cadmium accumulation in all tissues occurred in the first 28 days (Appendix 3.9). Gill and haemolymph lead concentrations were generally significantly higher than those of the hepatopancreas for all collection days (Appendix 3.8; Figure 8.5). Bonferroni pair-wise comparisons of within tissue lead concentration differences showed the majority of the accumulation in all tissues occurred in the first 28 days (Appendix 3.9). The selenium concentration in the gills of the organisms from CC1 and CC2 are significantly higher than the other two tissues after the first 14 days but were generally no different in selenium concentration after this, whereas, the CB organisms had significantly higher selenium concentrations in the hepatopancreas and haemolymph than the gill at day 14 with little difference after this (Appendix 3.8; Figure 8.5). Bonferroni pair-wise comparisons of within tissue metal concentration differences showed the majority of the selenium accumulation in all tissues occurred in the first 28 days (Appendix 3.9). In general the significant zinc tissue accumulation pattern for all sites on each of the collection days was gill > hepatopancreas > haemolymph, on day 56 at sites CC1 and CB this pattern changed to gill > haemolymph > hepatopancreas (Appendix 3.8; Figure 8.6).

Bonferroni pair-wise comparisons of within tissue zinc concentration differences showed a steady pattern of accumulation in each of the tissues over the course of the exposure (Appendix 3.9; Figure 8.6). The accumulation of copper was fairly even between the gill and haemolymph for each collection time at all the sites with the gill tending to slightly higher copper concentrations after 42 days but the differences were not significant. The hepatopancreas tissue had significantly lower copper concentrations than the other two tissues at all sites at all collection times (Appendix 3.8; Figure 8.6). Bonferroni pair-wise comparisons of within tissue copper concentration differences showed the majority of the accumulation in all tissues occurred after 42 days with some loss of copper occurring to day 56 (Appendix 3.9; Figure 8.6).

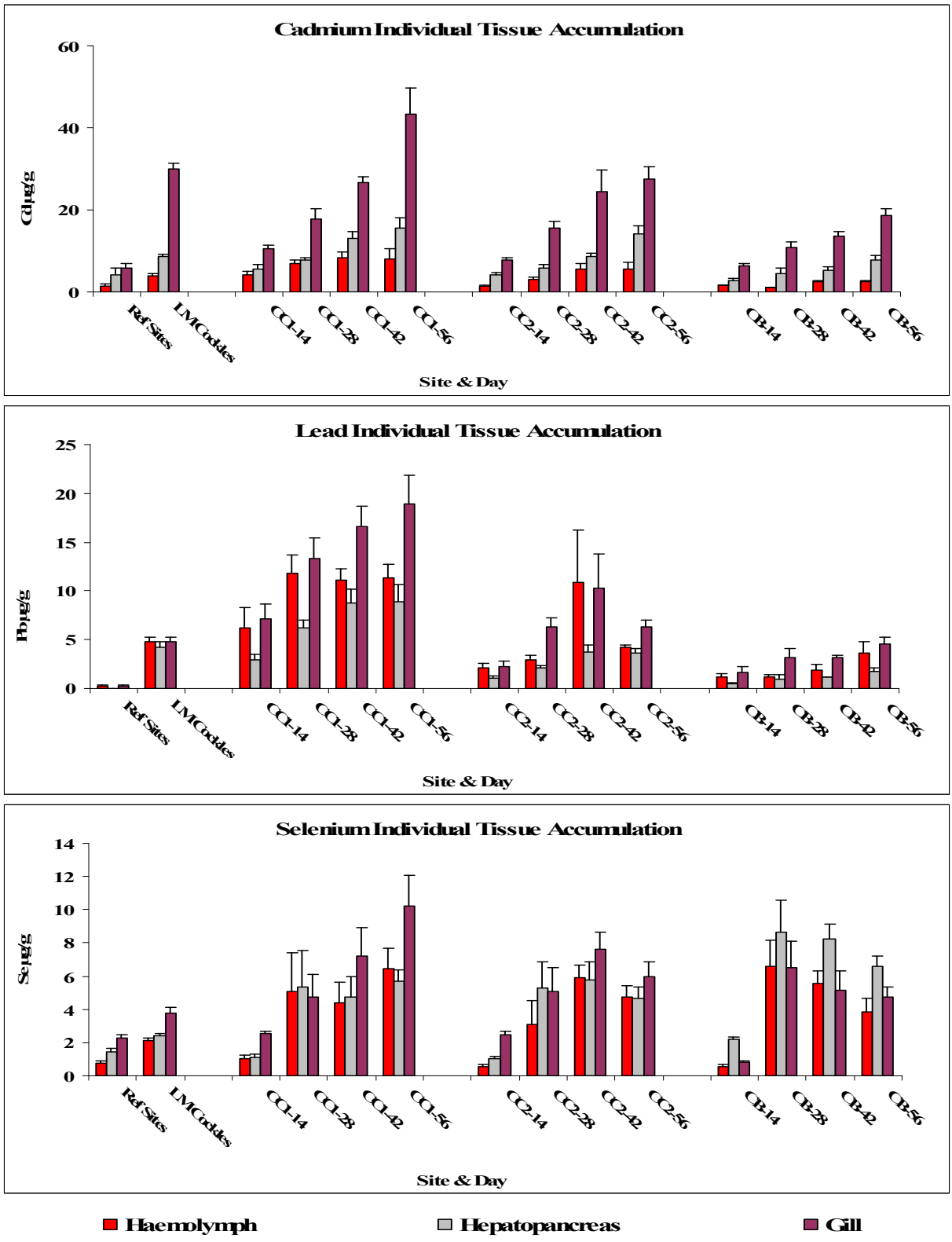


Figure 8.5: Cadmium, lead and selenium accumulation in gill hepatopancreas and haemolymph tissues of *A. trapezia* at 2 weekly intervals over 56 days of exposure to sediments from a metal contamination gradient in Lake Macquarie. CC1 = Cockle Ck. Site 1, CC2 = Cockle Ck. Site 2, CB = Cockle Bay site. Tissue metal concentrations from unexposed organisms (Ref) and the Lake Macquarie native metal exposed *A. trapezia* (LM) are also shown. Mean ± SE, n = 9.

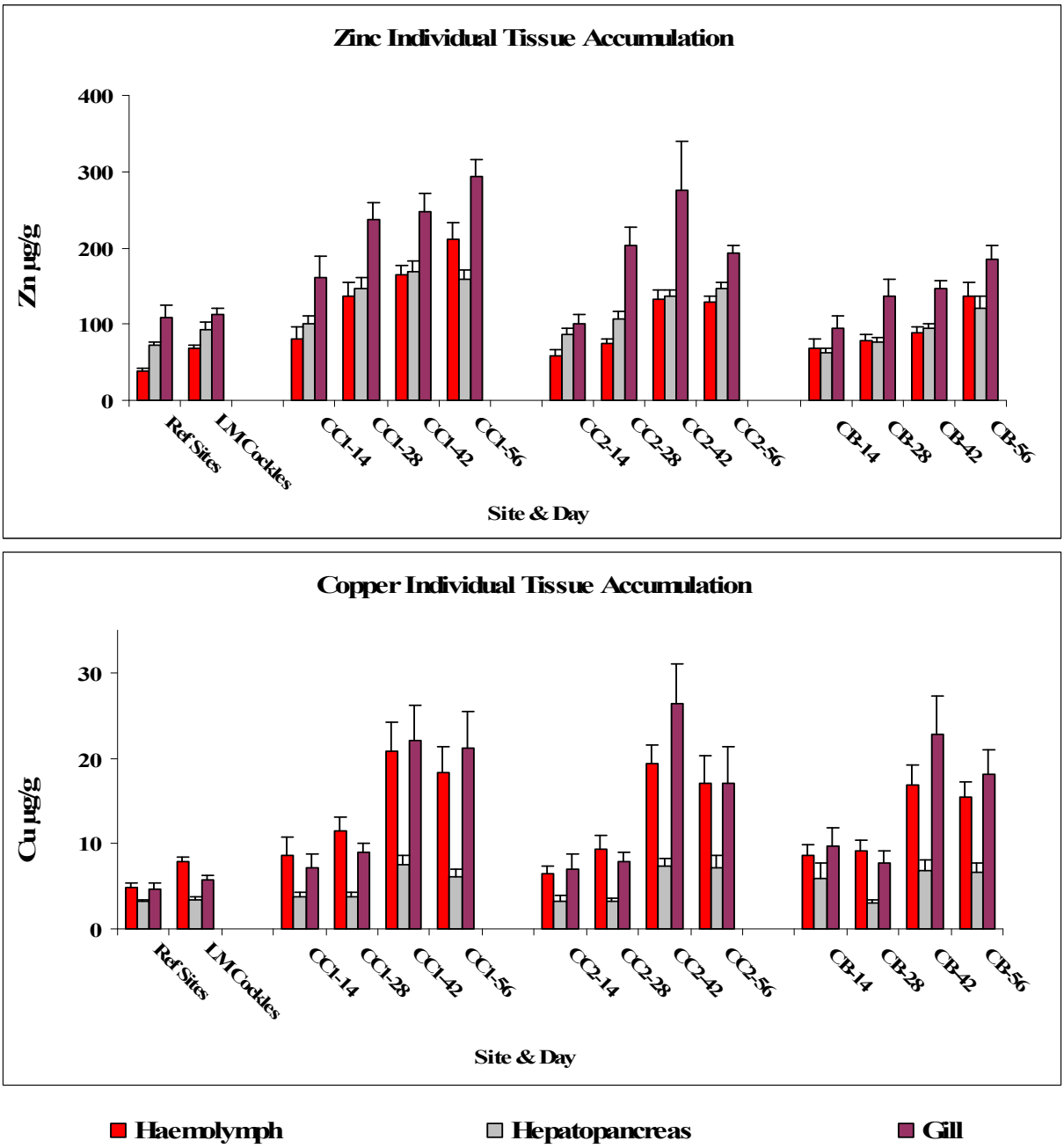


Figure 8.6: Zinc and copper accumulation in gill hepatopancreas and haemolymph tissues of *A. trapezia* at 2 weekly intervals over 56 days of exposure to sediments from a metal contamination gradient in Lake Macquarie. CC1 = Cockle Ck. Site 1, CC2 = Cockle Ck. Site 2, CB = Cockle Bay site. Tissue metal concentrations from unexposed organisms (Ref) and the Lake Macquarie native metal exposed *A. trapezia* (LM) are also shown.
Mean ± SE, n = 9.

8.3.3 Subcellular Tissue Metal Distribution

8.3.3.1 *Cadmium*

Gill

Between 47 and 69 % of the total gill cadmium burden was recovered in the fractions (Table 8.2). The majority of the recovered cadmium, up to 75 %, was in the biologically detoxified metal (BDM) fraction for all sites (Table 8.2). Twenty to 26 % of the remaining cadmium was in the biologically active metal (BAM) fraction with only 8 - 10 % in the nuclei+cellular debris fraction (Table 8.2). While the percentage cadmium distribution in the fractions did not differ much between sites, the total cadmium burden associated with the fractions increased in organisms in line with their increased cadmium exposure (Table 8.2). The BAM fractions of the CC1 organisms had 15 times more cadmium than the control organisms and 3 times that of organisms from the other two sites, while the organisms from the CC2 and CB sites had equal amounts of cadmium in their BAM fractions and 5 fold more than the control organisms (Table 8.2). The majority of cadmium in the BDM was in the heat stable metallothionein like proteins (MTLP) fraction for all sites (Table 8.3). The percentage distribution of cadmium within the BAM fractions was similar for the organisms from sites CC2 and CB with 43 – 46 % in the mitochondrial fraction 32 – 35 % in the lysosomal+microsomal fraction and the remainder in the heat sensitive protein (HSP) fraction (Figure 8.7; Table 8.3). The CC1 organisms had a higher percentage of cadmium in the mitochondrial fraction, 60 %, with the remainder equally distributed between the other two fractions (Figure 8.7; Table 8.3). Control organisms also had the highest percentage of their BAM cadmium burden in the mitochondria, 47 %, with the remainder evenly distributed between the other two fractions (Figure 8.7; Table 8.3). The percentage distribution of cadmium in the gill tissue of *A. trapezia* from the three metal contaminated sites was very similar to that of the organisms exposed to cadmium spiked sediments (Figure 5.4; Table 5.2).

Hepatopancreas

The proportion of cadmium recovered in the hepatopancreas subcellular fractions was similar to the gill at around 50 % for sites CC2 and CB, while for the control and CC1 organisms it was higher at 82 and 76 % respectively (Table 8.2). The proportion of cadmium in the BDM fractions was reduced in all treatments compared to the gill tissue for all sites (Table 8.2).

There was a higher percentage of cadmium in the nuclei+cellular debris fraction and only a slight increase in the BAM fraction for the three metal contaminated sites while the control organisms had the same percentage of cadmium in the nuclei+cellular debris fraction and a higher percentage of cadmium in the BAM than the control gill tissue (Table 8.2; Figure 8.7). The CC1 and CC2 organisms had the same total cadmium burden in the BAM fraction and this was twice and 10 times respectively, greater than that of the CB and control organisms (Figure 8.7). The percentage distribution of cadmium within the fractions of the hepatopancreas BDM was the same as that of the gill tissue for all sites, with over 90 % in the MTLP fraction (Figure 8.7; Table 8.3). The metal contaminated sites had a very similar cadmium distribution between the BAM fractions to the gill tissue, with the highest percentage in the mitochondrial fraction (Figure 8.7; Table 8.3). The cadmium BAM distribution in the hepatopancreas of the control organisms was different to their gills, with the cadmium being fairly evenly distributed between the three fractions (Figure 8.7; Table 8.3). The percentage distribution of cadmium in the hepatopancreas tissue of *A. trapezia* from the three metal contaminated sites was very similar to that of the organisms exposed to cadmium spiked sediments (Figure 5.4; Table 5.2).

Table 8.2: Cadmium, lead, selenium, zinc and copper concentrations (µg wet mass) in gill and hepatopancreas tissues and the total metal with percentage of each metal recovered from subcellular fractions of *A. trapezia* after 56 days of exposure to sediments from a metal contamination gradient in Lake Macquarie. Metal subcellular concentrations (µg wet mass) and percentage distribution of total recovered metal in fractions is grouped as nuclei+cellular debris and biologically active and detoxified metal (Figure 3.7). Mean ± SD, n = 2.

Cadmium	**Gill**				**Hepatopancreas**			
	Control	CC1	CC2	CB	Control	CC1	CC2	CB
Total Tissue Cadmium (µg)	**0.1 ± 0.01**	**2.3 ± 0.7**	**1.6 ± 0.03**	**0.9 ± 0.1**	**0.04 ± 0.03**	**0.7 ± 0.2**	**0.8 ± 0**	**0.3 ± 0.1**
Total Recovered Cadmium (µg)	**0.1 ± 0**	**1.3 ± 0.7**	**0.8 ± 0.03**	**0.4 ± 0.02**	**0.03 ± 0.03**	**0.5 ± 0.1**	**0.4 ± 0.1**	**0.2 ± 0.1**
Proportion of total recovered in fractions (%)	69 ± 9	55 ± 14	52 ± 1	47 ± 4	82 ± 16	76 ± 1	50 ± 12	56 ± 0.3
Cadmium Distribution								
Nuclei + Cellular debris (µg)	**0.005 ± 0**	**0.1 ± 0.1**	**0.1 ± 0.01**	**0.04 ± 0.01**	**0.003 ± 0**	**0.1 ± 0.03**	**0.1 ± 0.01**	**0.03 ± 0.01**
Nuclei + Cellular debris (%)	8 ± 1	9 ± 0.1	8 ± 1	10 ± 2	8 ± 4	14 ± 2	14 ± 2	13 ± 0.5
Biologically Active Metal (µg)	**0.02 ± 0**	**0.3 ± 0.1**	**0.1 ± 0**	**0.1 ± 0.01**	**0.01 ± 0.01**	**0.1 ± 0.01**	**0.1 ± 0.02**	**0.05 ± 0.01**
Biologically Active Metal (%)	26 ± 7	19 ± 1	18 ± 0.01	20 ± 2	46 ± 15	21 ± 6	19 ± 0.3	24 ± 1
Biologically Detoxified Metal (µg)	**0.04 ± 0.01**	**0.9 ± 0.5**	**0.6 ± 0.03**	**0.3 ± 0.02**	**0.02 ± 0.02**	**0.3 ± 0.1**	**0.3 ± 0.1**	**0.1 ± 0.04**
Biologically Detoxified Metal (%)	66 ± 8	72 ± 1	74 ± 1	70 ± 1	46 ± 11	65 ± 4	67 ± 2	63 ± 2

Lead	**Gill**				**Hepatopancreas**			
	Control	CC1	CC2	CB	Control	CC1	CC2	CB
Total Tissue Lead (µg)	**0.04 ± 0.02**	**1.4 ± 0.8**	**0.7 ± 0.4**	**0.3 ± 0.01**	**0.02 ± 0.01**	**0.5 ± 0.3**	**0.5 ± 0.3**	**0.1 ± 0.01**
Total Recovered Lead (µg)	**0.03 ± 0.03**	**1 ± 0.7**	**0.2 ± 0.1**	**0.2 ± 0**	**0.02 ± 0.01**	**0.5 ± 0.1**	**0.2 ± 0.1**	**0.1 ± 0**
Proportion of total recovered in fractions (%)	88 ± 16	63 ± 17	41 ± 16	50 ± 0.02	62 ± 4	100 ± 23	51 ± 6	59 ± 4
Lead Distribution								
Nuclei + Cellular debris (µg)	**0.004 ± 0**	**0.1 ± 0.1**	**0.02 ± 0**	**0.02 ± 0.01**	**0.001 ± 0**	**0.1 ± 0.1**	**0.03 ± 0**	**0.01 ± 0**
Nuclei + Cellular debris (%)	15 ± 9	14 ± 2	9 ± 3	14 ± 4	15 ± 14	16 ± 6	13 ± 4	16 ± 2
Biologically Active Metal (µg)	**0.02 ± 0.01**	**0.2 ± 0.2**	**0.04 ± 0.02**	**0.03 ± 0**	**0.01 ± 0.01**	**0.1 ± 0.1**	**0.04 ± 0.01**	**0.02 ± 0**
Biologically Active Metal (%)	55 ± 22	18 ± 4	18 ± 4	20 ± 2	57 ± 7	27 ± 9	19 ± 2	26 ± 4
Biologically Detoxified Metal (µg)	**0.01 ± 0.02**	**0.6 ± 0.5**	**0.2 ± 0.04**	**0.1 ± 0.01**	**0.01 ± 0.01**	**0.3 ± 0.1**	**0.1 ± 0.05**	**0.05 ± 0**
Biologically Detoxified Metal (%)	30 ± 31	68 ± 12	73 ± 12	66 ± 6	28 ± 2	57 ± 13	68 ± 6	58 ± 1

Selenium	**Gill**				**Hepatopancreas**			
	Control	CC1	CC2	CB	Control	CC1	CC2	CB
Total Tissue Selenium (µg)	**0.3 ± 0.2**	**0.4 ± 0.1**	**0.4 ± 0.1**	**0.4 ± 0.03**	**0.3 ± 0.1**	**0.3 ± 0.2**	**0.4 ± 0.1**	**0.4 ± 0.04**
Total Recovered Selenium (µg)	**0.1 ± 0.02**	**0.1 ± 0.02**	**0.1 ± 0.02**	**0.1 ± 0.02**	**0.1 ± 0.01**	**0.1 ± 0.05**	**0.1 ± 0.02**	**0.1 ± 0.01**
Proportion of total recovered in fractions (%)	35 ± 14	31 ± 10	24 ± 2	23 ± 3	31 ± 5	46 ± 12	27 ± 0.3	28 ± 0.3
Selenium Distribution								
Nuclei + Cellular debris (µg)	**0.02 ± 0.01**	**0.04 ± 0.02**	**0.03 ± 0.01**	**0.03 ± 0**	**0.01 ± 0.01**	**0.03 ± 0.03**	**0.03 ± 0.01**	**0.03 ± 0**
Nuclei + Cellular debris (%)	23 ± 10	30 ± 17	34 ± 4	30 ± 1	14 ± 6	23 ± 12	25 ± 4	21 ± 3
Biologically Active Metal (µg)	**0.06 ± 0**	**0.06 ± 0.02**	**0.04 ± 0.01**	**0.05 ± 0.01**	**0.06 ± 0**	**0.07 ± 0**	**0.06 ± 0**	**0.07 ± 0.01**
Biologically Active Metal (%)	67 ± 13	52 ± 16	46 ± 6	54 ± 0.1	70 ± 13	65 ± 19	58 ± 13	66 ± 6
Biologically Detoxified Metal (µg)	**0.01 ± 0**	**0.02 ± 0**	**0.02 ± 0.01**	**0.02 ± 0**	**0.01 ± 0.01**	**0.02 ± 0.02**	**0.02 ± 0.01**	**0.01 ± 0**
Biologically Detoxified Metal (%)	10 ± 4	19 ± 0.5	19 ± 5	16 ± 1	16 ± 5	12 ± 8	19 ± 12	13 ± 3

Zinc	**Gill**				**Hepatopancreas**			
	Control	CC1	CC2	CB	Control	CC1	CC2	CB
Total Tissue Zinc (µg)	**5.4 ± 1.4**	**13 ± 4**	**8 ± 3**	**7.3 ± 0.3**	**6 ± 0.1**	**6 ± 2**	**5.8 ± 0.1**	**6.7 ± 0.7**
Total Recovered Zinc (µg)	**4 ± 0.1**	**9 ± 4**	**4.8 ± 2**	**3.8 ± 0.4**	**4.1 ± 1.3**	**5 ± 1**	**3.5 ± 0.7**	**3.9 ± 0.3**
Proportion of total recovered in fractions (%)	77 ± 18	64 ± 10	60 ± 5	53 ± 5	68 ± 21	89 ± 8	61 ± 14	58 ± 1
Zinc Distribution								
Nuclei + Cellular debris (µg)	**0.3 ± 0.1**	**0.7 ± 0.5**	**0.3 ± 0.1**	**0.3 ± 0.1**	**0.5 ± 0.4**	**0.6 ± 0.1**	**0.3 ± 0.1**	**0.4 ± 0.1**
Nuclei + Cellular debris (%)	9 ± 2	8 ± 2	5 ± 1	7 ± 1	12 ± 6	11 ± 1	10 ± 1	11 ± 1
Biologically Active Metal (µg)	**0.8 ± 0.1**	**1.3 ± 0.5**	**0.8 ± 0.3**	**0.7 ± 0.01**	**0.9 ± 0.1**	**1 ± 0.1**	**0.6 ± 0.1**	**0.9 ± 0.1**
Biologically Active Metal (%)	21 ± 3	16 ± 1	17 ± 0.2	18 ± 2	24 ± 4	20 ± 7	18 ± 0.5	22 ± 0.1
Biologically Detoxified Metal (µg)	**3 ± 0.3**	**7 ± 3**	**4 ± 1.3**	**3 ± 0.3**	**3 ± 0.8**	**4 ± 1.4**	**3 ± 0.5**	**3 ± 0.2**
Biologically Detoxified Metal (%)	70 ± 5	76 ± 0.5	78 ± 1	75 ± 1	70 ± 6	69 ± 9	72 ± 0.1	67 ± 1

Copper	**Gill**				**Hepatopancreas**			
	Control	CC1	CC2	CB	Control	CC1	CC2	CB
Total Tissue Copper (µg)	**1 ± 0.2**	**1.4 ± 0.4**	**0.8 ± 0.2**	**1 ± 0.5**	**1 ± 0.4**	**1 ± 0**	**0.9 ± 0.2**	**0.8 ± 0.1**
Total Recovered Copper (µg)	**0.7 ± 0.3**	**1.3 ± 0.3**	**0.8 ± 0.2**	**1.1 ± 0.3**	**0.5 ± 0.1**	**0.7 ± 0**	**0.7 ± 0.01**	**0.9 ± 0.04**
Proportion of total recovered in fractions (%)	68 ± 37	93 ± 7	98 ± 37	124 ± 33	58 ± 27	72 ± 6	79 ± 18	113 ± 11
Copper Distribution								
Nuclei + Cellular debris (µg)	**0.08 ± 0.01**	**0.17 ± 0.4**	**0.11 ± 0.03**	**0.15 ± 0.05**	**0.09 ± 0.05**	**0.14 ± 0.01**	**0.11 ± 0.02**	**0.15 ± 0.03**
Nuclei + Cellular debris (%)	13 ± 7	13 ± 0	14 ± 1	14 ± 1	18 ± 11	19 ± 2	15 ± 2	17 ± 3
Biologically Active Metal (µg)	**0.24 ± 0.05**	**0.28 ± 0.24**	**0.11 ± 0.03**	**0.19 ± 0.08**	**0.17 ± 0.02**	**0.22 ± 0.19**	**0.11 ± 0**	**0.17 ± 0.01**
Biologically Active Metal (%)	37 ± 7	20 ± 14	14 ± 1	17 ± 3	32 ± 7	29 ± 24	15 ± 0.1	19 ± 3
Biologically Detoxified Metal (µg)	**0.4 ± 0.2**	**0.8 ± 0.02**	**0.6 ± 0.1**	**0.8 ± 0.2**	**0.3 ± 0.1**	**0.4 ± 0.1**	**0.5 ± 0**	**0.6 ± 0.2**
Biologically Detoxified Metal (%)	50 ± 13	66 ± 14	72 ± 2	69 ± 4	50 ± 17	52 ± 12	71 ± 2	63 ± 1

