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

2. Lead spiked sediments

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Abstract

The composition of near shore marine environments is increasingly being altered by contaminants from human activities. The ability of lead, which has no known biological function, to mimic biologically essential metals makes it one of the most toxic to marine biota. The relationship between lead exposure, dose and response was investigated in Anadara trapezia exposed for 56 days to lead spiked sediment (100 µg/g and 300 µg/g dry mass). Lead tissue concentrations of the 300 µg/g exposed A. trapezia doubled in the last 2 weeks of the exposure with final lead tissue concentrations of exposed organisms of (1 µg/g and 12 µg/g respectively). Tissue lead accumulation of exposed organisms followed the pattern haemolymph > gill > hepatopancreas during much of the 56 day exposure. Between 30 to 69 % of accumulated lead in the gill and hepatopancreas was detoxified and fairly evenly distributed between the metal rich granule and the metallothionein like protein fractions. Approximately half of the biologically active lead in both tissues was in the mitochondrial fraction which showed increased cytochrome c oxidase activity in lead exposed organisms. There was a reduction in GPx activity, an associated increase in total glutathione concentrations and reduced GSH:GSSG ratios due to a build up of oxidised glutathione. These changes in the glutathione pathway were reflected in the total antioxidant capacity of lead exposed A. trapezia which were significantly reduced compared to control organisms. Increased lead exposure significantly increased lipid peroxidation, lysosomal destabilisation and frequency of micronuclei. A significant exposure – dose – response relationship for A. trapezia exposed to lead enriched sediments indicates that elevated sediment lead concentrations have the potential to lead to increased biologically active lead burdens and impairment of the antioxidant reduction capacity leading to a series of associated effects from lipid peroxidation to cellular perturbation and genotoxic damage.

Keywords: Lead, Biomarkers, Biologically active metal, Biologically detoxified metal, Oxidative stress, Lysosomal stability, Lipid peroxidation, Micronuclei
1. Introduction

Coastal bays and estuaries in Australia are under increasing pressure to assimilate toxicants, with 90% of the population inhabiting coastal areas and most industry situated in close proximity (ABS, 1996). Common metals released in significant quantities include lead, zinc, cadmium, copper and selenium from metal refining and power generation activities (Peters et al., 1999; Roach, 2005). Lead is accumulated by a range of marine organisms and while it has no known biological function, its ability to mimic biologically important metals such as calcium, iron and zinc makes it one of the most toxic metals in the marine environment (Company et al., 2011). Its accumulation can adversely affect cellular function as lead has a high affinity for sulphhydryl groups and affects enzyme activity (Dafre et al., 2004). Lead is in many respects similar to calcium and because of competition affects mitochondrial respiration, neurological functions, protein synthesis and red cell formation (Landis et al., 2011). 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; Viarengo et al., 1989). Lead intake also results in the production of reactive oxygen species that result in depletion of cell antioxidant defense systems, destabilization of cell membranes and DNA damage (El-Ashmawy et al., 2006; Farmand et al., 2005; Shalan et al., 2005; Weber et al., 1991). Physiological effects of metals strongly depend on their intracellular localisation and binding within organelles (Sokolova et al., 2005). Lead accumulation in mitochondria and other organelles may therefore result in serious disturbances of tissue energy balance and eventually cell death (Morris et al., 2005), energetic changes (Einsporn and Koehler, 2008) and genotoxic damage (Monteiro et al., 2011), and ultimately impairment of growth, reproduction and survival (Weis et al., 2001).

