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3 **Exposure-dose-response of *Tellina deltoidalis* to metal contaminated estuarine sediments**

4 **1. Cadmium spiked sediments**

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17

18 **Abstract**

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

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

37

38 1 Introduction

39 Cadmium sediment concentrations up to 50 µg/g dry mass have been reported in
40 contaminated Australian estuaries (Roach, 2005). The Australian guidelines for Fresh and
41 Marine Water Quality (ANZECC and ARMCANZ, 2000) interim sediment quality high
42 effects guideline for cadmium is 10 µg/g dry mass. Cadmium is accumulated in high
43 concentrations by a range of marine organisms and has no known biological function (ECB,
44 2007) but at low concentrations may adversely affect cellular function (Ercal *et al.*, 2001;
45 Sokolova, 2004; Sokolova *et al.*, 2004). Physiological effects and toxicity of metals strongly
46 depend on their intracellular localisation and binding to organelles and ligands (Sokolova *et*
47 *al.*, 2005). Cadmium, for example, affects the bioenergetics of oyster mitochondria in vitro
48 and in vivo at low concentrations, leading to reduced coupling and impaired ability to produce
49 ATP (Sokolova, 2004).

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

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

70 length with their siphons extending up to the sediment surface and ingest deposited organic
71 material and sand grains (Beesley *et al.*, 1998). The purpose of this study was to examine the
72 exposure - dose - response relationship to cadmium spiked sediments in *T. deltoidalis* using
73 28 day sediment bioaccumulation tests (Ingersoll *et al.*, 2000), to develop useful biomarkers
74 of effect and evaluate their potential for sediment toxicity testing. The concentrations of
75 cadmium chosen for the sediment spiking were the high sediment quality guideline
76 concentration (10 µg/g dry mass) and the highest sediment cadmium concentrations
77 previously measured in contaminated estuarine sediments (50 µg/g dry mass). The study
78 examines total cadmium tissue accumulation and subcellular tissue cadmium distribution to
79 determine the total cadmium fraction stored in metabolically sensitive organelles. Oxidative
80 stress was determined by measuring total antioxidant scavenging capacity of cells, cellular
81 concentrations of oxidised and reduced glutathione, glutathione peroxidase activity and the
82 extent of lipid peroxidation. Cellular damage was determined by measuring lysosomal
83 membrane stability and a micronucleus assay used to assess genotoxic damage.

84 2 Materials and Methods

85 2.1 Organism and sediment collection

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

95

96 2.2 Sediment cadmium spiking

97 Sediments were sieved through a 2 mm stainless steel sieve to remove large pieces of organic
98 matter and organisms prior to the addition of metals. Sub samples of the collected sediments
99 were measured for moisture content and grain size. To produce a sediment matrix which was
100 suitable for organism burrowing and feeding, sediment was mixed with clean beach sand so
101 that the 63 µm fraction was not greater than 20% mass/mass. To ensure added cadmium was
102 rapidly adsorbed and strongly bound to the sediment particles a method developed by

103 (Simpson *et al.*, 2004) for producing metal spiked marine sediments, was followed. Wet
104 sediment was added to mixing containers. CdCl₂, (AR grade Merck), was added to
105 concentrations of 10 mg/kg and 50 mg/kg dry mass of sediment. All containers were topped
106 up with clean deoxygenated sea water and the final mixture was completely deoxygenated by
107 bubbling with nitrogen for 2 hours. Head spaces of containers were filled with nitrogen prior
108 to sealing. Any pH adjustments were made immediately after the addition of cadmium using
109 1M NaOH, (AR grade BDH), prepared in seawater. pH was checked weekly and maintained
110 at 7 - 8.2. Sediments were maintained at room temperature 22 - 25°C and mixed on a Cell-
111 production Roller Apparatus (Belco, USA) for several hours each day. The time required for
112 equilibration of added metals is affected by the sediment properties, equilibration pH and the
113 concentration and properties of the metal (Simpson *et al.*, 2004). To determine when the
114 added cadmium was completely bound to sediment particles pore waters were collected and
115 acidified to 1% with nitric acid (AristaR, BDH, Australia) and cadmium measured using an
116 ELAN[®] 6000 ICP-MS (PerkinElmer SCIEX, USA). Once pore water cadmium
117 concentrations had fallen below instrument detection limits 0.001 µg/l the sediment was ready
118 for use. Time to full absorption was 4 - 6 weeks. Unspiked sediments were treated in the
119 same way and used for control treatments. Sediment cadmium concentrations were measured
120 by ICP-MS after digestion of 0.2 g of lyophilised sediment in 3 ml of nitric acid (AristaR,
121 BDH, Australia) in polyethylene 50 ml centrifuge tubes for 60 minutes at 115°C (Maher *et al.*,
122 2003). Cadmium in NRCC Certified Reference Materials, BCSS-1 marine sediment
123 measured along with samples was 0.22 ± 0.10 µg/g (n = 10) and in agreement with certified
124 values 0.25 ± 0.04 µg/g. Sediment cadmium concentrations were measured prior to and at the
125 end of the 28 day exposure period, concentrations were < 0.001, 10.0 ± 0.6 and 50 ± 2 µg/g
126 on each occasion.

127 **2.3 *Microcosm experiment design***

128 Procedures for conducting the exposures were adapted from the test method for conducting 28
129 day sediment bioaccumulation tests (Ingersoll *et al.*, 2000). Spiked and control sediments
130 (500 g wet wt.) were placed in each of three replicate 770 ml polypropylene containers
131 (Chanrol # 01C30, Australia) per treatment. The containers were filled with fresh seawater
132 adjusted to a salinity of 28‰. Containers were placed in random order on a tray in an
133 incubator set at 22°C with a day / night light cycle of 14 / 10 hours to reflect spring / summer
134 conditions. Aeration was introduced and the treatments were left for 24 hours to allow them
135 settle and the temperature to equilibrate. *T. deltoidalis* were then introduced to each treatment
136 container. Organisms were not given supplementary food and surface water was changed

137 weekly during the 28 day exposure period. Aquaria were continually aerated using an air
138 pump with valves on each line to regulate air flow so oxygen saturation $\approx 100\%$ were
139 maintained in each aquarium but sediments were not agitated. Due to the natural buffering
140 capacity of sea water and associated sediments pH remained relatively constant at pH 7.8-8.0
141 in all aquaria throughout the 28 days of exposure. This is similar to results of other studies of
142 this type (King *et al.*, 2006; Strom *et al.*, 2011). Total tissue cadmium bioaccumulation was
143 measured at intervals of 3, 7, 14, 21 and 28 days. A day 0 measurement was made using
144 organisms from the acclimation tanks to give the background cadmium concentration. All
145 organisms were placed in fresh seawater 28‰ with no sediment for 24 hours to allow
146 depuration of ingested sediment particles, prior to cadmium analysis.