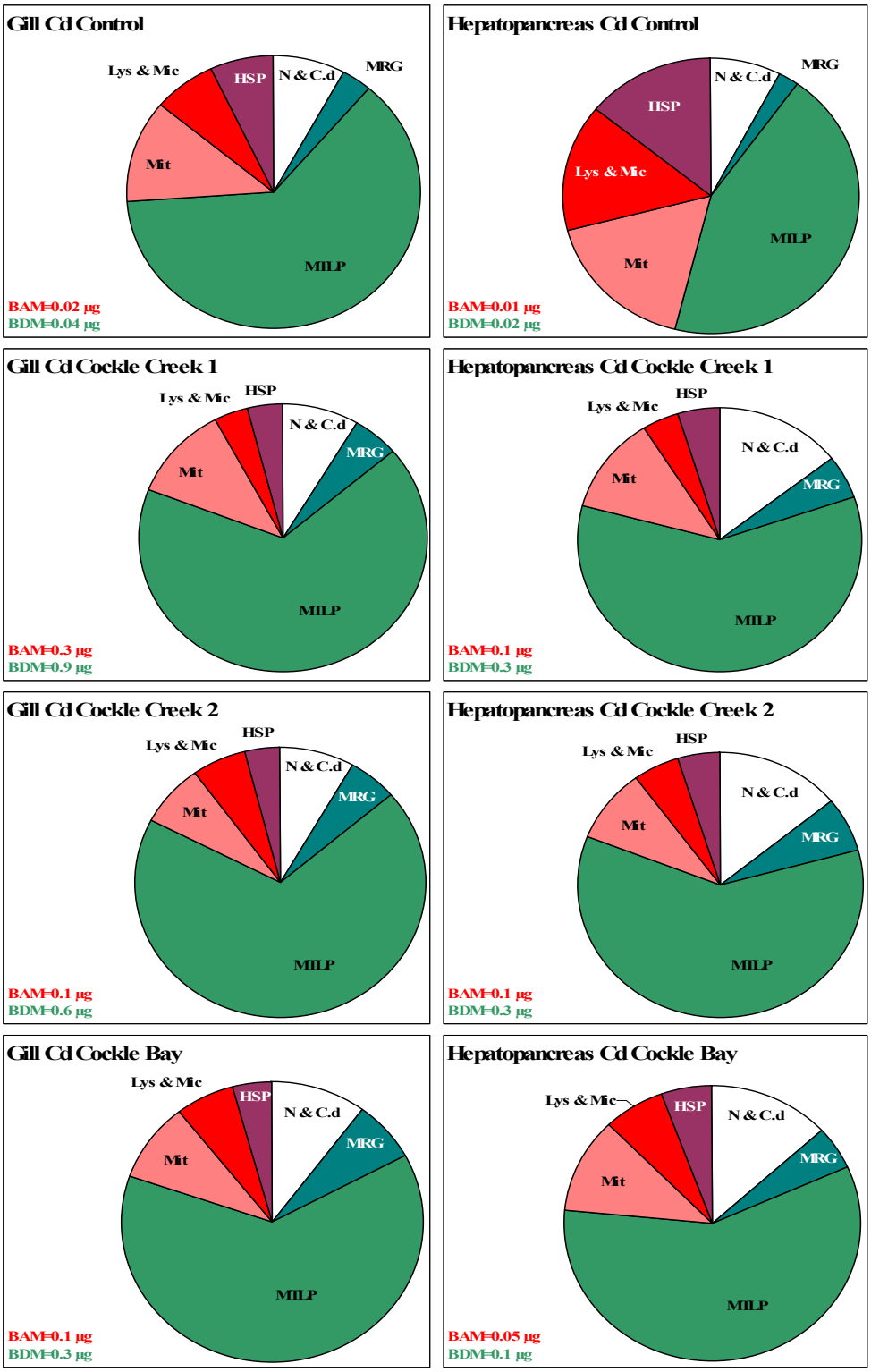


Figure 8.7: Distribution (%) of cadmium in each of the subcellular fractions of *A. trapezia* gill and hepatopancreas tissues following 56 days of exposure to sediments from a metal contamination gradient in Lake Macquarie. Subcellular fractions are: nuclei + cellular debris (N & C.d); metal rich granules (MRG); heat stable metallothionein like proteins (MTLP); mitochondria (Mit); lysosomes + microsomes (Lys & Mic); heat sensitive proteins (HSP). Red fractions ( ) make up the biologically active metal (BAM), green fractions ( ) make up the biologically detoxified metal (BDM). Mean ± SD, n = 2.

Table 8.3: Cadmium, lead, selenium, zinc and copper percentage distribution in the nuclei+cellular debris, biologically detoxified (BDM) and active (BAM) metal fractions of *A. trapezia* after 56 days of exposure to sediments from a metal contamination gradient in Lake Macquarie, with the percentage metal each of the fractions within contributes to the BDM and BAM. Mean ± SD, n = 2.

Cadmium	**Gill**				**Hepatopancreas**			
	Control	CC1	CC2	CB	Control	CC1	CC2	CB
Nuclei + Cellular debris % of total	**8**	**9**	**8**	**10**	**8**	**14**	**14**	**13**
BDM % of total	**66**	**72**	**74**	**70**	**46**	**65**	**67**	**63**
Metal Rich Granules % of BDM	5	7	7	10	5	8	10	8
Heat Stable MT Like Proteins % of BDM	95	93	93	90	95	92	90	92
BAM % of total	**26**	**19**	**18**	**20**	**46**	**21**	**19**	**24**
Mitochondria % of BAM	47	60	43	46	37	58	47	49
Lysosomes + Microsomes % of BAM	26	20	35	32	33	19	29	28
Heat Sensitive Proteins % of BAM	27	20	22	22	30	23	24	23
Lead	**Gill**				**Hepatopancreas**			
	Control	CC1	CC2	CB	Control	CC1	CC2	CB
Nuclei + Cellular debris % of total	**15**	**14**	**9**	**14**	**15**	**16**	**13**	**16**
BDM % of total	**30**	**68**	**73**	**66**	**28**	**57**	**68**	**58**
Metal Rich Granules % of BDM	28	19	32	28	33	24	31	26
Heat Stable MT Like Proteins % of BDM	72	81	68	72	67	76	69	74
BAM % of total	**55**	**18**	**18**	**20**	**57**	**27**	**19**	**26**
Mitochondria % of BAM	49	65	48	46	47	76	55	51
Lysosomes + Microsomes % of BAM	27	17	27	25	22	11	17	14
Heat Sensitive Proteins % of BAM	25	18	25	29	31	13	28	35
Selenium	**Gill**				**Hepatopancreas**			
	Control	CC1	CC2	CB	Control	CC1	CC2	CB
Nuclei + Cellular debris % of total	**23**	**30**	**34**	**30**	**17**	**23**	**25**	**21**
BDM % of total	**10**	**19**	**19**	**16**	**14**	**12**	**17**	**13**
Metal Rich Granules % of BDM	100	100	100	100	100	100	100	100
Heat Stable MT Like Proteins % of BDM	0	0	0	0	0	0	0	0
BAM % of total	**67**	**52**	**46**	**54**	**70**	**65**	**58**	**66**
Mitochondria % of BAM	57	56	39	40	45	47	41	36
Lysosomes + Microsomes % of BAM	14	16	26	24	23	19	20	18
Heat Sensitive Proteins % of BAM	28	28	35	36	32	34	39	46
Zinc	**Gill**				**Hepatopancreas**			
	Control	CC1	CC2	CB	Control	CC1	CC2	CB
Nuclei + Cellular debris % of total	**9**	**8**	**5**	**7**	**12**	**11**	**10**	**11**
BDM % of total	**70**	**76**	**78**	**75**	**64**	**69**	**72**	**67**
Metal Rich Granules % of BDM	9	8	9	13	6	12	13	13
Heat Stable MT Like Proteins % of BDM	91	92	91	87	94	88	87	87
BAM % of total	**21**	**16**	**17**	**18**	**24**	**20**	**18**	**22**
Mitochondria % of BAM	61	53	39	43	48	60	43	45
Lysosomes + Microsomes % of BAM	22	24	38	35	33	17	33	33
Heat Sensitive Proteins % of BAM	17	23	23	22	19	23	24	22
Copper	**Gill**				**Hepatopancreas**			
	Control	CC1	CC2	CB	Control	CC1	CC2	CB
Nuclei + Cellular debris % of total	**13**	**13**	**14**	**14**	**18**	**19**	**15**	**17**
BDM % of total	**50**	**66**	**72**	**69**	**50**	**52**	**71**	**66**
Metal Rich Granules % of BDM	17	49	12	32	12	49	9	25
Heat Stable MT Like Proteins % of BDM	83	51	88	68	88	51	91	75
BAM % of total	**37**	**20**	**14**	**17**	**32**	**29**	**15**	**17**
Mitochondria % of BAM	52	73	37	57	53	73	36	54
Lysosomes + Microsomes % of BAM	25	16	43	29	27	11	41	28
Heat Sensitive Proteins % of BAM	24	12	20	14	20	16	23	18

8.3.3.2 *Lead*

Gill

Lead recovery in the fractions varied from as low as 41 % in the CC2 organisms to 88 % in the control gill tissue with around 70 % of the recovered lead in the BDM fractions in the metal exposed organisms (Table 8.2). The metal exposed organisms had around 20 % of the recovered lead in the BAM fractions which was less than half the percentage of lead in the BAM fractions of control organisms (Table 8.2). The total lead burden within the BAM fractions of the CC1, CC2 and CB organisms was, 10 times, twice and 1.5 times, respectively, greater than that of the control organisms (Figure 8.8). The majority of the BDM was in the MTLP of organisms from all sites (Table 8.3; Figure 8.8). The CC1 organisms had a higher percentage of lead in the MTLP fraction and a lower percentage in the MRG fraction than organisms from the other sites (Table 8.3; Figure 8.8). This is the reverse of what was observed for *A. trapezia* in the lead spiking experiment where the lead percentage increased in the MRG fraction and decreased in the MTLP with increased lead exposure (Figure 6.4; Table 6.2). Of the BAM fractions, the mitochondrial fraction contained the highest percentage of lead in organisms from all sites with the remainder fairly evenly distributed between the lysosome+microsome and HSP fractions (Table 8.3; Figure 8.8). This is in agreement with the results from the *A. trapezia* lead spiking experiment (Figure 6.4; Table 6.2).

Hepatopancreas

The lowest lead recovery from the fractions was 51 % in the CC2 organisms with the highest 100 % in the CC1 organisms (Table 8.2). As was seen for the gill tissue, the greatest percentage of lead in the hepatopancreas tissue of all the metal exposed organisms was in the BDM fractions, although the percentage was lower with generally a correspondingly higher percentage in the BAM compared to that of the gills (Table 8.2). The percentage in the nuclei+cellular debris fraction was about the same as that found in the gill (Table 8.2). The total lead burden within the hepatopancreas BAM fractions of the CC1, CC2 and CB organisms was, 10 times, 4 times and twice, respectively, greater than that of the control organisms (Table 8.2). The majority of the BDM was in the MTLP of organisms from all treatments with only about a quarter in the MRG fraction and there was no difference in the fraction percentages between sites (Table 8.3; Figure 8.8).

The pattern seen in the gills, of a decreasing percentage in the MRG fraction with increased lead exposure, was also present in the hepatopancreas (Table 8.3; Figure 8.8). The distribution of lead in the hepatopancreas BAM fractions was similar to that found for the gills in that the mitochondria of organisms from all sites contained the highest percentage of lead, however, the lead distribution differed from the gills in the other two fractions with the HSP having a higher percentage than the lysosome+microsome fraction (Table 8.3; Figure 8.8). The lead distribution pattern of the hepatopancreas HSP and lysosome+microsomes (Table 8.3) was the reverse of the pattern seen in the organisms in the lead spiking experiment (Table 6.2).

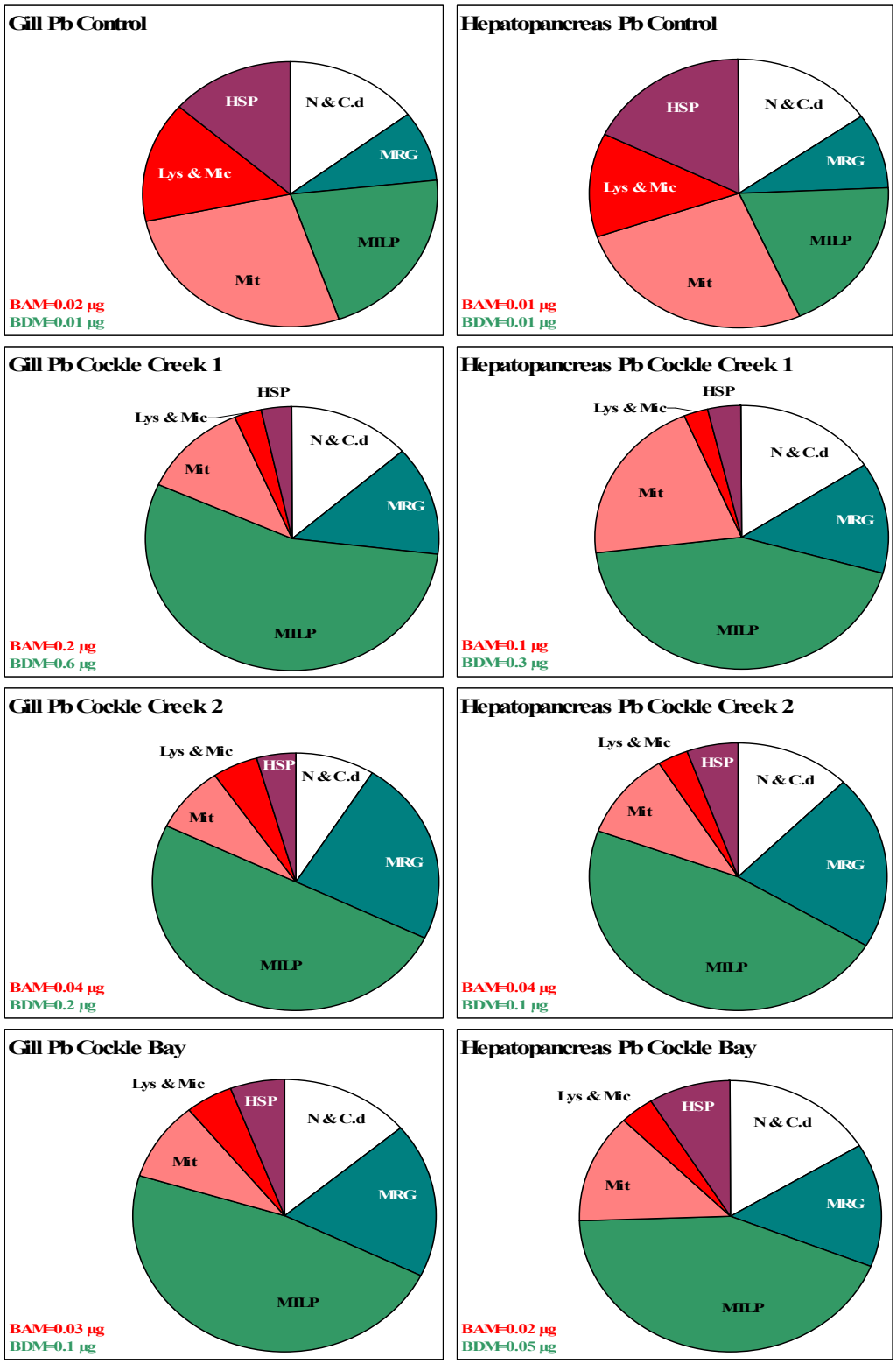


Figure 8.8: Distribution (%) of lead in each of the subcellular fractions of *A. trapezia* gill and hepatopancreas tissues following 56 days of exposure to sediments from a metal contamination gradient in Lake Macquarie. Subcellular fractions are: nuclei + cellular debris (N & C.d); metal rich granules (MRG); heat stable metallothionein like proteins (MTLP); mitochondria (Mit); lysosomes + microsomes (Lys & Mic); heat sensitive proteins (HSP). Red fractions ( ) make up the biologically active metal (BAM), green fractions ( ) make up the biologically detoxified metal (BDM). Mean ± SD, n = 2.

8.3.3.3 *Selenium*

Gill

Only a low percentage of the total selenium burden, 23 to 35 %, was recovered in the gill tissue fractions (Table 8.2). Of the recovered selenium, ≈ 50 % was in the BAM fractions, ≈30 % was in the nuclei+cellular debris fraction and only 16 to 19 % was in the BDM fraction of the metal exposed *A. trapezia.* This distribution is in general agreement with the distribution found in the *A. trapezia* selenium spiked sediment study (Table 7.1). The percentage of selenium recovered in the BAM fractions of the metal exposed organisms was less than the control organisms and the selenium recovered within the gill BAM fractions of the CC1, CC2 and CB organisms was, equal to or lower than that of the control organisms (Table 8.2). All the BDM selenium was in the MRG fraction of organisms from all sites (Figure 8.9; Table 8.3). This differs from the *A. trapezia* selenium spiked sediment study where up to 50 % of the BDM of the selenium exposed organisms was in the MTLP fraction (Table 7.1). Selenium distribution among the BAM fractions was mitochondria > HSP > lysosomes+microsomes for each treatment with some differences in the percentage values between sites (Table 8.3), which is the same as the BAM distribution pattern of the *A. trapezia* selenium spiked sediment study (Table 7.2).

Hepatopancreas

Selenium recoveries in the hepatopancreas fractions were also low but slightly better than the gill at between 27 and 46 % (Table 8.2). This is in general agreement with the *A. trapezia* selenium spiked sediment study recoveries (Table 7.1). The majority, 58 to 70 %, of the recovered selenium was in the BAM fractions followed by the nuclei+cellular debris with 14 to 25 % and only 12 to 19 % of the selenium was in the BDM fractions (Table 8.2). This differs from the selenium distribution pattern of the hepatopancreas from the *A. trapezia* selenium spiked sediment study which had the greatest percentage recovered in the nuclei+cell debris fraction followed by the BAM while the percentage in the BDM was about the same as this study (Tables 7.2 & 8.2). The selenium distribution between fractions within the BDM was the same as in the gill with 100 % in the MRG fraction (Figure 8.9) but differed from the *A. trapezia* selenium spiked sediment study hepatopancreas which had around 30 % of the BDM selenium in the MTLP fraction (Table 7.2).

The selenium distribution in the hepatopancreas BAM fractions also followed the same pattern as found in the gills (Table 8.3) and this is in agreement with BAM distribution pattern of the *A. trapezia* selenium spiked sediment study (Table 7.2).

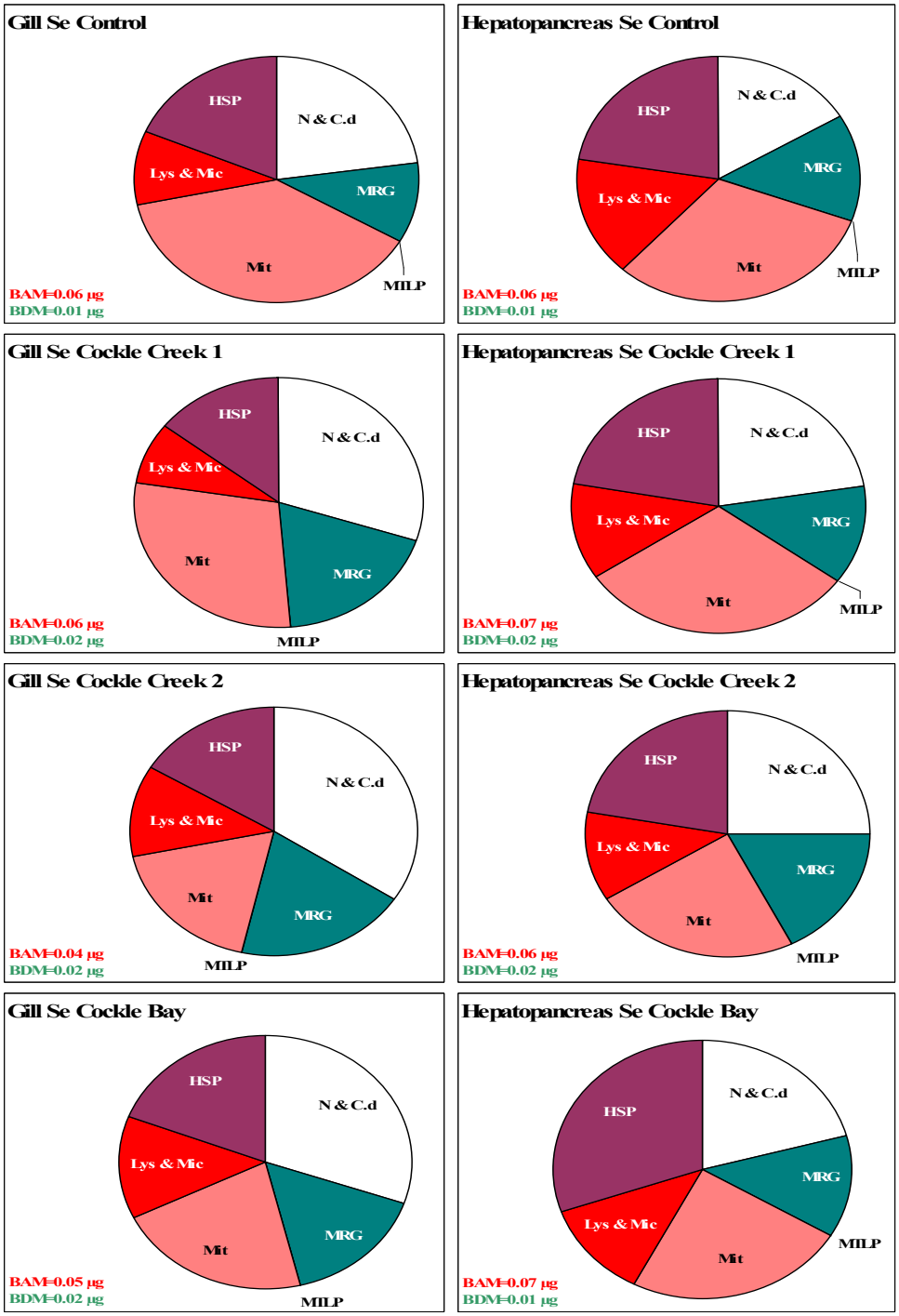


Figure 8.9: Distribution (%) of selenium in each of the subcellular fractions of *A. trapezia* gill and hepatopancreas tissues following 56 days of exposure to sediments from a metal contamination gradient in Lake Macquarie. Subcellular fractions are: nuclei + cellular debris (N & C.d); metal rich granules (MRG); heat stable metallothionein like proteins (MTLP); mitochondria (Mit); lysosomes + microsomes (Lys & Mic); heat sensitive proteins (HSP). Red fractions ( ) make up the biologically active metal (BAM), green fractions ( ) make up the biologically detoxified metal (BDM), n = 2.

8.3.3.4 *Zinc*

Gill

Zinc recovery in the gill tissue fractions ranged between 55 and 77 % (Table 8.2). Of the zinc recovered in the fractions, 70 to 78 % was in the BDM fractions followed by the BAM fractions with 16 to 21 % and the nuclei+cellular debris with only 5 to 9 % (Table 8.2). The percentage of the zinc recovered in the BAM fractions of the CC1 organisms was only slightly lower than that of the reference, CC2 and CB organisms but the zinc burden within the CC1 organism fractions was more than 1.5 times that of organisms from the other two sites (Table 8.2). The majority, 87 to 92 % of the BDM was in the MTLP fraction with only 9 to 13 % in the MRG fraction for all sites (Table 8.3; Figure 8.10). The distribution of zinc within the BAM fractions was in the order mitochondria > lysosome+microsome > HSP for organisms from all sites with some minor variations in the percentage values between sites (Table 8.3).

Hepatopancreas

Recovery of zinc in the hepatopancreas tissue fractions was slightly better than the gill recoveries ranging from 58 to 89 % (Table 8.2) and similar to the gills the distribution of recovered zinc was greatest in the BDM fractions ranging from 67 to 72 %, followed by the BAM 18 to 24 % with only 10 to 12 % recovered in the nuclei+cellular debris fractions (Table 8.2). There was very little difference in the zinc burden recovered in the BAM fractions between sites (Table 8.2). The BDM zinc distribution was the same as in the gills with the majority, 87 to 94 % of the BDM in the MTLP fraction and only 6 to 13 % in the MRG fraction for all sites (Table 8.3; Figure 8.10). Similarly the hepatopancreas BAM zinc distribution among fractions followed the same pattern as the gills with 43 to 60 % in the mitochondria followed by the lysosome/microsome fraction with 17 to 33 % and the HSP with 19 to 24 % (Table 8.3; Figure 8.10).

