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

3. Selenium spiked sediments

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Pages: 25

Tables: 2

Figures: 5

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20/7/12

Abstract

Selenium enters near shore marine environments from the activities of coal-fired power stations. Although selenium is an essential element, at elevated concentrations it can cause genotoxic damage. The relationship between selenium exposure dose and response was investigated in *Anadara trapezia* exposed to selenium spiked sediment (5 μg/g and 20 μg/g dry mass) for 56 days. *A. trapezia* reached an equilibrium selenium tissue concentration (2 μg/g and 10 μg/g respectively) by day 42. Gills had significantly more selenium than the hepatopancreas and haemolymph. Between 12 to 21% of accumulated selenium in the gill and hepatopancreas was detoxified and in the metal rich granule. Most of the biologically active selenium in both tissues was in the mitochondrial fraction. Glutathione peroxidase activity and mean total glutathione concentrations for selenium exposed organisms were not significantly different to controls. The ratio of reduced to oxidised glutathione and the total antioxidant capacity were significantly reduced in selenium exposed organisms compared to control organisms. Increased selenium exposure resulted in significant increases in lipid peroxidation, lysosomal destabilisation and an increased frequency of micronuclei. A significant exposure – dose – response relationship for *A.trapezia* exposed to selenium enriched sediments indicates that elevated sediment selenium concentrations can increased biologically active selenium burdens and cause impairment of cellular processes and cell integrity.

Keywords: Selenium; Biomarkers; Subcellular fractionation; Biologically active selenium; Biologically detoxified selenium; Oxidative stress; Lysosomal stability; Lipid peroxidation, Micronuclei.

1. Introduction

In Australia, 70 % of power generation is from the burning of coal that contains significant quantities of selenium (ARCARP, 2006). Selenium leaching from fly ash enters coastal bays, accumulates in sediments (Peters *et al.*, 1999a), benthic dwelling animals (Peters *et al.*, 1999b) and biomagnifies in aquatic food webs (Barwick and Maher, 2003). 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 is involved in the reduction of peroxides in the glutathione cycle and the protection of cell membranes from damage due to lipid peroxidiation (Lemly, 1998). Selenium is chemically similar to sulfur and animals cannot discriminate between the two elements in biochemical processes. Substitution of selenium for sulfur results in dysfunctional proteins and enzymes. At elevated concentrations, selenium can cause genotoxic damage (Hodson, 1988; Hoffman, 2002; Micallef and Tyler, 1987).

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. A multi-biomarker approach at several levels of biological organisation has advantages over the use of a single biomarker and provides 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.*, 2003; Smolders *et al.*, 2004). The oxidative system offers a range of general response and effect biomarkers which have been shown to be sensitive to selenium through perturbations in the glutathione cycle (Hoffman, 2002; Palace *et al.*, 2004; Winston, 1991). The measurement of a suite of biomarkers within this system, from oxidative enzymes to lipid peroxidation offers a weight of evidence approach to assessing molecular level exposure and effects. As lysosomes play a role in metal sequestration and detoxification (Viarengo, 1989) and are also susceptible to oxidative damage (Regoli *et al.*, 1998) the measurement of their integrity is a useful biomarker of effect at the cellular level, while a measure of DNA damage aids in completing the picture of a series of early adverse reactions resulting from exposure to selenium which may have the potential to lead to higher order effects.

The purpose of this study was to establish the exposure - dose - response of *Anadara trapezia*, a common sediment dwelling estuarine bivalve which satisfies most of the basic requirements to be an effective biomonitor of contaminants (Phillips and Rainbow, 1994), to sediments with known concentrations of selenium. The exposure time was 56 days and the development of useful biomarkers of effect was undertaken, with a view to determining whether they would be useful organisms for assessing selenium bioavailability and toxicity in estuarine ecosystems. There are currently no Australian selenium sediment guideline concentrations, so sediment selenium exposure concentrations 5 and 20 μg/g dry mass are based on concentrations previously measured in contaminated Australian estuarine sediments (Peters, 1997; Roach, 2005). Organism internal exposure was measured by total selenium burden in gill and hepatopancreas tissues and in haemolymph. Internal tissue doses were further examined by subcellular fractionation of tissues to determine what fraction of the total selenium taken up was in a metabolically available form. Oxidative stress was assessed 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. *Anadara trapezia* have a cream to white heavy ribbed equivalve shell and ranges in length from 30 – 80 mm. A strong elongate foot is used to move the organism around and burrow into sediment as well as anchoring it in place (Sullivan, 1961). *A. trapezia* is a filter feeder which has no siphon to extend beyond its shell so it never buries entirely below the sediment surface as it must keep its posterior end exposed to enable feeding (Beesley *et al.*, 1998). *A. trapezia* used in the laboratory exposure experiments were collected from sediment beds in 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

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. The water temperature was maintained at 22°C with a day / night light cycle of 14 / 10 hours to reflect spring / summer conditions and aerated to maintain high oxygen saturation levels ≈ 100 %. 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 and pH which was measured daily remained relatively constant at pH 7.8-8.0.

