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

2   **3. Selenium spiked sediments**

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15

Abstract

17 The metalloid selenium is an essential element which at slightly elevated concentrations is

18 toxic and mutagenic. In Australia the burning of coal for power generation releases selenium

19 into estuarine environments where it accumulates in sediments. The relationship between

20 selenium exposure, dose and response was investigated in the deposit feeding, benthic, marine

21 bivalve *Tellina deltoidalis*. Bivalves were exposed in microcosms for 28 days to individual

22 selenium spiked sediments, 0, 5 and 20 µg/g dry mass. *T. deltoidalis* accumulated selenium

23 from spiked sediment but not in proportion to the sediment selenium concentrations. The

24 majority of recovered subcellular selenium was associated with the nuclei and cellular debris

25 fraction, probably as protein bound selenium associated with plasma and selenium bound

26 directly to cell walls. Selenium exposed organisms had increased biologically detoxified

27 selenium burdens which were associated with both granule and metallothionein like protein

28 fractions, indicating selenium detoxification. Half of the biologically active selenium was

29 associated with the mitochondrial fraction with up to 4 fold increases in selenium in exposed

30 organisms. Selenium exposed *T. deltoidalis* had significantly reduced GSH:GSSG ratios

31 indicating a build-up of oxidised glutathione. Total antioxidant capacity of selenium exposed

32 *T. deltoidalis* was significantly reduced which corresponded with increased lipid peroxidation,

33 lysosomal destabilisation and micronuclei frequency. Clear exposure – dose – response

34 relationships have been demonstrated for *T. deltoidalis* exposed to selenium spiked sediments,

35 supporting its suitability for use in selenium toxicity tests using sub-lethal endpoints.

36

37 **Keywords:** Biomarkers, subcellular selenium, oxidative stress, lysosomes, lipid peroxidation,

38 micronuclei, bivalve.

39

40 1 **Introduction**

41 Selenium is an essential element within a fairly narrow concentration range, above which it is

42 both mutagenic and toxic and below which selenium deficiency occurs (Hodson, 1988;

43 Hoffman, 2002). Selenium studies which examined selenium dietary requirements, in the

44 trout *Salmo gairdneri* showed that plasma glutathione peroxidase homeostasis was maintained

45 at intakes of up to 1.25 µg/g dry food and toxicity occurred at 13 µg/g dry food. The authors

46 speculated that dietary concentrations in excess of 3 µg/g in dry food over long time periods

47 might be toxic. (Hilton et al., 1980; Hodson et al., 1980; Hodson and Hilton, 1983; Hicks et

48 al., 1984). Eisler (2000) and Puls (1994) have reported similar responses in other fish species,

49 birds and mammals in relation to selenium dietary requirements, deficiency, and toxicity.

50 Selenium is released into aquatic environments through industrial activity such as metal

51 smelting, overflow and leaching from ash dams and stack emissions associated with coal fired

52 power stations and through sewage effluent (Davies and Linkson, 1991; Peters et al., 1999a).

53 Sediments contain most of the total estuarine selenium inventory because of sorption and/or

54 precipitation mechanisms (Peters et al., 1999a). Selenium biotransformation,

55 bioaccumulation, and transfer through both sediment and water column foodwebs constitute

56 major biogeochemical pathways in aquatic ecosystems (Lemly, 1999; Fan et al., 2002;

57 Hamilton, 2004; Luoma and Rainbow, 2008; Maher et al., 2010). To acquire sufficient

58 essential elements from environments with low ambient concentrations, aquatic organisms

59 have evolved highly efficient uptake mechanisms, coupled with detoxification storage and

60 excretion strategies (Phillips and Rainbow, 1989). Selenium accumulation by sediment

61 dwelling deposit feeding bivalves may be from the interstitial water, sediment ingestion or

62 from food (Luoma and Rainbow, 2005). The route of uptake may influence the organism's

63 metal handling and therefore its toxicity (Rainbow, 2007). Physiological effects and toxicity

64 of metals strongly depend on their intracellular localisation and binding to organelles and

65 ligands (Sokolova et al., 2005) and selenium appears to be bound and incorporated differently

66 according to the selenium species (Ewan, 1989; Burk, 1991; Hortensia et al., 2006).

67 To understand the fate and effects of such toxicants in aquatic environments the causal

68 relationships between contaminant exposure, internal dose and associated biological effects

69 need to be established (Widdows and Donkin, 1992; Adams et al., 2011). The evaluation of

70 contaminant exposure, uptake and ecotoxicological effects is now an essential component of

71 sediment quality assessment in Australia (Simpson et al., 2005) and toxicity data for local

72 species along with suitable routine test protocols is necessary to develop relevant local

73 exposure dose response toxicity guidelines. The current developments in ecotoxicological

74 assessment are moving to the evaluation of sub lethal endpoints for determining toxicant

75 guideline exposure concentrations. To this end the development of biomarkers of exposure

76 and effect for application in environmental assessment have been progressively developed and

77 refined for a range of toxicants and aquatic species (Cajaraville et al., 2000; Adams, 2001;

78 van der Oost et al., 2003; Galloway et al., 2004; Moore et al., 2004; Farmer, 2006; Batley et

79 al., 2007; Damiens et al., 2007; Hagger et al., 2009; Taylor and Maher, 2010). Biomarker

80 measurements can provide evidence that organisms have been exposed to contaminants at

81 levels that exceed their detoxification and repair capacity establishing links between toxicant

82 exposure and ecologically relevant effects (Koeman et al., 1993). Proteins contain the

83   majority of selenium in organisms and of the known selenoproteins, cellular and plasma

84   glutathione peroxidase, which is involved in redox metabolism, has the highest selenium

85   content (Burk, 1991; Fan et al., 2002). The oxidative system has been shown to be sensitive

86   to selenium through perturbations in the glutathione cycle (Hoffman, 2002). Lysosomes are

87   involved in contaminant sequestration and are also susceptible to oxidative damage

88   (Viarengo, 1989; Winston et al., 1996; Ringwood et al., 2002), while the frequency of

89   micronuclei occurrence is an effective measure of DNA damage (Burgeot et al., 1996;

90   Bolognesi et al., 2004).

91   *Tellina deltoidalis* is a sediment dwelling bivalve which is widely distributed in coastal

92   estuaries around Australia where it lives buried in the sediments at a depth several times the

93   shell length, of between 15 - 25 mm, and extends its siphons to the sediment surface to feed

94   (Beesley et al., 1998). It satisfies most of the basic requirements to be an effective biomonitor

95   being hardy, representative of the area of interest and an accumulator of bioavailable metals