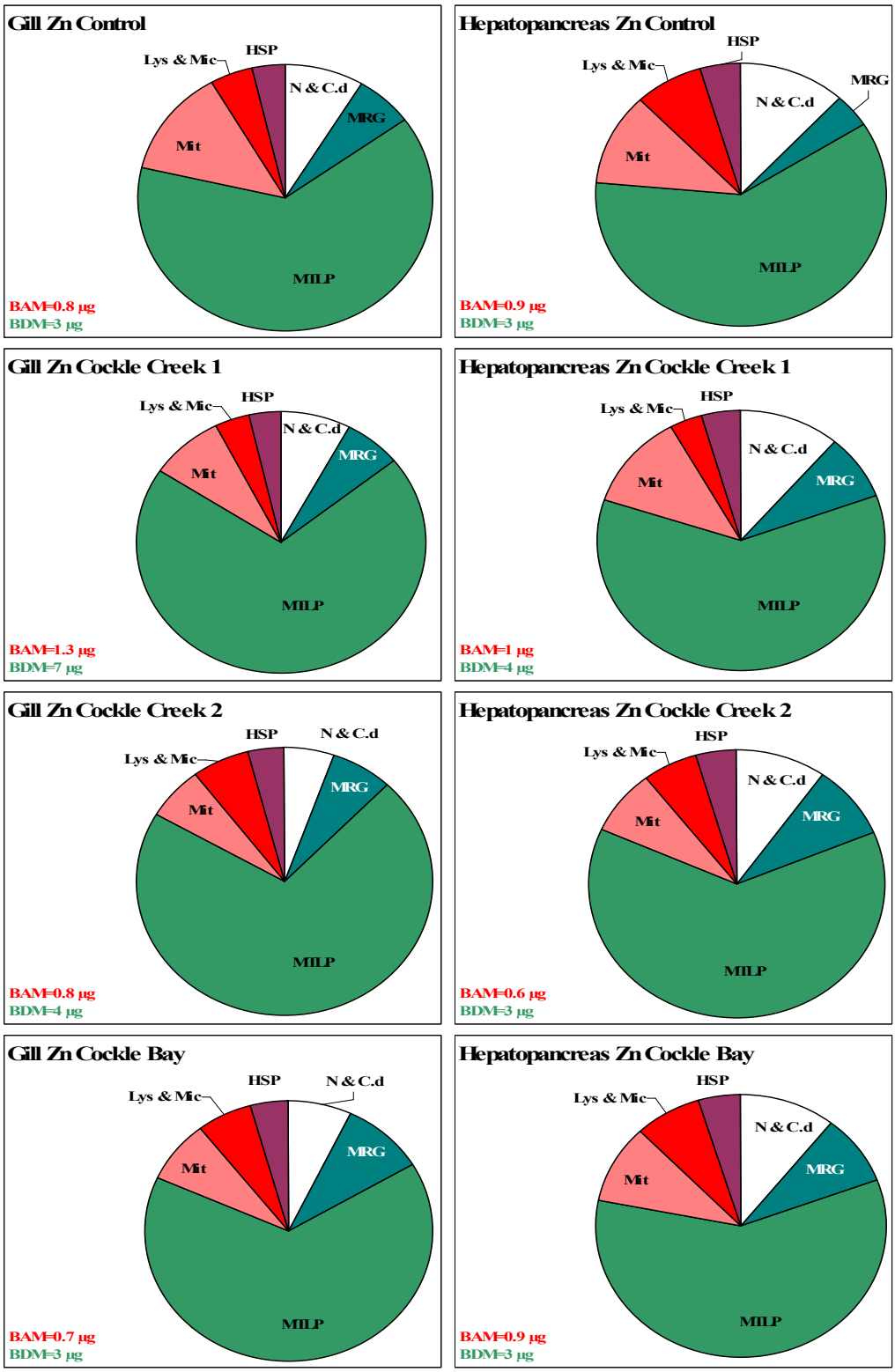


Figure 8.10: Distribution (%) of zinc in each of the subcellular fractions of *A. trapezia* gill and hepatopancreas tissues following 56 days of exposure to sediments from a metal contamination gradient in Lake Macquarie. Subcellular fractions are: nuclei + cellular debris (N & C.d); metal rich granules (MRG); heat stable metallothionein like proteins (MTLP); mitochondria (Mit); lysosomes + microsomes (Lys & Mic); heat sensitive proteins (HSP). Red fractions ( ) make up the biologically active metal (BAM), green fractions ( ) make up the biologically detoxified metal (BDM), n = 2.

8.3.3.5 *Copper*

Gill

Copper recoveries in the gill tissue fractions were between 68 and 100 % of the total copper burden (Table 8.2). As was seen for zinc, the majority of recovered copper was in the BDM. In the control organisms, 50 % of the copper was in the BDM fraction, this was followed by the BAM with 37 % and only 13 % was in the nuclei+cellular debris tissue fraction (Table 8.2). The organisms from the metal exposed sites had 67 to 72 % of their copper in the BDM fractions with the remainder fairly evenly distributed between the BAM and nuclei+cellular debris fraction (Table 8.2). There was very little difference in the copper burden recovered in the BAM fractions between sites (Table 8.2). The majority of the copper in the BDM was in the MTLP fraction for all sites except CC1 where it was evenly distributed between the MTLP and the MRG fraction (Table 8.3; Figure 8.11). The copper burden in the BAM fractions was greatest in the mitochondria followed by the lysosome+microsome fraction and the HSP for all sites except CC2 where there was slightly more copper in the lysosome+microsome fraction than the mitochondria (table 8.3; Figure 8.11).

Hepatopancreas

Copper recoveries in hepatopancreas tissues ranged between 58 and 100 % of the total copper (Table 8.2). The distribution pattern of recovered copper was the same as seen in the gills with the majority of copper in the BDM for all sites (Table 8.2). The control and CC1 organisms had a higher percentage of copper in the BAM fraction 32 and 29 % respectively than in the nuclei+cellular debris fraction while the CC2 and CB organisms had the remaining copper evenly distributed between the two (Table 8.2). There was very little difference in the copper burden recovered in the BAM fractions between sites (Table 8.2). The percentage distribution of copper between fractions within the BDM and BAM of the hepatopancreas tissues were the similar to those seen in the gills (Table 8.3; Figure 8.11).

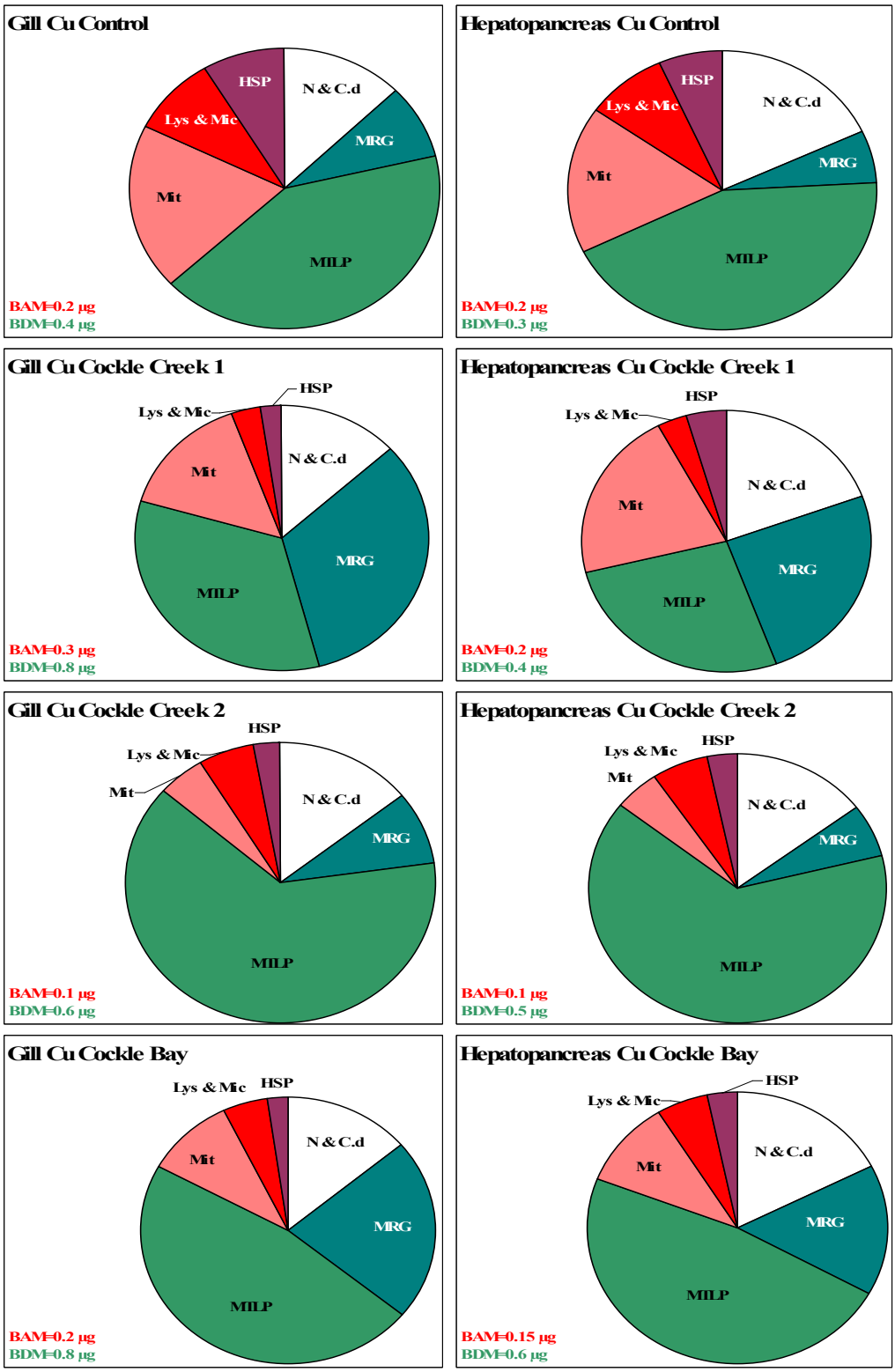


Figure 8.11: Distribution (%) of copper in each of the subcellular fractions of *A. trapezia* gill and hepatopancreas tissues following 56 days of exposure to sediments from a metal contamination gradient in Lake Macquarie. Subcellular fractions are: nuclei + cellular debris (N & C.d); metal rich granules (MRG); heat stable metallothionein like proteins (MTLP); mitochondria (Mit); lysosomes + microsomes (Lys & Mic); heat sensitive proteins (HSP). Red fractions ( ) make up the biologically active metal (BAM), green fractions ( ) make up the biologically detoxified metal (BDM), n = 2.

8.3.4 Activity of Marker Enzymes

8.3.4.1 *Whole Tissues*

The activity of the lysosomal enzyme, acid phosphatase (AP), in whole tissues were greater in the hepatopancreas than in the gill tissues for all sites (Figure 8.12). The mitochondrial enzyme, cytochrome c oxidase (CcO), activity was greater in the gill than in the hepatopancreas tissues for all sites (Figure 8.12). AP activity was reduced in the gills and increased in the hepatopancreas tissues of organisms from CC1 compared to the reference, CC2 and CB organisms (Figure 8.12). CcO activity was increased in the gill and hepatopancreas tissues of CC1, CC2 and CB organisms compared to the reference organisms (Figure 8.12).

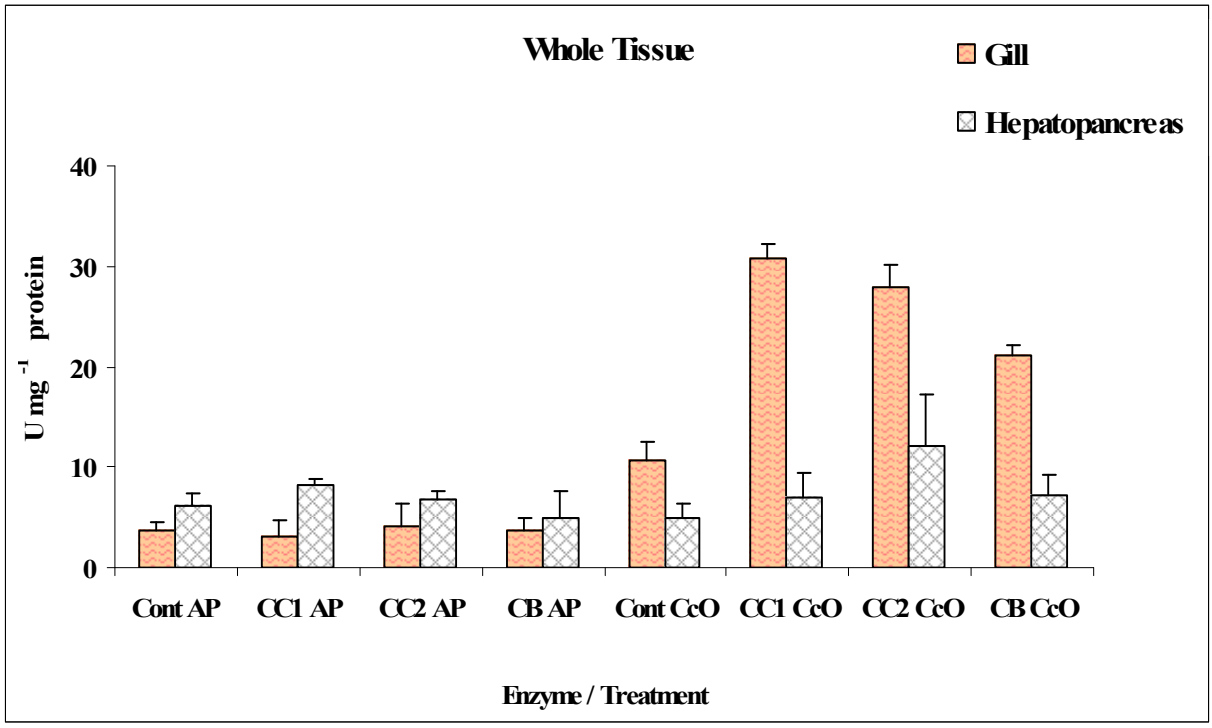


Figure 8.12: Specific marker enzyme activity, for lysosomes (acid phosphatase (AP)) and mitochondria (cytochrome c oxidase (CcO)) in whole gill and hepatopancreas tissue of *A. trapezia* exposed to sediments from a metal contamination gradient in Lake Macquarie for 56 days. Cont = control organisms, CC1 = Cockle Ck. Site 1, CC2 = Cockle Ck. Site 2, CB = Cockle Bay site. Mean ± SD, n = 2.

8.3.4.2 *Subcellular Fractions*

Gill and Hepatopancreas

Activity of lysosomal and mitochondrial marker enzymes AP and CcO in the subcellular fractions of gill and hepatopancreas tissues from all sites indicate that the CP 3 fractions of both tissues were enriched with mitochondria while the CP 4 fractions were enriched with lysosomes (Figure 8.13). There was some carry over of mitochondria into the CP 4 fractions and some lysosomal enzyme activity was present in the CP 3 fractions (Figure 8.13).

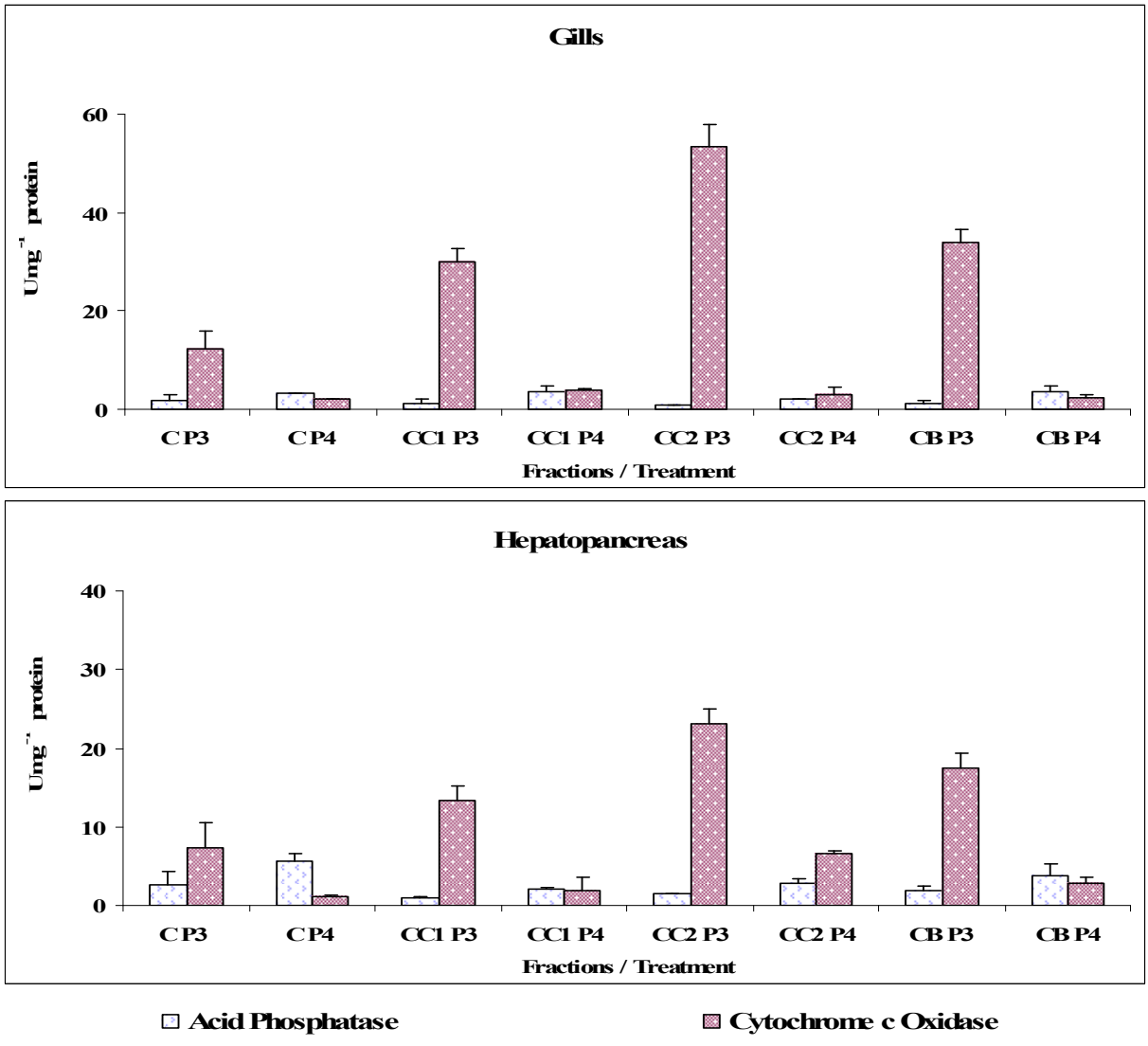


Figure 8.13: Specific marker enzyme activity, for lysosomes (acid phosphatase (AP)) and mitochondria (cytochrome c oxidase (CcO)) in the mitochondrial fraction (P3) and lysosomal+microsomal fraction (P4) following fractionation of gill and hepatopancreas tissue of *A. trapezia* exposed to sediments from a metal contamination gradient in Lake Macquarie for 56 days. C = control organisms, CC1 = Cockle Ck. Site 1, CC2 = Cockle Ck. Site 2, CB = Cockle Bay site. Mean ± SD, n = 2.

8.3.5 Enzymatic Biomarker – Total Antioxidant Capacity

The total antioxidant capacity (TAOC) was reduced in metal exposed *A. trapezia* compared to the reference organisms (Figure 8.14). ANOVA showed that site was significant in determining TAOC (Appendix 3.10). Pair-wise comparisons showed that the organisms from site CC1 had a significantly lower TAOC than the organisms from all other sites (Figure 8.14; Appendix 3.11). The TAOC of organisms from sites CC2, CB and LM native *A. trapezia* were significantly lower than the reference organisms and higher than the CC1 organisms but they were not significantly different from each other (Figure 8.14; Appendix 3.11). The regression analysis shows TAOC and tissue metal concentration had a significant negative relationship, the TAOC in organisms exposed for 56 days to metal contaminated sediments decreased as tissue metal concentration increased (Figure 8.14). The LM native *A. trapezia* did not fit this pattern; they had a lower TAOC capacity relative to their tissue metal concentration than the laboratory exposed organisms (Figure 8.14).

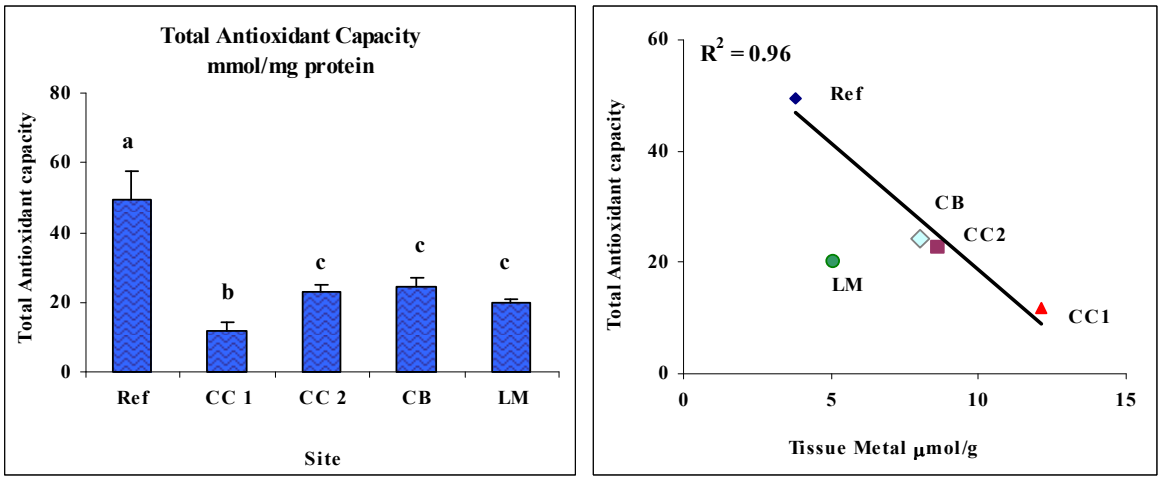


Figure 8.14: Activity of the antioxidant system of *A. trapezia* with associated regression of mean tissue metal (µmol/g) versus biomarker response, for Ref (◆),CB (◇), CC2 (■) and CC1 (▲). LM (●) is shown but not included in the regression. Following 56 days of exposure to sediments from a metal contamination gradient in Lake Macquarie. Mean ± SE, n = 9 (metal); n = 18 (TAOC). Different letters indicate significant differences between means (Bonferroni test; $p < 0.05$).

8.3.6 Oxidative Damage Biomarker – Thiobarbituric Acid Reactive Substances

Thiobarbituric acid reactive substances (TBARS) increased in *A. trapezia* with increased exposure to metals (Figure 8.15). ANOVA showed that site significantly influenced TBARS concentration (Appendix 3.10). Bonferroni pair-wise comparisons showed the CC1, CC2 and LM native organisms had significantly higher TBARS concentrations than the reference organisms but were not significantly different to each other or to organisms from the CB site (Figure 8.15; Appendix 3.11). While the TBARS concentrations of the CB organisms were not significantly higher than the reference organisms they were not significantly lower than the other metal exposed organisms (Figure 8.15; Appendix 3.11). The regression analysis shows TBARS concentrations and tissue metal concentrations had a significant positive relationship, the TBARS concentrations in organisms exposed for 56 days to metal contaminated sediments increased as tissue metal concentrations increased (Figure 8.15). The LM native *A. trapezia* did not fit this pattern; they had higher TBARS concentrations relative to their tissue metal concentrations than the laboratory exposed organisms (Figure 8.15).

8.3.7 Cellular Biomarker – Lysosomal Stability

The percentage of unstable lysosomes increased in organisms exposed to metal contaminated sediments compared to reference organisms (Figure 8.15). ANOVA showed site significantly influenced lysosomal stability (Appendix 3.10). Bonferroni pair-wise comparisons showed the CC1 and CC2 organisms had significantly more unstable lysosomes than the reference organisms but were not significantly different to each other (Figure 8.15; Appendix 3.11). The CC1 organisms had significantly more unstable lysosomes than the CB and LM native *A. trapezia* while the CC2 organisms had significantly more unstable lysosomes than the LM native *A. trapezia* but were not significantly different to the CB organisms (Figure 8.15; Appendix 3.11). The LM native A. *trapezia* did not have significantly more unstable lysosomes than the reference organisms or significantly less than the CB organisms but did have significantly lower lysosomal instability than the CC1 and CC2 organisms (Figure 8.15; Appendix 3.11).

