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Exposure-dose-response of *Tellina deltoidalis* to metal contaminated estuarine sediments 1. Cadmium spiked sediments

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Abstract

Cadmium is a ubiquitous environmental metal contaminant which is accumulated into cellular membranes via calcium channels, where it binds to biomolecules and affects membrane system function. The relationship between cadmium exposure, dose and response was investigated in the benthic, deposit feeding, marine bivalve *Tellina deltoidalis,* using 28 day microcosm spiked cadmium exposures. Tissue cadmium reached equilibrium with the exposure concentration. Half the accumulated cadmium was detoxified and with increased exposure more was converted into metal rich granules. Most biologically active cadmium was in the mitochondrial fraction, with up to 7200 fold cadmium increases in exposed organisms. Cadmium exposed *T. deltoidalis* generally had reduced glutathione peroxidase enzyme activity. An increase in total glutathione concentrations, due to a build up of oxidised glutathione, was indicated by the reduced to oxidised glutathione ratio. All cadmium exposed *T. deltoidalis* had reduced total antioxidant capacity which corresponded with increased lipid peroxidation, lysosomal destabilisation and micronuclei frequency. Clear exposure-dose-response relationships have been demonstrated for *T. deltoidalis* exposed to cadmium spiked sediments, supporting this organism's suitability for laboratory or insitu evaluation of sediment cadmium toxicity.

Keywords: Biomarkers, cadmium, sediments, subcellular, oxidative stress, lysosomes, micronuclei, bivalve

1 Introduction

Cadmium sediment concentrations up to 50 µg/g dry mass have been reported in contaminated Australian estuaries (Roach, 2005). The Australian guidelines for Fresh and Marine Water Quality (ANZECC and ARMCANZ, 2000) interim sediment quality high effects guideline for cadmium is 10 µg/g dry mass. Cadmium is accumulated in high concentrations by a range of marine organisms and has no known biological function (ECB, 2007) but at low concentrations may adversely affect cellular function (Ercal *et al.*, 2001; Sokolova, 2004; Sokolova *et al.*, 2004). Physiological effects and toxicity of metals strongly depend on their intracellular localisation and binding to organelles and ligands (Sokolova *et al.*, 2005). Cadmium, for example, affects the bioenergetics of oyster mitochondria in vitro and in vivo at low concentrations, leading to reduced coupling and impaired ability to produce ATP (Sokolova, 2004).

An understanding of the fate and effects of cadmium in aquatic environments requires that the causal relationships between cadmium exposure, internal dose and associated biological effects be established (Widdows and Donkin, 1992). Biomarker measurements provide information which cannot be obtained through measurements of contaminants in the environmental media or tissue concentrations and have the potential to provide evidence that organisms have been exposed to contaminants at levels that exceed their detoxification and repair capacity, thereby, establishing the link between toxicant exposure and ecologically relevant effects (Koeman *et al.*, 1993). Biomarkers within the oxidative system have been shown to be sensitive to metals through perturbations in the redox cycle and other oxidative pathways (Chandran *et al.*, 2005; Company *et al.*, 2004; Li *et al.*, 2006; Maity *et al.*, 2008; Regoli *et al.*, 1998b; Regoli and Principato, 1995). Lysosomes are involved in metal management and are also susceptible to oxidative damage; the measurement of their integrity, therefore, offers a useful biomarker of effect at the cellular level (Ringwood *et al.*, 2002; Viarengo, 1989; Winston *et al.*, 1996). The frequency of micronuclei occurrence provides evidence of genotoxic effects (Bolognesi *et al.*, 2004; Burgeot *et al.*, 1996).

Tellina deltoidalis is a sediment dwelling bivalve which satisfies most of the basic requirements to be an effective bioindicator being sedentary, hardy, relatively abundant, and an accumulator of metal with sufficient tissue for analysis (Phillips and Rainbow, 1994). It has a thin laterally compressed shell with equilateral valves and adult shell length is generally between 20 - 30 mm. Live animals bury in the sediment to a depth several times their shell

length with their siphons extending up to the sediment surface and ingest deposited organic material and sand grains (Beesley *et al.*, 1998). The purpose of this study was to examine the exposure - dose - response relationship to cadmium spiked sediments in *T. deltoidalis* using 28 day sediment bioaccumulation tests (Ingersoll *et al.*, 2000), to develop useful biomarkers of effect and evaluate their potential for sediment toxicity testing. The concentrations of cadmium chosen for the sediment spiking were the high sediment quality guideline concentration (10 µg/g dry mass) and the highest sediment cadmium concentrations previously measured in contaminated estuarine sediments (50 µg/g dry mass). The study examines total cadmium tissue accumulation and subcellular tissue cadmium distribution to determine the total cadmium fraction stored in metabolically sensitive organelles. Oxidative stress was determined by measuring total antioxidant scavenging capacity of cells, cellular concentrations of oxidised and reduced glutathione, glutathione peroxidase activity and the extent of lipid peroxidation. Cellular damage was determined by measuring lysosomal membrane stability and a micronucleus assay used to assess genotoxic damage.

2 Materials and Methods

2.1 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^{o}C until use. *Tellina. deltoidalis* were collected from Durras Lake and Lake Tabourie, NSW in July 2005 and January 2006, placed in coolers with sediment and water from the collection sites for transportation. Organisms were maintained for a maximum of two weeks at 22^{o}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 cadmium 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 metals. 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 cadmium was rapidly adsorbed and strongly bound to the sediment particles a method developed by

(Simpson *et al.*, 2004) for producing metal spiked marine sediments, was followed. Wet sediment was added to mixing containers. $CdCl_2$, (AR grade Merck), was added to concentrations of 10 mg/kg and 50 mg/kg dry mass of sediment. All containers were topped up with clean deoxygenated sea water and the final mixture was completely deoxygenated by bubbling with nitrogen for 2 hours. Head spaces of containers were filled with nitrogen prior to sealing. Any pH adjustments were made immediately after the addition of cadmium 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 cadmium was completely bound to sediment particles pore waters were collected and acidified to 1% with nitric acid (AristaR, BDH, Australia) and cadmium measured using an ELAN® 6000 ICP-MS (PerkinElmer SCIEX, USA). Once pore water cadmium concentrations had fallen below instrument detection limits 0.001 µg/l the sediment was ready for use. Time to full absorption was 4 - 6 weeks. Unspiked sediments were treated in the same way and used for control treatments. Sediment cadmium 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). Cadmium in NRCC Certified Reference Materials, BCSS-1 marine sediment measured along with samples was 0.22 ± 0.10 µg/g (n = 10) and in agreement with certified values 0.25 ± 0.04 µg/g. Sediment cadmium concentrations were measured prior to and at the end of the 28 day exposure period, concentrations were < 0.001, 10.0 ± 0.6 and 50 ± 2 µg/g on each occasion.

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^{o}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. *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 cadmium 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 cadmium concentration. All organisms were placed in fresh seawater 28‰ with no sediment for 24 hours to allow depuration of ingested sediment particles, prior to cadmium analysis.

2.4 Cadmium Measurements

2.4.1 Total Cadmium

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. Cadmium 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 cadmium analysis. The measured CRM cadmium value; 4.4 ± 0.6 µg/g (n = 50) was not significantly different from the certified value 4.15 ± 0.38 µg/g.

2.4.2 Subcellular cadmium

The subcellular tissue cadmium 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°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 cadmium 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), 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 cadmium fractions while the granule and heat stable metallothionein like proteins were grouped as biologically detoxified cadmium fractions (Figure 1). The supernatant from the granule pellet isolation contained the cellular debris (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, mitochondrial and lysosome+microsome pellets using commercial colorimetric assays (CYTOC-OX1 Sigma-Aldrich, USA and CS0740 Sigma-Aldrich, USA respectively). This showed that the activity of both enzymes was greater than that of the control organisms in the cadmium exposed organisms and that the mitochondrial fraction was enriched with mitochondria, and the lysosome+microsome fraction with lysosomes (Supplementary Figure 1). 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 cadmium was as previously described above. The measured CRM cadmium value; 3.8 ± 0.5 µg/g (n = 5) were in good agreement with certified value 4.15 ± 0.38 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°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 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°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 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 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 (cadmium exposure concentration) on organism tissue cadmium accumulation. A Mixed Linear Model ANOVA was used to analyse the effects of treatment (cadmium 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 cadmium and mean tissue cadmium concentrations and means of effects variables antioxidant capacity, lipid peroxidation, lysosomal stability and micronuclei frequency were calculated using EXCEL™ *v* 2003.

3 Results

3.1 Cadmium accumulation

Cadmium accumulation by *Tellina deltoidalis* was dependent on time and sediment cadmium concentration, ($p < 0.001$; Supplementary Table 1). Cadmium tissue concentrations were in

302 the order 50 µg/g > 10 µg/g > control for each analysis time and at day 28 were equal to that
303 of the sediment concentrations in both treatments (Figure 2). The 10 µg/g treatment
304 organisms had the highest cadmium concentration at day 28, while the 50 µg/g treatment
305 organisms had the highest cadmium concentration at day 21 and then a decrease to day 28
306 (Figure 2). The regression between cadmium sediment concentration and organism tissue
307 cadmium concentration after 28 days showed a significant (r = 1.0) positive relationship
308 (Figure 2).

309 **3.1.1 Subcellular tissue cadmium**

310 Approximately half of the total cadmium was recovered in the fractions (Table 1). Around
311 half of the cadmium was in the biologically detoxified fractions (Table 1), with a greater
312 percentage of cadmium in the metallothionein like protein fraction than in the metal rich
313 granule fraction in the control and 10 µg/g cadmium exposed organisms (Table 2). The 50
314 µg/g cadmium exposed organisms had more cadmium in the metal rich granule fraction than
315 in the metallothionein like protein fraction (Figure 3; Table 2). The percentage of cadmium
316 recovered in the biologically active metal fractions of each of the 10 and 50 µg/g cadmium
317 treatments was less than half that of the control, however, the total cadmium burden (µg)
318 within these fractions was 100 and 280 times, respectively, greater in the cadmium exposed
319 organisms (Table 1). The 10 and 50 µg/g cadmium exposed organisms had 59 and 72%
320 respectively of the recovered biologically active metal in the mitochondrial fraction with the
321 remainder fairly equally distributed between the lysosome+microsome and heat sensitive
322 protein fractions (Figure 3; Table 2). In the control organisms it was equally distributed
323 between the heat sensitive proteins and lysosome+microsome fractions with only 3% in the
324 mitochondrial fraction (Figure 3; Table 2).