147 **2.4 Cadmium Measurements**

148 **2.4.1 Total Cadmium**

149 Lyophilised ground tissue was microwave digested in 1 ml of nitric acid (AristaR BDH,
150 Australia) in a 630 watt oven (CEM MDS-2000, USA) for two min at 630 W, two min 0 W,
151 and 45 min at 315 W (Baldwin *et al.*, 1994). Prior to analysis samples were diluted with
152 deionised water to 1% v/v HNO₃, and an ICP-MS mixed 7-element internal standard (EM
153 Science) was added to monitor for variations due to instrument drift and/or matrix effects.
154 Cadmium was measured using an ELAN[®] 6000 ICP-MS (PerkinElmer, SCIEX) following the
155 method of Maher *et al.* (2001). NRCC Certified Reference Material, NIST 1566a oyster
156 tissue and acid blanks were routinely digested and diluted in the same way as the samples and
157 analysed along with them to verify accuracy and precision of cadmium analysis. The
158 measured CRM cadmium value; $4.4 \pm 0.6 \mu\text{g/g}$ (n = 50) was not significantly different from
159 the certified value $4.15 \pm 0.38 \mu\text{g/g}$.

160 **2.4.2 Subcellular cadmium**

161 The subcellular tissue cadmium distribution was examined in tissues of day 28 exposed
162 organisms using a procedure adapted from Sokolova *et al.* (2005) and Wallace *et al.* (2003).
163 The dissected tissues were placed in polypropylene vials, snap frozen in liquid nitrogen and
164 stored at -80°C until processed. The tissue was thawed and minced on ice with a blade. A
165 sub sample, approximately 0.1 g wet wt., was taken for total tissue cadmium analysis. The
166 remainder, approximately 0.5 g wet wt., was homogenised in Ca²⁺ / Mg²⁺ free saline buffer
167 pH 7.35 on ice using an IKA[®] Labortechnik Ultra-turrax-T25 homogeniser equipped with an
168 S25-UT dispersing tool at $9,500 \text{ rpm}^{-1}$ (Janke & Kunkel, Germany). Homogenised tissue
169 was subjected to differential centrifugation and tissue digestion procedures according to the

170 protocol outlined in Taylor and Maher (2010), using an Eppendorf 5804R centrifuge and a
171 Himac CP90WX preparative ultracentrifuge (Hitachi, Japan). The mitochondria, lysosomes
172 plus microsomes and heat sensitive protein pellets were grouped as biologically active
173 cadmium fractions while the granule and heat stable metallothionein like proteins were
174 grouped as biologically detoxified cadmium fractions (Figure 1). The supernatant from the
175 granule pellet isolation contained the cellular debris (Figure 1). To determine the
176 mitochondrial and lysosomal content of the fractions obtained the activity of enzymes specific
177 for these organelles, cytochrome *c* oxidase and acid phosphatase, respectively, were measured
178 in each of the total tissue, mitochondrial and lysosome+microsome pellets using commercial
179 colorimetric assays (CYTOC-OX1 Sigma-Aldrich, USA and CS0740 Sigma-Aldrich, USA
180 respectively). This showed that the activity of both enzymes was greater than that of the
181 control organisms in the cadmium exposed organisms and that the mitochondrial fraction was
182 enriched with mitochondria, and the lysosome+microsome fraction with lysosomes
183 (Supplementary Figure 1). Fractions were acidified to 10% v/v with nitric acid (AristaR
184 BDH, Australia) and placed in a water bath at 80°C for 4 hours. NIST CRM 1566a oyster
185 tissue, buffer and acid blanks were digested and diluted in the same way as the samples and
186 analysed along with them. Analysis of cadmium was as previously described above. The
187 measured CRM cadmium value; $3.8 \pm 0.5 \mu\text{g/g}$ ($n = 5$) were in good agreement with certified
188 value $4.15 \pm 0.38 \mu\text{g/g}$.

189 **2.5 Biomarker measurements**

190 **2.5.1 Total antioxidant capacity and lipid peroxidation**

191 Tissues were homogenised on ice in a 5 mM potassium phosphate buffer containing 0.9%
192 (w/v) sodium chloride and 0.1% (w/v) glucose, pH 7.4 (1:5 w/v) using a motorised
193 microcentrifuge pellet pestle, sonicated on ice for 15 seconds at 40 V (VibraCell™ Sonics
194 Materials, USA) and centrifuged, in a 5804R centrifuge (Eppendorf, Germany), at 10,000 x g
195 for 15 minutes at 4°C (Cayman, 2011). The supernatant was stored at -80°C until analysis.
196 Total antioxidant capacity was measured using an assay based on the ability of the tissue
197 lysate antioxidant system to inhibit the oxidation of ABTS (2,2'-azino-di-[3-
198 ethylbenzthiazoline sulphonate]) to ABTS^{•+} by metmyoglobin in the presence of hydrogen
199 peroxide. This was compared with the antioxidant capacity of a standard, Trolox (Cayman,
200 2011). The amount of ABTS^{•+} produced was measured by the suppression of absorbance at
201 750 nm and is proportional to the final total antioxidant capacity concentration, expressed in
202 millimolar Trolox equivalents. Samples were pipetted into a 96 well plate with
203 metmyoglobin and ABTS. The reactions were initiated with a 441 μl solution of hydrogen

204 peroxide. The plate was shaken for 5 minutes at 25°C and absorbance was read at 750 nm on
205 a BioRad Benchmark Plus microplate spectrophotometer. The Thiobarbituric Acid Reactive
206 Substances (TBARS) assay was used to measure lipid peroxidation by measuring the
207 malondialdehyde (MDA) concentration in each tissue lysate. The end product of lipid
208 peroxidation, MDA, forms a 1:2 adduct with TBARS and produces a colour reaction that can
209 be read spectrophotometrically at 532 nm and compared to an MDA standard curve
210 (ZepoMetrix, 2011). The samples were incubated in a solution of sodium dodecyl sulphate,
211 thiobarbituric acid and sodium hydroxide dissolved in acetic acid at 95°C for 60 minutes.
212 After cooling on ice and centrifuging at 3000 rpm for 10 minutes at room temperature, the
213 colour reaction was measured, on a BioRad Benchmark Plus microplate spectrophotometer at
214 532 nm.