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 selenium. 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 % by mass. Wet sediment was added to glass mixing containers and Na_2SeO_3 (AR grade Sigma-Aldrich, USA) added at concentration of 0, 5 and 20 mg/kg dry mass of sediment. To ensure added selenium 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 the jars. Any pH adjustments were made immediately after the addition of the Na_2SeO_3 using 1M NaOH (AR grade BDH, Aust) prepared in seawater. pH was checked weekly and maintained at 7.5 - 8.2. 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 Na_2SeO_3 was completely bound to sediment particles, pore waters were collected, acidified to 1 % v/v with nitric acid (AristaR, BDH) and selenium measured using an ELAN® 6000 ICP-MS (PerkinElmer SCIEX, USA) (Maher *et al.*, 2001). Once pore water selenium concentrations had fallen below instrument detection limits 0.001 μg/l the sediment was ready for use. The time until full adsorption was four weeks. Sediment selenium concentrations were measured by ICP-MS after

digestion of 0.2 g of lyophilised sediment in 3 ml of nitric acid (AristaR, BDH, Aust) in polyethylene 50 ml centrifuge tubes for 60 minutes at 115°C (Maher *et al.*, 2003). Selenium in NRCC Certified Reference Materials, BCSS-1 marine sediment measured along with samples was 0.41 ± 0.01 µg/g and in agreement with certified values 0.43 ± 0.06 µg/g. Exposure sediment selenium concentrations were measured prior to and at the end of the 56 day organism exposure period, sediment concentrations were < 0.001, 5.00 ± 0.05 and 20 ± 1 µg/g 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 six replicate 12 litre 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 aquariums were left for 24 hours to allow them settle and the temperature to equilibrate. Twelve to fourteen *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 of aquarium water 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). Selenium tissue accumulation was measured in haemolymph, gill and hepatopancreas of two organisms from each treatment replicate at 14 day intervals to investigate the pattern of selenium accumulation over time. After 56 days; selenium subcellular distribution was measured in one organism from two treatment replicates; oxidative enzyme biomarker assays were measured on two organisms from each treatment replicate; lysosomal stability and micronuclei were measured on one organism from each replicate. Mortalities were low during the 56 day exposure with the loss of only four individuals from the control and 20 µg/g treatments.

2.4. *Selenium Measurements*

2.4.1. *Total selenium*

Lyophilised ground tissues were microwave digested in 1 ml of nitric acid (AristaR BDH, Aust) in a 630 W microwave oven (CEM MDS-2000, USA) for two min at 630 W, two min 0 W, and 45 min at 315 W (Baldwin *et al.*, 1994). Analysis of selenium was as previously described above. 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 selenium values 2.1 ± 0.3 μg/g were in good agreement with certified values 2.21 ± 0.24 μg/g.

2.4.2. *Subcellular Selenium*

The subcellular tissue selenium distribution was examined in gill and hepatopancreas tissues of day 56 exposed *A. trapezia* using a procedure adapted from Sokolova *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, Germany). Homogenised tissue was subjected to differential centrifugation and tissue digestion procedures according to the protocol outlined in Taylor and Maher (2012) using an Eppendorf 5804R centrifuge and a Himac CP90WX preparative ultracentrifuge (Hitachi, Japan). The mitochondria, lysosomes plus microsomes and heat sensitive protein pellets were grouped as biologically active selenium fractions. The granule pellet and final supernatant containing heat stable metallothionein like proteins were grouped as biologically detoxified selenium fractions. The supernatant from the granule pellet isolation, contained the nuclei and cellular debris (Wallace *et al.*, 2003). 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 phosphatise, respectively, were measured in each of the total tissue, mitochondrial and lysosome+microsome pellets using commercial colorimetric assays (CYTOC-OX1 Sigma-Aldrich, USA and CS0740 Sigma-Aldrich, USA, respectively). This showed that the mitochondrial fraction was enriched with mitochondria, and the lysosome+microsome fraction with lysosomes (Supp Figure 2). Fractions were acidified to 10 % v/v with nitric acid (AristaR

BDH, Aust) 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 selenium was as previously described above.

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 on ice using a (VibraCell™ Sonics Materials USA) sonic probe 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 (Cayman, 2011). The supernatant was stored at -80°C until analysis of total antioxidant capacity (TAOC), lipid peroxidation (TBARS) and protein. TAOC was measured using an assay based on the ability of the tissue lysate antioxidants 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, 2011). The amount of $ABTS^{\bullet+}$ produced was measured by the suppression of absorbance at 750 nm and is proportional to the final total antioxidant capacity concentration, expressed in millimolar Trolox equivalents. Samples were pipetted into a 96 well plate with metmyoglobin and ABTS. The reactions were initiated with a 441 µl solution of hydrogen peroxide. The plate was shaken for 5 minutes at 25°C and absorbance was read at 750 nm on a BioRad Benchmark Plus microplate spectrophotometer. 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 (ZepoMetrix, 2011). The samples were incubated in a solution of sodium dodecyl sulphate, thiobarbituric acid and sodium hydroxide dissolved in acetic acid at 95°C for 60 minutes. After cooling on ice and centrifuging at 3000 rpm for 10 minutes at room temperature,

the colour reaction was measured, on a BioRad Benchmark Plus microplate spectrophotometer at 532 nm.

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 in HCl (Calbiochem®, Merck, Germany) was added to GSSG tissue homogenates to remove GSH, prior to the addition of buffer and production of the final supernatant. The remaining GSSG is then reduced to GSH and determined by the reaction with Ellman's reagent (Calbiochem, 2004). 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 based on one 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 (Calbiochem®, Merck, Germany). The samples were acidified by the addition of a 5 % solution of metaphosphoric acid, vortexed for 15 seconds and centrifuged at 1000 x g for 10 minutes at room temperature. The metaphosphoric acid extracts were diluted with a sodium phosphate buffer and mixed at room temperature in 1 ml cuvettes with DTNB and glutathione reductase 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.

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. 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. Assay buffer (50 mM Tris-HCl, pH 7.6, 5 mM EDTA) was added to sample wells of a flat bottomed 96 well plate with a co-substrate mixture (NADPH, glutathione and GR) (2:1 v/v). Samples were added to each well and the reaction was initiated by the addition 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 at 25°C on a BioRad Benchmark Plus microplate spectrophotometer. 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°C.