325 ***3.2 Biomarkers***

326 The antioxidant capacity (TAOC) was significantly reduced ($p \leq 0.001$; Supplementary Table
327 3a) in cadmium exposed organisms compared to that of unexposed organisms, however, the
328 antioxidant capacity of each of the high and low metal treatments were not significantly
329 different to each other (Figure 4A; Supplementary Table 3b). The activity of the glutathione
330 peroxidase enzyme and the total glutathione concentrations were reduced in both cadmium
331 treatments compared to the control organisms (Figure 4B), however, the difference was not
332 significant ($p > 0.05$; Supplementary Table 3b). The ratio of reduced and oxidised glutathione
333 was reduced in cadmium exposed organisms compared to that of unexposed organisms
334 (Figure 4B). The difference was significant for 50 µg/g cadmium exposed organisms ($p \leq$

335 0.05; Supplementary Table 3b) compared to controls. Thiobarbituric acid reactive substances
336 (TBARS) were higher in cadmium exposed organisms than in unexposed organisms (Figure
337 5A) the 10 µg/g cadmium exposed organisms did not have significantly higher TBARS than
338 the control while the 50 µg/g cadmium exposed organisms did ($p \leq 0.05$; Supplementary
339 Table 3b) and the treatments were not significantly different to each other (Figure 5A).
340 Lysosomal stability and micronuclei frequency significantly decreased ($p \leq 0.001$
341 Supplementary Tables 3a & 3b) with exposure to increased cadmium concentrations (Figure
342 5B & C). Regression analysis showed that the reduced TAOC within cells had a negative
343 relationship with the effects measures of TBARS ($r = 0.49$), lysosomal stability ($r = 0.98$) and
344 micronuclei frequency ($r = 0.89$) for cadmium exposed organisms. There was a positive
345 relationship between TBARS and lysosomal stability ($r = 0.99$) and micronuclei frequency (r
346 $= 0.99$).

347 # 4 Discussion

348 ## *4.1 Cadmium accumulation and subcellular distribution*

349 ### 4.1.1 Whole organism

350 Bivalve molluscs which accumulate metals in direct proportion to their ambient
351 concentrations have been described as weak accumulators of these metals (Luoma and
352 Rainbow, 2008). *T .deltoidalis* cadmium uptake in these experiments fits this pattern (Figure
353 2). The slight decrease in tissue cadmium between day 21 and 28 in the 50 µg/g exposed
354 organisms suggests that they may have reached equilibrium with their exposure environment
355 by day 21. This is at odds with modelling work on cadmium accumulation in *T. deltoidalis*
356 tissues by King *et al.* (2005) which concluded that > 40 days would be required for *T.*
357 *deltoidalis* to reach a steady-state concentration with their cadmium exposure environment.
358 Bioturbation of sediments by deposit feeders leads to dispersal of metal oxides by mechanical
359 actions through burrowing as well as via bioresuspension when fluid faecal pellets are ejected
360 into the water column (Meysman *et al.*, 2006). Atkinson (2007) showed that *T. deltoidalis*
361 caused bioturbation of sediments which increased metal release compared to unmixed
362 sediments. Surprisingly their study which exposed *T. deltoidalis* to sediment containing
363 concentrations of 25 µg/g of cadmium for 21 days achieved a final cadmium tissue
364 concentration of only 5 µg/g dry mass. The sediment used was highly silty < 63 µm = 73%
365 compared to this experiment < 63 µm = 30% which may have affected feeding and burrowing
366 behaviour. Their sediment also had significant concentrations of copper, lead and zinc so
367 metal interactions may have affected the individual metal bioavailability and uptake kinetics

(Moolman *et al.*, 2007). In estuarine and marine environments cadmium is relatively soluble due to strong complexation by chloride (Stumm and Morgan, 1996). The even pattern of cadmium accumulation over time (Figure 2) and positive linear sediment tissue cadmium concentration relationship for the two treatments suggests the sediment bound cadmium was readily resuspended and bioavailable and the major exposure route remained the same over the course of the experiment. King *et al.* (2005) concluded this was largely via sediment ingestion, although, the model developed, using *T. deltoidalis,* was based on 24 hour exposure and depuration experiments and the authors felt that sediment retained in the gut and shell may have resulted in an overestimation of the tissue concentrations from sediment exposure compared to water and food exposures used.

4.1.2 Subcellular cadmium distribution

T. deltoidalis detoxified about 50% of accumulated cadmium (Figure 3; Table 1). The percentage converted to metal rich granules (MRG) increased from 23% to 60% with increased cadmium exposure (Table 2). The formation of MRG has been associated with increased metal tolerance in marine organisms (Wallace *et al.*, 1998). George (1983b) found that granules of cadmium exposed *Mytilus edulis* contained high concentrations of protein, calcium and sulphur which he postulated may be due to an increase in lysosomal protein degradation, due to enzyme inactivation by intracellular cadmium, causing an increase in intracellular protein turnover. The reduction in the percentage of cadmium in the lysosomal fraction of the exposed versus control organisms and the increase in cadmium in the MRG fraction of the 50 µg/g exposed *T. deltoidalis* tends to support the route of cadmium transfer from the biologically active cadmium lysosomal fraction to the MRG fraction (Table 2). Metallothionein plays a key role in metal detoxification and the relationship between metallothioneins and transport of metal to sites of MRG production is also likely to be important (Wallace *et al.*, 1998). Cadmium bound to metallothionein in the kidney of the mussel *Mytilus edulis* became incorporated in the granules (George, 1983a). Cadmium bound to metallothionein in the digestive gland of *Mytilus galloprovincialis* becomes incorporated into lysosomes and is transformed into insoluble thionein polymers, a likely precursor of MRG (Viarengo *et al.*, 1987). It is likely that a combination of these two transfer routes are operating in *T. deltoidalis* as seen in the change in the percentage distribution of cadmium in the lysosomal, MTLP and MRG fractions with increasing cadmium exposure (Figure 3, Table 2).

The mitochondrial enzyme cytochrome *c* oxidase and lysosomal acid phosphatase activity in the total homogenates shows an increase in both organelles with increased cadmium

exposure, particularly in the mitochondria which is also seen in the mitochondria and lysosomal fractions (Supplementary Figure 1). This suggests that both organelles are responding to the accumulation of cadmium within the cells. The majority of cadmium in the biologically active metal fractions of the cadmium exposed *T. deltoidalis* was in the mitochondrial fraction (Figure 3; Table 2). Cadmium burdens in the freshwater bivalve *Pyganodon grandis* were also found to be higher in the mitochondria than the lysosome+microsome fraction with increased cadmium exposure (Bonneris *et al.*, 2005). Li *et al.* (2003) showed that cadmium could directly lead to dysfunction of mitochondria including inhibition of respiration, loss of transmembrane potential and the release of cytochrome *c* oxidase. Mitochondrial cadmium in *T. deltoidalis* from the 10 µg/g and 50 µg/g exposures increased to 2000 and 7200, respectively, times that of the control organisms (Table 2). Extensive Cd^{2+} accumulation in mitochondria mediated by Ca^{2+} voltage dependant channels has previously been reported by Li *et al.* (2000; 2003). The increased mitochondrial cadmium observed in *T. deltoidalis* is also in agreement with studies on cadmium subcellular distribution following increased exposure in oysters *Crassostrea virginica* (Sokolova *et al.*, 2005). Mitochondrial function was also found to be highly sensitive to cadmium at physiological and environmentally relevant low concentrations in oysters (Sokolova, 2004). Cadmium has been shown to have a high affinity for mitochondria, it is capable of inhibiting respiration and oxidative phosphorylation and interfering with the 1-hydroxylation of vitamin D (Fowler and Mahaffey, 1978). Lysosomes are involved in numerous functions including, nutrition, tissue repair, cellular defence, turnover of membranes, organelles and proteins as well as in the sequestration and metabolism of toxins, such as organic xenobiotics and metals and are an important target organelle for metal toxicity (Ringwood *et al.*, 1998). While the percentage of cadmium in the lysosomal+microsomal fraction of the 10 µg/g and 50 µg/g cadmium exposed organisms was slightly less than half that of the controls (Table 2) the cadmium associated with them was 46 and 100, respectively, times greater than that of the controls. Lysosomes have been identified as the metal cation homeostasis mechanism which sits between soluble binding ligands such as metallothionein and the formation of insoluble precipitates such as metal rich granules (Viarengo and Nott, 1993). While lysosomal cadmium uptake may reflect sequestration and detoxification of the metal, it can also lead to adverse effects when the handling capacity of the lysosomes is overwhelmed (Sokolova *et al.*, 2005; Viarengo *et al.*, 1987). This fraction also contained the microsomal component of the cell. Since this includes fragmented endoplasmic reticulum, which is generally responsible for protein synthesis and transport; if cadmium in this fraction was associated with microsomes rather than lysosomes then this could be indicative of toxicity (Bonneris *et al.*,

2005). Similar amounts of cadmium were also associated with the heat sensitive proteins (HSP) of the 10 µg/g and 50 µg/g cadmium exposed organisms compared to the controls as those found in the lysosome+microsome fraction (Table 2). The HSP fraction contains enzymes, high and low molecular weight proteins and other target molecules which are sensitive to metals (Wallace *et al.*, 2003). The increased binding of cadmium to this fraction may therefore have implications for toxicity. The percentage of cadmium within the nuclei+cellular debris fraction of 10 µg/g cadmium exposed organisms was double, and in the 50 µg/g two and a half times the control cadmium. This fraction was not included in the biologically active and detoxified cadmium compartments as it contains tissue fragments, cell membranes and other cellular components of unknown consequence in terms of function, as well as the nucleic material (Wallace *et al.*, 2003). Binding of cadmium to cell membranes could result in toxicity, however, if the cadmium in this fraction was bound to less sensitive fractions within the cellular debris this could reduce binding to more sensitive cell components (Lucu and Obersnel, 1996). Metallothionein has been shown to be present in the nucleus of gill, digestive gland, gonad and posterior adductor muscle of *Mytilus edulis* in equal or lower concentrations than the corresponding tissue cytosol (del Castillo and Robinson, 2008). If this is also the case for *T. deltoidalis*, cadmium in the nuclei+cellular debris fraction may be associated with nuclear metallothionein and so detoxified. The effectiveness of cadmium detoxification relies on binding to metallothioneins transfer across cell membranes, compartmentalisation within lysosomes and the formation of metal rich granules. The presence of increased cadmium associated with these fractions shows that these processes were occurring, however, increases in cadmium within the mitochondrial fraction and to a lesser extent the heat sensitive protein fraction indicates the detoxification and storage capacity was exceeded suggesting the potential for cadmium toxicity.

