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Exposure-dose-response of *Tellina deltoidalis* to metal contaminated estuarine sediments. 2. Lead spiked sediments

Anne M. Taylor* and William A. Maher

Ecochemistry Laboratory, Institute for Applied Ecology, University of Canberra, Canberra, ACT 2601, Australia

*corresponding author:
Email: anne.taylor@canberra.edu.au
Phone: +61 2 62063805

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Abstract

Lead accumulation in estuarine sediments, as a result of activities such as mining and ore smelting, and through urban runoff is a continuing problem in the increasingly developed world. Marine organisms accumulate lead, which is known to be highly toxic to biological processes and to degrade organism and ecosystem health. Here the relationship between lead exposure, dose and response was investigated in the sediment dwelling, deposit feeding, marine bivalve *Tellina deltoidalis*. Bivalves were exposed in the laboratory to individual lead spiked sediments at 100 and 300 μg/g dry mass, for 28 days and accumulated total tissue lead concentrations of 96 and 430 μg/g, respectively. Subcellular fractionation indicated that around 70% of accumulated lead was detoxified, three quarters of which was converted into metal rich granules. The majority of biologically active lead was associated with the mitochondrial fraction with up to a 128 fold increase in lead burden in exposed organisms compared to controls. This indicates active lead detoxification which at these exposures was unable to prevent significant lead burdens accumulating in sensitive organelles. With increased lead exposure *T. deltoidalis* showed a suppression in glutathione peroxidase activity, total glutathione concentration and reduced GSH:GSSG ratios, however, these differences were not significant. Lead exposed *T. deltoidalis* had a significantly reduced total antioxidant capacity which corresponded with increased lipid peroxidation, lysosomal

destabilisation and micronuclei frequency. The exposure-dose-response relationship demonstrated for lead exposed *T. deltoidalis* supports its potential for the development of sub-lethal endpoints in lead toxicity assessment.

Keywords: Biomarkers, subcellular lead, oxidative stress, lysosomes, lipid peroxidation, micronuclei, bivalve.

1 Introduction

Lead is released into the environment through a variety of human activities including lead ore mining and smelting, alkyl-lead petroleum combustion, coal burning, waste incineration, production and storage of lead-acid batteries, leaded glass, lead oxide pigments and ferroalloys, metal fabricating industries, cement manufacture and urban runoff (Ãlvarez-Iglesias *et al.*, 2012; ARCARP, 2006; Chiaradia *et al.*, 1997; Cook and Gale, 2005; Genaidy *et al.*, 2009; Renberg *et al.*, 2002). In Australia the majority of the population is located along coastlines, the bays and estuaries of which are repositories for sediment bound lead (ABS, 1996; ANZECC and ARMCANZ, 2000; Carroll, 1996; Chiaradia *et al.*, 1997).
The accumulation of lead by marine organisms has implications for both ecological and human health at both individual and population levels (Clark, 2001; Gnassia-Barelli and Romeo, 1993). Although it has no known biological function lead is accumulated by marine organisms through similar pathways as essential elements such as calcium, iron and zinc making it one of the more toxic metals in these environments (Company *et al.*, 2011; 2008). The metal exposure route, through either food or water, affects the organism's metal handling and potentially its toxicity (Rainbow, 2007). Deposit feeding bivalves are of particular interest as biomonitors as they are exposed to contaminants through water and food and directly through ingested fine grain sediment particles containing organic material (Griscom and Fisher, 2004; Lee *et al.*, 2000; 1998).
Whole sediment toxicity tests are used worldwide as an integral part of toxicity assessment as they assess contaminant exposure from several exposure routes; including those bound to sediments, from pore waters and in overlying waters (Adams *et al.*, 2005; ASTM, 2010; Ingersoll *et al.*, 2000). In Australia, aquatic toxicity tests have been established (ANZECC and ARMCANZ, 2000) but the use of whole sediment toxicity tests has been hampered by a lack of established test procedures and knowledge of species sensitivity to contaminants. Early investigations of suitable Australian organisms' sensitivity to metal contamination in

whole sediment toxicity tests have included polychaetes, amphipods and bivalves, of which, the bivalve *Tellina deltoidalis* was highly sensitive to zinc and copper (King *et al.*, 2004; 2005). *T. deltoidalis* is a deposit feeding bivalve which satisfies most of the basic requirements to be an effective biomonitor, they are: hardy, accumulate metal and have sufficient tissue for analysis (King *et al.*, 2010; 2004; 2005; Taylor and Maher, 2013); and are widely distributed around Australian coastal estuaries, living in sediments at a depth several times their 15 – 25 mm shell length with siphons extended to the surface to feed (Beesley *et al.*, 1998).

The development of Australian guidelines for sediment quality assessment, which includes contaminant exposure, uptake and ecotoxicological effects, identified *T. deltoidalis* as a suitable species for whole sediment toxicity testing and a protocol for its use is included in the Australian Handbook for Sediment Quality Assessment (Simpson *et al.*, 2005). The assessment of ecotoxicological effects of contaminants is moving from the use of mortality as an endpoint to the use of biomarkers of sub lethal effects and there is a need, in understanding organism sensitivity to contaminants, to develop suitable biomarkers which can be included in routine testing. The use of biomarkers enables links to be made between external exposure and an internal toxicant dose, which has exceeded the organism's detoxification and repair capacity, allowing ecologically relevant effect concentrations to be determined (Adams *et al.*, 2011; Depledge, 1992). Biomarkers of exposure and effect for use in aquatic toxicological assessment have been progressively developed and refined for a range of species and toxicants worldwide (Bergayou *et al.*, 2009; Bocchetti *et al.*, 2008; Cajaraville *et al.*, 2000; Damiens *et al.*, 2007; Domouhtsidou *et al.*, 2004; Durou *et al.*, 2007; Galloway *et al.*, 2004; Lam, 2009; Lam and Gray, 2003; Taylor and Maher, 2010; Viarengo *et al.*, 2007).

Lead has a high affinity for sulfhydryl, forming mercaptides with the sulfhydryl group of cysteine and inhibiting several enzymes having functional sulfhydryl groups, thereby initiating oxidative stress responses (Dafre *et al.*, 2004; Ercal *et al.*, 2001; Gurer and Ercal, 2000). Changes in the oxidative pathway including increased reactive oxygen by-products and suppression or increased expression of oxygen reduction enzymes have been shown for a number of lead exposed molluscs (Alcutt and Pinto, 1994; Cossu *et al.*, 2000; Dafre *et al.*, 2004; Jing *et al.*, 2007; Taylor and Maher, 2012). Lead is also thought to interact with a variety of cellular lipids altering the lipid composition of cellular membranes and increased susceptibility to lipid peroxidation (Campana *et al.*, 2003; Mateo *et al.*, 2003). This can result in perturbations in cell membrane integrity, permeability and function increasing lysosomal destabilisation (Einsporn and Koehler, 2008; Ercal *et al.*, 2001; Gurer and Ercal,

2000). Lead has been shown to interact directly with DNA by covalent binding of Pb^{2+} to DNA (Hong, 2007) producing micronuclei, through either the breakage of chromosomes or dysfunction of the mitotic spindle apparatus (Monteiro *et al.*, 2011; Winter, 2007). The micronuclei induction assay which can detect these small masses of cytoplasmic chromatin, present outside the main nucleus, has proven useful in assessing the genotoxic effects of a range of compounds in fish and other aquatic organisms (Bolognesi *et al.*, 2004; Kalpaxis *et al.*, 2004; Udroiu, 2006).

The purpose of this study was to examine the exposure-dose-response relationship to lead in *T. deltoidalis* using a 28 day sediment bioaccumulation test (ASTM, 2010; Ingersoll *et al.*, 2000), to develop useful biomarkers of effect, with a view to determining their suitability for laboratory sediment metal toxicity tests using sub lethal endpoints. Two lead sediment exposure concentrations were used, 100 mg/kg based on the high value for lead sediment concentrations from the ANZECC and ARMCANZ (2000) Interim Sediment Quality Guidelines and 300 mg/kg based on concentrations previously measured in lead contaminated Australian estuarine sediments (Roach, 2005). The study examines lead accumulation and subcellular tissue lead distribution. Biomarkers of lead exposure effects were total antioxidant capacity, and the associated oxidative enzymes; total, reduced and oxidised glutathione and glutathione peroxidise. The cellular effects measure lysosomal destabilisation and a genotoxic measure, micronuclei occurrence were also measured in a weight of evidence approach to establishing lead sub lethal toxic effects.

2 Materials and Methods

2.1 Organism and sediment collection

Sediments were collected from a NSW Department of Environmental and Climate Change reference site in Durras Lake NSW, and stored at 4°C until use. *Tellina deltoidalis* of 15 – 20 mm in size were collected from Durras Lake and Lake Tabourie, NSW in July 2005 and January 2006 and placed in coolers with sediment and water from the collection sites for transportation. Organisms were maintained for a maximum of two weeks at 22°C in uncontaminated sediments, depth 15 cm, in glass aquaria with filtration and aeration to allow acclimation before experimentation. Overlying water used in aquaria was collected from coastal waters near Murramurrang National Park, NSW and adjusted from 35‰ to 28‰ with deionised water to match the salinity of the estuarine water from which organisms were collected.