Lead concentrations in sediments of up to 300 μg/g dry mass have been reported in contaminated Australian estuaries (Roach, 2005). The Australian interim sediment quality guidelines for Fresh and Marine Water Quality (ANZECC and ARMCANZ, 2000) low and high effects for lead, are 50 and 220 μg/g dry mass. To establish relationships between sediment lead exposure, organism dose and biological response for benthic dwelling molluscs, a common benthic marine bivalve mollusc, *Anadara trapezia* was exposed for 56 days to different
concentrations of lead spiked estuarine sediments in laboratory aquaria and compared to unexposed organisms. *Anadara trapezia* is a common sediment dwelling estuarine bivalve which satisfies most of the basic requirements to be an effective biomonitor (Phillips and Rainbow, 1994). It has a cream to white heavy equivvalve shell with broad flat radial ribs which in mature organisms ranges in length from 300 – 800 mm. Unlike other marine bivalves the haemolymph of *A. trapezia* contains haemoglobin as a respiratory pigment (Sullivan, 1961). *A. trapezia* is a filter feeder which has no siphon to extend beyond its shell, therefore, it never buries entirely below the sediment surface as it must keep its posterior end exposed to enable feeding. (Beesley *et al.*, 1998). The concentrations of lead chosen for the sediment spiking were an intermediate value between the low and high sediment quality guideline concentration (100 μg/g dry mass) and the highest sediment lead concentrations previously measured in contaminated Australian estuarine sediments (300 μg/g dry mass). Organism internal exposure was measured by total lead burden while internal tissue dose was examined by subcellular fractionation of whole tissues to determine what fraction of the total accumulated lead was 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

Estuarine 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 on the south coast of NSW. They were placed in coolers with sediment and water from the collection sites for transportation. Organisms were 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 Murramurang National Park, NSW and adjusted from 35 %o to 30 %o with filtered deionised water adjusted to pH 7.8 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 lead. 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, PbCl₂ (AR grade Sigma-Aldrich, USA), added to a concentration of 0, 100 and 300 mg/kg dry mass of sediment. To ensure added lead was rapidly adsorbed and strongly bound to the sediment particles a method developed by (Simpson *et al.*, 2004) was followed. Briefly, 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 PbCl₂ using 1M NaOH, (AR grade BDH), prepared in seawater. pH was checked weekly and maintained at 7.5 - 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°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 PbCl₂ was completely bound to sediment
particles, pore waters were collected, acidified to 1 % (v/v) with nitric acid (AristaR, BDH, Australia) and lead measured using an ELAN® 6000 ICP-MS (PerkinElmer SCIEX, USA). Once pore water lead 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 lead concentrations were measured prior to and at the end of the 56 exposure period, concentrations were < 0.001, 100.0 ± 5.1 and 300.0 ± 10.3 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 and allowed to settle for 24 hours. The containers were filled with seawater adjusted to a salinity of 30 ‰. Containers were placed in a 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. Aquaria were continually aerated using an air pump with valves on each line to regulate air flow such that oxygen saturation levels (≈ 100 ‰), were maintained in individual aquaria but sediments were not agitated. Due to the natural buffering capacity of sea water and associated sediments pH remained relatively constant at pH 7.8-8.0 in all aquaria throughout the 56 days of exposure. This is similar to results of other studies of this type (King et al., 2006; Strom et al., 2011). Lead 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 lead accumulation over time. Lead subcellular distribution and biomarker assays were measured after 56 days to determine end point effects.
2.4. Lead Measurements

2.4.1. Total lead
Lyophilised ground gill/mantle, hepatopancreas and haemolymph tissue was microwave digested in 1 ml of nitric acid (Aristar BDH, Australia) in a 630 W microwave oven (CEM MDS-2000, USA) for 2 min at 630 W, 2 min 0 W, and 45 min at 315 W (Baldwin et al., 1994). Lead concentrations were measured using the ELAN® 6000 ICP-MS (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 lead values were: 21 ± 4 and 0.36 ± 0.02 μg/g respectively were in good agreement with certified values (22.7 ± 3.4 and 0.37 ± 0.014 μg/g respectively). Whole organism tissue metal concentrations were calculated from individual tissue metal concentrations and the total tissue mass.