The regression analysis shows lysosomal destabilisation and tissue metal concentration had a significant positive relationship, the percentage of unstable lysosomes in organisms exposed for 56 days to metal contaminated sediments increased as tissue metal concentration increased (Figure 8.15). The LM native *A. trapezia* also fitted this pattern (Figure 8.15).

8.3.8 Genotoxic Biomarker – Micronuclei Frequency

The frequency of micronuclei increased in organisms exposed to metal contaminated sediments compared to reference organisms (Figure 8.15). ANOVA showed site significantly influenced micronuclei frequency (Appendix 3.10). Bonferroni pair-wise comparisons showed the organisms from the metal exposure sites CC1, CC2 and CB all had significantly more micronuclei than the reference organisms and they were all significantly different to each other, with the CC1 > CC2 > CB percentage occurrence of micronuclei (Figure 8.15; Appendix 3.11). The regression analysis shows micronuclei frequency and tissue metal concentration had a significant positive relationship, the frequency of micronuclei in organisms exposed for 56 days to metal contaminated sediments increased as tissue metal concentrations increased (Figure 8.15). Micronuclei analysis was not done on LM native *A. trapezia.*

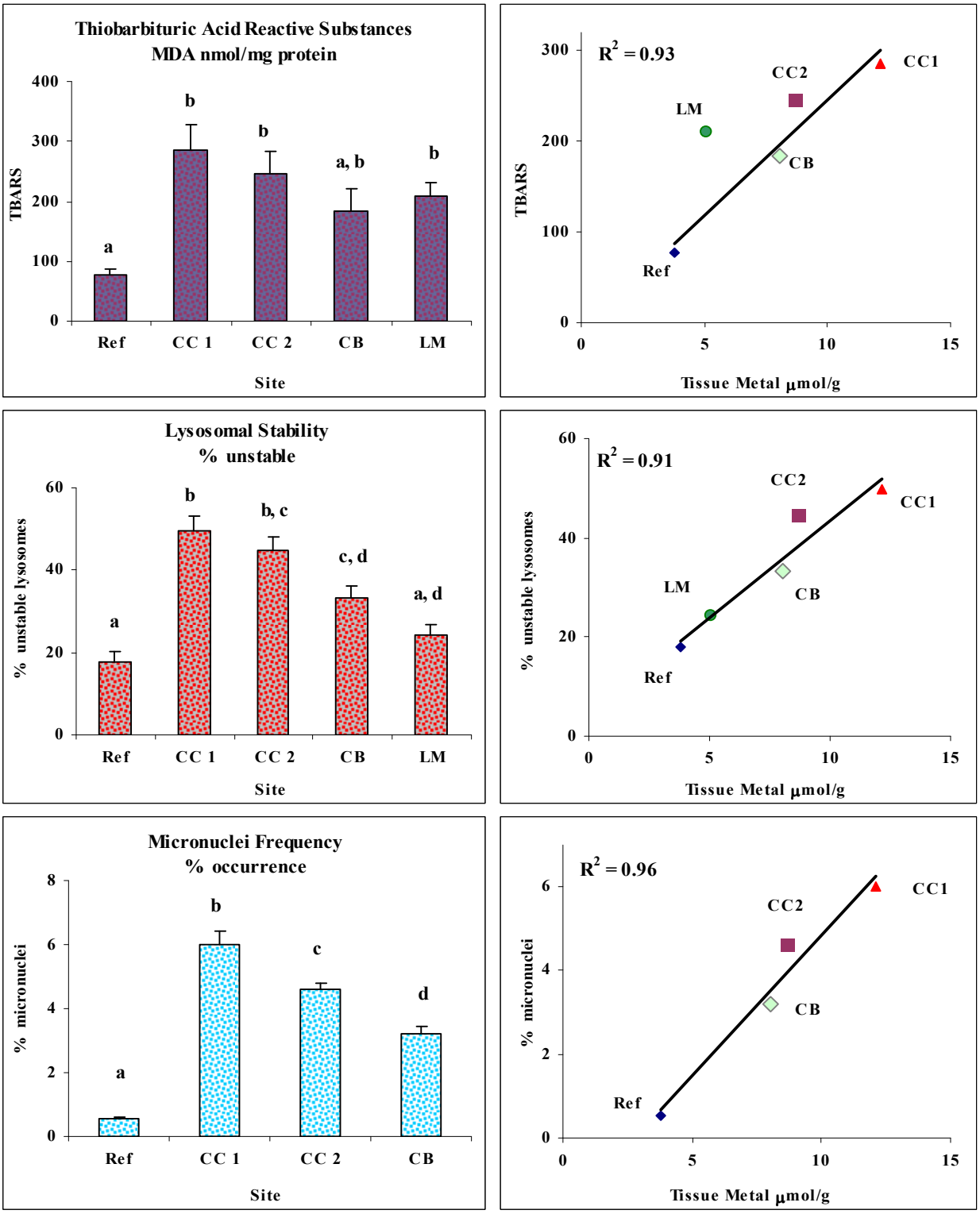


Figure 8.15: Changes in oxidative damage, cellular and genotoxic biomarkers of *A. trapezia* with associated regression of mean tissue metal (µmol/g) versus biomarker response for, Ref (◆), CB (◇), CC2 (■) and CC1 (▲). LM (●) is shown but not included in the regression. Following 56 days of exposure to sediments from a metal contamination gradient in Lake Macquarie. Mean ± SE, n = 9 (metal, lysosomal stability and micronuclei frequency); n = 18 (TBARS). Different letters indicate significant differences between means (Bonferroni test; $p < 0.05$).

8.3.9 Physiological Biomarker – Condition Index

Condition index was calculated for reference organisms, Lake Macquarie native *A. trapezia* and at fortnightly intervals for the CC1, CC2 and CB organisms (Figure 8.16). *A. trapezia* from the CC1, CC2 and CB exposures had higher or equal condition index ratios to the reference organisms on days 14 to 42. By day 56 the condition index of the CC1, CC2 and CB organisms was lower than the reference organisms (Figure 8.16). The LM native *A. trapezia* had a higher condition index ratio than the reference organisms, the CB organisms, the day 14 and 56 CC2 organisms and the day 56 CC1 organisms (Figure 8.16). ANOVA with Bonferroni pair-wise comparisons showed that the condition of the CC1 and CC2 *A. trapezia* was significantly poorer on day 56 than on day 28 when it was at its best, there were no other significant differences (Figure 8.16).

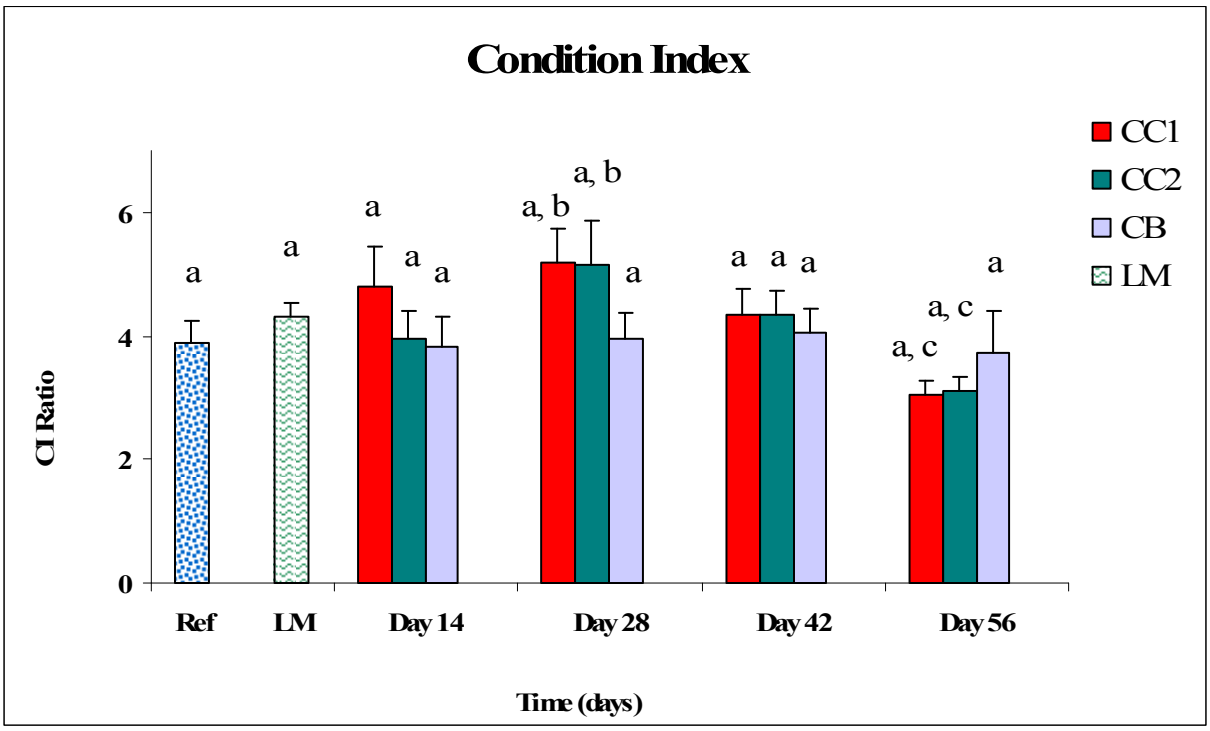


Figure 8.16: Condition index (CI) of *A. trapezia* at 2 weekly intervals over 56 days of exposure to sediments from a cadmium, lead, selenium, zinc and copper contamination gradient in Lake Macquarie. CC1 = Cockle Ck. Site 1, CC2 = Cockle Ck. Site 2, CB = Cockle Bay site. Condition index of unexposed organisms (Ref) and the Lake Macquarie native metal exposed *A. trapezia* (LM) are also shown. Mean ± SE, n = 9. Different letters indicate significant differences between means (Bonferroni test; $p < 0.05$).

8.3.10 Relationship between Biomarkers

8.3.10.1 *Enzymatic with Oxidative Damage, Cellular and Genotoxic Effects*

Regression analysis shows that TAOC within cells had significant negative relationships with the effects indices TBARS, lysosomal destabilisation and micronuclei frequency (Figure 8.17). As sediment metal exposure increased, organism capacity to neutralise reactive oxygen species was reduced with a consequent increase in cell damage. The LM native *A. trapezia,* which are shown to enable comparison with the 56 day laboratory exposed organisms but not included in the regression analysis, fitted the pattern for TBARS but not that for lysosomes which had lower instability than the laboratory exposed organisms for the TAOC reduction measured (Figure 8.17).

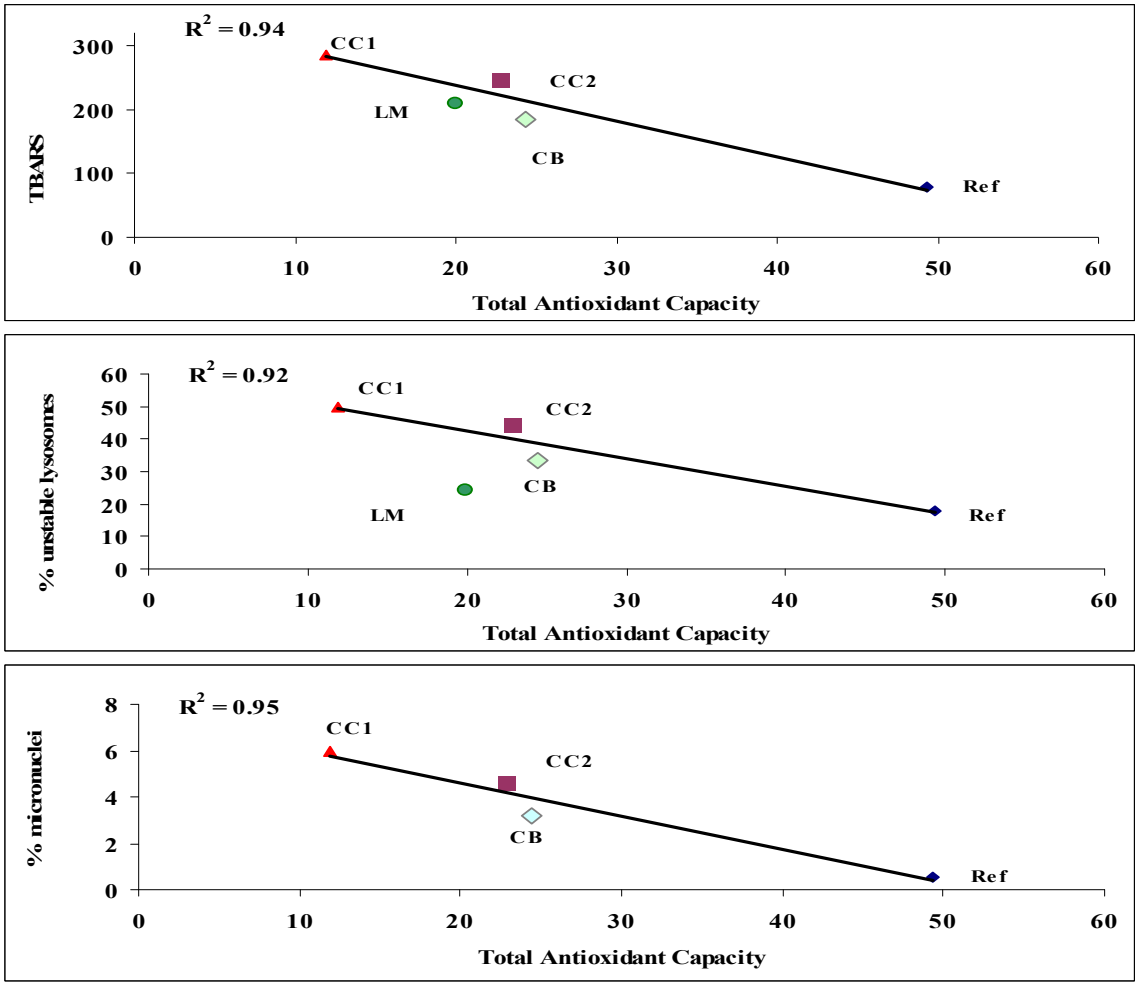


Figure 8.17: Regressions of site means of total antioxidant capacity with biomarker responses, TBARS, lysosomal stability and micronuclei frequency for Ref (◆), CB (◇), CC2 (■) and CC1 (▲). LM (●) is shown but not included in the regressions. Following 56 days of exposure to sediments from a metal contamination gradient in Lake Macquarie. Mean n = 9 (lysosomal stability, micronucli frequency; n = 18 (TAOC, TBARS).

8.3.10.2 *Oxidative Damage with Cellular and Genotoxic Effects*

The regression analysis shows a significant positive relationship between TBARS concentration and the effects indices lysosomal stability and micronuclei frequency (Figure 8.18). As metal exposure increased, the peroxidation of lipids increased, damage to lysosomal membranes increased and the frequency of genotoxic damage to nuclei increased (Figure 8.18). The LM native *A. trapezia* shown do not fit the pattern for lysosomal destabilisation which was lower than that of the laboratory exposed organisms for the TBARS concentration measured (Figure 8.18).

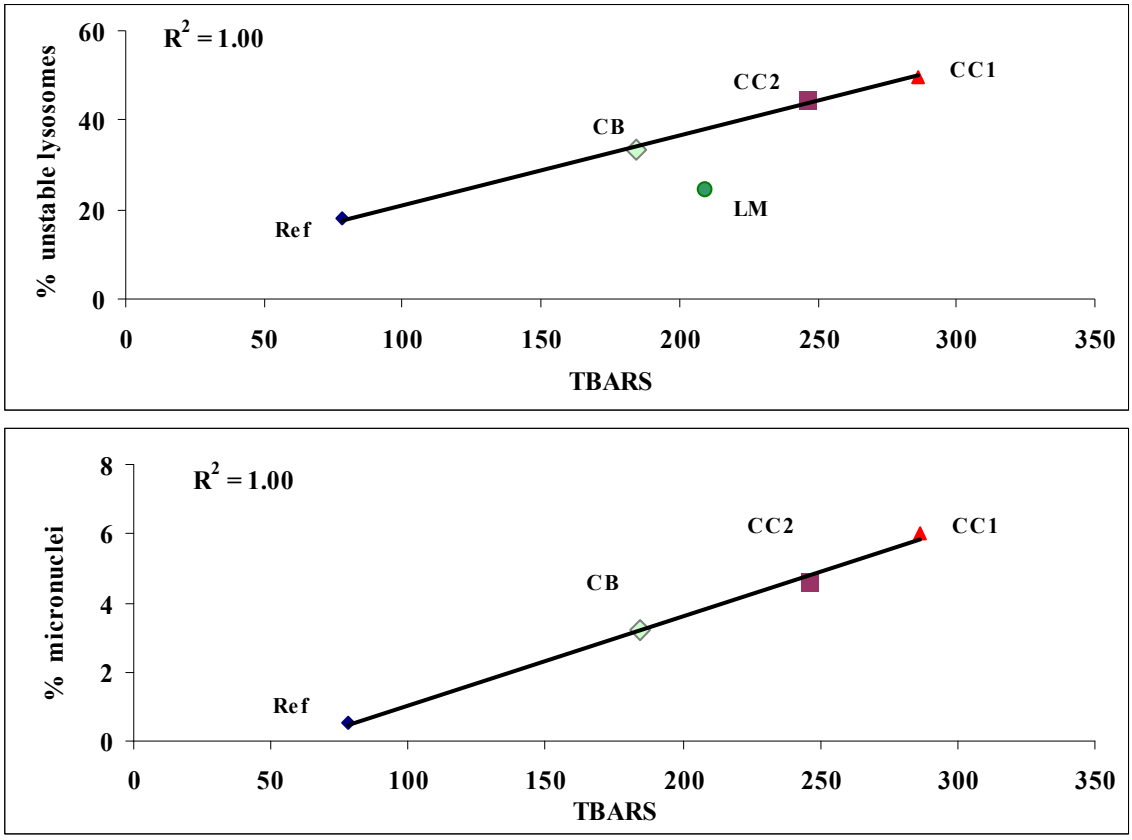


Figure 8.18: Regressions of site means of TBARS and biomarker responses, lysosomal stability and micronuclei frequency for Ref (◆), CB (◇), CC2 (■) and CC1 (▲). LM (●) is shown but not included in the regressions. Following 56 days of exposure to sediments from a metal contamination gradient in Lake Macquarie. Mean n = 9 (lysosomal stability, micronucli frequency; n = 18 (TBARS).

8.4 Discussion

8.4.1 Sediment Metal Gradient

A clear metal contamination gradient, decreasing in concentration from the zinc/lead smelter drainage inflow point into Cockle Creek down the Creek and into Cockle Bay was measured in the sediments collected from sites CC1, CC2 and CB (Figures 3.3 & 8.1; Table 8.1). Metal concentrations were in the order; zinc > lead > copper > cadmium > selenium for the CC1, CB and LM sites (Figure 8.1; Table 8.1). The CC2 site had slightly higher cadmium than copper concentrations but otherwise also fitted this metal concentration pattern (Figure 8.1; Table 8.1). Positive correlations between the metals at sites CC1, CC2 and CB suggests that the metals originated from a common source. Metals from point sources such as the zinc/lead smelter are generally mobilised by runoff during rain and enter estuarine environments either dissolved in water or adsorbed to chelating agents, primarily clay particles and organic matter. Once runoff enters estuarine waters salinity influences complexation causing flocculation of particles which then precipitate out and accumulate in benthic sediments (Du Laing *et al.*, 2009). The stability of metal complexes and the movement of metals into and out of sediments is influenced by the sediment geochemistry, such as the presence of acid-volatile sulphides which form stable metal sulphide precipitates in sediments (Lee *et al.*, 2000) and oxidation of sediments which alters the metal redox state (Simpson *et al.*, 2004). A portion of the metals complexed to sediments and organic matter are moved down the Creek into the Bay and redeposited over time, particularly during high flow generated during storms where sediments are actively resuspended and transported and anoxic sediments are reoxidised releasing metals into the water column (Du Laing *et al.*, 2007). The CC2 site situated where Cockle Creek enters the Bay is a point at which flow is slowed as the Creek channel widens. Sediments are deposited here, while the CB site which is across the Bay from the Creek mouth, captures the remaining fine sediments as the flow moves across the Bay and around the small peninsular (Figure 3.3). These three sites, therefore, show a decreasing metal concentration gradient from the metal entry point at CC1, down the Creek to the Bay entrance at CC2 and across the Bay to CB (Figures 3.3 & 8.1). The inflow site from the zinc/lead smelter, CC1, had significantly higher concentrations of lead selenium zinc and copper than both the downstream sites and significantly higher cadmium than the CB site, while only lead and selenium were significantly higher at the CC2 site than the CB site (Appendix 3.2).

This suggests that the mobility of the individual metals is different and this is most likely related to the size of the particles the metals are complexed to, the stability of the various metal complexes and metal solubility. Metals bound to finer particles for example would be carried further than those bound to coarser ones, which may explain why the concentrations of copper measured in the CC2 site sediments was lower than that found at the CB site (Figure 8.1). Although the < 63 µm fraction of the sediments at the CB sites was lower than at the CC2 site, those fine particles which were carried from the Creek and deposited across the Bay may have been largely organic complexes which are important in copper transportation (Du Laing *et al.*, 2009). A change in redox state resulting from oxidation can alter pH which in turn affects mobility of metals such as zinc, lead and cadmium (Du Laing *et al.*, 2009; Simpson *et al.*, 2004). Metals such as cadmium can be mobilised as chloride complexes, cadmium and zinc are carried by iron and manganese hydroxides, while an increase in salinity increases the major cations which compete with these metals for binding sites(Du Laing *et al.*, 2009). The LM site which is in an embayment quite close to the CB site had significantly higher concentrations of zinc lead and copper than the CC2 and CB sites and significantly higher selenium and equal cadmium concentrations to the nearby CB site (Appendix 3.2; Figure 3.3). This site is in a narrow bay, which while it is situated to capture the sediment laden inflow from Cockle Creek has minimal flushing, so metals which build up over time are not readily removed. Lower flushing may also lead to increased anoxia resulting in reduction and changes in complexation of copper, zinc, cadmium and lead as well as fluctuations in salinity which affect metal mobility. The LM site also has seagrass beds which stabilise sediments, and take up and cycle metals within the ecosystem. The < 63 µm fraction of the sediments at the LM site was 15 % (mass/mass) which was higher than at the other three sites. As metals are largely bound to this sediment fraction the elevated metal concentrations at this site compared the CC2 and CB sites may also be related to the sediment composition. The very high concentrations of zinc measured in the sediments collected at the CC1 and LM sites compared to those at the CB site and previous studies in Cockle Bay by Burt *et al*, (2007) and Roach (2005) (Table 8.1) could have resulted from newly deposited sediment following extreme flooding in the weeks prior to sampling. With the exception of zinc at these two sites, the sediment metal concentrations measured in this study are generally within the range (Table 8.1) of concentrations recorded in previous studies of Cockle Bay (Batley, 1987; Burt *et al.*, 2007; Roach, 2005).

Previous studies have also established the zinc/lead smelter as a point source of cadmium, lead, zinc and copper to the Lake (Batley, 1987; Roach, 2005), and power generation by coal fired power stations as a source of selenium (Carroll, 1996; Peters *et al.*, 1999a). The concentrations of cadmium and zinc at all the sites sampled in Lake Macquarie exceeded the ANZECC and ARMCANZ (2000) high interim sediment guideline concentrations, while lead exceeded the high value at the two Cockle Creek and LM sites and copper exceeded the low value at the CC1 and LM sites (Table 8.1). Exposure of *A. trapezia* to these sediments is likely to result in significant tissue metal accumulation and impairment at the cellular and subcellular level.

8.4.2 Tissue Metal Accumulation and Subcellular Distribution

8.4.2.1 *Whole Organism Metal Accumulation*

Total Tissue Metal

The relationship between sediment and final tissue metal concentrations across the three sites is positive but non-linear, as sediment metal increased tissue metal also increased but not proportionally (Figure 8.3a). Tissue metal concentrations were below ambient sediment metal concentrations in *A. trapezia* from all exposures (Figure 8.3a). This is most likely due to cellular binding sites for metals being saturated and therefore limiting the rate at which metal can be accumulated, detoxified and stored or excreted. Saturation of binding sites and a subsequent loss of linearity in zinc uptake with increased zinc exposure concentrations has been observed in *Littorina littorea* (Simkiss and Mason, 1984). It may also be indicative of antagonistic metal:metal interactions limiting individual metal bioavailability (Moolman *et al*., 2007). Alternatively it may reflect a high proportion of the sediment metal not being bioavailable to *A. trapezia*. The native *A. trapezia* did not fit the sediment tissue metal regression, having far lower tissue metal concentrations for the sediment exposure concentrations than the laboratory exposed *A. trapezia* (Figure 8.3a). This suggests an adaptive response by the chronically exposed organisms and this appears to be for the essential metals zinc, copper and selenium but not for cadmium and lead (Figure 8.3 b-f). There were strong positive relationships between tissue metal concentrations for all metals except copper with lead and cadmium which suggests coaccumulation of most metals via common pathways (Figure 8.4). Burt *et al,* (2007) also found positive tissue metal relationships for zinc with cadmium and lead and between cadmium and lead for *A. trapezia* after 90 days of exposure to mixed metal contaminated sediments in Lake Macquarie.

