Exposure-dose-response of *Anadara trapezia* to metal contaminated estuarine sediments

1. Cadmium spiked sediments

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Abstract

The relationship between cadmium exposure dose and response was investigated in *Anadara trapezia* exposed to cadmium spiked sediment (10 µg/g and 50 µg/g dry mass) for 56 days. *A. trapezia* reached an equilibrium cadmium tissue concentration (13 µg/g and 25 µg/g respectively) by day 42. Gills accumulated significantly more cadmium than the hepatopancreas and haemolymph. After 56 days exposure between 46 to 73% of accumulated gill and hepatopancreas cadmium was detoxified and in the metallothionein like protein fraction. Approximately half of the biologically active cadmium in both tissues was in the mitochondrial fraction which has the potential to cause dysfunction in mitochondrial activity. Cadmium exposed *A. trapezia* generally had reduced GPx activity with an associated increase in total glutathione concentrations and reduced GSH:GSSG ratios due to a build up of oxidised glutathione. The changes in the glutathione pathway were reflected in the Total Antioxidant Capacity of cadmium exposed *A. trapezia* which were significantly reduced compared to 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. The significant exposure – dose – response relationship for *A.trapezia* exposed to cadmium enriched sediments indicates that elevated sediment cadmium concentrations have the potential to lead to increased biologically active cadmium burdens and impairment of individual *A. trapezia* at cellular and subcellular levels.

Keywords: Biomarkers, Biologically active metal, Biologically detoxified metal, Oxidative stress, Lipid peroxidation, Lysosomal stability, Micronuclei

1. Introduction

In Australia, 90% of the population lives in coastal areas and most industry is situated near coastal bays and estuaries (ABS, 1996). Metals released into aquatic systems bind to particles and accumulate in estuarine sediments, which become the main repositories and therefore potential sources of contaminants to sediment dwelling organisms such as molluscs (Byrne and O'Halloran, 2001). Common metals released in significant quantities include cadmium, lead and selenium from metal refining and power generation activities (Peters *et al.*, 1999b; Roach, 2005). Burrowing and feeding by benthic organisms resuspends and releases contaminants, increasing their biological availability (Atkinson *et al.*, 2007; Peters *et al.*, 1999a). Cadmium is a metal which is accumulated in high concentrations by a range of marine organisms and has no known biological function (ECB, 2007) but at low concentrations may adversely affect cellular function (Ercal *et al.*, 2001; Sokolova, 2004; Sokolova *et al.*, 2004).

Physiological effects of metals strongly depend on their intracellular localisation and binding within organelles (Sokolova *et al.*, 2005). 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.*, 2005), energetic changes (Cherkasov *et al.*, 2006; Chora *et al.*, 2009) and ultimately impairment of growth, reproduction and survival (Weis *et al.*, 2001). Cadmium also causes general oxidative stress, resulting in cellular and genotoxic damage (Stegeman *et al.*, 1992; van der Oost *et al.*, 2003; Winston and Di Giulio, 1991).

In Australia, cadmium concentrations up to 50 µg/g dry mass have been reported in contaminated Australian estuaries (Roach, 2005). The Australian guidelines for Fresh and Marine Water Quality (ANZECC and ARMCANZ, 2000) interim sediment quality high effects guideline for cadmium is 10 µg/g dry mass. To establish relationships between sediment

cadmium exposure, organism dose and biological response for benthic dwelling molluscs, a common benthic marine bivalve mollusc, *Anadara trapezia* was exposed to different concentrations of cadmium spiked sediments in laboratory aquaria for fifty six days and compared to unexposed organisms. The concentrations of cadmium chosen for the sediment spiking were the high sediment quality guideline concentration (10 µg/g dry mass) and the highest sediment cadmium concentrations previously measured in contaminated estuarine sediments (50 µg/g dry mass). Organism internal exposure was measured by total cadmium burden while internal doses were examined by subcellular fractionation of whole tissues to determine what fraction of the total metal taken up was stored in a metabolically available form. Oxidative stress was determined by measuring total antioxidant scavenging capacity of cells, cellular concentrations of oxidised and reduced glutathione, glutathione peroxidase activity and the extent of lipid peroxidation. Cellular damage was determined by measuring lysosomal membrane stability and a micronucleus assay used to assess genotoxic damage.

2. Materials and Methods

2.1. Sediment and Anadara trapezia collection

Sediments were collected from a NSW Department of Environmental and Climate Change reference site in Durras Lake NSW. *A. trapezia* used in the laboratory exposure experiments were collected from Burrill Lake, an uncontaminated estuary on the south coast of NSW (Chenhall *et al.*, 1992; Jones *et al.*, 2003). Cadmium was measured in five organisms prior to the exposure to establish background tissue cadmium concentrations, mean tissue cadmium concentration was 1.10 ± 0.25 µg/g. *A. trapezia* were transported in coolers with sediment and water from the collection sites and maintained for a maximum of two weeks, in clean sediments, 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 ‰ with deionised water to match the salinity of the estuarine water from which organisms were collected.

2.2. Sediment spiking

Sediments were sieved through a 2 mm stainless steel sieve to remove large pieces of organic matter and organisms prior to the addition of cadmium. Sub samples of the collected sediments were measured for moisture content and grain size. To create a sediment matrix suitable for organism burrowing and feeding, fine sediment was mixed with clean beach sand so that the 63 µm fraction was not greater than 20 % mass/mass. Wet sediment was added to glass mixing containers and, $CdCl_2$, (AR grade Merck), added to a concentration of 0, 10 and 50 mg/kg dry mass of sediment. To ensure added cadmium was rapidly adsorbed and strongly bound to the sediment particles spiking was done under nitrogen, following a method developed by (Simpson *et al.*, 2004). To determine when the added $CdCl_2$ was completely bound to sediment particles, pore waters were collected, acidified to 1 % (v/v) with nitric acid (AristaR, BDH) and cadmium measured using an ELAN® 6000 ICP-MS (PerkinElmer SCIEX). Once pore water cadmium concentrations had fallen below instrument detection limits (0.001 µg/L) the sediment was ready for use. Time to full adsorption was 4 to 6 weeks. Sediment cadmium concentrations were

measured prior to and at the end of the 56 exposure period, concentrations were < 0.001, 10.0 ± 0.1 and 50 ± 1 µg/L on each occasion.