96   (Phillips, 1990; Phillips and Rainbow, 1994). The suitability of *T. deltoidalis* for use in whole

97   sediment toxicity tests has been investigated by King et al. (2004; 2005; 2010) who found

98   they were tolerant of a wide range of sediment types and salinities and easy to handle in a

99   laboratory setting, while being sensitive to metal contamination. A protocol for the use of *T.*

100   *deltoidalis* in whole-sediment acute toxicity tests has been included in the Australian

101   Handbook for Sediment Quality Assessment (Simpson et al., 2005).

102   The purpose of this study was to examine the exposure - dose - response relationship to

103   selenium in *T. deltoidalis* using 28 day sediment bioaccumulation tests (USEPA, 2000;

104   ASTM-E1688-10, 2010) to develop useful biomarkers of effect, and further evaluate their

105   potential for sediment toxicity testing in Australia using sublethal endpoints. There are no

106   Australian sediment quality guideline concentrations for selenium so the exposure

107   concentrations, 5 and 20 μg/g, chosen where based on those previously measured in

108   contaminated Australian estuarine sediments (Peters et al., 1999a; Roach, 2005). Internal

109   selenium exposure was measured in whole tissues, and subcellular tissue fractionation used to

110   determine the active and detoxified selenium. Biomarker measurements of oxidative stress

111   included total antioxidant scavenging capacity of cells, total glutathione concentrations, the

112   ratio of reduced to oxidised glutathione, glutathione peroxidise and the extent of lipid

113   peroxidation. Cellular damage was assessed using a lysosomal destabilisation assay and

114   DNA damage through the presence of micronuclei. Measurement of enzymatic biomarkers in

115   the glutathione cycle along with the cellular and genotoxic biomarkers of lysosomal

116   membrane integrity and micronuclei occurrence provides a weight of evidence approach for

117 selenium toxicity at the individual organism level which may indicate the potential for
118 population level effects.

119 2   **Materials and Methods**

120 *2.1   Organism and sediment collection*
121 Sediments were collected from a NSW Department of Environmental and Climate Change
122 reference site in Durras Lake NSW, and stored at 4°C until use. *Tellina. deltoidalis* of 15 – 20
123 mm in size were collected from Durras Lake and Lake Tabourie, NSW in July 2005 and
124 January 2006 and placed in coolers with sediment and water from the collection sites for
125 transportation. Organisms were maintained for a maximum of two weeks at 22°C in
126 uncontaminated sediments, depth 15 cm, in glass aquaria with filtration and aeration to allow
127 acclimation before experimentation. Overlying water used in aquaria was collected from
128 coastal waters near Murramurrang National Park, NSW and adjusted from 35‰ to 28‰ with
129 deionised water to match the salinity of the estuarine water from which organisms were
130 collected.
131

132 *2.2   Sediment selenium spiking*
133 Sediments were sieved through a 2 mm stainless steel sieve to remove large pieces of organic
134 matter and organisms prior to the addition of selenium. Sub samples of the collected
135 sediments were measured for moisture content and grain size. To ensure the sediment matrix
136 was suitable for organism burrowing and feeding, sediment was mixed with clean beach sand
137 so that the 63 μm fraction was not greater than 20% mass/mass. To ensure added selenium
138 was rapidly adsorbed and strongly bound to the sediment particles a method developed by
139 (Simpson et al., 2004) for producing metal spiked marine sediments, was followed. Wet
140 sediment was added to mixing containers. Na_2SeO_3, (AR grade Sigma-Aldrich) was added to
141 concentrations of 5 and 20 mg/kg dry mass of sediment. All containers were topped up with
142 clean deoxygenated sea water and the final mixture was completely deoxygenated by
143 bubbling with nitrogen for 2 hours. Head spaces of containers were filled with nitrogen prior
144 to sealing. Any pH adjustments were made immediately after the addition of the selenium
145 using 1M NaOH, (AR grade BDH), prepared in seawater, checked weekly and maintained at
146 7 - 8.2. Sediments were mixed on a Cell-production Roller Apparatus (Belco, USA) for
147 several hours each day. Sediments were maintained at room temperature 22 - 25°C. The time
148 required for equilibration of added metals will be affected by the sediment properties,
149 equilibration pH and the concentration and properties of the metal (Simpson et al., 2004).

150   To determine when the added selenium was completely bound to sediment particles, pore

151   waters were collected and acidified to 1% v/v with nitric acid (AristaR, BDH, Australia) and

152   selenium was measured using an ELAN$^®$ 6000 ICP-MS (PerkinElmer SCIEX, USA). Once

153   pore water selenium concentrations had fallen below instrument detection limits 0.001 µg/l

154   the sediment was ready for use. Time to full absorption was 4 to 6 weeks. Unspiked

155   sediments were treated in the same way and used for control treatments. Sediment selenium

156   concentrations were measured by ICP-MS after digestion of 0.2 g of lyophilised sediment in 3

157   ml of nitric acid (AristaR, BDH, Australia) in polyethylene 50 ml centrifuge tubes for 60

158   minutes at 115°C (Maher et al., 2003). Selenium in NRCC Certified Reference Materials,

159   BCSS-1 marine sediment measured along with samples was 0.41 ± 0.1 µg/g ($n = 10$) and in

160   agreement with certified values 0.43 ± 0.06 µg/g. Sediment selenium concentrations were

161   measured prior to and at the end of the 28 day exposure period. Pre exposure concentrations

162   were < 0.001, 5.00 ± 0.05 and 20 ± 1 µg/g and post exposure were < 0.001, 5.00 ± 0.15 and

163   19 ± 2 µg/g.

164

165   *2.3   Microcosm Experiment Design*

166   Procedures for conducting the exposures were adapted from the test method for conducting 28

167   day sediment bioaccumulation tests (Ingersoll et al., 2000). Spiked and control sediments

168   (500 g wet mass) were placed in each of three replicate 770 ml polypropylene containers

169   (Chanrol # 01C30, Australia) per treatment. The containers were filled with fresh seawater

170   adjusted to a salinity of 28‰. Containers were placed in random order on a tray in an

171   incubator set at 22°C with a day / night light cycle of 14 / 10 hours to reflect spring / summer

172   conditions. Aeration was introduced and the treatments were left for 24 hours to allow them

173   settle and the temperature to equilibrate. Fifteen *T. deltoidalis* were then introduced to each

174   treatment container. Organisms were not given supplementary food and surface water was

175   changed weekly during the 28 day exposure period. Aquaria were continually aerated using

176   an air pump with valves on each line and fine tubing to each container to regulate air flow so

177   oxygen saturation $\approx 100\%$ were maintained in overlying water of each aquarium but

178   sediments were not agitated. Due to the natural buffering capacity of sea water and associated

179   sediments, pH remained relatively constant at 7.8-8.0 in all aquaria throughout the 28 days of

180   exposure. This is similar to results of other studies of this type (King et al., 2006; Strom et

181   al., 2011). Total tissue selenium bioaccumulation was measured at intervals of 3, 7, 14, 21

182   and 28 days. A day 0 measurement was made using organisms from the acclimation tanks to

183 give the background selenium concentration. All organisms were placed in fresh seawater at
184 salinity 28‰ with no sediment for 24 hours (King et al., 2004; Simpson et al., 2005; Atkinson
185 et al., 2007; King et al., 2010) to allow depuration of ingested sediment particles, prior to
186 selenium analysis. All assays were done on whole tissues of individual organisms.