Cadmium

Cadmium concentrations increased steadily over the 56 day exposure in tissues of *A. trapezia* exposed to sediments from each of the three sites (Figure 8.2). Tissue cadmium concentrations on day 56 showed a positive relationship with sediment cadmium concentrations although the relationship was not proportional (Figure 8.3b). Final tissue cadmium concentrations of the CC1 exposed organisms were about one eigth of the sediment concentration, the CC2 organisms reached tissue cadmium concentrations which were one third of the sediment cadmium concentration while the CB organisms had two fifths the sediment concentration (Figure 8.3b). The LM *A. trapezia* collected from Lake Macquarie had the same sediment cadmium exposure as the CB organisms but had a tissue cadmium concentration which was three fifths their exposure concentration (Figure 8.3b). While the organisms exposed in the laboratory to the Lake Macquarie sediments had a similar pattern of steady cadmium accumulation over time to that observed in the cadmium spiked sediment experiment, the final cadmium tissue to sediment concentration in the Lake Macquarie sediment exposed organisms was lower (Figures 5.1 & 8.2). The difference is probably related to both differences in physiochemical sediment characteristics altering cadmium bioavailability and competition for cellular binding sites with the other metals present in the Lake Macquarie sediments. Since cadmium and zinc have similar physicochemical properties, both can be biologically antagonistic (Moolman *et al.*, 2007). Competitive interactions between cadmium and zinc for example have been shown to reduce cadmium accumulation in the marine bivalves *Mytilus edulis & galloprovincialis* and *Mulina lateralis* (Simkiss and Mason, 1984). The cadmium accumulation observed in this experiment supports the view that *A. trapezia* are net accumulators of cadmium.

Lead

Lead tissue accumulation was continuous in organisms exposed to sediments from each of the sites throughout the 56 days of exposure (Figure 8.2). Lead tissue concentrations on day 56 of the exposure showed a positive relationship with sediment lead concentrations which was proportional across the sites (Figure 8.3c). Final lead tissue concentrations were only one hundredth the sediment lead concentrations in organisms from the CC1 and CB sediments and were one fiftieth the exposure concentration in the CC2 sediment exposed organisms (Figure 8.3c). The LM organisms did not fit the regression, as they had a lower tissue lead concentration relative to their exposure concentration than the laboratory exposed organisms (Figure 8.3c).

The *A. trapezia* exposed to the Lake Macquarie sediments, like those exposed in the laboratory to lead spiked sediments, had similarly low final lead tissue concentrations, relative to the exposure concentrations, of between one fiftieth and one hundredth the exposure concentration after 56 days of exposure (Figures 8.2 & 6.1). While there are differences in the final tissue to sediment concentrations between the lead spiked, mixed metal and native exposed organisms the pattern of lower than ambient lead concentrations in *A. trapezia* tissues is consistent and has previously been observed (Burt *et al.*, 2007). This suggests that *A. trapezia* have some ability to regulate lead, although the lead spiked sediment study indicates that in certain situations the regulatory systems may become overwhelmed. As the zinc concentrations in the Lake Macquarie sediments were high, competition for cellular binding sites between zinc and lead may have resulted in preferential binding of zinc thereby reduced total bound lead. The low lead tissue concentrations observed in *A. trapezia* may also be due to low lead bioavailability in the sediments.

Selenium

Selenium tissue concentrations increased between the 14th and 28th day of the exposure and did not vary much at any site after this (Figure 8.2). The final tissue selenium concentration of the CC1 organisms was not much greater than that of the organisms from the other two sites despite the higher selenium sediment concentration (Figures 8.1 & 8.2). The regression shows a positive relationship between tissue and sediment selenium but the relationship is poor (Figure 8.3d). The selenium tissue accumulation in the organisms from all the Lake Macquarie sediment laboratory exposures was considerably higher, between 23 and 1.2 times, than that of the exposure concentration. The LM organisms had lower selenium tissue concentrations for the selenium sediment exposure than the laboratory exposed organisms but this was still twice that of the sediments. The selenium spiked sediment exposed *A. trapezia* on the other hand had selenium tissue concentrations which were only half to two fifths that of the exposure concentrations (Figure 7.2). There appears to be no pattern in selenium tissue accumulation between the *A. trapezia* from the different selenium sediment exposures, however, the results of the selenium spiked and Lake Macquarie sediment laboratory exposures do seem to indicate that *A. trapezia* has the ability to partially regulate selenium accumulation. Despite selenium sediment concentrations which ranged from 0.2 to 20 µg/g (mean 5.5 ± 7 µg/g) across all of the exposures the range of tissue concentrations among organisms after 56 days of exposure was only 1.4 to 6.3 µg/g (mean 4 ± 2 µg/g) (Figures 7.2 & 8.2; Table 8.1).

The low absolute value of the regression coefficient (Figure 8.3d) lends further support to this conclusion, although the range of sedimentary values in this experiment is low. These results are somewhat at odds with Jolley *et al.* (2004) who found *A. trapezia* from a single population were able to maintain almost constant internal selenium concentrations which co-varied with the sediment concentration. Burt *et al*. (2007) also found that *A. trapezia* reached an equilibrium selenium tissue concentration after 60 days exposure in Lake Macquarie, which was lower than the sediment selenium concentration. Peters *et al*., (1999b) and Barwick and Maher (2003) on the other hand both found native *A. trapezia* from Lake Macquarie NSW had selenium tissue concentrations up to 6 times their selenium exposure concentration, however, as was seen in the sediment exposure experiments I conducted the ratio of tissue selenium to sediment exposure reduced with increasing exposure concentration (Figures 7.2 & 8.2; Table 8.1). The exposure route is likely to be the significant influence in the selenium accumulation observed. As selenium accumulation is primarily from food (Hamilton, 2002; Luoma and Rainbow, 2005) the availability of different food sources and the rate at which they assimilate selenium will affect the apparent bioavailability of selenium to *A. trapezia.* Luoma *et al,* (1992) for example found the estuarine benthic bivalve *Macoma balthica* assimilated selenium with 86 % efficiency from diatoms, 22 % efficiency from sediments and selenite was taken up from solution slowly. The higher than ambient selenium tissue concentrations in some of the laboratory exposures compared to those observed in field exposure experiments (Barwick and Maher, 2003; Burt *et al.*, 2007; Jolley *et al.*, 2004; Peters *et al.*, 1999b) probably relate to increased exposure via food in a closed system with only weekly half water changes compared to constant flushing in an open water environment.

Zinc

Organisms from all sites had significant zinc accumulation over the exposure period (Figure 8.2; Appendix 3.5). The regression shows a positive relationship between tissue and sediment zinc concentrations for the laboratory exposed *A. trapezia* with final zinc tissue concentrations between one fifth and one tenth of the sediment concentration (Figure 8.3e). The LM native *A. trapezia* did not fit the regression having far lower zinc tissue concentrations for the zinc sediment exposure than the laboratory exposed *A. trapezia* (Figure 8.3e). This could be due to an adaptive response by the native population following chronic exposure over generations to high concentrations of zinc. Alternatively, the very high concentrations measured in the sediments collected at the CC1 and LM sites, compared to those at the CB site and in previous studies in Cockle Bay by Burt *et al*, (2007) and Roach

(2005) (Table 8.1), could have resulted from newly deposited sediment following extreme flooding in the weeks prior to sampling.

Thus the zinc sediment exposure history of these organisms might not be accurately reflected in the sediment concentrations measured at the time of collection. Even if this were the case, however, the sediment concentrations measured in previous studies of Cockle Bay were similar to those at the CC2 and CB sites in this study (Table 8.1) but the zinc tissue concentrations of the resident *A. trapezia* population were still well below those measured in the organisms exposed to the CC2 and CB sediments in the laboratory and closer to those of the reference organisms (Figure 8.3e). Zinc tissue concentrations, including those of the reference organisms, exposed in this experiment were similar to that of the *A. trapezia* exposed to elevated sediment zinc concentrations in Lake Macquarie for 90 days by Burt *et al*, (2007). The differences in total zinc concentration observed between previously unexposed *A. trapezia* and the native *A. trapezia* exposed to similar zinc concentrations may be due to population genetic differences, as previously observed by Burt *et al*., (2007), in *A. trapezia* from different locations, varying bioavailability of zinc in the different sediments or an antagonistic interaction with cadmium and or lead (Moolman *et al.*, 2007). The lower than ambient tissue zinc concentrations observed in *A. trapezia* in these experiments and that of Burt *et al*., (2007) suggest that they have the ability to partially regulate zinc tissue concentrations.

Copper

There was no significant difference in the copper tissue concentrations of the laboratory exposed *A. trapezia* among the sites despite the CC1 sediments having four times more copper than the other two sites (Figure 8.2; Table 8.1; Appendix 3.4). Copper tissue concentrations increased over time in organisms from all sites, to a concentration which was significantly higher than the reference organism tissue concentrations by day 42 (Figure 8.2; Appendix 3.5). Organisms from all sites had a decrease in tissue copper concentration between day 42 and 56 (Figure 8.2). This suggests there is a regulatory response to copper exposure in *A. trapezia.* The tissue sediment copper concentration regression was not significant, and as was the case with zinc, the resident *A. trapezia* had far lower copper tissue concentrations for the copper sediment exposure than the laboratory exposed *A. trapezia* and did not have significantly more copper than the reference organisms (Figure 8.3f). The chronically exposed *A. trapezia* appear to have a well developed regulatory response to copper accumulation compared to the organisms with no previous copper exposure.

8.4.2.2 *Individual Tissue Metal Accumulation*

There were significant differences in metal accumulation between tissues for all metals at all sites (Figures 8.5; 8.5; Appendix 3.6). In general the distribution of cadmium between tissues was gill > hepatopancreas > haemolymph (Figure 8.4).

This is the same as the cadmium distribution between tissues observed in the cadmium spiked sediment laboratory exposures (Figure 5.3; Section 5.3.1.2). Lead distribution between tissues was generally gill > haemolymph > hepatopancreas (Figure 8.5) which was also the case in the lead spiked laboratory exposed *A. trapezia* (Figure 6.3; Section 6.3.1.2). The distribution of selenium was fairly even between the tissues. The gill tissue tended to dominate after 42 days at the CC1 and CC2 sites which had higher selenium sediment concentrations (Figure 8.5). This is in agreement with what was seen in the selenium spiked sediment experiments (Figure 7.3; Section 7.3.1.2). At the lower selenium sediment exposure of the CB site the hepatopancreas had the highest selenium concentrations throughout the exposure period (Figure 8.5). The sediment selenium concentration at this site was only one tenth that of the CC2 site but the total selenium tissue concentrations of the CB organisms were equal to those of the CC2 organisms (Figure 8.2). The high selenium concentrations in the hepatopancreas tissues compared to the gill and haemolymph of the CB organisms may point to a higher dietary input of selenium for these organisms than for those from the other exposures. The distribution of zinc generally followed the pattern gill > hepatopancreas ≥ haemolymph (Figure 8.6). Extracellular binding of zinc to mucus in bivalve gills is thought to promote epithelial uptake by trapping and concentrating the metal thereby establishing a diffusion gradient to favour zinc uptake with rapid transport across the epithelia into the blood (Marigómez *et al.*, 2002). Copper was fairly evenly distributed between the gill and the haemolymph with the haemolymph tending to have higher concentrations for the first 28 days of the exposure and the gill higher after this while the hepatopancreas had the lowest copper concentrations throughout the exposure period (Figure 8.6). The high concentrations in the haemolymph suggests binding of copper to haemoglobin which is a respiratory pigment in *A. trapezia* (Sullivan, 1961). An increase in dissolved copper concentrations resulting from bioturbation induced oxidation of bound copper could explain the higher copper concentrations in the gills relative to the other tissues in the later part of the exposure. The lower copper concentration in the hepatopancreas relative to the other tissues tends to support dissolved copper as the dominant source of absorbed copper.

Bonneris *et al.* (2005) found gill tissue copper concentrations of the freshwater bivalve *Pyganodon grandis* from contaminated lakes were always higher than the hepatopancreas tissue concentrations but a positive relationship with tissue copper and ambient dissolved copper was only established for the hepatopancreas.

8.4.2.3 *Subcellular Tissue Metal Distribution*

Cadmium

There were no differences in the percentage subcellular distribution of cadmium in the organisms exposed to mixed metal contaminated sediments to those exposed to cadmium spiked sediments (Figures 5.4 & 8.7). Roughly three quarters of the recovered cadmium in the gill and hepatopancreas of the metal exposed organisms was in the BDM fractions indicating active cadmium detoxification (Table 8.2). Of this the majority was in the MTLP fraction with very little in the MRG fraction (Figure 8.7). The induction of MTLP by bivalves in response to cadmium exposure has been widely reported (Bebianno *et al.*, 1994; Chan *et al.*, 2002; Giguere *et al.*, 2003; Roesijadi, 1996). The proportion in the nuclei+cellular debris fraction was slightly greater in the hepatopancreas than the gills (Figure 8.7). A certain amount of the cadmium in the hepatopancreas tissue would be derived from food and particulate matter and therefore the cadmium assimilation process may be different resulting in more cadmium being bound to cell walls in this tissue. The cadmium burden in the BAM fractions of the metal exposed organisms was 5 to 15 times greater than that of the control organisms (Figure 8.7). About half of the BAM was in the mitochondrial fraction with the remainder fairly evenly distributed between the HSP and lysosome+microsome fractions (Table 8.3). The large percentage of cadmium in the mitochondria has implications for toxicity. The activity of the mitochondrial enzyme cytochrome c oxidase was increased in the tissues of the metal exposed organisms (Figure 8.12). Increased activity of this enzyme was also seen in the cadmium, lead and selenium spiked sediment exposed *A. trapezia* and has been observed in the liver of cadmium exposed mice associated with mitochondrial perturbations (Li *et al.*, 2003). Increased mitochondrial cadmium with increased tissue burdens has been observed in freshwater bivalves (Bonneris *et al.*, 2005) and oysters (Sokolova *et al.*, 2005a). Small increases in mitochondrial cadmium have been found to lead to impairment of mitochondrial processes in oysters (Sokolova *et al.*, 2004).

Lead

The majority, 57 – 73 %, of the recovered lead in the metal exposed organisms was in the BDM fractions, twice the percentage recovered in the control organisms indicating effective detoxification of lead was occurring (Table 8.3). Very little of the BDM was in the MRG fraction with the majority in the MTLP fraction of both tissues from organisms from all treatments (Table 8.3). This was different to that found in the lead spiked sediment exposed organisms. While the total percentage of lead in BDM fraction of lead spiked sediment exposed organisms was similar to that of the mixed metal exposed organisms the distribution of lead was more even between the MRG and MTLP fractions (Table 6.2). As discussed previously (Section 6.3.1.3) while it is presumed that MTLP bind and transport lead, no specific protein for molluscs has so far been described. The lead burden in the BAM fractions of the metal exposed organisms was 1.5 to 10 times greater than that of the control organisms (Figure 8.8). Around half of the BAM lead was in the mitochondria of the CC2 and CB organisms while the CC1 organisms which were exposed to the highest sediment lead concentrations had 65 % in the gill and 76 % in the hepatopancreas tissue in the mitochondria (Table 8.3). The pattern of increasing lead in the mitochondria with increased lead exposure is similar to that seen in the lead spiked sediment exposed organisms (Table 6.2) and indicates that lead detoxification is incomplete and the potential for lead toxicity is present.

Selenium

The subcellular distribution of selenium in the organisms exposed in the mixed metal experiment was different to that seen in the selenium spiked sediment experiments (Figures 7.4 & 8.9). The percentage of selenium recovered in the nuclei+cellular debris fraction of the mixed metal exposed organisms like the selenium spiked sediment organisms was greater than that of the controls, however, the percentage increase was not as great (Tables 7.1 & 8.3). The reasons for this are probably two fold; with the exception of the CC1 exposed organisms the selenium exposure concentrations were lower in the mixed metal exposure, and this with the synergistic and or antagonistic effects of the other metals may have influenced uptake and handling strategies. As was seen in the selenium spiked sediment exposed organisms, the selenium burden in the BDM fractions of the mixed metal exposed organisms was about twice that of the control organisms in both tissues (Figures 7.4 & 8.9). The distribution differed, however, as the entire amount in the mixed metal exposed organisms was in the MRG fraction with none in the MTLP fraction (Figures 7.4 & 8.9).

As the selenium burden of the metal exposed organisms increased in this fraction compared to the control organisms it appears that selenium detoxification was occurring. Selenium associated with MRG has not been reported for marine molluscs. Sulphur binding of selenium is a possible pathway for incorporation into MRG structures as sulphur is known to be a component of zinc and cadmium granules in *M. edulis* (George, 1983a). Unlike the selenium spiked sediment exposed organisms, which had higher selenium burdens than the control organisms in the BAM fraction of both tissues, the selenium burden in the BAM fraction of the gill tissues of the mixed metal exposed organisms was equal to or lower than the control organisms and in the hepatopancreas tissues were equal to or only slightly higher than the control organisms (Figures 7.4 & 8.9). While the BAM selenium burden was lower the percentage distribution of selenium in the BAM fractions of the mixed metal exposed organisms was similar to that seen in organisms from the selenium spiked sediment exposures (Tables 7.1 & 8.3). The BAM selenium subcellular distribution was not different to the controls and as the selenium burdens were not all that different either it suggests that selenium detoxification was effective in the mixed metal exposed organisms and the BAM measured was probably not above metabolic requirements.

Zinc

The subcellular distribution pattern of zinc in the gill and hepatopancreas tissues was similar (Table 8.2; Figure 8.10). The majority 64 – 78 % of zinc was in the BDM fraction in all treatments with 87 – 94 % of this in the MTLP fraction (Table 8.3; Figure 8.10). While the percentage distribution of zinc did not change much the total BDM zinc burden increased with increased zinc exposure, particularly in the gills, indicating zinc detoxification was occurring (Table 8.2; Figure 8.10). Zinc increased in the MTLP fraction and decreased in the MRG fraction with increased zinc exposure (Table 8.3). This is in contrast to the zinc burden in the gills of the freshwater bivalve *Pyganodon grandis* which increased in the MRG fraction with increased zinc exposure while zinc in the MTLP remained relatively constant (Bonneris *et al.*, 2005). The bivalves in the Bonneris *et al*. (2005) study were chronically exposed organisms collected from contaminated lakes rather than the short term exposures conducted here which may account for the different patterns of detoxification observed. The percentage of zinc in the BAM of the metal exposed *A. trapezia* was slightly lower than in the control organisms of both tissues with the hepatopancreas having slightly higher BAM zinc than the gills (Table 8.3; Figure 8.10). The BAM zinc burden of the exposed organisms was not different to that of the controls (Table 8.2; Figure 8.10).

The marine bivalves *Macoma balthica* and *Potamocorbula amurensis* exposed for 14 days to dissolved zinc and cadmium both partitioned zinc differently to each other and to the *A. trapezia* in this experiment (Wallace *et al.*, 2003). Only a small proportion of the zinc was in the BDM fractions of *M. balthica* and *P. amurensis*. *M. balthica* had the majority of accumulated zinc in the nuclei+cellular debris fraction while *P. amurensis* had the highest percentage in the BAM fractions. The exposure route in the Wallace *et al.* (2003) study being entirely from dissolved metal, was markedly different to this study which included both particulate and food zinc exposure, this with the shorter exposure time may account for the lower BDM observed by Wallace *et al.* (2003). The differences between these three species highlights the different metal handling strategies of separate species and demonstrates the need to investigate metal tissue distribution on a case by case basis and not make generalisations between species. The results indicate that *A. trapezia* has some capacity to regulate and was able to effectively detoxify zinc. The potential for zinc toxicity can therefore be ruled out.

Copper

The percentage of copper in the BDM fractions was in the order CC2 > CB > CC1 > control for both tissues with between 52 – 72 % detoxified (Table 8.2; Figure 8.11). The distribution pattern for copper between the MRG and MTLP fractions was very different between treatments with the organisms exposed to the CC1 sediments, which had 3 – 4 times the copper exposure of the CC2 and CB organisms (Table 8.1), having an equal distribution of copper between the two fractions while organisms from the other treatments had the majority of the copper in the MTLP (Table 8.3; Figure 8.11). This pattern of copper distribution in the BDM fractions suggests effective detoxification of copper by *A. trapezia* which is further supported by the BAM copper burden which was less than or equal to that of the control organisms in the CC2 and CB metal exposed organisms and only slightly higher in the CC1 exposed organisms (Table 8.2; Figure 8.11). Bonneris *et al.*, (2005) found the chronically copper exposed freshwater bivalve *P. grandis* had the majority of its accumulated subcellular copper in the MRG fraction in both gill and hepatopancreas tissues with significant increases in MRG copper burden with increased copper exposure. Increases in BAM copper burden were only observed in the hepatopancreas tissues and these were modest. The dominance of the MTLP in the majority of the copper exposed *A. trapezia*, in contrast to the dominance of the MRG in the copper exposed *P. grandis*, may relate to the nature of the exposures, short term versus chronic, saline versus fresh water and or species differences.

Although the copper burden in the BAM portion of the copper exposed organisms was not greatly different between treatments the distribution of copper among the fractions was (Table 8.3). The most notable feature is the increase in the percentage of copper in the mitochondrial fraction of the CC1 and to a lesser extent the CB organisms compared to the other two fractions (Table 8.3). In the control and the lowest copper exposure CC2, the copper distribution was similar with around half in the mitochondrial fraction and the remainder fairly evenly distributed between the lysosomes+microsomes and the HSP. Increased copper associated mitochondria following increased copper exposure has also been found in gill and hepatopancreas tissues of the marine gastropod *Nassarius reticulatus* (Kaland *et al.*, 1993). Reduction of membrane permeability is one of the major modes of toxic action of copper (Brown *et al.*, 2004). Copper induced reduction of lysosomal stability has been shown in other invertebrates (Ringwood *et al.*, 1998a; Viarengo, 1989) and may explain the increased copper in the mitochondria which has the potential to induce toxic effects through increased redox cycling producing excess ROIs.

8.4.3 Enzymatic Biomarker – Total Antioxidant Capacity

Exposure to the Lake Macquarie mixed metal contaminated sediments significantly reduced the TAOC of *A. trapezia* compared to that of the reference organisms and the effect on organisms from the CC1 site which accumulated the highest tissue metal was significantly greater than that of organisms from all other sites (Figure 8.14). The sensitivity of the oxidative system to metals has previously been demonstrated in a range of marine bivalves: oysters *Crassostrea virginica* Ringwood *et al.*, (1998b; 2004); mussels *Mytilus galloprovincialis* (Camus *et al.*, 2004; Frenzilli *et al.*, 2004; Moncheva *et al.*, 2004; Regoli, 2000; Regoli *et al.*, 2004; Regoli and Principato, 1995); *Mytella guyanensis* (Aloisio Torres *et al.*, 2002); *Bathymodiolus azoricus* (Company *et al.*, 2004). In the laboratory exposed *A. trapezia*, the reduction in TAOC showed a strong correlation with tissue metal concentrations (Figure 8.14), indicating that the increased metal dose progressively reduced the organism's capacity to reduce ROIs. Compared to the *A. trapezia* controls, only cadmium and lead showed increased BAM burdens in organisms from all sites, while BAM selenium burdens were no different and zinc and copper BAM burdens were only greater in the CC1 organisms (Table 8.2).

The reduction in TAOC seen in these organisms must, therefore, have been largely due to the effects of cadmium and lead. Significant effects of cadmium and lead on *A. trapezia* TAOC have previously been demonstrated in the single spiked metal exposures (Figures 5.7 and 6.7). The application of the same TAOC assay technique used in this study, to mussels *Mytilus galloprovincialis* exposed to cadmium and lead has also demonstrated an increased inhibition in the total antioxidant scavenging capacity with increased tissue cadmium and lead concentrations (Gorinstein *et al.*, 2005). Although zinc and copper were only elevated in the BAM fraction of the CC1 organisms these redox active metals may have increased the cycling and production of oxygen intermediates (Winston and Di Giulio, 1991) during the process of their uptake and detoxification, thereby contributing to the net reduction in the TAOC of metal exposed organisms.