2.3. *Microcosm experiments*

Procedures for conducting the exposures were adapted from methods recommended for conducting sediment bioaccumulation tests (Ingersoll *et al.*, 2000). Sediment (1000 g wet wt.) was placed in each of 3 replicate 12 L polystyrene aquariums per treatment. Containers were filled with seawater adjusted to a salinity of 30 ‰ and placed in random order in a water bath 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 *A. trapezia* were added to each treatment aquarium. Organisms were fed daily with a commercial powdered complete food suitable for marine bivalves (Sera Micron, Germany), made up in seawater. Half water changes were done twice weekly. Samples of aquaria waters were collected twice weekly, acidified to 1 % (v/v) with nitric acid (AristaR, BDH) and cadmium measured using an ELAN® 6000 ICP-MS (PerkinElmer SCIEX). Cadmium concentrations were below instrument detection limits (0.001 µg/L) at all measurement times. Organisms were exposed for 56 days. Cadmium tissue accumulation was measured in haemolymph, gill and hepatopancreas of 2 organisms from each treatment replicate at 14 day intervals to investigate the pattern of cadmium accumulation over time. Cadmium subcellular distribution and biomarker assays were measured after 56 days to determine end point effects.

2.4. *Cadmium Measurements*

2.4.1. *Total cadmium*

Lyophilised ground tissue was microwave digested in nitric acid (AristaR BDH) in a 630 W microwave oven (CEM MDS-2000) for 2 min at 630 W, 2 min at 0 W, and 45 min at 315 W (Baldwin *et al.*, 1994). Cadmium concentrations were measured using an ELAN® 6000 ICP-MS (PerkinElmer, SCIEX) (Maher *et al.*, 2001). 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. The measured cadmium

values for CRMs: (0.22 ± 0.10 and 4.4 ± 0.6 μg/g respectively) were in good agreement with certified values (0.25 ± 0.04 and 4.15 ± 0.38 μg/g respectively).

2.4.2. Subcellular Cadmium

The subcellular tissue cadmium distribution was examined in gill and hepatopancreas tissues of day 56 exposed *A. trapezia* using a procedure adapted from Soklova *et al.*, (2005) and Wallace *et al.*, (2003). The dissected tissues were placed in polypropylene vials, snap frozen in liquid nitrogen and stored at -80°C until processed. The tissue was homogenised in Ca^{2+} / Mg^{2+} free saline buffer on ice using an IKA® Labortechnick Ultra-turrax-T25 homogeniser equipped with an S25-UT dispersing tool at 9,500 rpm (Janke & Kunkel). Homogenised tissue was subjected to differential centrifugation and tissue digestion procedures according to the protocol outlined in Taylor and Maher (2010) using an Eppendorf 5804R centrifuge and a Himac CP90WX preparative ultracentrifuge (Hitachi, Japan). Two organelle pellets, mitochondria (P3) and lysosomes and microsomes (P4) and one enzyme pellet, heat sensitive proteins (P5), were grouped as biologically active metal (BAM) fractions while the granule pellet, (P2) and final supernatant, containing heat stable metallothionein like proteins (S5) were grouped as biologically detoxified metal (BDM) fractions. The supernatant, (S2) contained the nuclei and cellular debris (Wallace *et al.*, 2003). 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 using commercial colourimetric assays (CYTOC-OX1 Sigma-Aldrich, USA and CS0740 Sigma-Aldrich, USA respectively). This showed that the P3 fraction was enriched with mitochondria, and the P4 with lysosomes (Supp Figure 2). Fractions were acidified to 10 % v/v with nitric acid (AristaR BDH) and placed in a water bath at 80°C for 4 hours. 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 cadmium was as previously described above.

2.5. *Enzymatic and Oxidative Damage Biomarkers*

2.5.1. *Total Antioxidant Capacity & Lipid Peroxidation*

Tissue lysates were produced by homogenisation on ice in a 5 mM potassium phosphate buffer containing 0.9 % w/v sodium chloride and 0.1 % w/v glucose, pH 7.4 (1:5 w/v) using a motorised microcentrifuge pellet pestle, sonicating for 15 seconds at 40 V and centrifuging, in a 5804R centrifuge (Eppendorf, Germany), at 10,000 x g for 15 minutes at 4°C. The supernatant was stored at -80°C until analysis. Total antioxidant capacity (TAOC) was measured using an assay based on the ability of the tissue lysate antioxidant system to inhibit the oxidation of ABTS® (2,2'-Azino-di-[3-ethylbenzthiazoline sulphonate]) to ABTS $^{®\bullet+}$ by metmyoglobin in the presence of hydrogen peroxide. This was compared with the antioxidant capacity of a standard, Trolox (Cayman Chemicals). The Thiobarbituric Acid Reactive Substances (TBARS) assay was used to measure lipid peroxidation by measuring the malondialdehyde (MDA) concentration in each tissue lysate. The end product of lipid peroxidation, MDA, forms a 1:2 adduct with TBARS and produces a colour reaction that can be read spectrophotometrically at 532 nm and compared to an MDA standard curve (ZeptoMetrix Corporation).