187 *2.4   Selenium Measurements*

188 **2.4.1   Total selenium**

189 Lyophilised ground tissue ≈ 0.1 g was digested in 1 ml of nitric acid (AristaR BDH,
190 Australia) in polytetra-fluroacetate digestion vessels, in a 630 watt microwave oven (CEM
191 MDS-2000, USA) for 2 min at 630 W, 2 min 0 W, and 45 min at 315 W (Baldwin et al.,
192 1994). Prior to analysis samples were diluted with deionised water to 1% v/v HNO_3, and an
193 ICP-MS mixed 7-element internal standard (EM Science) was added to monitor for variations
194 due to instrument drift and/or matrix effects. Selenium was measured using an ELAN® 6000
195 ICP-MS (PerkinElmer, SCIEX) following the method of Maher et al. (2001). NRCC
196 Certified Reference Material, NIST 1566a oyster tissue and acid blanks were routinely
197 digested and diluted in the same way as the samples and analysed along with them to verify
198 accuracy and precision of selenium analysis. The measured CRM mean selenium value; 2.1 ±
199 0.3 µg/g ($n = 50$) was not significantly different from the certified value 2.21 ± 0.24 µg/g.
200

201 **2.4.2   Subcellular selenium**
202 The subcellular tissue selenium distribution was examined in tissues of day 28 exposed
203 organisms using a procedure adapted from Sokolova et al. (2005) and Wallace et al. (2003).
204 The dissected tissues were placed in polypropylene vials, snap frozen in liquid nitrogen and
205 stored at -80°C until processed. The tissue was thawed and minced on ice with a blade. A
206 sub sample, ≈ 0.1 g wet wt., was taken for total tissue selenium analysis. The remainder, ≈
207 0.5 g wet wt., was homogenised in Ca^{2+} / Mg^{2+} free saline buffer pH 7.35 on ice using an
208 IKA® Labortechnick Ultra-turrax-T25 homogeniser equipped with an S25-UT dispersing tool
209 at 9,500 rpmin^{-1} (Janke & Kunkel, Germany). Homogenised tissue was subjected to
210 differential centrifugation and tissue digestion procedures according to the protocol outlined
211 in Taylor (2009), using an Eppendorf 5804R centrifuge and a Himac CP90WX preparative
212 ultracentrifuge (Hitachi, Japan). The mitochondria, lysosomes-microsomes and heat sensitive
213 protein pellets were grouped as biologically active selenium fractions while the granule and
214 heat stable metallothionein like proteins were grouped as biologically detoxified selenium

215 fractions (Taylor and Maher, 2013). The supernatant from the granule pellet isolation

216 contained the nuclei-cellular debris. To determine the mitochondrial and lysosomal content of

217 the fractions obtained the concentration of enzymes specific for these organelles, cytochrome

218 *c* oxidase and acid phosphatase, respectively, were measured in each of the total tissue,

219 mitochondrial and lysosome-microsome pellets using commercial colorimetric assays

220 (CYTOC-OX1 Sigma-Aldrich, USA and CS0740 Sigma-Aldrich, USA, respectively). This

221 showed that the mitochondrial fraction was enriched with mitochondria and in the lysosome-

222 microsome fraction there was some enrichment of lysosomes compared to the mitochondrial

223 fraction (Supplementary Figure 1). Fractions were acidified to 10% v/v with nitric acid

224 (AristaR BDH, Australia) and placed in a water bath at 80°C for 4 hours. NIST CRM 1566a

225 oyster tissue, buffer and acid blanks were digested and diluted in the same way as the samples

226 and analysed along with them. Analysis of selenium was as previously described above. The

227 measured CRM selenium value 2.25 ± 0.3 µg/g ($n = 5$) were in good agreement with certified

228 value 2.21 ± 0.24 µg/g.

229

230 *2.5   Biomarker Measurements*

231 **2.5.1   Total antioxidant capacity and lipid peroxidation**

232 Tissues were homogenised on ice in a 5 mM potassium phosphate buffer containing 0.9%

233 (w/v) sodium chloride and 0.1% (w/v) glucose, pH 7.4 (1:5 w/v) using a motorised

234 microcentrifuge pellet pestle, sonicated on ice for 15 seconds at 40 V (VibraCell™ Sonics

235 Materials, USA) and centrifuged, in a 5804R centrifuge (Eppendorf, Germany), at 10,000 x g

236 for 15 minutes at 4°C (Cayman, 2011). The supernatant was stored at -80°C until analysis.

237 Total antioxidant capacity was measured using an assay based on the ability of the tissue

238 lysate antioxidant system to inhibit the oxidation of ABTS (2,2'-azino-di-[3-

239 ethylbenzthiazoline sulphonate]) to ABTS $^{\bullet+}$ by metmyoglobin in the presence of hydrogen

240 peroxide. This was compared with the antioxidant capacity of a standard, Trolox (Cayman,

241 2011). Samples were pipetted into a 96 well plate with metmyoglobin and ABTS. The

242 reactions were initiated with a 441 µl solution of hydrogen peroxide. The plate was shaken

243 for 5 minutes at 25°C and the amount of ABTS$^{\bullet+}$ produced was measured by the suppression

244 of absorbance at 750 nm on a BioRad Benchmark Plus microplate spectrophotometer. This is

245 proportional to the final total antioxidant capacity concentration, expressed in millimolar

246 Trolox equivalents. The Thiobarbituric Acid Reactive Substances (TBARS) assay was used

247 to measure lipid peroxidation by measuring the malondialdehyde (MDA) concentration in

248 each tissue lysate. The end product of lipid peroxidation, MDA, forms a 1:2 adduct with

249 TBARS and produces a colour reaction that can be read spectrophotometrically at 532 nm and

250 compared to an MDA standard curve (ZepoMetrix, 2011). The samples were incubated in a

251 solution of sodium dodecyl sulphate, thiobarbituric acid and sodium hydroxide dissolved in

252 acetic acid at $95^{o}C$ for 60 minutes. After cooling on ice and centrifuging at 3000 rpm for 10

253 minutes at room temperature, the colour reaction was measured, on a BioRad Benchmark Plus

254 microplate spectrophotometer at 532 nm.