The native *A. trapezia* from a contaminated site in Lake Macquarie did not fit the tissue metal TAOC regression (Figure 8.14) as they had a far greater inhibition of the TAOC for the total tissue metal concentration than the laboratory exposed organisms. The tissue metal concentrations of the native organisms were generally lower for the essential elements zinc, copper and selenium, equal for lead and higher for cadmium for the exposure concentrations than that of the laboratory exposed organisms (Figure 8.3), reflecting the different metal handling strategies of chronically versus previously unexposed organisms. The lower concentrations of the redox metals in the native organisms seems to suggest that increased redox cycling would not have been present, and as lead and selenium were not different the higher cadmium concentrations must have been largely responsible for the reduced TAOC. There was insufficient material to enable subcellular fractionation of native *A. trapezia* tissues so information on the BAM burdens is not available. Given the lower TAOC it is possible that these chronically exposed organisms had a greater percentage of the accumulated tissue metal in the BAM fractions. Other factors such as salinity, temperature and stress related to collection and transportation from Lake Macquarie may also have contributed to the reduction in TAOC of these organisms

8.4.4 Oxidative Damage Biomarker – Thiobarbituric Acid Reactive Substances

The concentration of TBARS increased in all the metal exposed *A. trapezia* compared to the reference organisms, with only the CB exposed organisms not being significantly higher (Figure 8.15). In the laboratory exposed organisms the increase in TBARS showed a strong correlation with tissue metal concentrations (Figure 8.15), indicating that the increased tissue metal dose progressively increased the concentration of lipid peroxidative products. Significant effects of lead on *A. trapezia* TBARS has previously been demonstrated in the single spiked lead exposures (Figure 6.8). The increased lead BAM burden measured in the laboratory metal exposed *A. trapezia* compared to the control organisms (Table 8.2) may, therefore, also have contributed directly to the increased TBARS through interaction with oxyhaemoglobin, leading to peroxidative hemolysis in the red blood cell membranes (Ercal *et al*., 2001). Increased lipid peroxidation is a widely reported consequence of excess ROS production in marine bivalves (Company *et al.*, 2004; de Almeida *et al.*, 2004; Winston and Di Giulio, 1991), and the correlation between the TAOC and TBARS of these organisms supports this as a major contributor to the enhancement of TBARS with increased tissue metal concentrations (Figure 8.17).

The native *A. trapezia* did not conform to the regression of tissue metal and TBARS of the laboratory exposed organisms, as they had a far higher TBARS concentration for the total tissue metal concentration than the laboratory exposed organisms (Figure 8.15). The tissue lead concentrations of the native organisms were higher than the CC2 and CB but lower than the CC1 laboratory exposed organisms (Figures 8.2 & 8.3), which may have contributed to the increased TBARS production (Figure 8.15). The greatly reduced TAOC, which may have been related to elevated tissue lead and cadmium concentrations, appears to have had a direct influence on the increased TBARS in the native organisms. This can be seen by the good fit of the native organisms to the regression of TAOC and TBARS of the laboratory exposed organisms (Figure 8.17), and supports the idea that increased lipid peroxidation is a direct consequence of increased ROS production and impaired antioxidant scavenging capacity.

8.4.5 Cellular Biomarker – Lysosomal Stability

The pattern of lysosomal destabilization in the laboratory exposed *A. trapezia* followed the same trend as the TBARS concentration with increased tissue metal resulting in increased lysosomal destabilisation (Figure 8.15). The lysosomal destabilisation was significantly greater in all metal exposed organisms than the reference organisms (Figure 8.15; Appendix 3.11). The organisms from the CB exposure had 33 % destabilised lysosomes which places them in the concern range of the Ringwood *et al*., (2003) criteria, while the CC1 had 50 % and CC2 45 % destabilised placing them both in the stressed range. Ringwood *et al*., (2003) reports that lysosomal destabilisation of over 40 % in oysters *Crassostrea virginica* may have irreversible effects resulting in significant impairment of normal cellular function and potentially result in mortality. This suggests that organisms from these exposures may be unable to recover even if removed from the sediments. The lysosomal destabilisation of the laboratory exposed *A. trapezia* was strongly correlated with both TAOC and TBARS (Figures 8.17 & 8.18), indicating an increase in metal burden in the BAM fractions initiated a reduction in TAOC with a consequent increase in lipid peroxidation which had a detrimental effect on the stability of the lysosomal membrane.

The native *A. trapezia* conformed well to the regression of tissue metal and lysosomal destabilisation of the laboratory exposed organisms, having a similar percentage of destabilised lysosomes for the total tissue metal concentration as the laboratory exposed organisms (Figure 8.15). They had only 24 % of lysosomes destabilised which places them in the normal range with the reference organisms, from which they were not significantly different (Figure 8.15). This result suggests that while reduced TAOC and increased TBARS would be expected to contribute to an increase in lysosomal destabilisation, in this case it did not, which is evident from the poor fit of the native organisms to the regressions of both the TAOC and TBARS with lysosomal destabilisation (Figures 8.17 & 8.18). Differences in biomarker responses between chronically and previously unexposed organisms have also been observed in other studies (Bacanskas *et al.*, 2004; Bard, 2000; Bolognesi *et al.*, 2004; Ferreira *et al.*, 2008), and are thought to be due to genetic differences conferring enhanced abilities on native organisms' toxicant handling capacities and strategies which have developed over time in response to exposure, or in other words natural selection in action.

In this case it is not clear why the reduced TAOC and increased TBARS did not cause a significant increase in lysosomal destabilisation. The lack of significant lysosomal destabilisation tends to support the notion that the severity of the TAOC and TBARS responses in the native *A. trapezia* were due to short term effects related to collection and transport rather than metal exposure alone and these effects were not translated into membrane damage because of the short time frame.

8.4.6 Genotoxic Biomarker – Micronuclei Frequency

There was a significant degree of genotoxic damage in *A. trapezia* from the metal exposures compared to the reference organisms (Figure 8.15). The occurrence of micronuclei increased with increased tissue metal concentrations and there was a strong correlation between the two (Figure 8.15). Micronuclei induction after exposure to metals has been reported in mussels *M. galloprovincialis* (Bolognesi *et al.*, 2004; Bolognesi *et al.*, 1999; Dailianis *et al.*, 2003; Gorbi *et al.*, 2008; Regoli *et al.*, 2004) and has been attributed to high mortality rates, cell death, inhibitory effects on cell division discharges and inhibition of valve closure responses (Dailianis *et al*., 2003). The increased occurrence of micronuclei was strongly correlated with the decrease in TAOC and the increase in TBARS (Figures 8.17 & 8.18), indicating that an increase in ROS contributed to an increase in genotoxic damage. Significant associations between perturbed oxyradical metabolism and increased micronuclei frequency have been also measured in metal exposed mussels (Gorbi *et al.*, 2008; Regoli *et al.*, 2004). There was significant micronuclei induction in each of the single metal spiked sediment laboratory exposures (Figures 5.8, 6.8 & 7.8). The percentage of micronuclei in the mixed metal exposures, however, was far higher than those exposed to the single metals (Figure 8.15). The mix of metals appears to have had a synergistic effect on micronuclei induction in these organisms. This synergistic effect was also evident in a generally greater reduction in TAOC, higher TBARS concentrations and a greater degree of lysosomal destabilisation relative to the reference organisms compared to the single metal exposures (Figures 5.7, 5.8, 6.7, 6.8, 7.7, 7.8, 8.14 & 8.15). The native *A. trapezia* were not measured for micronuclei frequency.

8.4.7 Physiological Biomarker – Condition Index

The condition index (CI) of *A. trapezia,* expressed as the ratio of soft tissue dry mass (g) to valve dry mass (g), was used as a physiological measure of organism response to tissue metal accumulation. *A. trapezia* from the CB site sediment exposure, which were exposed to the lowest sediment metal concentrations, did not show any change in CI during the exposure (Figure 8.16). *A. trapezia* exposed to sediments from sites CC1 and CC2 had improved CIs after the first 28 days of the exposure followed by a significant fall in the CI ratio to day 56 (Figure 8.16). A decline in CI associated with cadmium accumulation in the sediment dwelling marine bivalve *Macoma balthica* experimentally exposed to cadmium for 5 weeks (Duquesne *et al.*, 2004) and to a range of metals accumulated by the mussel *Mytilus edulis* environmentally exposed (Mubiana *et al.*, 2006) have been reported. Both of these studies found organism size influenced metal accumulation and CI, with smaller individuals being more sensitive to metal exposure. The *A. trapezia* used in this study ranged in size, with valve mass between 10 and 50 g (mean 30 g). The different sized organisms were evenly distributed between treatments so that the range of valve mass means between treatments was only 20 to 25 g. Despite this, the size of individual *A. trapezia* may have been a factor influencing CI response to metal accumulation. The increased CI in the CC1 and CC2 exposed organisms up to day 28 may be a reflection of increased metabolic activity associated with metal accumulation and detoxification activities. The subsequent reduction in the CI of these organisms by the end of the exposure period may be related to the effects of metal toxicity as seen by the increased metal accumulation and the perturbations to the antioxidant system. The lack of control organisms measured over time makes it difficult to say with any confidence that the decline in CI is due to metal exposure rather than other experimental effects, although the organisms exposed to site CB sediments, with the lowest metal exposure, did not show any loss in CI over time and apart from the different sediment metal concentrations were exposed to the same conditions. As a means to assess whether the biochemical, cellular and genotoxic effects measured after the 56 day exposure were expressed at the physiological level the CI lends some support to the use of these cellular and subcellular measures as useful indicators of higher order effects in individuals which might have implications for population health.

8.5 Summary and Conclusions

A clear metal contamination gradient decreasing in concentration from site CC1 to CC2 and CB was measured with metal concentrations in the order; zinc > lead > copper > cadmium > selenium. Positive correlations between metals from the three sites indicated that they originated from a common source. Cadmium, zinc and lead exceeded the high and copper the low ANZECC and ARMCANZ (2000) interim sediment guideline concentrations and exposure of *A. trapezia* is, therefore, likely to result in significant tissue metal accumulation and impairment of organism health. Sediment and final tissue metal concentrations across the three sites were positively correlated but the relationship was non-linear. As metal exposure concentrations increased the tissue metal increase was not proportional which is most likely due to saturation of metal binding sites at the higher exposures, or a reflection of metal bioavailability in the sediments. The native *A. trapezia* showed an adaptive response to the essential elements zinc, copper and selenium with far lower tissue concentrations for the sediment exposure than the laboratory exposed organisms but not for lead and cadmium which were the same or higher. The patterns of cadmium, lead and selenium accumulation in the mixed metal laboratory exposed *A. trapezia* were similar to those observed for *A. trapezia* in the spiked sediment exposures. Overall results indicate *A. trapezia* is a net accumulator of cadmium, is able to regulate lead, selenium and zinc to a degree and has a well developed regulatory system for copper. There were significant concentrations of copper and lead in the haemolymph which was probably associated with haemoglobin. The gill generally had the highest metal concentrations, associated with dissolved metal uptake, while selenium concentrations in the hepatopancreas of the CB exposed organisms were significantly greater than the other tissues, indicating an increased contribution of dietary selenium. The subcellular distribution of cadmium and lead followed the distribution seen in the single metal spiked exposures with increased burdens of these metals in the BAM fractions compared to unexposed organisms. There were modest increases in mitochondrial zinc and copper burdens of the CC1 exposed organisms but not in the CC2 or CB organisms, and no increase of selenium BAM burdens in organisms from any exposure. There were significant correlations between TAOC reduction and increased TBARS, lysosomal destabilisation and micronuclei frequency associated with increased tissue metal.

Increased BAM lead and cadmium burdens probably account for the cellular perturbations observed, although the presence of significant sediment concentrations of the redox active metals zinc and copper may have contributed to an increase in the production of ROIs during the process of their uptake and detoxification. The native *A. trapezia* TAOC and TBARS responses were higher than predicted by the laboratory exposures for their total tissue metal concentrations. This points to lead and cadmium, which were significantly accumulated by these organisms, being responsible for the effects, which is supported by the increased BAM burdens of these metals in the laboratory exposed *A. trapezia.* Alternatively collection and transport of the native organisms may have had a short term effect on their antioxidant reduction system which is supported by the low percentage of destabilised lysosomes which was close to that of the reference organisms. The CI of the *A. trapezia* from the higher sediment metal exposure sites CC1 and CC2 showed an initial increase followed by a significant reduction in CI ratio by the end of the exposure. As a means to assess whether the biochemical, cellular and genotoxic effects were expressed at the physiological level the CI lends some support to the use of these cellular and subcellular measures as useful indicators of higher order effects in individuals which could have implications for population health.

9 Synopsis

The major findings relating to each of the objectives (Section 1.4) of the studies are presented along with an assessment of the efficacy of the biomarkers used to demonstrate cellular and subcellular effects of metals on *T. deltoidalis* and *A. trapezia,* to determine whether significant exposure – dose – response relationships for sediment metal contamination have been demonstrated for these organisms.

9.1 Metal Spiked Sediment Studies

9.1.1 Sediment and Tissue Metal Relationships

9.1.1.1 *Tellina deltoidalis*

T. deltoidalis accumulated cadmium, lead and selenium at all spiked metal exposure concentrations. Equilibrium tissue concentrations were reached at between 21 and 28 days of exposure which were equal to that of the sediment exposure concentrations in all treatments except the 300 µg/g lead and 5 µg/g selenium exposures where organisms had higher tissue metal than the sediment metal concentrations. These higher than ambient tissue metal concentrations suggest regulatory mechanisms were overwhelmed and or an increase in metal availability through food sources over the course of the exposure. Previous metal exposure experiments with these organisms showed lower than ambient tissue metal concentrations over a similar exposure time (Atkinson *et al.*, 2007; King *et al.*, 2005), which may be due to differences in sediment matrix and or antagonistic effects of other metals in the sediment used in these studies.

9.1.1.2 *Anadara trapezia*

A. trapezia accumulated cadmium, lead and selenium at all spiked metal exposure concentrations. Tissue cadmium concentrations reached equilibrium by day 42 of the exposure which were up to 1.2 times higher than the sediment cadmium concentrations, indicating *A. trapezia* is a net accumulator of cadmium. Lead appeared to reach equilibrium tissue concentrations by day 42 which were significantly lower than sediment concentrations, suggesting either a low bioavailability of lead or partial regulation, however, there was a doubling of lead tissue concentrations in the final two weeks of the exposure which points to regulatory mechanisms being overwhelmed.

As final tissue lead concentrations were still well below sediment lead concentrations it is possible that *A. trapezia* have some capacity to regulate lead but at the lead exposure concentrations in this study this was progressively overwhelmed. A background tissue selenium concentration of around 1 to 2 µg/g is presumed to be *A. trapezias'* metabolic requirement for this metal, as demonstrated for control organisms in this and in a previous study (Jolley *et al*., 2004). In the spiked sediment selenium exposures selenium tissue concentrations reached equilibrium within 14 days, at about half in the 5 µg/g exposure and one fifth in the 20 µg/g exposure of the sediment selenium concentration, with no appreciable increase after this. This accumulation pattern suggests *A. trapezia* has some ability to regulate selenium dose and may be a partial regulator of this metal. Previous studies of *A. trapezia* metal accumulation patterns have shown lower than ambient cadmium accumulation and similar lead and selenium accumulation patterns to those observed in these studies (Burt *et al.*, 2007; Jolley *et al.*, 2004).

9.1.2 Individual Tissue Metal Accumulation Relationships

9.1.2.1 *Anadara trapezia*

The majority of cadmium accumulation in *A. trapezia* is explained by the gill which had significantly more cadmium than the other tissues at all collection times, indicating dissolved cadmium was the major source. This is supported by previous studies of cadmium accumulation by filter feeding bivalves (Bebianno *et al.*, 1993; Chan *et al.*, 2002). The haemolymph contributed significantly to whole tissue lead concentrations. This may be related to the high affinity for lead to bind to haemoglobin which is a respiratory pigment in *A. trapezia* (Jin *et al.*, 2008; Sullivan, 1961). Gill lead became more significant in the final 2 weeks of the exposure when lead tissue concentrations doubled, suggesting that lead detoxification systems were overwhelmed and indiscriminate lead binding in gill tissues was probably occurring. Gill selenium contributed the greatest proportion of the whole tissue selenium indicating dissolved selenium was a major uptake pathway. The contribution of the haemolymph, particularly in the first 14 days supports this, as dissolved metal is transported in haemolymph. The contribution of the hepatopancreas became more significant in the later part of the exposure indicating increased dietary exposure which is an important source of accumulated selenium (Hamilton, 2004; Luoma and Rainbow, 2008).

9.1.3 Subcellular Tissue Metal Distribution

9.1.3.1 *Tellina deltoidalis*

T. deltoidalis detoxified around 50 % of accumulated cadmium and 70 % of lead. Organisms with higher cadmium exposure converted a higher percentage of BDM cadmium to MRG, 60 %, than those with lower cadmium exposure, 25 %. The conversion of lead to MRG was 75 % of the total BDM in organisms from both lead treatments, indicating active detoxification of these metals by *T. deltoidalis*. The majority of BAM cadmium and lead was associated with the mitochondrial fraction in *T. deltoidalis* from all exposures, with increases in cadmium burden in this organelle up to 7200 fold and lead 154 fold in exposed organisms compared to controls. This shows that despite active detoxification processes significant cadmium and lead concentrations were reaching sensitive organelles. The subcellular distribution of selenium followed a very different pattern to that of cadmium and lead. The majority of recovered selenium was associated with the nuclei+cellular debris fraction probably as protein bound selenium associated with plasma and selenium bound directly to cell walls, and so potentially effectively removed from active sites. The percentage of selenium in the BDM fractions of selenium exposed organisms increased, indicating detoxification of accumulated selenium was occurring. The BDM selenium was associated with both MRG and MTLP fractions. Neither process has previously been described in bivalves for selenium, however, recent tissue fractionation studies reported; crustaceans had 40 – 60 % (Zang and Wang, 2006), and oligochaetes 1 – 2 % (Dubois and Hare, 2009), of accumulated selenium in the MRG fraction. This supports the existence of selenium detoxification processes in these organisms which have similarities with those previously described for other metals by George (1983b). Increases in mitochondrial selenium burdens of exposed organisms, up to a 7 fold from controls, were only modest compared to those of lead and cadmium. The increase is not unexpected given the involvement of selenium in the glutathione peroxidase enzyme which is active in the oxygen reduction processes carried out in mitochondria, however, selenium toxicity can occur at concentrations which are only slightly greater than those that are required (Palace *et al*, 2004). Overall the subcellular distribution of all three metals indicates active metal detoxification processes which at these exposure concentrations were unable to prevent significant metal burdens from accumulating in sensitive organelles.

This indicates that *T. deltoidalis* exposed to these sediment metal concentrations is a net accumulator, a metal accumulation category which includes invertebrates that do not regulate and those in which regulatory mechanisms have broken down (Rainbow *et al.*, 1990).

9.1.3.2 *Anadara trapezia*

Metal spiked sediment exposed *A. trapezia* detoxified between 50 and 70 % of accumulated cadmium and lead with a higher percentage detoxified in the gill than the hepatopancreas tissues for both metals, reflecting the higher gill metal tissue burdens. The majority of BDM cadmium of both tissues was in the MTLP fraction with very little cadmium converted to MRG. Accumulated BDM lead was fairly evenly distributed between the MRG and MTLP fractions with a slightly higher percentage converted to MRG in the gill. This indicates active detoxification of these metals was occurring in both tissues of *A. trapezia*. Selenium subcellular distribution was similar in both tissues of *A. trapezia* to the selenium distribution in *T. deltoidalis*. A high percentage of recovered selenium was in the cell nuclei+cellular debris fraction. There was increased selenium detoxification in selenium exposed organisms but the total percentage of BDM selenium was low, 12 – 22 % of the total recovered selenium. More of the BDM selenium in the hepatopancreas was converted to MRG than in the gills suggesting different selenium accumulation and detoxification pathways may be operating in the two tissues. The BAM mitochondrial burdens in the metal exposed organisms increased in both tissues; cadmium up to 84 fold, lead up to 50 fold and selenium up to 7 fold. The majority of BAM cadmium and lead was in the mitochondrial fractions of both tissues, with the exception of the hepatopancreas of the 300 μg/g lead exposed organisms which and the majority of BAM in the lysosome+microsome fraction. Gill tissue had the majority of selenium in the mitochondria while in the hepatopancreas the lysosome+microsome fraction dominated. These differences suggest different uptake routes and metal handling processes are operating in the two tissues at these concentrations for these metals. The subcellular distribution of the three metals shows active metal detoxification with different metabolic pathways in the gill and hepatopancreas for the individual metals. Increased BAM burdens indicate detoxification mechanisms were unable to prevent significant concentrations of metal reaching sensitive organelles. *A. trapezia* appears to have some capacity to regulate selenium in that tissue accumulation did not increase after the first 14 days of the exposure, however, very little of the selenium which was accumulated was detoxified so that increased selenium would still have reached critical molecular targets.

9.1.4 Biomarker Responses

9.1.4.1 *Tellina deltoidalis*

T. deltoidalis in all metal exposures had significantly reduced TAOC compared to control organisms. An examination of glutathione cycling showed cadmium accumulation in the mitochondria depleted the total glutathione concentration and reduced the activity of GPx, leading to a significant reduction in the ratio if GSH:GSSG. Lead had a similar effect but it was far less pronounced and not significantly different to the controls. Selenium enhanced the GPx activity and total glutathione concentrations which had the effect of increasing GSSG production to significantly reduce the GSH:GSSG ratio. While the different metals affected the various components of the glutathione cycle differently they all produced a significant reduction in the organism's overall capacity to reduce reactive oxygen species. The TAOC is, therefore, probably a more useful indicator of the effect of metal accumulation on the oxidative system than the individual enzyme measurements. *T. deltoidalis* showed oxidative damage at all metal concentrations, although the TBARS concentrations were only significantly higher in organisms from the 50 µg/g cadmium, 300 µg/g lead and both selenium treatments compared to control organisms. There was significant lysosomal destabilisation at all metal exposures. When compared with criteria for assessing the significance of the percentage of lysosomal destabilisation developed by Ringwood *et al*, (2003) the cadmium exposed *T. deltoidalis* were in the stressed – concern range, the lead and selenium exposed were in the stressed range. The selenium exposed organisms had the highest percentage of destabilised lysosomes. Significant genotoxic damage was observed with *T. deltoidalis* from all metal exposures having higher numbers of micronuclei than the control organisms.

9.1.4.2 *Anadara trapezia*

A. trapezia had significantly reduced TAOC in all metal exposures compared to control organisms. Examination of glutathione cycling showed lead and selenium accumulation in the mitochondria reduced the activity of GPx and increased the total glutathione concentration. The increase in glutathione was probably due to increased oxidised glutathione concentrations as evidenced by the significant reduction in the ratio if GSH:GSSG. Cadmium had a similar effect but it was far less pronounced and only the GSH:GSSG ratio of the 50 µg/g cadmium exposed organisms was significantly lower than the controls.

This result is surprising given the high mitochondrial cadmium burdens. While the different metals affected the various components of the glutathione cycle somewhat differently they all produced a significant reduction in the overall oxygen reduction capacity. The TAOC is, therefore, probably a more useful indicator of the effect of metal accumulation on the oxidative system than the individual enzyme measurements. Oxidative damage was evident in organisms from all metal exposures but TBARS concentrations were only significantly higher than controls in the lead and selenium exposed organisms. There was significant lysosomal destabilisation at all metal exposures. Based on the Ringwood *et al*, (2003) criteria for the significance of lysosomal destabilisation the cadmium, lead and selenium exposed *A. trapezia* were all in the stressed range. The selenium exposed organisms again had the highest percentage of destabilised lysosomes. There was significant genotoxic damage in all metal exposed organisms. This was greatest in the cadmium and selenium exposed organisms which had similar percentages of micronuclei.

9.1.5 Exposure – Dose – Response

Significant exposure – dose – response relationships have been demonstrated for both *T. deltoidalis* and *A. trapezia* to sediments spiked at realistic environmental cadmium, lead and selenium contamination concentrations for these metals in Australia. Metal accumulation in *T. deltoidalis* occurred at all concentrations. Equilibrium tissue concentrations reached were generally equal to the exposure concentrations with the exception of the 5 µg/g exposed organisms which had significantly higher selenium tissue concentrations than the sediment selenium concentration. Metal accumulation in *A. trapezia* occurred at all metal exposure concentrations. Cadmium tissue concentrations in *A. trapezia* reached an equilibrium which was greater than sediment concentrations. Lead tissue concentrations were lower than exposure concentrations and selenium tissue concentrations about equal to ambient concentrations. Detoxification of all metals was evident in both *T. deltoidalis* and *A. trapezia* but detoxification capacity was exceeded for all metals leading to significant metal accumulation in sensitive organelles. The significant relationships between TAOC reduction and increased TBARS, lysosomal destabilisation and micronuclei frequency and between increased TBARS and lysosomal destabilisation and micronuclei frequency indicates that increased tissue metal dose and BAM burdens caused significant impairment of the antioxidant reduction capacity which resulted in a cascade of effects from lipid peroxidation to cellular perturbation and genotoxic damage.

The suite of interrelated biomarkers offers a weight of evidence approach for demonstrating adverse effects of cadmium, lead and selenium tissue accumulation in *T. deltoidalis* and *A. trapezia*.