2.5.2. *Reduced:Oxidised Glutathione Ratio & Glutathione Peroxidase*

Tissue lysates were produced by homogenisation on ice in a 50 mM Tris-HCl buffer containing 5 mM EDTA and 1 mM DTT, pH 7.5 (1:5 w/v) using the technique outlined above. A thiol scavenging agent 1-methyl-2-vinyl-pyridium trifluoromethane sulfonate (M2VP) was added to supernatant for oxidised glutathione (GSSG) to remove GSH. Supernatants were stored at -80°C until analysis. Total glutathione activity (GSH+2GSSG) and 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, read over 3 minutes at intervals of 15 seconds (Calbiochem®, Merck, Germany). Glutathione peroxidise activity (GPx) was measured using a coupled reaction with glutathione reductase (GR) (Cayman Chemicals). The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm, read for 5 minutes at intervals of 30 seconds. Samples were compared with a bovine erythrocyte GPx positive control.

2.5.3. *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 was read at 485 nm excitation and 620 nm emission, on a Luminoskan Ascent Fluorescence Plate Reader (Thermo Electrical Corp., USA). Bovine serum (BSA) calibration curve standards used were made up in sample buffer.

2.6. *Cellular and Genotoxic Biomarkers*

2.6.1. *Lysosomal Stability*

Lysosomal stability was assessed in hepatopancreas tissue using a method developed by (Ringwood *et al.*, 2003) for oysters. The assay uses neutral red (NR) dye retention to assess 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 whereas in cells with damaged lysosomal membranes it leaks out into the cytoplasm. Cells were incubated in neutral red for 1 hour and one hundred cells per slide counted using a light microscope with 40 x lens and scored as stable or unstable. Two slides per sample were counted.

2.6.2. *Micronuclei Frequency*

The frequency of micronuclei was measured in gill tissue using an assay 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. Tissue preparation for the collection of cells was the same as that used for the neutral red retention assay. A drop of cell suspension in Carnoy's solution was placed on a slide and air dried. DAPI (# 32670 Sigma, USA) 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.

2.7. Statistical analyses

A Mixed Linear Model analysis of variance with repeated measures (ANOVA) (SPSS *v* 14.0) was used to analyse the effects of time and treatment on whole organism tissue cadmium accumulation, and treatment on TAOC, GSH+2GSSG, GSH:GSSG ratio, GPx, TBARS, lysosomal stability and micronuclei frequency. (Supp Tables 1 – 3). Regressions of sediment cadmium and mean tissue cadmium concentrations and means of effects variables TAOC, TBARS, lysosomal stability and micronuclei frequency were calculated using EXCEL™ *v* 2003.

3. Results

3.1. Cadmium accumulation

Cadmium accumulation by *A. trapezia* was dependent on exposure time and sediment cadmium concentration ($p \leq 0.001$; Supplementary Tables 1 & 2a). Tissue cadmium concentrations were in the order 50 µg/g > 10 µg/g > control for each analysis time (Figure 1). The tissue cadmium concentration of the control organisms remained the same over the course of the exposure (Figure 1). The pattern of cadmium accumulation over time was similar in organisms from both cadmium treatments (Figure 1). 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 1). Regression between cadmium concentrations in sediments and organisms after 56 days shows a significant positive ($r = 0.93$), but not proportional, relationship (Figure 1). Cadmium accumulation was significantly different between tissues ($p \leq 0.001$; Supplementary Table 2b) with tissue cadmium concentrations being generally in the order gills > hepatopancreas > haemolymph (Figure 2).

3.1.1. Subcellular Tissue Cadmium

Between 45 and 70 % of the total gill and 42 and 82 % of the total hepatopancreas cadmium was recovered in the fractions (Table 1). Between 66 and 73 % of recovered gill and 46 and 67 % of hepatopancreas cadmium was in the biologically detoxified metal (BDM) fraction (Table 1; Figure 3). The majority of BDM in both tissues was in the metallothionein like protein (MTLP) fraction for all treatments (Figure 3). The highest percentage of cadmium in the biologically

active metal (BAM) fractions of both tissues in 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 3; Table 2). The cadmium in the BAM fractions of the control *A. trapezia* was fairly equally distributed among the three fractions (Figure 3; Table 2). Total cadmium burden in the BDM and BAM fractions of both tissues increased with the cadmium exposure (Table 1; Figure 3).

3.2. Biomarkers

TAOC of cells was significantly reduced ($p \leq 0.001$; Supplementary Table 3a) in both the cadmium treatments, relative to the control organisms (Figure 4). GPx activity was reduced in both the cadmium treatments, relative to the controls (Figure 4) but this was not significant ($p > 0.05$; Supplementary Table 3b). Mean total glutathione (GSH + 2GSSG) concentrations were not significantly different for controls or treatments (Figure 4; Supplementary Table 3a). The mean ratio of reduced to oxidised glutathione in the 50 μg/g cadmium exposed organisms were 0.25 of, and significantly different ($p \leq 0.01$; Supplementary Table 3b) to those of controls (Figure 4). Although mean TBARS increased with exposure to increased sediment cadmium concentrations (Figure 5) they were not significantly different ($p > 0.05$; Supplementary Table 3b) to control organisms. Lysosomal stability and micronuclei frequency significantly decreased ($p \leq 0.001$ Supplementary Tables 3a & 3b) with exposure to increased cadmium concentrations (Figure 5). Regression analysis showed that the reduced TAOC within cells had a negative relationship with the effects measures of TBARS ($r = 0.79$), lysosomal stability ($r = 0.96$) and micronuclei frequency ($r = 0.88$) for cadmium exposed organisms (Figures 4 & 5). There was a positive relationship between TBARS and lysosomal stability ($r = 0.93$) and micronuclei frequency ($r = 0.98$) (Figure 5).