2.4.2. Subcellular Lead
The subcellular tissue lead distribution was examined in gill/mantle 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 Ca²⁺/Mg²⁺ free saline buffer on ice using an IKA® Labortechnik Ultra-turrax-T25 homogeniser equipped with an S25-UT dispersing tool at 9,500 rpm (Janke & Kunkel, Germany). Homogenised tissue was subjected to differential centrifugation and tissue digestion procedures according to the protocol outlined in Taylor and Maher (2010) using a 5804R centrifuge (Eppendorf, Germany) and a Himac CP90WX preparative ultracentrifuge (Hitachi, Japan) (Figure 1). 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 (Figure 1). 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. Lead analysis was as previously described.

2.5. Enzymatic and Oxidative Damage Biomarkers

All enzymatic biomarkers were measured in gill tissue, an active site for metal accumulation (Ringwood et al., 2003).

2.5.1. Total Antioxidant Capacity & Lipid Peroxidation

Dissected tissue was homogenised 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). 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°C. The supernatant was stored at 80°C until analysis of total antioxidant capacity (TAOC), lipid peroxidation (TBARS) and protein analysis. TAOC was measured using an assay based on the ability of the tissue lysate antioxidant system to inhibit the oxidation of ABTS® (2,2'-Azinodi-[3-ethylbenzthiazoline sulphonate]) to ABTS®+ by metmyoglobin in the presence of hydrogen peroxide. This was compared with the antioxidant capacity of a standard, Trolox (Cayman Chemicals, USA). 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, USA).

2.5.2. Reduced:Oxidised Glutathione Ratio & Glutathione Peroxidase

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) on ice and supernatants produced using the technique outlined above. A thiol scavenging agent 1-methyl-2-vinyl-pyridium trifluoromethane sulfonate (M2VP)
was added to GSSG tissue homogenates to remove GSH, prior to the addition of buffer and production of the final supernatant. Supernatants were stored at 80°C until analysis of reduced glutathione (GSH), glutathione peroxidise (GPx) and protein (Calbiochem, 2004). 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, USA). 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. Under conditions where GPx activity is rate limiting, the rate of decrease in the A₃₄₀ is directly proportional to the GPx activity in the sample. 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

The cellular biomarker lysosomal stability was measured in the hepatopancreas, one of the most important sites of contaminant deposition and effects, which has large cells with numerous lysosomes which act as an important detoxification pathway in these organisms (Ringwood et al., 2003). The genotoxic biomarker micronuclei frequency was measured in gill tissues of A. trapezi which are another important site for metal uptake and accumulation.

2.6.1. 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 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. Minced tissue was shaken in CMFS buffer pH 7.35 salinity 30 % on a reciprocating shaker at 100 rpm for 20 minutes. Trypsin (T4799 Sigma, USA), 325 μl at 1 mg/ml in CMFS buffer, was added and samples shaken for a further 20 minutes. Cells were then collected by centrifuging samples through a 20 μm screen 250 - 500 g at 15°C for 5 - 15 minutes. Cells were incubated in neutral red (Sigma, USA), 0.04 mg/ml in CMFS for 1 hour and one hundred cells per slide were counted using a light microscope with 40x lens and scored as stable or unstable. Two slides per sample were counted.

2.6.2. 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 plane 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 the cell suspension was placed on a slide and air dried. A drop of the 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, Japan) with the appropriate filter for DAPI, excitation wavelength 350 nm magnification 40x. 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 lead accumulation, and treatment on TAOC, GSH+2GSSG, GSH:GSSG ratio, GPx, TBARS, lysosomal stability and micronuclei frequency. (Supp Tables 1 – 3). Regressions of sediment lead and mean tissue lead concentrations and means of effects variables TAOC, TBARS, lysosomal stability and micronuclei frequency were calculated using EXCEL™ v 2003.
3. Results