2.2 Sediment lead 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 produce a sediment matrix which was suitable for organism burrowing and feeding, sediment was mixed with clean beach sand so that the 63 µm fraction was not greater than 20% mass/mass. To ensure added lead was rapidly adsorbed and bound to the sediment particles the procedure developed by (Simpson *et al.*, 2004) for producing metal spiked marine sediments, was followed. Wet sediment was added to mixing containers. $PbCl_2$, (AR grade Sigma-Aldrich), was added to concentrations of 100 mg/kg and 300 mg/kg dry mass of sediment. All containers were topped up with clean deoxygenated sea water and the final mixture was completely deoxygenated by bubbling with nitrogen for 2 hours. Head spaces of containers were filled with nitrogen prior to sealing. Any pH adjustments were made immediately after the addition of lead using 1M NaOH, (AR grade BDH), prepared in seawater. pH was checked weekly and maintained at 7 - 8.2. Sediments were maintained at room temperature 22 - 25^{o}C and mixed on a Cell-production Roller Apparatus (Belco, USA) for several hours each day. The time required for equilibration of added metals is affected by the sediment properties, equilibration pH and the concentration and properties of the metal (Simpson *et al.*, 2004). To determine when the added lead was completely bound to sediment particles, pore waters were collected and 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 - 6 weeks. Unspiked sediments were treated in the same way and used for control treatments. Sediment lead concentrations were measured by ICP-MS after digestion of 0.2 g of lyophilised sediment in 3 ml of nitric acid (AristaR, BDH, Australia) in polyethylene 50 ml centrifuge tubes for 60 minutes at 115^{o}C (Maher *et al.*, 2003). Lead in NRCC Certified Reference Materials, BCSS-1 marine sediment measured along with samples was 21 ± 4 µg/g (n = 10) and in agreement with certified values 22.7 ± 3.4 µg/g. Pre exposure sediment lead concentrations were < 0.001, 100 ± 4.5 and 300 ± 8 µg/g and post exposure were < 0.001, 101 ± 5 and 298 ± 10 µg/g.

2.3 Microcosm experiment design

Procedures for conducting the exposures were adapted from the test method for conducting 28 day sediment bioaccumulation tests (Ingersoll *et al.*, 2000). Spiked and control sediments

(500 g wet wt.) were placed in each of three replicate 770 ml polypropylene containers (Chanrol # 01C30, Australia) per treatment. The containers were filled with fresh seawater adjusted to a salinity of 28‰. Containers were placed in random order on a tray in an incubator set at 22°C with a day / night light cycle of 14 / 10 hours to reflect spring / summer conditions. Aeration was introduced and the treatments were left for 24 hours to allow them settle and the temperature to equilibrate. Fifteen *T. deltoidalis* were then introduced to each treatment container. Organisms were not given supplementary food and surface water was changed weekly during the 28 day exposure period. Aquaria were continually aerated using an air pump with valves on each line to regulate air flow so oxygen saturation ≈ 100% were maintained in each aquarium 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 28 days of exposure. This is similar to results of other studies of this type (King *et al*., 2006; Strom *et al*., 2011). Total tissue lead bioaccumulation was measured at intervals of 3, 7, 14, 21 and 28 days. A day 0 measurement was made using organisms from the acclimation tanks to give the background lead concentration. All organisms were placed in fresh seawater 28‰ with no sediment for 24 hours to allow depuration of ingested sediment particles, prior to lead analysis. Visual inspection under a dissecting microscope showed gut clearance was achieved in this timeframe. All assays were undertaken on whole tissues of individual organisms.

2.4 Lead measurements

2.4.1 Total lead

Lyophilised ground tissue was microwave digested in 1 ml of nitric acid (AristaR BDH, Australia) in a 630 watt 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). Prior to analysis samples were diluted with deionised water to 1% v/v HNO_3, and an ICP-MS mixed 7-element internal standard (EM Science) was added to monitor for variations due to instrument drift and/or matrix effects. Lead was measured using an ELAN® 6000 ICP-MS (PerkinElmer, SCIEX) following the method of Maher *et al*. (2001). NRCC Certified Reference Material, NIST 1566a oyster tissue and acid blanks were routinely digested and diluted in the same way as the samples and analysed along with them to verify accuracy and precision of lead analysis. The measured CRM mean lead value; 0.36 ± 0.02 µg/g (n = 50) was not significantly different from the certified value 0.37 ± 0.014 µg/g.

2.4.2 Subcellular lead

The subcellular tissue lead distribution was examined in tissues of day 28 exposed organisms 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^{o}C until processed. The tissue was thawed and minced on ice with a blade. A sub sample, approximately 0.1 g wet wt., was taken for total tissue lead analysis. The remainder, approximately 0.5 g wet wt., was homogenised in Ca^{2+} / Mg^{2+} free saline buffer pH 7.35 on ice using an IKA® Labortechnick Ultra-turrax-T25 homogeniser equipped with an S25-UT dispersing tool at 9,500 $rpmin^{-1}$ (Janke & Kunkel, Germany). Homogenised tissue was subjected to differential centrifugation and tissue digestion procedures according to the protocol outlined in Taylor and Maher (2010; 2013), using an Eppendorf 5804R centrifuge and a Himac CP90WX preparative ultracentrifuge (Hitachi, Japan). The mitochondria (P3), lysosomes+microsomes (P4) and heat sensitive protein (P5) pellets were grouped as biologically active lead fractions while the metal rich granule (P2) and heat stable metallothionein like proteins (S5) were grouped as biologically detoxified lead fractions (Supplementary Figure 1). The supernatant from the granule pellet isolation (S2) contained the nuclei and cellular debris (Supplementary Figure 1). To determine the mitochondrial and lysosomal content of the fractions obtained the activity of enzymes specific for these organelles, cytochrome *c* oxidase and acid phosphatase, respectively, were measured in each of the total tissue homogenate, mitochondrial (P3) and lysosome+microsome (P4) pellets using commercial colorimetric assays (CYTOC-OX1 Sigma-Aldrich, USA and CS0740 Sigma-Aldrich, USA, respectively). In the total homogenate both the mitochondrial enzyme cytochrome *c* oxidase and the lysosomal enzyme acid phosphatase showed an increase in concentration with increased lead exposure (Supplementary Figure 2). The mitochondrial fractions had higher cytochrome *c* oxidase concentrations than the lysosome fractions indicating mitochondrial enrichment of this fraction (Supplementary Figures 1 & 2). The lysosome+microsome fractions had slightly higher acid phosphatase concentrations than the mitochondrial fractions indicating some enrichment of lysosomes in this fraction with some carryover of mitochondria. All fractions were acidified to 10% v/v with nitric acid (AristaR BDH, Australia) and placed in a water bath at 80^{o}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 lead was as previously described above. The measured CRM lead value; 0.38 ± 0.05 µg/g (n = 5) was in good agreement with certified value 0.37 ± 0.014 µg/g.

2.5 Biomarker measurements

2.5.1 Total antioxidant capacity and lipid peroxidation

Tissues were homogenised on ice in a 5 mM potassium phosphate buffer containing 0.9% (w/v) sodium chloride and 0.1% (w/v) glucose, pH 7.4 (1:5 w/v) using a motorised microcentrifuge pellet pestle, sonicated on ice for 15 seconds at 40 V (VibraCell™ Sonics Materials, USA) and centrifuged, in a 5804R centrifuge (Eppendorf, Germany), at 10,000 x g for 15 minutes at 4^{o}C (Cayman, 2011). The supernatant was stored at -80^{o}C until analysis. Total antioxidant capacity was measured using an assay based on the ability of the tissue lysate antioxidant system to inhibit the oxidation of ABTS (2,2'-azino-di-[3-ethylbenzthiazoline sulphonate]) to ABTS $^{\bullet+}$ by metmyoglobin in the presence of hydrogen peroxide. This was compared with the antioxidant capacity of a standard, Trolox (Cayman, 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^{o}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^{o}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 and glutathione peroxidase

Tissue lysates were produced by homogenisation on ice in a 50 mM Tris-HCl buffer containing 5 mM EDTA and 1 mM DTT, pH 7.5 (1:5 w/v) using the technique outlined above. A thiol scavenging agent 1-methyl-2-vinyl-pyridium trifluoromethane sulfonate 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^{o}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^{o}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^{o}C.

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 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 used were made up in sample buffer.