4. Discussion

4.1. Cadmium Accumulation and Subcellular Distribution

4.1.1. Whole Organism

Cadmium exposed *A. trapezia* in this experiment accumulated higher tissue cadmium concentrations (Figure 1), than those reported 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 the Burt *et al.*, (2007) 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). 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 metal bioavailability and route of exposure. 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 2, Supplementary Table 2b). 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). 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 (Figure 2, Supplementary Table 2b). 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.*, 2005). 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 2). 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.

4.1.2. Subcellular Cadmium Distribution

Induction of MTLP during the metabolism of cadmium has been reported for a range of bivalves (Bebianno *et al.*, 1994; Chan *et al.*, 2002; Giguere *et al.*, 2003; Roesijadi, 1996). The majority of detoxified cadmium in *A. trapezia* in this study was in the MTLP fraction with very little sequestered in the MRG fraction (Tables 1& 2; Figure 3). 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 this.

The increased mitochondrial cadmium observed in this study (Table 2, Figure 3) is in agreement with studies on cadmium subcellular distribution following increased cadmium exposure in oysters *Crassostrea virginica* (Sokolova *et al.*, 2005) and the freshwater bivalve *Pyganodon grandis* (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; 2003), who showed that cadmium could directly lead to dysfunction of mitochondria including inhibition of respiration, loss of transmembrane potential and the release of cytochrome c oxidase. The associated increase in activity of the mitochondrial enzyme cytochrome c oxidase with an increase in mitochondrial cadmium in *A. trapezia* in this study (Supplementary Figures 1 & 2) suggests mitochondrial function impairment is probable. Lysosomal destabilisation following cadmium exposure has been demonstrated for other marine bivalves (Bolognesi *et al.*, 1999; Ringwood *et al.*, 1998). 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. The increase in cadmium burden in the lysosomal+microsome fraction of both tissues of cadmium exposed *A. trapezia* (Tables 1 & 2) with an associated increase in the activity of the lysosomal marker enzyme acid phosphatase (Supplementary Figures 1 & 2) and the reduced lysosomal

membrane stability (Figure 5) indicates that this was a probable pathway for cadmium toxicity in these organisms. Cadmium associated with the HSP fraction accounted for around one quarter of the BAM cadmium (Table 2; Figure 3). Increased cadmium in this fraction may include binding to metal sensitive enzymes and proteins which has implications for toxicity (Wallace *et al.*, 2003). The small proportion of the recovered cadmium in the hepatopancreas nuclei and cell debris fraction of the cadmium exposed organisms (Table 1; Figure 3) 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, leading to cadmium toxicity.

4.2. Enzymatic Biomarkers – Oxidative Enzymes

The significantly reduced TAOC of *A. trapezia* after 56 days cadmium exposure compared to control organisms (Figure 4) is in agreement with previous studies. The 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 *A. trapezia's* total antioxidant system, showed that activity of the GPx enzyme was reduced, the concentration of total glutathione (GSH+2GSSG) was increased slightly in the 50 µg/g sediment cadmium treatment and the ratio of GSH:GSSG reduced in cadmium exposed organisms (Figure 4). As the mitochondria is the primary site of ATP production, which includes the cycling of oxygen, the increased cadmium in this organelle (Tables 1 & 2) has clearly affected the

glutathione cycling, particularly in the 50 μg/g sediment 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 effect 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).

4.3. Oxidative Damage Biomarker – Thiobarbituric Acid Reactive Substances

The trend of increasing TBARS concentration with cadmium exposure (Figure 5) has previously been demonstrated in a study of the cadmium-handling strategy of the chronically exposed freshwater bivalve *Pyganodon grandis* which linked higher cadmium exposure to increased cytosolic and mitochondrial cadmium with increased TBARS in gill tissue fractions (Bonneris *et al.*, 2005). A 30 day cadmium accumulation study in the marine bivalve *Perna viridis* also showed TBARS increased as tissue cadmium increased in gill tissue (Prakash and Rao, 1995). *A. trapezia* gill tissue TBARS showed a similar trend for lipid peroxidation with increased cadmium exposure in the cytosolic and mitochondrial fractions (Figure 5). The TBARS concentration was negatively correlated with the TAOC (Figures 4 & 5), indicating the cadmium induced perturbations in the antioxidant reduction system influenced the build up of lipid peroxidation by-products.

4.4. Cellular Biomarker – Lysosomal Stability

Lysosomes are the target for a wide range of toxic chemicals including metals which may affect these organelles directly and indirectly through the enhanced formation of oxygen radicals and 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 (Figure 5, Supplementary Table 3b). Based on the Ringwood *et al.*, (2003) criteria for oysters the percentage of destabilised lysosomes of the cadmium exposed *A. trapezia* (Figure 5) places

them in the concern / stressed range for organism physiological function. 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.*, 2005) and was also observed in the *A. trapezia* in this study (Table 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; 2002). Lysosomal destabilisation of the cadmium exposed *A. trapezia* was strongly negatively correlated with TAOC (Figures 4 & 5), and positively correlated with TBARS (Figure 5). This supports a cadmium induced reduction in the TAOC resulting in an increase in reactive oxygen species (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).

4.5. Genotoxic Biomarker – Micronuclei Frequency

Micronuclei are small intracytoplasmic masses of chromatin resulting from chromosomal breakage or aneuploidy during cell division and the micronucleus assay is one of the most promising techniques to identify genetic alterations in organisms exposed to toxicants (Bolognesi *et al.*, 2004). The increased micronuclei frequency of the cadmium exposed *A. trapezia* (Figure 5) was negatively correlated with TAOC (Figures 4 & 5) and positively correlated with TBARS (Figure 5) supporting the pathway of genotoxic damage via the increase in ROS resulting from cadmium inhibition of the ROS detoxification capacity. 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).