4.2 Enzymatic biomarkers – oxidative enzymes

Marine invertebrates exposed to elevated concentrations of redox inactive metal ions such as cadmium are susceptible to intracellular fluxes of reactive oxygen species through direct interactions with cellular molecules which generate ROS by inducing cell signalling pathways (Leonard *et al.*, 2004) or deplete the cell's major sulfhydryl reserves (Ercal *et al.*, 2001). The capacity to reduce reactive oxygen species was significantly reduced in *T. deltoidalis* in both cadmium treatments compared to the control organisms (Figure 4; Supplementary Table 3b). A reduced oxyradical scavenging capacity in mussels exposed to a range of contaminants has also been reported: *Mytilus galloprovincialis*: sewage, agricultural, industrial and oil tanker effluents (Camus *et al.*, 2004), metals and PAHs (Frenzilli *et al.*, 2004; Regoli, 2000; Regoli

et al., 2004); *Modiolus modiolus*: cadmium (Dovzhenko *et al.*, 2005). An antioxidant capacity reduction in cadmium exposed *T. deltoidalis* (Figure 4A) indicates a breakdown in the reactive oxygen species detoxification pathway with the potential for higher order effects. An investigation of glutathione cycling and the glutathione peroxidase enzyme (GPx) which are involved in the reduction of oxyradicals was undertaken to further investigate the mode of action of accumulated metal on the ROS reduction pathway of *T. deltoidalis*.

Glutathione peroxidase activity and the total glutathione were reduced in the cadmium treated organisms compared to the control organisms (Figure 4B) but the reduction was not significant (Supplementary Table 3b). When the ratio of reduced glutathione (GSH) to oxidised glutathione (GSSG) is considered, a significant reduction was seen in the cadmium exposed organisms compared to the controls (Figure 4B; Supplementary Table 3b). Reduced glutathione is a tripeptide of glutamine, cysteine and glycine which provides reducing equivalents for the GPx catalysed reduction of hydrogen peroxide to water and the respective alcohol. During this process GSH becomes oxidised glutathione which is recycled into GSH by glutathione reductase and NADPH (Tietze, 1969). In healthy cells the ratio is usually high, however, when cells are exposed to increased oxidative stress the ratio of GSH to GSSG decreases as a consequence of GSSG accumulation (Wataha *et al.*, 2000). The responsiveness of antioxidants to specific toxicants is difficult to predict and a high degree of variability has been reported in early work on marine bivalves relating to the class of chemical, exposure, organism type and phase in biological cycle (Regoli and Principato, 1995; Ribera *et al.*, 1989; Viarengo *et al.*, 1991; Viarengo *et al.*, 1989). The use of single antioxidant enzyme measurements to interpret exposure effects, therefore, may give inconclusive or contradictory results. The total glutathione concentration has been investigated in marine bivalves exposed to metal contamination in numerous studies and has been reported as both increasing (Camus *et al.*, 2004; Frenzilli *et al.*, 2004; Regoli *et al.*, 2004; Regoli *et al.*, 1998a) and decreasing (de Almeida *et al.*, 2004; Regoli, 1998; Regoli *et al.*, 1998b; Regoli and Principato, 1995), indicating that glutathione may be up-regulated in response to metal contamination but at different concentrations and varying physiochemical conditions may be depleted when the system is overwhelmed. The suppression of total glutathione concentrations in *T. deltoidalis* by cadmium (Figure 4B) may be related to the high affinity of this molecule for metals, resulting in either a stable coordination complex or the oxidation of GSH to GSSG which may be subsequently excreted from the cell more rapidly than it can be reconverted to the reduced form by glutathione reductase (Meister, 1989). GSH cadmium complexes are not detectable with the methods used here. Despite the tendency for excess GSSG to be actively excreted from cells the decreased ratio of reduced to oxidised glutathione detected in the cadmium

exposed organisms (Figure 4B) indicates that despite reduced GPx activity oxidation of glutathione was occurring. The freshwater bivalve *Unio tumidus* exposed to a wide mix of contaminants including PAHs, persistent organics and metals showed a decrease in the GSH:GSSG ratio (Cossu *et al.*, 2000; Cossu *et al.*, 1997). The GSH:GSSG ratio has not been used widely in environmental toxicological work but may be a more useful indicator of the oxidative status than total glutathione concentrations. Regoli *et al*. (2002) recommends an integrative approach to the use of antioxidant measurements in ecotoxicology where the individual antioxidant parameters are used in understanding modes of toxic action of a stressor and integrated with the total antioxidant capacity to provide a more holistic assessment of the overall biological significance of the variations. The suppression of GPx, GSH and the GSH:GSSG ratio, together with the significantly reduced TAOC, in response to cadmium exposure clearly indicates cadmium induced impairment of the antioxidant system.

4.3 Oxidative damage biomarker – thiobarbituric acid reactive substances

Thiobarbituric acid reactive substances TBARS are a measure of lipid peroxidation which is a widely recognised consequence of excess oxyradical production (Winston and Di Giulio, 1991). Like the oxygen reduction system the lipid production process is a complex sequence of biochemical reactions, broadly defined as oxidative deterioration of polyunsaturated fatty acids, which results in the production of highly reactive and unstable lipid radicals and a variety of lipid degradation products, the most abundant of which is malondialdehyde, which can alter the structure of cell membranes (Viarengo, 1989). The process of lipid peroxidation destabilises cell membranes which can lead to loss of lysosomal integrity and the leaking of the lysosomal contents into the cytoplasm (Winston *et al.,* 1991). The TBARS concentration was increased in *T. deltoidalis* from both cadmium treatments compared to the control organisms, however, the difference was only significant in the 50 µg/g cadmium exposed organisms (Figure 5A; Supplementary Table 3b). Other cadmium exposed marine bivalves have also shown reduced antioxidant enzyme activity and a consequent increase in lipid peroxidation (Chelomin *et al.*, 2005; Company *et al.*, 2004; de Almeida *et al.*, 2004; Legeay *et al.*, 2005). The TBARS concentration was highly negatively correlated with the antioxidant capacity, indicating that the progressive reduction in the capacity to reduce ROS and their subsequent increase directly influenced the build up of lipid peroxidation by-products.

4.4 Cellular biomarker – lysosomal stability

Lysosomes are intracellular organelles that contain acid hydrolases for the digestion of cellular waste: including excess or damaged organelles; food particles; viruses and bacteria.

The lysosomal interior is more acidic pH ≈ 4.8 than the cytosol pH ≈ 7.2 and it is enclosed in a single membrane which stabilises the low pH by pumping protons from the cytosol via proton pumps and ion channels. Metals can also enter lysosomes via these channels, or more usually as protein complexes with metallothioneins, and it is thought that from here they are then formed into granules for storage or excretion (Marigómez *et al.*, 2002).

Metal accumulation in the lysosomes can induce lipid peroxidation through redox cycling or by direct reaction with cellular molecules to generate ROS. This can destabilise the lysosomal membrane causing the contents to leak out into the cytosol thereby reducing the cells capacity to remove waste which will ultimately lead to cell death (Viarengo *et al.*, 1987). The 10 µg/g cadmium exposed *T. deltoidalis* had 33% and the 50 µg/g 43% lysosomal destabilisation. This was significantly higher than the control organisms which had less than 10% of lysosomes destabilised (Figure 5B; Supplementary Table 3b). Based on lysosomal destabilisation criteria developed by, Ringwood *et al*. (2003) for *Crassostrea virginica* the cadmium exposed *T. deltoidalis* score in the concern / stressed range.

Cadmium accumulation has been linked to lysosomal destabilisation in other marine bivalves: mussels *Mytilus galloprovincialis* (Regoli *et al.*, 2004; Viarengo and Nott, 1993) and oysters *Crassostrea virginica* Ringwood *et al*. (2004; 2002). The lysosomal destabilisation was strongly negatively correlated with antioxidant capacity and positively correlated with TBARS. This suggests that increased cadmium accumulation in metal sensitive tissue fractions initiated a reduction in the capacity to reduce ROS which may have both directly damaged lysosomal membrane but also induced lipid peroxidation which also induced lysosomal membrane destabilisation.