2.6 Cellular and Genotoxic Biomarkers

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 *x* g at 15^{o}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 40*x* 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^{o}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 40*x*. 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 (lead exposure concentration) on organism tissue lead accumulation. A Mixed Linear Model ANOVA was used to analyse the effects of treatment (lead exposure concentration) on the effect measurement variables antioxidant capacity, total glutathione, GSH:GSSG ratio, glutathione peroxidase, lipid peroxidation, lysosomal stability and micronuclei frequency (Supplementary Tables 1 – 3). Regressions of sediment lead and mean tissue lead concentrations and means of effects variables antioxidant capacity, lipid peroxidation, lysosomal stability and micronuclei frequency were calculated using EXCEL™ *v* 2003 (Supplementary Table 4).

3 **Results**

3.1 Lead accumulation

Lead accumulation by *Tellina deltoidalis* was dependent on time and sediment lead concentration, ($p < 0.001$; Supplementary Tables 1 & 2). Lead tissue concentrations were in the order 300 µg/g > 100 µg/g > control for each analysis time and at day 28 were equal to or above that of the sediment concentrations in both treatments (Figure 1). The 100 µg/g treatment organisms had the highest lead concentration at day 28, while the 300 µg/g treatment organisms had the highest lead concentration at day 21 and with a small but not significant decrease to day 28 (Figure 1). The regression between sediment lead concentration and organism tissue lead concentration after 28 days showed a significant ($r^2 = 0.72$; $p \leq 0.0001$; $n = 41$) positive relationship (Supplementary Table 4).

3.1.1 Subcellular tissue lead

Between 72 and 92% of the total lead was recovered in the fractions (Table 1). Of the lead recovered around 70% was in the biologically detoxified metal fractions for all treatments (Table 1). Within the biologically detoxified fraction, the lead was fairly equally distributed between the metallothionein like protein fraction and the metal rich granule fraction in the control organisms, while in the lead exposed organisms 74% was in the metal rich granule fraction (Figure 2; Table 2). The percentage of lead recovered in the biologically active metal fractions of each of the 100 and 300 µg/g lead treatments was about half that of the

control, however, the total lead burden (µg) within these fractions was 28 and 100 times, respectively, greater in the lead exposed organisms (Table 1). The percentage of biologically active lead in the mitochondrial fraction increased to from 50 to 67% with increased lead exposure. The remainder of the biologically active lead in the exposed organisms was fairly equally distributed between the lysosome+microsome and heat sensitive protein fractions with the balance towards the lysosomal fraction, while the control organisms had 3.5 times more lead in the heat sensitive protein fraction than the lysosomal fraction (Figure 2; Table 2).

3.2 Biomarkers

The antioxidant capacity (TAOC) was significantly reduced ($p \leq 0.01$; Supplementary Table 3a) in lead exposed organisms compared to that of unexposed organisms, however, the antioxidant capacity of each of the high and low lead treatments were not significantly different to each other (Figure 3A; Supplementary Table 3b). The activity of the glutathione peroxidase enzyme, the total glutathione concentrations and ratio of reduced and oxidised glutathione were reduced in both lead treatments compared to the control organisms (Figure 3B), however, the difference was not significant ($p > 0.05$; Supplementary Table 3b) Thiobarbituric acid reactive substances (TBARS) were higher in lead exposed organisms than in unexposed organisms (Figure 4A). The 100 µg/g lead exposed organisms did not have significantly higher TBARS than the controls while the 300 µg/g lead exposed organisms had significantly higher concentrations than both the controls ($p \leq 0.001$) and the 100 µg/g lead exposed organisms ($p \leq 0.05$; Supplementary Table 3b). Lysosomal stability decreased and micronuclei frequency increased significantly ($p \leq 0.001$ Supplementary Tables 3a & 3b) with exposure to increased lead concentrations (Figure 4B & C). Regression analysis showed that when increased lead exposure reduced TAOC within cells there was a corresponding increase in the other effects measures; TBARS ($r^2 = 0.57$; $p \leq 0.0001$; $n = 29$), lysosomal destabilisation ($r^2 = 0.54$; $p \leq 0.001$; $n = 18$) and micronuclei frequency ($r^2 = 0.52$; $p \leq 0.001$; $n = 18$) (Supplementary Table 4). As TBARS increased there was a corresponding increase in lysosomal destabilisation ($r^2 = 0.55$; $p \leq 0.001$; $n = 18$) and micronuclei frequency ($r^2 = 0.51$; $p \leq 0.001$; $n = 18$) (Supplementary Table 4).

4 **Discussion**

4.1 Lead Accumulation and Subcellular Distribution

4.1.1 Whole organism

Lead as Pb^{2+} acts as a Ca^{2+} analogue, and is taken up presumably through high affinity Ca uptake mechanisms (Macdonald *et al.*, 2002). The *T. deltoidalis* exposed to 100 µg/g lead showed a rapid accumulation of lead in the first three days of exposure followed by small increases over the remainder of the 28 day exposure to reach a final tissue concentration equal to that of the spiked sediment (Figure 1). Mussels *Mytilus galloprovincialis* transplanted in a lead polluted area also reached a steady state of tissue lead after four weeks exposure (Regoli and Orlando, 1993). The organisms exposed to 300 µg/g of lead also accumulated lead rapidly over the first 3 days and reached a tissue concentration very close to that of the spiked sediment in this time (Figure 1). The subsequent drop in tissue lead between day seven and day fourteen (Figure 1) in these organisms suggests a regulatory response involving excretion. By day 21, the lead tissue concentration was almost double the exposure concentration but had again decreased to a concentration which was 1.5 times greater than the exposure concentration by day 28 (Figure 1). Rapid accumulation of lead and slow excretion has been observed in mussels, *Mytilus galloprovincialis* (Regoli and Orlando, 1994) and clams, *Ruditapes philippinarum* (Blasco and Puppo, 1999). This pattern of pulses of uptake and loss could be due to binding sites being temporarily saturated, following which lead is bound to metallothioneins and transferred into lysosomes with some excretion then occurring, this in turn would 'free up' binding sites for further lead accumulation (Marigómez *et al.*, 2002; Rainbow, 2002; Rainbow, 2007). A study by Atkinson *et al.* (2007) which exposed *T. deltoidalis* to sediment containing concentrations of 314 µg/g of lead for 21 days achieved a final lead tissue concentration of only 55 µg/g dry mass. The zinc, copper and cadmium which the sediment also contained may have competed for binding sites, with preferential binding of zinc and copper reducing the lead binding (Rainbow, 2007). Alternatively, despite their observation that bioturbation by *T. deltoidalis* increased lead bioavailability compared to undisturbed sediments, lead in their experiment may have been less bioavailable than in the present study due to different physicochemical properties of the sediment and overlying waters used.

4.1.2 Subcellular lead distribution

Around 70% of the accumulated lead was in the biologically detoxified fractions of organisms from all treatments (Figure 2, Table 1). With lead exposure the proportion of the detoxified metal in the metal rich granules increased from 47% to nearly three quarters of the

total (Table 2). A high proportion of lead was found to be associated with the metal rich granules of lead exposed mussels, *Mytilus galloprovincialis* (Regoli and Orlando, 1994), and the variegated scallop, *Chlamys varia* (Bustamante and Miramand, 2005). Simkiss and Taylor, (1989) describe two pathways of granule formation in marine organisms, one based on orthophosphate and the other on pyrophosphate. They suggest that these are derived from adenosine triphosphate during normal cell metabolism and that many marine organisms have the ability to switch between the two metabolic pathways of granule production which is related to the physical properties of the cell membranes. Some metals, such as lead, appear to enter the deposits by displacing calcium into the cytoplasm which in extreme conditions can result in cell death, but in less severe treatments it is presumed that small amounts of calcium are released inducing exocytosis and excretion of the granules. This mechanism may explain the fluctuation in lead tissue concentrations over the course of the exposure (Figure 1). Metallothioneins are a class of inducible proteins which have a high metal binding capacity and can detoxify excess metals that have entered cells (Viarengo and Nott, 1993). The turnover rate of metallothioneins in the oyster *Crassostrea virginica* is about 4 - 5 days (Roesijadi and Robinson, 1994). It has been shown in mussels that metallothionein binding of cellular metal can reduce interaction of metals with lysosomal membranes thus protecting them from destabilisation (Viarengo *et al.*, 1987), however, it has also been demonstrated that metallothionein bound metals can be internalised into the lysosomes (Viarengo and Nott, 1993). It is likely that a combination of the two transfer routes of metallothioneins and lysosomes to granules are operating in *T. deltoidalis* as seen in the change in the distribution of lead in these three fractions with increasing lead exposure (Figure 2, Table 2). The activity of mitochondrial and lysosomal marker enzymes cytochrome *c* oxidase and acid phosphatase, in the total tissue homogenates shows an increase in activity of both organelles with increased lead exposure (Supplementary Figure 2). This is also seen in the mitochondria (P3) and lysosomal (P4) fractions (Supplementary Figures 1 & 2) and suggests a response in both organelles to the accumulation of lead within the cells. The percentage of lead in the biologically active metal compartment of the exposed organisms was quite low, 7% in the 100 μg/g and 9% in the 300 μg/g compared to 17% in the controls (Table 1), a further clear indication that detoxification processes were operating at these lead exposures. Within the biologically active metal compartment the percentage of lead in the mitochondrial fraction increased from 50% to 67% with exposure (Table 2), which in the 100 μg/g and 300 μg/g lead exposed organisms corresponded to a 29 and 128 fold increase, respectively, in mitochondrial lead burden, compared to the control organisms. Lead is known to accumulate