2.5.3. Protein

All tissue lysates used for enzymatic assays were analysed for protein concentration and enzyme concentration / activity was normalised to mg^{-1} of protein in the sample. 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 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 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 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. The rinsed cells were fixed in Carnoy's solution (methanol:glacial acetic acid 3:1) and stored at 4°C until counted. A drop of the fixed 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 (ANOVA) (SPSS *v* 14.0) was used to simultaneously analyse the effects of time (day) and treatment (selenium exposure concentration) on organism tissue selenium accumulation. A Mixed Linear Model ANOVA (SPSS *v* 14.0) was used analyse the effect of treatment (selenium exposure concentration) on the effect measurement variables TAOC, total glutathione, GSH:GSSG ratio, GPx, TBARS, lysosomal stability and micronuclei frequency (Supp Tables 1 – 3). Regressions of sediment selenium and mean tissue selenium concentrations and means of effects variables TAOC, TBARS, lysosomal stability and micronuclei frequency were calculated using EXCEL™ *v* 2003.

3. Results

3.1. Selenium accumulation

Selenium accumulation by *A. trapezia* was dependent on time and sediment selenium concentration ($p \leq 0.0005$; Supplementary Table 1). Tissue selenium concentrations reflected the sediment exposure concentrations and were in the order 20 μg/g > 5 μg/g > control for each analysis time (Figure 1). The tissue selenium concentration of the control organisms remained the same over the course of the exposure (Figure 1). The pattern of selenium accumulation was similar in organisms from both selenium treatments, both accumulated selenium over the first 14 days with very slight increases over the next 28 days and then a slight decrease to day 56 (Figure 1). Regression between selenium concentrations in sediments and organisms after 56 days shows a significant positive ($r = 0.97$), but not proportional, relationship (Figure 1). Selenium accumulation was significantly different between tissues ($p \leq 0.0005$; Supplementary Table 2) with tissue selenium concentrations in the order gills > hepatopancreas ≥ haemolymph (Figure 2).

3.1.1. Subcellular Tissue Selenium

Between 35 and 50 % of the total gill and 31 and 59 % of the total hepatopancreas selenium was recovered in the subcellular tissue fractions (Table 1). Of the selenium recovered in the gill fractions between 16 and 21 % was in the biologically detoxified selenium fraction and fairly equally distributed between the metal rich granule (MRG) and metallothoinein like protein (MTLP) fractions. The hepatopancreas selenium distribution was similar with between 12 and 16 % in the biologically detoxified selenium fractions with three quarters in the MRG and the remainder in the MTLP fraction (Table 2; Figure 3). The biologically detoxified selenium in both control tissues was in the metal rich granule (MRG) fraction (Figure 3). The highest percentage of selenium in the biologically active selenium fractions of tissues in controls and treatments was generally in the mitochondria (35-62 %) with the remainder distributed between the heat sensitive proteins (HSP) and lysosome+microsome fractions (Figure 3; Table 2).

3.2. Biomarkers

The total antioxidant capacity of cells were significantly reduced ($p \leq 0.0005$; Supplementary Table 3a) in both the selenium treatments, relative to the control organisms (Figure 4).

Glutathione peroxidase activity and mean total glutathione (GSH + 2GSSG) concentrations for treatments were not significantly different to controls (Figure 4, $p \leq 0.0005$; Supplementary Table 3b). The ratio of reduced to oxidised glutathione was significantly reduced in selenium exposed organisms (Figure 4, P> 0.05; Supplementary Table 3b). Lipid peroxidation (TBARS) significantly increased with exposure to increased sediment selenium concentrations (Figure 5, P> 0.05; Supplementary Table 3b). Lysosomal stability decreased and micronuclei frequency significantly increased with exposure to increased selenium concentrations (Figure 5, Supplementary Tables 3a & 3b). Regression analysis showed that the reduced total antioxidant capacity within cells had a negative relationship with the effects measures of TBARS ($r = 0.88$), lysosomal stability ($r = 0.95$) and micronuclei frequency ($r = 0.88$) for selenium exposed organisms (Figures 4 & 5). There was a positive relationship between TBARS and lysosomal stability ($r = 0.99$) and micronuclei frequency ($r = 1.00$) (Figure 5).

4. Discussion

4.1. Selenium Accumulation and Subcellular Distribution

4.1.1. Whole Organism and individual tissues

The background selenium concentration of the *A. trapezia* used was approximately 1 µg/g (Figure 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. 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 population and the sediment exposure concentration. Populations from areas with higher selenium sediment exposure maintained higher tissue selenium concentrations. This is further supported by Burt *et al.* (2007) who transplanted *A. trapezia* from an uncontaminated environment to sites with elevated selenium, in Lake Macquarie NSW. Within three months they increased their selenium tissue concentrations from 2 µg/g to a concentration of 4 – 5 µg/g approaching that of the indigenous population. Selenium exposed *A. trapezia* in this experiment appeared to reach an equilibrium tissue concentration at both exposure concentrations which was lower than the exposure concentration with the organisms at the higher exposure accumulating higher selenium concentrations (Figure

1) suggesting some capacity for *A. trapezia* to regulate selenium, relative to their exposure environment.

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). Dietary selenium has also been established as a major selenium exposure route in aquatic organisms (Hamilton, 2002; Hamilton, 2004; Lemly, 1999b; Luoma and Rainbow, 2008). The pattern of tissue accumulation observed in the 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 or equal selenium concentrations relative to the hepatopancreas tissues (Figure 2). The hepatopancreas tissues contributed more to the total selenium in the latter part of the exposure period suggesting an increase in the dietary selenium contribution, probably combined with internal transport of accumulated dissolved selenium to the digestive system (Viarengo and Nott, 1993). Bacteria and marine algae, which were present in the microcosms, are 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)

4.1.2. 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 selenium exposed organisms (Table 1; Figure 3). 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 could account for the high proportion of selenium associated with the debris fraction.