215 **2.5.2 Reduced:oxidised glutathione ratio and glutathione peroxidase**

216 Tissue lysates were produced by homogenisation on ice in a 50 mM Tris-HCl buffer
217 containing 5 mM EDTA and 1 mM DTT, pH 7.5 (1:5 w/v) using the technique outlined
218 above. A thiol scavenging agent 1-methyl-2-vinyl-pyridium trifluoromethane sulfonate in
219 HCl (Calbiochem[®], Merck, Germany) was added to GSSG tissue homogenates to remove
220 GSH, prior to the addition of buffer and production of the final supernatant. The remaining
221 GSSG is then reduced to GSH and determined by the reaction with Ellman's reagent
222 (Calbiochem, 2004). Supernatants were stored at -80°C until analysis of reduced glutathione
223 (GSH), glutathione peroxidase (GPx) and protein (Calbiochem, 2004). The ratio of reduced to
224 oxidised glutathione (GSH:GSSG) was measured using an enzymatic method based on one
225 developed by (Tietze, 1969). The method uses Ellman's reagent (5,5'-dithiobis-
226 (2-nitrobenzoic acid) (DTNB) which reacts with GSH to form a colour which is detected at
227 412 nm (Calbiochem[®], Merck, Germany). The samples were acidified by the addition of a
228 5% solution of metaphosphoric acid, vortexed for 15 seconds and centrifuged at 1000 x g for
229 10 minutes at room temperature. The metaphosphoric acid extracts were diluted with a
230 sodium phosphate buffer and mixed at room temperature in 1 ml cuvettes with DTNB and
231 glutathione reductase enzyme at (1:1:1 v/v/v). The reaction was initiated with
232 β - nicotinamide adenine dinucleotide phosphate (NADPH) and absorbance read at 412 nm for
233 3 minutes at intervals of 15 seconds on a Unicam Helios Gamma UV-Vis spectrophotometer
234 (Spectronic, UK). Absorbance rates were calculated and GSH and GSSG concentrations
235 calculated using a 6 point GSH calibration curve. A GSSG buffer blank was run for
236 interference correction.

237 Glutathione peroxidase activity (GPx) was measured using a coupled reaction with glutathione
238 reductase (GR) (Cayman Chemicals, USA). The oxidation of NADPH to NADP⁺ is
239 accompanied by a decrease in absorbance at 340 nm. Under conditions where GPx activity is
240 rate limiting, the rate of decrease in the A₃₄₀ is directly proportional to the GPx activity in the
241 sample. Assay buffer 50 mM Tris-HCl, pH 7.6, 5 mM EDTA was added to sample wells of a
242 flat bottomed 96 well plate with a co-substrate mixture NADPH, glutathione and GR (2:1
243 v/v). Samples were added to each well and the reaction was initiated by the addition of
244 cumene hydroperoxide. The plate was shaken briefly and the decrease in absorbance read at
245 340 nm for 5 minutes at intervals of 30 seconds at 25°C on a BioRad Benchmark Plus
246 microplate spectrophotometer. Rates were calculated and samples were compared with a
247 bovine erythrocyte GPx positive control. Buffer blanks run with the samples were used to
248 correct for interferences and GPx activity was calculated using the NADPH extinction
249 coefficient, adjusted for the pathlength of the solution, of 0.00373 μM⁻¹. One unit is defined
250 as the amount of enzyme that will cause oxidation of 1.0 nmol of NADPH to NADP⁺ per
251 minute at 25°C.

252 **2.5.3 Protein**

253 All tissue lysates used for enzymatic assays were analysed for protein concentration and
254 enzyme concentration / activity is expressed as mg⁻¹ of protein in the sample. The
255 FluoroProfile™ (Sigma #FP0010, Sigma-Aldrich, USA) protein assay used is a fluorescent
256 assay based on Epiccoconone, a biodegradable natural product. The fluorescence intensity
257 was read at 485 nm excitation and 620 nm emission, on a Luminoskan Ascent Fluorescence
258 Plate Reader (Thermo Electrical Corp., USA). Bovine serum (BSA) calibration curve
259 standards used were made up in sample buffer.

260 **2.6 Cellular and genotoxic biomarkers**

261 **2.6.1 Lysosomal stability**

262 Lysosomal stability was assessed using a method developed by (Ringwood *et al.*, 2003) for
263 oysters. The assay uses neutral red (NR) dye retention to assess the integrity of the lysosomal
264 membrane. Cells incubated in neutral red accumulate the lipophilic dye in the lysosomes.
265 Healthy cells retain the dye in the lysosomes whereas in cells with damaged lysosomal
266 membranes it leaks out into the cytoplasm. Minced tissue was shaken in CMFS buffer pH
267 7.35 salinity 30 ‰ on a reciprocating shaker at 100 rpm for 20 minutes. Trypsin (T4799
268 Sigma, USA), 325 μl at 1 mg/ml in CMFS buffer, was added and samples shaken for a further
269 20 minutes. Cells were then collected by centrifuging samples through a 20 μm screen 250 -

270 500 g at 15°C for 5 - 15 minutes. Cells were incubated in neutral red (Sigma, USA), 0.04
271 mg/ml in CMFS for 1 hour and one hundred cells per slide were counted using a light
272 microscope with 40x lens and scored as stable or unstable. Two slides per sample were
273 counted.

274 **2.6.2 Micronuclei frequency**

275 The micronuclei assay used was based on a technique developed on the mussel *Mytilus*
276 *galloprovincialis* (Gorbi *et al.*, 2008). The assay uses DAPI (4',6-diamidine-2'-phenylindole
277 dihydrochloride), a fluorescent dye specific for nucleic material, to stain the nuclei.
278 Micronuclei are defined as small round structures less than one third the diameter and in the
279 same optical plan as the main nucleus, with a boundary distinct from the nuclear boundary.
280 Tissue preparation for the collection of cells was the same as that used for the neutral red
281 retention assay. The rinsed cells were fixed in Carnoy's solution (methanol:glacial acetic acid
282 3:1) and stored at 4°C until counted. A drop of the fixed cell suspension was placed on a slide
283 and air dried. A drop of the DAPI (# 32670 Sigma, USA) working solution was added to
284 each slide and a cover-slip added. Slides were incubated in the dark for 5 minutes and
285 observed under an inverted epifluorescent microscope (Nikon, Eclipse TE 300, Japan) with
286 the appropriate filter for DAPI, excitation wavelength 350 nm magnification 40x. Two slides
287 per sample were counted with 1000 cells per slide scored as micronuclei present or absent.

288 **2.7 Statistical analyses**

289 A Mixed Linear Model analysis of variance (ANOVA) (SPSS v 14.0) was used to
290 simultaneously analyse the effects of time (day) and treatment (cadmium exposure
291 concentration) on organism tissue cadmium accumulation. A Mixed Linear Model ANOVA
292 was used to analyse the effects of treatment (cadmium exposure concentration) on the effect
293 measurement variables antioxidant capacity, total glutathione, GSH:GSSG ratio, glutathione
294 peroxidase, lipid peroxidation, lysosomal stability and micronuclei frequency (Supplementary
295 Tables 1 – 3). Regressions of sediment cadmium and mean tissue cadmium concentrations
296 and means of effects variables antioxidant capacity, lipid peroxidation, lysosomal stability
297 and micronuclei frequency were calculated using EXCEL™ v 2003.