5. Conclusions

A significant exposure – dose – response relationship for cadmium has been established in this study which indicates that increased tissue cadmium dose and BAM cadmium 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 tissue accumulation in *A. trapezia*.

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Table 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. Total cadmium in subcellular fractions (µg wet mass) and percentage distribution of total recovered cadmium fractions are grouped as nuclei+cellular debris and biologically active and detoxified metal (Figure 4).
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 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(s)

Figure Captions

Figure 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 2: 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.

Figure 3: 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.

Figure 4: 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 between means (Bonferroni test; $p < 0.05$).

Figure 5: 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 between means (Bonferroni test; $p < 0.05$).

Figure 1

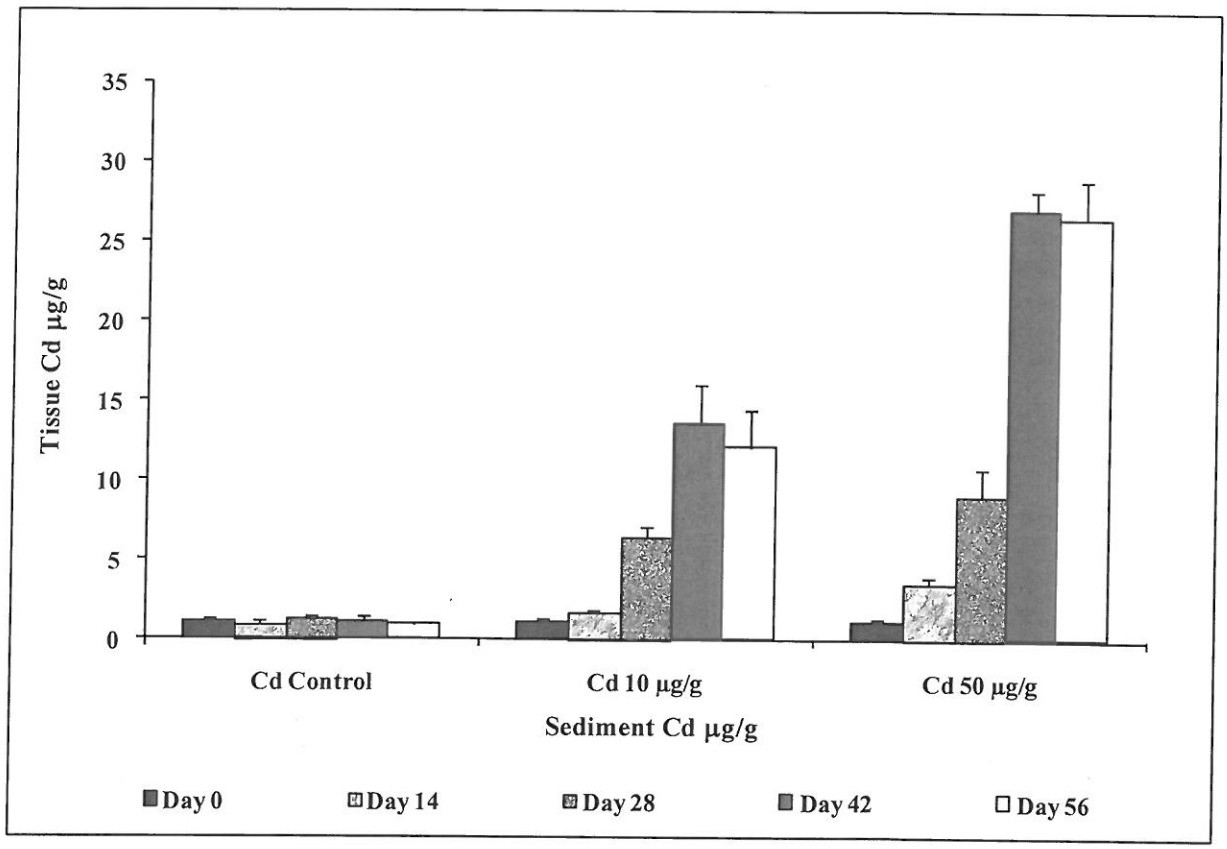

35
30
25
20
15
10
5
0
Tissue Cd µg/g
Cd Control
Cd 10 µg/g
Cd 50 µg/g
Sediment Cd µg/g
Day 0
Day 14
Day 28
Day 42
Day 56