It would, therefore, be comprised of both biologically detoxified and active selenium. Only a small percentage of the accumulated selenium was recovered in the biologically detoxified selenium fractions in the exposed organisms (Figure 3). The distribution within the detoxified selenium fractions differed between tissues. There was an even distribution in the gill tissues and a higher proportion in the metal rich granules (MRG) than the metallothionein like proteins (MTLP) in the hepatopancreas tissues (Table 1). Selenium associated with metallothioneins 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 metallothioneins 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, 1983). The lack of selenium in the MTLP of the control organisms (Figure 3) 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, or could represent a previous exposure history in which excess selenium was detoxified and stored. If this is the case the observation that selenium 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 time scale. 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 biologically active selenium fraction (Table 1). This supports the view that the selenium measured in these organisms represents the metabolic requirement (DeNicola Cafferky *et al.*, 2006). The percentage of selenium in the biologically active selenium fractions of organisms from all treatments was far greater than the percentage in the biologically detoxified selenium fractions indicating that *A. trapezias'* capacity to detoxify or store selenium

is limited. More than half of the selenium in the biologically active selenium component of the gill tissues was in the mitochondrial fraction (Figure 3; Table 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 cytochrome *c* oxidase in the gill tissues compared to the hepatopancreas tissues (Supplementary Figures 1 and 2) shows the gill 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 heat sensitive protein fraction (Figure 3; Table 1). The higher binding of selenium with heat sensitive proteins in the hepatopancreas tissue may be due to the incorporation of selenium into the hepatopancreas; via assimilation of food and sediment, as well as through haemolymph transfer and directly from water (Fan *et al.*, 2002). The increased selenium concentrations in the biologically active selenium fractions of the exposed organisms has implications for adverse effects in *A. trapezia*, as there is only a narrow concentration range at which selenium is required for metabolic processes and beyond this it is highly toxic (Hamilton, 2004).

4.2. 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 total antioxidant capacity (TAOC) in *A. trapezia* from both selenium treatments to less than half that of the control organisms (Figure 4). The final gill biologically active 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 biologically active selenium burdens was also seen for lead exposure in *A. trapezia* (Taylor and Maher, 2012) and like lead it is possible that there is a critical selenium concentration that impairs TAOC which may be lower than these concentrations. The glutathione peroxidase (GPx) enzyme activity, in which selenium plays a major role, was reduced in *A. trapezia* from both treatments but neither was significantly lower than control organisms (Figure 4). 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 biologically active selenium burden having a toxic inhibitory effect on GPx formation. The total glutathione concentration was enhanced in *A. trapezia* from both treatments but neither was significantly higher than control organisms (Figure 4). The significantly reduced GSH:GSSG ratio in selenium exposed *A. trapezia*, indicates that increased total glutathione was largely comprised of oxidised glutathione. Significantly reduced ratios of GSH:GSSG have previously been measured in selenium exposed mallard ducks (Hoffman, 2002). Oxidised glutathione can react with protein sulfhydryls, contributing to total and protein bound thiol depletions (Hoffman, 2002), but equally excess GSSG may be excreted from cells more rapidly than it can be reconverted back to the reduced form by glutathione reductase (Meister, 1989). Selenite in high concentrations can also deplete GSH, inhibiting the release of selenium metabolites (Magos and Webb, 1980).

4.3. Oxidative Damage Biomarker – Thiobarbituric Acid Reactive Substances

Increased lipid peroxidation has been 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). There was a trend of increasing lipid peroxidation with increased selenium exposure which was significantly higher in organisms from both selenium treatments than in the control organisms (Figure 5). The negative relationship between total antioxidant capacity and lipid peroxidation with increased selenium exposure supports the existence of a link between increased ROS and the production of lipid peroxidation products.

4.4. 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 5). This level of lysosomal destabilisation indicates significant selenium toxicity and puts them well into the stressed range of the criteria developed for the oyster *C. virginica* by Ringwood *et al.* (2003). The increased lysosomal destabilisation with increased selenium burdens follows the same pattern as seen for lipid peroxidation (Figure 5) and the positive relationship between these two

biomarkers indicates peroxidative damage was probably a significant pathway for lysosomal destabilisation. Heat sensitive cytosolic proteins of the selenium exposed *A. trapezia* had significantly increased selenium burdens (Table 1) 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).

4.5. Genotoxic Biomarker – Micronuclei Frequency

The significant increase in micronuclei with increased selenium exposure showed a relationship with both total antioxidant capacity reduction and increased TBARS (Figure 5) indicating that an increase in reactive oxygen species probably 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. The tetragenic tendency of this element suggests there is a potential for this level of exposure to have consequences for population viability.

5. Conclusions

The metabolic selenium requirement of *A. trapezia* appears to be in the order of 1 to 2 μg/g dry tissue mass. A large percentage of the accumulated selenium was associated with the cellular debris. 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 detoxified a

small percentage of accumulated selenium which suggests a limited detoxification and storage capacity for this element. The high proportion in the nuclei+cellular debris potentially bound to cell walls could, however, effectively be detoxified. The differences in selenium distribution in the biologically active selenium fractions of the two tissues indicate two major exposure routes, dissolved selenium in the gill and dietary selenium in the hepatopancreas. There was a significant reduction in the total antioxidant capacity in *A. trapezia* from both selenium treatments. Glutathione peroxidase reduction was reflected in increased total glutathione concentrations which the GSH:GSSG ratios indicate was due to a build up of oxidised glutathione. Organisms with reduced total antioxidant capacity showed increased lipid peroxidation, lysosomal destabilisation and micronuclei frequency. This research supports a significant exposure - dose - response relationship for selenium in *A. trapezia.* Identification of these relationships for selenium exposure at these levels of biological organisation should enable a greater understanding of the mechanisms of stress responses to this toxicant. If such relationships can be demonstrated in field exposed organisms this could ultimately aid in developing improved predictive capability for ecological risk assessment, allowing better informed decisions regarding remedial actions to be made.