4.5 Genotoxic biomarker – micronuclei frequency

Micronuclei are small, intracytoplasmic masses of chromatin resulting from chromosomal breakage or aneuploidy during cell division. As an index of chromosomal damage the micronucleus test is based on the enumeration of downstream aberrations after DNA damage and gives a time-integrated response to toxic exposure. The micronucleus test is a fast and sensitive test to detect genomic damage due to both clastogenic effects and alterations to the mitotic spindle (Migliore *et al.*, 1987). It has been used in bivalves to examine the genotoxicity of a range of chemicals including metals (Bolognesi *et al.*, 2004; Burgeot *et al.*, 1996; Scarpato *et al.*, 1990; Williams and Metcalfe, 1992). The 10 µg/g cadmium exposed *T. deltoidalis* had significantly more micronuclei than the control organisms and the 50 µg/g cadmium exposed *T. deltoidalis* had significantly more micronuclei than both the control and the 10 µg/g cadmium exposed organisms (Figure 5C; Supplementary Table 3b). Increased

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

5 Conclusions

Cadmium exposed *T. deltoidalis* accumulated cadmium over 28 days and reached equilibrium tissue concentrations which were equal to that of the sediment cadmium exposure concentrations. Approximately 50% of accumulated cadmium was detoxified. The percentage converted to metal rich granules increased from 23% to 60% with increased cadmium exposure. The majority of biologically active cadmium in exposed organisms was in the mitochondrial fraction which was significantly higher than the control organisms. This was associated with an increase in the activity of the mitochondrial cytochrome *c* oxidase enzyme. The antioxidant capacity and ratio of GSH:GSSG of cadmium exposed *T. deltoidalis* was significantly reduced compared to control organisms. The impairment of the oxidative system initiated significant cellular damage. Lipid peroxidation increased, contributing to significant lysosomal destabilisation and increased frequency of micronuclei. The significant exposure – dose – response relationships for cadmium established in this study indicate that sediment cadmium at these concentrations has the potential to lead to increased biologically active cadmium burdens and impairment of individual *T. deltoidalis* at a cellular and subcellular level. This has implications for higher order effects which may impact on population viability in the long term. *T. deltoidalis* has good attributes as a bioindicator, being hardy, abundant, easy to work with in the laboratory and a net accumulator of cadmium, reaching tissue metal equilibrium over a 28 day exposure period.

607 They were sensitive to metals as seen by the enzymatic and cellular biomarkers and would,
608 therefore, be suitable for sediment metal toxicity tests.
609
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614

615 **References**

616 ANZECC, ARMCANZ (2000) National Water Quality Management Strategy Paper No 4. In.
617 (Australian Govt. Publisher)
618 Atkinson CA, Jolley DF, Simpson SL (2007) Effect of overlying water pH, dissolved oxygen, salinity
619 and sediment disturbances on metal release and sequestration from metal contaminated marine
620 sediments. *Chemosphere* **69**, 1428-1437.
621 Baldwin S, Deaker M, Maher W (1994) Low-volume microwave digestion of marine biological
622 tissues for the measurement of trace elements. *Analyst* **119**, 1701-1704.
623 Beesley PL, Ross GJB, Wells A (Eds) (1998) 'Mollusca: The Southern Synthesis ' Fauna of Australia
624 Vol. 5 (CSIRO Publishing: Melbourne)
625 Bolognesi C, Frenzilli G, Lasagna C, Perrone E, Roggieri P (2004) Genotoxicity biomarkers in
626 *Mytilus galloprovincialis*: wild versus caged mussels. *Mutation Research/Fundamental and*
627 *Molecular Mechanisms of Mutagenesis* **552**, 153-162.
628 Bonneris E, Perceval O, Masson S, Hare L, Campbell PGC (2005) Sub-cellular partitioning of Cd, Cu
629 and Zn in tissues of indigenous unionid bivalves living along a metal exposure gradient and
630 links to metal-induced effects. *Environmental Pollution* **135**, 195-208.
631 Burgeot T, Woll S, Galgani F (1996) Evaluation of the micronucleus test on *Mytilus galloprovincialis*
632 for monitoring applications along French coasts. *Marine Pollution Bulletin* **32**, 39-46.
633 Calbiochem (2004) GSH/GSSG Ratio Assay Kit User Protocol 371757.
634 Camus L, Pampanin DM, Volpato E, Delaney E, Sanni S, Nasci C (2004) Total oxyradical scavenging
635 capacity responses in *Mytilus galloprovincialis* transplanted into the Venice lagoon (Italy) to
636 measure the biological impact of anthropogenic activities. *Marine Pollution Bulletin* **49**, 801-808.
637 Cayman (2011) Antioxidant Assay Kit User Protocol 709001.
638 Cerutti PA (1985) Prooxidant states and tumor promotion. *Science* **227**, 375-381.
639 Chandran R, Sivakumar AA, Mohandass S, Aruchami M (2005) Effect of cadmium and zinc on
640 antioxidant enzyme activity in the gastropod, *Achatina fulica*. *Comparative Biochemistry and*
641 *Physiology, Part C* **140**, 422-426.
642 Chelomin VP, Zakhartsev MV, Kurilenko AV, Belcheva NN (2005) An in vitro study of the effect of
643 reactive oxygen species on subcellular distribution of deposited cadmium in digestive gland of
644 mussel *Crenomytilus grayanus*. *Aquatic Toxicology* **73**, 181-189.

Company R, Serafim A, Bebianno MJ, Cosson R, Shillito B, Fiala-Medioni A (2004) Effect of cadmium, copper and mercury on antioxidant enzyme activities and lipid peroxidation in the gills of the hydrothermal vent mussel *Bathymodiolus azoricus*. *Marine Environmental Research* **58**, 377-381.

Cossu C, Doyotte A, Babut M, Exinger A, Vasseur P (2000) Antioxidant Biomarkers in Freshwater Bivalves, *Unio tumidus*, in Response to Different Contamination Profiles of Aquatic Sediments. *Ecotoxicology and Environmental Safety* **45**, 106-121.

Cossu C, Doyotte A, Jacquin MC, Babut M, Exinger A, Vasseur P (1997) Glutathione Reductase, Selenium-Dependent Glutathione Peroxidase, Glutathione Levels, and Lipid Peroxidation in Freshwater Bivalves,*Unio tumidus*,as Biomarkers of Aquatic Contamination in Field Studies. *Ecotoxicology and Environmental Safety* **38**, 122-131.

de Almeida EA, Miyamoto S, Bainy ACD, de Medeiros MHG, Di Mascio P (2004) Protective effect of phospholipid hydroperoxide glutathione peroxidase (PHGPx) against lipid peroxidation in mussels *Perna perna* exposed to different metals. *Marine Pollution Bulletin* **49**, 386-392.

del Castillo E, Robinson WE (2008) Nuclear and cytosolic distribution of metallothionein in the blue mussel *Mytilus edulis* L. *Comparative Biochemistry and Physiology, Part B* **151**, 46-51.

Dovzhenko NV, Kurilenko AV, Bel'cheva NN, Chelomin VP (2005) Cadmium-induced oxidative stress in the bivalve mollusk *Modiolus modiolus*. *Russian Journal of Marine Biology* **31**, 309-313.

ECB (2007) 'European Union Risk Assessment Report: Cadmium Oxide and Cadmium Metal. Institute for Health and Consumer Protection, European Chemicals Bureau, Ispra p. 678.'

Ercal N, Gurer-Orhan H, Aykin-Burns N (2001) Toxic metals and oxidative stress part 1: mechanisms involved in metal induced oxidative damage. *Current Topics in Medicinal Chemistry* **1**, 529-539.

Fowler BA, Mahaffey KR (1978) Interactions among lead, cadmium, and arsenic in relation to porphyrin excretion patterns. *Environmental Health Perspectives* **25**, 87-90.

Frenzilli G, Bocchetti R, Pagliarecci M, Nigro M, Annarumma F, Scarcelli V, Fattorini D, Regoli F (2004) Time-course evaluation of ROS-mediated toxicity in mussels, *Mytilus galloprovincialis*, during a field translocation experiment. *Marine Environmental Research* **58**, 609-613.

George S (1983a) Heavy metal detoxication in *Mytilus* kidney - an *in vitro* study of Cd- and Zn-binding to isolated tertiary lysosomes. *Comparative Biochemistry and Physiology* **76C**, 59-65.

George S (1983b) Heavy metal detoxication in the mussel *Mytilus edulis* - composition of Cd-containing kidney granules (tertiary lysosomes). *Comparative Biochemistry and Physiology* **76C**, 53-57.

Gorbi S, Virno Lamberti C, Notti A, Benedetti M, Fattorini D, Moltedo G, Regoli F (2008) An ecotoxicological protocol with caged mussels, *Mytilus galloprovincialis,* for monitoring the impact of an offshore platform in the Adriatic sea. *Marine Environmental Research* **65**, 34-39.

Ingersoll CG, Burton GA, Dawson TD, Dwyer FJ, Ireland DS, Kemble NE, Mount DR, Norburg-King TJ, Sibley PK, Stahl L (2000) Methods for Measuring the Toxicity and Bioaccumulation of Sediment-associated Contaminants with Freshwater Invertebrates In. (Ed. SaTW Offices of