Figure 2

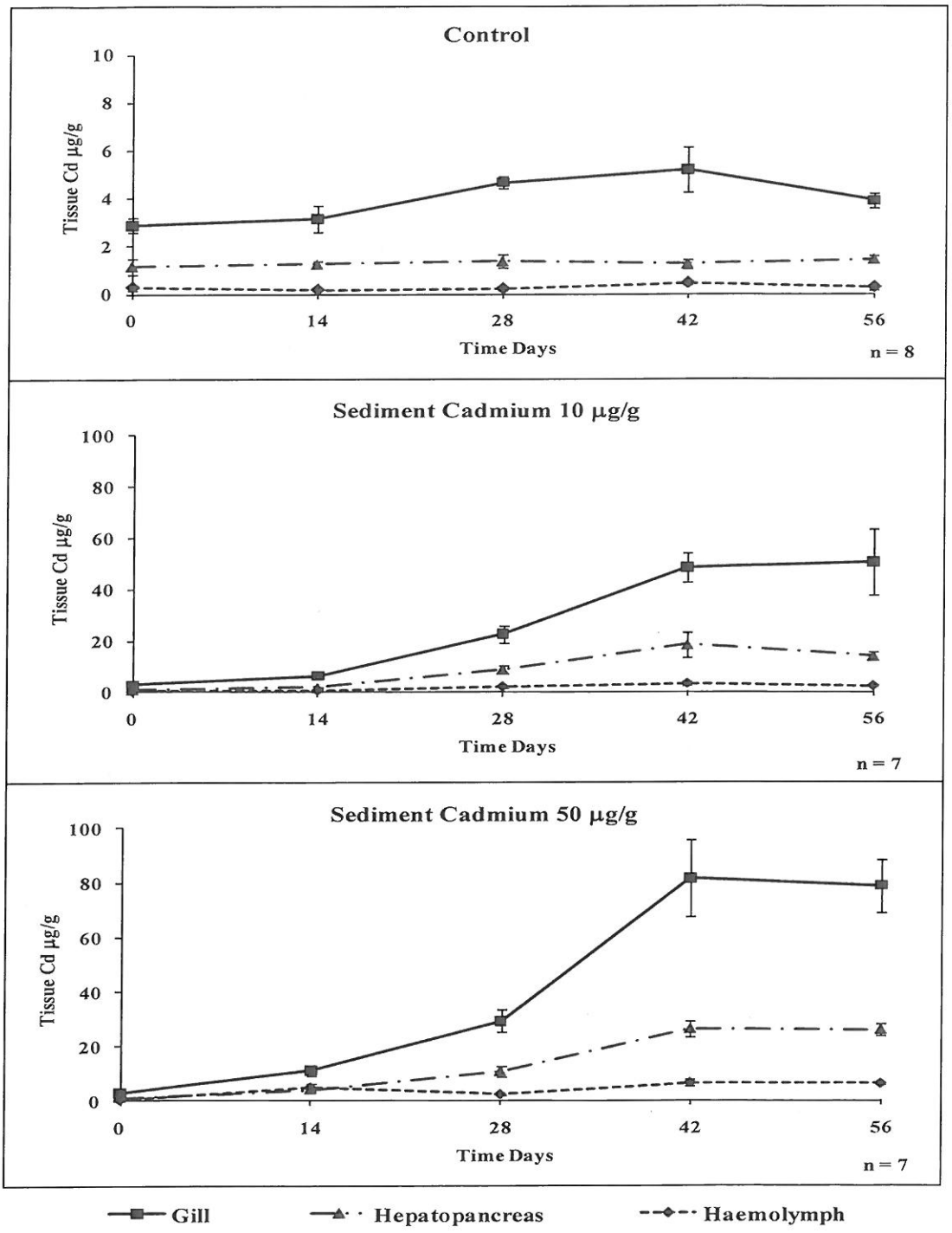

Control
Tissue Cd µg/g
10
8
6
4
2
0
0
14
28
42
56
Time Days
n = 8
Sediment Cadmium 10 µg/g
Tissue Cd µg/g
100
80
60
40
20
0
0
14
28
42
56
Time Days
n = 7
Sediment Cadmium 50 µg/g
Tissue Cd µg/g
100
80
60
40
20
0
0
14
28
42
56
Time Days
n = 7
Gill
Hepatopancreas
Haemolymph