in mitochondria causing swelling indicative of nephrotoxicity. It is thought to inhibit the synthesis of a variety of enzymes in the mitochondria and may cause cell death by impairment of energy production in this organelle system (Fowler and Mahaffey, 1978). The percentage of lead in the lysosomal+microsomal fraction of the 100 µg/g lead exposed organisms was close to three times that of the controls while the 300 µg/g lead exposed organisms had only a slightly higher percentage than that of the controls (Table 2). The lead associated with them, however, was 63 and 141, respectively, times greater than that of the controls. It is thought that metals in the lysosomes may bind to lipofuscins, which are lipid peroxidation end products in the lysosomes, as insoluble lipoprotein granules (George, 1983a; George, 1983b). In most cells of marine invertebrates these lipofuscin granules are usually excreted by exocytosis (George, 1983b). Thus, lead associated with lysosomes may be in the process of detoxification, however, unbound lead associated with this fraction can lead to the formation of oxygen derived free radicals in the cells which if not fully detoxified can start a sequence of lipid peroxidation reactions (Viarengo and Nott, 1993). The percentage of lead associated with the heat sensitive protein fraction and nuclei and cell debris in the lead exposed organisms (Table 2) is indicative of a detoxification system struggling with the excess lead. The high percentage of lead in the biologically detoxified metal fraction is clear evidence that an efficient system of lead binding and detoxification was present and active; however, the increased lead burden associated with the biologically active metal fractions indicates that the metal sensitive organelles were exposed to potentially toxic concentrations of lead as the tissue concentrations increased.

4.2 Enzymatic biomarkers – oxidative enzymes

The antioxidant capacity of the lead exposed *T. deltoidalis* was significantly reduced in both treatments compared to the control organisms (Figure 3A; Supplementary Table 3b). While the 300 µg/g lead exposed organisms had a slightly higher antioxidant capacity than the 100 µg/g lead exposed organisms, the difference was not significant (Figure 3A; Supplementary Table 3b). This is a surprising result as the 300 µg/g lead exposed organisms had four times the lead burden of the 100 µg/g lead exposed organisms in the biologically active metal fraction, of which 67% was associated with the mitochondria (Table 2; Figure 2). *Mytilus edulis* exposed to lead accumulated it in granules in the lysosomes and nucleus causing impairment of lysosomal function, a reduction of the mitochondrial cristae and deformation of the nuclear envelope (Einsporn and Koehler, 2008). There was some suppression of the total glutathione concentration and glutathione peroxidase activity in the 300 µg/g lead

exposed *T. deltoidalis* but this was not significant (Figure 3B). The glutathione concentration and glutathione peroxidase activity in the 100 µg/g lead exposed organisms were no different to the control organisms and neither lead exposure resulted in a significant reduction in the GSH:GSSG ratio (Figure 3B). Studies of lead induced oxidative stress in rats showed lead was bound to glutathione leading to a decreased in the GSH:GSSG ratio (Tandon *et al.*, 2002). Lead exposure in the brown mussel *Perna perna* decreased the GSH:GSSG ratio (Dafre *et al.*, 2004). Exposure of *Perna perna* to 200 mg/L of lead caused the depletion of GSH after 12 hours and increased GPx activity after 120 hours (de Almeida *et al.*, 2004). Exposure of the Sydney cockle *Anadara trapezia* to 100 and 300 µg/g of sediment lead reduced GPx concentrations, increased total GSH and significantly reduced the GSH:GSSG ratio (Taylor and Maher, 2012). It has been suggested that GSH may protect cells from accumulating lead by forming insoluble sulphide adducts with lead and excreting these complexes (Alcutt and Pinto, 1994). A response such as this would be expected to deplete GSH, reducing antioxidant potential thereby increasing susceptibility to oxidative stress. This may have occurred in the early part of the *T. deltoidalis* lead exposure but was not apparent after 28 days. Earthworms, *Lampito mauritii*, exposed to 75, 150 and 300 mg/kg of lead spiked soil showed a significant decrease in the GSH:GSSG ratio at day two and seven of the exposure but returned to pre-exposure GSH:GSSG levels by day 14 and remained so until the end of the exposure at day 28 (Maity *et al.*, 2008). An adaptive response by *L. mauritii* to lead exposure may have occurred, however, the lead tissue dose was not measured so it is unclear whether the measured oxidative response was linked to an increased metal dose or if the return to the pre-exposure concentrations of glutathione was due to a cessation of lead uptake. Although lead has the capacity to indirectly influence the oxidative system by reacting directly with cellular molecules to generate reactive oxygen species our results indicate that the glutathione reduction system of *T. deltoidalis* was not significantly affected by tissue lead accumulation. The inhibitory effects of lead on various other antioxidant enzymes, which can impair cell antioxidant defences rendering them more vulnerable to oxidative attacks (Gurer and Ercal, 2000), was demonstrated by the reduced antioxidant capacity (Figure 3A) suggesting that overall the antioxidant system was impaired with a probable reactive oxygen species increase and the potential for lipid peroxidation.

4.3 Oxidative damage biomarker – thiobarbituric acid reactive substances

Thiobarbituric acid reactive substances (TBARS) increased in *T. deltoidalis* from both lead treatments compared to the control organisms, however, the difference was only significant in

the 300 µg/g lead exposed organisms which had significantly higher lipid peroxidation than both the control and the 100 µg/g lead exposed organisms (Figure 4A; Supplementary Table 3b). The marine bivalve *Perna viridis* exposed to 13 µg/l of lead over 30 days had a linear accumulation of lead into the tissues and enhanced TBARS concentrations relative to controls were seen in all tissues but this response was not linear over the 30 days (Prakash and Rao, 1995). The brown mussel *Perna perna* exposed to 200 mg/l of lead showed no significant increase in TBARS over 120 hours despite significant changes to antioxidant enzymes (de Almeida *et al.*, 2004). De Almeida *et al*. (2004) suggest that this may be due to elevated concentrations of the enzyme phospholipid hydroperoxide glutathione peroxidase, which is highly reactive towards oxidised phospholipids, having a protective effect against lipid peroxidation. *T. deltoidalis* lipid peroxidation was highly correlated with the antioxidant capacity ($r^2 = 0.57$; $p \leq 0.0001$). Ercal *et al*. (2001) suggest that as lead cannot initiate lipid peroxidation on membrane lipids directly it might induce oxidative stress by interacting with oxyhaemoglobin, leading to peroxidative hemolysis in red blood cell membranes. As *T. deltoidalis* do not have haemoglobin, this pathway for lipid peroxidation is not available thereby reducing the total lipid peroxidation potential of lead in these organisms. It appears from the range of different TBARS responses seen for lead exposures that lipid peroxidation alone may not be strong indicator for lead toxicity in the majority of marine invertebrates, however, when viewed together with the antioxidant capacity a pattern of lead induced oxidative perturbation in *T. deltoidalis* is apparent.

4.4 Cellular biomarker – lysosomal stability

The lead exposed *T. deltoidalis* had significantly higher lysosomal destabilisation than the control organisms (Figure 4B; Supplementary Table 3a). The 100 µg/g lead exposed *T. deltoidalis* had 40% and the 300 µg/g 48% lysosomal destabilisation which, using the Ringwood *et al*. (2003) criteria, would class them as stressed. Ercal *et al*. (2001) suggests that lead alters the lipid composition of cellular membranes which may alter membrane integrity, permeability and function. *Mytilus galloprovincialis* transplanted in a lead contaminated area of the North Tyrrhenian Sea accumulated lead over time and showed a severe disturbance in lysosomal membrane stability with increased tissue lead (Regoli and Orlando, 1993). *A. trapezia* exposed to the same sediment lead concentrations as the *T. deltoidalis* in this experiment were highly stressed, with significant lysosomal destabilisation, up to 61% (Taylor and Maher, 2012). The *T. deltoidalis* lysosomal destabilisation was negatively correlated with antioxidant capacity, ($r^2 = 0.54$; $p \leq 0.001$) and positively

correlated, ($r^2 = 0.55$; $p \leq 0.001$) with lipid peroxidation. Despite the limited increase in lipid peroxidation of the 100 µg/g lead exposed *T. deltoidalis* the lysosomal destabilisation of organisms from both treatments was at a similar level (Figures 4A & B), suggesting that lead interaction with the lysosomal membrane leading to its destabilisation was probably occurring via more than just the lipid peroxidation pathway.