Acknowledgements

We thank F. Krikowa 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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Table(s)

Table 1: Selenium (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. Subcellular selenium(μg wet mass) and percentage distribution of total recovered selenium fractions are grouped as nuclei+cellular debris and biologically active and detoxified selenium (Figure 3).

	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	0.40 ± 0.05	0.4 ± 0.1
Total Recovered Selenium (μg)	0.10 ± 0.02	0.2 ± 0.1	0.80 ± 0.05	0.10 ± 0.01	0.10 ± 0.01	0.20 ± 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 Selenium (μg)	0.06 ± 0	0.09 ± 0.04	0.40 ± 0.02	0.05 ± 0	0.06 ± 0	0.08 ± 0.01
Biologically Active Selenium (%)	67 ± 13	37 ± 7	49 ± 1.4	70 ± 13	37 ± 6	37 ± 5
Biologically Detoxified Selenium (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 Selenium (%)	10 ± 4	21 ± 3	16 ± 3	16 ± 5	16 ± 3	12 ± 2

Mean ± SD, $n = 2$.

Table 2: Mean percentage of selenium in the debris, biologically detoxified selenium (BDM) and biologically active selenium (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(s)

Figure Captions

Figure 1: Selenium accumulation in whole tissue of *A. trapezia* at 2 week intervals over 56 days of exposure to sediments containing selenium at 0 (control), 5 & 20 µg/g dry mass. Mean ± SE, n = 8, 12 and 8 respectively. Day 0 are unexposed organisms n=5.

Figure 2: Selenium accumulation in gill, hepatopancreas and haemolymph tissues of *A. trapezia* at 2 week intervals for 56 days exposure to sediments spiked with selenium at; 0 (control), 5 and 20 µg/g dry mass. Mean ± SE, n = 8, 12 and 8 respectively. Day 0 are unexposed organisms, n=5.

Figure 3: Distribution (%) of selenium in 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 & 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 selenium (BAM), green fractions make up the biologically detoxified selenium (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 selenium spiked sediments: 0 Se (control), Se 5 µg/g; and Se 20 µg/g dry mass. Mean ± SE, n = 8, 12 and 8 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* gill, hepatopancreas and gill tissues respectively following 56 days 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 between means (Bonferroni test; $p < 0.05$).

Figure 1

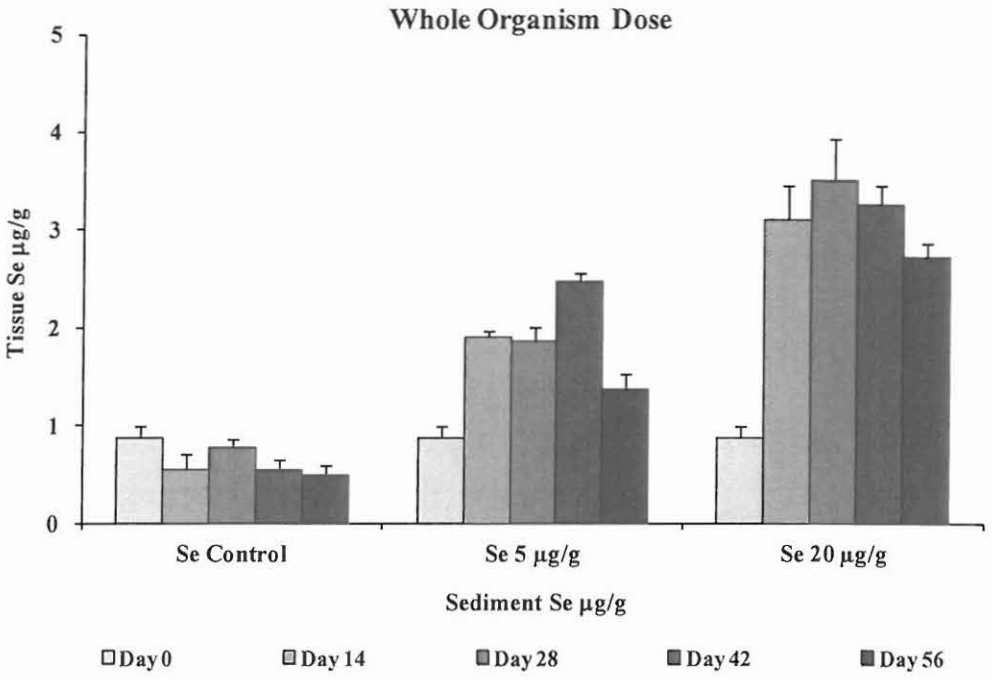

Whole Organism Dose
Tissue Se µg/g
5
4
3
2
1
0
Se Control
Se 5 µg/g
Se 20 µg/g
Sediment Se µg/g
Day 0
Day 14
Day 28
Day 42
Day 56