685 Research and Development Mid-Continent Ecology Department). (U.S. Environmental
686 Protection Agency)
687 King CK, Gale SA, Hyne RV, Stauber JL, Simpson SL, Hickey CW (2006) Sensitivities of Australian
688 and New Zealand amphipods to copper and zinc in waters and metal-spiked sediments.
689 *Chemosphere* **63**, 1466-1476.
690 King CK, Simpson SL, Smith SV, Stauber JL, Bately GE (2005) Short-term accumulation of Cd and
691 Cu from water, sediment and algae by the amphipod *Melita plumulosa* and the bivalve *Tellina*
692 *deltoidalis*. *Marine Ecology Progress Series* **287**, 177-188.
693 Koeman JH, Kohler-Gunther A, Kurelec B, Riviere JL, Versteeg D, Walker CH (1993) Applications
694 and Objectives of Biomarker Research. In 'Biomarkers Research and Application in the
695 Assessment of Environmental Health'. (Eds DB Peakall and LR Shugart) pp. 1-13)
696 Legeay A, Achard-Joris M, Baudrimont M, Massabuau J-C, Bourdineaud J-P (2005) Impact of
697 cadmium contamination and oxygenation levels on biochemical responses in the Asiatic clam
698 *Corbicula fluminea*. *Aquatic Toxicology* **74**, 242-253.
699 Leonard SS, Harris GK, Shi X (2004) Metal-induced oxidative stress and signal transduction. *Free*
700 *Radical Biology and Medicine* **37**, 1921-1942.
701 Li M, Hu C, Zhu Q, Chen L, Kong Z, Liu Z (2006) Copper and zinc induction of lipid peroxidation
702 and effects on antioxidant enzyme activities in the macroalga *Pavlova viridis*
703 (Prymnesiophyceae). *Chemosphere* **62**, 565-572.
704 Li M, Kondo T, Zhao Q-L, Li F-J, Tanabe K, Arai Y, Zhou Z-C, Kasuya M (2000) Apoptosis Induced
705 by Cadmium in Human Lymphoma U937 Cells through Ca2+-calpain and Caspase-
706 Mitochondria- dependent Pathways. *J. Biol. Chem.* **275**, 39702-39709.
707 Li M, Xia T, Jiang C-S, Li L-J, Fu J-L, Zhou Z-C (2003) Cadmium directly induced the opening of
708 membrane permeability pore of mitochondria which possibly involved in cadmium-triggered
709 apoptosis. *Toxicology* **194**, 19-33.
710 Lucu C, Obersnel V (1996) Cadmium influx across isolated *Carcinus* gill epithelium Interection of
711 lanthanum and calcium with cadmium influxes. *Journal of Comparative Physiology B:*
712 *Biochemical, Systematic, and Environmental Physiology* **166**, 184-189.
713 Luoma SN, Rainbow PS (2008) 'Metal Contamination in Aquatic Environments: Science and Lateral
714 Management.' (Cambridge University Press: Cambridge)
715 Maher W, Forster S, Krikowa F, Snitch P, Chapple G, Craig P (2001) Measurement of trace elements
716 and phosphorus in marine animal and plant tissues by low-volume microwave digestion and
717 ICP-MS. *Atomic Spectroscopy* **22**, 361-370.
718 Maher W, Krikowa F, Kirby J, Townsend AT, Snitch P (2003) Measurement of trace elements in
719 marine environmental samples using solution ICP-MS. Current and future applications.
720 *Australian Journal of Chemistry* **56**, 103-116.
721 Maity S, Roy S, Chaudhury S, Bhattacharya S (2008) Antioxidant responses of the earthworm
722 *Lampito mauritii* exposed to Pb and Zn contaminated soil. *Environmental Pollution* **151**, 1-7.
723 Marigómez I, Soto M, Cajaraville MP, Angulo E, Giamberini L (2002) Cellular and subcellular
724 distribution of metals in molluscs. *Microscopy Research and Technique* **56**, 358-392.

Meister A (1989) On the biochemistry of glutathione. In 'Glutathione Centennial: molecular perspectives and clinical implications'. (Eds N Taniguchi, T Higashi, S Sakamoto and A Meister) pp. 3-22. (Academic Press: San Diego)

Meysman FJR, Middelburg JJ, Heip CHR (2006) Bioturbation: a fresh look at Darwin's last idea. *Trends in Ecology and Evolution* **21**, 688-695.

Migliore L, Barale R, Belluomini D, Cognetti AG, Loprieno N (1987) Cytogenetic damage induced in human lymphocytes by adriamycin and vincristine: A comparison between micronucleus and chromosomal aberration assays. *Toxicology in Vitro* **1**, 247-254.

Moolman L, Van Vuren JHJ, Wepener V (2007) Comparative studies on the uptake and effects of cadmium and zinc on the cellular energy allocation of two freshwater gastropods. *Ecotoxicology and Environmental Safety* **68**, 443-450.

Phillips DJH, Rainbow PS (1994) 'Biomonitoring of Trace Aquatic Contaminants.' (Chapman & Hall: London)

Regoli F (1998) Trace metals and antioxidant enzymes in gills and digestive gland of the Mediterranean mussel *Mytilus galloprovincialis*. *Archives of Environmental Contamination and Toxicology* **34**, 48-63.

Regoli F (2000) Total oxyradical scavenging capacity (TOSC) in polluted and translocated mussels: a predictive biomarker of oxidative stress. *Aquatic Toxicology* **50**, 351-361.

Regoli F, Frenzilli G, Bocchetti R, Annarumma F, Scarcelli V, Fattorini D, Nigro M (2004) Time-course variations of oxyradical metabolism, DNA integrity and lysosomal stability in mussels, *Mytilus galloprovincialis*, during a field translocation experiment. *Aquatic Toxicology* **68**, 167-178.

Regoli F, Gorbi S, Frenzilli G, Nigro M, Corsi I, Focardi S, Winston GW (2002) Oxidative stress in ecotoxicology: from the analysis of individual antioxidants to a more integrated approach. *Marine Environmental Research* **54**, 419-423.

Regoli F, Hummel H, Amiard - Triquet C, Larroux C, Sukhotin A (1998a) Trace metals and variations of antioxidant enzymes in arctic bivalve populations. *Archives of Environmental Contamination and Toxicology* **35**, 594-601.

Regoli F, Nigro M, Orlando E (1998b) Lysosomal and antioxidant responses to metals in the Antarctic scallop *Adamussium colbecki*. *Aquatic Toxicology* **40**, 375-392.

Regoli F, Principato G (1995) Glutathione, glutathione-dependent and antioxidant enzymes in mussel, *Mytilus galloprovincialis*, exposed to metals under field and laboratory conditions: implications for the use of biochemical biomarkers. *Aquatic Toxicology* **31**, 143-164.

Ribera D, Narbonne JF, Daubeze M, Michel X (1989) Characterisation, tissue distribution and sexual differences of some parameters related to lipid peroxidation in mussels. *Marine Environmental Research* **28**, 279-283.

Ringwood AH, Conners DE, Hoguet J (1998) Effects of natural and anthropogenic stressors on lysosomal destabilization in oysters *Crassostrea virginica*. *Marine Ecology Progress Series* **166**, 163-171.

764 Ringwood AH, Hoguet J, Keppler C, Gielazyn M (2004) Linkages between cellular biomarker
765 responses and reproductive success in oysters - *Crassostrea virginica*. *Marine Environmental*
766 *Research* **58**, 151-155.
767 Ringwood AH, Hoguet J, Keppler CJ (2002) Seasonal variation in lysosomal destabilization in
768 oysters, *Crassostrea virginica*. *Marine Environmental Research* **54**, 793-797.
769 Ringwood AH, Hoguet J, Keppler CJ, Gielazyn ML, Ward CH, Rourk AR (2003) 'Cellular
770 Biomarkers (Lysosomal Destabilization, Glutathione & Lipid Peroxidation) in Three Common
771 Estuarine Species: A Methods Handbook.' (Marine Resources Research Institute, Sth.
772 Carolina Dept. Nat. Res. Charleston)
773 Roach A (2005) Assessment of metals in sediments from Lake Macquarie, New South Wales,
774 Australia, using normalisation models and sediment quality guidelines. *Marine Environmental*
775 *Research* **59**, 453-472.
776 Scarpato R, Migliore L, Alfinito-Cognetti G, Barale R (1990) Induction of micronuclei in gill tissue of
777 *Mytilus galloprovincialis* exposed to polluted marine waters. *Marine Pollution Bulletin* **21**,
778 74-80.
779 Simpson SL, Angel BM, Jolley DF (2004) Metal equilibration in laboratory-contaminated (spiked)
780 sediments used for the development of whole-sediment toxicity tests. *Chemosphere* **54**, 597-609.
781 Sokolova IM (2004) Cadmium effects on mitochondrial function are enhanced by elevated
782 temperatures in a marine poikilotherm, *Crassostrea virginica* Gmelin (Bivalvia: Ostreidae). *J*
783 *Exp Biol* **207**, 2639-2648.
784 Sokolova IM, Evans S, Hughes FM (2004) Cadmium-induced apoptosis in oyster hemocytes involves
785 disturbance of cellular energy balance but no mitochondrial permeability transition. *J Exp Biol*
786 **207**, 3369-3380.
787 Sokolova IM, Ringwood AH, Johnson C (2005) Tissue-specific accumulation of cadmium in
788 subcellular compartments of eastern oysters *Crassostrea virginica* Gmelin (Bivalvia:
789 Ostreidae). *Aquatic Toxicology* **74**, 218-228.
790 Strom D, Simpson SL, Bately GE, Jolley DF (2011) The influence of sediment particle size and
791 organic carbon on toxicity of copper to benthic invertebrates in oxic/suboxic surface
792 sediments. *Environmental Toxicology and Chemistry* **30**, 1599-1610.
793 Stumm W, Morgan JJ (1996) 'Aquatic chemistry: Chemical equilibria and rates in natural waters,.'
794 (John Wiley: New York)
795 Taylor A, Maher W (2010) Establishing Metal Exposure - Dose - Response Relationships in Marine
796 Organisms: Illustrated with a Case Study of Cadmium Toxicity in *Tellina deltoidalis*. In 'New
797 Oceanography Research Developments: Marine Chemistry, Ocean Floor Analyses and Marine
798 Phytoplankton'. (Ed. L Martorino, and Puopolo, K.) pp. 1 - 57. (Nova Science: New York)
799 Tietze F (1969) Enzymatic method for quantitative determination of nanogram amounts of total and
800 oxidised glutathione: Applications to mammalian blood and other tissues. *Analytical*
801 *Biochemistry* **27**, 502-522.
802 Viarengo A (1989) Heavy metals in marine invertebrates: mechanisms of regulation and toxicity at the
803 cellular level. *Rev. Aquat. Sci.* **1**, 295-317.