298 **3 Results**

299 **3.1 Cadmium accumulation**

300 Cadmium accumulation by *Tellina deltoidalis* was dependent on time and sediment cadmium
301 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

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

378 **4.1.2 Subcellular cadmium distribution**

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

402 exposure, particularly in the mitochondria which is also seen in the mitochondria and
403 lysosomal fractions (Supplementary Figure 1). This suggests that both organelles are
404 responding to the accumulation of cadmium within the cells. The majority of cadmium in the
405 biologically active metal fractions of the cadmium exposed *T. deltoidalis* was in the
406 mitochondrial fraction (Figure 3; Table 2). Cadmium burdens in the freshwater bivalve
407 *Pyganodon grandis* were also found to be higher in the mitochondria than the
408 lysosome+microsome fraction with increased cadmium exposure (Bonneris *et al.*, 2005). Li
409 *et al.* (2003) showed that cadmium could directly lead to dysfunction of mitochondria
410 including inhibition of respiration, loss of transmembrane potential and the release of
411 cytochrome *c* oxidase. Mitochondrial cadmium in *T. deltoidalis* from the 10 µg/g and 50 µg/g
412 exposures increased to 2000 and 7200, respectively, times that of the control organisms
413 (Table 2). Extensive Cd²⁺ accumulation in mitochondria mediated by Ca²⁺ voltage dependant
414 channels has previously been reported by Li *et al.* (2000; 2003). The increased mitochondrial
415 cadmium observed in *T. deltoidalis* is also in agreement with studies on cadmium subcellular
416 distribution following increased exposure in oysters *Crassostrea virginica* (Sokolova *et al.*,
417 2005). Mitochondrial function was also found to be highly sensitive to cadmium at
418 physiological and environmentally relevant low concentrations in oysters (Sokolova, 2004).
419 Cadmium has been shown to have a high affinity for mitochondria, it is capable of inhibiting
420 respiration and oxidative phosphorylation and interfering with the 1-hydroxylation of vitamin
421 D (Fowler and Mahaffey, 1978). Lysosomes are involved in numerous functions including,
422 nutrition, tissue repair, cellular defence, turnover of membranes, organelles and proteins as
423 well as in the sequestration and metabolism of toxins, such as organic xenobiotics and metals
424 and are an important target organelle for metal toxicity (Ringwood *et al.*, 1998). While the
425 percentage of cadmium in the lysosomal+microsomal fraction of the 10 µg/g and 50 µg/g
426 cadmium exposed organisms was slightly less than half that of the controls (Table 2) the
427 cadmium associated with them was 46 and 100, respectively, times greater than that of the
428 controls. Lysosomes have been identified as the metal cation homeostasis mechanism which
429 sits between soluble binding ligands such as metallothionein and the formation of insoluble
430 precipitates such as metal rich granules (Viarengo and Nott, 1993). While lysosomal
431 cadmium uptake may reflect sequestration and detoxification of the metal, it can also lead to
432 adverse effects when the handling capacity of the lysosomes is overwhelmed (Sokolova *et al.*,
433 2005; Viarengo *et al.*, 1987). This fraction also contained the microsomal component of the
434 cell. Since this includes fragmented endoplasmic reticulum, which is generally responsible
435 for protein synthesis and transport; if cadmium in this fraction was associated with
436 microsomes rather than lysosomes then this could be indicative of toxicity (Bonneris *et al.*,

437 2005). Similar amounts of cadmium were also associated with the heat sensitive proteins
438 (HSP) of the 10 µg/g and 50 µg/g cadmium exposed organisms compared to the controls as
439 those found in the lysosome+microsome fraction (Table 2). The HSP fraction contains
440 enzymes, high and low molecular weight proteins and other target molecules which are
441 sensitive to metals (Wallace *et al.*, 2003). The increased binding of cadmium to this fraction
442 may therefore have implications for toxicity. The percentage of cadmium within the
443 nuclei+cellular debris fraction of 10 µg/g cadmium exposed organisms was double, and in the
444 50 µg/g two and a half times the control cadmium. This fraction was not included in the
445 biologically active and detoxified cadmium compartments as it contains tissue fragments, cell
446 membranes and other cellular components of unknown consequence in terms of function, as
447 well as the nucleic material (Wallace *et al.*, 2003). Binding of cadmium to cell membranes
448 could result in toxicity, however, if the cadmium in this fraction was bound to less sensitive
449 fractions within the cellular debris this could reduce binding to more sensitive cell
450 components (Lucu and Obersnel, 1996). Metallothionein has been shown to be present in the
451 nucleus of gill, digestive gland, gonad and posterior adductor muscle of *Mytilus edulis* in
452 equal or lower concentrations than the corresponding tissue cytosol (del Castillo and
453 Robinson, 2008). If this is also the case for *T. deltoidalis*, cadmium in the nuclei+cellular
454 debris fraction may be associated with nuclear metallothionein and so detoxified. The
455 effectiveness of cadmium detoxification relies on binding to metallothioneins transfer across
456 cell membranes, compartmentalisation within lysosomes and the formation of metal rich
457 granules. The presence of increased cadmium associated with these fractions shows that these
458 processes were occurring, however, increases in cadmium within the mitochondrial fraction
459 and to a lesser extent the heat sensitive protein fraction indicates the detoxification and
460 storage capacity was exceeded suggesting the potential for cadmium toxicity.

461 **4.2 Enzymatic biomarkers – oxidative enzymes**

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

471 *et al.*, 2004); *Modiolus modiolus*: cadmium (Dovzhenko *et al.*, 2005). An antioxidant
472 capacity reduction in cadmium exposed *T. deltoidalis* (Figure 4A) indicates a breakdown in
473 the reactive oxygen species detoxification pathway with the potential for higher order effects.
474 An investigation of glutathione cycling and the glutathione peroxidase enzyme (GPx) which
475 are involved in the reduction of oxyradicals was undertaken to further investigate the mode of
476 action of accumulated metal on the ROS reduction pathway of *T. deltoidalis*.
477 Glutathione peroxidase activity and the total glutathione were reduced in the cadmium treated
478 organisms compared to the control organisms (Figure 4B) but the reduction was not
479 significant (Supplementary Table 3b). When the ratio of reduced glutathione (GSH) to
480 oxidised glutathione (GSSG) is considered, a significant reduction was seen in the cadmium
481 exposed organisms compared to the controls (Figure 4B; Supplementary Table 3b). Reduced
482 glutathione is a tripeptide of glutamine, cysteine and glycine which provides reducing
483 equivalents for the GPx catalysed reduction of hydrogen peroxide to water and the respective
484 alcohol. During this process GSH becomes oxidised glutathione which is recycled into GSH
485 by glutathione reductase and NADPH (Tietze, 1969). In healthy cells the ratio is usually
486 high, however, when cells are exposed to increased oxidative stress the ratio of GSH to GSSG
487 decreases as a consequence of GSSG accumulation (Wataha *et al.*, 2000). The responsiveness
488 of antioxidants to specific toxicants is difficult to predict and a high degree of variability has
489 been reported in early work on marine bivalves relating to the class of chemical, exposure,
490 organism type and phase in biological cycle (Regoli and Principato, 1995; Ribera *et al.*, 1989;
491 Viarengo *et al.*, 1991; Viarengo *et al.*, 1989). The use of single antioxidant enzyme
492 measurements to interpret exposure effects, therefore, may give inconclusive or contradictory
493 results. The total glutathione concentration has been investigated in marine bivalves exposed
494 to metal contamination in numerous studies and has been reported as both increasing (Camus
495 *et al.*, 2004; Frenzilli *et al.*, 2004; Regoli *et al.*, 2004; Regoli *et al.*, 1998a) and decreasing (de
496 Almeida *et al.*, 2004; Regoli, 1998; Regoli *et al.*, 1998b; Regoli and Principato, 1995),
497 indicating that glutathione may be up-regulated in response to metal contamination but at
498 different concentrations and varying physiochemical conditions may be depleted when the
499 system is overwhelmed. The suppression of total glutathione concentrations in *T. deltoidalis*
500 by cadmium (Figure 4B) may be related to the high affinity of this molecule for metals,
501 resulting in either a stable coordination complex or the oxidation of GSH to GSSG which may
502 be subsequently excreted from the cell more rapidly than it can be reconverted to the reduced
503 form by glutathione reductase (Meister, 1989). GSH cadmium complexes are not detectable
504 with the methods used here. Despite the tendency for excess GSSG to be actively excreted
505 from cells the decreased ratio of reduced to oxidised glutathione detected in the cadmium