Figure 3

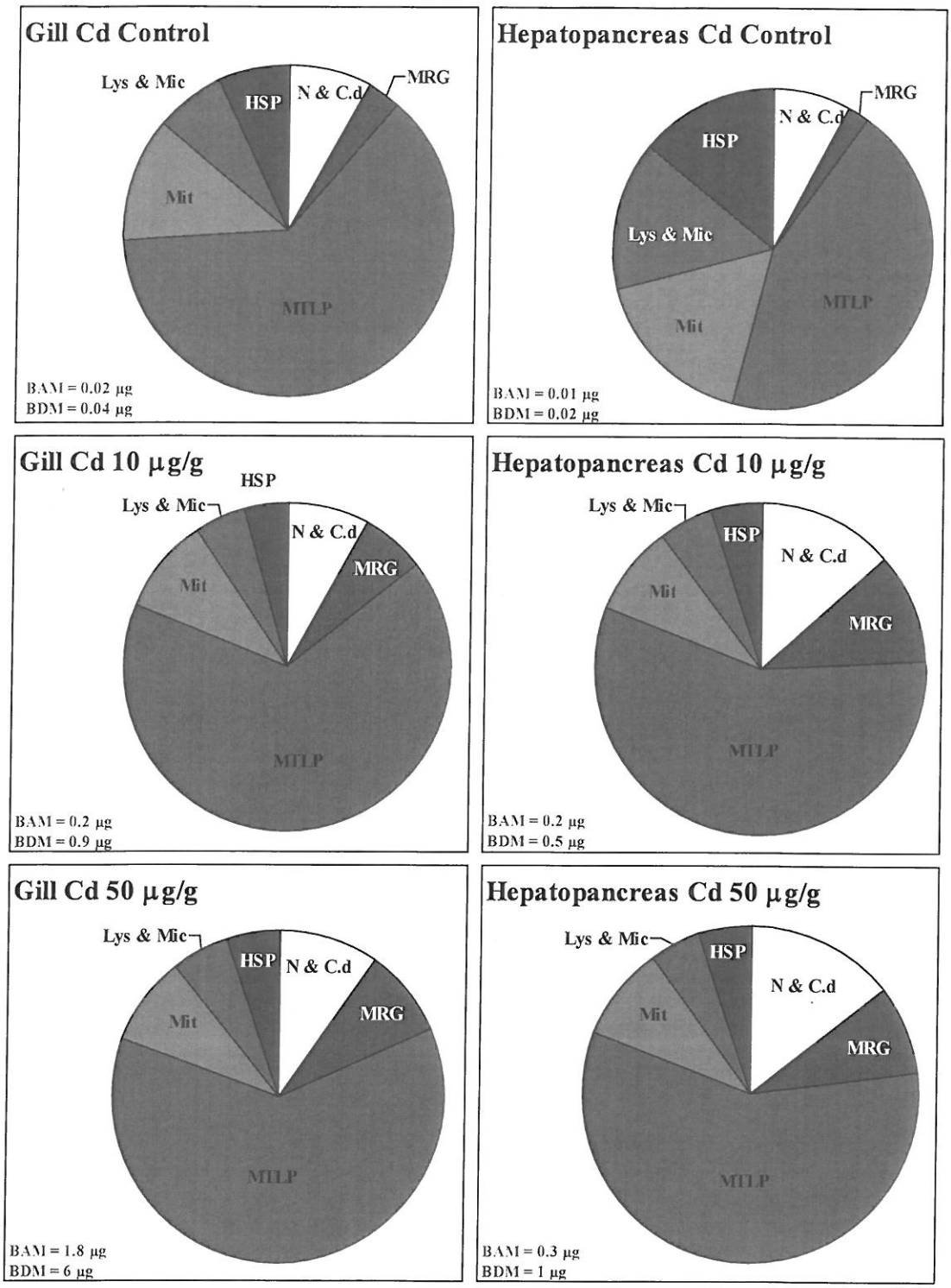

Gill Cd Control
Lys & Mic
HSP
N & C.d
MRG
Mit
MTLP
BAM = 0.02 µg
BDM = 0.04 µg
Hepatopancreas Cd Control
MRG
N & C.d
HSP
Lys & Mic
MTLP
Mit
BAM = 0.01 µg
BDM = 0.02 µg
Gill Cd 10 µg/g
HSP
Lys & Mic
N & C.d
MRG
Mit
MTLP
BAM = 0.2 µg
BDM = 0.9 µg
Hepatopancreas Cd 10 µg/g
Lys & Mic
HSP
N & C.d
Mit
MRG
MTLP
BAM = 0.2 µg
BDM = 0.5 µg
Gill Cd 50 µg/g
Lys & Mic
HSP
N & C.d
Mit
MRG
MTLP
BAM = 1.8 µg
BDM = 6 µg
Hepatopancreas Cd 50 µg/g
Lys & Mic
HSP
N & C.d
Mit
MRG
MTLP
BAM = 0.3 µg
BDM = 1 µg