804 Viarengo A, Canesi L, Pertica M, Livingstone DR (1991) Seasonal variations in the antioxidant
805 defence system and lipid peroxidation of the digestive gland of mussels. . *Comparative*
806 *Biochemistry and Physiology* **100C**, 187-190.
807 Viarengo A, Moore MN, Mancinelli G, Mazzucotelli A, Pipe RK, Farrar SV (1987) Metallothioneins
808 and lysosomes in metal toxicity and accumulation in marine mussels: the effect of cadmium in
809 the presence and absence of phenanthrene. *Marine Biology* **94**, 251-257.
810 Viarengo A, Nott JA (1993) Mechanisms of heavy metal cation homeostasis in marine invertebrates.
811 *Comparative Biochemistry and Physiology Part C: Comparative Pharmacology* **104**, 355-372.
812 Viarengo A, Pertica M, Canesi L, Accomando R, Mancinelli G, Orunesu M (1989) Lipid peroxidation
813 and level of antioxidant compounds (GSH, vitamin E) in the digestive gland of mussels of
814 three different age groups exposed to anaerobic and aerobic conditions. *Marine Environmental*
815 *Research* **28**, 291-295.
816 Wallace WG, Lee BG, Luoma SN (2003) Subcellular compartmentalization of Cd and Zn in two
817 bivalves. I. Significance of metal-sensitive fractions (MSF) and biologically detoxified metal
818 (BDM). *Marine Ecology Progress Series* **249**, 183-197.
819 Wallace WG, Lopez GR, Levinton JS (1998) Cadmium resistance in an oligochaete and its effect on
820 cadmium trophic transfer to an omnivorous shrimp. *Marine Ecology Progress Series* **172**,
821 225-237.
822 Wataha JC, Lewis JB, Lockwood PE, Rakich DR (2000) Effect of dental metal ions on glutathione
823 levels in THP-1 human monocytes. *Journal of Oral Rehabilitation* **27**, 508-516.
824 Widdows J, Donkin P (1992) Mussels and Environmental Contaminants: Bioaccumulation ans
825 Physiological Aspects. In 'The Mussel *Mytilus*: Ecology, Physiology, Genetics and Culture'.
826 (Ed. E Gosling) pp. 383-424. (Elsevier Science: Amsterdam)
827 Williams RC, Metcalfe CD (1992) Development of an in vivo hepatic micronucleus assay with
828 rainbow trout. *Aquatic Toxicology* **23**, 193-202.
829 Winston GW, Di Giulio RT (1991) Prooxidant and antioxidant mechanisms in aquatic organisms.
830 *Aquatic Toxicology* **19**, 137-161.
831 Winston GW, Moore MN, Kirchin MA, Soverchia C (1996) Production of reactive oxygen species by
832 Hemocytes from the marine mussel, *Mytilus edulis*: Lysosomal localization and effect of
833 xenobiotics,. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology*
834 *and Endocrinology* **113**, 221-229.
835 ZepoMetrix (2011) Oxitek TBARS Assay Kit User Protocol ZMC Catalog # 0801192.

836 **Figure Captions**

837 **Figure 1** Procedure for subcellular fractionation of *T. deltoidalis* tissues by differential
838 centrifugation. The shaded boxes show details of the centrifugation and digestion / heating
839 steps used to obtain the specific fractions. The final fractions, four pellets P2, P3, P4 & P5
840 and two supernatants S2 & S5 are grouped as: biologically detoxified (BDM) P2 & S5;
841 biologically active (BAM) P3, P4 & P5 metals or S2 which contains metal associated with
842 dissolved tissues.
843
844 **Figure 2:** Tissue cadmium concentrations (µg/g dry mass) of *T. deltoidalis* exposed to
845 cadmium spiked sediments of 0 (control), Cd 10 and 50µg/g dry mass. Mean ± SE, $n = 12$.
846 Day 0 are unexposed organisms $n = 6$.
847
848 **Figure 3:** Distribution (%) of cadmium in each of the subcellular fractions of *T. deltoidalis*
849 following 28 days exposure to cadmium spiked sediments. Subcellular fractions are:
850 nuclei+cellular debris (N & C.d); metal rich granules (MRG); heat stable, metallothionein like
851 proteins (MTLP); mitochondria (Mit); lysosomes microsomes (Lys & Mic); heat sensitive
852 proteins (HSP). Stippled fractions ( ) make up the biologically active cadmium (BAM),
853 dashed fractions ( ) make up the biologically detoxified cadmium (BDM), $n = 2$**.**
854
855 **Figure 4:** Antioxidant enzyme biomarkers of *T. deltoidalis* after 28 days exposure to
856 cadmium spiked sediments of 0 (control), Cd 10 and Cd 50µg/g dry mass . Mean ± SE, $n =$
857 12 for all except GPx (glutathione peroxidise) $n = 6$. GSH+2GSSG (total glutathione);
858 GSH/GSSG (ratio of reduced to oxidised glutathione). Different letters indicate significant
859 differences between means (Bonferroni test; $p < 0.05$).
860
861 **Figure 5:** Changes in oxidative damage biomarkers: MDA (lipid peroxidation); cellular
862 (lysosomal destabilisation); and genotoxic (micronuclei) of *T. deltoidalis* after 28 days
863 exposure to cadmium spiked sediments, 0 (control), Cd 10 and Cd 50µg/g dry mass. Mean ±
864 SE $n = 12$. Different letters indicate significant differences between means (Bonferroni test;
865 $p < 0.05$).

Figure 1

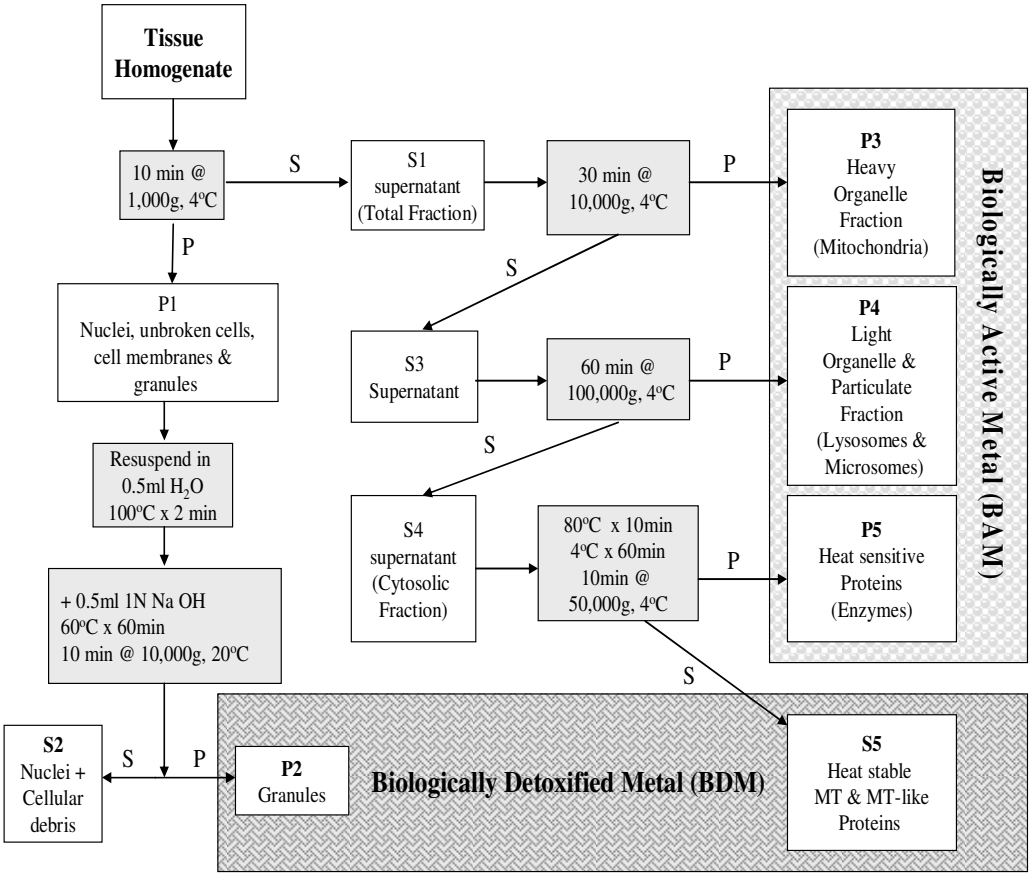

Tissue Homogenate
10 min @ 1,000g, 4ºC
S
S1 supernatant (Total Fraction)
30 min @ 10,000g, 4ºC
P
P3 Heavy Organelle Fraction (Mitochondria)
P
P1 Nuclei, unbroken cells, cell membranes & granules
S
S3 Supernatant
60 min @ 100,000g, 4ºC
P
P4 Light Organelle & Particulate Fraction (Lysosomes & Microsomes)
S
Resuspend in 0.5ml H2O 100ºC x 2 min
S4 supernatant (Cytosolic Fraction)
80ºC x 10min 4ºC x 60min 10min @ 50,000g, 4ºC
P
P5 Heat sensitive Proteins (Enzymes)
Biologically Active Metal (BAM)
+ 0.5ml 1N Na OH 60ºC x 60min 10 min @ 10,000g, 20ºC
S
S2 Nuclei + Cellular debris
S
P
P2 Granules
Biologically Detoxified Metal (BDM)
S5 Heat stable MT & MT-like Proteins