9.2 Lake Macquarie Cadmium, Lead, Selenium, Zinc and Copper Contamination Gradient Sediment Study

9.2.1 Metal Contamination Gradient

A clear sediment metal contamination gradient, decreasing in concentration from the zinc/lead smelter drainage inflow down Cockle Creek into and across Cockle Bay was established with metal concentrations decreasing in the order zinc > lead > copper > cadmium > selenium. Positive correlations between metals at each of the sites suggest a common source. Cadmium and zinc concentrations exceeded the ANZECC and ARMCANZ (2000) high guideline concentrations, the two Cockle Creek and LM sites exceeded the lead high guideline value and copper exceeded the low value at the CC1 and LM sites. *A. trapezia* exposed to these sediments are likely to show significant metal exposure – dose - response relationships.

9.2.2 Sediment Tissue Metal Relationships and Subcellular Metal Distribution

The relationship between sediment and final tissue metal concentrations was positive but non-linear. Organisms at the higher metal exposures had higher tissue metal concentrations but the increase was not proportional with exposure concentrations. *A. trapezia* from all sites had final equilibrium tissue metal concentrations which were lower than the ambient concentrations except for selenium, probably due to saturation of binding sites and or antagonistic metal interactions. Cadmium and lead accumulation patterns and subcellular distribution were similar to those observed for *A. trapezia* in the spiked sediment experiments, with increased mitochondrial burdens of these two metals. Selenium tissue accumulation patterns were also similar to the spiked sediment experiment but final tissue selenium concentrations were higher than the exposure concentrations, while in the spiked exposures they were lower. The subcellular distribution of selenium also differed from the spiked selenium exposures, in that all the BDM selenium was in the MRG fraction and there was no increase in the BAM selenium burden, indicating effective detoxification of selenium in *A. trapezia* exposed to the Lake Macquarie sediments.

Zinc tissue concentrations increased significantly with increased sediment concentrations to about one fifth to one tenth the exposure concentration while copper tissue concentrations did not differ among sites. The tissue distribution of zinc followed the same pattern as cadmium with gills explaining most of the total tissue zinc concentrations while copper tissue distribution was similar to that of lead with the copper binding to haemoglobin in the haemolymph and both haemolymph and gills having higher concentrations than the hepatopancreas. This points to dissolved zinc and copper being more important than dietary input. The majority of accumulated zinc; 64 – 78 % and copper; 52 – 72 %, was in the BDM fractions of metal exposed organisms indicating effective zinc and copper detoxification which is further supported by the BAM zinc and copper burdens which were only slightly elevated in the CC1 exposed organisms and not different in the CC2 and CB organisms. The native *A. trapezia* collected from a contaminated site in Lake Macquarie did not fit the sediment tissue metal regression of the laboratory exposed organisms, having lower tissue metal concentrations for the exposure concentrations than the laboratory exposed *A. trapezia*. This suggests an adaptive response by these organisms to chronic metal exposure and appears to be for the essential metals zinc, copper and selenium, which were significantly lower than the laboratory exposed organisms at the same sediment metal exposure concentrations, but not for cadmium and lead which were higher.

9.2.3 Biomarker Responses

The TAOC was significantly reduced in all metal exposed organisms compared to the reference *A. trapezia.* As only lead and cadmium BAM burdens were increased in these organisms the TAOC reduction must be largely due to the effects of these metals. Although copper and zinc BAM burdens were only elevated slightly in the CC1 exposed organisms these redox active metals may have increased the cycling and production of ROIs during their uptake and detoxification and so contributed to the net reduction in TAOC of metal exposed organisms. The native *A. trapezia* had a greater inhibition of the TAOC than the laboratory exposed organisms which may be explained by the high cadmium tissue concentrations, or may relate to other stress factors such as transport and storage prior to analysis. TBARS concentrations were increased in all metal exposed *A. trapezia*. The native organisms also had higher TBARS concentrations for the total tissue metal than the laboratory exposed organisms which probably relates to the very reduced TAOC. There was significant lysosomal destabilisation in *A. trapezia* from all metal exposures which correlated well with the metal exposure concentrations.

A. trapezia from the CC1 and CC2 exposures were in the stressed range, the CB in the concern range and the native *A. trapezia* in the normal range (Ringwood *et al*., 2003). A higher percentage of destabilised lysosomes would have been anticipated in the native organisms given the high TBARS and this result suggests that short term effects relating to collection and transport may have played a greater role in the TAOC and TBARS than metal exposure effects which were not manifested in membrane damage. Frequency of micronuclei increased significantly with increased metal tissue dose. The enzymatic and cellular perturbations lead to a reduction in the physiological condition of the CC1 and CC2 metal exposed *A. trapezia* indicating the subcellular effects measures are useful early indicators of potential later organism and population level effects.

9.2.4 Exposure – Dose – Response

A clear exposure – dose – response relationship has been demonstrated for *A. trapezia* exposed to Lake Macquarie metal contaminated sediments. Accumulation of cadmium and lead exceeded detoxification capacity leading to an increase of these metals in sensitive areas of cells. Laboratory exposed *A. trapezia* showed significant relationships for total metal tissue concentration and TAOC reduction with increased TBARS, lysosomal destabilisation and micronucleus frequency indicating the increased BAM cadmium and lead produced an increase in ROIs which were unable to be effectively reduced, leading to an increase in oxidative damage, cellular membrane and genotoxic damage. The cascade of interrelated response indices shows a clear response relationship with the increased metal dose. The reduction in physiological condition in the organisms with the highest tissue metal doses suggests the response goes beyond subcellular perturbations to whole organism and potentially population effects. Chronically metal exposed native Lake Macquarie *A. trapezia* did not show a clear metal exposure – dose - response relationship. Accumulation of the essential elements zinc, copper and selenium appeared to be regulated while cadmium and lead were not. TAOC was significantly reduced and TBARS significantly increased compared to reference organisms but lysosomal stability and condition were not significantly affected. If short term effects relating to collection and transport of these organisms account for the TAOC and TBARS results then it is apparent that the native population of *A. trapezia* has adapted to chronic metal exposure and there are no implications for population level effects in these organisms.

9.3 Recommendations

T. deltoidalis has good attributes as a biomonitor, being hardy, abundant, easy to work with in the laboratory and a net accumulator of cadmium, lead and selenium, reaching tissue metal equilibrium over a 28 day exposure period. They were sensitive to metals as seen by the cascade of enzymatic and cellular effects and would therefore be suitable for sediment metal toxicity tests. Although they are small they have sufficient tissue for analysis but also require less sediment and smaller aquaria than the larger *A. trapezia*, making them easier to use in laboratory tests. As they are deposit feeders supplementary feeding is not required because they gain sufficient food from the ingested sediment, provided the organic fraction is not too low. Their small size and relatively fragile shells make them unsuitable for field exposures as they are easily damaged with rough handling, the cage mesh size would need to be very small to contain them and buried to allow burrowing. *T. deltoidalis* can also be difficult to find as it lives wholly buried in sediment. Digging and sieving is required to collect them which is time consuming, can be very 'hit and miss' and digging damages estuarine seagrass beds. If they were to be regularly used in laboratory sediment toxicity testing consideration should be given to breeding them in aquarium systems.

A. trapezia also has good attributes for metal biomonitoring they are hardy, relatively abundant and accumulated metals from both individually metal spiked and mixed metal sediments over 56 days. They were also sensitive to metals as seen by the cascade of enzymatic, cellular, genotoxic and physiological effects and would therefore be suitable for sediment metal toxicity tests. They are a more robust and larger organism than *T. deltoidalis* and with the exception of selenium appear to take longer to reach tissue metal equilibrium. They only bury into the top 6 – 8 cm of sediment and as larger mesh size can be used for cages these can be easily worked into the sediment to this depth to allow for this. *A. trapezia* would, therefore, probably be suitable for use in longer term caged experiment exposures in metal contaminated estuarine environments. *A. trapezia* is less suitable than *T. deltoidalis* for use in the laboratory as they require larger sediment volumes, more water and supplementary feeding, however, they are easier to collect than *T. deltoidalis* as they live on the sediment surface in shallow estuarine waters and can be readily 'picked'.

The downside to this is that they are a commercial species and are a popular eating mollusc with may groups so there can be competition for them, knowledge of where populations are can be a closely guarded secret and there is a bag limit so a large group of friends or a difficult to obtain scientific collection permit is required to collect sufficient organisms for experimentation.

If they were to be used in routine toxicity testing, the sensitivity of *T. deltoidalis* and *A. trapezia* to other toxicants including other metals, PCBs, PAHs, dioxins, pesticides and to mixtures of xenobiotics would need to be established and this may require the development of other biomarker responses specific for the different toxicants. The suite of biomarkers used in this study are good general biomarkers of exposure and effect and may be suitable for measuring responses to other contaminants. The biomarkers used show a cascade of metal exposure effects which are inter-linked providing a more powerful picture of response to metals than a series of unrelated biomarker measurements. The examination of the glutathione cycling responses to metal dose while they provided a greater insight into the antioxidant system are expensive and time consuming and for further work the activity of the total antioxidant system is probably more relevant in establishing metal dose response which together with lipid peroxidation and lysosomal stability would be sufficient for establishing metal effects. The condition index measured in the mixed metal exposed *A. trapezia* indicated possible higher order effects which linked the earlier enzymatic and cellular responses to organism health and potentially population effects. An examination of CI over a longer term exposure would be useful to better understand this link. An examination of cellular energetics such as cellular energy allocation would also help in linking these effects measures with organism and population health.

Experimental caged exposures of *A. trapezia* in metal contaminated estuarine sediments need to be undertaken to determine whether the metal exposure – dose – response relationships established in these studies can also be measured in a natural environment where conditions such as salinity, light, temperature water movement and other physicochemical properties, which were controlled in the laboratory exposures, influence the relationships.

Further work is required on native chronically exposed *A. trapezia* to look at subcellular metal distribution to understand how they are adapted for managing increased tissue doses of cadmium and lead. Whether they show genotoxic damage, which could have implications for reproduction, also needs to be investigated. Resident organisms may look healthy but a reduced reproductive capacity or gamete survival has implications for populations in the long term.

Complimentary exposure experiments looking at the effects of changes in salinity and temperature in the absence of metals are also needed to see if these factors also affect the biomarker responses. Climate change will have implications for temperature and salinity effects as they may influence biomarkers on their own but also have the potential to increase metal bioavailability. Confounding effects of contamination and climate change will need to be explored.

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11 Appendices

Appendix 1

Statistical analysis results tables for *Tellina deltoidalis*.

1. Tissue Metal Accumulation

Appendix 1.1: Mixed linear model ANOVA of tissue metal accumulation for collection day and treatment. Metal log_{10} for *T. deltoidalis* whole tissue.

		Cadmium		Lead		Selenium	
Source	**df**	**F**	**p**	**F**	**p**	**F**	**p**
Treatment	2	211	***	301	***	97	***
Day	4	32	***	6	***	2	ns
Day*Treatment	8	7	***	2	ns	8	***

*** $p < 0.0005$ ns $p > 0.05$

Appendix 1.2: Pair-wise comparisons, with Bonferroni adjustment for multiple comparisons, showing significant differences (p) between treatments. Metal log_{10} for *T. deltoidalis* whole tissue. None = day 0 unexposed organisms.

Treatments					
Cadmium	**p**	**Lead**	**p**	**Selenium**	**p**
none - control	ns	none - control	ns	none - control	ns
none - Cd 10	**	none - Pb 100	***	none - Se 5	**
none - Cd 50	***	none - Pb 300	***	none - Se 20	***
control - Cd 10	***	control - Pb 100	***	control - Se 5	***
control - Cd 50	***	control - Pb 300	***	control - Se 20	***
Cd 10 - Cd 50	***	Pb 100 - Pb 300	***	Se 5 - Se 20	ns

*** $p \leq 0.001$ ** $p \leq 0.01$ * $p \leq 0.05$ ns $p > 0.05$

Appendix 1.3: Pair-wise comparisons, with Bonferroni adjustment for multiple comparisons, showing significant differences (p) between treatments for each collection day. Metal $\log_{10}$ for *T. deltoidalis* whole tissue.

	Treatments					
Day	**Cadmium**	**p**	**Lead**	**p**	**Selenium**	**p**
3	Control - Cd 10	**	Control - Pb 100	***	Control - Se 5	ns
	Control - Cd 50	***	Control - Pb 300	***	Control - Se 20	***
	Cd 10 - Cd 50	***	Pb 100 - Pb 300	***	Se 5 - Se 20	ns
7	Control - Cd 10	***	Control - Pb 100	***	Control - Se 5	**
	Control - Cd 50	***	Control - Pb 300	***	Control - Se 20	***
	Cd 10 - Cd 50	***	Pb 100 - Pb 300	***	Se 5 - Se 20	ns
14	Control - Cd 10	***	Control - Pb 100	***	Control - Se 5	***
	Control - Cd 50	***	Control - Pb 300	***	Control - Se 20	***
	Cd 10 - Cd 50	***	Pb 100 - Pb 300	**	Se 5 - Se 20	ns
21	Control - Cd 10	***	Control - Pb 100	***	Control - Se 5	***
	Control - Cd 50	***	Control - Pb 300	***	Control - Se 20	***
	Cd 10 - Cd 50	***	Pb 100 - Pb 300	***	Se 5 - Se 20	ns
28	Control - Cd 10	***	Control - Pb 100	***	Control - Se 5	***
	Control - Cd 50	***	Control - Pb 300	***	Control - Se 20	***
	Cd 10 - Cd 50	***	Pb 100 - Pb 300	***	Se 5 - Se 20	ns

*** $p \leq 0.001$ ** $p \leq 0.01$ ns $p > 0.05$

Appendix 1.4: Pair-wise comparisons, with Bonferroni adjustment for multiple comparisons, showing significant differences (p) within treatments for each collection day. Metal log_{10} for *T. deltoidalis* whole tissue. Highlighted day comparisons are between collection times.

Days	Cadmium Treatments			Lead Treatments			Selenium Treatments		
	Control	Cd 10	Cd 50	Control	Pb 100	Pb 300	Control	Se 5	Se 20
	p	**p**	**p**	**p**	**p**	**p**	**p**	**p**	**p**
0 - 3	ns	*	***	ns	***	***	ns	**	***
0 - 7	ns	**	***	ns	***	***	ns	*	***
0 - 14	ns	**	***	ns	***	***	ns	**	***
0 - 21	ns	***	***	ns	***	***	ns	***	***
0 - 28	ns	***	***	ns	***	***	ns	***	***
3 - 7	ns	ns	*	ns	ns	ns	ns	ns	ns
3 - 14	ns	ns	***	ns	ns	ns	ns	ns	ns
3 - 21	ns	**	***	ns	ns	ns	ns	***	ns
3 - 28	ns	***	***	ns	ns	ns	***	**	ns
7 - 14	ns	ns	**	ns	ns	ns	ns	ns	ns
7 - 21	ns	*	***	ns	ns	ns	ns	***	ns
7 - 28	ns	***	***	ns	ns	ns	ns	**	ns
14 - 21	ns	ns	ns	ns	ns	***	ns	**	ns
14 - 28	ns	***	*	ns	ns	**	**	*	ns
21 - 28	ns	ns	ns	ns	ns	ns	ns	ns	ns

*** $p \leq 0.001$ ** $p \leq 0.01$ * $p \leq 0.05$ ns $p > 0.05$

2. Enzyme and Cellular Effects

Appendix 1.5: Mixed linear model ANOVA of effects for treatment of *T. deltoidalis* tissue. Lysosomes and micronuclei were calculated on arsin transformed data, all other effects are calculated on $\log_{10}$ transformed data. AOC: antioxidant capacity; GPx: glutathione peroxidase; GSH+2GSSG: total glutathione; GSH:GSSG: ratio of reduced to oxidised glutathione; TBARS: total thiobarbituric acid reactive substances; lysosomes: % unstable lysosomes; micronuclei: % micronucleus frequency.

Source

Treatment (df 2)	**Cadmium**		**Lead**		**Selenium**	
Effect	**F**	**p**	**F**	**p**	**F**	**p**
AOC	36	***	9.8	**	13	**
GPx	0.8	ns	2.4	ns	0.5	ns
GSH+2GSSG	3.5	ns	1.5	ns	1	ns
GSH:GSSG	7.2	*	3.4	*	10	**
TBARS	7.2	*	11	***	9	*
Lysosomes	23	***	53	***	227	***
Micronuclei	62	***	17	***	30	***

*** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$; ns $p > 0.05$

Appendix 1.6: Pair-wise comparisons, with Bonferroni adjustment for multiple comparisons, of effects for treatment of *T. deltoidalis* tissue. Lysosomes and micronuclei were calculated on arsin transformed data, all other effects are calculated on $\log_{10}$ transformed data. AOC: antioxidant capacity; GPx: glutathione peroxidase; GSH+2GSSG: total glutathione; GSH:GSSG: ratio of reduced to oxidised glutathione; TBARS: total thiobarbituric acid reactive substances; lysosomes: % unstable lysosomes; micronuclei: % micronucleus frequency.

	Effects Measures						
Treatments	**AOC**	**GPx**	**GSH+2GSSG**	**GSH:GSSG**	**TBARS**	**Lysosomes**	**Micronuclei**
Cadmium	**p**	**p**	**p**	**p**	**p**	**p**	**p**
Control - Cd 10	**	ns	ns	ns	ns	***	**
Control - Cd 50	***	ns	ns	*	*	***	***
Cd 10 - Cd 50	ns	ns	ns	ns	ns	ns	**
Lead	**p**	**p**	**p**	**p**	**p**	**p**	**p**
Control - Pb 100	*	ns	ns	ns	ns	***	**
Control - Pb 300	*	ns	ns	ns	***	***	***
Pb 100 - Pb 300	ns	ns	ns	ns	*	ns	ns
Selenium	**p**	**p**	**p**	**p**	**p**	**p**	**p**
Control - Se 5	**	ns	ns	*	*	***	**
Control - Se 20	**	ns	ns	*	*	***	***
Se 5 - Se 20	ns	ns	ns	ns	ns	***	**

*** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$; ns $p > 0.05$

Appendix 2

Statistical analysis results tables for *Anadara trapezia* spiked sediment studies.

1. Whole Organism Tissue Metal Accumulation

Appendix 2.1: Mixed linear model ANOVA of tissue metal accumulation for collection day and treatment. Metal $\log_{10}$ for *A. trapezia* whole organism tissue.

		Cadmium		Lead		Selenium	
Source	**df**	**F**	**p**	**F**	**p**	**F**	**p**
Treatment	2	178	***	96	***	227	***
Day	3	75	***	2	ns	4	**
Treatment*Day	6	14	***	2	*	1	ns

*** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$; ns $p > 0.05$

Appendix 2.2: Pair-wise comparisons, with Bonferroni adjustment for multiple comparisons, showing significant differences (p) between treatments. Metal $\log_{10}$ for *A. trapezia* whole organism tissue. None = day 0 unexposed organisms.

Treatments					
Cadmium	**p**	**Lead**	**p**	**Selenium**	**p**
none - control	ns	none - control	ns	none - control	ns
none - Cd 10	***	none - Pb 100	*	none - Se 5	***
none - Cd 50	***	none - Pb 300	***	none - Se 20	***
control - Cd 10	***	control - Pb 100	**	control - Se 5	***
control - Cd 50	***	control - Pb 300	***	control - Se 20	***
Cd 10 - Cd 50	*	Pb 100 - Pb 300	***	Se 5 - Se 20	**

*** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$; ns $p > 0.05$

Appendix 2.3: Pair-wise comparisons, with Bonferroni adjustment for multiple comparisons, showing significant differences (p) between treatments for each collection day. Metal $\log_{10}$ for *A. trapezia* whole organism tissue.

	Treatments					
Day	**Cadmium**	**p**	**Lead**	**p**	**Selenium**	**p**
14	Control - Cd 10	**	Control - Pb 100	ns	Control - Se 5	***
	Control - Cd 50	***	Control - Pb 300	***	Control - Se 20	***
	Cd 10 - Cd 50	**	Pb 100 - Pb 300	***	Se 5 - Se 20	***
28	Control - Cd 10	***	Control - Pb 100	**	Control - Se 5	***
	Control - Cd 50	***	Control - Pb 300	***	Control - Se 20	***
	Cd 10 - Cd 50	ns	Pb 100 - Pb 300	***	Se 5 - Se 20	***
42	Control - Cd 10	***	Control - Pb 100	ns	Control - Se 5	***
	Control - Cd 50	***	Control - Pb 300	***	Control - Se 20	***
	Cd 10 - Cd 50	*	Pb 100 - Pb 300	***	Se 5 - Se 20	ns
56	Control - Cd 10	***	Control - Pb 100	**	Control - Se 5	***
	Control - Cd 50	***	Control - Pb 300	***	Control - Se 20	***
	Cd 10 - Cd 50	*	Pb 100 - Pb 300	***	Se 5 - Se 20	***

*** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$; ns $p > 0.05$

Appendix 2.4: Pair-wise comparisons, with Bonferroni adjustment for multiple comparisons, showing significant differences (p) within treatments for each collection day. Metal $\log_{10}$ for *A. trapezia* whole tissue. Highlighted day comparisons are between 2 weekly collection times.

Days	**Cadmium Treatments**			**Lead Treatments**			**Selenium Treatments**		
	Control	Cd 10	Cd 50	Control	Pb 100	Pb 300	Control	Se 5	Se 20
	p	**p**	**p**	**p**	**p**	**p**	**p**	**p**	**p**
0 - 14	ns	ns	***	ns	ns	***	ns	***	***
0 - 28	ns	***	***	ns	ns	***	ns	***	***
0 - 42	ns	***	***	ns	ns	***	ns	***	***
0 - 56	ns	***	***	ns	*	***	ns	***	***
14 - 28	ns	***	***	ns	ns	ns	*	ns	ns
14 - 42	ns	***	***	ns	ns	ns	ns	ns	ns
14 - 56	ns	***	***	ns	ns	**	ns	ns	ns
28 - 42	ns	***	***	ns	ns	ns	ns	ns	ns
28 - 56	ns	**	***	ns	ns	*	***	ns	ns
42 - 56	ns	ns	ns	ns	ns	*	ns	ns	ns

*** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$; ns $p > 0.05$

2. Individual Tissues Metal Accumulation

Appendix 2.5: Repeated measures mixed linear ANOVA, of tissue metal accumulation for collection day, treatment and tissues. Metal $\log_{10}$ for *A. trapezia* individual tissues.

		Cadmium		Lead		Selenium	
Source	**df**	**F**	**p**	**F**	**p**	**F**	**p**
Treatment	2	192	***	166	***	259	***
Day	3	50	***	4	*	5	**
Tissue	2	740	***	52	***	314	***
Treatment*Day	6	9	***	3	*	1	ns
Treatment*Tissue	4	6	***	18	***	11	***
Day*Tissue	6	4	***	6	***	3	*
Treatment*Day*Tissue	12	3	**	3	**	1	ns

*** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$; ns $p > 0.05$

Appendix 2.6: Pair-wise comparisons, with Bonferroni adjustment for multiple comparisons, showing significant differences (p) within treatments between tissues. Metal $\log_{10}$ for *A. trapezia* individual tissues. None = day 0 unexposed organisms.

Tissues	**Cadmium Treatments**			
	None	Control	Cd 10	Cd 50
	p	**p**	**p**	**p**
Gill - Hepatopancreas	***	***	***	***
Gill - Haemolymph	***	***	***	***
Hepatopancreas - Haemolymph	***	***	***	***
	Lead Treatments			
	None	Control	Pb 100	Pb 300
	p	**p**	**p**	**p**
Gill - Hepatopancreas	ns	ns	***	***
Gill - Haemolymph	ns	ns	ns	***
Hepatopancreas - Haemolymph	ns	ns	**	***
	Selenium Treatments			
	None	Control	Se 5	Se 20
	p	**p**	**p**	**p**
Gill - Hepatopancreas	ns	***	***	***
Gill - Haemolymph	***	***	***	***
Hepatopancreas - Haemolymph	***	***	*	ns

*** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$; ns $p > 0.05$

Appendix 2.7: Pair-wise comparisons, with Bonferroni adjustment for multiple comparisons, showing significant differences (p) between tissues for each treatment at each collection day. Metal log_{10} for *A. trapezia* individual tissues. None = day 0 unexposed organisms.