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

518 **4.3 Oxidative damage biomarker – thiobarbituric acid reactive substances**

519 Thiobarbituric acid reactive substances TBARS are a measure of lipid peroxidation which is a
520 widely recognised consequence of excess oxyradical production (Winston and Di Giulio,
521 1991). Like the oxygen reduction system the lipid production process is a complex sequence
522 of biochemical reactions, broadly defined as oxidative deterioration of polyunsaturated fatty
523 acids, which results in the production of highly reactive and unstable lipid radicals and a
524 variety of lipid degradation products, the most abundant of which is malondialdehyde, which
525 can alter the structure of cell membranes (Viarengo, 1989). The process of lipid peroxidation
526 destabilises cell membranes which can lead to loss of lysosomal integrity and the leaking of
527 the lysosomal contents into the cytoplasm (Winston *et al.*, 1991). The TBARS concentration
528 was increased in *T. deltoidalis* from both cadmium treatments compared to the control
529 organisms, however, the difference was only significant in the 50 µg/g cadmium exposed
530 organisms (Figure 5A; Supplementary Table 3b). Other cadmium exposed marine bivalves
531 have also shown reduced antioxidant enzyme activity and a consequent increase in lipid
532 peroxidation (Chelomin *et al.*, 2005; Company *et al.*, 2004; de Almeida *et al.*, 2004; Legeay
533 *et al.*, 2005). The TBARS concentration was highly negatively correlated with the
534 antioxidant capacity, indicating that the progressive reduction in the capacity to reduce ROS
535 and their subsequent increase directly influenced the build up of lipid peroxidation by-
536 products.

537 **4.4 Cellular biomarker – lysosomal stability**

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

540 The lysosomal interior is more acidic $\text{pH} \approx 4.8$ than the cytosol $\text{pH} \approx 7.2$ and it is enclosed in
541 a single membrane which stabilises the low pH by pumping protons from the cytosol via
542 proton pumps and ion channels. Metals can also enter lysosomes via these channels, or more
543 usually as protein complexes with metallothioneins, and it is thought that from here they are
544 then formed into granules for storage or excretion (Marigómez *et al.*, 2002).
545 Metal accumulation in the lysosomes can induce lipid peroxidation through redox cycling or
546 by direct reaction with cellular molecules to generate ROS. This can destabilise the
547 lysosomal membrane causing the contents to leak out into the cytosol thereby reducing the
548 cells capacity to remove waste which will ultimately lead to cell death (Viarengo *et al.*, 1987).
549 The 10 $\mu\text{g/g}$ cadmium exposed *T. deltoidalis* had 33% and the 50 $\mu\text{g/g}$ 43% lysosomal
550 destabilisation. This was significantly higher than the control organisms which had less than
551 10% of lysosomes destabilised (Figure 5B; Supplementary Table 3b). Based on lysosomal
552 destabilisation criteria developed by, Ringwood *et al.* (2003) for *Crassostrea virginica* the
553 cadmium exposed *T. deltoidalis* score in the concern / stressed range.
554 Cadmium accumulation has been linked to lysosomal destabilisation in other marine bivalves:
555 mussels *Mytilus galloprovincialis* (Regoli *et al.*, 2004; Viarengo and Nott, 1993) and oysters
556 *Crassostrea virginica* Ringwood *et al.* (2004; 2002). The lysosomal destabilisation was
557 strongly negatively correlated with antioxidant capacity and positively correlated with
558 TBARS. This suggests that increased cadmium accumulation in metal sensitive tissue
559 fractions initiated a reduction in the capacity to reduce ROS which may have both directly
560 damaged lysosomal membrane but also induced lipid peroxidation which also induced
561 lysosomal membrane destabilisation.

562 **4.5 Genotoxic biomarker – micronuclei frequency**

563 Micronuclei are small, intracytoplasmic masses of chromatin resulting from chromosomal
564 breakage or aneuploidy during cell division. As an index of chromosomal damage the
565 micronucleus test is based on the enumeration of downstream aberrations after DNA damage
566 and gives a time-integrated response to toxic exposure. The micronucleus test is a fast and
567 sensitive test to detect genomic damage due to both clastogenic effects and alterations to the
568 mitotic spindle (Migliore *et al.*, 1987). It has been used in bivalves to examine the
569 genotoxicity of a range of chemicals including metals (Bolognesi *et al.*, 2004; Burgeot *et al.*,
570 1996; Scarpato *et al.*, 1990; Williams and Metcalfe, 1992). The 10 $\mu\text{g/g}$ cadmium exposed *T.*
571 *deltoidalis* had significantly more micronuclei than the control organisms and the 50 $\mu\text{g/g}$
572 cadmium exposed *T. deltoidalis* had significantly more micronuclei than both the control and
573 the 10 $\mu\text{g/g}$ cadmium exposed organisms (Figure 5C; Supplementary Table 3b). Increased

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

588 5 Conclusions

589 Cadmium exposed *T. deltoidalis* accumulated cadmium over 28 days and reached equilibrium
590 tissue concentrations which were equal to that of the sediment cadmium exposure
591 concentrations. Approximately 50% of accumulated cadmium was detoxified. The
592 percentage converted to metal rich granules increased from 23% to 60% with increased
593 cadmium exposure. The majority of biologically active cadmium in exposed organisms was
594 in the mitochondrial fraction which was significantly higher than the control organisms. This
595 was associated with an increase in the activity of the mitochondrial cytochrome *c* oxidase
596 enzyme. The antioxidant capacity and ratio of GSH:GSSG of cadmium exposed *T.*
597 *deltoidalis* was significantly reduced compared to control organisms. The impairment of the
598 oxidative system initiated significant cellular damage. Lipid peroxidation increased,
599 contributing to significant lysosomal destabilisation and increased frequency of micronuclei.
600 The significant exposure – dose – response relationships for cadmium established in this
601 study indicate that sediment cadmium at these concentrations has the potential to lead to
602 increased biologically active cadmium burdens and impairment of individual *T. deltoidalis* at
603 a cellular and subcellular level. This has implications for higher order effects which may
604 impact on population viability in the long term. *T. deltoidalis* has good attributes as a
605 bioindicator, being hardy, abundant, easy to work with in the laboratory and a net
606 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

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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 ($\mu\text{g/g}$ dry mass) of *T. deltoidalis* exposed to
845 cadmium spiked sediments of 0 (control), Cd 10 and $50\mu\text{g/g}$ dry mass. Mean \pm 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 and 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 $50\mu\text{g/g}$ dry mass . Mean \pm SE, $n =$
857 12 for all except GPx (glutathione peroxidase) $n = 6$. 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 $50\mu\text{g/g}$ dry mass. Mean \pm
864 SE $n = 12$. Different letters indicate significant differences between means (Bonferroni test;
865 $p < 0.05$).