Figure 2

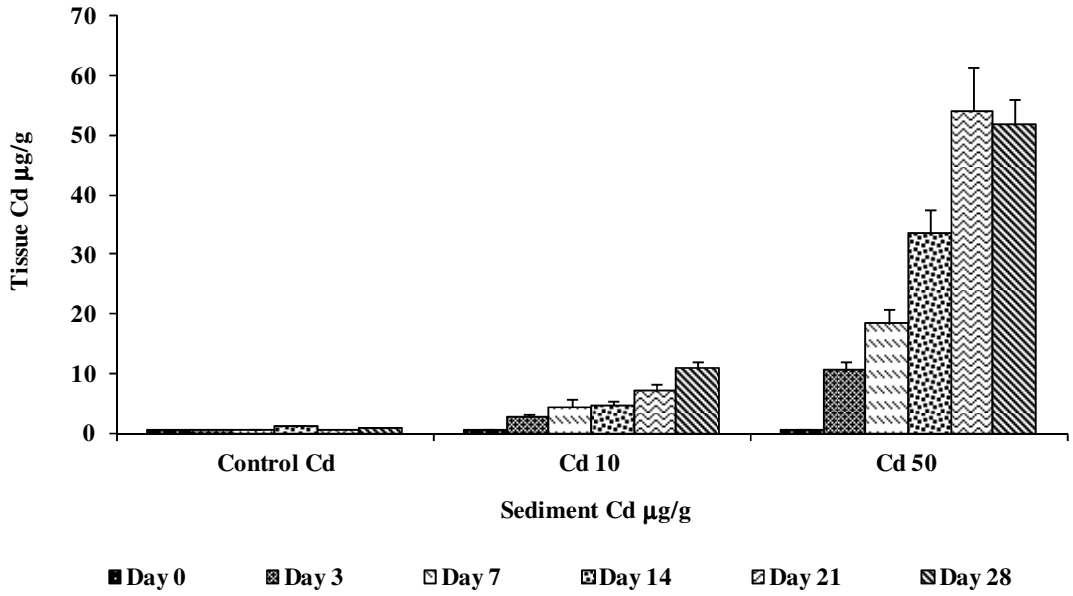

70
60
50
40
30
20
10
0
Tissue Cd µg/g
Control Cd
Cd 10
Cd 50
Sediment Cd µg/g
Day 0
Day 3
Day 7
Day 14
Day 21
Day 28

Figure 3

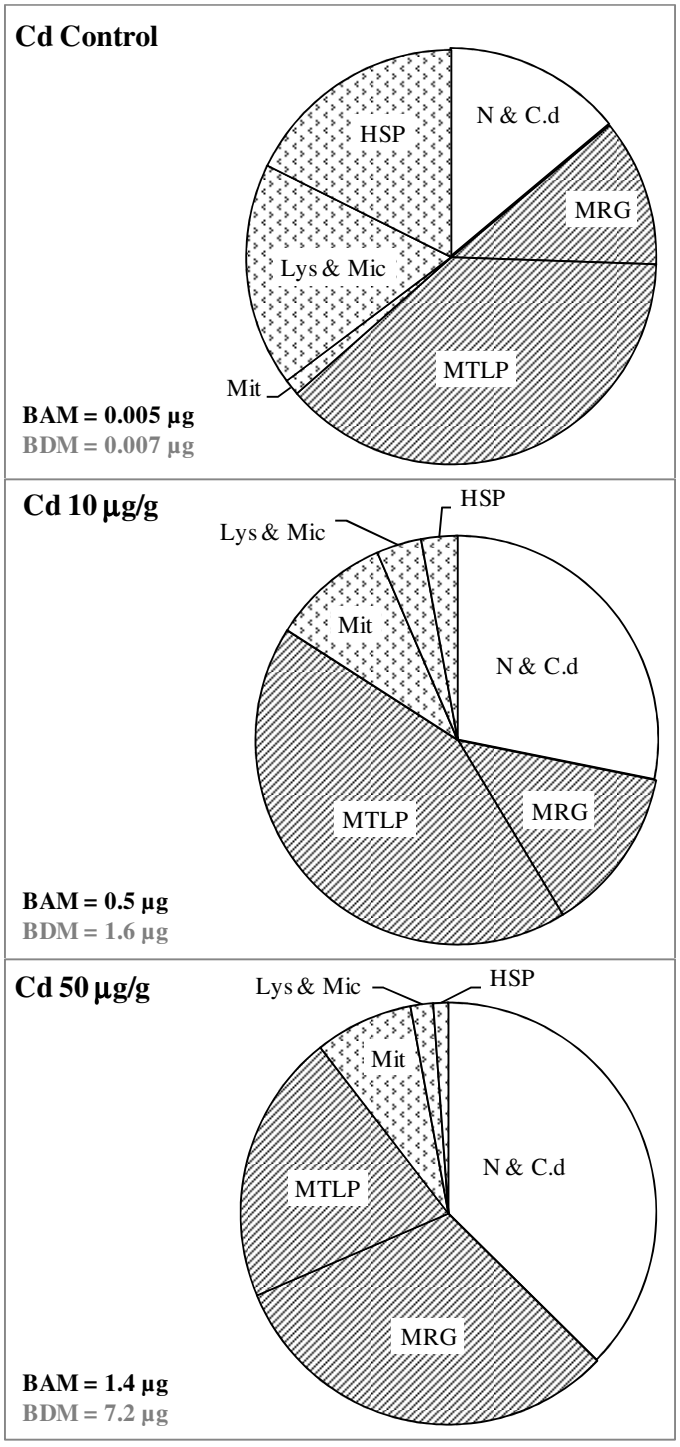
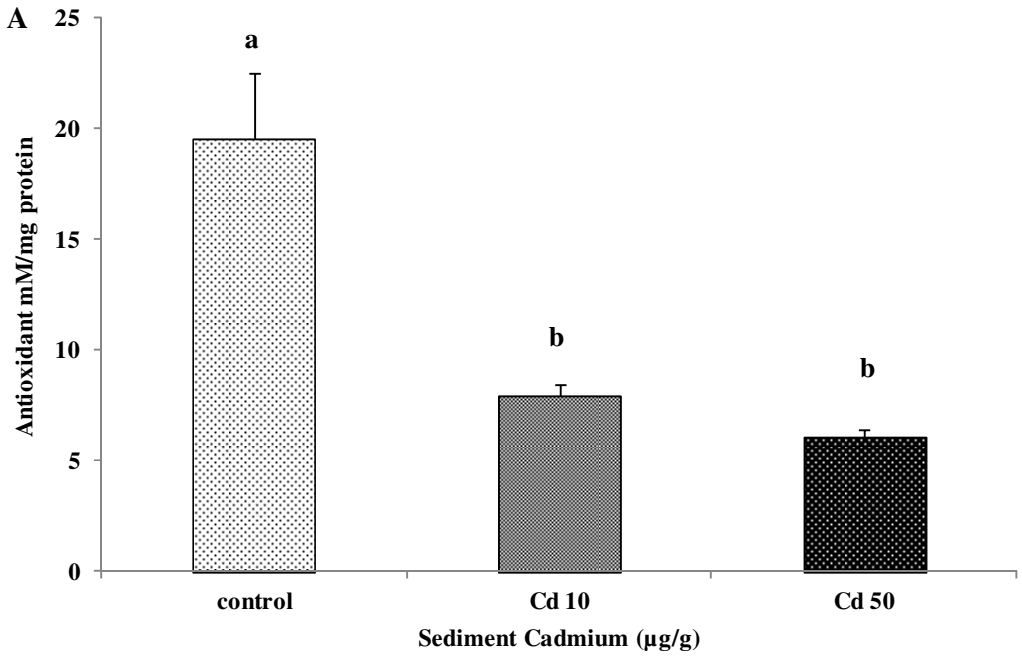

Cd Control
HSP
N & C.d
MRG
Lys & Mic
MTLP
Mit
BAM = 0.005 µg
BDM = 0.007 µg
Cd 10 µg/g
HSP
Lys & Mic
Mit
N & C.d
MTLP
MRG
BAM = 0.5 µg
BDM = 1.6 µg
Cd 50 µg/g
Lys & Mic
HSP
Mit
N & C.d
MTLP
MRG
BAM = 1.4 µg
BDM = 7.2 µg

Figure 4


A
Antioxidant mM/mg protein
25
20
15
10
5
0
a
b
b
control
Cd 10
Cd 50
Sediment Cadmium (µg/g)

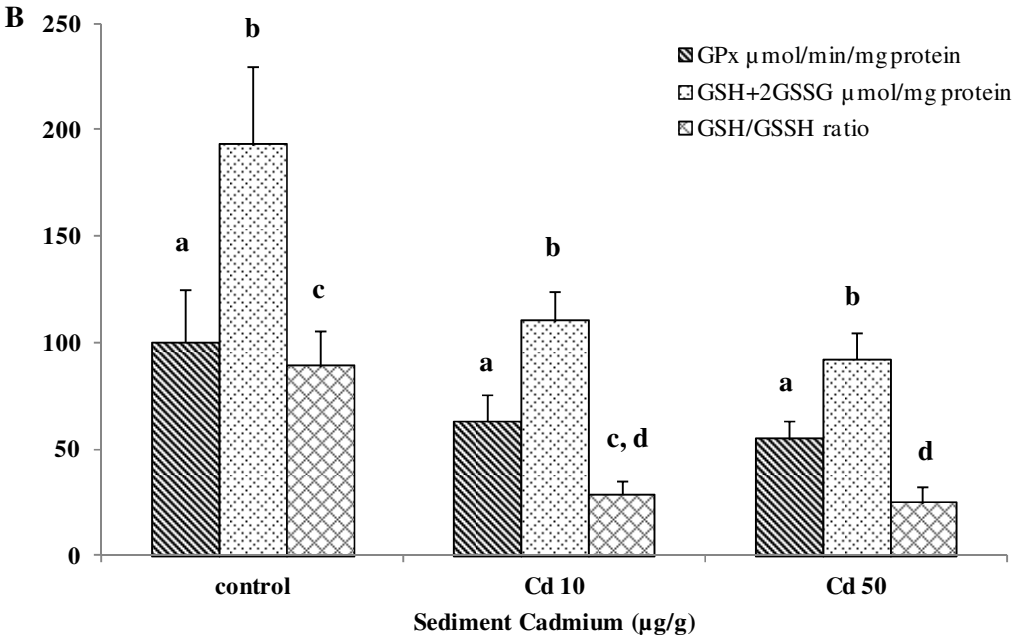

B
250
200
150
100
50
0
GPx µmol/min/mg protein
GSH+2GSSG µmol/mg protein
GSH/GSSH ratio
a
b
c
a
b
c, d
a
b
d
control
Cd 10
Cd 50
Sediment Cadmium (µg/g)