3.1. Lead accumulation

Lead accumulation by *A. trapezia* was dependent on sediment lead concentration (*p* ≤ 0.001; Supplementary Table 1). Tissue lead concentrations were in the order 300 μg/g > 100 μg/g > control for each analysis time (Figure 2). The tissue lead concentration of the control organisms remained the same over the course of the exposure (Figure 2). The lead exposed organisms reached the highest tissue concentrations at day 56, with a rapid increase from day 42 to day 56 in the 300 μg/g exposure suggesting increased lead bioavailability (Figure 2). Final tissue concentrations were considerably lower than that of the exposure concentrations, the 100 μg/g and the 300 μg/g lead exposed organisms had 0.01 and 0.04 respectively, that of the sediment lead. Regression between lead concentrations in sediments and organisms after 56 days shows a significant positive (*r* = 0.89), but not proportional, relationship (Figure 2). Tissue lead accumulation of exposed organisms generally followed the pattern haemolymph > gill/mantle > hepatopancreas during the 56 day exposure. At day 56 lead accumulation in the 300 μg/g exposed organisms was significantly different between tissues (*p* ≤ 0.001; Supplementary Table 2b) with tissue lead concentrations at day 56 being in the order gill/mantle > hepatopancreas > haemolymph (Figure 3). This was not the case for the 100 μg/g exposed organisms where at day 56 lead was significantly lower in the hepatopancreas than the other tissues (*p* ≤ 0.001; 0.01; Supplementary Table 2b) which were not significantly different from each other (Figure 3).

3.1.1. Subcellular Tissue Lead

In lead exposed *A. trapezia*, between 44 and 64 % of the total gill/mantle and 74 and 90 % of the total hepatopancreas lead was recovered in the fractions (Table 1). Of the lead recovered, between 66 and 69 % in the gill/mantle and 49 and 56 % in the hepatopancreas, was in the biologically detoxified metal (BDM) fraction (Table 1; Figure 4). The BDM in both tissues was distributed fairly evenly between the metallothionein like proteins (MTLP) and metal rich granule (MRG) fractions for all treatments (Figure 4). Both the percentage of lead and total lead burden in the MRG fraction increased with lead exposure with an associated decrease in the MTLP fraction (Table 2). The highest percentage of lead in the biologically active metal (BAM) fractions was in the mitochondria ≈ 50 % with the remainder equally distributed between the
heat sensitive proteins (HSP) = 25% and lysosome+microsome fractions ≈ 25% in all tissues and treatments, with the exception of the 300 μg/g exposed hepatopancreas tissue where the lysosome+microsome fraction had ≈ 50% with the remainder fairly evenly distributed between the mitochondria and HSP (Figure 4; Table 2).

3.2. Biomarkers

Cell TAOC was significantly reduced (p ≤ 0.001; Supplementary Table 3a) in both the lead treatments, relative to the control organisms (Figure 5). GPx activity was reduced in lead exposed organisms relative to the controls (Figure 5) and in the 100 μg/g lead treatment this was significant (p ≤ 0.01; Supplementary Table 3b). Mean total glutathione (GSH + 2GSSG) concentrations of lead exposed A. trapezia were higher than but not significantly different to controls (Figure 5). The mean ratio of reduced to oxidised glutathione in the 100 and 300 μg/g lead exposed organisms were both 0.5 of and significantly lower (p ≤ 0.01; Supplementary Table 3b) than those of controls (Figure 5). Mean TBARS increased with exposure to increased sediment lead concentrations (Figure 6) and both were significantly different (p ≤ 0.05; Supplementary Table 3b) to control organisms. Lysosomal stability significantly decreased and micronuclei frequency significantly increased with exposure to increased lead concentrations (Figure 6, Supplementary Tables 3a & 3b). Regression analysis showed that the reduced TAOC within cells had a negative relationship with the effects measures of TBARS (r = 0.99), lysosomal stability (r = 0.91) and micronuclei frequency (r = 0.92) for lead exposure (Figures 5 & 6). There was a positive relationship between TBARS and lysosomal stability (r = 0.84) and micronuclei frequency (r = 0.97) (Figure 6).

4. Discussion

4.1. Lead Accumulation and Subcellular Distribution

4.1.1. Whole Organism and individual tissues

The most striking feature of the lead accumulation pattern was the doubling of lead tissue concentration between the 42nd and 56th day in organisms from the 300 μg/g lead treatment (Figure 2). The lack of significant lead accumulation prior to the 42nd day and lower than ambient lead tissue concentrations gained (Figure 2) are indicative of low lead bioavailability.
The change in accumulation 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 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 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.