255 **2.5.2   Reduced:oxidised glutathione ratio and glutathione peroxidase**

256 Tissue lysates were produced by homogenisation on ice in a 50 mM Tris-HCl buffer

257 containing 5 mM EDTA and 1 mM DTT, pH 7.5 (1:5 w/v) using the technique outlined

258 above. A thiol scavenging agent 1-methyl-2-vinyl-pyridium trifluoromethane sulfonate in

259 HCl (Calbiochem®, Merck, Germany) was added to GSSG tissue homogenates to remove

260 GSH, prior to the addition of buffer and production of the final supernatant. The remaining

261 GSSG is then reduced to GSH and determined by the reaction with Ellman's reagent

262 (Calbiochem, 2004). Supernatants were stored at $-80^{o}C$ until analysis of reduced glutathione

263 (GSH), glutathione peroxidise (GPx) and protein (Calbiochem, 2004). The ratio of reduced to

264 oxidised glutathione (GSH:GSSG) was measured using an enzymatic method based on one

265 developed by (Tietze, 1969). The method uses Ellman's reagent 5,5'-dithiobis-2-nitrobenzoic

266 acid (DTNB) which reacts with GSH to form a colour which is detected at 412 nm

267 (Calbiochem®, Merck, Germany). The samples were acidified by the addition of a 5%

268 solution of metaphosphoric acid, vortexed for 15 seconds and centrifuged at 1000 x g for 10

269 minutes at room temperature. The metaphosphoric acid extracts were diluted with a sodium

270 phosphate buffer and mixed at room temperature in 1 ml cuvettes with DTNB and glutathione

271 reductase enzyme at (1:1:1 v/v/v). The reaction was initiated with ß‑nicotinamide adenine

272 dinucleotide phosphate (NADPH) and absorbance read at 412 nm for 3 minutes at intervals of

273 15 seconds on a Unicam Helios Gamma UV‑Vis spectrophotometer (Spectronic, UK).

274 Absorbance rates were calculated and GSH and GSSG concentrations calculated using a 6

275 point GSH calibration curve. A GSSG buffer blank was run for interference correction.

276 Glutathione peroxidise activity (GPx) was measured using a coupled reaction with glutathione

277 reductase (GR) (Cayman Chemicals, USA). The oxidation of NADPH to $NADP^{+}$ is

278 accompanied by a decrease in absorbance at 340 nm. Under conditions where GPx activity is

279 rate limiting, the rate of decrease in the A_{340} is directly proportional to the GPx activity in the

280 sample. Assay buffer 50 mM Tris-HCl, pH 7.6, 5 mM EDTA was added to sample wells of a

281   flat bottomed 96 well plate with a co-substrate mixture NADPH, glutathione and GR (2:1

282   v/v). Samples were added to each well and the reaction was initiated by the addition of

283   cumene hydroperoxide. The plate was shaken briefly and the decrease in absorbance read at

284   340 nm for 5 minutes at intervals of 30 seconds at 25°C on a BioRad Benchmark Plus

285   microplate spectrophotometer. Rates were calculated and samples were compared with a

286   bovine erythrocyte GPx positive control. Buffer blanks run with the samples were used to

287   correct for interferences and GPx activity was calculated using the NADPH extinction

288   coefficient, adjusted for the pathlength of the solution, of 0.00373 μM^{-1}. One unit is defined

289   as the amount of enzyme that will cause oxidation of 1.0 nmol of NADPH to $NADP^{+}$ per

290   minute at 25°C.

291   **2.5.3   Protein**

292   All tissue lysates used for enzymatic assays were analysed for protein concentration and

293   enzyme concentration / activity is expressed as mg^{-1} of protein in the sample. The

294   FluoroProfile™ (Sigma #FP0010, Sigma-Aldrich, USA) protein assay used is a fluorescent

295   assay based on Epiccoconone, a biodegradable natural product. The fluorescence intensity

296   was read at 485 nm excitation and 620 nm emission, on a Luminoskan Ascent Fluorescence

297   Plate Reader (Thermo Electrical Corp., USA). Bovine serum (BSA) calibration curve

298   standards used were made up in sample buffer.

299   *2.6   Cellular and Genotoxic Biomarkers*

300   **2.6.1   Lysosomal Stability**

301   Lysosomal stability was assessed using a method developed by (Ringwood et al., 2003) for

302   oysters. The assay uses neutral red (NR) dye retention to assess the integrity of the lysosomal

303   membrane. Cells incubated in neutral red accumulate the lipophilic dye in the lysosomes.

304   Healthy cells retain the dye in the lysosomes whereas in cells with damaged lysosomal

305   membranes it leaks out into the cytoplasm. Minced tissue was shaken in CMFS buffer pH

306   7.35 salinity 30‰ on a reciprocating shaker at 100 rpm for 20 minutes. Trypsin (T4799

307   Sigma, USA), 325 µl at 1 mg/ml in CMFS buffer, was added and samples shaken for a further

308   20 minutes. Cells were then collected by centrifuging samples through a 20 µm screen at 250

309   - 500 x g at 15°C for 5 - 15 minutes. Cells were incubated in neutral red (Sigma, USA), 0.04

310   mg/ml in CMFS for 1 hour and one hundred cells per slide were counted using a light

311   microscope with 40x lens and scored as stable or unstable, based on dye retained in the

312   lysosomes or present in the cytosol, respectively. Two slides per sample were counted.

2.6.2 Micronuclei Frequency

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

2.7 Statistical analyses

A Mixed Linear Model analysis of variance (ANOVA) (SPSS *v* 14.0) was used to simultaneously analyse the effects of time (day) and treatment (selenium exposure concentration) on organism tissue selenium accumulation. A Mixed Linear Model ANOVA was used to analyse the effects of treatment (selenium 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 selenium and mean tissue selenium concentrations and means of effects variables antioxidant capacity, lipid peroxidation, lysosomal stability and micronuclei frequency were calculated using EXCEL™ *v* 2003 (Supplementary Table 4).