Figure 2

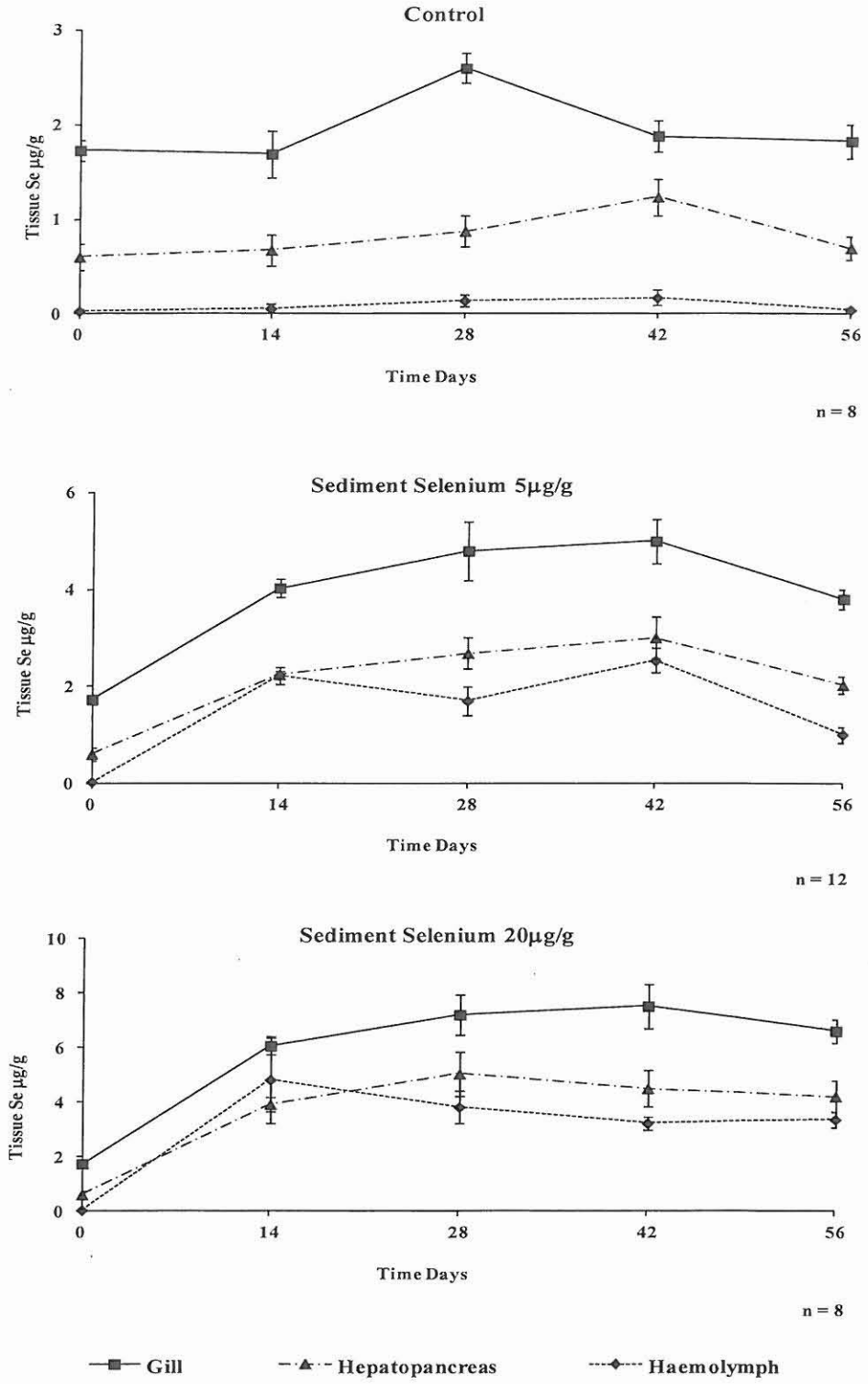

Control
Tissue Se µg/g
Time Days
n = 8
Sediment Selenium 5µg/g
Tissue Se µg/g
Time Days
n = 12
Sediment Selenium 20µg/g
Tissue Se µg/g
Time Days
n = 8
Gill
Hepatopancreas
Haemolymph