4.5 Genotoxic biomarker – micronuclei frequency

T. deltoidalis exposed to lead had a significantly higher micronuclei frequency than the control organisms but they were not significantly different to each other (Figure 4C; Supplementary Table 3b). The frequency of micronuclei in the lead exposed *T. deltoidalis* was much lower than that of these organisms when exposed to cadmium, which is generally in keeping with the pattern seen for the other stress indices (Taylor and Maher, 2013). This suggests that cadmium is a genotoxic agent exerting a direct influence on the induction of micronuclei, perhaps in concert with ROS, while lead had a more indirect influence perhaps via ROS alone. Lead, for example, has been shown to affect the expression of 2 nuclear transcription factors: NF-kB and HIF-1 which may lead to cell cycle arrest, apoptosis or interruption to gene transcription, while cadmium affects 3: NF-kB, and AP-1 and p53 which help protect cells from carcinogenesis (Leonard *et al.*, 2004). The frequency of micronuclei in the lead exposed *T. deltoidalis* was negatively correlated with antioxidant capacity ($r^2 = 0.52$; $p \leq 0.001$) and positively correlated with lipid peroxidation ($r^2 = 0.51$; $p \leq 0.001$) indicating that an increase in ROS likely contributed to an increase in genotoxic damage.

5 **Conclusions**

The response of *T. deltoidalis* to lead exposure differed between sediment lead exposure concentrations. Accumulation was rapid at both exposures in the first three days, however, the 100 µg/g lead exposed organisms continued to accumulate slowly over the remaining time reaching an equilibrium tissue with sediment lead concentration, while the 300 µg/g lead exposed organisms had a pattern of pulses of uptake and loss over the remainder of the exposure suggesting saturation of binding sites may have occurred. In lead exposed organisms around 70% of accumulated lead was detoxified with 74% converted to metal rich granules and the remainder in the metallothionein like protein fraction. The exposure – dose showed a strong relationship for lead with significant increases in biologically active lead burdens. The dose-response relationships were mixed, with no significant changes in the

glutathione enzymes measured but a significant reduction in the total antioxidant capacity. There were strong correlations between antioxidant capacity and lipid peroxidation, lysosomal destabilisation and increased micronuclei and between lipid peroxidation and lysosomal destabilisation and increased micronuclei frequency. The mixed response to lead observed for these effects biomarkers supports the multibiomarker approach, to both investigate the mechanisms of toxicological effect and by using interlinked biomarkers to demonstrate effects. The exposure-dose-response relationships demonstrated in this experiment for lead in *T. deltoidalis* indicates that lead exposure leads to significant tissue lead accumulation and increased biologically active lead burdens with associated cellular perturbations. These results show *T. deltoidalis* is sensitive to lead toxicity and would be a suitable organism for use in marine sediment lead toxicity assessment.

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Figure Captions

Figure 1: Tissue lead concentrations (µg/g dry mass) of *T. deltoidalis* exposed to lead spiked sediments of 0 (control), Pb 100 and 300µg/g dry mass. Mean ± SE, $n = 12$. Day 0 are unexposed organisms $n = 6$.

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

Figure 3: Antioxidant enzyme biomarkers of *T. deltoidalis* after 28 days exposure to lead spiked sediments of 0 (control), Pb100 and Pb 300µg/g dry mass. Mean ± SE, $n = 12$. **3A**: TAOC (total antioxidant capacity); **3B**: GPx (glutathione peroxidise); GSH+2GSSG (total glutathione); GSH/GSSG (ratio of reduced to oxidised glutathione). Different letters indicate significant differences between means (Bonferroni test; $p < 0.05$).

Figure 4: Changes in oxidative damage biomarkers: **4A**: MDA (lipid peroxidation); **4B**: cellular (lysosomal destabilisation); and **4C**: genotoxic (micronuclei) of *T. deltoidalis* after 28 days exposure to lead spiked sediments, 0 (control), Pb 100 and Pb 300µg/g dry mass. Mean ± SE $n = 12$. Different letters indicate significant differences between means (Bonferroni test; $p < 0.05$).

Figure 1

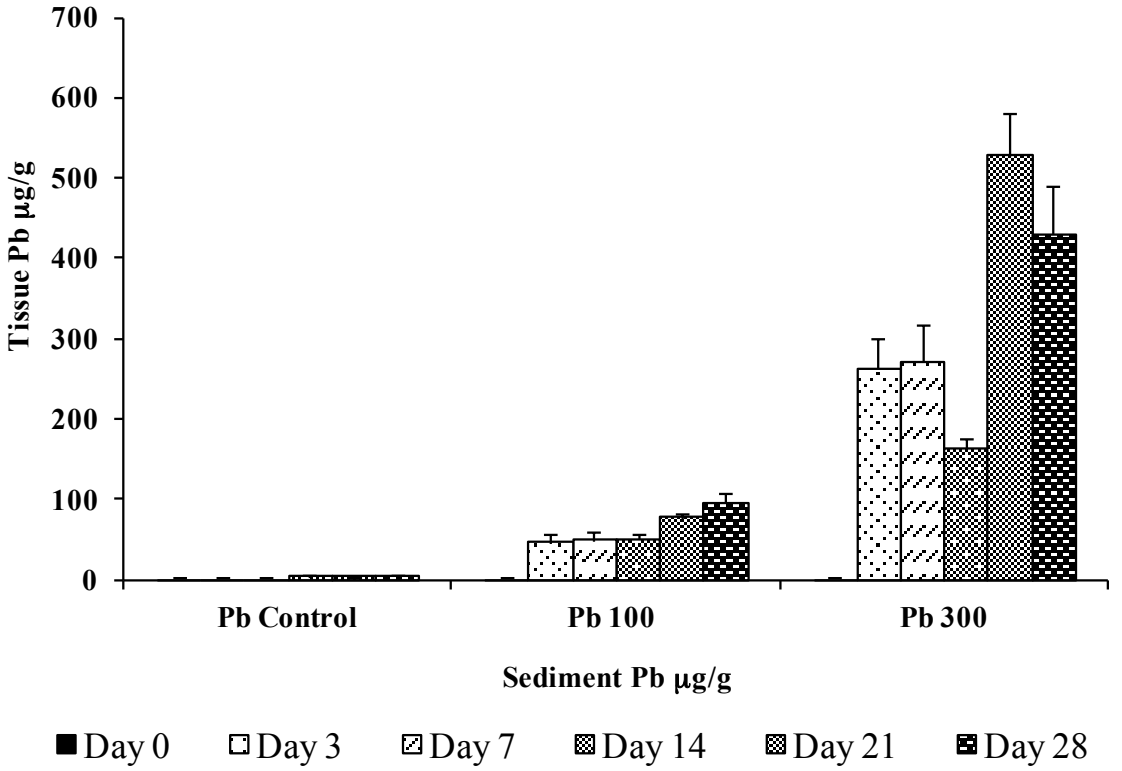

700
600
500
400
300
200
100
0
Tissue Pb µg/g
Pb Control
Pb 100
Pb 300
Sediment Pb µg/g
Day 0
Day 3
Day 7
Day 14
Day 21
Day 28