3 Results

3.1 Selenium Accumulation

Selenium tissue concentrations in organisms from both selenium treatments differed significantly from the unexposed and control organisms ($p \le 0.0005$) but not from each other (Figure 1; Supplementary Tables 1 & 2). The highest selenium concentrations for both treatments were at day 21 with a slight but not significant decrease to day 28 (Figure 1). At day 28, selenium tissue concentrations in the both treatments were higher than the selenium

345   spiked sediment with the 5 µg/g treatment organisms having 5 times and the 20 µg/g 1.5 times

346   the sediment selenium concentration (Figure 1). After 28 days of exposure there was a

347   significant positive relationship ($r = 0.46$; $p \leq 0.0001$; $n = 41$) between sediment and organism

348   tissue selenium concentrations (Supplementary Table 4).

349   **3.1.1   Subcellular Tissue Selenium Distribution**

350   Approximately 75% the total selenium from the organisms exposed to sediment

351   concentrations of 5 and 20 µg/g selenium was recovered in the fractions with 82% recovered

352   from the controls (Table 1). Of the recovered selenium, up to 60% was in the nuclei-cell

353   debris fraction in the selenium treatments (Table 1). The biologically active selenium burden

354   was 1.8 and 2.8 times respectively, greater in the 5 and 20 µg/g selenium exposed organisms

355   than the controls (Table 1). The mitochondrial fraction contained the highest percentage of

356   biologically active selenium in the 5 and 20 µg/g selenium exposed organisms, followed by

357   the heat sensitive protein fraction, with only a small percentage in the lysosome-microsome

358   fraction (Figure 2; Table 2). The control organisms also had the lowest percentage of

359   biologically active selenium in the lysosome-microsome fraction while the heat sensitive

360   protein fraction had the highest percentage with slightly less in the mitochondria (Figure 2;

361   Table 2). The majority of biologically detoxified selenium was in the granule fraction

362   accounting for 97% in the control organisms and 66 and 77%, respectively, in the 5 and

363   20µg/g selenium exposed organisms (Figure 2; Table 2). Selenium exposed organisms had a

364   higher concentration of the mitochondrial enzyme cytochrome *c* oxidase than the control

365   organisms (Supplementary Figure 1). At the highest selenium exposure the concentration of

366   the lysosomal enzyme acid phosphatase was increased in both whole tissue and in the

367   lysosome-microsome fraction (Supplementary Figure 1).

368   ***3.2   Biomarkers***

369   The total antioxidant capacity (TAOC) of the selenium exposed organisms was significantly

370   reduced ($p \leq 0.01$; Supplementary Table 3a) compared to that of unexposed organisms,

371   however, the TAOC of each of the selenium treatments were not significantly different to

372   each other (Figure 3A; Supplementary Table 3b). Compared to control organisms the

373   glutathione peroxidase (GPx) activity and total glutathione concentrations were enhanced in

374   the selenium treatments (Figure 3B) but the difference was not significant ($p > 0.05$;

375   Supplementary Table 3b). The ratio of reduced and oxidised glutathione was significantly

376   reduced in selenium exposed organisms compared to that of unexposed organisms ($p \leq 0.01$;

Supplementary Table 3a); however, the ratios of each of the selenium treatments were not significantly different to each other (Figure 3B). Thiobarbituric acid reactive substances were significantly higher in selenium exposed organisms than in unexposed organisms ($p \leq 0.05$; Supplementary Table 3a). The organisms from the selenium exposures both had significantly higher lipid peroxidation (TBARS) than the controls but not from each other (Figure 4A; Supplementary Table 3b). Selenium exposed organisms had significantly more unstable lysosomes and a higher frequency of micronuclei than the control organisms ($p \leq 0.001$; Supplementary Tables 3a). The 20 µg/g selenium exposed organisms had significantly more unstable lysosomes and a higher frequency of micronuclei than both the control and 5 µg/g selenium exposed organisms ($p \leq 0.001$; Supplementary Tables 3b; Figures 5B & C). Regression analysis showed that when selenium exposure reduced the TAOC within cells this corresponded with an increase in the effects measures of TBARS ($r = 0.37$; $p \leq 0.0001$; $n = 36$), lysosomal destabilisation ($r = 0.41$; $p \leq 0.01$; $n = 18$) and micronuclei frequency ($r = 0.51$; $p \leq 0.001$; $n = 18$) (Supplementary Table 4). As TBARS increased there was a corresponding increase in lysosomal destabilisation ($r = 0.32$; $p \leq 0.01$; $n = 18$) and the frequency of micronuclei ($r = 0.35$; $p \leq 0.01$; $n = 18$) (Supplementary Table 4).

4   Discussion

4.1   Selenium Accumulation and Subcellular Distribution

4.1.1   Whole tissue

The variation in tissue selenium concentrations over time in the control organisms was in the order of a few micrograms per gram (Figure 1). As selenium is an essential element some basal concentration is expected (Hamilton, 2004), so this would be indicative of natural variation. Selenium accumulation was rapid during the first three days of exposure in organisms from both selenium treatments, with the 5 µg/g selenium exposed organisms accumulating twice the exposure concentration and the 20 µg/g equalling it in this time (Figure 1). A similar equilibrium tissue concentration for organisms from both treatments appears to have been reached after four weeks (Figure 1). Peters et al. (1999b) found native *T. deltoidalis* exposed to sediment selenium concentrations of 3.4 µg/g in Lake Macquarie NSW accumulated tissue concentrations of 32 µg/g which is a considerably higher exposure to tissue selenium ratio than observed for the *T. deltoidalis* in this experiment. Selenite is taken up rapidly by the aquatic microflora and fauna that is consumed directly by deposit feeding bivalves from the sediment surface or as part of the detritus. In addition selenite

409   adsorbed to sediment particles may be ingested (Fan et al., 2002; Hamilton, 2004). *T.*

410   *deltoidalis* in our experiments may have had a change in selenium exposure route from an

411   initial direct absorption from ingested sediment particles to a later additional dietary exposure

412   as microfauna and flora, present in the natural sediments and water used, assimilated selenium

413   and were consumed. The major route of selenium uptake in aquatic systems is via food rather

414   than as the free ions in solution (Luoma and Rainbow, 2008), therefore the final greater than

415   ambient selenium tissue concentrations observed in this experiment after 21 days exposure

416   may be related to the generation of a source of dietary selenium.