Figure 3

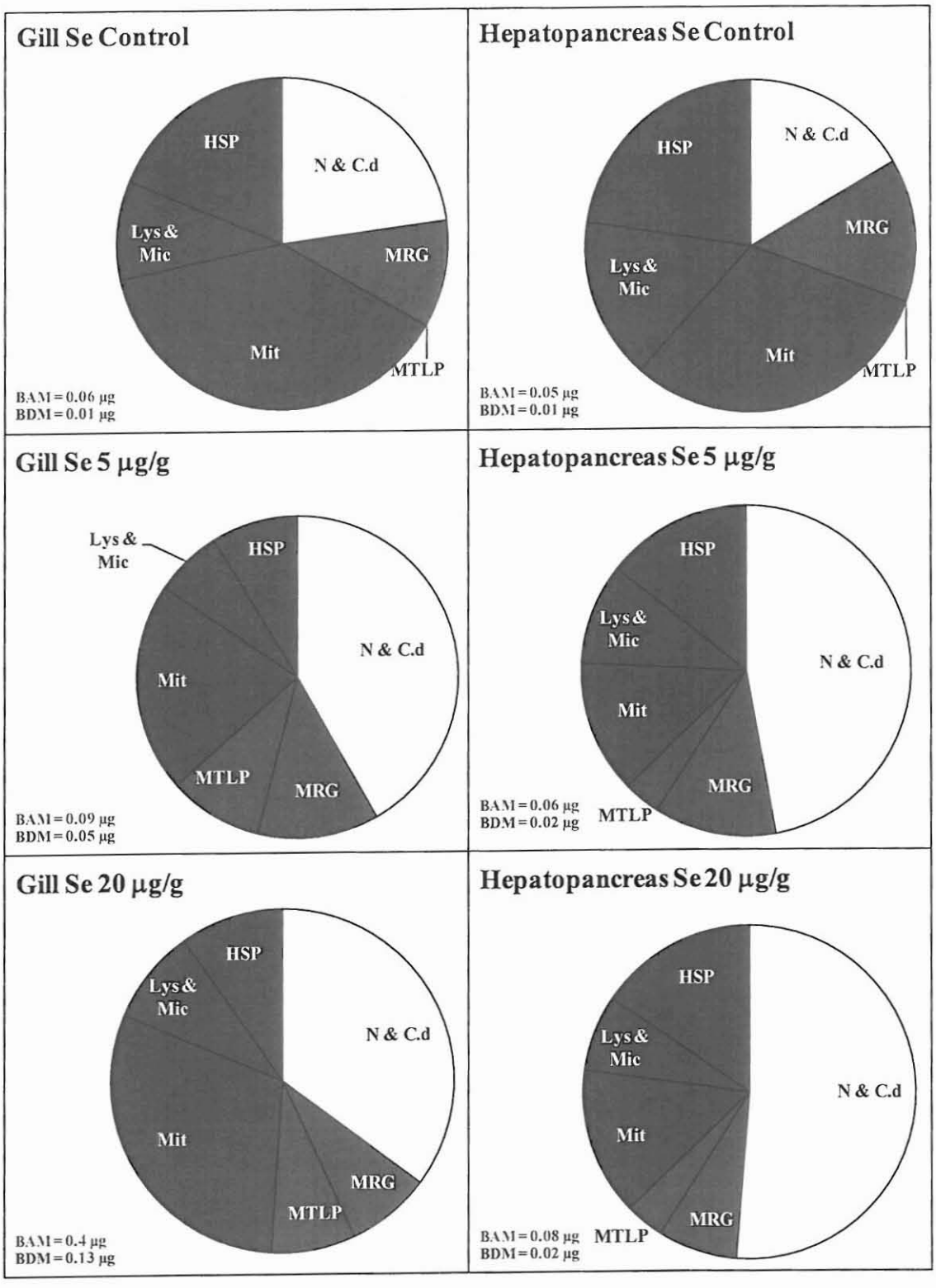
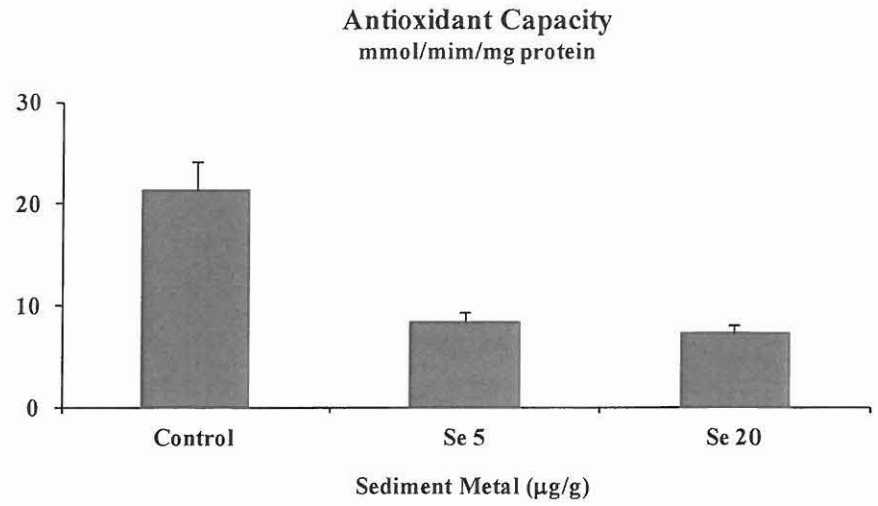

Gill Se Control
HSP
N & C.d
Lys & Mic
MRG
Mit
MTLP
BAM = 0.06 µg
BDM = 0.01 µg
Hepatopancreas Se Control
HSP
N & C.d
MRG
Lys & Mic
Mit
MTLP
BAM = 0.05 µg
BDM = 0.01 µg
Gill Se 5 µg/g
Lys & Mic
HSP
N & C.d
Mit
MTLP
MRG
BAM = 0.09 µg
BDM = 0.05 µg
Hepatopancreas Se 5 µg/g
HSP
Lys & Mic
N & C.d
Mit
MRG
MTLP
BAM = 0.06 µg
BDM = 0.02 µg
Gill Se 20 µg/g
HSP
Lys & Mic
N & C.d
Mit
MRG
MTLP
BAM = 0.4 µg
BDM = 0.13 µg
Hepatopancreas Se 20 µg/g
HSP
Lys & Mic
N & C.d
Mit
MRG
MTLP
BAM = 0.08 µg
BDM = 0.02 µg

Figure 4


Antioxidant Capacity
mmol/mim/mg protein
30
20
10
0
Control
Se 5
Se 20
Sediment Metal (µg/g)