Figure 1

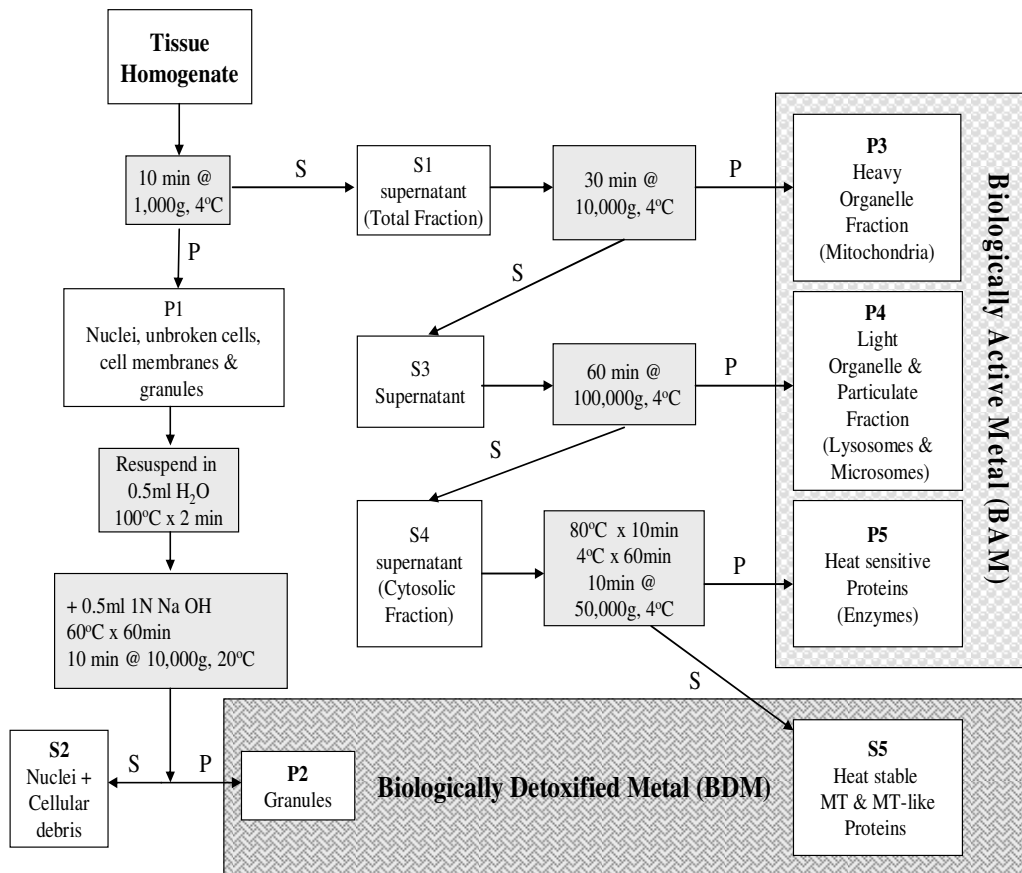


Figure 2

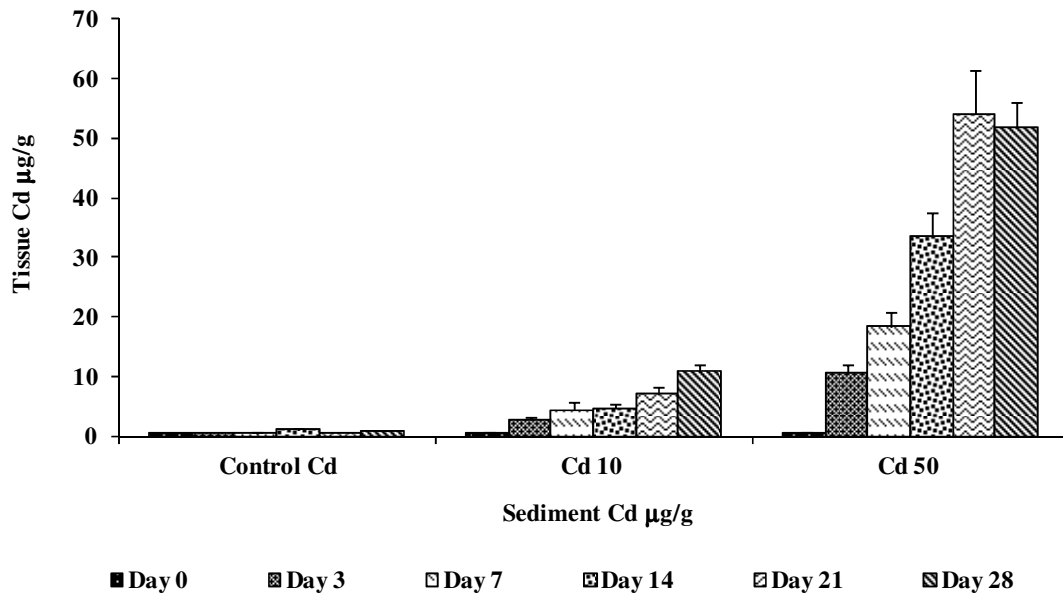


Figure 3