Figure 2

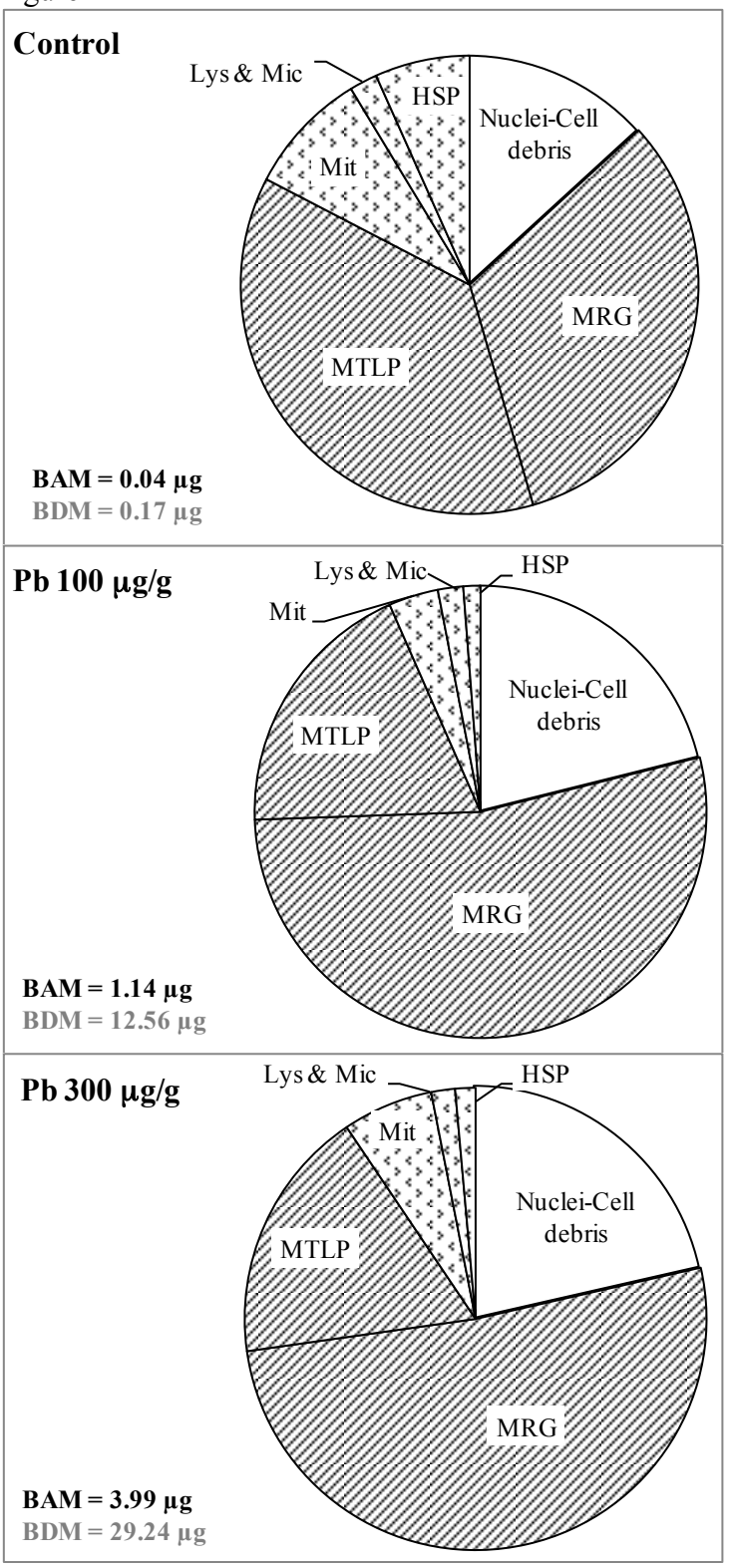
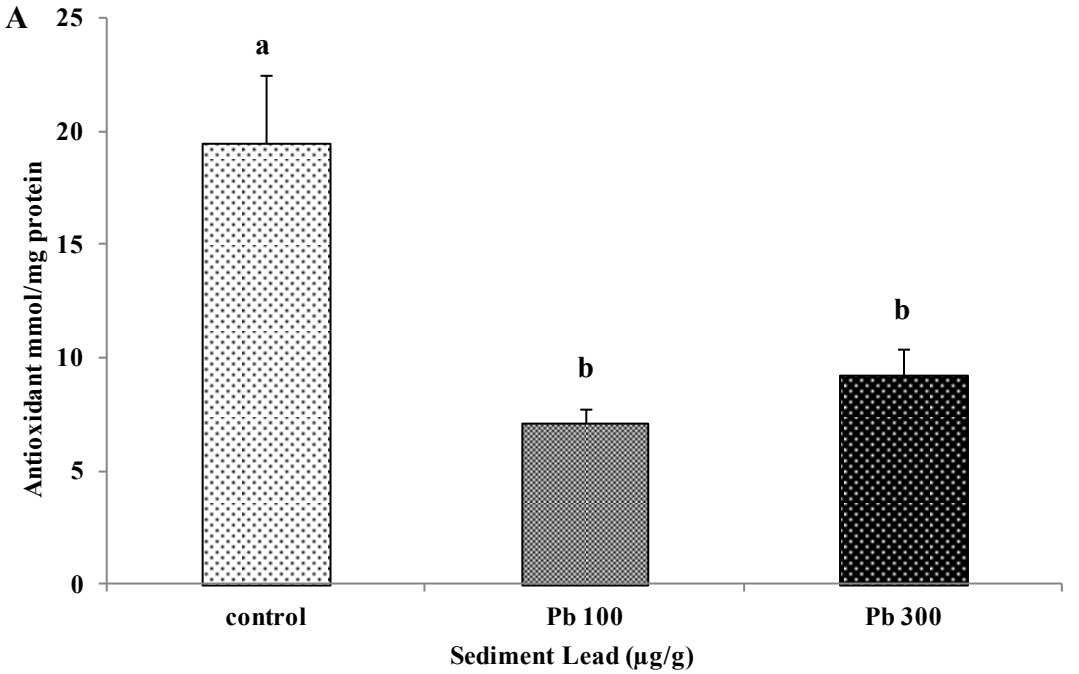

Control
Lys & Mic
HSP
Nuclei-Cell debris
Mit
MRG
MTLP
BAM = 0.04 µg
BDM = 0.17 µg
Pb 100 µg/g
Lys & Mic
HSP
Mit
Nuclei-Cell debris
MTLP
MRG
BAM = 1.14 µg
BDM = 12.56 µg
Pb 300 µg/g
Lys & Mic
HSP
Mit
Nuclei-Cell debris
MTLP
MRG
BAM = 3.99 µg
BDM = 29.24 µg

Figure 3


A
25
20
15
10
5
0
Antioxidant mmol/mg protein
a
b
b
control
Pb 100
Pb 300
Sediment Lead (µg/g)

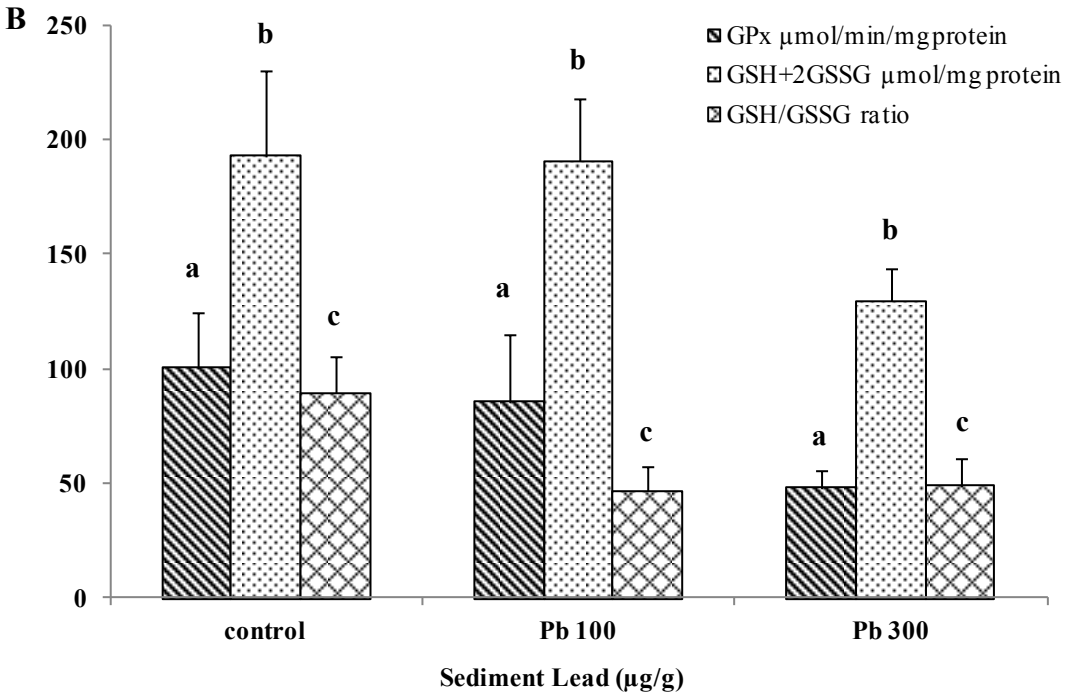

B
250
200
150
100
50
0
GPx µmol/min/mgprotein
GSH+2GSSG µmol/mg protein
GSH/GSSG ratio
a
b
c
a
b
c
a
b
c
control
Pb 100
Pb 300
Sediment Lead (µg/g)