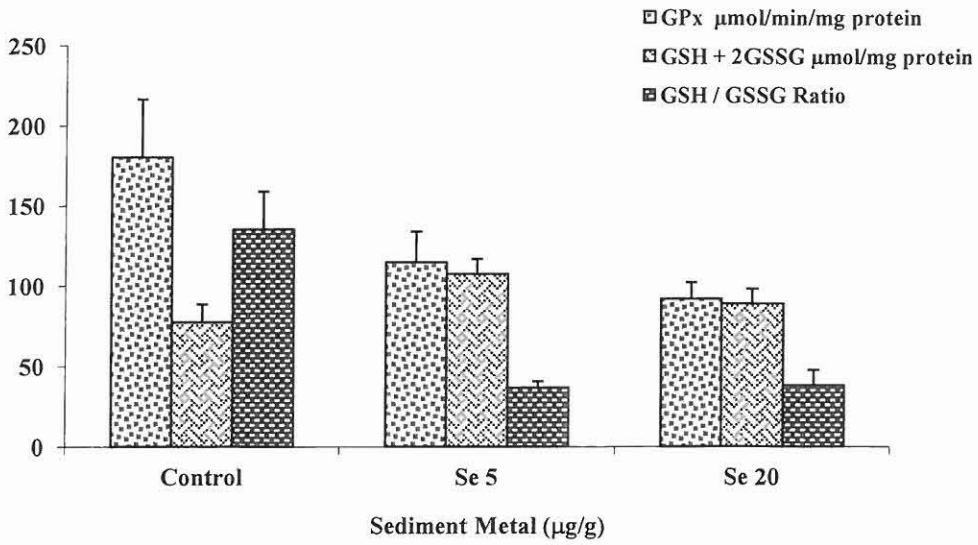
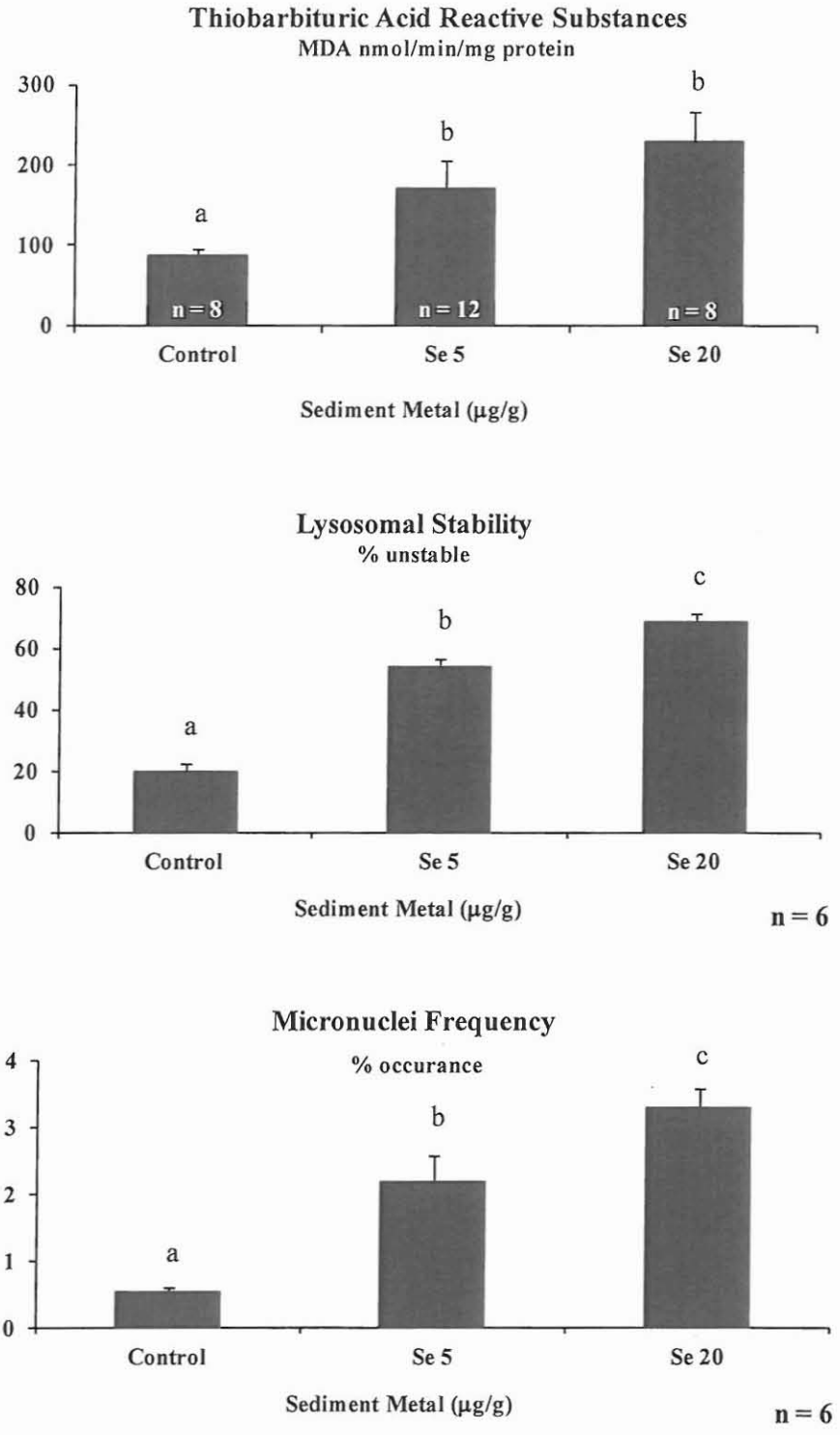

GPx µmol/min/mg protein
GSH + 2GSSG µmol/mg protein
GSH / GSSG Ratio
250
200
150
100
50
0
Control
Se 5
Se 20
Sediment Metal (µg/g)

Figure 5


Thiobarbituric Acid Reactive Substances
MDA nmol/min/mg protein
300
200
100
0
a
b
b
n = 8
n = 12
n = 8
Control
Se 5
Se 20
Sediment Metal (µg/g)
Lysosomal Stability
% unstable
80
60
40
20
0
a
b
c
Control
Se 5
Se 20
Sediment Metal (µg/g)
n = 6
Micronuclei Frequency
% occurance
4
3
2
1
0
a
b
c
Control
Se 5
Se 20
Sediment Metal (µg/g)
n = 6

Supplementary Material
Click here to download Supplementary Material: Supp se final.docx

Highlights

>We describe an exposure-dose-response approach to assessing selenium exposure in *Anadara trapezia*. > The selenium accumulation was both dissolved via gills and dietary via hepatopancreas. > The majority of accumulated selenium was associated with cellular debris or as biologically active selenium. > The small proportion in metallothionein like proteins and granules suggests a limited detoxification capacity for selenium. > Increased selenium dose resulted in reduced antioxidant capacity with an associated increase in lipid peroxidation, increased lysosomal destabilisation and genotoxic damage. > Elevated sediment selenium concentrations significantly impaired *A. trapezia* cellular processes and may threaten the health of sediment dwelling organisms.>