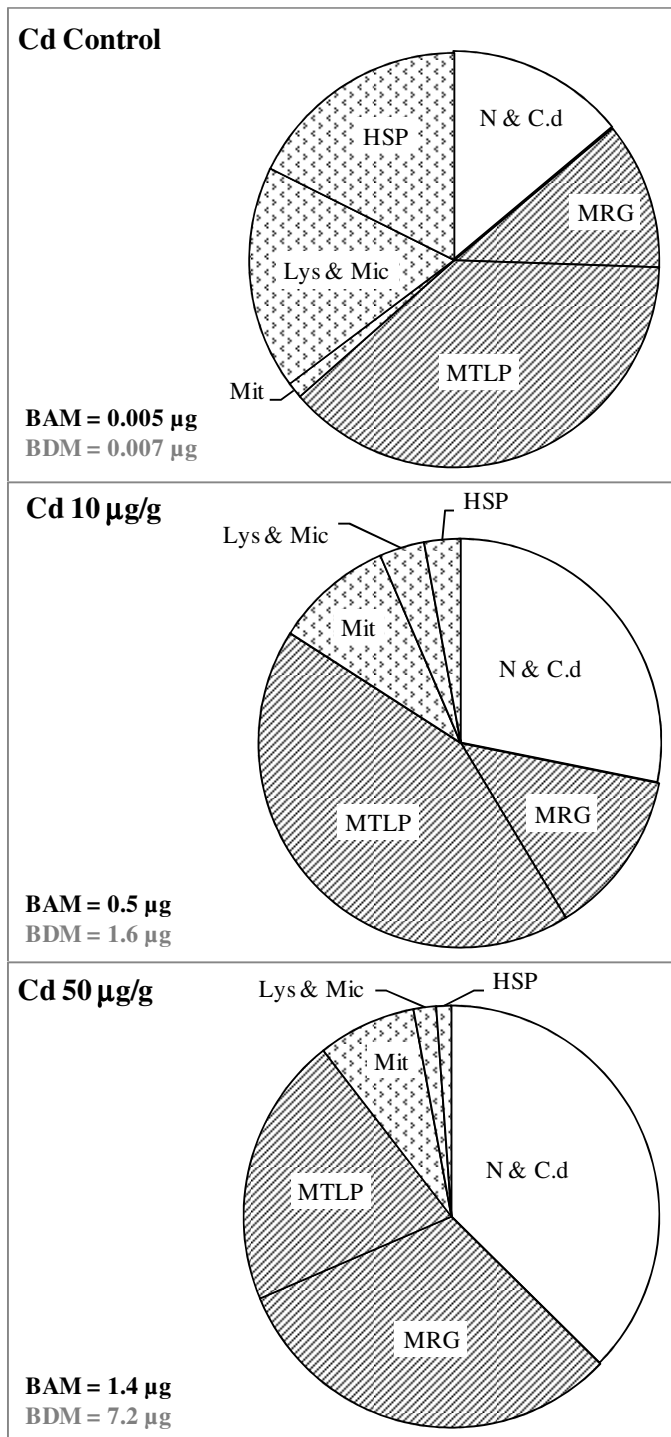


Figure 4

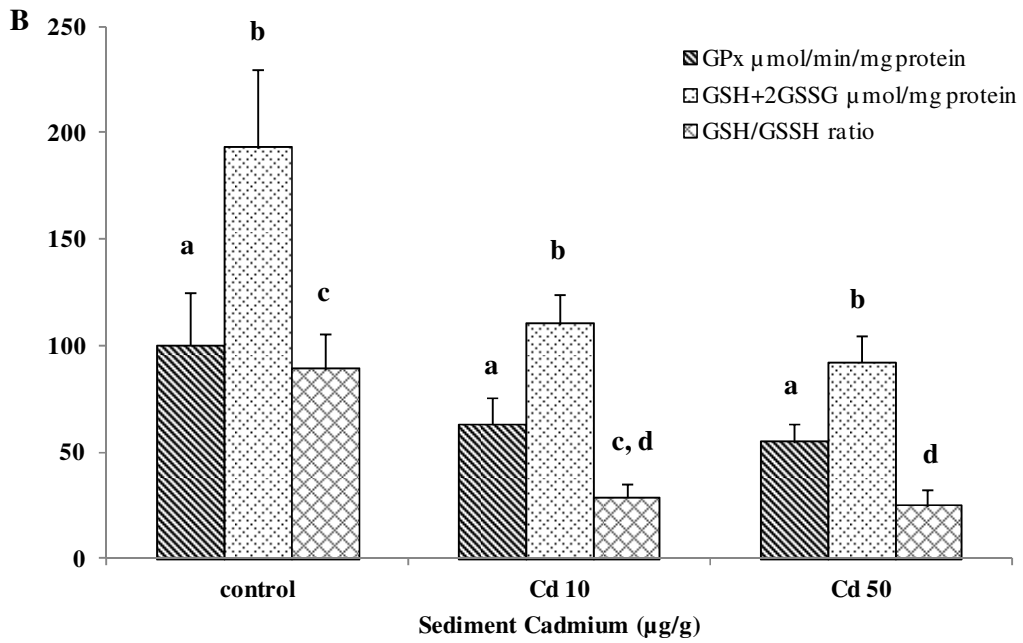
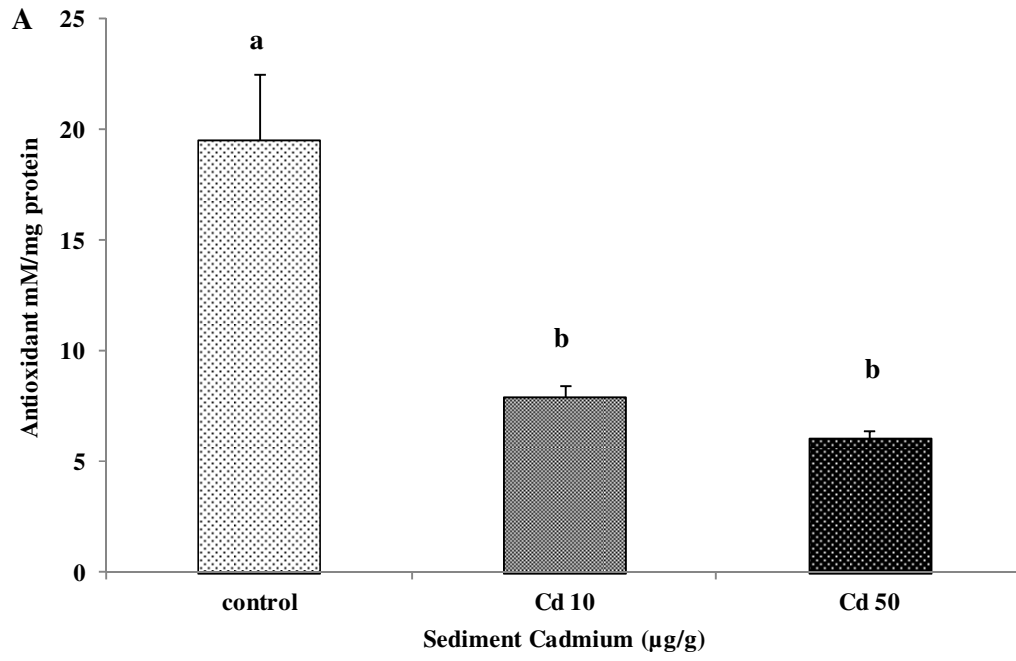