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 higher concentrations of lead in the haemolymph relative to the other two tissues for much of the exposure time may be related to the affinity for lead to bind to haemoglobin. Previous bivalve studies have found that gills accumulated 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 tissue in the present study so would have contributed to the gill lead concentrations measured. In particular the rapid increase in the gill/mantle 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 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.

4.1.2. Subcellular Lead Distribution

Metallothionein 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; Roesijadi, 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. The percentage of lead in the BOM fractions of organisms from both lead treatments more than doubled in the gill/mantle tissues and roughly doubled in the hepatopancreas tissues compared to these tissues in the control organisms with a fairly even distribution of lead between the MRG and MTLP indicating lead detoxification (Table 1). 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 heat stable MTLP fraction of lead exposed organisms in this study indicates the presence of a lead binding protein in *A. trapezia*. The increased lead associated with the MRG fraction in the hepatopancreas of the lead exposed *A. trapezia* (Figure 4) 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 gill/mantle 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.

Higher acid phosphatase activity (Supplementary Figure 1) 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., 2005). The epithelium of the hepatopancreatic diverticula of bivalves contain digestive cells which are characterised by a well developed endo-lysosomal 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 higher lead concentrations in the haemolymph relative to the other tissues during the much of the exposure (Figure 3). A higher percentage of lead in the lysosomal+microsome fraction of the hepatopancreas of exposed organisms compared to the gill/mantle was also seen (Figure 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 cytochrome c oxidase in the gill/mantle 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 (Supplementary Figure 1). This was associated with a considerable increase in lead concentration (Table 1) in the mitochondrial fraction of both tissues of the lead exposed organisms. The tendency for lead associated with the BAM fractions in the lead exposed A. trapezia to increase in line with the increase in lead concentrations of whole tissue indicates that lead is not being completely detoxified and the potential for lead toxicity exists. 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).
4.2. Enzymatic Biomarkers – Oxidative Enzymes

Lead exposed *A. trapezia* had significantly reduced TAOC which may be related to the increased lead burdens in the mitochondrial fraction affecting oxygen reduction in this organelle (Figure 5). The TAOC of the lead exposed organisms was virtually the same, despite the gill/mantle total and mitochondrial lead burdens being higher in organisms from the 300 μg/g lead exposure (Figures 3 & 4). It is possible that there is a critical lead concentration at which TAOC is impaired that may actually be lower than the lead concentrations measured here. 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 Bocchetti *et al.*, (2008) study. The GPx activity of *A. trapezia* was also significantly reduced in the lead exposed organisms, compared to the controls (Figure 5). This was 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 5; Supplementary Table 3b). Decreased GSH:GSSG ratios in response to increased tissue lead has previously been demonstrated in earthworms *Lampito mauritii* after 2 and 7 days exposure to soil spiked with 75 to 300 mg/kg Pb²⁺ (Maity *et al.*, 2008) demonstrating lead’s capacity to disrupt the antioxidant system.

4.3. Oxidative Damage Biomarker – Thiobarbituric Acid Reactive Substances

A lead induced increase in TBARS associated with increased GSSG concentrations has previously 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). The TBARS concentration of *A. trapezia* was highly negatively correlated with TAOC (Figure 6) which supports an interaction between increased ROS and lipid peroxidation. 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 of 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).

4.4. Cellular Biomarker – Lysosomal Stability

Lysosomal destabilisation in the *A. trapezia* exposed to lead was negatively correlated with TAOC, and positively correlated with TBARS concentration suggesting that the destabilisation of the lysosomal membrane was probably twofold: direct attack from ROS; and through unstable lipid radicals, from excess oxyradical production, destabilising the lysosomal membrane (Figures 5 & 6). 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 and would be expected to cause significant impairment of physiological functions including growth and reproduction, potentially resulting in mortality (Ringwood et al., 2003). Severe disturbance in lysosomal membrane stability has previously 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* was found to cause specific alterations including 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).