Figure 4

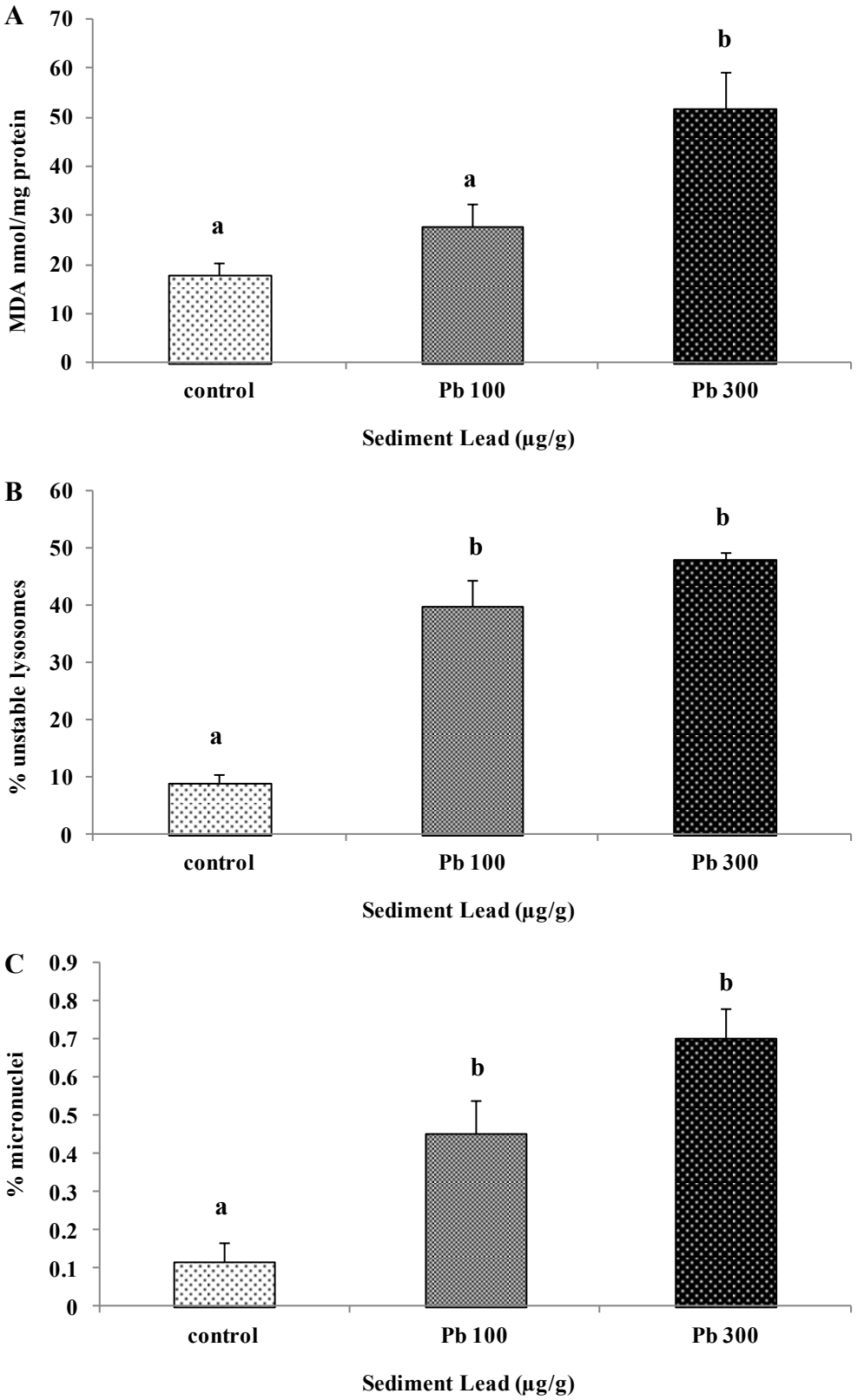

A
MDA nmol/mg protein
70
60
50
40
30
20
10
0
a
a
b
control
Pb 100
Pb 300
Sediment Lead (µg/g)
B
% unstable lysosomes
60
50
40
30
20
10
0
a
b
b
control
Pb 100
Pb 300
Sediment Lead (µg/g)
C
% micronuclei
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
a
b
b
control
Pb 100
Pb 300
Sediment Lead (µg/g)

Table 1: Total lead concentrations (µg wet mass) in whole tissue and subcellular fractions with the percentage of total lead recovered in all fractions of *T. deltoidalis* after 28 days exposure to lead spiked sediments. Lead subcellular concentrations (µg wet mass) and percentage distribution of total recovered lead fractions are grouped as debris and biologically active and biologically detoxified lead (Fig. 3).

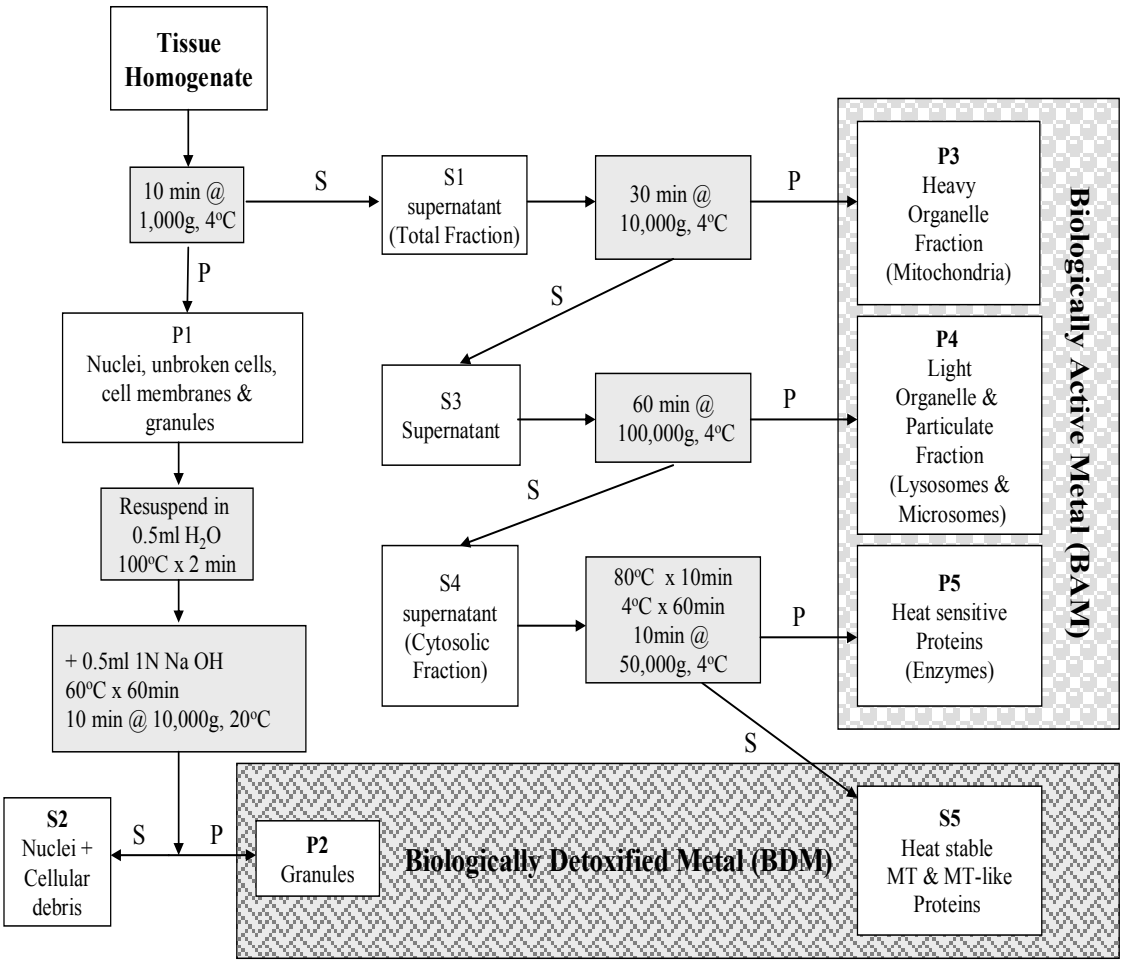
	Sediment Treatments (µg/g)		
	control	Pb 100	Pb 300
Total Tissue Lead (µg)	**0.34 ± 0.08**	**20 ± 3**	**46 ± 6**
Total Recovered Lead (µg)	**0.25 ± 0.05**	**17 ± 7**	**42 ± 5**
Proportion of total recovered in fractions (%)	72 ± 0.2	89 ± 20	92 ± 4
Lead Subcellular Distribution			
Nuclei + Cellular debris (µg)	**0.032 ± 0.01**	**3.7 ± 1.5**	**9.1 ± 1**
Nuclei + Cellular debris (%)	13 ± 3	21 ± 0.5	21 ± 1
Biologically Active Metal (µg)	**0.04 ± 0.01**	**1.1 ± 0.6**	**4 ± 0.3**
Biologically Active Metal (%)	17 ± 1.7	7 ± 0.5	9 ± 0.4
Biologically Detoxified Metal (µg)	**0.17 ± 0.03**	**12.6 ± 5.3**	**29 ± 4**
Biologically Detoxified Metal (%)	70 ± 5	72 ± 0.02	70 ± 0.6