Figure 5

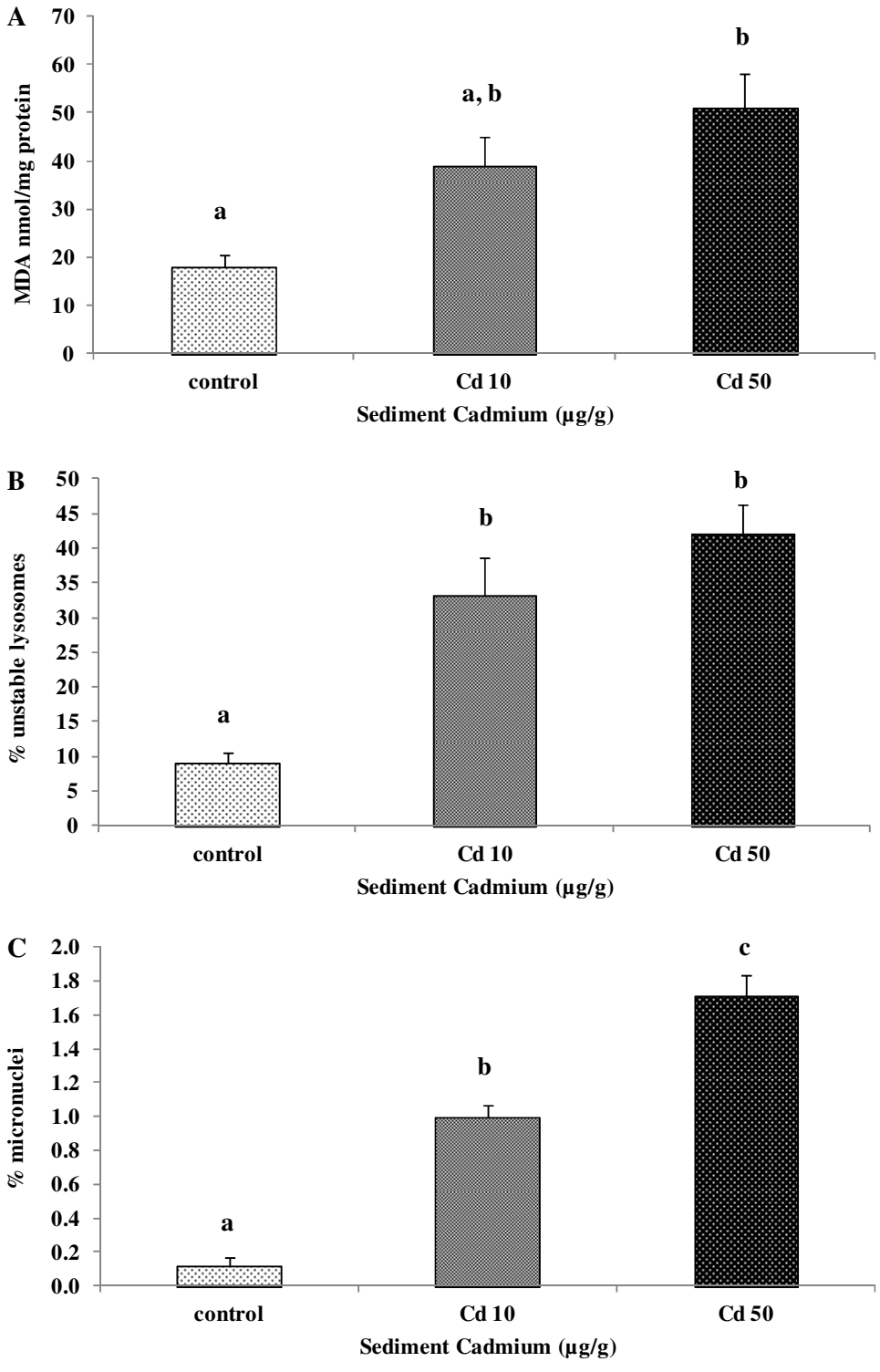

A
MDA nmol/mg protein
70
60
50
40
30
20
10
0
a
a, b
b
control
Cd 10
Cd 50
Sediment Cadmium (µg/g)
B
% unstable lysosomes
50
45
40
35
30
25
20
15
10
5
0
a
b
b
control
Cd 10
Cd 50
Sediment Cadmium (µg/g)
C
% micronuclei
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
a
b
c
control
Cd 10
Cd 50
Sediment Cadmium (µg/g)

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

	Sediment Treatments (µg/g)		
	control	Cd 10	Cd 50
Total Tissue Cadmium (µg)	**0.04 ± 0.01**	**5.9 ± 0.02**	**26 ± 7**
Total Recovered Cadmium (µg)	**0.01 ± 0**	**3 ± 0.4**	**14 ± 2**
Proportion of total recovered in fractions (%)	38 ± 8	50 ± 7	56 ± 21
Cadmium Distribution			
Nuclei + Cellular debris (µg)	**0.002**	**0.8 ± 0.1**	**5.2 ± 0.9**
Nuclei + Cellular debris (%)	14 ± 3	28 ± 0.5	37 ± 2
Biologically Active Metal (µg)	**0.005**	**0.5 ± 0.1**	**1.4 ± 0.2**
Biologically Active Metal (%)	36 ± 12	16 ± 5	11 ± 2
Biologically Detoxified Metal (µg)	**0.007**	**1.6 ± 0.2**	**7.2 ± 1**
Biologically Detoxified Metal (%)	50 ± 5	56 ± 2	52 ± 3

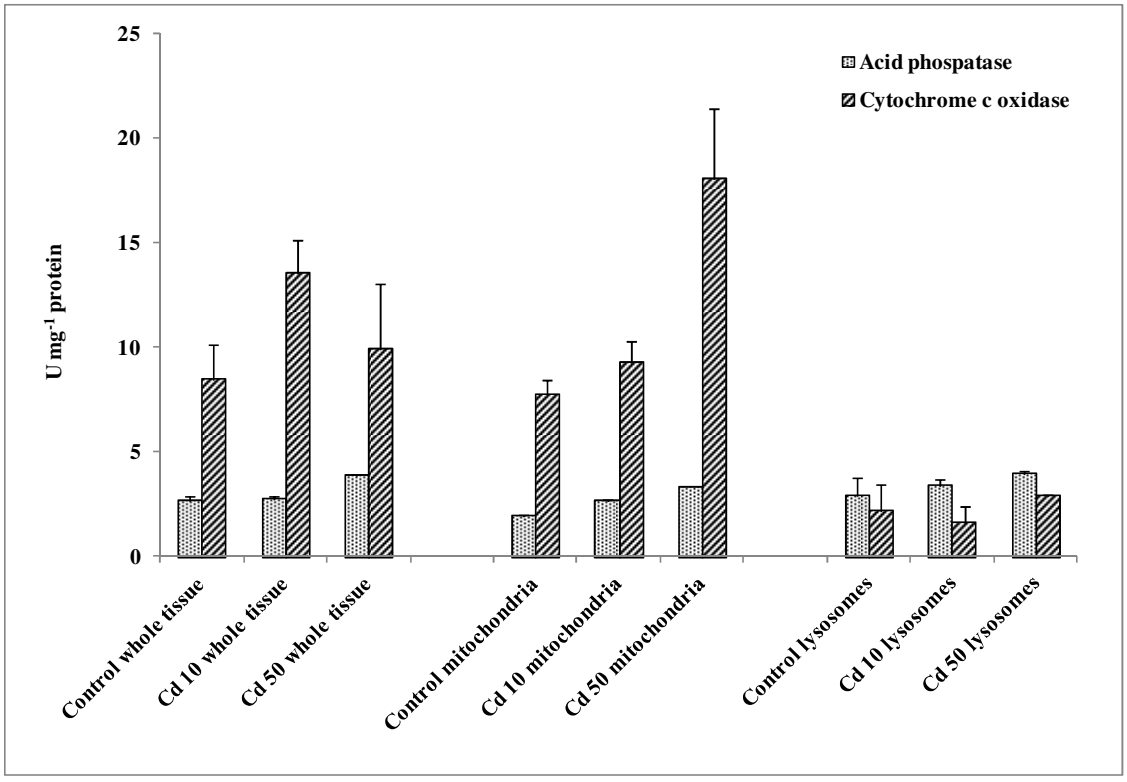
Mean ± SD, $n = 2$

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

	Sediment Treatments (µg/g)		
	control	Cd 10	Cd 50
Nuclei + Cellular debris % of total	**14**	**28**	**37**
Biologically Detoxified Metal % of total	**50**	**56**	**52**
Metal Rich Granules % of BDM	23	24	60
Heat Stable MT Like Proteins % of BDM	77	76	40
Biologically Active Metal % of total	**36**	**16**	**11**
Mitochondria % of BAM	3	60	72
Lysosomes + Microsomes % of BAM	48	22	17
Heat Sensitive Proteins % of BAM	49	18	11

Supplementary Material

Supplementary Figure 1: 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 cadmium spiked sediments at 0 (Control), 10 and 50 µg/g dry mass after 28 days. Mean ± SD, $n = 2$.



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

		Cadmium	
Source	**df**	**F**	**p**
Treatment	2	211	***
Day	4	32	***
Day*Treatment	8	7	***

*** $p < 0.001$

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

Treatments	
Cadmium	**p**
none - control	ns
none - Cd 10	**
none - Cd 50	***
control - Cd 10	***
control - Cd 50	***
Cd 10 - Cd 50	***

*** $p \leq 0.001$, ** $p \leq 0.01$, 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	**Cadmium**		
	df	**F**	**p**
TAOC	2	36	***
GPx	2	0.8	ns
GSH+2GSSG	2	3.5	ns
GSH:GSSG	2	7.2	*
TBARS	2	7.2	*
Lysosomes	2	23	***
Micronuclei	2	62	***

*** $p \leq 0.001$, * $p \leq 0.05$, *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**
Cadmium	**p**	**p**	**p**	**p**	**p**	**p**	**p**
Control - Cd 10	**	ns	ns	ns	ns	***	**
Control - Cd 50	***	ns	ns	*	*	***	***
Cd 10 - Cd 50	ns	ns	ns	ns	ns	ns	**

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