417   **4.1.2   Subcellular selenium distribution**

418   A large proportion of the selenium recovered in the subcellular fractions was in the nuclei-

419   cellular debris fraction, increasing from 36% in the controls to 56 and 60%, respectively, in

420   the 5 and 20 µg/g exposed organisms (Table 1; Figure 2). Selenite is bound to plasma

421   proteins for transport to tissues. It has been suggested that selenite is taken up by

422   haemolymph, reduced to selenide, released into the plasma and rapidly bound by plasma

423   proteins (Ewan, 1989). The majority of accumulated selenate and selenomethionine occurs in

424   the plasma (Ewan, 1989). Selenomethionine has been shown to bind to glutathione

425   peroxidase extracellularly as well as intracellularly (Burk, 1991). Mycelia of the fungus

426   *Pleurotus ostreatus* enriched with selenium had 56% of accumulated selenium associated

427   with the cell wall (Hortensia et al., 2006). A combination of protein bound selenium

428   associated with plasma and selenium bound directly to cell walls would account for the high

429   proportion of selenium associated with this fraction and therefore it would be comprised of

430   both biologically active and detoxified selenium. Of the remaining selenium recovered in the

431   fractions, the control organisms had 11% and the exposed organisms 20% in the detoxified

432   selenium fractions (Table 1; Figure 2), but the distribution within this portion differed. The

433   control organisms had most selenium in the granule fraction while the 5 and 20 µg/g exposed

434   organisms had 66 and 77%, respectively, in the granules with the remainder in the

435   metallothionein like proteins (Table 2). Selenium associated with metallothionein like

436   proteins has not previously been reported in aquatic organisms, although there is evidence

437   from mammalian studies that suggests selenite exposure induces metallothionein production

438   (Iwai et al., 1988; Chen and Whanger, 1994). The majority of selenium not associated with

439   selenoproteins of the glutathione peroxidase family has been found bound to selenoamino

440   acids and other low molecular weight selenium compounds analogous to metallothioneins

441   and it is presumed that these act as storage and transport proteins and intermediaries in the

442 synthesis of selenoproteins (Akesson and Srikumar, 1994). The metallothionein like protein

443 fraction may therefore represent a pool of detoxified selenium. Like metallothioneins,

444 selenium associated with granules has not previously been described. The operational

445 fraction defined as granules in this procedure has been examined in fractions obtained from

446 cadmium exposed oligochaetes by Wallace et al. (1998), with a compound microscope, and

447 shown to contain numerous metal rich granules of varying sizes. The fraction obtained in the

448 present study using the same technique as described by Wallace et al. (1998) was not

449 examined visually for granules so it can only be assumed that the fraction contained

450 detoxified selenium rich concretions. Using a similar fractionation procedure Zhang and

451 Wang (2006) found 40% and 60% of accumulated selenium in crustaceans and bivalves,

452 respectively, was associated with the granule fractions, while Dubois and Hare (2009)

453 obtained only 1 - 2% of selenium in the granule fractions of the oligochaete *Tubifex tubifex*

454 and the insect *Chironomus riparius*. George, (1983) showed that granules of cadmium

455 exposed *Mytilus edulis* contained high concentrations of protein, calcium and sulphur.

456 Selenium is known to substitute for sulphur in proteins as it has similarities with the

457 chemistry of sulphur (Ewan, 1989). The presence of selenium in a protein is always related to

458 the presence of sulphur, the selenium atom is either incorporated in the place of a sulphur

459 atom in a sulphur amino acid, or it is attached to the sulphur atoms of cysteine residues

460 (Ganther, 1974). It is possible that selenium is incorporated into granule like structures via a

461 similar process to that postulated for cadmium by George (1983), due to an increase in

462 lysosomal protein degradation, following enzyme inactivation by intracellular selenium,

463 causing an increase in intracellular protein turnover. Alternatively the granule fraction may

464 represent selenium associated with incompletely digested tissue and cell debris in the NaOH

465 digestion step of the fractionation procedure (Taylor and Maher, 2013). The fractionation

466 procedure used by Zhang and Wang (2006) which found 40 and 60% of selenium in

467 crustaceans and bivalves associated with the granule fraction used a shorter NaOH digestion

468 step, 10 minutes rather than the 60 minutes used in this study, so incomplete digestion of the

469 tissue and cell debris fraction in their study is also a possibility. If this is the case then it is

470 still a reasonable assumption that a fair proportion of this fraction represents detoxified

471 selenium. The increased percentage of selenium associated with the detoxified selenium

472 fractions (Figure 2) demonstrates that selenium detoxification processes are operating.

473 The concentration of the mitochondrial enzyme cytochrome *c* oxidase was increased in the

474 total homogenate and mitochondrial fractions of the selenium exposed organisms

475 (Supplementary Figure 1) indicating an increased response in this organelle to selenium

476   accumulation. This is in agreement with the organelle selenium distribution results (Table 2;

477   Figure 2) which show a 3 and 4 fold increase, respectively, in mitochondrial selenium in the 5

478   and 20 µg/g exposed organisms. As selenium is an essential component of the glutathione

479   peroxidase enzyme it is expected that it will be present in the mitochondria where oxygen

480   reduction and cellular energy production occurs, however, selenium toxicity can arise at

481   concentrations only slightly greater than those that are required (Palace et al., 2004). The

482   percentage of selenium in the heat sensitive protein fraction was slightly lower than that of the

483   mitochondria in the selenium exposed organisms and higher in the controls (Table 2). This

484   fraction contains enzymes, high and low molecular weight proteins and other target molecules

485   which are sensitive to metals (Wallace et al., 2003). The increased binding of selenium in this

486   fraction is not unexpected as selenium is largely associated with protein complexes (Ganther,

487   1974). The activity of the lysosomal enzyme acid phosphatase was only increased in the 20

488   µg/g exposure while the 5 µg/g organisms remained the same as the control organisms

489   (Supplementary Figure 1). The percentage of biologically active selenium in the lysosomal-

490   microsomal fraction of the selenium exposed organisms was around half that of the controls

491   (Table 2) but the selenium concentration was 1.1 and 1.6 times higher in the 5 and 20 µg/g

492   treatments, respectively, than the controls. The microsomal component of the cell includes

493   fragmented endoplasmic reticulum, which is generally responsible for protein synthesis and

494   transport, selenium in this fraction may be associated with microsomes rather than lysosomes

495   which could be indicative of essential activity but equally could have implications for toxicity

496   (Bonneris et al., 2005).