Mean ± SD, $n = 2$

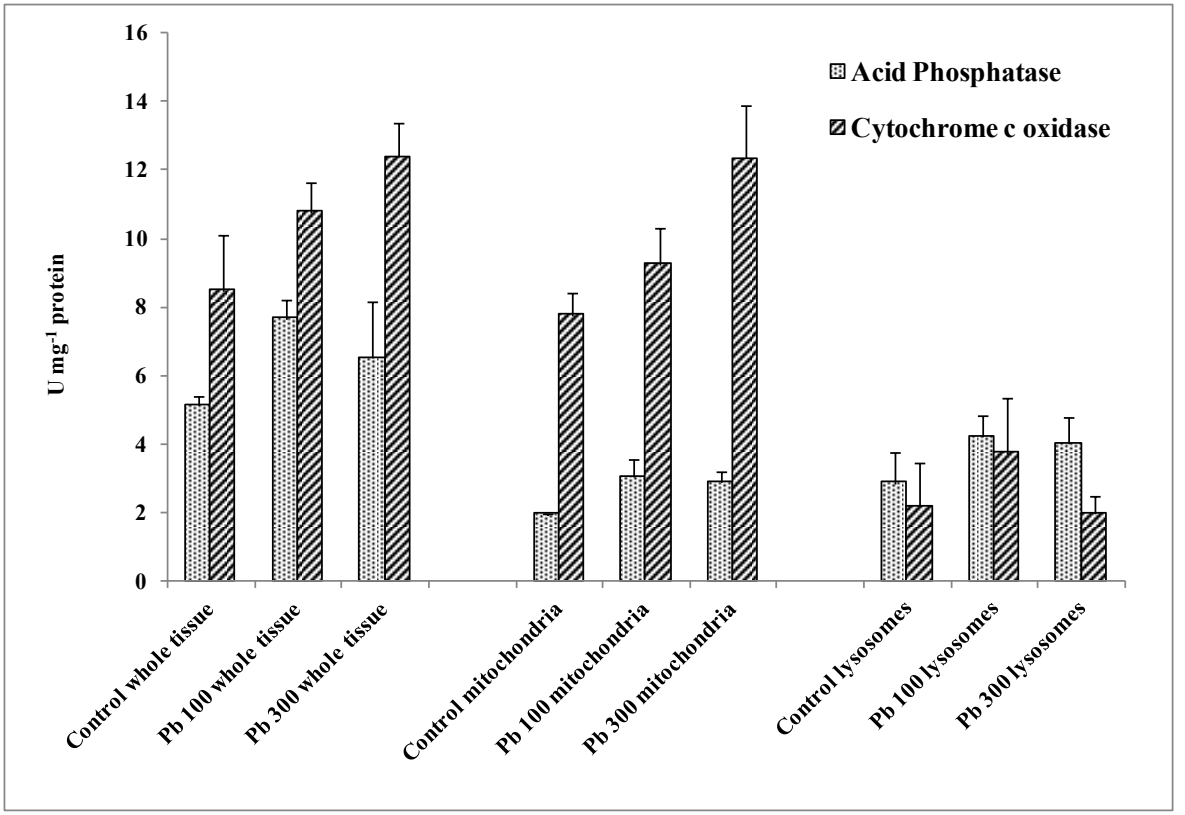
Table 2: Mean percentage of lead in the debris, biologically detoxified metal (BDM) and biologically active metal (BAM) with the percentage of lead each of the fractions contributes to BDM or BAM of *T. deltoidalis* subcellular fractions after 28 days exposure to lead spiked sediments, $n = 2$.

	Sediment Treatments (µg/g)		
	control	Pb 100	Pb 300
Nuclei + Cellular debris % of total	**13**	**21**	**21**
Biologically Detoxified Metal % of total	**70**	**72**	**70**
Metal Rich Granules % of BDM	47	74	74
Heat Stable MT Like Proteins % of BDM	53	26	26
Biologically Active Metal % of total	**17**	**7**	**9**
Mitochondria % of BAM	50	54	67
Lysosomes + Microsomes % of BAM	11	27	18
Heat Sensitive Proteins % of BAM	39	19	15

Supplementary 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 metal (BDM) P2 & S5; biologically active metal (BAM) P3, P4 & P5 metals or S2 which contains metal associated with dissolved tissues.



Supplementary Figure 2: Activity of specific marker enzymes for lysosomes (acid phosphatase) and mitochondria (cytochrome *c* oxidase) in whole tissue; and mitochondria and lysosome+microsome fractions following subcellular fractionation of whole tissue of *T. deltoidalis* exposed to lead spiked sediments at 0 (Control), 100 and 300 µg/g dry mass after 28 days. Mean ± SD, $n = 2$.



Statistical Analysis

A Mixed Linear Model analysis of variance (ANOVA) (SPSS *v* 14.0) was used to simultaneously analyse the effects of time (day) and treatment (lead exposure concentration) on whole organism tissue lead accumulation. Multiple pair-wise comparisons were also performed to determine where the significant differences lay between and within the treatments on each of the analysis days. As a high number of tests were performed, a Bonferroni correction for experiment wise error rate was applied to all calculations to determine the significance level (Sokal and Rohlf, 1995). A preliminary investigation of the data was done to examine the distribution of residuals using a non-parametric Kolmogorov-Smirnov Z Test. Where the population distribution was found to be non-normal a $\log_{10}$ transformation was performed prior to analysis. A Mixed Linear Model ANOVA (SPSS *v* 14.0) was used analyse the effect of treatment (lead exposure concentration) on the effect measurement variables total antioxidant capacity (TAOC), glutathione peroxidase, total glutathione, reduced and oxidised glutathione ratio, thiobarbituric acid reactive substances (TBARS), lysosomal stability and micronuclei frequency. Tank(treatment) was run as a covariance parameter in all analysis to determine whether the tank replicates were contributing to the treatment variance, in all cases the variance estimate was found to be ≤ 0.01. Arsine transformation was used for lysosomal stability and micronuclei frequency data as these values were percentages.

Supplementary Table 1: Mixed linear model ANOVA of tissue lead accumulation for collection day and treatment. Lead log_{10} for *T. deltoidalis* whole tissue.

		Lead	
Source	**df**	**F**	**p**
Treatment	2	301	***
Day	4	6	***
Day*Treatment	8	2	ns

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

Supplementary Table 2: Pair-wise comparisons, with Bonferroni adjustment for multiple comparisons, showing significant differences (p) between treatments. Lead log_{10} for *T. deltoidalis* whole tissue. None = day 0 unexposed organisms

Treatments	
Lead	**p**
none - control	ns
none - Pb 100	***
none - Pb 300	***
control - Pb 100	***
control - Pb 300	***
Pb 100 - Pb 300	***

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

Supplementary Table 3a: Mixed linear model ANOVA of effects for treatment of *T. deltoidalis* tissue. Lysosomes and micronuclei were calculated on arsine transformed data, all other effects are calculated on log_{10} transformed data. TAOC: antioxidant capacity; GPx: glutathione peroxidase; GSH+2GSSG: total glutathione; GSH:GSSG: ratio of reduced to oxidised glutathione; TBARS: total thiobarbituric acid reactive substances; lysosomes: % unstable lysosomes; micronuclei: % micronucleus frequency.

Source		**Lead**	
	df	**F**	**p**
TAOC	2	9.8	**
GPx	2	2.4	ns
GSH+2GSSG	2	1.5	ns
GSH:GSSG	2	3.4	*
TBARS	2	11	***
Lysosomes	2	53	***
Micronuclei	2	17	***

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

Supplementary Table 3b: Pair-wise comparisons, with Bonferroni adjustment for multiple comparisons, of effects for treatment of *T. deltoidalis* tissue. Lysosomes and micronuclei were calculated on arsine transformed data, all other effects are calculated on log_{10} transformed data. TAOC: antioxidant capacity; GPx: glutathione peroxidase; GSH+2GSSG: total glutathione; GSH:GSSG: ratio of reduced to oxidised glutathione; TBARS: total thiobarbituric acid reactive substances; lysosomes: % unstable lysosomes; micronuclei: % micronuclei frequency.

	Effects Measures						
Treatments	**TAOC**	**GPx**	**GSH+2GSSG**	**GSH:GSSG**	**TBARS**	**Lysosomes**	**Micronuclei**
Lead	**p**	**p**	**p**	**p**	**p**	**p**	**p**
Control - Pb 100	*	ns	ns	ns	ns	***	**
Control - Pb 300	*	ns	ns	ns	***	***	***
Pb 100 - Pb 300	ns	ns	ns	ns	*	ns	ns

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

Supplementary Table 4: Regression analysis of relationships between sediment and tissue lead, between the effects measure total antioxidant capacity and TBARS, Lysosomal stability and micronuclei frequency and between TBARS and Lysosomal stability and micronuclei frequency

Regression	**Lead**					
	N	**df**	**F**	**R^2**	**p**	**p**
Sediment lead *vs* Tissue lead	41	39	99.84	0.72	0.0000	****
TAOC *vs* TBARS	29	27	35.9	0.57	0.0000	****
TAOC *vs* lysosomal stability	18	16	18.6	0.54	0.0005	***
TAOC *vs* micronuclei frequency	18	16	17.3	0.52	0.0007	***
TBARS *vs* lysosomal stability	18	16	19.9	0.55	0.0004	***
TBARS *vs* micronuclei frequency	18	16	16.7	0.51	0.0009	***

**** $p \leq 0.0001$; *** $p \leq 0.001$

References

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