4.5. Genotoxic Biomarker – Micronuclei Frequency

The significant increase in micronucleus frequency in the lead exposed *A. trapezia* compared to control organisms (Figure 6) 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 and positively correlated with TBARS 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 contaminated 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.

5. Conclusions

Final tissue concentrations were considerably lower than that of the sediment lead exposure concentrations and 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 low lead bioavailability. The doubling of gill/mantle tissue lead concentrations in the final two weeks of the exposure in organisms from the highest lead treatment indicates lead bioavailability increased at this time. The higher concentrations of lead in the haemolymph relative to the other two tissues for much of the exposure time may be related to the affinity of lead binding to haemoglobin. The higher lead burden in the gill/mantle 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 perturbations in the antioxidant system, with increasing lead exposure, were associated with increases in lipid peroxidation, lysosomal destabilisation and micronuclei frequency.
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 impair *A. trapezia* reproductive success.

**Acknowledgements**

We thank F. Krikova for metal analysis, S. Foster for assistance with aquarium set up and K. & C. Taylor for assistance with organism collection and sample preparation. Funding from the Ecochemistry Laboratory and the NSW Environmental Trust is acknowledged. This paper is dedicated to Tony Roach (1962-2011) a sadly missed friend and research collaborator.
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induction by Cd, Cu, Hg and Zn in European flounder -- calibration for environmental

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ecotoxicological protocol with caged mussels, Mytilus galloprovincialis, for monitoring the

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Table 1: Lead concentrations (µg wet mass) in gill/mantle and hepatopancreas tissue and the total lead, with percentage recovered from subcellular fractions of _A. trapezia_ after 56 days exposure to lead spiked sediments. Total lead in subcellular fractions (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 4).

<table>
<thead>
<tr>
<th></th>
<th>Gill/Mantle</th>
<th>Hepatopancreas</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pb control</td>
<td>Pb 100 µg/g</td>
</tr>
<tr>
<td>Total Tissue Lead (µg)</td>
<td>0.04 ± 0.02</td>
<td>3.4 ± 0.6</td>
</tr>
<tr>
<td>Total Recovered Lead (µg)</td>
<td>0.03 ± 0.03</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>Proportion of total recovered in fractions (%)</td>
<td>88 ± 16</td>
<td>44 ± 15</td>
</tr>
<tr>
<td><strong>Lead Distribution</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nuclei + Cellular debris (µg)</td>
<td>0.004 ± 0</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td>Nuclei + Cellular debris (%)</td>
<td>15 ± 9</td>
<td>19 ± 7</td>
</tr>
<tr>
<td>Biologically Active Metal (µg)</td>
<td>0.02 ± 0.01</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>Biologically Active Metal (%)</td>
<td>55 ± 22</td>
<td>15 ± 7</td>
</tr>
<tr>
<td>Biologically Detoxified Metal (µg)</td>
<td>0.01 ± 0.02</td>
<td>1 ± 0.2</td>
</tr>
<tr>
<td>Biologically Detoxified Metal (%)</td>
<td>30 ± 31</td>
<td>66 ± 0.5</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th></th>
<th>Gill/Mantle</th>
<th>Hepatopancreas</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pb control</td>
<td>Pb 100 µg/g</td>
</tr>
<tr>
<td>Nuclei + Cellular debris % of total</td>
<td>15</td>
<td>19</td>
</tr>
<tr>
<td>BDM % of total</td>
<td>30</td>
<td>66</td>
</tr>
<tr>
<td>Metal Rich Granules % of BDM</td>
<td>28</td>
<td>55</td>
</tr>
<tr>
<td>Heat Stable MT Like Proteins % of BDM</td>
<td>72</td>
<td>45</td>
</tr>
<tr>
<td>BAM % of total</td>
<td>55</td>
<td>15</td>
</tr>
<tr>
<td>Mitochondria % of BAM</td>
<td>49</td>
<td>52</td>
</tr>
<tr>
<td>Lysosomes + Microsomes % of BAM</td>
<td>27</td>
<td>24</td>
</tr>
<tr>
<td>Heat Sensitive Proteins % of BAM</td>
<td>25</td>
<td>24</td>
</tr>
</tbody>
</table>
Figure Captions

**Figure 1:** 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.

**Figure 2:** Lead accumulation in whole tissue of *A. trapezia* at 2 weekly intervals over 56 days of exposure to sediments containing lead at 0 (control), 100 & 300 μg/g dry mass. Mean ± SE, n = 8, 7 and 7 respectively. Day 0 are unexposed organisms, n=5.