497   ### *4.2   Enzymatic Biomarkers – Oxidative Enzymes*
498   Selenium is an essential element involved in the reduction of peroxide in the glutathione cycle

499   (Micallef and Tyler, 1987; Hodson, 1988; Hoffman, 2002). Total antioxidant capacity was

500   significantly reduced in the selenium exposed *T. deltoidalis* compared to the control

501   organisms but there was no difference in antioxidant capacity between selenium treatments

502   (Figure 3A). The similarity in the antioxidant response between selenium treatments may be

503   explained by the similarity in the final selenium tissue concentrations between the two

504   treatments (Figure 1). Changes in activity and concentration of enzymes within the

505   glutathione cycle indicate an imbalance in the intracellular glutathione redox status. The

506   activity of the glutathione peroxidase (GPx) enzyme was enhanced in the *T. deltoidalis* from

507   both selenium exposures although not significantly compared to control organisms (Figure

508   3B). The increase in GPx activity may have increased the rate of oxidation of GSH as seen in

509 the increased GSH+2GSSG concentration and the significantly reduced GSH:GSSG ratio

510 (Figure 3B; Supplementary Table 3b). The increase in GSSG may also be a result of the

511 direct reaction of selenite with GSH, which has been shown in the trout *Oncorhynchus mykiss*

512 to produce increased reactive oxygen species, a sharply decreased GSH/GSSG ratio and

513 increased membrane lipid peroxidation (Misra and Niyogi, 2009). Studies in mallard ducks

514 showed that increased dietary and subsequent selenium tissue concentrations resulted in

515 increases in plasma and hepatic GPx activity and GSH concentrations, followed by a dose-

516 dependent decrease in the ratio of hepatic GSH to GSSG concentrations which ultimately led

517 to increased hepatic lipid peroxidation (Hoffman, 2002). Excess GSSG can react with protein

518 sulfhydryls, contributing to the total thiol and protein bound thiol depletions, by the formation

519 of mixed glutathione:protein disulphides. Formation of mixed disulphides may be part of a

520 significant mechanism in regulating metabolic activity as well as the integrity of the cell

521 membranes in response to oxidative stress (Hoffman, 2002). An examination of

522 selenomethionine metabolism in embryos of the trout *Oncorhynchus mykiss* showed oxidative

523 stress, which appeared to be generated by methioninase enzyme activity, liberating

524 methylselenol from L-Selenomethionine (Palace et al., 2004). The methylselenol is able to

525 undergo redox cycling in the presence of glutathione producing superoxide and likely

526 accounts for oxidative stress measured in aquatic organisms environmentally exposed to

527 excess selenomethionine (Palace et al., 2004). Although the sediment in this study was

528 spiked with sodium selenite, which is readily bioaccumulated by animals and bound to

529 proteins following assimilation into cells, animals do not have the capacity to transform it into

530 selenomethionine (Suzuki and Ogra, 2002; Suzuki et al., 2006). Marine algae and bacteria,

531 however, are known to convert selenite mainly into selenomethionine (Fan et al., 2002; Orr et

532 al., 2006) and this secondary pathway of dietary derived selenium may have resulted in

533 selenomethionine exposure for *T. deltoidalis* during the course of the experiment.

534 *4.3   Oxidative Damage Biomarker – Thiobarbituric Acid Reactive Substances*
535 Thiobarbituric acid reactive substances (TBARS) are a measure of lipid peroxidation, a

536 widely recognised consequence of excess oxyradical production which destabilises cell

537 membranes leading to loss of lysosomal integrity and the leaking of the lysosomal contents

538 into the cytoplasm (Winston, 1991; Winston and Di Giulio, 1991). The concentration of

539 TBARS increased significantly in *T. deltoidalis* from both selenium treatments compared to

540 the control organisms (Figure 4A). Increased hepatic lipid peroxidation related to effects of

541 accumulated selenium on glutathione metabolism have been measured in a number of wild

542   aquatic birds, including their hatchlings and eggs (Hoffman, 2002). The TBARS

543   concentration of the selenium exposed organisms was highly negatively correlated with the

544   total antioxidant capacity, indicating that the increased tissue selenium resulted in a reduction

545   in the capacity to reduce reactive oxygen species. It is likely that their subsequent increase

546   directly influenced the build-up of lipid peroxidation by-products.

547   *4.4   Cellular Biomarker – Lysosomal Stability*

548   Metal accumulation in the lysosomes can induce lipid peroxidation through redox cycling or

549   by direct reaction with cellular molecules to generate reactive oxygen species (Ercal et al.,

550   2001). This can destabilise the lysosomal membrane causing the contents to leak out into the

551   cytosol thereby reducing the cells capacity to remove waste which will ultimately lead to cell

552   death (Viarengo et al., 1987). Similar to *T. deltoidalis* exposed to cadmium and lead (Taylor

553   and Maher, 2013; 2014), the selenium exposed *T. deltoidalis* had significantly higher

554   lysosomal destabilisation than the control organisms (Figure 4B). The 5 µg/g selenium

555   exposed *T. deltoidalis* were in the 'concern range' with 30% destabilised lysosomes, while the

556   20 µg/g selenium exposed organisms would be classed as 'stressed' with 68% lysosomal

557   destabilisation based on the Ringwood et al. (2003) criteria. The biologically active selenium

558   burden of the 20 µg/g selenium exposed organisms was ≈ 1.6 times that of the 5 µg/g

559   selenium exposed organisms and this may account for the significantly higher lysosomal

560   membrane damage. The lysosomal fraction of the selenium exposed organisms did not have a

561   marked selenium burden increase, the majority of active selenium was associated with the

562   mitochondrial and heat sensitive protein fractions (Table 2). Selenium binding to molecules

563   present in the heat sensitive proteins of the cytosol may contribute to the total thiol and

564   protein bound thiol depletions, which may be part of a significant mechanism in regulating

565   metabolic activity as well as the integrity of the cell membranes in response to oxidative stress

566   (Hoffman, 2002).

567   *4.5   Genotoxic Biomarker – Micronuclei Frequency*

568   The micronuclei test is a sensitive test to detect genomic damage due to both clastogenic

569   effects and alterations to the mitotic spindle (Migliore et al., 1987). It has been used in

570   bivalves to examine the genotoxicity of a range of chemicals (Scarpato et al., 1990; Williams

571   and Metcalfe, 1992; Burgeot et al., 1996; Bolognesi et al., 2004). The occurrence of

572   micronuclei increased significantly with selenium exposure (Figure 4C). Induction of

573   micronuclei in response to selenium bioaccumulation has not previously been investigated in

574   bivalves, however, these results fit the pattern found for metal induced genotoxic damage as

575   increased frequency of micronuclei shown for *Mytilus galloprovincialis* (Dailianis et al.,

576   2003; Bolognesi et al., 2004; Kalpaxis et al., 2004; Gorbi et al., 2008) and for *T. deltoidalis*

577   exposed to cadmium and lead (Taylor and Maher, 2013; 2014). Increased micronuclei

578   frequency in response to selenium exposure has been observed in fish erythrocyte cells (al

579   Sabti, 1994) and mice bone marrow (Itoh and Shimada, 1996). The frequency of micronuclei

580   in the selenium exposed *T. deltoidalis* corresponded with a decrease in antioxidant capacity

581   and an increase in lipid peroxidation (Supplementary Table 4) indicating that an increase in

582   ROS contributed to an increase in genotoxic damage, either through interaction of reactive

583   oxygen intermediates and lipid peroxidation products with DNA or direct interaction of

584   selenium with cellular macromolecules forming adducts, alkaline labile sites and strand

585   breaks (Regoli et al., 2004).