Day	Tissue	Treatment	p	Treatment	p	Treatment	p
0	Gill - Hepatopancreas	None	***	None	ns	None	ns
	Gill - Haemolymph		***		ns		***
	Hepatopancreas - Haemolymph		***		ns		***
14	Gill - Hepatopancreas	Cd Control	***	Pb Control	ns	Se Control	***
	Gill - Haemolymph		***		ns		***
	Hepatopancreas - Haemolymph		***		ns		***
	Gill - Hepatopancreas	Cd 10 µg/g	***	Pb 100 µg/g	*	Se 5 µg/g	***
	Gill - Haemolymph		***		ns		***
	Hepatopancreas - Haemolymph		***		*		ns
	Gill - Hepatopancreas	Cd 50 µg/g	***	Pb 300 µg/g	***	Se 20 µg/g	***
	Gill - Haemolymph		***		ns		**
	Hepatopancreas - Haemolymph		ns		***		ns
28	Gill - Hepatopancreas	Cd Control	***	Pb Control	ns	Se Control	***
	Gill - Haemolymph		***		ns		***
	Hepatopancreas - Haemolymph		***		ns		***
	Gill - Hepatopancreas	Cd 10 µg/g	***	Pb 100 µg/g	ns	Se 5 µg/g	***
	Gill - Haemolymph		***		ns		***
	Hepatopancreas - Haemolymph		***		ns		*
	Gill - Hepatopancreas	Cd 50 µg/g	***	Pb 300 µg/g	***	Se 20 µg/g	**
	Gill - Haemolymph		***		ns		***
	Hepatopancreas - Haemolymph		***		***		ns
42	Gill - Hepatopancreas	Cd Control	***	Pb Control	ns	Se Control	***
	Gill - Haemolymph		***		ns		***
	Hepatopancreas - Haemolymph		***		ns		**
	Gill - Hepatopancreas	Cd 10 µg/g	***	Pb 100 µg/g	**	Se 5 µg/g	**
	Gill - Haemolymph		***		ns		***
	Hepatopancreas - Haemolymph		***		ns		ns
	Gill - Hepatopancreas	Cd 50 µg/g	***	Pb 300 µg/g	***	Se 20 µg/g	***
	Gill - Haemolymph		***		ns		***
	Hepatopancreas - Haemolymph		***		ns		ns
56	Gill - Hepatopancreas	Cd Control	***	Pb Control	ns	Se Control	***
	Gill - Haemolymph		***		ns		***
	Hepatopancreas - Haemolymph		***		ns		***
	Gill - Hepatopancreas	Cd 10 µg/g	***	Pb 100 µg/g	***	Se 5 µg/g	***
	Gill - Haemolymph		***		ns		***
	Hepatopancreas - Haemolymph		***		ns		ns
	Gill - Hepatopancreas	Cd 50 µg/g	***	Pb 300 µg/g	***	Se 20 µg/g	***
	Gill - Haemolymph		***		***		***
	Hepatopancreas - Haemolymph		***		**		ns

*** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$; ns $p > 0.05$

Appendix 2.8: Pair-wise comparisons, with Bonferroni adjustment for multiple comparisons, showing significant differences (p) within tissues for each treatment at each collection day. Metal $\log_{10}$ for *A. trapezia* individual tissues. Yellow highlighted day comparisons are between 2 weekly collection times.

Tissue	Day	Cadmium Treatments			Lead Treatments			Selenium Treatments		
		Control	Cd 10	Cd 50	Control	Pb 100	Pb 300	Control	Se 5	Se 20
		p	p	p	p	p	p	p	p	p
Gill	0 - 14	ns	ns	***	ns	ns	***	ns	***	***
	0 - 28	ns	***	***	ns	ns	***	ns	***	***
	0 - 42	ns	***	***	ns	*	***	ns	***	***
	0 - 56	ns	***	***	ns	**	***	ns	***	***
	14 - 28	ns	***	***	ns	ns	ns	**	ns	ns
	14 - 42	ns	***	***	ns	ns	ns	ns	ns	ns
	14 - 56	ns	***	***	ns	ns	***	ns	ns	ns
	28 - 42	ns	**	***	ns	ns	ns	ns	ns	ns
	28 - 56	ns	**	***	ns	**	***	ns	ns	ns
	42 - 56	ns	ns	ns	ns	ns	***	ns	ns	ns
Hepatopancreas	0 - 14	ns	ns	***	ns	ns	***	ns	ns	***
	0 - 28	ns	***	***	ns	ns	***	ns	**	***
	0 - 42	ns	***	***	ns	ns	***	ns	**	***
	0 - 56	ns	***	***	ns	ns	***	ns	ns	***
	14 - 28	ns	***	**	ns	ns	ns	ns	ns	ns
	14 - 42	ns	***	***	ns	ns	ns	ns	ns	ns
	14 - 56	ns	***	***	ns	ns	***	ns	ns	ns
	28 x 42	ns	ns	**	ns	ns	ns	ns	ns	ns
	28 - 56	ns	ns	**	ns	ns	***	ns	ns	ns
	42 - 56	ns	ns	ns	ns	ns	***	ns	ns	ns
Haemolymph	0 - 14	ns	ns	***	ns	ns	***	ns	***	***
	0 - 28	ns	***	***	ns	ns	***	ns	***	***
	0 - 42	ns	***	***	ns	ns	***	ns	***	***
	0 - 56	ns	***	***	ns	ns	***	ns	***	***
	14 - 28	ns	**	ns	ns	ns	ns	ns	ns	ns
	14 - 42	*	***	ns	ns	ns	ns	ns	ns	ns
	14 - 56	ns	***	ns	ns	ns	ns	ns	ns	ns
	28 - 42	ns	ns	*	ns	ns	ns	ns	ns	ns
	28 - 56	ns	ns	*	ns	ns	ns	ns	ns	ns
	42 - 56	*	ns	ns	ns	ns	ns	ns	ns	ns

*** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$; ns $p > 0.05$

3. Enzyme and Cellular Effects

Appendix 2.9: Mixed linear model ANOVA of effects for treatment of *A. trapezia* tissue. Lysosomes and micronuclei were calculated on arsine transformed data, all other effects are calculated on $\log_{10}$ transformed data. TAOC: total antioxidant capacity; GPx: glutathione peroxidase; GSH+2GSSG: total glutathione; GSH:GSSG: ratio of reduced to oxidised glutathione; TBARS: thiobarbituric acid reactive substances; lysosomes: % unstable lysosomes; micronuclei: % micronucleus frequency.

Source	**Treatment (df 2)**					
	Cadmium		**Lead**		**Selenium**	
Effects	**F**	**p**	**F**	**p**	**F**	**p**
AOC	12	***	14	***	18	**
GPx	1	ns	5	**	2.7	ns
GSH+2GSSG	0.6	ns	2.4	ns	2.3	ns
GSH:GSSG	8	**	8	**	10	***
TBARS	1.8	ns	4.1	*	2.7	*
Lysosomes	17	**	16	***	77	***
Micronuclei	20	**	17	**	23	***

*** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$; ns $p > 0.05$

Appendix 2.10: Pair-wise comparisons, with Bonferroni adjustment for multiple comparisons, of effects for treatment of *A. trapezia* tissue. Lysosomes and micronuclei were calculated on arsine transformed data all other effects are calculated on $\log_{10}$ transformed data. TAOC: total antioxidant capacity; GPx: glutathione peroxidase; GSH+2GSSG: total glutathione; GSH:GSSG: ratio of reduced to oxidised glutathione; TBARS: thiobarbituric acid reactive substances; lysosomes: % unstable lysosomes; micronuclei: % micronucleus frequency.

	Effects Measures						
Treatments	**AOC**	**GPx**	**GSH+2GSSG**	**GSH:GSSG**	**TBARS**	**Lysosomes**	**Micronuclei**
Cadmium	**p**	**p**	**p**	**p**	**p**	**p**	**p**
Control - Cd 10	**	ns	ns	ns	ns	*	*
Control - Cd 50	***	ns	ns	**	ns	**	**
Cd 10 - Cd 50	ns	ns	ns	ns	ns	ns	ns
Lead	**p**	**p**	**p**	**p**	**p**	**p**	**p**
Control - Pb 100	**	**	ns	**	*	*	**
Control - Pb 300	***	ns	ns	**	*	***	**
Pb 100 - Pb 300	ns	ns	ns	ns	ns	ns	ns
Selenium	**p**	**p**	**p**	**p**	**p**	**p**	**p**
Control - Se 5	**	ns	ns	**	*	***	**
Control - Se 20	**	ns	ns	***	*	***	***
Se 5 - Se 20	ns	ns	ns	ns	ns	**	*

*** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$; ns $p > 0.05$

Appendix 3

Statistical analysis results tables for *Anadara trapezia* Lake Macquarie sediment studies.

1. Sediment Metal Concentrations

Appendix 3.1: ANOVA, mixed linear model with Bonferroni adjustment for multiple comparisons, of sediment metal at each site and of *A. trapezia* whole organism tissue metal accumulation for each collection day and site. Metal $\log_{10}$.

		Sediments	**Tissues**		
	Source	Site	Site	Day	Site*Day
	df	4	2	3	6
Cadmium	**F**	303	20	44	0.4
	p	***	**	***	ns
Lead	**F**	180	46	20	1
	p	***	***	***	ns
Selenium	**F**	184	0.07	42	2
	p	***	ns	***	ns
Zinc	**F**	165	27	36	1
	p	***	***	***	ns
Copper	**F**	137	0.4	27	0.7
	p	***	ns	***	ns

*** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$; ns $p > 0.05$

Appendix 3.2: Pair-wise comparisons, with Bonferroni adjustment for multiple comparisons, showing significant differences (p) between sites. Metal $\log_{10}$ for sediment metal concentrations. Ref = unexposed organism collection sites, CC1 = Cockle Ck. Site 1, CC2 = Cockle Ck. Site 2, CB = Cockle Bay site, LM = Lake Macquarie native metal exposed *A. trapezia* collection site.

	Cadmium	**Lead**	**Selenium**	**Zinc**	**Copper**
Sites	**p**	**p**	**p**	**p**	**p**
Ref - CC1	***	***	***	***	***
Ref - CC2	***	***	***	**	***
Ref - CB	*	***	ns	*	***
Ref - LM	**	***	***	***	***
CC1 - CC2	ns	**	**	*	***
CC1 - CB	**	***	***	**	***
CC1 - LM	***	***	***	ns	ns
CC2 - CB	ns	*	**	ns	ns
CC2 -LM	**	*	ns	***	***
CB - LM	ns	**	**	***	***

*** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$; ns $p > 0.05$

2. Whole Organism Tissue Metal Accumulation

Appendix 3.3: Pair-wise comparisons, with Bonferroni adjustment for multiple comparisons, showing significant differences (p) in metal concentration between sites. Metal $\log_{10}$ for *A. trapezia* whole tissue. Ref = day 0 unexposed organisms, CC1 = Cockle Ck. Site 1, CC2 = Cockle Ck. Site 2, CB = Cockle Bay site.

	Cadmium	Lead	Selenium	Zinc	Copper
Sites	**p**	**p**	**p**	**p**	**p**
Ref - CC1	***	***	ns	***	***
Ref - CC2	***	***	ns	**	***
Ref - CB	*	***	ns	*	***
CC1 - CC2	ns	**	ns	*	ns
CC1 - CB	**	***	ns	**	ns
CC2 - CB	ns	*	ns	ns	ns

*** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$; ns $p > 0.05$

Appendix 3.4: Pair-wise comparisons, with Bonferroni adjustment for multiple comparisons, showing significant differences (p) in metal concentration between sites for each collection day. Metal $\log_{10}$ for *A. trapezia* whole tissue. CC1 = Cockle Ck. Site 1, CC2 = Cockle Ck. Site 2, CB = Cockle Bay site.

		Cadmium	Lead	Selenium	Zinc	Copper
Day	**Sites**	**p**	**p**	**p**	**p**	**p**
14	CC1 - CC2	ns	**	ns	ns	ns
	CC1 - CB	**	***	ns	**	ns
	CC2 - CB	ns	ns	ns	ns	ns
28	CC1 - CC2	ns	**	ns	*	ns
	CC1 - CB	**	***	ns	***	ns
	CC2 - CB	ns	**	ns	ns	ns
42	CC1 - CC2	ns	ns	ns	ns	ns
	CC1 - CB	***	***	ns	***	ns
	CC2 - CB	*	***	ns	**	ns
56	CC1 - CC2	ns	**	ns	*	ns
	CC1 - CB	***	***	ns	**	ns
	CC2 - CB	*	ns	ns	ns	ns

*** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$; ns $p > 0.05$

Appendix 3.5: Pair-wise comparisons, with Bonferroni adjustment for multiple comparisons, showing significant differences (p) in metal concentration within sites for each collection day. Metal $\log_{10}$ for *A. trapezia* whole tissue. CC1 = Cockle Ck. Site 1, CC2 = Cockle Ck. Site 2, CB = Cockle Bay site. Yellow highlighted day comparisons are between 2 weekly collection times.

Days	**Cadmium**			**Lead**			**Selenium**			**Zinc**			**Copper**		
	CC1	CC2	CB	CC1	CC2	CB	CC1	CC2	CB	CC1	CC2	CB	CC1	CC2	CB
	p	**p**	**p**	**p**	**p**	**p**	**p**	**p**	**p**	**p**	**p**	**p**	**p**	**p**	**p**
0 x 14	**	ns	ns	***	***	*	ns	ns	ns	*	ns	ns	ns	ns	ns
0 x 28	***	**	ns	***	***	***	ns	ns	*	***	**	ns	ns	ns	ns
0 x 42	***	***	**	***	***	***	ns	*	*	***	***	*	***	***	***
0 x 56	***	***	***	***	***	***	**	*	ns	***	***	***	***	***	***
14 x 28	ns	**	ns	*	*	ns	*	**	***	**	**	ns	ns	ns	ns
14 x 42	***	***	***	**	***	*	***	***	***	***	***	**	***	***	*
14 x 56	***	***	***	**	***	***	***	***	***	***	***	***	***	***	ns
28 x 42	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	ns	*	***	**
28 x 56	***	**	**	ns	ns	*	**	ns	ns	ns	ns	**	ns	*	**
42 x 56	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

*** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$; ns $p > 0.05$

3. Individual Tissue Metal Accumulation

Appendix 3.6: Repeated measures mixed linear ANOVA, of tissue metal accumulation for collection day, site and tissue type. Metal $\log_{10}$ for *A. trapezia* individual tissues.

		Cadmium		Lead		Selenium		Zinc		Copper	
Source	**df**	**F**	**p**	**F**	**p**	**F**	**p**	**F**	**p**	**F**	**p**
Site	2	26	***	47	***	0.1	ns	17	**	0.4	ns
Day	3	42	***	27	***	49	***	45	***	26	***
Tissue	2	491	***	55	***	67	***	111	***	332	***
Site*Day	6	0.6	ns	0.7	ns	2	ns	1.2	ns	0.7	ns
Site*Tissue	4	6	***	1.4	ns	0.7	ns	3.3	**	0.5	ns
Day*Tissue	6	2	ns	2	ns	7	***	3.5	**	7	***
Site*Day*Tissue	12	2	ns	1	ns	0.5	ns	1.2	ns	0.7	ns

*** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$; ns $p > 0.05$

Appendix 3.7: Pair-wise comparisons, with Bonferroni adjustment for multiple comparisons, showing significant differences (p) within sites between tissue types. Metal log_{10} for *A. trapezia* individual tissues. Ref = day 0 unexposed organisms, CC1 = Cockle Ck. Site 1, CC2 = Cockle Ck. Site 2, CB = Cockle Bay site.

Tissues	**Sites**			
	Cadmium			
	Ref	CC1	CC2	CB
	p	**p**	**p**	**p**
Gill x Hepatopancreas	**	***	***	***
Gill x Haemolymph	***	***	***	***
Hepatopancreas x Haemolymph	**	***	***	***
	Lead			
	Ref	CC1	CC2	CB
	p	**p**	**p**	**p**
Gill x Hepatopancreas	ns	***	***	***
Gill x Haemolymph	ns	*	**	***
Hepatopancreas x Haemolymph	ns	***	**	***
	Selenium			
	Ref	CC1	CC2	CB
	p	**p**	**p**	**p**
Gill x Hepatopancreas	ns	***	***	***
Gill x Haemolymph	***	***	***	***
Hepatopancreas x Haemolymph	ns	ns	ns	ns
	Zinc			
	Ref	CC1	CC2	CB
	p	**p**	**p**	**p**
Gill x Hepatopancreas	*	***	***	***
Gill x Haemolymph	***	***	***	***
Hepatopancreas x Haemolymph	***	ns	***	ns
	Copper			
	Ref	CC1	CC2	CB
	p	**p**	**p**	**p**
Gill x Hepatopancreas	*	***	***	***
Gill x Haemolymph	ns	ns	ns	ns
Hepatopancreas x Haemolymph	**	***	***	***

*** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$; ns $p > 0.05$

Appendix 3.8: Pair-wise comparisons, with Bonferroni adjustment for multiple comparisons, showing significant differences (p) between tissue types for each site on each collection day. Metal $\log_{10}$ for *A. trapezia* individual tissues. Ref = day 0 unexposed organisms, CC1 = Cockle Ck. Site 1, CC2 = Cockle Ck. Site 2, CB = Cockle Bay site.

		Cadmium		Lead		Selenium		Zinc		Copper	
Day	**Tissue**	**Site**	**p**	**Site**	**p**	**Site**	**p**	**Site**	**p**	**Site**	**p**
0	Gill x Hepatopancreas	Ref	**	Ref	ns	Ref	ns	Ref	*	Ref	*
	Gill x Haemolymph		***		ns		***		***		ns
	Hepatopancreas x Haemolymph		**		ns		ns		***		**
14	Gill x Hepatopancreas	CC1	***	CC1	**	CC1	***	CC1	**	CC1	***
	Gill x Haemolymph		***		ns		***		***		ns
	Hepatopancreas x Haemolymph		ns		*		ns		*		***
	Gill x Hepatopancreas	CC2	**	CC2	**	CC2	***	CC2	ns	CC2	***
	Gill x Haemolymph		***		ns		***		***		ns
	Hepatopancreas x Haemolymph		***		*		*		***		***
	Gill x Hepatopancreas	CB	***	CB	***	CB	***	CB	*	CB	***
	Gill x Haemolymph		***		ns		***		*		ns
	Hepatopancreas x Haemolymph		**		***		ns		ns		***
28	Gill x Hepatopancreas	CC1	***	CC1	*	CC1	ns	CC1	***	CC1	***
	Gill x Haemolymph		***		ns		ns		***		ns
	Hepatopancreas x Haemolymph		ns		*		ns		ns		***
	Gill x Hepatopancreas	CC2	***	CC2	***	CC2	ns	CC2	***	CC2	***
	Gill x Haemolymph		***		**		**		***		ns
	Hepatopancreas x Haemolymph		**		ns		ns		**		***
	Gill x Hepatopancreas	CB	***	CB	***	CB	ns	CB	***	CB	***
	Gill x Haemolymph		***		**		ns		***		ns
	Hepatopancreas x Haemolymph		***		ns		ns		ns		***
42	Gill x Hepatopancreas	CC1	***	CC1	*	CC1	ns	CC1	**	CC1	***
	Gill x Haemolymph		***		ns		ns		**		ns
	Hepatopancreas x Haemolymph		*		ns		ns		ns		***
	Gill x Hepatopancreas	CC2	***	CC2	**	CC2	ns	CC2	***	CC2	***
	Gill x Haemolymph		***		ns		ns		***		ns
	Hepatopancreas x Haemolymph		*		ns		ns		ns		***
	Gill x Hepatopancreas	CB	***	CB	***	CB	**	CB	**	CB	***
	Gill x Haemolymph		***		**		ns		***		ns
	Hepatopancreas x Haemolymph		***		ns		ns		ns		***
56	Gill x Hepatopancreas	CC1	***	CC1	**	CC1	*	CC1	***	CC1	***
	Gill x Haemolymph		***		ns		ns		*		ns
	Hepatopancreas x Haemolymph		***		ns		ns		ns		***
	Gill x Hepatopancreas	CC2	**	CC2	ns	CC2	ns	CC2	ns	CC2	***
	Gill x Haemolymph		***		ns		ns		**		ns
	Hepatopancreas x Haemolymph		***		ns		ns		ns		***
	Gill x Hepatopancreas	CB	***	CB	***	CB	ns	CB	***	CB	***
	Gill x Haemolymph		***		ns		ns		ns		ns
	Hepatopancreas x Haemolymph		***		*		ns		ns		***

*** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$; ns $p > 0.05$

Appendix 3.9: Pair-wise comparisons, with Bonferroni adjustment for multiple comparisons, showing significant differences (p) within tissue type for each site on each collection day. Metal $\log_{10}$ for *A. trapezia* individual tissues. CC1 = Cockle Ck. Site 1, CC2 = Cockle Ck. Site 2, CB = Cockle Bay site. Colour highlighted day comparisons are between 2 weekly collection times.

Tissue	Day	Cadmium			Lead			Selenium			Zinc			Copper		
		CC1	CC2	CB	CC1	CC2	CB	CC1	CC2	CB	CC1	CC2	CB	CC1	CC2	CB
		p	p	p	p	p	p	p	p	p	p	p	p	p	p	p
Gill	0 x 14	**	ns	ns	***	**	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	0 x 28	***	***	**	***	***	**	ns	ns	*	***	*	ns	ns	ns	ns
	0 x 42	***	***	***	***	***	**	*	**	**	***	***	ns	***	***	***
	0 x 56	***	***	***	***	***	***	**	ns	*	***	*	ns	***	***	***
	14 x 28	ns	**	*	ns	**	ns	ns	ns	***	*	**	ns	ns	ns	ns
	14 x 42	***	***	***	**	***	**	**	***	***	*	***	*	***	***	**
	14 x 56	***	***	***	**	**	***	***	**	***	***	***	***	***	**	ns
	28 x 42	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	***	***
	28 x 56	***	**	*	ns	ns	ns	***	ns	ns	ns	ns	ns	*	ns	**
	42 x 56	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Hepatopancreas	0 x 14	ns	ns	***	***	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	0 x 28	***	*	***	***	**	**	ns	ns	*	**	ns	ns	ns	ns	ns
	0 x 42	***	***	***	***	***	**	ns	**	ns	***	**	**	**	**	ns
	0 x 56	***	***	***	***	**	***	**	*	*	***	**	***	ns	*	ns
	14 x 28	ns	ns	ns	ns	ns	*	*	**	***	**	ns	ns	ns	ns	ns
	14 x 42	ns	ns	ns	**	**	**	**	***	***	***	***	**	*	**	ns
	14 x 56	ns	ns	ns	**	***	***	***	***	***	***	***	***	ns	**	ns
	28 x 42	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	**	**
	28 x 56	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	**	ns	**	**
	42 x 56	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Haemolymph	0 x 14	**	ns	ns	***	ns	ns	ns	ns	ns	**	ns	ns	ns	ns	ns
	0 x 28	***	**	ns	***	**	ns	*	ns	**	***	**	**	**	ns	ns
	0 x 42	***	***	ns	***	***	ns	**	***	***	***	***	***	***	***	***
	0 x 56	***	***	*	***	**	*	***	***	**	***	***	***	***	***	***
	14 x 28	ns	ns	ns	*	ns	ns	*	**	***	**	ns	ns	ns	ns	ns
	14 x 42	ns	ns	ns	*	***	ns	**	***	***	***	***	ns	***	***	*
	14 x 56	ns	ns	ns	*	*	**	***	***	***	***	***	***	**	***	ns
	28 x 42	ns	ns	ns	ns	ns	ns	ns	*	ns	ns	**	ns	ns	**	ns
	28 x 56	ns	ns	ns	ns	ns	**	ns	ns	ns	*	**	**	ns	ns	ns
	42 x 56	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

*** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$; ns $p > 0.05$

4. Enzyme and Cellular Effects

Appendix 3.10: Mixed linear model ANOVA of effects for site of *A. trapezia* tissue. Lysosomes (% unstable) and micronuclei (% frequency) are calculated on arsine transformed data; TAOC (total antioxidant capacity) and TBARS (thiobarbituric acid reactive substances), are calculated on log_{10} transformed data.

Source	**Site**		
Effects	**df**	**F**	**p**
TAOC	4	14	***
TBARS	4	6.4	***
Lysosomes	4	19	***
Micronuclei	3	68	***

*** $p \leq 0.001$

Appendix 3.11: Pair-wise comparisons, with Bonferroni adjustment for multiple comparisons, of effects between sites for *A. trapezia* tissue. Lysosomes (% unstable) and micronuclei (% frequency) are calculated on arsine transformed data; TAOC (total antioxidant capacity) and TBARS (thiobarbituric acid reactive substances), are calculated on log_{10} transformed data. Ref = unexposed organisms, CC1 = Cockle Ck. Site 1, CC2 = Cockle Ck. Site 2, CB = Cockle Bay site, LM = Lake Macquarie native metal exposed *A. trapezia*.

	Effects Measures			
Sites	**TAOC**	**TBARS**	**Lysosomes**	**Micronuclei**
	p	**p**	**p**	**p**
Ref - CC1	***	***	***	***
Ref - CC2	**	***	***	***
Ref - CB	*	ns	**	***
Ref - LM	**	*	ns	--
CC1 - CC2	***	ns	ns	**
CC1 - CB	***	ns	**	***
CC1 - LM	***	ns	***	--
CC2 - CB	ns	ns	ns	**
CC2 - LM	ns	ns	***	--
CB - LM	ns	ns	ns	--

*** $p \leq 0.001$; ** $p \leq 0.01$; * $p \leq 0.05$; ns $p > 0.05$; -- no data