**Figure 3:** Lead accumulation in gill/mantle, hepatopancreas and haemolymph tissues of *A. trapezia* at 2 weekly intervals for 56 days exposure to sediments spiked with lead at; 0 (control), 100 and 300 μg/g dry mass. Mean ± SE. Day 0 are unexposed organisms, n=5.

**Figure 4:** Distribution (%) of lead in the subcellular fractions of *A. trapezia* gill/mantle 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.

**Figure 5:** 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* gill tissue following 56 days of exposure to lead spiked sediments: 0 Pb (control), Pb 100 μg/g; and Pb 300 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 6:** Changes in oxidative damage, cellular and genotoxic biomarkers of *A. trapezia* gill, hepatopancreas and gill tissues respectively 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).
Figure 2

Whole Organism Dose

Tissue Lead µg/g

<table>
<thead>
<tr>
<th>Sediment Pb µg/g</th>
<th>Pb Control</th>
<th>Pb 100 µg/g</th>
<th>Pb 300 µg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 28</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Day 42</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 56</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 3

Control

Sediment Lead 100 µg/g

Sediment Lead 300 µg/g

--- Gill/Mantle
- - - Hepatopancreas
- - - Haemolymph
Figure 5

**Total Antioxidant Capacity**

mmol/mg protein

![Bar chart showing total antioxidant capacity for control, Pb 100, and Pb 300 conditions.]

- **control**
- **Pb 100**
- **Pb 300**

**Sediment Metal (µg/g)**

**Legend:**
- □ GPx
- ■ GSH + 2GSSG
- □ GSH / GSSG Ratio

μmol/mg protein

![Bar chart showing antioxidant capacity with different metal concentrations.]

- **control**
- **Pb 100**
- **Pb 300**

**Sediment Metal (µg/g)**
Figure 6

Thiobarbituric Acid Reactive Substances
MDA nmol/mg protein

<table>
<thead>
<tr>
<th>Sediment Metal (μg/g)</th>
<th>control</th>
<th>Pb 100</th>
<th>Pb 300</th>
</tr>
</thead>
<tbody>
<tr>
<td>n = 8</td>
<td>a</td>
<td>b</td>
<td>b</td>
</tr>
</tbody>
</table>

Lysosomal Stability
% unstable

<table>
<thead>
<tr>
<th>Sediment Metal (μg/g)</th>
<th>control</th>
<th>Pb 100</th>
<th>Pb 300</th>
</tr>
</thead>
<tbody>
<tr>
<td>n = 6</td>
<td>a</td>
<td>b</td>
<td>b</td>
</tr>
</tbody>
</table>

Micronuclei Frequency
% occurrence

<table>
<thead>
<tr>
<th>Sediment Metal (μg/g)</th>
<th>control</th>
<th>Pb 100</th>
<th>Pb 300</th>
</tr>
</thead>
<tbody>
<tr>
<td>n = 6</td>
<td>a</td>
<td>b</td>
<td>b</td>
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</tbody>
</table>
Highlights

We describe an exposure-dose-response approach to assessing lead exposure in *Anadara trapezia*. The haemolymph was significant in lead accumulation. Accumulated lead was detoxified in metallothionein like proteins and granules or as active metal in mitochondria. Increased lead dose resulted in reduced antioxidant capacity with an associated increase in lipid peroxidation, increased lysosomal destabilisation and genotoxic damage. Elevated sediment lead concentrations can significantly impair *A. trapezia* cellular processes.