Figure 4

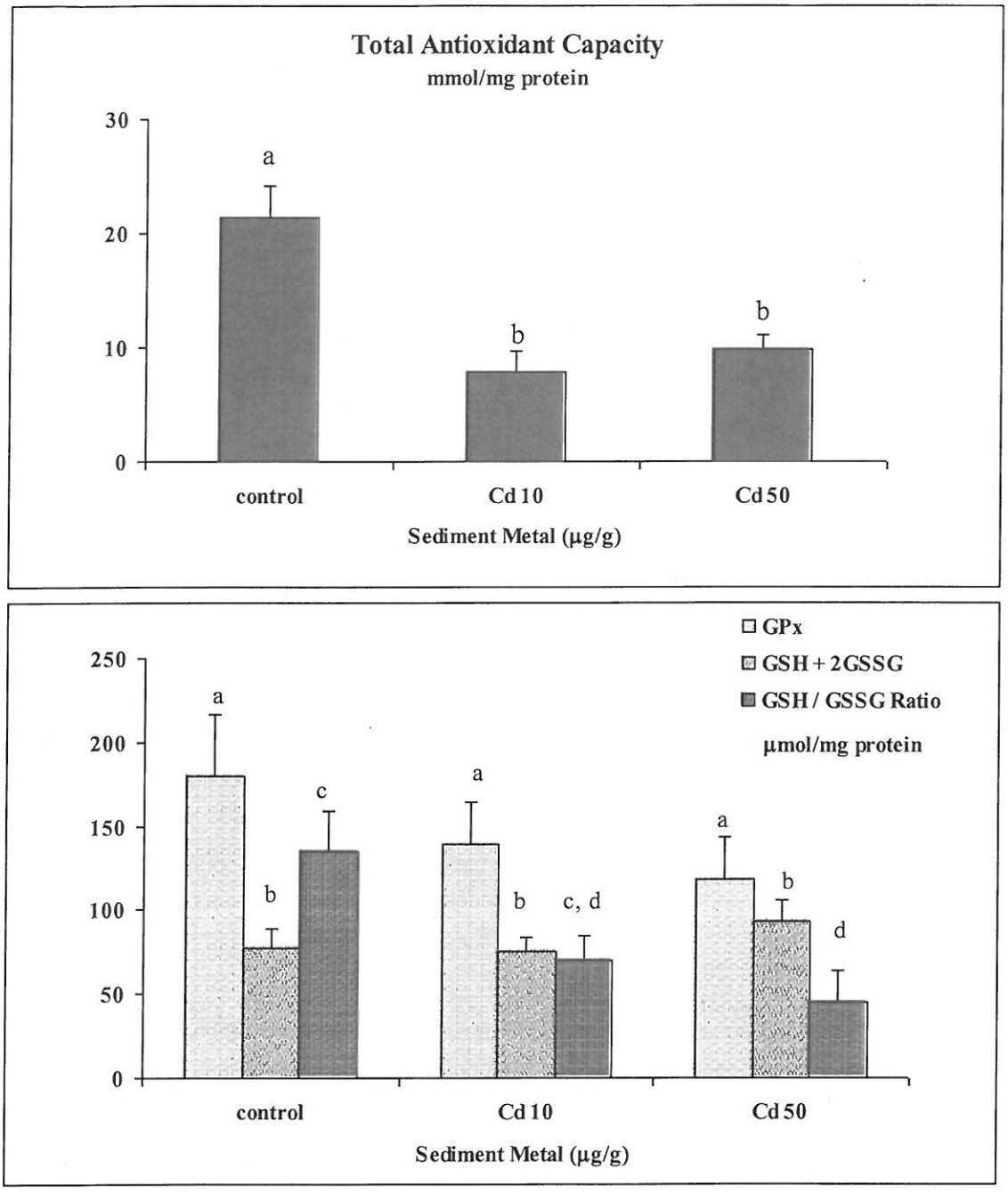
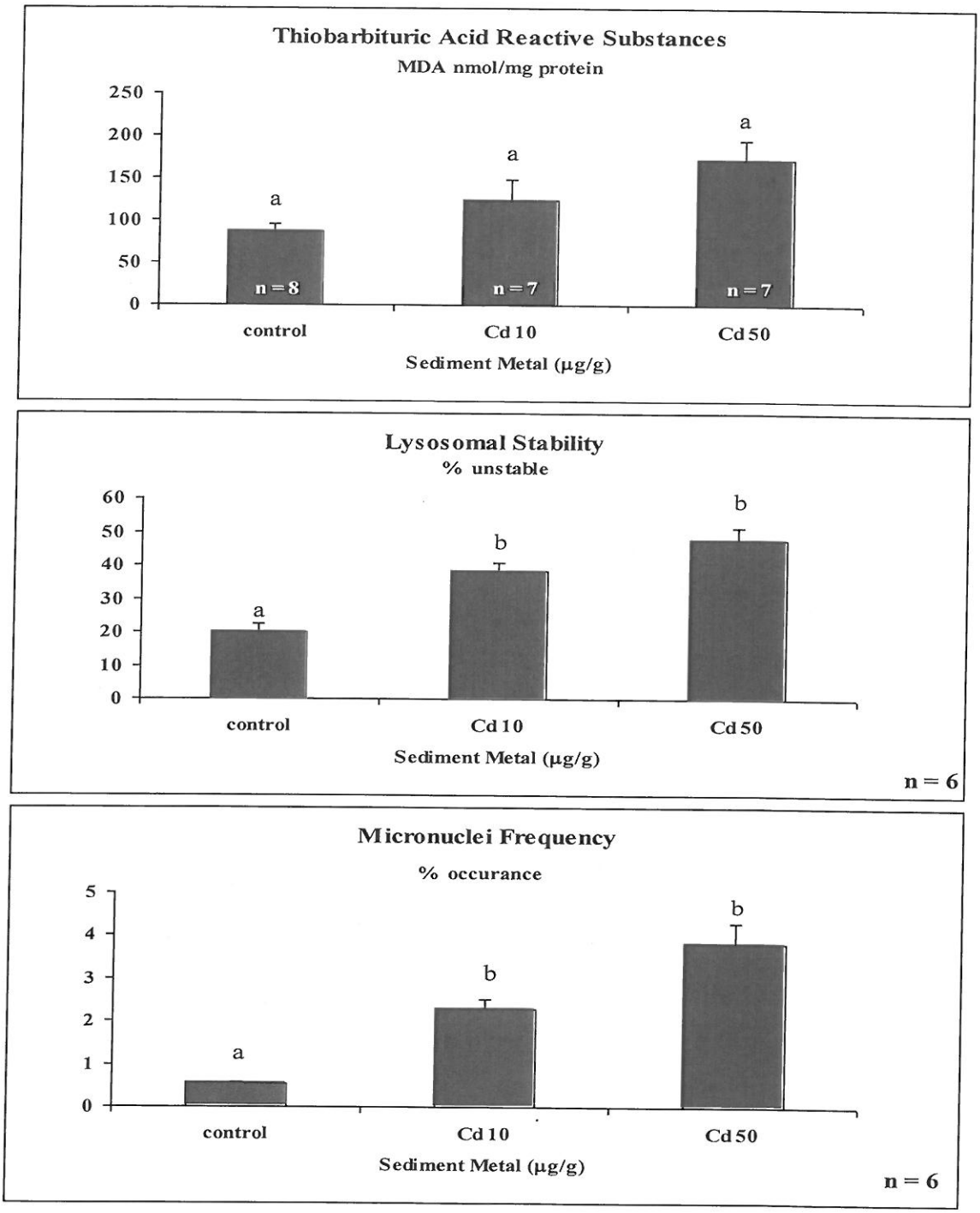

Total Antioxidant Capacity
mmol/mg protein
30
20
10
0
a
b
b
control
Cd 10
Cd 50
Sediment Metal (µg/g)
GPx
GSH + 2GSSG
GSH / GSSG Ratio
µmol/mg protein
250
200
150
100
50
0
a
b
c
a
b
c, d
a
b
d
control
Cd 10
Cd 50
Sediment Metal (µg/g)

Figure 5


Thiobarbituric Acid Reactive Substances
MDA nmol/mg protein
250
200
150
100
50
0
a
a
a
n = 8
n = 7
n = 7
control
Cd 10
Cd 50
Sediment Metal (µg/g)
Lysosomal Stability
% unstable
60
50
40
30
20
10
0
a
b
b
control
Cd 10
Cd 50
Sediment Metal (µg/g)
n = 6
Micronuclei Frequency
% occurance
5
4
3
2
1
0
a
b
b
control
Cd 10
Cd 50
Sediment Metal (µg/g)
n = 6

Supplementary Material
Click here to download Supplementary Material: Cd supp revision.docx

Highlights

>We describe an exposure-dose-response approach to assessing cadmium exposure in *Anadara trapezia*. > Accumulated cadmium was detoxified in metallothionein like proteins or as active metal in mitochondria. > Increased cadmium dose resulted in a reduction in total antioxidant capacity, increased lysosomal stability and genotoxic damage. > Elevated sediment cadmium concentrations can lead to increased biologically active cadmium and impairment of *A. trapezia*.