586   **5   Summary and Conclusions**

587   This study has demonstrated a significant exposure – dose – response relationship for

588   selenium in *T. deltoidalis*. Exposure to selenium contaminated sediments resulted in selenium

589   bioaccumulation but not in proportion to the sediment selenium concentrations. Up to 60% of

590   the accumulated selenium was in the nuclei-cellular debris fraction probably comprised of a

591   combination of protein bound selenium associated with plasma and selenium bound directly

592   to cell walls and therefore effectively removed from active sites within the cell. The

593   percentage of selenium increased in the biologically detoxified fraction of selenium exposed

594   organisms and was associated with both granules and metallothionein like proteins, which has

595   not previously reported for marine bivalves. Selenium associated with low molecular weight

596   proteins is likely to act as storage and transport and intermediaries in the synthesis of

597   selenoproteins and therefore may represent a pool of detoxified selenium. The strong

598   association of selenium with sulphur is a likely mechanism for the incorporation of selenium

599   into granules as has been demonstrated for cadmium. Biologically active selenium burdens

600   increased with selenium exposure and this led to impairment of the antioxidant system which

601   may have initiated the observed increase in lipid peroxidation, lysosomal destabilisation and

602   micronuclei frequency.

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
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Figure & Table Legends

Figure 1: Tissue selenium concentrations (µg/g dry mass) of *T. deltoidalis* exposed to selenium spiked sediments of 0 (control), Se 5 and 20µg/g dry mass. Mean ± SE, $n = 12$. Day 0 are unexposed organisms $n = 6$. Different letters indicate significant differences between means within treatments among collection day (Bonferroni test: $p < 0.05$).

Figure 2: Distribution (%) of selenium in each of the subcellular fractions of *T. deltoidalis* following 28 days exposure to selenium spiked sediments. Subcellular fractions are: Nuclei-cellular debris; granules; heat stable, metallothionein like proteins (MTLP); mitochondria (Mit); lysosomes- microsomes (Lys & Mic); heat sensitive proteins (HSP). Stippled fractions (▒▒▒) make up the biologically active selenium (BA), dashed fractions (▨▨) make up the biologically detoxified selenium (BD), $n = 2$.

Figure 3: Antioxidant enzyme biomarkers of *T. deltoidalis* after 28 days exposure to selenium spiked sediments of 0 (control), Se 5 and Se 20µg/g dry mass. Mean ± SE, $n = 12$. **3A**: TAOC (Total Antioxidant Capacity); **3B**: GPx (glutathione peroxidise); GSH+2GSSG (total glutathione); GSH/GSSG (ratio of reduced to oxidised glutathione). Different letters indicate significant differences between means (Bonferroni test; $p < 0.05$).

Figure 4: Changes in oxidative damage biomarkers: **4A**: MDA (lipid peroxidation); **4B**: cellular (lysosomal destabilisation); and **4C**: genotoxic (micronuclei) of *T. deltoidalis* after 28 days exposure to selenium spiked sediments, 0 (control), Se 5 and Se 20µg/g dry mass. Mean ± SE $n = 12$. Different letters indicate significant differences between means (Bonferroni test; $p < 0.05$).

Table 1: Total selenium concentrations (µg/g wet mass) in whole tissue and subcellular fractions with the percentage of total selenium recovered in all fractions of *T. deltoidalis* after 28 days exposure to selenium spiked sediments. Selenium subcellular concentrations (µg/g wet mass) and percentage distribution of total recovered selenium fractions are grouped as nuclei-cellular debris and biologically active and biologically detoxified selenium. Mean ± SE, $n = 2$.

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

Table 1:

	Sediment Treatments (µg/g)		
	control	Se 5	Se 20
Total Tissue Selenium (µg/g)	**0.42 ± 0.07**	**1.9 ± 0.5**	**3.4 ± 0.7**
Total Recovered Selenium (µg/g)	**0.34 ± 0.01**	**1.4 ± 0.5**	**2.5 ± 0.2**
Proportion of total recovered in fractions (%)	82 ± 12	75 ± 4	74 ± 10
Selenium Subcellular Distribution			
Nuclei - Cellular debris (µg/g)	**0.12 ± 0.004**	**0.78 ± 0.21**	**1.5 ± 0.2**
Nuclei - Cellular debris (%)	36 ± 1	56 ± 5	60 ± 4
Biologically Active Selenium (BA) (µg/g)	**0.18 ± 0.001**	**0.32 ± 0.1**	**0.51 ± 0.05**
Biologically Active Selenium (%)	53 ± 0.8	23 ± 1	20 ± 4
Biologically Detoxified Selenium (BD) (µg/g)	**0.04 ± 0.0003**	**0.29 ± 0.14**	**0.51 ± 0.08**
Biologically Detoxified Selenium (%)	11 ± 0.5	21 ± 4	20 ± 2

1020 Mean ± SD, $n = 2$

1021
1022 **Table 2**:

	Sediment Treatments (µg/g)		
	control	Se 5	Se 20
Nuclei - Cellular debris % of total	**36 ± 1**	**56 ± 5**	**60 ± 4**
Biologically Detoxified Selenium % of total	**11 ± 0.5**	**21 ± 4**	**20 ± 2**
Selenium Rich Granules % of BD	97 ± 0.5	66 ± 5	77 ± 6
Heat Stable MT Like Proteins % of BD	3 ± 0.5	34 ± 4	23 ± 3
Biologically Active Selenium % of total	**53 ± 0.8**	**23 ± 1**	**20 ± 4**
Mitochondria % of BA	34 ± 4	53 ± 1	48 ± 2
Lysosomes - Microsomes % of BA	22 ± 0.1	14 ± 2	12 ± 0.4
Heat Sensitive Proteins % of BA	44 ± 4	34 ± 1	40 ± 3

1023

Figure 1

1027   **Figure 2**

Control

HSP

Nuclei-Cell debris

Lys & Mic

Mit

Granules

MTLP

BA = 0.18 µg/g
BD = 0.04 µg/g

1028

Se 5 µg/g

Lys & Mic

HSP

Mit

Nuclei-Cell debris

MTLP

Granules

BA = 0.32 µg/g
BD = 0.29 µg/g

1029

Se 20 µg/g

Lys & Mic

HSP

Mit

MTLP

Nuclei-Cell debris

Granules

BA = 0.51 µg/g
BD = 0.50 µg/g

1030

31

1031 **Figure 3**

32

Figure 4