Figure 5

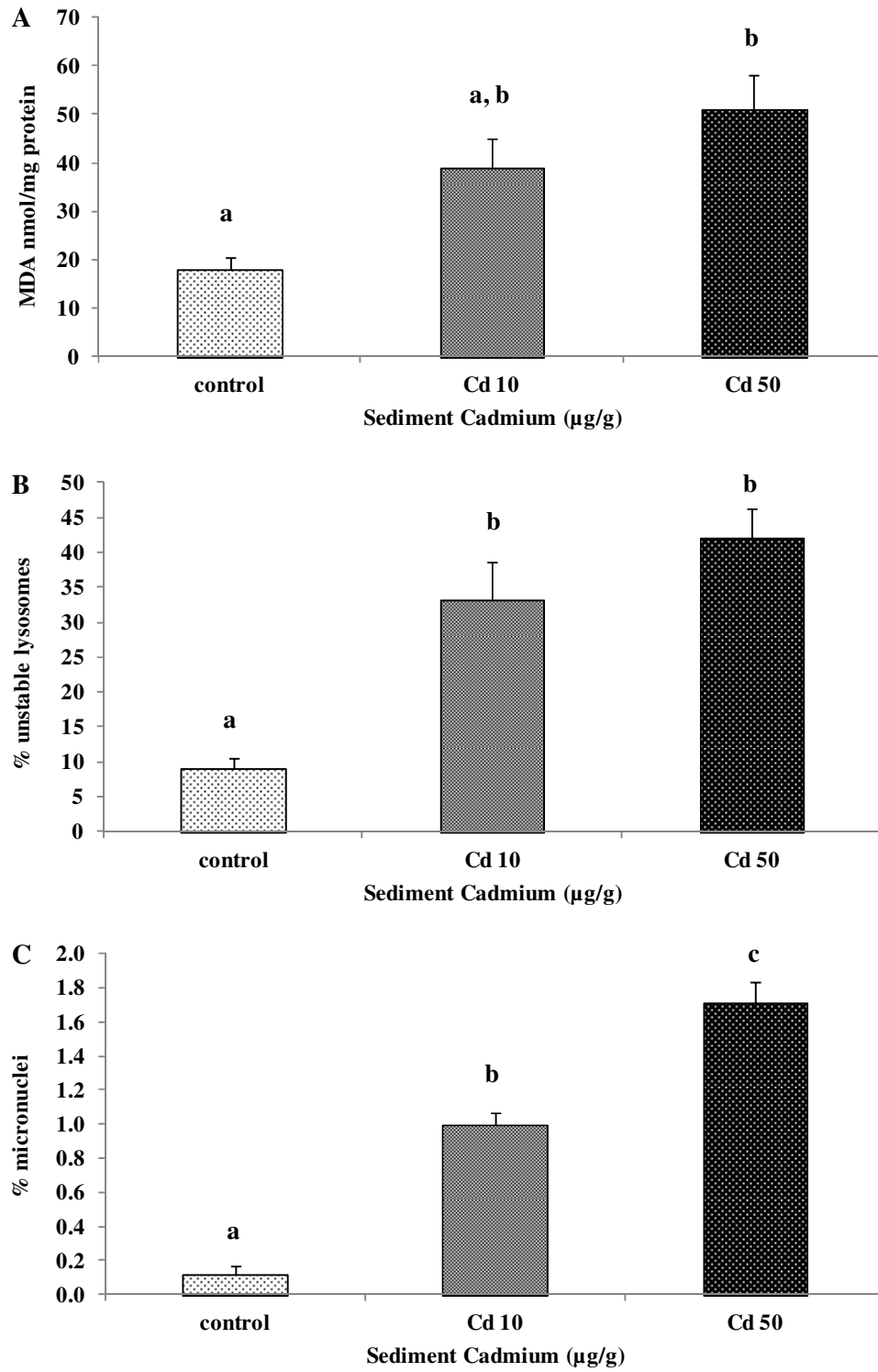


Table 1: Total cadmium concentrations (μg wet mass) in whole tissue and subcellular fractions with the percentage of total cadmium recovered in all fractions of *T. deltoidalis* after 28 days exposure to cadmium spiked sediments. Cadmium subcellular concentrations (μg wet mass) and percentage distribution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

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

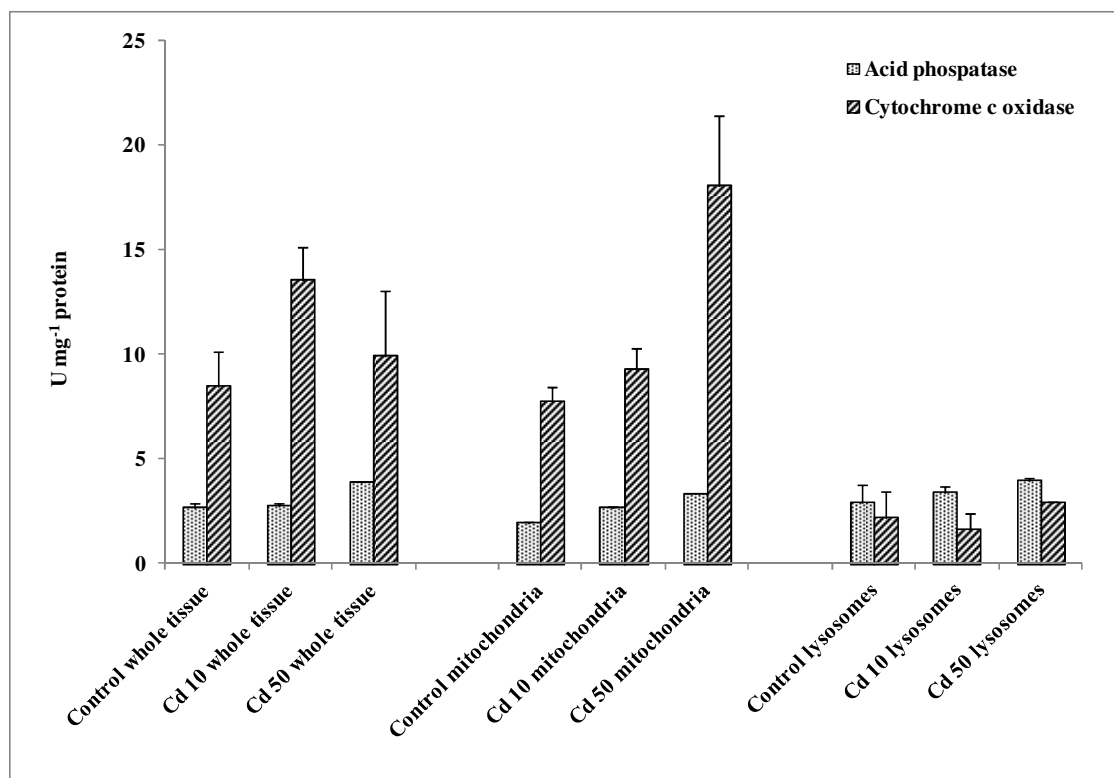
Mean \pm SD, $n = 2$

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 ($\mu\text{g/g}$)		
	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 $\mu\text{g/g}$ dry mass after 28 days. Mean \pm 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.

Source	Cadmium		
	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 log₁₀ 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 log₁₀ 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.

Treatments	Effects Measures						
	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 $p > 0.05$